



UNIVERSITÀ DI PARMA

UNIVERSITÀ DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN SCIENZA E TECNOLOGIA DEI MATERIALI

XXXVI CICLO

**Fluorescent Organic Nanoparticles for
Bioimaging: from Spectroscopy to *In Vitro*
and *Ex Vivo* Applications**

Coordinatore

Prof. Enrico Dalcanale

Relatore

Prof.ssa Cristina Sissa

Dottorando

Andrea Delledonne

Anni accademici 2020/21 - 2022/2023

E poi la luce, luce che trasforma il mondo in un giocattolo.

Fabrizio De Andre', Un Ottico

Contents

List of Acronyms	iii
List of Publications	v
Introduction	1
1 Polymeric unimer micelles: nanocarriers for fluorescent probes	5
1.1 Introduction	5
1.1.1 Materials selection and chapter outline	6
1.2 Self-Assembly of unimer micelles in water	8
1.3 Encapsulation of Coumarin-153 in unimer micelles	10
1.3.1 UV-Vis Spectroscopy in Aqueous Media	10
1.3.2 Fluorescence anisotropy study	11
1.3.3 Solvatochromic study	14
1.4 Bioimaging experiments in porcine sclera	15
1.5 Cytotoxicity study	19
1.6 Conclusions and perspectives	20
1.7 Materials and Methods	21
1.7.1 Preparation of unimer micelles suspension	21
2 Förster resonance energy transfer in CTAB & TPGS micelles: a comparison	23
2.1 Introduction	23
2.2 Aggregation of DiI and DiD	26
2.3 CTAB and TPGS micelles in aqueous suspension	28
2.4 Spectroscopic properties of dye-loaded micelles	30
2.4.1 Single-dye loaded micelles	30
2.4.2 FRET micelles	34
2.4.3 Mixed single dye-loaded micelles	37
2.5 Bioimaging experiments in porcine sclera	39
2.5.1 Single dye-loaded micelles	39
2.5.2 FRET micelles	43
2.6 Conclusions and perspectives	47
2.7 Materials and Methods	49
2.7.1 Preparation of DiI and DiD aggregates	49
2.7.2 Aggregates in the gel	49
2.7.3 Preparation of dye-Loaded CTAB and TPGS micelles	50
3 Fluorescent Quatsomes for bioimaging applications	53
3.1 Introduction	53
3.2 Quatsomes decorated with a hydrophilic donor and a hydrophobic acceptor	57
3.2.1 Spectroscopic properties of EoY and RhB at varying pH	60
3.2.2 Physicochemical characterization of Quatsomes	62
3.2.3 Encapsulation efficiency estimation	64
3.2.4 Optical spectroscopy and FRET	67
3.2.5 Two-Photon Microscopy of Quatsomes suspensions	70
3.2.6 Effect of the preparation procedure on EosinY-decorated Quatsomes	71
3.3 DiR-loaded MKC-Quatsomes for specific targeting	72
3.3.1 Spectroscopic characterization	78
3.3.2 <i>In vitro</i> confocal imaging experiments	84
3.4 Conclusions and perspectives	92

3.5	Materials and Methods	94
3.5.1	Preparation of dye-loaded Quatsomes	94
3.5.2	Photobleaching experiments	97
3.5.3	<i>In vitro</i> experiments	98
4	Two-photon microscopy applied to drug delivery systems	101
4.1	Introduction	101
4.2	TPGS micelles for drug delivery applications	104
4.2.1	TPGS micelles for ocular delivery	105
4.2.2	Skin retention of TPGS micelles	112
4.3	Nanostructured lipid carriers for enhanced transscleral delivery	116
4.4	Buccal permeation of polysaccharide high molecular weight compounds	121
4.5	Conclusions and perspectives	124
4.6	Materials and Methods	125
4.6.1	Preparation of NR-loaded TPGS micelles	125
4.6.2	Caprylic acid pre-treatment of buccal mucosa	125
	General Conclusions	127
	Experimental Part	129
T.1	Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS)	129
T.2	Linear spectroscopic measurements	130
T.3	<i>Ex-Vivo</i> permeation experiments	132
T.4	Two-Photon Microscopy setup	132
	APPENDICES	135
A	Two-Photon Microscopy of blank tissues	137
A.1	TPA and SHG	137
A.2	Ocular tissues	139
A.2.1	Sclera	140
A.2.2	Cornea	142
A.3	Skin	144
B	TPA spectroscopic measurements	147
B.1	TPA cross section estimation	147
B.2	Examples of TPA spectroscopic measurements	148
	Bibliography	151

List of Acronyms

η	Viscosity
τ	Fluorescence lifetime
τ_c	Rotational correlation time
ε	Dielectric constant
D_h	Hydrodynamic Diameter
K_b	Boltzmann constant
n	Refractive index
r	Fluorescence anisotropy
R_g	Radius of Gyration
V_h	Hydrodynamic volume
ATRP	Atom Transfer Radical Polymerization
BAK	BenzAlKonium chloride
C153	Coumarin153
CMC	Critical Micellar Concentration
cryoTEM	cryogenic Transmission Electron Microscopy
CTAB	CetylTriMethylAmmonium Bromide
DD	Descanned Detection
DELOS-SUSP	Depressurization of an Expanded Liquid Organic Solution-Suspension
DiD	1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine perchlorate
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DiR	1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide
DLS	Dynamic Light Scattering
ELS	electrophoretic light scattering
EoY	Eosin Y
FA	perFluorohexylethyl Acrylate
FON	Fluorescent Organic Nanoparticle

HITC	1,1',3,3,3',3'-Hexamethylindotricarbocyanine iodide
MKC	MyristalKonium Chloride
MW	Molecular Weight
N_{agg}	Aggregation number
NDD	Non-Descanned Detection
NIR	Near-Infrared
NLC	Nanostructured Lipid Carrier
NMR	Nuclear Magnetic Resonance spectroscopy
NP	Nanoparticle
NR	Nile Red
NV	Nanovesicle
PBS	Phosphate-Buffered Saline
PdI	Polydispersity Index
PEGMA	oligo(Ethylene Glycol) Methyl ether methAcrylate
QS	Quatsome
RhB	Rhodamine B isothiocyanate
RL	Ringer's Lactate
SANS	Small Angle Neutron Scattering
SAXS	Small Angle X-ray Scattering
SCNPs	Single-Chain Nanoparticles
SEM	Standard Error of the Mean
SHG	Second Harmonic Generation
SUV	Small Unilamellar Vesicle
TPA	Two-Photon Absorption
TPEF	Two-Photon Excited Fluorescence
TPGS	Tocopherol Polyethylene Glycol 1000 Succinate
TPM	Two-Photon Microscopy

List of Publications

- ▶ A. Delledonne, et al. Amphiphilic Fluorinated Unimer Micelles as Nanocarriers of Fluorescent Probes for Bioimaging. *ACS Applied Nano Materials*, 2023, 6, 15551-15562;
- ▶ F. Bertocchi, et al. Aggregates of Cyanine Dyes: When Molecular Vibrations and Electrostatic Screening Make the Difference. *The Journal of Physical Chemistry C*, 2023, 127(21), 10185–10196;
- ▶ Felipe M. González-Fernández, et al. Nanostructured Lipid Carriers for Enhanced Transscleral Delivery of Dexamethasone Acetate: Development, Ex Vivo Characterization and Multiphoton Microscopy Studies. *Pharmaceutics*, 2023, 15, 407;
- ▶ A. Delledonne, et al. Luminescence properties of mixed-ligand MOFs containing fluorene scaffolds functionalized with isonicotinoyl arms. *CrystEngComm*, 2023, 25, 2085-2095;
- ▶ K. Swathi, et al. From symmetry breaking to symmetry swapping: is Kasha’s rule violated in multi-branched phenyleneethynylenes?. *Chemical Science*, 2023, 14, 1986-1996;
- ▶ A. Fantini, et al. Buccal Permeation of Polysaccharide High Molecular Weight Compounds: Effect of Chemical Permeation Enhancers. *Pharmaceutics*, 2022, 15, 129;
- ▶ M. Spedicati, et al. Biomimetic design of bioartificial scaffolds for the in vitro modelling of human cardiac fibrosis. *Frontiers in Bioengineering and Biotechnology*, 2022, 10, 2106;
- ▶ M. Ghezzi, et al. Cyclosporine-loaded micelles for ocular delivery: Investigating the penetration mechanisms. *Journal of Controlled Release*, 2022, 349, 744–755;
- ▶ A. Delledonne, et al. Bis-isonicotinoyl linkers containing polyaromatic scaffolds: synthesis, structure and spectroscopic properties. *Phys. Chem. Chem. Phys.*, 2022, 24, 1191-1201;
- ▶ M. Ghezzi, et al. Improvement of Imiquimod Solubilization and Skin Retention via TPGS Micelles: Exploiting the Co-Solubilizing Effect of Oleic Acid. *Pharmaceutics*, 2021, 13, 1476;
- ▶ A. Delledonne, et al. Increasing resonance energy transfer upon dilution: a counterintuitive observation in CTAB micelles. *J. Mater. Chem. C.*, 2021, 9, 10952–10964;
- ▶ R. Schettini, et al. Crystal structures and photoluminescence properties of chromium(III) complexes with 2-thenoyltrifluoroacetone ligand. *J. Mol. Struct.*, 2021, 1245, 131023.

Introduction

In the vast landscape of nanotechnology, nanoparticles (NPs) reign supreme, offering various applications in disparate scientific fields, including biomedicine,[1] electronics,[2] construction materials[3] and catalysis.[4] The sub-micrometer size of the particles enables the appearance of unique features, significantly different from bulk materials, granting high surface-area-to-volume ratio and specific chemical properties, that, in the field of biomedical science, make NPs exceptionally valuable for drug delivery,[5] bioimaging,[6] and diagnostics.[7] These applications, when combined synergistically, result to attractive therapeutic systems co-delivering therapeutic and imaging functions.[8] According to their components, NPs are usually classified as inorganic, polymer-based, lipid-based or protein-based.[9, 10] The research work presented in this thesis is focused on organic systems formed by lipids or polymers, presented in Figure 1, and labelled with fluorescent dyes in order to obtain fluorescent organic nanoparticles (FONs).

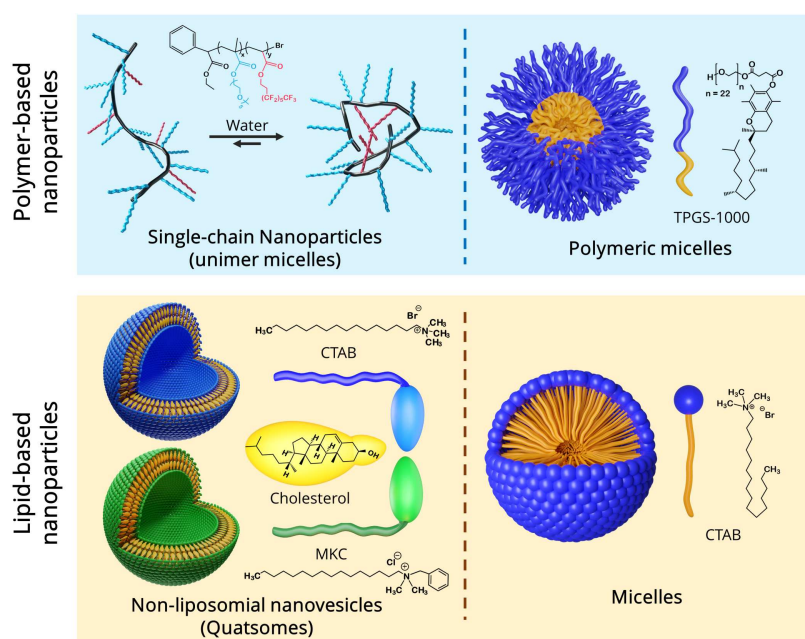


Figure 1: Schematic representation of the organic nanoparticles studied in this thesis, classified with respect to their composition: polymer-based nanoparticles (single-chain nanoparticles and polymeric micelles); lipid-based nanoparticles (Quatsomes and micelles). The molecular structures of the components are also shown.

Several approaches can be employed to introduce fluorescent functionalities in a nanoparticulate system.[10] A straightforward strategy concerns the use of fluorescent molecules as primary building blocks of the FON structure. This can be achieved by forming nanovesicles from fluorescent amphiphiles[11] or organic NPs from self-associated fluorescent molecules.[12] In this work, small fluorescent molecules are loaded into non-fluorescent NPs, exploiting non-covalent interactions (hydrophobic or electrostatic interactions). The fluorescent labelling can be directly incorporated during the preparation of the sample or by post-processing of preformed nanoparticles.

Detailed information on the fluorophores encapsulated into FONs has been retrieved making use of advanced optical spectroscopic techniques,

invaluable tools for studying a fluorescent probe and its interactions with the nanocarrier with minimal alterations of the supramolecular assembly environment. UV–Vis absorption and emission spectra can be extremely useful to assess:

¹: provided that the emission spectrum of one fluorophore spectrally overlaps the absorption spectrum of the other.

- ▶ dye aggregation, usually due to the high local concentration that can be achieved within the NPs, leading to excitonic effects (at short interchromophoric distances, typically on the order of 4–20 Å).[13] When the intermolecular interactions are strong, the spectra result different from those of the isolated fluorophore, showing the typical characteristics of aggregation (J- or H-aggregates, according to the specific molecular packing);[14]
- ▶ occurrence of FRET, i.e. the exchange of excitation energy from one fluorophore to another, occurring when loading the NPs with multiple fluorophores and their distance is on the order of 20–100 Å¹;
- ▶ fluorescence quantum yield, a crucial parameter for bioimaging applications;
- ▶ polarity of the local environment, when a solvatochromic dye is embedded in the particles. The spectral positions of absorption and emission bands, as well as bandhapes, are highly sensitive to the polarity of the surroundings in solvatochromic probes.[15]

Steady-state and time-resolved fluorescence anisotropy are powerful techniques for studying the mobility of small fluorophores interacting with NPs and obtaining precise information on the FONs size and shape. Fluorescence anisotropy is a measure of how strongly the emitted light is polarized after polarized excitation. In common non viscous solvents, the emission of small fluorophores is usually (almost) completely depolarized, while FONs, being “large” objects, typically give rise to a partially polarized emission. Fluorescence anisotropy has been extensively exploited in this work to assess the effective encapsulation of small fluorophores and their rotational motion inside the nanocarrier.

This work also demonstrates that preliminary and detailed spectroscopic characterization of dye-loaded NPs is always necessary when they are adopted for investigations in complex environments, such as biological tissues. Eventual variations of the emission bandshape or FRET efficiency can be analyzed to study the interactions of the FONs with the biological samples, their diffusion and their integrity. The efficacy of FONs as fluorescent bioimaging probes has been tested in different biological environments. *Ex vivo* thick tissues have been analyzed with two-photon microscopy, exploiting the intrinsic advantages that this technique offers for the collection of images in depth. On the other hand, confocal fluorescence microscopy has been selected to investigating *in vitro* cell cultures.

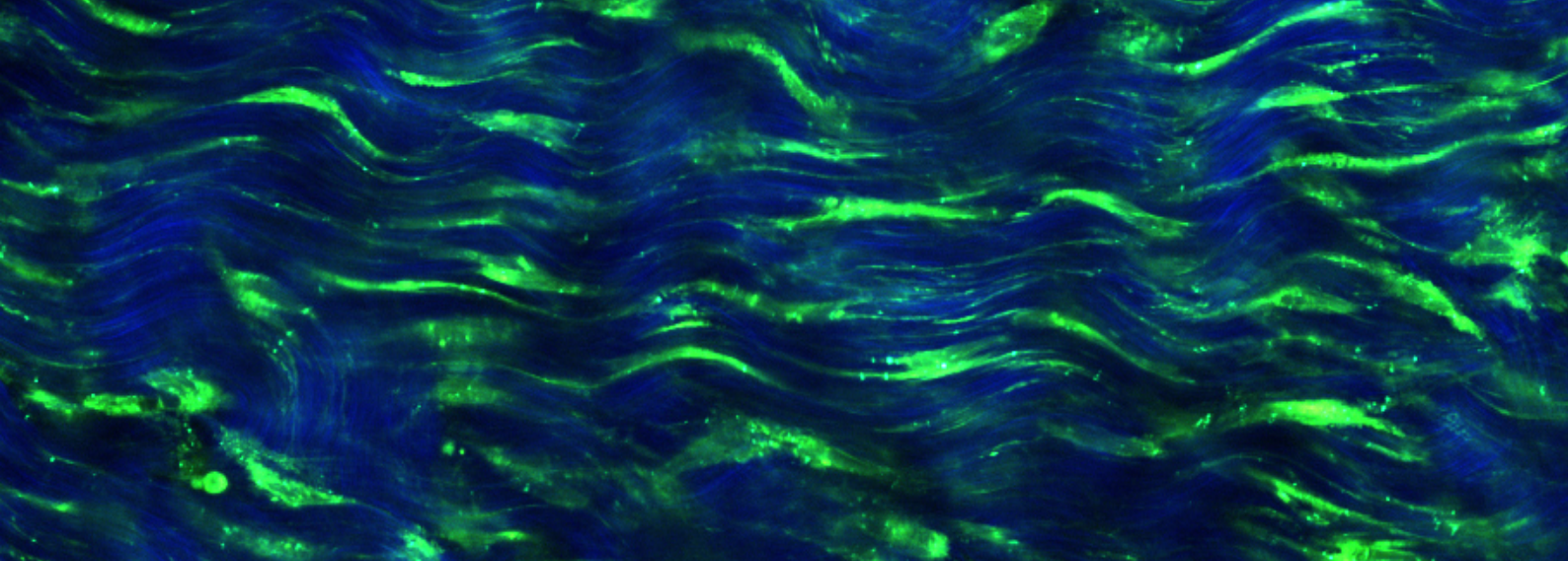
The thesis is organized into four chapters: Chapter 1, 2 and 3 present the preparation, spectroscopic characterization, and evaluation of the bioimaging capabilities of different FONs families exhibiting increasing complexity. In Chapter 1, self-folded unimer micelles derived from an amphiphilic random copolymer loaded with a simple hydrophobic fluorophore are discussed. Chapter 2 concerns the encapsulation of two different cyanine dyes into common polymeric or lipidic micelles,

assessing the presence of FRET phenomena in the system. In Chapter 3, Quatsomes, a class of non-liposomal vesicles with an elaborate supramolecular structure are studied, by investigating the labelling with multiple fluorophores characterized by different physico-chemical properties or targeted surface functionalization. Chapter 4 collects the results obtained by combined spectroscopic and microscopic investigations of NPs specifically designed for drug delivery. In this context, the images and spectroscopic data retrieved from two-photon microscopy proved to be of major support to address the nanocarriers' fate inside the tissues and corroborate quantification data concerning their permeation and retention.

The work has been carried out in collaboration with different research groups:

- ▶ the group of Prof. E. Martinelli, University of Pisa, Italy, that synthesized polymeric unimer micelles presented in Chapter 1;
- ▶ the Nanomol-Bio group of Prof. N. Ventosa (ICMAB-CSIC) and Nanomol Technologies S.L., Spain, that collaborated to the research presented in Chapter 3 about Quatsomes;
- ▶ the group of Prof. S. Pescina and Prof. S. Nicoli, Food and Drug Department of the University of Parma, which provided the *ex vivo* animal tissue samples, contributed to the development of the experimental set-up for the microscopic analysis, provided the expertise and facilities necessary for the permeation experiments, and prepared the NPs designed for drug delivery discussed in Chapter 4;
- ▶ the group of Prof. K. Belfield, New Jersey Institute of Technology, USA, that was involved in the confocal imaging of cell lines addressed in Chapter 3.

Part of this work was also developed in the framework of the European Project Micro4Nano, which funded several secondments to Spain and to USA.



1 Polymeric unimer micelles: nanocarriers for fluorescent probes

1.1 Introduction

Small organic fluorophores are of primary interest in biomedical science, medical diagnostics and imaging.[16–18] However, their use in biological environments often faces several inherent challenges, such as: low water solubility, aggregation-caused quenching,[19] photostability[20] and toxicity[21]. To address these limitations, extensive research has been dedicated to the development of polymeric fluorescent micro/-nanoparticles, relying on diverse strategies. These include copolymerization of monomers containing fluorescent groups, functionalization of non-fluorescent block copolymer micelles, as well as the utilization of dendrimers, cross-linked polymer particles, and conjugated fluorescent polymers.[10, 22, 23]

The nanoscale features can improve the efficacy of agents used in both bioimaging and nanomedicine, leading to brighter emission due to the presence of multiple fluorophores, reduced photobleaching, enhanced biocompatibility and biodistribution. These aspects potentially allow also the combination of imaging and drug delivery, in order to obtain theranostic agents.[22, 24] However, the size and surface properties of the particles play a major role in determining their fate after administration, especially for in vivo applications.[25] NPs with diameter lower than 10 nm generally show increased cellular uptake, traversal of intact membranes and are rapidly cleared through extravasation or renal clearance, which is extremely important in the case of non biodegradable materials.[24, 26]

Among the variety of available nanomaterials, single-chain polymer nanoparticles (SCNPs) exploit the folding of single polymer chains through covalent, dynamic covalent, or non-covalent intramolecular interactions to achieve extremely small systems, reaching sizes as small

1.1 Introduction	5
1.2 Self-Assembly of unimer micelles in water	8
1.3 Encapsulation of Coumarin-153 in unimer micelles . . .	10
1.4 Bioimaging experiments in porcine sclera	15
1.5 Cytotoxicity study	19
1.6 Conclusions and perspectives	20
1.7 Materials and Methods . .	21

as 3 nm.[27] This feature makes them promising polymeric NPs for applications in nanomedicine, sensing, catalysis, and various other fields.[28] When the single chain folding is promoted by solvophobic interactions in a selective solvent, typically water, it can lead to the formation of globular structures. These nanoobjects are characterized by hydrophobic compartments surrounded by a hydrophilic shell, and are generally referred to as unimer micelles.[29, 30] The unimer micelles feature distinct domains with tunable polarity and affinity, making them responsive to external stimuli (such as temperature or light)[31–33] and able to accommodate small molecules like fluorophores or drugs, avoiding additional synthetic steps for particle functionalization or intricate encapsulation protocols.[34–36] Moreover, unimer micelles are expected to be stable at higher concentrations and to exhibit a more globular structure compared to covalently crosslinked SCNPs.[37]

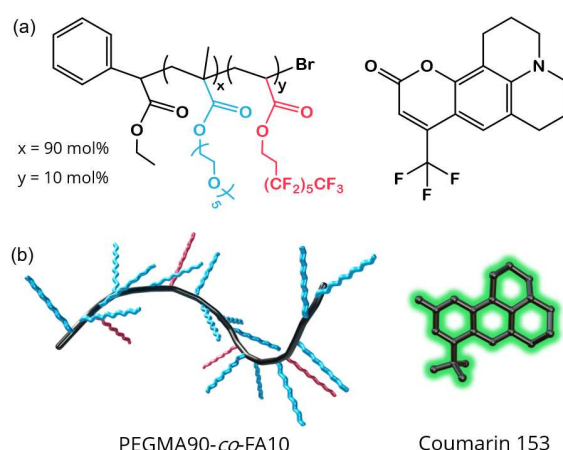
In this chapter, we present a novel application of spontaneously self-folded unimer micelles derived from an amphiphilic random copolymer as nanocarriers for a hydrophobic fluorescent probe. The fluorescent nanocarriers were also tested for bioimaging purposes in multiphoton microscopy. The work was carried out in collaboration with the group of Prof. Elisa Martinelli, University of Pisa, who synthesized and characterized the copolymer for the formation of unimer micelles. Tissue permeation experiments and cytotoxicity studies were performed at the Food and Drug department of University of Parma by Prof. Silvia Pescina and Prof. Annalisa Bianchera, respectively.

1.1.1 Materials selection and chapter outline

1: The presence of PEG groups have gained increasing interest in the last two decades not only due to the high biocompatibility but also because they confer to the system additional features such as thermoresponsive aggregation.[38]

Figure 1.1 shows the building blocks of the fluorescent nanocarrier: a (meth)acrylic random copolymer with oligoethyleneglycol¹ and perfluorohexylethyl side chains (PEGMA90-co-FA10) was chosen to encapsulate Coumarin 153 (C153), a commercial fluorophore.

Figure 1.1: Molecular structures [Panel(a)] and schematic representation [Panel(b)] of the random copolymer PEGMA90-co-FA10 ($x = 90$, $y = 10$ mol %) and Coumarin 153 (C153).



The selected copolymer is characterized by a relatively small content of hydrophobic FA counts (10 mol%) to ensure its complete solubility in water, but sufficient to promote the copolymer self-assembly into unimer micelles. The formation of unimer micelles for the PEGMA90-co-FA10 copolymer was already demonstrated by our collaborators at University

of Pisa, by dynamic light scattering (DLS) and small angle neutron scattering (SANS).[31]

On the other hand, C153 was selected as an extensively employed fluorescent probe across various applications, such as bioimaging[39] and the study of micellar systems.[40] Moreover, the following features of C153 make it an ideal candidate for the preparation of a fluorescent nanocarrier for bioimaging:

- ▶ scarce water solubility, promoting efficient encapsulation in the nanoparticles;
- ▶ good fluorescence quantum yield, essential for bioimaging;
- ▶ solvatofluorochromic behavior, facilitating the investigation of the local microenvironment of the fluorescent probe;
- ▶ small molecular dimensions, crucial for studying encapsulation through fluorescence anisotropy measurements.

In Section 1.2 we propose a procedure for the preparation of unimer micelles (both plain and C153-loaded) that is simple, avoids covalent bonding and is less chemically demanding, compared to the formation of fluorescent SCNPs by multistep intramolecular crosslink.[41] In Section 1.3, a detailed spectroscopic characterization of the C153-loaded unimer micelles is conducted to explore the efficiency of dye loading, the emissive features of the fluorescent nanocarrier, and the mobility of the dye within the micelles.² Section 1.4 addresses the capacity of C153-loaded unimer micelles to transport small hydrophobic molecules during tissue permeation, particularly in the context of topical administration. Topical administration (i.e., skin, eyes, mucous membranes) is the favored route for local delivery owing to its convenience and affordability[44] and offers a non-invasive approach for bioimaging within tissues. We selected *ex vivo* porcine scleral tissue as testing biological material and two-photon microscopy to analyze the permeation results, being it a powerful tool for the in-depth imaging of biological tissues. Cytotoxicity studies are reported in Section 1.5. The efficient permeation of the C153-loaded unimer micelles in sclera was demonstrated, enabling the direct visualization of the fluorescent probe in the whole tissue and paving the way for diverse applications, including drug delivery and sensing.

²: C153 was adopted as a probe for measuring steady-state and time-resolved fluorescence anisotropy, providing important information about the mobility of the dye interacting with the nanocarrier. C153 was employed for similar purposes also in previous studies.[40, 42, 43]

1.2 Self-Assembly of unimer micelles in water

The amphiphilic random copolymer PEGMA90-co-FA10 (Figure 1.1) was synthesized by the group of Prof. E. Martinelli (University of Pisa) through atom transfer radical polymerization (ATRP) of hydrophobic perfluorohexylethyl acrylate (FA) with a hydrophilic oligo(ethylene glycol) methyl ether methacrylate containing ~ 5 repeating units (PEGMA) following a literature procedure.[45, 46] A water-soluble copolymer was obtained, with large hydrophilic character (90% mol PEGMA units, $M_n = 21\,000\text{ g mol}^{-1}$ by ^1H NMR) and narrow monomodal molecular weight distribution (Dispersity, $\bar{D} = 1.21$ from GPC analysis).

The self-assembly behavior in aqueous solution of amphiphilic random copolymers with perfluoroalkyl and oligooxyethylene side chains, such as the copolymer PEGMA90-co-FA10 studied in this thesis, was previously investigated in great detail as a function of copolymer concentration and temperature by different and complementary techniques, including DLS, NMR relaxometry, SANS and SAXS analyses, and integrated with molecular dynamics simulations.³[31, 45–48] The self-folding of the macromolecular chains is driven by the hydrophobic intramolecular interactions of the fluorinated side chains when the copolymer is dissolved in water.[31] Such a self-assembly mechanism was also supported by computational studies of the folding trajectory of a typical PEGMA-co-FA copolymer in water in terms of decrease in R_g , especially when compared to the size of the unfolded copolymer in a non-selective organic solvent, and the evolution of the solvent-accessible surface area.⁴ A notable advantage of employing the folding of a single-chain entity, instead of a supramolecular aggregate, is the formation of unimer micelles characterized by remarkably low size, with a typical hydrodynamic diameter (D_h) of less than 8 nm and a gyration radius (R_g) of approximately 2 nm.

Recently, the incorporation and controlled release of a highly hydrophobic drug (combretastatin A-4) in PEGMA-co-FA unimer micelles has been achieved as a proof of concept of their application in the biomedical field.[49] Additionally, the formation of segregated hydrophobic domains within the micelle itself were probed via the incorporation of fluorescent organic molecules and counits.[45, 47] Building on these findings, we loaded PEGMA90-co-FA10 unimer micelles with C153, following a thin-film re-hydration procedure. The resulting unimer micelles suspension was characterized by final concentrations of 5 g/L ($\sim 238\text{ }\mu\text{M}$) and 15 μM for PEGMA90-co-FA10 and C153, respectively. Technical details on the experimental procedure are reported in Section 1.7.1. C153-loaded unimer micelles were prepared in both distilled water and saline solution in order to have a suitable medium for biological applications.

The self-assembly of the copolymer was verified by DLS at room temperature; technical details regarding these measurements and a brief description of the technique are reported in Section T.1. Figure 1.2 shows the size distributions obtained from DLS measurements for the unloaded copolymer in distilled water and for the C153-loaded unimer micelles in saline solution (the concentration of the copolymer is the same in the two samples): the intensity size distribution shows two populations for both samples, one having a D_h of $\sim 5\text{ nm}$ and the other of $\sim 130\text{ nm}$. The presence of a population having $D_h \sim 5\text{ nm}$ is consistent with the

3: In particular, SANS profiles of copolymer PEGMA90-co-FA10 in D_2O at different concentrations (1–10 g/L) and as a function of temperature demonstrated the existence of non-aggregated single polymer chains with an $R_g \sim 2\text{ nm}$ (from an ellipsoidal fitting) below 45°C . The bell-shaped Kratky plots elaborated from SANS data proved that, independently of copolymer concentration, the single-chain unimers exhibited a self-folded, compact, and low flexible, globular conformation with size (i.e., pseudo-Guinier radius) of $\approx 3\text{ nm}$.

4: The surface area exposed to the selective solvent (water) showed a general reduction, which was mainly related to the FA component gradually becoming less and less exposed to the aqueous environment.[45]

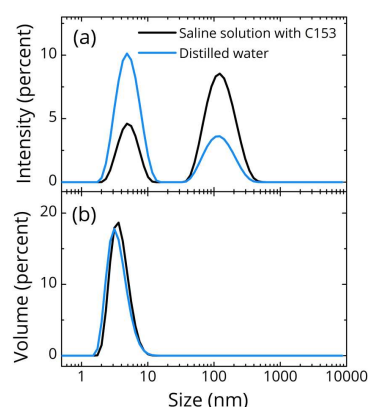


Figure 1.2: Intensity [panel (a)] and volume [panel (b)] size distributions of unimer micelles in distilled water without dye (blue lines, total polymer concentration: 5 g/L) and in saline solution after the loading with C153 (black lines, total polymer concentration: 5 g/L, total dye concentration: 15 μM).

formation of unimer micelles in both samples as expected from the previous studies on self-folding of PEGMA-co-FA analogue copolymers.[31, 46, 48] A second population of larger particles ($D_h \sim 130$ nm) is also usually detected in the intensity distribution,[45, 46] but it is absent in the volume distribution (Figure 1.2b) indicating a negligible tendency of the copolymer to self-assemble into larger multi-chain aggregates.⁵ Although minimal, such a contribution is more pronounced for the suspension in saline solution containing C153 as the relative intensity of the associated peak is higher (Figure 1.2a), suggesting that the presence of the C153 dye and/or a change in ionic force promotes the formation of aggregates. The presence of C153 might favor the aggregation of different polymeric chains acting as a physical cross-linker, as previously observed when loading of a hydrophobic drug[49] and a fluorinated agrochemical[34] in similar systems.

Moreover, it was recently shown that NaCl aqueous solutions in the range of the physiological concentration favor the dehydration of the oxyethylene side chains, which results in a reduced solubility of the copolymer, with a slight decrease in the value of the solution cloud point.[48]

Other polymeric suspensions have been prepared in distilled water changing the experimental conditions, in some cases using a preparation procedure different from thin film re-hydration. This second preparation procedure is described in detail in Section 1.7.1 and involves the direct addition of a small volume of C153 concentrated solution in acetone (final acetone concentration in the suspension: $\sim 0.1\%$ v/v) to a suspension of PEGMA90-co-FA10 in water (5 g/L), obtaining a final C153 concentration of 5 or 30 μ M.

All the volume size distributions that we obtained do not show any significant difference and clearly indicate that neither C153, nor NaCl (9 g/L) or the preparation procedure significantly impact the structure of unimer micelles (see Table 1.1 and Figure 1.3 for a summary of DLS results obtained with all the prepared polymeric suspensions).

5: The minor role of multi-chain aggregates in comparison to unimer micelles was previously confirmed through SANS measurements of a PEGMA90-co-FA10 solution in D_2O . These measurements revealed the presence of only unimer micelles at lower temperatures ($< 45^\circ\text{C}$). As the temperature exceeded 45°C , the self-folded unimers began to form larger multi-chain particles, with a weight fraction of larger aggregates that increased gradually up to 98% at 60°C . [31]

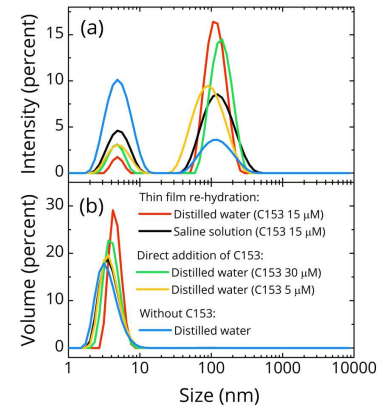


Figure 1.3: Intensity [panel (a)] and volume [panel (b)] size distributions of the unimer micelles suspensions obtained with different preparation method, medium and dye concentration (total polymer concentration: 5 g/L).

Table 1.1: DLS results of PEGMA90-co-FA10 suspensions as the average of three measurements with the corresponding standard error of the measurement mean (SEM). Measurements were performed at 25°C as described in Section T.1.

Sample	Peak 1 mean D_h^a (nm)	Peak 1 area intensity (%)	Peak 2 mean D_h^a (nm)	Peak 2 area intensity (%)	Peak 1 area volume (%)	Peak 2 area volume (%)
Saline solution with C153 (15 μ M)	137 ± 2	72.7 ± 0.1	5.23 ± 0.02	27.3 ± 0.1	0	100
Distilled water	131 ± 1	30.5 ± 0.1	5.31 ± 0.07	69.5 ± 0.1	0	100
Distilled water with C153 (15 μ M)	119 ± 1	94.5 ± 0.1	4.91 ± 0.04	5.5 ± 0.1	0	100
Distilled water with C153 (5 μ M, direct addition)	102 ± 1	81.9 ± 0.1	5.23 ± 0.03	18.1 ± 0.1	0	100
Distilled water with C153 (30 μ M, direct addition)	144 ± 1	86.6 ± 0.1	4.79 ± 0.02	13.4 ± 0.1	0	100

^a estimated from the mean size value of the corresponding peak in the intensity distribution.

1.3 Encapsulation of Coumarin-153 in unimer micelles

1.3.1 UV-Vis Spectroscopy in Aqueous Media

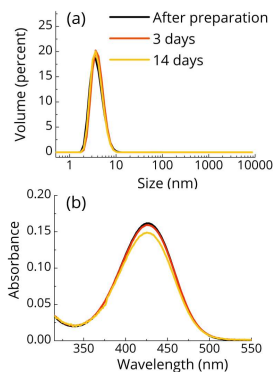


Figure 1.4: Panel (a): size distributions by volume of a C153-loaded micelles suspension in saline solution acquired after different time periods, up to 14 days from preparation. Panel (b): Absorption over time variations of C153-loaded micelles.

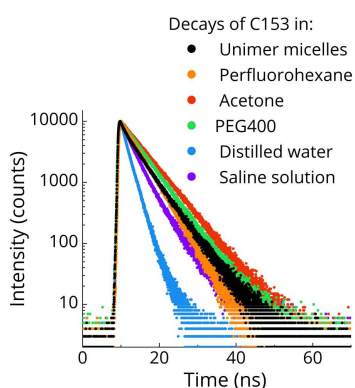


Figure 1.5: Emission decays of C153 in different environments: unimer micelles in aqueous suspension (black line, $\lambda_{em} = 515$ nm), perfluorohexane (orange line $\lambda_{em} = 440$ nm), acetone (red line $\lambda_{em} = 515$ nm), PEG400 (green line, $\lambda_{em} = 515$ nm), distilled water (blue line, $\lambda_{em} = 550$ nm), and saline solution (purple line, $\lambda_{em} = 515$ nm).

Figure 1.6: Panel(a): comparison between the absorption spectra of C153-loaded unimer micelles (polymer concentration: 5 g/L, total theoretical dye concentration: 15 μ M), C153-saturated unimer micelles (polymer concentration: 5 g/L, total theoretical dye concentration: 0.5 g/L), and C153-saturated saline solution. Panel(b): comparison between normalized absorption and emission spectra of C153-loaded unimer micelles in distilled water and saline solution.

C153 is a strongly hydrophobic dye, and its solubility in water is very poor.[43] The maximum absorbance obtained in C153-saturated saline solution is 0.008 at 431 nm (maximum of the low-energy absorption band of C153 in water), as reported in Figure 1.6a. In the presence of the copolymer, for a relatively low dye concentration of 15 μ M (theoretical value), the absorbance of the suspension is significantly increased and reaches the value of 0.15, also maintaining good stability over time up to 2 weeks after preparation (Figure 1.4b, the corresponding DLS results are reported in Figure 1.4a). The total polymer concentration for all the prepared suspensions has been kept at 5 g/L, ensuring optimal conditions for DLS experiments. More absorptive solutions can be obtained increasing the concentration of dye, as shown in Figure 1.6a, e.g. saturating the polymeric suspension with 0.5 g/L of C153. Further spectroscopic characterizations (and two-photon microscopy experiments) were performed on suspensions having low dye concentration (15 μ M) to avoid inner filter effects in fluorescence experiments and the formation of dimers or aggregates in the micelles, which could affect the spectroscopic properties of the fluorophore. UV-Vis and fluorescence spectra (Figure 1.6b), as well as lifetime decays (Table 1.2) of dye-loaded micelles prepared in water and in saline solution are superimposable; thus, the spectroscopic characterization discussed later on in the text is referred to the suspension in distilled water (see Section T.2 for technical information on these measurements).

The presence of the copolymer significantly contributes to preserving the good emissive properties of C153: the fluorescence quantum yield of C153-loaded micelles is 50%, a value comparable to those measured in other organic solvents.[50, 51] The fluorescence lifetime (τ) measured in the unimer micelles suspension is significantly higher than the values obtained from C153 in water or saline solution (Table 1.2 and Figure 1.5). These results confirm that unimer micelles are able to efficiently solubilize the C153 dye in an aqueous medium, without harming its valuable spectroscopic properties.

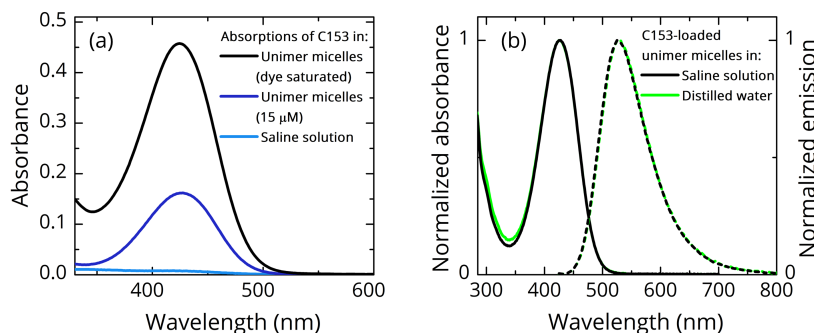


Table 1.2: Spectroscopic properties of C153 in different environments, obtained as described in Section T.2.

Solvent	$\lambda_{\text{abs}}^{\text{max}}$ (nm)	$\lambda_{\text{em}}^{\text{max}}$ (nm)	Stokes shift (cm^{-1})	Fluorescence quantum yield ϕ (%)	Fluorescence lifetime τ^a (ns)
perfluorohexane	383	438	3279	68	4.1
acetone	417	515	4563	78	5.6
PEG400	425	530	4661	68	4.9
unimer micelles in distilled water	425	530	4661	50	4.6 ^b
distilled water	431	541	4718	14 ^c	1.9
saline solution	431	541	4718	13 ^c	3.5

^a The corresponding lifetime decays are reported in Figure 1.5. Apart from C153 in acetone and perfluorohexane, the τ have been obtained by bi-exponential fitting and reported as the average lifetime: $\langle\tau\rangle = \frac{B_1\tau_1^2 + B_2\tau_2^2}{B_1\tau_1 + B_2\tau_2}$

^b The value is in line with the one obtained for C153-loaded unimer micelles in saline solution, that amounts to 4.4 ns.

^c The quantum yield value is in good agreement with the one for C153 in water reported in [50].

1.3.2 Fluorescence anisotropy study

Fluorescence anisotropy (r) is a powerful tool for investigating changes in the local environment when a small fluorescent molecule is embedded in a nanocarrier. Technical information concerning measurements of r is reported in Section T.2. Fluorescence anisotropy is related to the rotational correlation time (τ_c) and the fluorescence lifetime (τ) through the Perrin equation:

$$r = \frac{r_0}{1 + \frac{\tau}{\tau_c}} \quad (1.1)$$

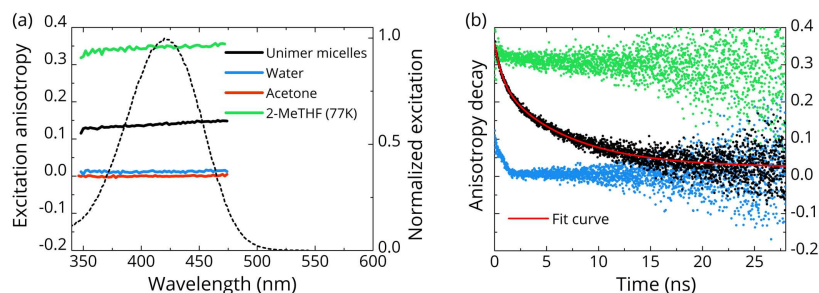
where r_0 is the intrinsic anisotropy of the dye that is measured in the absence of other depolarization sources, such as diffusion, scattering, and energy transfer.[52] The intrinsic anisotropy r_0 of C153 was measured in 2-MeTHF transparent glass at 77 K and resulted equal to 0.35 (Figure 1.7a), very close to the limiting value of anisotropy 0.4, in agreement with the literature data.[53] In non-viscous solvents such as water or acetone, the rotational correlation time is much shorter than the emission lifetime of C153 and, according to Equation 1.1, the Brownian motions of the molecule depolarize molecular fluorescence, resulting in anisotropy values close to zero. The anisotropy measured from the C153-loaded unimer micelle solution amounts to ~ 0.14 (Figure 1.7a), confirming that C153 is embedded in the micelles, which have a correlation time much higher with respect to the molecule due to their bigger dimensions. However, the anisotropy value of C153 measured from unimer micelle suspension is affected not only by the motion of the micelles but also by the motion of C153 within the micelles, and the separation of these two contributions is not trivial. From Equation 1.1, we calculated an effective orientational correlation time of 3 ns for C153 embedded in unimer micelles. The τ_c of a particle is defined as the time needed for a

rotation of 1 radian and it is related to the hydrodynamic volume (V_h) by the Stokes-Einstein equation for rotational diffusion:[42, 54]

$$\tau_c = \frac{\eta V_h}{k_b T} \quad (1.2)$$

where η is the medium viscosity, T is the temperature, and k_b is the Boltzmann constant. Equation 1.2 enables the estimation of the correlation time associated with the hydrodynamic diameter determined by DLS measurements (5 nm), which amounts to 16 ns when assuming a perfect spherical shape for the nanoassemblies. As expected, the rotational correlation time obtained by steady-state fluorescence anisotropy (3 ns) is lower than the rotational correlation time derived from DLS. This discrepancy arises because the former is strongly affected by the internal motion of the dyes loaded in the nanoassemblies, which further contributes to the depolarization of the emitted light and leads to an underestimation of the calculated τ_c .

Figure 1.7: Panel(a) Steady-state excitation anisotropy of C153 in different environments ($\lambda_{em} = 550$ nm). Panel(b) Time-resolved anisotropies of C153 in different environments, measured by exciting the sample at 405 nm and detecting emission at 500 nm (2-MeTHF at 77 K and unimer micelles) or 515 nm (water). The red solid line represents the best fitting of Equation 1.3 to the experimental fluorescence anisotropy decay obtained from C153-loaded unimer micelles.



Additional information concerning the rotational motion of the fluorescent probe can be extracted from time-resolved anisotropy decays reported in Figure 1.7b. In the case of C153 in vitrified 2-MeTHF at 77 K, the r value starts at 0.35 and remains constant during the entire fluorophore decay (up to ~ 20 ns), as expected due to the hindered rotational diffusion in this matrix. The anisotropy decay of C153 in water starts from a low value (~ 0.1) and rapidly decreases, pointing to fast depolarization even before the earliest accessible time with our experimental setup. When the fluorescent probe is loaded in the unimer micelles, the rotational motion is partially hindered (if compared to C153 in liquid solution), and the resulting anisotropy decay reaches a steady value slightly higher than zero ($r = 0.02$) after ~ 20 ns. This residual anisotropy (r_∞) suggests that the rotational diffusion angular range of C153 in its local environment is limited.[52]

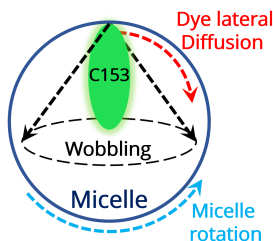


Figure 1.8: Scheme representing the three independent motions taken into account in the fitting model: dye translational diffusion along the spherical surface of the micelle, wobbling of the dye molecule in a restricted region (assumed for simplicity as a cone), and rotation of the micelle as a whole.[42]

Multiple models can be considered to describe the reorientation dynamics of a fluorophore within a micelle, according the number of exponentials needed to fit the anisotropy decay. Since the experimental anisotropy decay measured for the C153-loaded unimer micelles suspension is bi-exponential, we selected a model that accounts for restricted rotational motion (wobbling-in-cone model) and translational diffusion of the dye coupled with the rotation of the whole micelle (see Figure 1.8).[42] We assumed that only the encapsulated C153 contributes to the measured anisotropy, since C153 in water has both scarce solubility and low quantum yield. The following bi-exponential function was employed to describe the experimental anisotropy decay (Figure 1.7b, red line):

$$r(t) = (r_0 - r_\infty) \left(\beta e^{-\frac{t}{\tau_{\text{slow}}}} + (1 - \beta) e^{-\frac{t}{\tau_{\text{fast}}}} \right) + r_\infty \quad (1.3)$$

where τ_{slow} and τ_{fast} are time constants associated with the three kind of motions described before (wobbling, dye lateral diffusion and micelle rotation), all contributing to the orientational diffusion of the fluorescent probe. The parameter β represents the fractional contribution of the slower motion. The specific time constants associated to the three depolarization sources (Figure 1.8), that could be extracted from τ_{slow} and τ_{fast} ,^[42] are beyond the scope of this work.⁶

In order to obtain all the parameters in Equation 1.3, we simultaneously fitted the parallel and perpendicular polarized fluorescence decays (Equation 1.4 and Equation 1.5, respectively).

$$I_{\parallel}(t) = \frac{1}{3} I(t) [1 + 2r(t)] \quad (1.4)$$

$$I_{\perp}(t) = \frac{1}{3} I(t) [1 - r(t)] \quad (1.5)$$

where $r(t)$ is the anisotropy decay (described in Equation 1.3) and $I(t)$ is the total fluorescence intensity decay associated with the time constant reported in Table 1.2 for the C153-loaded unimer micelles suspension. The curves resulting from the fitting are presented in Figure 1.9, along with the weighted residual distributions. The optimized fitting parameters are listed in Table 1.3. Prior to commencing the fitting procedures, the parallel decay has been corrected by the G factor of our experimental set-up. Besides to visually assessing the randomness of the residual distributions and calculating the corresponding χ^2 , we employed an additional criterion to validate the quality of the fit: ensuring the agreement between the experimental value of steady-state anisotropy (r_{ss} , reported in Figure 1.7a) and the value calculated from the optimized fitting parameters with the following equation:^[42]

$$r_{ss} = r_0 \left[\beta \frac{\tau_{\text{slow}}}{\langle \tau \rangle + \tau_{\text{slow}}} + (1 - \beta) \frac{\tau_{\text{fast}}}{\langle \tau \rangle + \tau_{\text{fast}}} \right] \quad (1.6)$$

The resulting r_{ss} of 0.158 is in line with the experimental value (0.14). The larger time constant ($\tau_{\text{slow}} = 6.9$ ns) extracted from the fitting procedure can be assigned to the orientational diffusion of the nanocarrier as a whole, together with the lateral diffusion of the encapsulated dye along the curved surface of the micelle. The presence of the latter fluorescence depolarization mechanism justifies why τ_{slow} is significantly lower than the expected correlation time for the unimer micelles, obtained from Equation 1.2 and DLS results (16 ns). The shorter time constant ($\tau_{\text{fast}} = 0.89$ ns) is mainly associated with the restricted wobbling motion of C153 in its local environment, with contributions from both the orientational diffusion of the whole micelle and, once again, the translational diffusion of the dye.

We underline that the time constants resulting from the fitting procedure are effective quantities since the employed model function assumed the unimer micelles to be spherical, without accounting for their prolate shape.^[48]

6: Relations between the time constants of the fitting model:

$$\frac{1}{\tau_{\text{slow}}} = \frac{1}{\tau_M} + \frac{1}{\tau_D}$$

$$\frac{1}{\tau_{\text{fast}}} = \frac{1}{\tau_W} + \frac{1}{\tau_M} + \frac{1}{\tau_D}$$

where τ_M , τ_D and τ_W are the time constants associated to the rotations of the whole micelle, lateral diffusion of the dye and restricted wobbling motion of the dye, respectively.

Table 1.3: Optimized parameters obtained from the simultaneous fitting of parallel and perpendicular fluorescence decays.

	β	τ_{fast} (ns)	τ_{slow} (ns)
Fitting results	0.69	0.89	6.9
	r_0	r_∞	
Fitting results	0.33	0.02	

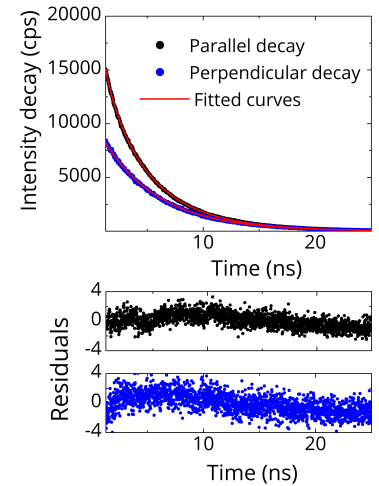


Figure 1.9: Above: Parallel and perpendicular fluorescence decay profiles of C153-loaded unimer micelles, measured by exciting the sample at 405 nm and detecting emission at 500 nm. The corresponding fitting curves are shown as overlaid red lines, while the weighted residual distributions are reported in the two panels below. The estimated values of χ^2 for the fitting of the parallel and perpendicular components are 0.99 and 1.86, respectively.

1.3.3 Solvatochromic study

Further information about the dye encapsulation can be retrieved from absorption and emission spectra of C153 collected in different solvents. C153 is a solvatochromic dye, and both the emission and absorption spectra shift to longer wavelengths when the polarity of the medium is increased, as a consequence of an intramolecular charge transfer transition.[55, 56] Figure 1.10 compares absorption and emission spectra of C153-loaded unimer micelles with spectra of C153 in perfluorohexane, acetone, PEG400, and water (see Table 1.2 for the corresponding Stokes shifts). Perfluorohexane is the nonpolar solvent that better mimics the hydrophobic core of the unimer micelles (FA units). Acetone is the polar organic solvent in which C153 is co-solubilized with the copolymer for the preparation of unimer micelles by re-hydration, while PEG400 was selected to mimic the oxyethylenic side chains of the copolymer that are located in the outer shell of the unimer micelles, at the interface with water.

Figure 1.10: Panel(a) Comparison between normalized absorption and emission spectra of C153 in different environments (the absorption spectrum in water is not reported for clarity due to the low signal/noise ratio). The emission spectra have been acquired using an excitation wavelength of 420 nm for acetone, PEG400, water and for the suspension of unimer micelles, and 380 nm for the perfluorohexane solution. Panel(b) Schematic representation of the self-folding mechanism of a PEGMA90-co-FA10 copolymer chain in water in the presence of C153 dye.

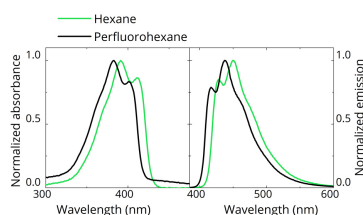
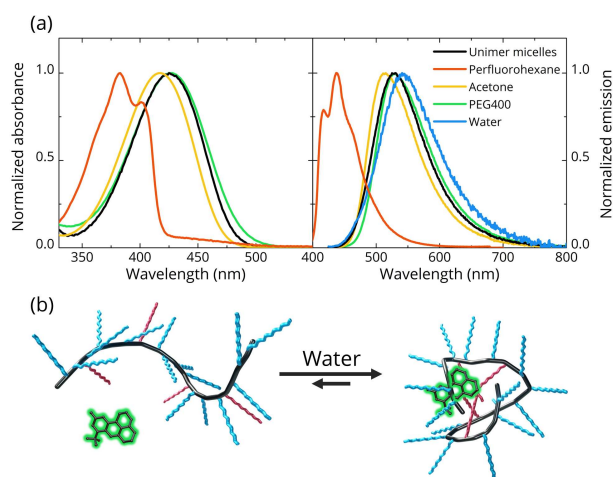


Figure 1.11: Comparison between normalized absorption and emission spectra of C153 in hexane (green lines) and perfluorohexane (black lines). The two solvents have similar dielectric constants ($\epsilon_{\text{PerfluoroHexane}} = 1.76$, $\epsilon_{\text{Hexane}} = 1.89$) but different refractive indices ($n_{\text{PerfluoroHexane}} = 1.25$, $n_{\text{Hexane}} = 1.38$). The observed spectral shifts are mainly attributed to the different refractive indices, and the solvent with the lower refractive index corresponds to blue-shifted bandshapes.

The emission and absorption profiles of C153 loaded in the unimer micelles (black lines in Figure 1.10) are similar to the ones collected in pure PEG400, and their spectral position is intermediate between those in acetone and water. The blue-shift observed in the nanoassemblies suspension in comparison to water confirms the encapsulation of the fluorescent dye in the micellar system. These results indicate that when C153 is loaded in the micelles is located in a less polar environment than water, but still quite polar being it comparable to that of non-hydrated PEG. The spectra collected in pure PEG should correspond to a less polar environment compared to the hydrated PEG chains typical of the micelles corona, since the dielectric constant (ϵ) is expected to be higher in the presence of water. However, the spectral position is also affected by the refractive index (n) of the medium, and the PEG/water mixtures are known for their lower values of n when compared to pure PEG.[57] The decrease of the medium refractive index leads to a blue shift of the absorption and emission bandshapes (see Figure 1.11), as opposed to the effects due to an increase of ϵ .

To sum up, unimer micelles are very complex systems in terms of local polarity and the precise localization of C153 is difficult to assess. In fact, although during the single-chain folding the perfluoroalkyl chains

tend to be buried in the core of the unimer micelles,[45] the existence of conformational and compositional constraints make their location in the inner compartment preferential, but not exclusive and PEG segments can also be present. The problem is further complicated by the refractive index of the local environment, which is difficult to predict and can play an important role in the absorption and emission spectral positions of the embedded C153.

In order to check if the preparation procedure can impact on the C153 location in the nanocarrier, we added a concentrated solution of C153 in acetone to a water suspension of self-assembled unimer micelles, and we obtained superimposable UV-Vis and emission spectra to those prepared by re-hydration, suggesting that the environment of C153 is not changed (the corresponding DLS results are reported in Table 1.1 and Figure 1.3, while the absorption and emission spectra are shown in Figure 1.12).

1.4 Bioimaging experiments in porcine sclera

Fluorescence microscopy techniques reconstruct the image of a sample through the detection of fluorescence signals. Fluorescence can be emitted by the sample itself, due to the presence of endogenous fluorophores (autofluorescence), or by a fluorescent probe which is added to the sample through a staining procedure. One of the major issues concerning the use of fluorescent probes is their compatibility with the biological environment, i.e., the solubility, the preservation of their emissive properties in aqueous media and their cytotoxic behavior.

After the careful spectroscopic and physico-chemical characterization of the C153-loaded unimer micelles, we tested their efficacy as fluorescent nanoprobes for two-photon microscopy (TPM). TPM is a powerful imaging technique that allows in-depth imaging, up to 1-2 mm, of biological tissues,[58] and hence requires an efficient permeation of the fluorescent probes. Additional technical information about the technique and our TPM setup are reported in Appendix A and Section T.4.

Ex vivo porcine scleral tissue was selected as testing biological material, being a robust recognized animal model for the study of the trans-scleral diffusion of drug addressing the posterior segment of the eye (see Section T.3 for details on *ex vivo* permeation experiments). Porcine sclera, similar to the human one, is mainly composed of randomly packed collagen fibers embedded in a proteoglycan matrix and traversed by elastic fibers and fibroblasts; the water content is approximately 70%.[59] Apart from its crucial physiological and mechanical functions, this tissue was selected to test the C153-loaded unimer micelles due to its structural simplicity and the absence of a strong autofluorescence signal. In fact, collagen fibers are detected owing to their intrinsic second harmonic generation (SHG) signal, which falls exactly at half of the exciting wavelength (i.e. in the blue channel when exciting at 830 nm), while the emission of C153 and the weak tissue autofluorescence are visualized in the green channel (See Appendix A for a detailed description of the blank tissue). A superposition of the three channels spectral ranges together with the spectrum profile of collagen SHG at 830 nm and the emission of C153-loaded unimer micelles in water is reported in Figure 1.13.

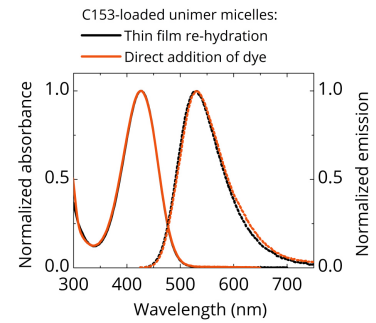


Figure 1.12: Comparison between normalized absorption and emission spectra of C153-loaded unimer micelles in saline solution (obtained with the thin film re-hydration methodology, total dye concentration: 15 μ M) and in distilled water (obtained by the direct addition procedure, total dye concentration: 30 μ M). Both suspensions are characterized by a polymer concentration of 5 g/L.

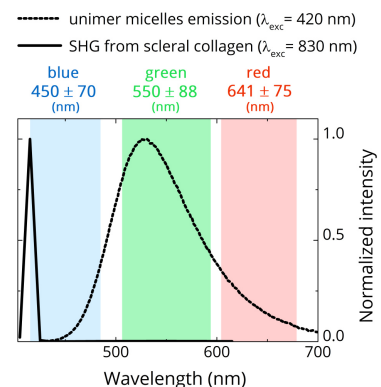


Figure 1.13: Spectral ranges of the three GaAsP detectors used to acquire the TPM images. The SHG signal from the scleral collagen and the emission of the unimer micelles loaded with C153 are also shown (continuous and dashed black lines, respectively). The excitation wavelength is reported in the legend.

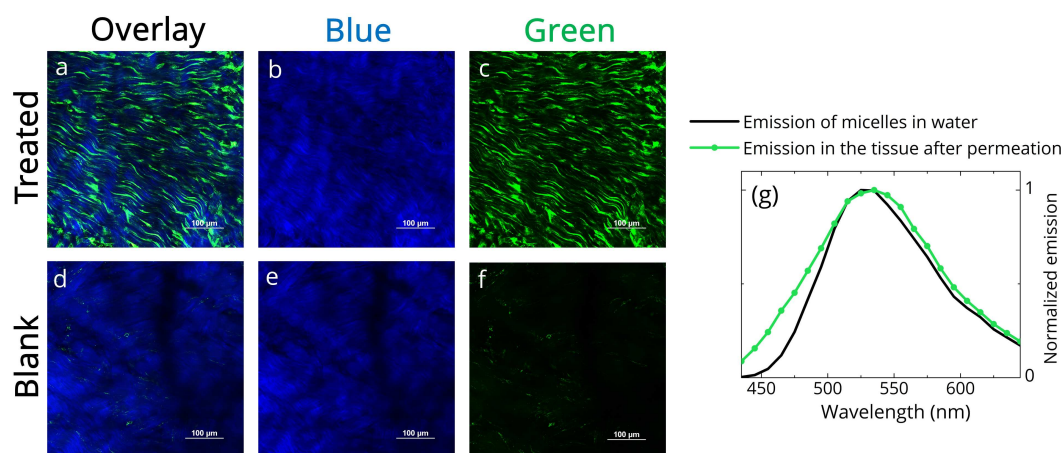


Figure 1.14: Panels(a-c) Sclera treated with unimer micelles in saline solution loaded with C153 (50 μm from the surface): (a) channels overlay, (b) blue channel, (c) green channel. Panel(d-f) Sclera treated with saline solution: (d) channels overlay, (e) blue channel, and (f) green channel. All the images were acquired exciting the sample at 830 nm and using the same detector gains. Panel(g) Comparison between the emission spectrum (excitation wavelength: 830 nm) acquired in correspondence with panel(a) focal plane and the emission spectrum of an aqueous suspension of unimer micelles loaded with C153. More images acquired from the stained and blank tissues are reported in Figure 1.16, together with the corresponding emission spectra.

Figure 1.14a-c presents TPM images of sclera after a 2-hours treatment with the C153-loaded unimer micelles suspension (for comparison, Figure 1.14d-f displays TPM images of non-stained, plain sclera). The strong signal detected in the green channel (Figure 1.14c) is mainly attributed to the emission of C153, as confirmed by the emission spectrum collected from the tissue (green line in Figure 1.14g), which is almost superimposable to the emission acquired from the C153-loaded unimer micelles in aqueous suspension (black line in Figure 1.14g). The comparison between the two aforementioned spectra suggests that the local environment of the dye is not significantly affected during the permeation, at least up to 50 μm of depth from the tissue surface. In Figure 1.16 are reported images (together with the corresponding emission spectra, Figure 1.16i) collected at different depths from both treated and blank porcine sclera. The spectral bandshapes that were measured from the treated sample corresponds to C153 fluorescence and are completely different from the autofluorescence of the blank tissue, demonstrating that the dye reached a depth up to at least 150 μm inside the sclera. The slight red-shift in the treated sclera emission profiles (Figure 1.16i, top panel), when recorded deeper in the tissue, can be mainly attributed to a stronger contribution from the autofluorescence signal (Figure 1.16i, bottom panel).

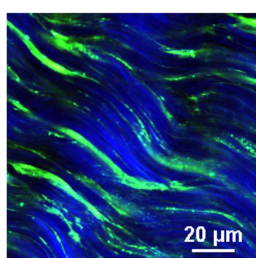


Figure 1.15: Channels overlay of a zoomed region of the tissue after treatment with C153-loaded unimer micelles (image size: 120 μm × 120 μm)

Unimer micelles permeate the sclera through the interfibrillar spaces since the signal from C153 arises mainly from the pores within the bundles of collagen fibers (see Figure 1.15), in a similar fashion to what we described for tocopherol polyethylene glycol 1000 succinate (TPGS) micelles (Section 4.2.1 and Section 2.5).[60] In order to have a complete overview of the dye distribution inside the tissue, a Z-scan was acquired on the stained sclera (Figure 1.17) and, for comparison, on the plain untreated sclera (Figure 1.20c,d). The efficiency of unimer micelles as nanocarriers for the fluorescent probe C153 is demonstrated by the dye distribution within the tissue: an intense signal from C153 is detected in the green channel up to 150 μm in depth, as already confirmed by the emission spectra reported in Figure 1.16i.

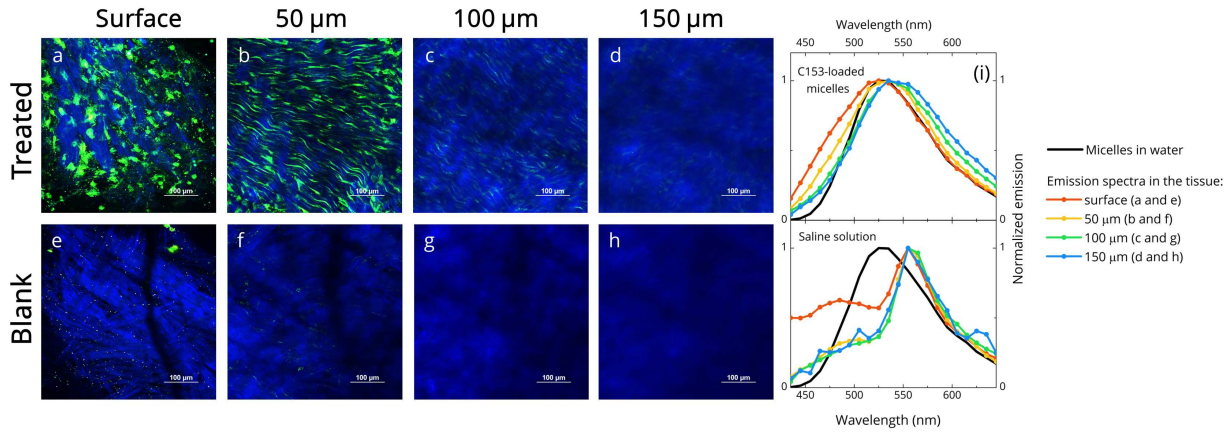


Figure 1.16: Panels(a-d): Sclera treated with unimer micelles in saline solution loaded with C153. (a) Surface, (b) 50 μm below the surface, (c) 100 μm below the surface, (d) 150 μm below the surface. Panels(e-h): Sclera treated with saline solution: (e) Surface, (f) 50 μm below the surface, (g) 100 μm below the surface, (h) 150 μm below the surface. The emission spectra collected from the focal planes of the images in panels a-h are reported in panel i. All the images and spectra have been recorded with an excitation wavelength of 830 nm. The images of the two tissues (when at the same depth) have been recorded with the same detector gains.

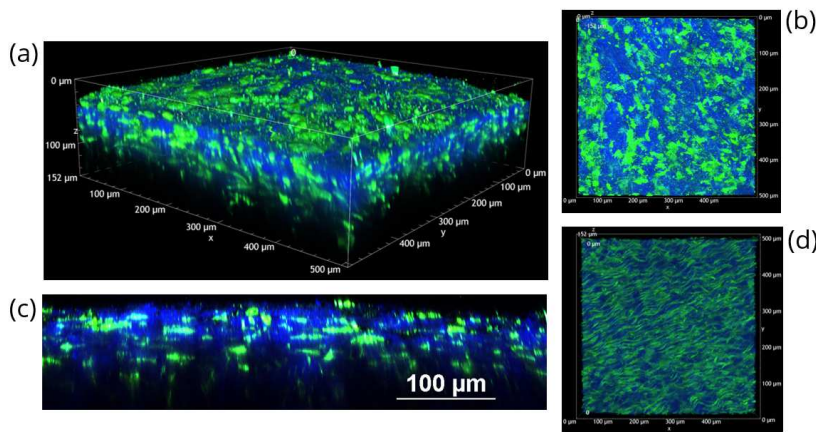


Figure 1.17: Panels(a-d) Volume rendering of porcine sclera treated with unimer micelles in saline solution loaded with C153, reconstructed from a Z-stack (Z-step: 1 μm , total depth: 152 μm): (a) 3D overview, (b) XY view, (c) XZ slice (512 $\mu\text{m} \times 152 \mu\text{m}$), and (d) -XY view. All images are acquired exciting the sample at 830 nm and using the same detector gains.

A control experiment was performed by treating the sclera with a solution of C153 in water⁷ (see Figure 1.18 for the corresponding absorption spectrum) in the absence of unimer micelles. Images are reported in Figure 1.20c-d: adopting the same experimental conditions, the signal from a solution of C153 in water is negligible in the absence of nanocarriers. This control confirms the positive role of unimer micelles as nanocarriers to promote the solubilization of a hydrophobic dye and its permeation across the biological tissue, as well as to preserve the emissive properties of the embedded fluorophore.

All the signals detected from the scleral tissue decreases rapidly at depths higher than 100 μm (Figure 1.17c) when the power of the excitation beam is kept constant, mainly due to scattering. However, the fluorescence signal of C153 is detected also from the opposite side of the sample, the choroidal interface (Figure 1.21), suggesting that C153 permeates through the whole sample in a relatively short time.⁸ The signal in the green channel is in fact much higher in the sample treated with the dye-loaded unimer micelles when compared to the blank one. Figure 1.19 reveals that, as C153 reaches this region of the tissue, the fluorophore tends to accumulate inside scleral fibroblasts. This is in contrast to the results obtained from the external side of the sclera, where the dye is primarily

⁷: In the absence of the nanocarriers the amount of dye solubilized in water decrease dramatically:

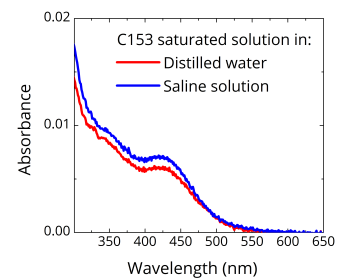


Figure 1.18: Comparison between the absorption spectra of distilled water and saline solution saturated with C153.

⁸: In order to study the choroidal side, the tissue has been flipped over to maximize the signal during the measurements.

localized in the aqueous interfibrillar pores.

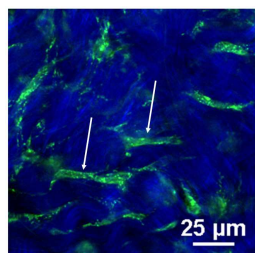


Figure 1.19: Channels overlay of a zoomed region of the tissue choroidal side after treatment with C153-loaded unimer micelles (image size: $150\ \mu\text{m} \times 150\ \mu\text{m}$). The fibroblasts labelling is highlighted by white arrows.

The emission spectra reported in Figure 1.21g confirm the presence of C153 in the choroidal side, but the convolution between the dye emission and the tissue autofluorescence is significant, because only a small amount of nanocarriers was able to fully cross the tissue during the permeation experiment. After the permeation experiments, emission spectra were recorded on the acceptor solutions of the Franz-cells, to assess if the signal of C153 could be detected, but no significant differences have been observed between the receiving solutions in contact with the blank and the stained tissues.

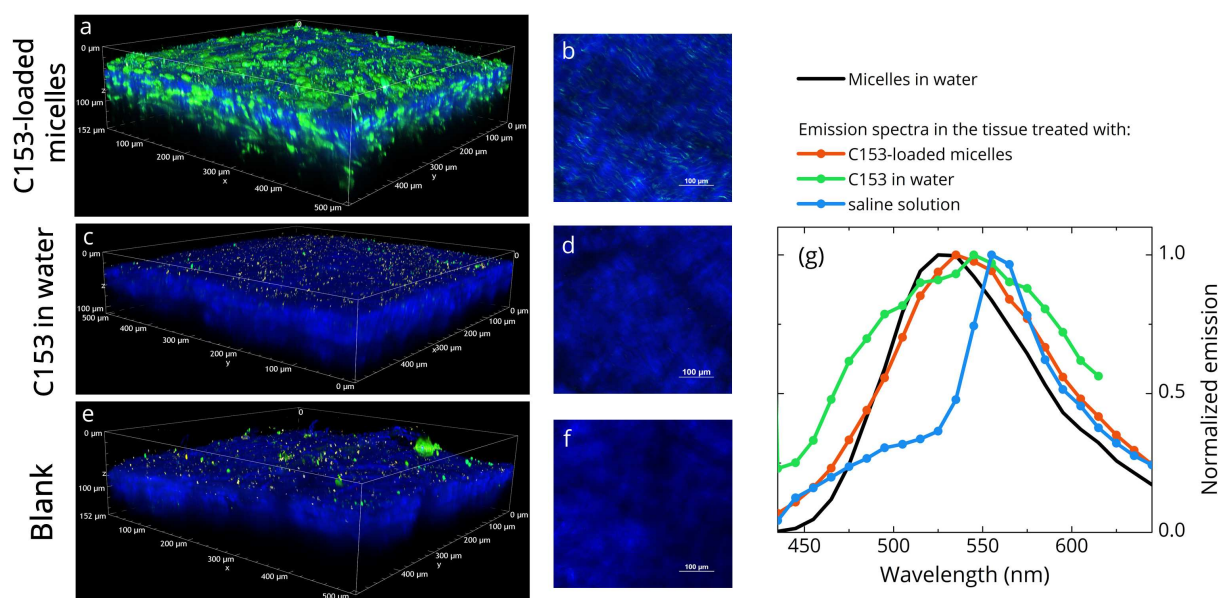


Figure 1.20: Comparison between Z-scans and images (acquired $100\ \mu\text{m}$ from the surface) of: sclera treated with C153-loaded unimer micelles [panels(a-b)], sclera treated with C153 in distilled water [panels(c-d)], and sclera treated with saline solution [panels(e-f)]. The emission spectra corresponding to the images in panels (b), (d), and (f) are reported in panel(g) together with the emission of an aqueous suspension of unimer micelles loaded with C153 (excitation wavelength: $830\ \text{nm}$).

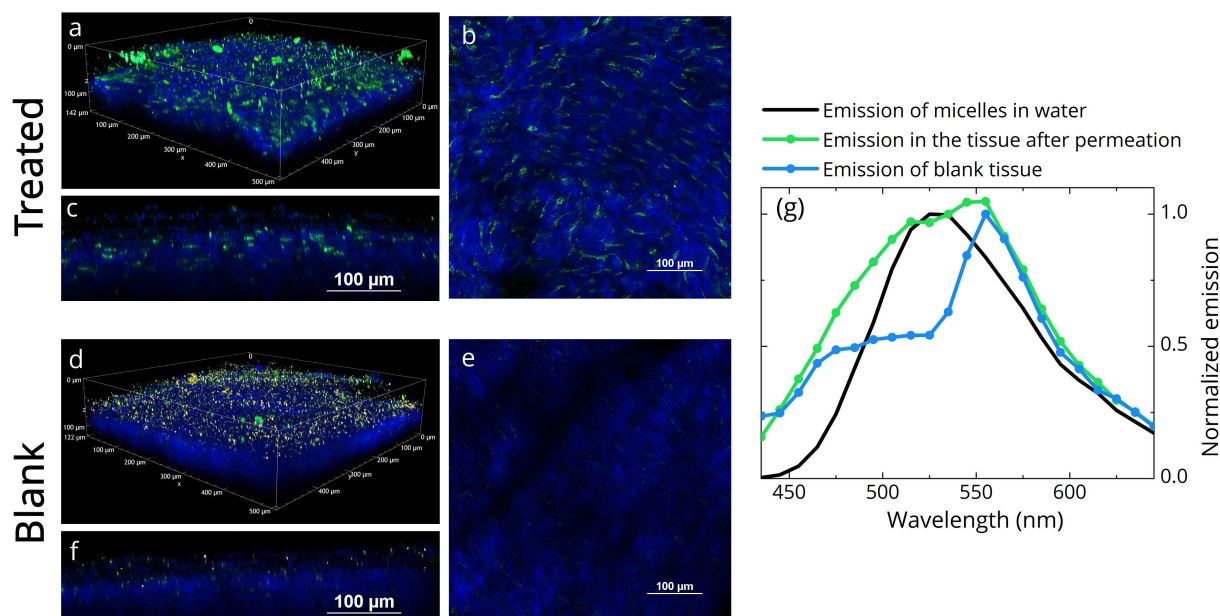


Figure 1.21: Volume renderings of sclera (choroid side) reconstructed from Z-stacks. Panels(a-c): Sclera treated with C153-loaded unimer micelles (Z-step: $1\ \mu\text{m}$, total depth: $142\ \mu\text{m}$): (a) 3D overview, (b) XY image acquired $50\ \mu\text{m}$ below the surface, (c) XZ slice ($512\ \mu\text{m} \times 142\ \mu\text{m}$). Panels(d-f): Sclera treated with saline solution (Z-step: $1\ \mu\text{m}$, total depth: $122\ \mu\text{m}$): (d) 3D overview, (e) XY image acquired $50\ \mu\text{m}$ below the surface, (f) XZ slice ($512\ \mu\text{m} \times 122\ \mu\text{m}$). All the images have been acquired exciting the sample at $830\ \text{nm}$ and using the same detector gains. Panel(g): Comparison between the emission spectra acquired in correspondence with image (b) and (e) focal planes and the emission of an aqueous suspension of unimer micelles loaded with C153 (excitation wavelength: $830\ \text{nm}$).

1.5 Cytotoxicity study

Since the unimer micelles were intended for bioimaging following topical application, their cytotoxicity profile *in vitro* was assessed by Prof. Annalisa Bianchera (Food and Drug Department, University of Parma). Particularly, HEK 293 and A549 cell lines, commonly used for cytotoxicity assessment, were chosen. After 3 hours of contact with PEGMA90-co-FA10, the MTT assay showed that cell viability was over 80% for concentrations up to $1.25\ \text{g/L}$ in both cell lines, as reported in Figure 1.22.

By increasing the polymer concentration up to $10\ \text{g/L}$, viability slightly decreased but never below 60% with respect to the control. Results presented here are in agreement with previously collected data, showing the non-cytotoxic character of structurally similar PEGylated/fluoroalkyl (meth)acrylic polymers toward Balb/3T3 clone A31 cells[49] and NIH 3T3 mouse embryonic fibroblast cells and human umbilical vein endothelial cells (HUVECs),[61] respectively.

Bearing in mind the safety profile of the PEGMA90-co-FA10, a precautionary concentration of the polymer should be set below $1.25\ \text{g/L}$. This concentration is compatible with the preparation of C153-loaded unimer micelles having the required spectroscopic properties for working as an efficient fluorescent probe.

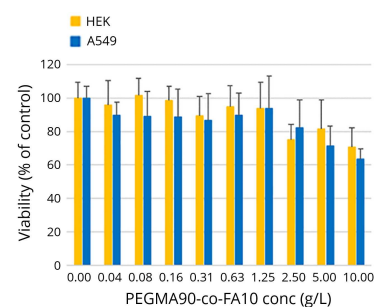


Figure 1.22: Cell viability of HEK (yellow) and A549 (blue) cultured with several concentrations of PEGMA90-co-FA10 for 3 h.

1.6 Conclusions and perspectives

In this chapter, we detailed the preparation, characterization, and application of C153-loaded PEGMA90-co-FA10 unimer micelles as fluorescent probes for bioimaging in topical administration. The successful preparation of dye-loaded micelles has been achieved through thin-film rehydration, obtaining a suspension of fluorescent unimer micelles with a straightforward procedure. A second simple preparation method, concerning direct addition of C153 in acetone to an aqueous suspension of self-assembled unimer micelles, has also been tested without observing any significant difference in the physico-chemical characterization.

The effective encapsulation of the C153 dye within the nanoassemblies was demonstrated through UV–Vis spectroscopy and fluorescence anisotropy, revealing distinct responses for the dye in water in the presence and absence of the polymer. Solvatochromic studies indicated that the dye is located in an environment less polar than water, yet still relatively polar and similar to non-hydrated PEG. Comparative analysis of absorptions, emissions and lifetimes of C153 in organic solvents, water, and water suspensions of unimer micelles confirmed the positive role of the polymeric nanocarriers in solubilizing C153 in water, while preserving its good emissive properties.

Two-photon microscopy analysis after the permeation in porcine sclera, demonstrated how C153-loaded unimer micelles can be visualized deep inside thick biological tissues. This study revealed that unimer micelles are particularly promising for *ex vivo* experiments in topical administration (such as eye and skin tissues), where the probe penetration into the tissue is a critical issue. The extremely small size of the nanoassemblies and their inherent structural adaptability can in fact facilitate the transport of hydrophobic dyes or drugs to deeper regions within the sample. Additionally, the same system holds potential interest for *in vitro* experiments with cell cultures. Cytotoxicity studies proved the biocompatibility of the nanomaterial for bioimaging purposes.

To the best of our knowledge, this study represents the first attempt to employ non-covalently crosslinked, self-folded unimer micelles as nanocarriers for fluorescent dyes in context of bioimaging. The work discussed in this chapter stands as proof of concept of the ability of unimer micelles to efficiently encapsulate small hydrophobic organic molecules, with potential applications spanning various areas of biomedical sciences.

The majority of the results presented in this chapter have been published in Ref. [62].

1.7 Materials and Methods

Coumarin153 was purchased from Sigma-Aldrich. Polyethylene glycol 400 (PEG400) was of pharmaceutical quality according to Ph.Eur.-USP. All the purchased chemicals have been employed without further purification.

1.7.1 Preparation of unimer micelles suspension

The dye-loaded unimer micelles employed for most of the experiments discussed in Chapter 1 were prepared adopting a thin-film re-hydration procedure (schematized in Figure 1.23). The copolymer PEGMA90-co-FA10 and the dye C153 were first dissolved in acetone. The solvent was then completely removed under vacuum, obtaining a thin film. An appropriate volume of distilled water or saline solution was added to obtain the final concentrations of 5 g/L ($\sim 238 \mu\text{M}$) and $15 \mu\text{M}$ for PEGMA90-co-FA10 and C153, respectively.

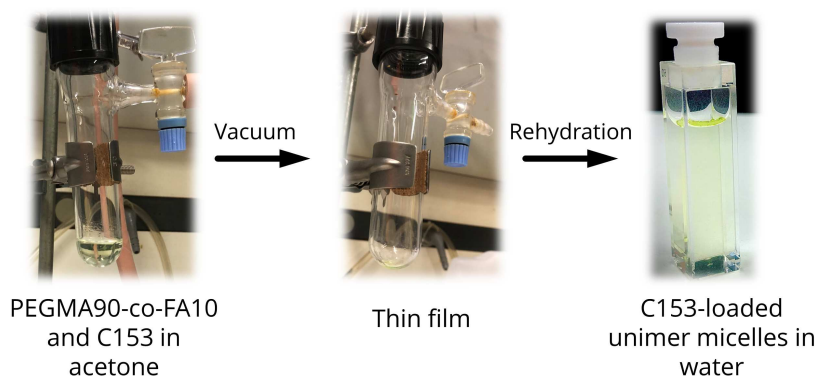
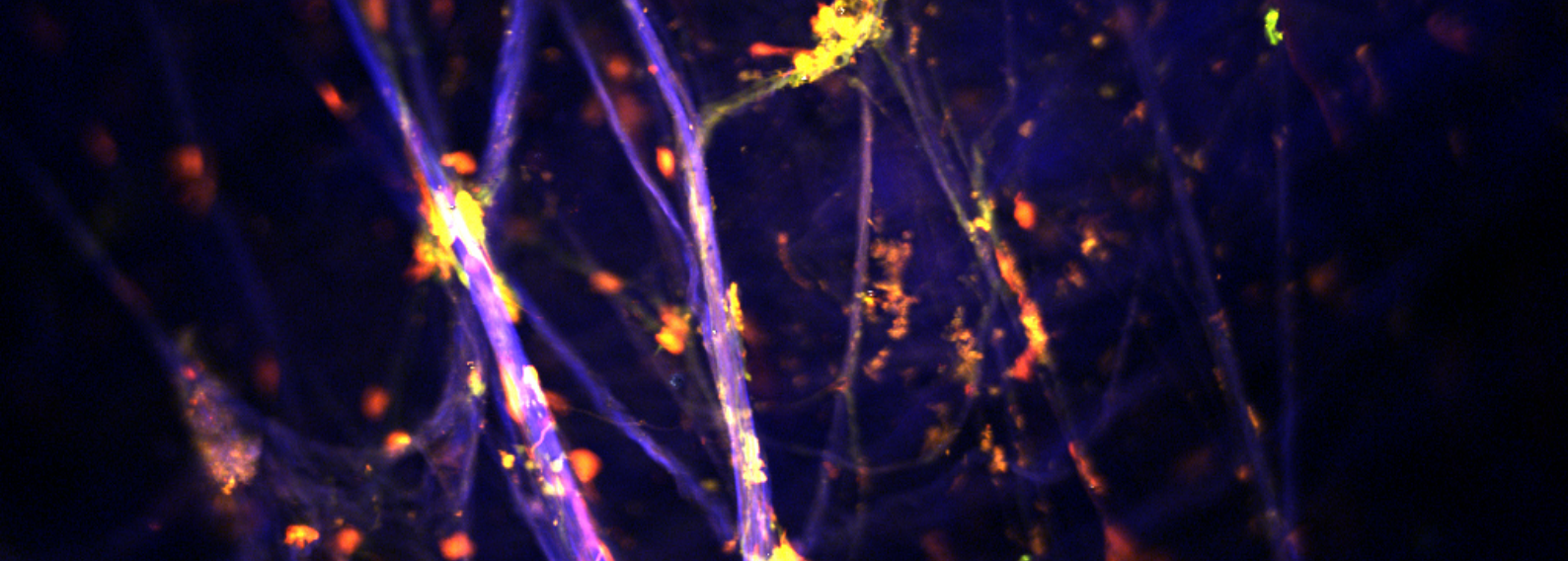


Figure 1.23: Thin film re-hydration procedure employed for the preparation of C153-loaded unimer micelles. After the re-hydration step, the suspension has been kept under magnetic stirring (1 h) and then filtered (hydrophilic PTFE, AISIMO $0.22 \mu\text{m}$).

A different preparation procedure of dye-loaded unimer micelles (direct addition) was tested for some control experiments: a small amount of a concentrated C153 solution in acetone was added (final acetone concentration in the suspension: $\sim 0.1\% \text{ v/v}$) to a suspension of PEGMA90-co-FA10 in water (5 g/L), obtaining a final C153 concentration of 5 or $30 \mu\text{M}$. All the obtained polymeric suspensions (prepared with the two procedures) were magnetically stirred for 1 h and then filtered (hydrophilic PTFE, AISIMO $0.22 \mu\text{m}$).

The control solution of C153 in distilled water employed for permeation experiments was obtained following the thin-film re-hydration procedure without adding the polymer. In the case of the control solution, the total theoretical concentration of C153 was $30 \mu\text{M}$, but the final concentration after the filtration (hydrophilic PTFE, AISIMO $0.22 \mu\text{m}$) is limited by the low water solubility of the dye.

The C153-saturated solutions in distilled water or saline solution used for spectroscopic measurements were prepared by adding 0.5 g/L of C153 to the solvent, magnetically stirring for 1 h, and then filtering the resulting suspension (hydrophilic PTFE, AISIMO $0.22 \mu\text{m}$).



2 Förster resonance energy transfer in CTAB & TPGS micelles: a comparison

2.1 Introduction

Förster Resonance Energy Transfer (FRET) is a well-known process where an excited dye, the energy donor, transfers its energy to another dye, the energy acceptor. This incoherent process relies on intermolecular electrostatic interactions and typically occurs within distances ranging from 1 to 10 nm.[52] A critical prerequisite for FRET is a sizable spectral overlap between the emission spectrum of the donor molecule and the absorption band of the acceptor dye. FRET governs many natural phenomena, and specifically it is at the heart of photosynthesis.[63, 64] It is widely exploited in biomedical studies, where the large dependence of the FRET efficiency on the distance between fluorescent energy donor and acceptor dyes is exploited to estimate distances at the nanoscale, making it a useful molecular ruler.[65] FRET also offers the possibility of investigating the stability of NPs in different environments and over time, a useful feature for pharmacokinetics and drug-delivery studies.[66]

In this chapter, we investigate two fluorescent dyes, DiI (1,10-dioctadecyl-3,3',30,30'-tetramethyl-indocarbocyanine perchlorate) and DiD (1,10-dioctadecyl-3,3',30,30'-tetramethyl-indodicarbocyanine perchlorate) (Figure 2.2), belonging to the family of indocarbocyanines, and their encapsulation in CTAB and TPGS micelles. As previously demonstrated in other colloidal systems,[67, 68] these two fluorophores are compatible for FRET, since the emission of DiI (energy donor) overlaps with the absorption of DiD (energy acceptor, see Figure 2.1).[69, 70] DiI and DiD are not soluble in water, and their chromophoric core is decorated with two long alkyl chains composed of eighteen carbon atoms, in order to make their interaction with lipophilic environments favorable. Accordingly, they are commercialized as lipophilic trackers for fluorescence microscopy, since they can be embedded into plasma membranes through hydrophobic

2.1 Introduction	23
2.2 Aggregation of DiI and DiD	26
2.3 CTAB and TPGS micelles in aqueous suspension . .	28
2.4 Spectroscopic properties of dye-loaded micelles	30
2.5 Bioimaging experiments in porcine sclera	39
2.6 Conclusions and perspectives	47
2.7 Materials and Methods . .	49

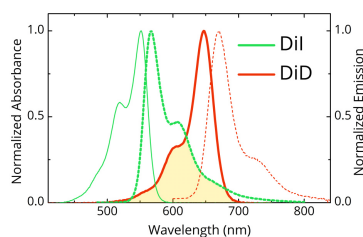
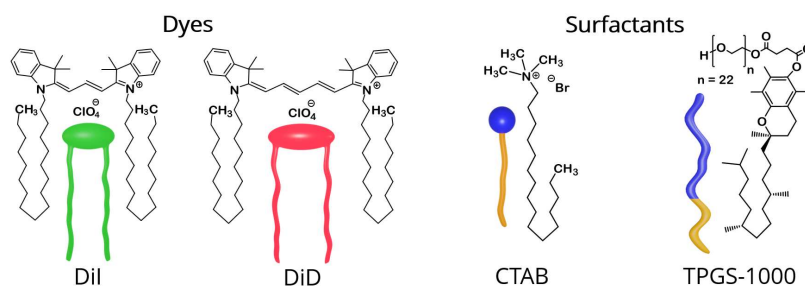


Figure 2.1: Absorption (continuous lines) and emission (dotted lines) normalized spectra of DiI and DiD in ethanol. The spectral overlap between the emission of DiI (energy donor) and the absorption of DiD (energy acceptor) is highlighted in yellow.

interactions with phospholipid bilayers.[71]

For our purposes, the hydrocarbon tails of DiI and DiD are of interest as they favor the interaction of the dye with the hydrophobic moieties of the surfactants CTAB and TPGS. CTAB (cetyltrimethylammonium bromide) is one of the most widely used surfactants in many industrial processes and everyday life.[72] The molecular structure of CTAB is characterized by a permanently positively charged quaternary ammonium polar head and an alkyl chain composed of sixteen carbons (Figure 2.2). TPGS (d- α -tocopheryl polyethylene glycol 1000 succinate) is a non-ionic polymeric surfactant soluble in water. It is a derivative of vitamin E, made up by a PEG 1000 chain linked to vitamin E through a succinate bond (Figure 2.2). TPGS has been used to prepare biocompatible and biodegradable polymeric micelles, employed as multifunctional nanocarriers and permeation enhancers in drug delivery applications.[73, 74] For both surfactants, the formation of micelles can occur only if the amphiphilic molecule concentration is above a specific concentration, known as critical micellar concentration (CMC) and if the temperature is higher than the so-called Kraft point.[75]

Figure 2.2: Molecular structures and schematic representation of the surfactants and dyes discussed in this chapter.



In Section 2.2, the spectroscopic behavior of DiI and DiD is firstly evaluated in aqueous environment: both dyes are not soluble in water, and show a clear tendency towards aggregation, that highly affects their useful spectroscopic properties.[76] We then study the behavior of CTAB and TPGS micelles loaded with DiI or/and DiD. Micellar nanostructures composed of amphiphilic molecules are thermodynamically stable colloidal structures above the CMC, while they can be easily destroyed upon dilution, as shown in Section 2.3. A detailed spectroscopic analysis (Section 2.4) demonstrates a very good affinity of both dyes for the two micellar environments, so that the dyes can be brought in aqueous suspensions without degrading their optical properties as expressed in organic solvents. Aggregation effects of both DiI and DiD, when loaded in micelles, have only marginal effects on absorption and emission spectral bandshapes. Above the CMC, for both TPGS and CTAB micelles loaded simultaneously with DiI and DiD, FRET is observed, suggesting that inside the micelles, intermolecular interactions are large enough to induce FRET, but they are not sufficiently strong to drive aggregation. The most intriguing result however is observed below the CMC: while in the presence of TPGS, FRET efficiency decreases, as expected when the two dyes move far apart, with CTAB an important increase of FRET efficiency is observed below the CMC. This somewhat counterintuitive observation suggests that the affinity of the dyes with CTAB is so large as to allow for the proximity of two dyes even after the disruption of the micelles. The different behavior described for the two micellar systems

upon dilution is also demonstrated in Section 2.5 after their permeation through a biological tissue, analyzed with multiphoton microscopy. Our results prove that a detailed spectroscopic characterization of the FRET nanoparticles is needed in order to reliably assess their integrity.

Part of the work presented in this chapter was performed in collaboration with Dr. Guillem Vargas-Nadal and in the context of Francesco Bertocchi's master thesis.

2.2 Aggregation of DiI and DiD

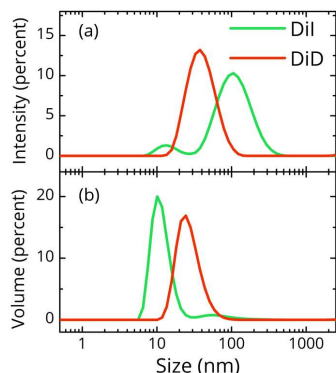


Figure 2.3: Size distributions by intensity [Panel(a)] and volume [Panel(b)] of DiI and DiD aggregates obtained three hours after preparation. These results are the average of three independent measurements.

Table 2.1: DLS data relative to DiI aggregates in water/ethanol 70/30 (*m/m*) and DiD aggregates in water/ethanol 90/10 (*m/m*). PDI indicates the polydispersity index of the suspension. The mean sizes of the peaks refer to the intensity distributions. Measurements were performed three hours after preparation.

Sample	Z-average (nm)	PdI	Peak 1 mean size (nm)	Peak 2 mean size (nm)
DiI aggregates	83.1 ± 0.8	0.461 ± 0.01	120 ± 5.4	14.1 ± 0.5
DiD aggregates	37.8 ± 0.8	0.234 ± 0.01	42.2 ± 1.5	—

and aggregates in water/ethanol mixtures are shown in Figure 2.4. The variations associated to the spectral bandshapes of DiI are consistent with J-aggregation: both absorption and emission are shifted to the red if compared to the dye in ethanol, the Stokes shift is negligible, and the ratio of the 0-1 vs the 0-0 vibronic band decreases.[77, 78] On the opposite, DiD spectra show H-aggregation: the absorption band is broader and blue-shifted compared to the monomer band and fluorescence is suppressed and strongly red-shifted. This behavior is consistent with the observation of a vibronically induced fluorescence from H-aggregates.[79] The formation of emissive J-aggregates and poorly emissive H-aggregates by DiI and DiD, respectively, has been already reported in the literature.[80, 81]

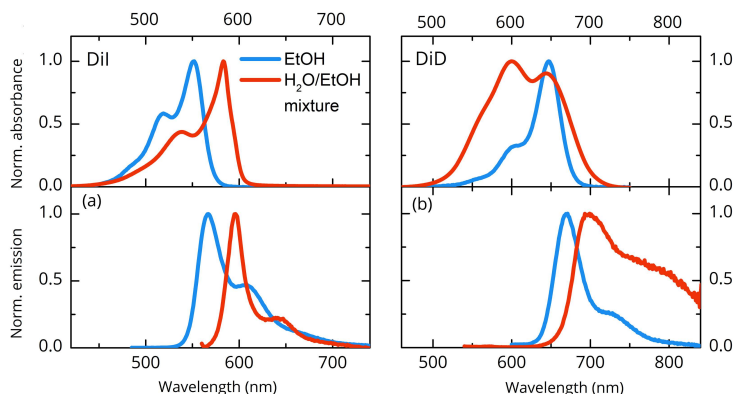


Figure 2.4: Absorptions (top) and emission spectra (bottom) of DiI [Panel(a)] and DiD [Panel(b)] in different environments: ethanol and water/ethanol mixtures. Water/ethanol mixtures with 70/30 and 90/10 (*m/m*) ratios have been employed to prepare DiI and DiD aggregates, respectively. Spectra of aggregates suspensions were collected three hours after preparation.

The good fluorescence of DiI allowed for the visualization of aggregates with via two-photon excited fluorescence (TPEF) imaging (the experi-

mental set-up is described in Section T.4).

Aggregates look very heterogeneous (Figure 2.5): NPs of different size and color were present. However, the continuous motion of the particles made difficult the collection of spectra from individual aggregates.

To slow down the Brownian motions, DiI aggregates were dispersed in an agarose hydrogel (see the Section 2.7.1 for the preparation procedure), a highly viscous medium that hinders the diffusion of NPs during the measurement. In this way, we collected images and spectra from immobilized aggregates. Absorption and emission spectra of DiI in the hydrogel are superimposable with those collected in water/EtOH mixtures (Figure 2.6), confirming that the hydrogel preparation does not significantly impact on the aggregation process.

Figure 2.7 shows a TPEF image of gel-suspended DiI aggregates collected with the two-photon microscope. Aggregates of different sizes and different colors are imaged, in agreement with the images collected on the liquid suspension (Figure 2.5) and in line with DLS data (Table 2.1), that point to polydisperse suspensions. The two bigger aggregates, labeled as “G” and “R” in Figure 2.7, were selected to collect single aggregate fluorescence spectra, shown in the left panel of Figure 2.7. Aggregate “G” is greenish, with an emission spectrum peaked at 585 nm, almost overlapping the spectrum collected on the bulk hydrogel or equivalently the spectrum collected from the liquid suspension. Aggregate “R” shows weak red fluorescence, as typical of H-aggregates,[82] with a broad spectrum, extending from ~600 nm downward (as described in Section T.4, the spectral detector of the TPM collects spectra in the 400-650 nm region). We conclude that DiI forms both H- and J-aggregates with distinctively different fluorescence spectra, and the overall emission of the liquid suspension and of the gel is largely dominated by the fluorescence of J-aggregates, which is much more intense than the emission from H-aggregates.

Further information on DiI and DiD aggregates, together with a detailed theoretical analysis which accounts for intermolecular interactions, molecular polarizability, vibronic effects, and environmental screening is reported in Ref. [76].

The study of DiI and DiD aggregation in aqueous environments highlights the importance of developing simple strategies to bring these carbocyanines into water without compromising their optical properties, essential for harnessing their full potential in bioimaging applications. Surfactants such as CTAB and TPGS are frequently employed to bring lipophilic molecules in water exploiting the formation of micelles, as described in next section.

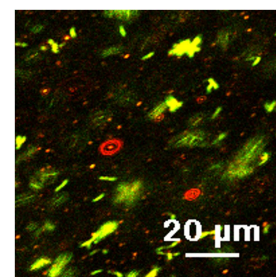


Figure 2.5: Two-photon excited fluorescence image of DiI aggregates suspended in the water/ethanol mixture. The image has been acquired exciting the sample at 1020 nm.

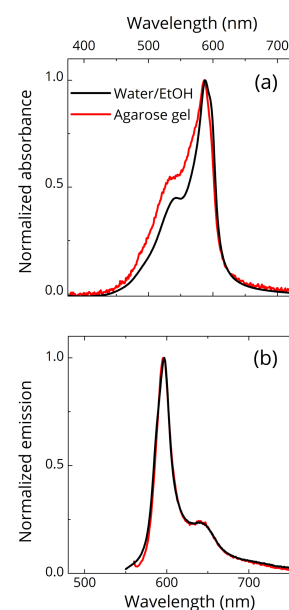


Figure 2.6: Comparison between absorption [Panel(a)] and emission [Panel(b)] spectra of DiI aggregates suspended in the water/ethanol mixture (black line) and in the agarose gel (red line).

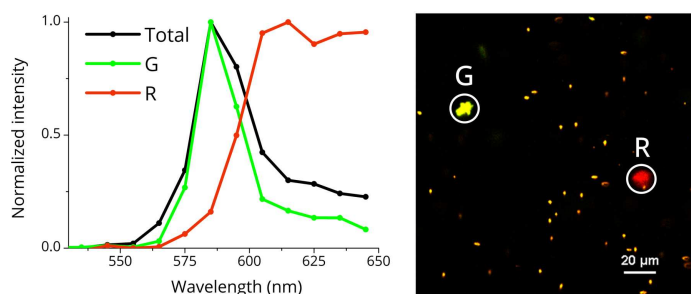


Figure 2.7: Left panel: two photon excited emissions spectra of single NP in the gel. Right panel: two-photon excited fluorescence image of the aggregates chosen for single aggregate spectra. Images and spectra have been acquired exciting the sample at 1020 nm

2.3 CTAB and TPGS micelles in aqueous suspension

CTAB and TPGS micelles were labeled with DiI and/or DiD, exploiting the poor solubility of the two carbocyanines in water. Thus, six types of micelles were prepared (according to the procedure reported in Section 2.7.3) and then characterized:

- CTAB micelles loaded with DiI (**C-m@I**)
- CTAB micelles loaded with DiD (**C-m@D**)
- CTAB micelles loaded with DiI and DiD (**C-m@I,D**)
- TPGS micelles loaded with DiI (**T-m@I**)
- TPGS micelles loaded with DiD (**T-m@D**)
- TPGS micelles loaded with DiI and DiD (**T-m@I,D**)

The composition associated with each formulation after preparation is reported in Table 2.2.

All the micellar suspensions were then diluted to different concentrations above and below the CMC (critical micellar concentration) of each surfactant. The CMC value reported in the literature for CTAB micelles is approximately 0.9 mM¹.^[83] The starting CTAB suspension (7 mM) has been diluted to values close to (2 and 1 mM) and below (0.5 and 0.25 mM) the CMC. For TPGS micelles, different CMC values are reported in the literature, amounting to 0.1 mM (0.02 %wt) or 0.02 mM (0.003 %wt) according to the measurement technique.² Different techniques may yield different results due to their sensitivity to specific aspects of micelle formation and to the different experimental conditions (such as temperature, pH, and the presence of salts). Moreover, the formation of TPGS micelles has been proved to occur gradually, without a clear break-point as in the case of more common surfactants.^[90]

For this reason, the 20 mM TPGS suspension was diluted to four different concentrations: 0.2, 0.05, 0.01, and 0.002 mM, reaching concentrations below both the CMC literature values (the 0.002 mM suspension was prepared only for T-m@I,D).

The dyes concentration was maintained sufficiently low to prevent extensive aggregation and inner filter effects. However, in the case of TPGS micelles, a higher concentration was employed to account for the higher dilution required to obtain suspensions below the CMC.

1: Some variability in the range 0.8 - 1.2 mM has been detected according to the technique employed for the CMC determination.^[83, 84]

2: Conductivity and solubility measurements resulted in a determined CMC of 0.1 mM,^[85-87] while a value of 0.02 mM has been reported through steady-state fluorometry (comparing the relative intensity of pyrene vibronic bands) and surface density.^[88, 89]

Table 2.2: Components concentrations associated with the prepared micellar formulations. All the samples were prepared in water with 12% of ethanol. The resulting micellar suspensions have been then diluted in order to reach the desired surfactant concentrations. The preparation procedure is described in Section 2.7.3.

Sample label	CTAB (mM)	TPGS (mM)	DiI (μ M)	DiD (μ M)
C-m@I	7	-	11	-
C-m@D	7	-	-	11
C-m@I,D	7	-	5.5	5.5
T-m@I	-	20	60	-
T-m@D	-	20	-	60
T-m@I,D	-	20	30	30

3: The electrophoretic mobility can be directly measured through the ELS technique, while the ζ -potential of the suspensions can be extrapolated employing the Helmholtz-Smoluchowsky approximation. More details on the DLS and ELS measurements and a brief description of the two techniques are reported in Section T.1.

In order to confirm the formation of micelles and verify if the presence of the two cyanines impacts on their size and structure, we performed DLS and ELS³ (electrophoretic light scattering) measurements, monitoring the

particle size distributions and ζ -potentials, respectively (results reported in Table 2.3 and Figure 2.8). Unfortunately, the samples containing DiD could not be measured because DiD strongly absorbs the incident light (633 nm) employed during the measurements.

The hydrodynamic diameters and ζ -potentials of plain CTAB and TPGS-micelles resulted in good agreement with the values reported in the literature [91, 92]. The presence of DiI had a negligible influence on the size of CTAB micelles, which amounted to a D_h of about 1 nm, while an increase in the size of TPGS micelles (from a D_h of 11.2 to 17.7 nm) was observed. This result can be attributed to the higher DiI/surfactant ratio in TPGS micelles compared to CTAB micelles. The ζ -potential of both CTAB and TPGS micelles is not significantly affected by the loading with DiI: the former is characterized by a positive value of about 25 mV, due to the permanent cationic character of the surfactant, while the latter is almost neutral, as expected given the absence of charged or ionizable groups.

Sample	Hydrodynamic diameter ^a (nm)	Apparent ζ -potential ^b (mV)
C-m	1.5 $\pm$ 0.1	24.5 $\pm$ 1.3
C-m@I	1.1 $\pm$ 0.1	24.7 $\pm$ 1.9
T-m	11.2 $\pm$ 0.1	-0.1 $\pm$ 0.1
T-m@I	17.7 $\pm$ 0.1	-1.6 $\pm$ 0.1

The almost negligible effect that the encapsulation of DiI has on size distributions and apparent ζ -potentials of CTAB and TPGS micelles is consistent with the low dye loadings employed. Thus, we can infer that also the loading with DiD dye did not alter significantly the hydrodynamic diameter and the apparent ζ -potential of the two nanocarriers.

TPGS micelles loaded with DiI (T-m@I) were also investigated upon dilution, at concentration of 0.05 mM and 0.01 mM, to verify if they are actually disrupted below the CMC values of 0.1 mM and 0.02 mM. The resulting suspensions were then analyzed through DLS to assess micelles integrity at these lower concentrations. Figure 2.9 shows that the size distribution by intensity (Figure 2.9a) and their corresponding correlograms (Figure 2.9b) are gradually deteriorated when the micelles are diluted at 0.05 and 0.01 mM, indicating a progressive disruption with the decrease of surfactant concentration. The correlogram associated to the 0.01 mM suspension present a very low intercept value, suggesting that the majority of micelles are broken at this TPGS concentration. Consequently, in the case of the TPGS surfactant, we considered 0.01 mM as the concentration below the CMC for the following analysis. The 0.05 mM suspensions also showed clear signs of micellar disaggregation but they were only taken into account when the dyes concentration of the 0.01 mM suspensions was too low to accurately measure absorption spectra and estimate some spectroscopic properties, such as fluorescence quantum yields and FRET efficiencies.

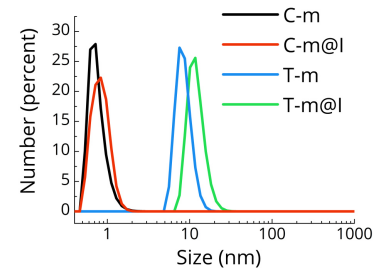


Figure 2.8: Particle size distributions by number of CTAB micelles (surfactant concentration: 7 mM) without (C-m) and with DiI (C-m@I) and TPGS micelles (surfactant concentration: 20 mM) without (T-m) and with DiI (T-m@I). These results are the average of three independent measurements.

Table 2.3: DLS and ELS results of CTAB and TPGS micelles suspensions in the presence and absence of DiI as the average of three measurements with the corresponding standard error of the measurement mean (SEM). Measurements were performed at 25°C as described in Section T.1.

^a Obtained from the corresponding peak in the particle size distribution by intensity

^b Apparent ζ -potential obtained employing the Helmholtz-Smoluchowski equation.

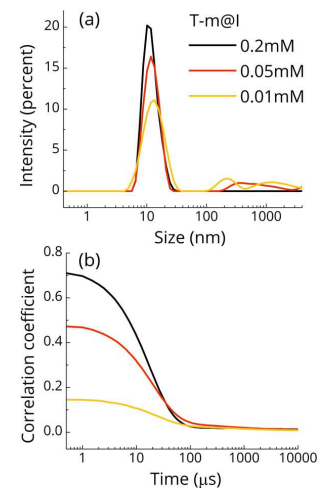


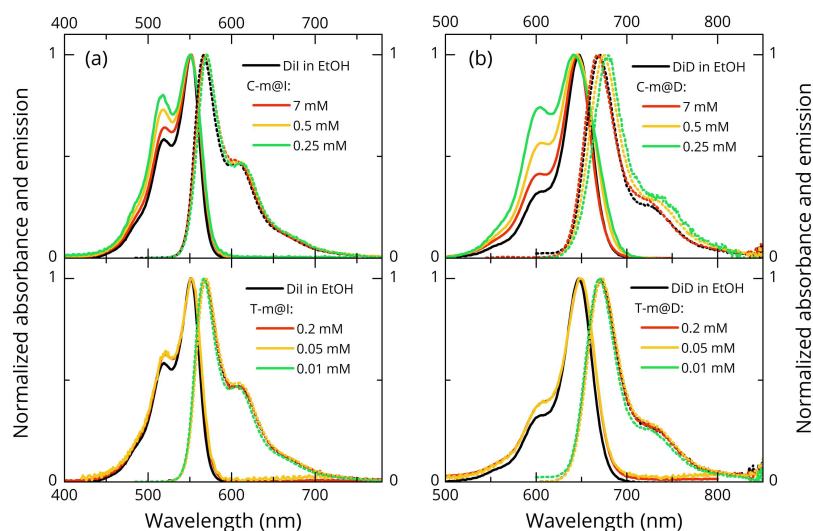
Figure 2.9: Size distributions by intensity [Panel(a)] and correlograms [Panel(b)] of the DiI-loaded TPGS micelles (T-m@I) at different concentrations. These results are the average of three independent measurements.

2.4 Spectroscopic properties of dye-loaded micelles

2.4.1 Single-dye loaded micelles

Absorption and emission spectra of CTAB and TPGS suspensions, loaded with either DiI or DiD at various surfactant concentrations, are shown in Figure 2.10. The spectra collected in both suspensions above the CMC contrast sharply with the ones recorded from the aggregated dyes in aqueous environment (described in Section 2.2), demonstrating the successful encapsulation of the dyes in the micelles. Notably, the interactions among the dyes, which strongly affect the optical properties in ethanol/water mixtures, are almost negligible in the micellar environment. Indeed, the absorption spectra in the CTAB and TPGS micelles are very similar to those obtained in ethanol solutions, showing only a modest increase of the relative intensity in the region of the 0–1 vibronic shoulder (Figure 2.10, top panels), a feature usually associated with the formation of weakly interacting H-aggregates.[80]

Figure 2.10: Absorption and emission spectra (continuous and dotted lines, respectively) of the four dye-loaded micelles at different surfactant concentrations. Panel (a): CTAB (top) and TPGS (bottom) micelles loaded with DiI (C-m@I and T-m@I, respectively); Panel (b): CTAB (top) and TPGS (bottom) micelles loaded with DiD (C-m@D and T-m@D, respectively). The emission spectra were collected exciting at 495 nm and 600 nm for samples containing DiI and DiD, respectively. The absorption spectrum of suspensions with a TPGS concentration lower than 0.05 could not be measured due to the low absorbance (< 0.1).



A summary of the optical properties of DiI and DiD loaded CTAB and TPGS suspensions at surfactant concentrations above and below the corresponding CMCs is reported in Table 2.4.

The molar extinction coefficients of DiI and DiD in micellar suspensions above the CMC are lower than in ethanol solutions, with a more pronounced effect for DiD (~20% decrease) than for DiI (~13% decrease) and only slight variations between the two surfactants. The fluorescence quantum yield of DiI is ~10% either in ethanol or in 1 mM CTAB suspension while amounts to 27% in TPGS 0.20 mM. For DiD, the fluorescence quantum yield slightly decreases from 37% in ethanol to 23% in 1 mM CTAB suspension while an increase to 43% was detected in 0.20 mM TPGS suspension. In CTAB suspensions, the decrease of ϕ with respect to ethanol is in line with the formation of weak H-aggregates in micelles, an effect somewhat more important for cyanine dyes with longer conjugated structures,[93] as already inferred from absorption bandshapes.[67, 94]

The high ϕ values detected for TPGS micelles do not exclude dye aggregation inside the nanocarriers, but may suggest a quite rigid environment for the two encapsulated dyes, that could reduce non-radiative decay processes.

Fluorescence lifetimes of DiI and DiD (Figure 2.11) are sizeably longer in TPGS above the CMC than in ethanol, confirming the reduction of non-radiative decay processes. In CTAB suspensions, the fluorescence lifetimes are comparable to those in ethanol.

Dye-loaded suspensions of the two surfactants show a completely different behavior below the corresponding CMC. Figure 2.10 shows absorption and emission spectra of DiI and DiD obtained diluting the 7 mM CTAB and 0.20 mM TPGS suspensions with distilled water. In the case of CTAB, the absorption intensity in the region of the 0–1 vibronic shoulder increases for both DiI and DiD below the CMC, suggesting a more important formation of aggregates as the dilution extent increases. The emission profile of DiI and DiD is barely affected by dilution below the CMC, indicating that in all cases the emission mainly comes from non-aggregated species. Moreover, the fluorescence lifetimes become shorter, and the quantum yield decreases both for DiI and DiD (Table 2.4). We ascribe these results to the formation of weak, poorly emissive H-aggregates. However, the aggregates formed in 0.5 and 0.25 mM CTAB suspensions are qualitatively different from those observed in mixed ethanol/water, since both dyes form weak H-aggregates (while DiI forms J-aggregates in ethanol/water mixtures) and intermolecular interactions remain weak, with only marginal spectroscopic effects.

We underline that these weak H-aggregates are only obtained upon dilution of preformed micellar systems containing an initial 11 μ M dye concentration. Indeed, when adding concentrated DiI in ethanol to a 0.5 mM CTAB suspension we obtain aggregates qualitatively similar to those observed in ethanol/water mixtures (Figure 2.12).

In the case of TPGS, no differences could be detected in the emission and absorption spectral bandshapes of the diluted suspensions. This observation, together with the calculated quantum yields (Table 2.4) and the excitation anisotropies (reported in the next section), suggests that TPGS efficiently solvates the dyes even if its micellar organization is lost, avoiding dye aggregation.

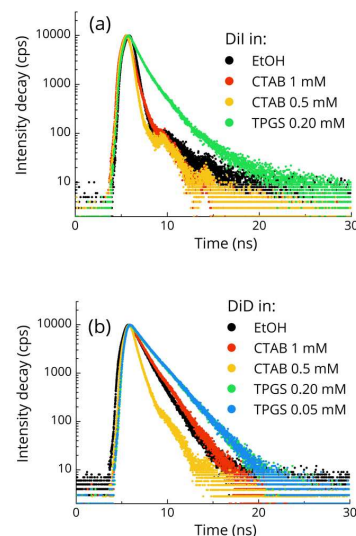


Figure 2.11: Emission decays of DiI [Panel(a)] and DiD [Panel(b)] in different environments: ethanol, CTAB 0.1 mM, CTAB 0.5 mM, TPGS 0.2 mM and TPGS 0.05 mM. All the samples have been excited at 375 nm, recording the emitted light at 570 nm for DiI and 670 nm for DiD.

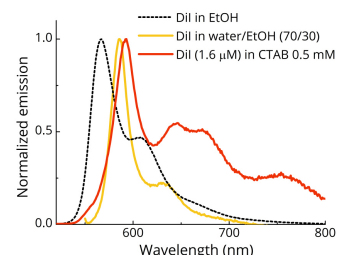


Figure 2.12: Emission spectra of DiI added to a solution of CTAB 0.5mM. The spectra collected in pure EtOH and water/ethanol 70/30 (*m/m*) mixture are shown for comparison.

Table 2.4: Spectroscopic properties of CTAB and TPGS micelles loaded with DiI and DiD.

	DiI			DiD		
	Molar extinction coefficient at $\lambda_{\max}$ ($M^{-1} \text{ cm}^{-1}$)	Fluorescence quantum yield ϕ (%)	Fluorescence lifetime $\langle \tau \rangle^a$ (ns)	Molar extinction coefficient at $\lambda_{\max}$ ($M^{-1} \text{ cm}^{-1}$)	Fluorescence quantum yield (%)	Fluorescence lifetime $\langle \tau \rangle^a$ (ns)
Ethanol ^b	140 000	10	0.4	246 000	37	1.31
C-m (above CMC) ^c	121 000	11	0.55	194 000	23	1.34
C-m (below CMC) ^d	108 000	6	0.30	163 000	5	0.65
T-m (above CMC) ^e	117 000	27	1.41	196 000	43	1.86
T-m (below CMC) ^f	97 000	29	-	179 000	45	1.84

^a The corresponding lifetime decays are reported in Figure 2.11. Lifetimes were fitted with a bi-exponential function. The average τ is reported as: $\langle \tau \rangle = \frac{B_1 \tau_1^2 + B_2 \tau_2^2}{B_1 \tau_1 + B_2 \tau_2}$ (ns)

^b From Ref. [67]

^c Molar extinction coefficients are estimated from a linear regression of the absorbance measured for CTAB suspensions at concentrations of 7 mM, 2 mM and 1 mM.

^d Both the extinction coefficient and the quantum yield change with the concentration, and the reported values refer to 0.5 mM CTAB solution.

^e Molar extinction coefficients are estimated from a linear regression of the absorbance measured for TPGS suspensions at concentrations of 20 mM, 4 mM, 0.8 mM (initial dye loading for the 20 mM suspension: 11 μM) and 0.2 mM (initial dye loading for the 20 mM suspension: 60 μM).

^f The data reported in the table refer to the suspension with 0.05 mM TPGS concentration.

Anisotropy study of single-dye loaded micelles

As described in Chapter 1, fluorescence anisotropy offers additional valuable information on the dye encapsulation. When the rotational motion of the dye competes with the fluorescence lifetimes, the fluorescence anisotropy decreases. The Perrin equation (Equation 1.1) relates the value of r to the rotational correlation time (τ_c) but it only accounts for the orientational motion as depolarization source and therefore does not apply to systems where other phenomena may contribute to depolarization, as in the case of homo and/or hetero FRET.[52] Fluorescence excitation anisotropy spectra of DiI and DiD in polyTHF, ethanol, CTAB and TPGS micellar environments above and below the CMC are shown in Figure 2.14. In all cases, the anisotropy is flat within the excitation band, suggesting that a single excited state is involved in the transition. The anisotropies measured in ethanol for DiI and DiD amount to $r \sim 0.17$ and 0.1, respectively. These values are much lower than those measured in polyTHF ($r \sim 0.33$), as expected due to the fast rotational diffusion in the low viscosity solvent. Table 2.5 lists rotational correlation times calculated using the Perrin equation (Equation 1.1). We set the intrinsic anisotropy r_0 to the value measured in polyTHF, assuming that in this viscous medium the rotational time is much longer than the fluorescence lifetime. For ethanol solutions and for micellar systems we got rotational times of the same order of magnitude as the fluorescence lifetime. In ethanol, in spite of its larger dimension, DiD has a lower r value than DiI due to its longer emission lifetime. Indeed a larger rotational correlation time is estimated for DiD than for DiI. In CTAB and TPGS suspensions, the excitation anisotropy is larger than in ethanol for either DiI or DiD. Since ethanol is more viscous than water, the larger r observed in water is attributed to a slower rotational diffusion, compatible with the encapsulation of the dyes inside the micelles. Usually, as discussed in Section

Table 2.5: Rotational correlation times, τ_c , calculated using the Perrin equation.

Sample	τ_c (ns)
DiI in EtOH	0.38
C-m@I	0.64
T-m@I	2.34
DiD in EtOH	0.53
C-m@D	1.13
T-m@D	2.46

1.3.2, the rotational time estimated from fluorescence anisotropy for an encapsulated dye is an effective quantity that accounts at least for two independent motions: the motion of the dye inside the micelles and the rotation of the micelle itself. The motion of dyes encapsulated in micelles is slower than in solution and the micelles themselves are big objects compared to molecules, with slower rotational diffusion (the modest increase in anisotropy observed for DiI in CTAB micelles when compared to the dye in ethanol is justified by the concomitant increase of lifetime). However, the larger size of TPGS micelles when compared to CTAB makes the contribution of the whole NPs rotations to the fluorescence depolarization almost negligible, being it associated to a τ_c much longer than the fluorescence decay of DiI and DiD.⁴ The marginal depolarization of DiI and DiD in both the micellar environments confirms minimal interchromophore interactions, and the depolarization is assigned only to the motion of the dye inside the micellar system.

The dilution of the CTAB micelles below the CMC is associated with a strong decrease in the anisotropy value, that results even lower than in ethanol. Since the rotational motion of the dyes in water in the presence of CTAB cannot be substantially faster than the monomer in ethanol, the observed large depolarization is safely ascribed to homo-FRET processes within the H-aggregates. The homo-FRET processes allows for the hopping of the excitation among different dyes, leading to depolarized emission.

Additional information on the depolarization sources can be obtained from time-resolved anisotropy of DiD in different environments, that we recorded for CTAB suspensions (the short lifetimes of DiI hinders these measurements with our experimental setup). Data in Figure 2.13 show that for DiD in ethanol and in the micellar environment (above the CMC) the initial anisotropy value, $r = 0.3$, is large and close to r_0 as measured in polyTHF. Over time, r decays rapidly towards full depolarization ($r = 0$) in ethanol, while a much slower decrease is observed in the micelles, where r reaches a steady value slightly higher than zero ($r = 0.07$) after ~ 10 ns, similarly to the behavior of C153 in the unimer micelles, as we described in Section 1.3.2. Indeed, also in this case the residual anisotropy at longer times suggests that the rotational diffusion of the dye in its local environment is limited, as a result of the encapsulation in a NP.

The behavior observed below the CMC (green trace in Figure 2.13) is qualitatively different: the anisotropy is very low ($r \sim 0.1$) at the earliest accessible time (~ 1 ns), confirming that depolarization in this case is due to fast homo-transfer processes. Overall, fluorescence anisotropy data fully support the hypothesis that in CTAB suspensions below the CMC, DiD and DiI form weak H-aggregates.

On the other hand, TPGS micelles did not show any sizable depolarization feature even below the CMC, indicating that the dyes are well solvated also after dilution. Additional investigation will be performed at lower TPGS concentration, in order to guarantee that the measurements are performed below the CMC (these measurements require a starting dye concentration much higher than the one used here).

4: A D_h of 11.2 nm, the value estimated for TPGS by DLS measurements, corresponds to a τ_c of ~ 160 ns (Equation 1.2), one order of magnitude higher than the τ of the two dyes.

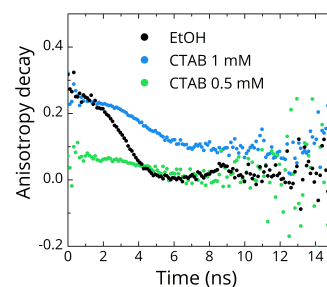


Figure 2.13: Time-resolved anisotropy of DiD in three different environments: ethanol, CTAB 1 mM and CTAB 0.5 mM. All the measurements have been performed exciting the sample at 590 nm and detecting the emission at 675 nm.

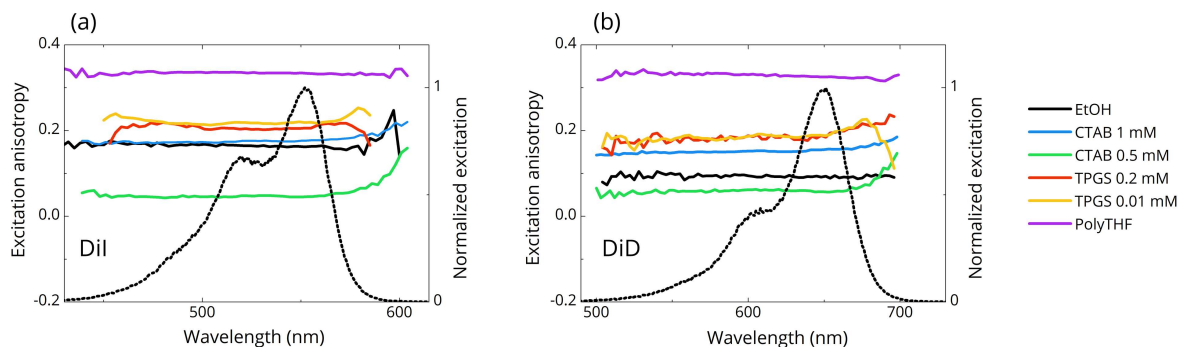


Figure 2.14: Steady-state excitation anisotropy of DiI [Panel(a)] and DiD [Panel(b)] in different environments ($\lambda_{em} = 650$ nm for DiI and 750 nm for DiD)

2.4.2 FRET micelles

In the previous sections we demonstrated the successful encapsulation of DiI or DiD in CTAB and TPGS micelles, with marginal aggregation phenomena for both kind of suspensions, as long as the surfactant concentration is kept above the CMC.

CTAB and TPGS micelles loaded with both DiI and DiD (1:1 ratio) have been prepared following the same procedure presented for single-dye loading (see Section 2.7.3). The absorption spectra of the resulting suspensions are reported in Figure 2.16 (top panels) and show, for both surfactants, a similar profile as in ethanol with a minor increase of intensity in the blue side of the band.

Emission spectra collected from both TPGS and CTAB micelles simultaneously loaded with DiI and DiD (Figure 2.16 bottom panels) support efficient FRET. By exciting the sample at 495 nm, where the absorption of the energy acceptor (DiD) is negligible, a sizeable emission of the acceptor is observed (for comparison, emission from a 1 : 1 DiI : DiD ethanol solution is also reported, where FRET is clearly negligible). Energy transfer was demonstrated also by the excitation spectra reported in Figure 2.15, where a clear contribution from the DiI absorption bandshape was present when detecting selectively the emission of DiD (at 750 nm). Both the acceptor band in emission and the donor contribution in the excitation spectra are more prominent for T-m@I,D, suggesting higher FRET efficiency in this system.

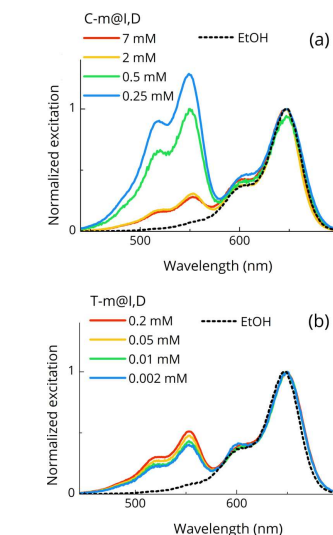
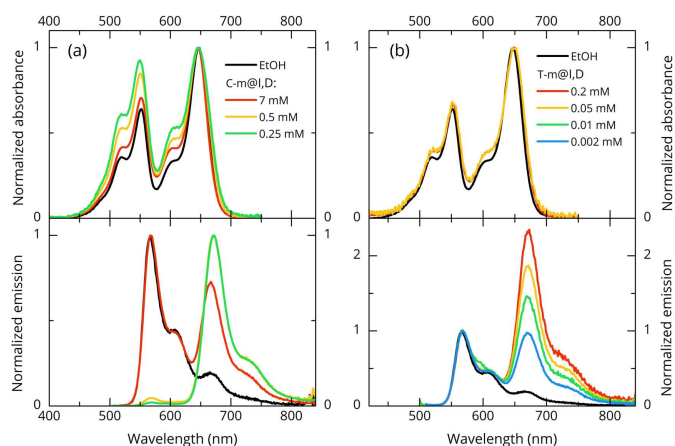
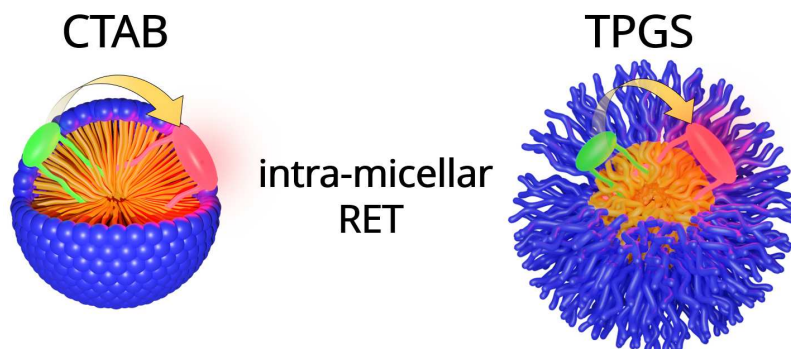


Figure 2.15: Excitation spectra of CTAB and TPGS suspensions loaded simultaneously with DiI and DiD at four different surfactant concentrations: panel(a) CTAB micelles, panel(b) TPGS micelles. All the reported spectra have been acquired detecting the light emitted at 750 nm, to minimize the contribution from the DiI emission. The spectra have been normalized to the peak of DiD. The excitation spectrum of DiI and DiD (1:1) dissolved in ethanol is reported for comparison ($\lambda_{em} = 750$ nm).

Figure 2.16: Absorption and emission spectra of micelles loaded simultaneously with DiI and DiD. Panel(a): CTAB micelles at three different surfactant concentrations. Panel (b) TPGS micelles at four different surfactant concentrations. Emission spectra were acquired exciting the sample at 495 nm, in order to selectively excite the energy donor. The emission spectra of CTAB micelles have been normalized to the emission peak of DiI.



Upon dilution of C-m@I,D suspensions with distilled water, minor effects are observed above the CMC (Figure 2.17): FRET signatures are similar for CTAB concentrations of 2 and 7 mM, and only slightly increase at 1 mM CTAB concentration, close to the CMC. The observed FRET features suggest that some micelles are loaded with at least two dyes (one DiI and one DiD), to guarantee interchromophore distances compatible with the energy transfer process. The marginal dependence of FRET upon dilution, at least for CTAB above the CMC, demonstrates that the phenomenon is independent of the distance among micelles. Accordingly, we describe FRET as an intramicellar phenomenon (Figure 2.18). At the same time, both TPGS and CTAB suspensions are characterized by weak perturbations of absorption and emission spectra, excluding the presence of strong excitonic interactions that indeed require very short intermolecular distances, typically less than 1 nm.



The fluorescence quantum yield of DiI (energy donor) decreases in the presence of DiD (energy acceptor), due to FRET, and at the same time, the emission of DiD becomes sizeable, with a quantum yield larger than 10% (Table 2.6). FRET efficiency has been estimated by comparing the fluorescence quantum yield of the donor dye (DiI) in the presence (ϕ_D , second column of Table 2.6) and in the absence of the acceptor⁵ (ϕ_{D_0}) in Table 2.4):[52]

$$\Phi_{FRET} = 1 - \frac{\phi_D}{\phi_{D_0}} \quad (2.1)$$

We estimated a FRET efficiency of 36% and 50% for C-m@I,D and T-m@I,D suspensions above the CMC, respectively. As expected from the emission and excitation spectra, the efficiency value determined for TPGS micelles is higher, probably due to the higher dye loading and DiI fluorescence quantum yield in this suspension⁶.

A strikingly different behavior has been observed when diluting the suspensions of the two surfactants below the corresponding CMC: the FRET efficiency of TPGS micelles decreases progressively with the extent of the dilution, while the FRET efficiency of the CTAB suspension considerably increases, reaching 88%, a value comparable to that measured in quatsomes.[67] The normalized emission spectra in Figure 2.16 (bottom panels) show indeed a decrease in the donor and in the acceptor emissions, respectively for CTAB and TPGS, but a proof of the efficiency

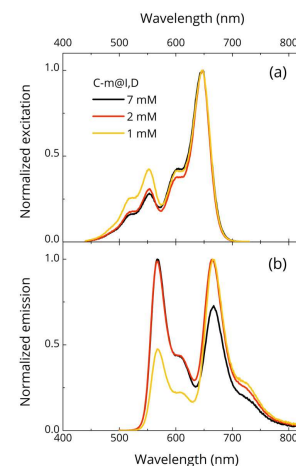


Figure 2.17: Excitation [Panel(a)] and emission [Panel(b)] spectra of CTAB suspensions loaded simultaneously with DiI and DiD at three different surfactant concentrations above the CMC. The excitation spectra have been acquired detecting the light emitted at 750 nm, while the emission spectra were collected exciting the sample at 495 nm.

Figure 2.18: Schematic representation of FRET as intramicellar phenomena when CTAB and TPGS micelles are loaded with DiI and DiD at equimolar ratios.

⁵: It is important to note that we cannot estimate FRET efficiency by comparing the emission lifetime of the donor in the presence and in the absence of the acceptor, since the lifetime of the energy donor is very short and its variation (specifically its decrease) due to FRET would be affected by very large errors.

⁶: The FRET efficiency values of both TPGS and CTAB micelles (above the CMC) are lower than those observed in larger organic NPs,[67, 95] most probably as a result of the lower dye loading.

variations are provided also from the excitation spectra in Figure 2.15. Specifically, for C-m@I,D below the CMC, the fluorescence quantum yield of the energy donor, DiI, becomes lower than 1%, while the one of the acceptor, DiD, increases to 16%. T-m@I,D shows the opposite behavior: the quantum yield of the energy donor slightly increases while the acceptor one decrease from 33 % to 26 %.

Table 2.6: FRET efficiencies and fluorescence quantum yields of C-m@I,D and T-m@I,D upon excitation of the donor (495 nm).

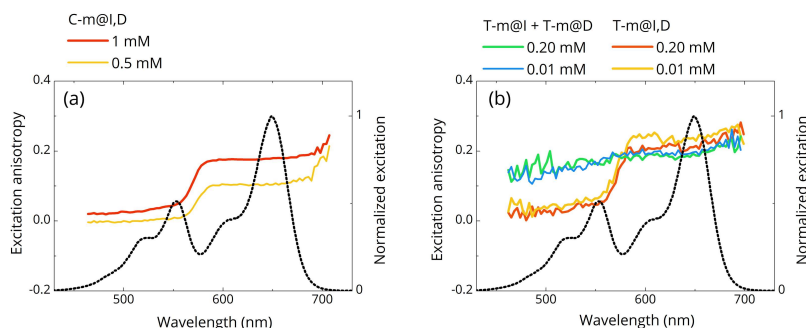
^a Estimated from the decrease of the fluorescence quantum yield of the energy donor.

Sample	DiI ($\lambda_{\text{exc}} = 495\text{nm}$)	DiD ($\lambda_{\text{exc}} = 495\text{nm}$)	Φ_{FRET}^a
C-m (1 mM)	7%	14%	36%
C-m (0.5 mM)	0.7%	16%	88%
T-m (0.2 mM)	14%	33%	50%
T-m (0.05 mM)	15%	26%	44%

A plausible explanation for the surprising and counterintuitive behavior of CTAB micelles when diluted below the CMC is that other clusters, different from micelles, composed of DiI, DiD, and CTAB are formed below the CMC, where FRET is promoted by an increased number of dyes in the same aggregate and/or short distances among the dyes in the FRET pair.

In the case of the TPGS, the micelles progressively loose their nanostructure after dilution and dyes that were loaded in the same particle tend to move far apart, decreasing FRET efficiency. Being DiI and DiD well solvated also below the CMC, aggregation phenomena are prevented in the presence of TPGS.

Figure 2.19: Steady-state excitation anisotropies of CTAB [Panel(a)] and TPGS micelles [Panel(b)] loaded with DiI and DiD. The measurements have been performed detecting selectively the emission of DiD ($\lambda_{\text{em}} = 750\text{ nm}$). Data both above and below the CMC are reported. In Panel(b) results from TPGS micelles loaded with DiI mixed with TPGS micelles loaded with DiD are shown.



Excitation anisotropies of C-m@I,D and T-m@I,D, both above and below the corresponding CMC, have been acquired detecting selectively the emission of DiD and are reported in Figure 2.19.

Above the CMC (red lines of Figure 2.19), the excitation anisotropy is almost constant within the excitation band of DiD for both C-m@I,D and T-m@I,D, with similar values to those in DiD-loaded micelles. The anisotropy decreases sharply in the region of the DiI absorption, a clear signature of FRET-driven depolarization. When diluting below the CMC (orange lines of Figure 2.19), no variations in the excitation anisotropy were detected for TPGS while lower values have been measured in the case of CTAB suspensions, due to increased FRET efficiency and homo-aggregation.

Temperature effect on CTAB micelles

The CMC increases with temperature,[96] so it's possible to move below the CMC of a surfactant by maintaining its concentration constant but increasing the temperature. For this reason, CTAB micelles were studied increasing the temperature of the suspension up to 55 °C.

Figure 2.20 shows the temperature dependence of the emission spectra of micelles loaded with both DiI and DiD. Above 45 °C, the DiI emission sizably decreases, pointing to increased FRET efficiency. A further proof of the same phenomenon comes from the excitation spectra (Figure 2.21): an increase of the signal observed in the region where the energy donor DiI absorbs was observed after increasing the temperature. The breakdown of micelles promoted by either increasing the temperature or by decreasing the CTAB concentration is in any case responsible for increased FRET efficiency.

The effect of the temperature will be tested in the future also for TPGS micelles.

2.4.3 Mixed single dye-loaded micelles

FRET is not detected upon mixing suspensions of single dye-loaded micelles containing the same surfactant: C-m@I with C-m@D and T-m@I with T-m@D, for surfactants concentrations larger than the corresponding CMC (Figure 2.23). This observation, validated up to 1 or 3 days from the sample preparation (Figure 2.22), confirms that the dye-loaded micelles are stable above the CMC, and the dyes do not show any tendency to aggregate once they are successfully encapsulated in the micelles. These results were expected, being FRET an intramolecular phenomenon and considering the extremely low loading of both CTAB and TPGS micelles. Taking into account the aggregation numbers of the two surfactants[90, 97], each micelle should be indeed loaded by at most a single dye, on a statistical basis. However, the homo-aggregation and FRET phenomena described in the previous sections (for single-dye loaded and FRET micelles, respectively) can be explained by aggregation of the dyes before entering the micelles. As we demonstrated in Ref. [98] through molecular dynamics simulations, a couple of dye molecules can enter the same micelles if they previously form a cluster in water, before interacting with the surfactant.

When the two mixed suspensions were diluted below the CMC of the corresponding surfactant, a strikingly different behavior emerges (Figure 2.23): while in the CTAB suspension an efficient FRET occurs, similarly to what happens after dilution of C-m@I,D, in the case of TPGS suspension no significant variations are detected in emission, absorption and excitation anisotropy (Figure 2.19) spectra. Upon dilution of the mixed TPGS suspensions no FRET was observed and the local environment of the loaded DiI and DiD dyes seems to remain unaffected, even at very low surfactant concentrations.

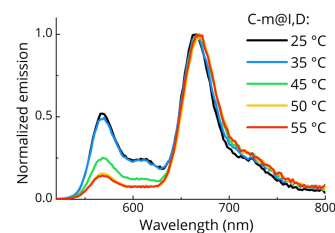


Figure 2.20: Emission spectra of micelles loaded with both DiI and DiD (CTAB 1 mM) as a function of temperature. All the reported spectra were acquired exciting the sample at 495 nm.

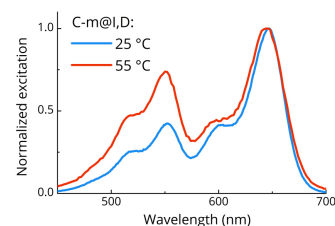


Figure 2.21: Excitation spectra of CTAB micelles loaded with both DiI and DiD at two different temperatures (concentration of CTAB: 1mM). Emitted light is collected at 750nm, where only the energy acceptor DiD is emitting.

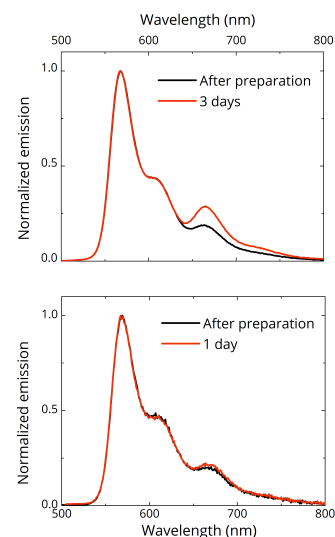
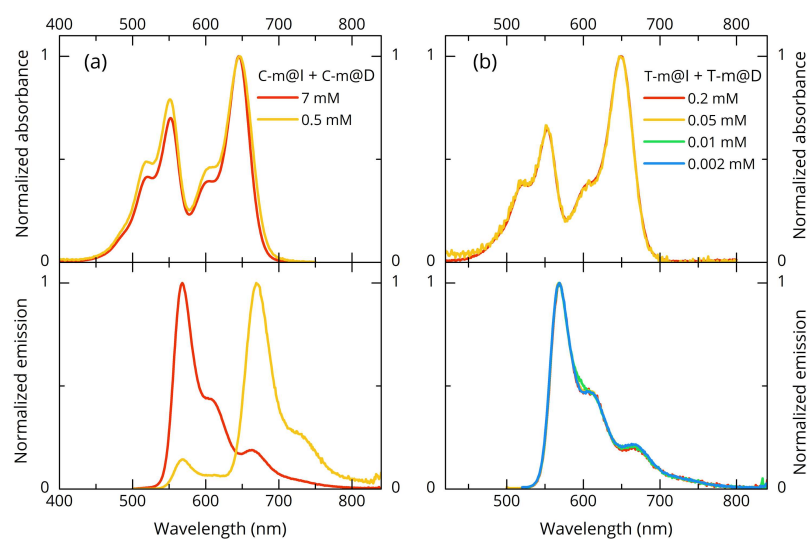


Figure 2.22: Emission spectra of surfactant suspensions obtained mixing the single-dye loaded micelles monitored over time. Top panel: CTAB micelles, bottom panel: TPGS micelles. All the reported spectra were acquired exciting the sample at 495 nm.

Figure 2.23: Absorption and emission spectra of surfactant suspensions obtained mixing the single-dye loaded micelles: a) CTAB micelles at two different surfactant concentrations. b) TPGS micelles at four different surfactant concentrations. All the emission spectra were acquired using an excitation wavelength of 495 nm.



2.5 Bioimaging experiments in porcine sclera

After the careful spectroscopic and physico-chemical characterization of the DiI/DiD-loaded CTAB and TPGS micelles in aqueous suspension, we tested their behavior in a biologically relevant environment. For this purpose, we selected *ex vivo* porcine scleral tissue, exploiting TPM as imaging technique (information concerning the setup can be found in Section T.4).

The emission of DiI is detected in both the green and red channels, with a much stronger contribution to the former, while DiD is a red emitter, and its fluorescence is detected in the red and far-red channels (Figure 2.24).

We started from the permeation of single dye-loaded micelles, which provide fundamental understanding of their interaction with the scleral tissue, and then moved to the FRET micelles, comparing the results obtained with the two different surfactants. Every suspensions was tested for permeation experiments in Franz- type diffusion cell with a contact time of 2 hours (Further details on the permeation experiments are reported in Section T.3).

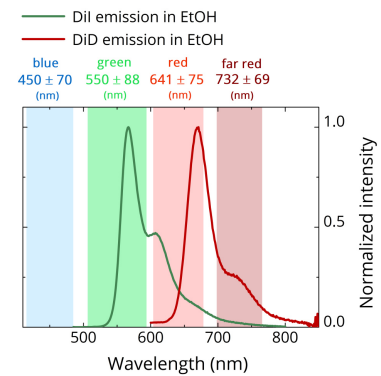


Figure 2.24: Spectral ranges of the three GaAsP and the multialkali detectors used to acquire the TPM images. The emission profiles of DiI and DiD are also shown for comparison (dark green and dark red lines, respectively).

2.5.1 Single dye-loaded micelles

CTAB micelles

Scleral tissue was exposed to the suspension of CTAB micelles loaded with DiI (C-m@I). In order to observe the emission of DiI and simultaneously collect the collagen SHG in the blue channel, an excitation wavelength of 950 nm was employed to record the images.

In Figure 2.26 are reported three images collected from sclera after the permeation of C-m@I. Figure 2.26a shows the surface of the tissue, with superficial collagen fibers (blue channel) and some globular structures, emitting mainly in the green channel and assigned to DiI fluorescence. The autofluorescence of the plain sclera in the green and red spectral regions is in fact negligible at the employed experimental conditions⁷. Similar globules were also observed below the surface, as demonstrated by Figure 2.26b, which was acquired 50 μm below the tissue surface and clearly shows the presence of these structures inside the porosities of the collagen matrix.

Figure 2.26c is collected at 400 μm below the scleral surface: at this depth, the strong scattering from the tissue drastically decrease the resolution of the image. The emission spectrum (Figure 2.25) clearly shows the presence of two signals: the SHG at half of the excitation wavelength (generated from the collagen fibers) and the of emission of DiI.

⁷: Due to the high emissivity of DiI both the laser power and detector gains were kept at very low values, to prevent the saturation of the green channel.

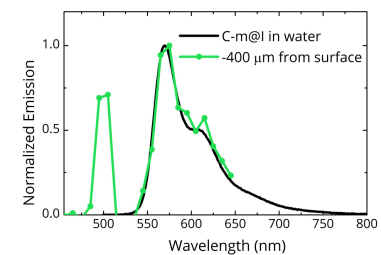


Figure 2.25: Comparison between emission spectra of the tissue collected 400 μm below the surface after the permeation of C-m@I (corresponding to image Figure 2.26c) and of an aqueous suspension of C-m@I. The green spectrum was collected exciting the sample at 1000 nm.

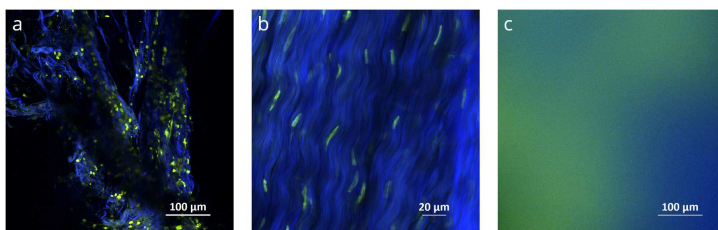


Figure 2.26: TPM images (channels overlay) acquired exciting the sclera treated with CTAB-micelles loaded with DiI (C-m@I) with an incident wavelength of 950 nm. Panel(a): Image showing the surface of the sclera; Panel(b): zoomed image (90 μm x 90 μm) taken 50 μm below the surface; Panel(c): Image taken 400 μm below the surface.

The globular structures that are observed at the surface and in the interfibrillar pores of the sclera are big assemblies of DiI and possibly CTAB. These globules are likely formed upon the disruption of the micelles, as a result of the electrostatic interaction between the surfactant and the tissue. In fact, CTAB has a permanent positive charge, while the scleral interfibrillar matrix is made up of negatively charged hydrated proteoglycans, consisting of a protein core with glycosaminoglycan (GAG) side chains of repeating disaccharide units (chondroitin, dermatan, keratan or heparin sulfate).[99, 100]

To better appreciate the distribution of DiI in the tissue, a Z-scan was performed (Figure 2.27), showing the presence of the DiI containing globules on the scleral surface and inside the collagen pores up to tenths of μm in depth. The emission of DiI is mainly localized in these structures, as can be observed from the green channel overview in Figure 2.27c.

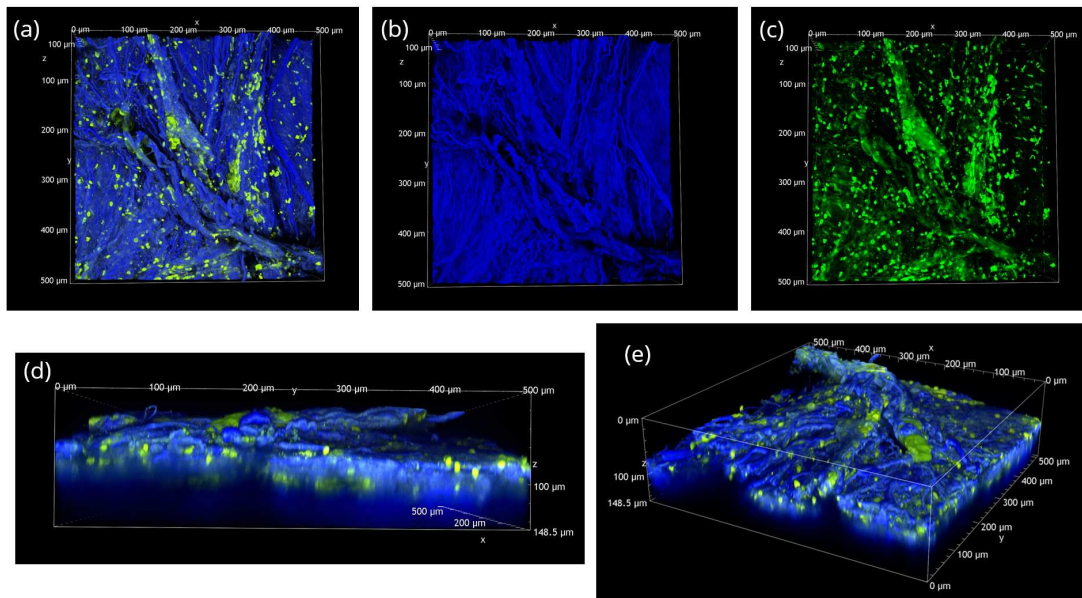


Figure 2.27: Volume renderings of the scleral tissue reconstructed from Z-stacks (z step: $1.5 \mu\text{m}$, total depth: $148.5 \mu\text{m}$): Panel(a): XY view of channels overlay; Panel(b): blue channel XY view; Panel(c): green channel XY view; panel(d): YZ view of channels overlay; Panel(e): 3D overview of channels overlay.

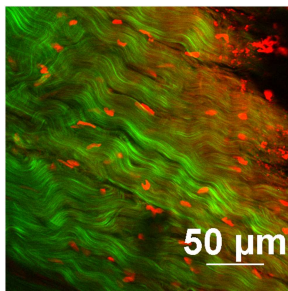


Figure 2.28: Channels overlay of a zoomed region of sclera after treatment with C-m@D (image size: $250 \mu\text{m} \times 250 \mu\text{m}$, excitation wavelength: 1150 nm).

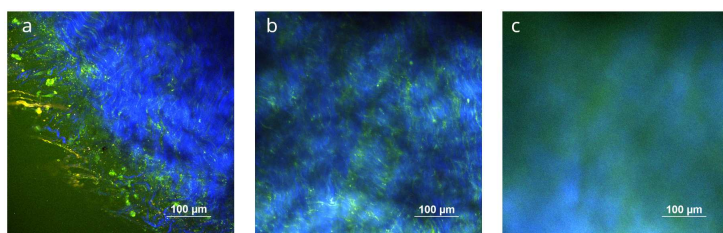
A similar scenario was observed after the permeation of CTAB micelles loaded with DiD (C-m@D). A scleral sample treated with C-m@D was excited at 1150 nm , in order to observe the collagen SHG signal in the green channel (575 nm) and the emission from the dye in the red and far-red ones. Figure 2.28 shows that, independently on the loaded carbocyanine dye, the CTAB micelles collapsed in globular aggregated structures in between the collagen fibers.

TPGS micelles

A sample of sclera was treated with TPGS micelles labeled with DiI (T-m@I). As discussed in the previous section, the sample was excited at 950 nm, to visualize DiI and the collagen SHG in the green and blue channels, respectively.

Figure 2.30 shows that DiI emission is much more homogeneously distributed when compared to what we observed with CTAB micelles in Figure 2.26, and the globular structures are absent. The green channel signal was detected both on the surface of the tissue and in the spaces between the collagen fibers, similarly to what we reported for the TPGS micelles when loaded with Nile red (Section 4.2.1)[101] and C153-loaded unimer micelles (Section 1.4).[62] The presence of well-solubilized DiI at different depths within the tissue, from the surface to 300 μm below, was confirmed by the emission spectra reported in Figure 2.29, which were recorded in correspondence to Figure 2.30 panels b and c.

TPGS is a neutral surfactant having a lower affinity towards scleral proteoglycan matrix, compared to positively charged CTAB. As a consequence, TPGS lowers the formation of dye/surfactant aggregates in the tissues. This result is in agreement with what we observed for permeation and diffusion studies of TPGS micelles loaded with Nile Red (see Ref. [101] and Section 4.2.1).



The Z-scan on the same sample revealed the presence of an intense and diffuse green signal above the scleral surface (Figure 2.32a and b).

The water solubility of DiI is extremely low, thus, this emission is assigned to a backdiffusion of DiI-loaded micelles to the aqueous solution in contact with the tissue and the microscope objective.

Figure 2.31 shows an image of the sclera treated with DiD-loaded TPGS and the corresponding emission spectrum. As discussed in the case of CTAB micelles, when exciting the sample at 1150 nm, DiD emission can be visualized in red channel while the collagen SHG falls within the green one. The emission of the dye mainly stems from the spaces between the collagen fibers, without presenting any substantial degree of aggregation, similarly to what was observed after the treatment with T-m@I. In the emission spectrum (Figure 2.31 top panel) both the SHG peak and the low-wavelength tail of the DiD emission spectrum (the rest of the bandshape falls outside our limited spectral range) are visible. The backdiffusion phenomenon has been observed also in the case of the DiD-loaded TPGS micelles, as demonstrated by the corresponding Z-scan (see Figure 2.33). The signal from either DiI or DiD was detected far below the scleral surface throughout all the scanned volume in the tissue (up to a depth of ~ 220 μm).

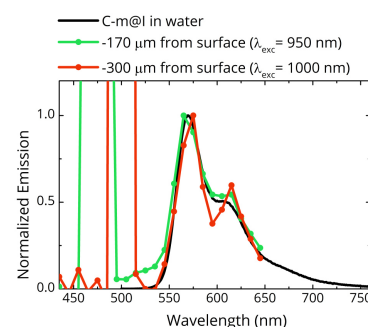


Figure 2.29: Comparison between emission spectra of the tissue collected 170 and 300 μm below the surface after the permeation of T-m@I (excitation wavelength: 1000 nm) and of an aqueous suspension of T-m@I.

Figure 2.30: TPM images (channels overlay) acquired exciting the sclera treated with TPGS-micelles loaded with DiI (T-m@I) at 950 nm. Panel(a): Image showing the surface of the sclera; Panel(b): zoomed image (90 μm x 90 μm) taken 50 μm below the surface; Panel(c): Image taken 400 μm below the surface.

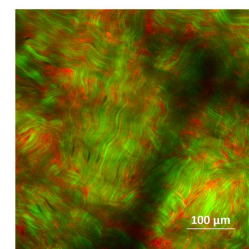
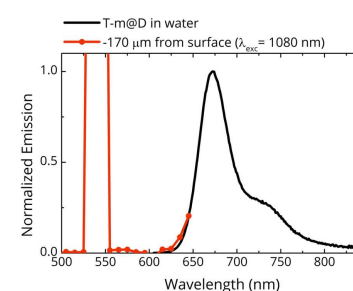


Figure 2.31: Top panel: comparison between emission spectra of the tissue collected 170 μm below the surface after the permeation of T-m@D (acquired in correspondence of the bottom panel, exciting at 1080 nm) and of an aqueous suspension of T-m@D. Bottom panel: channels overlay of sclera after treatment with T-m@D (excitation wavelength: 1150 nm).

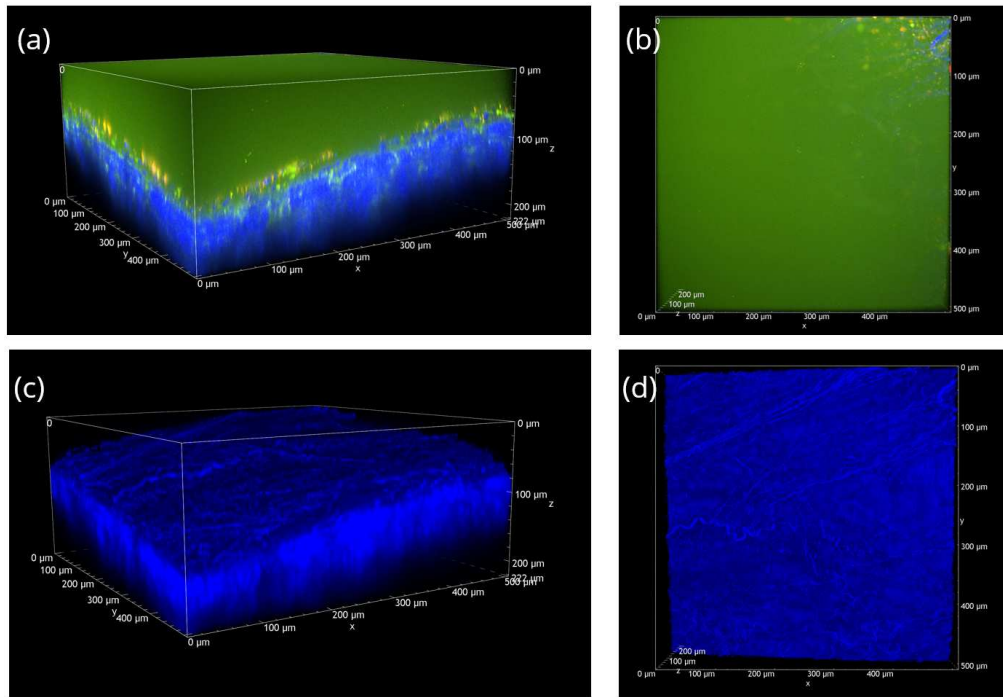


Figure 2.32: Volume renderings of the scleral tissue treated with T-m@I reconstructed from Z-stacks (z step: $1.2\ \mu\text{m}$, total depth: $222\ \mu\text{m}$): Panel(a): 3D overview of channels overlay; Panel(b): XY view of channels overlay; Panel(c): 3D overview of the blue channel; panel(d): XY view of the blue channel. Excitation wavelength: $950\ \text{nm}$.

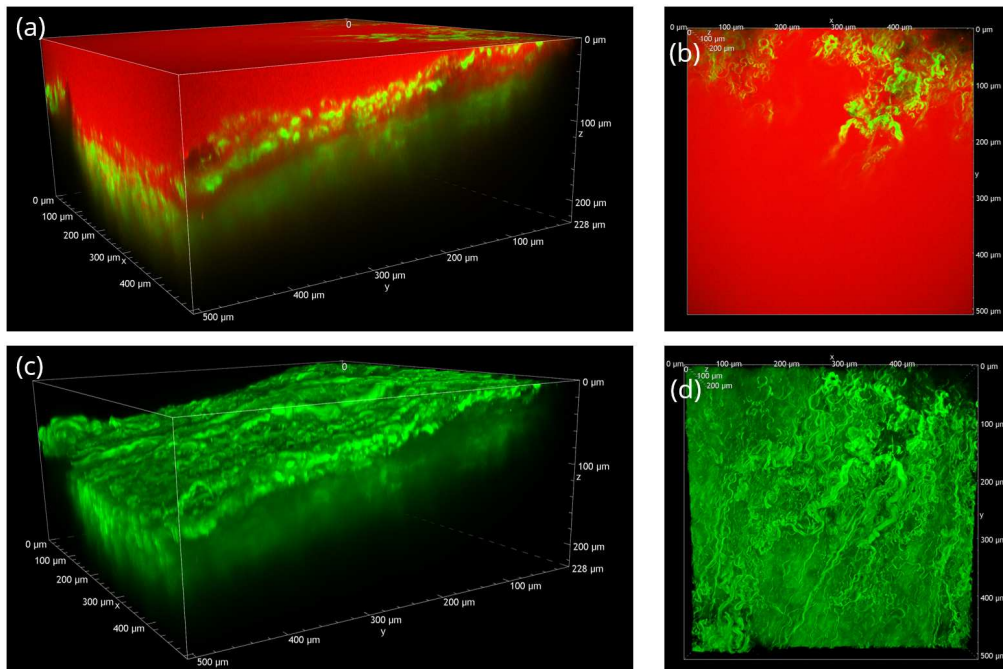


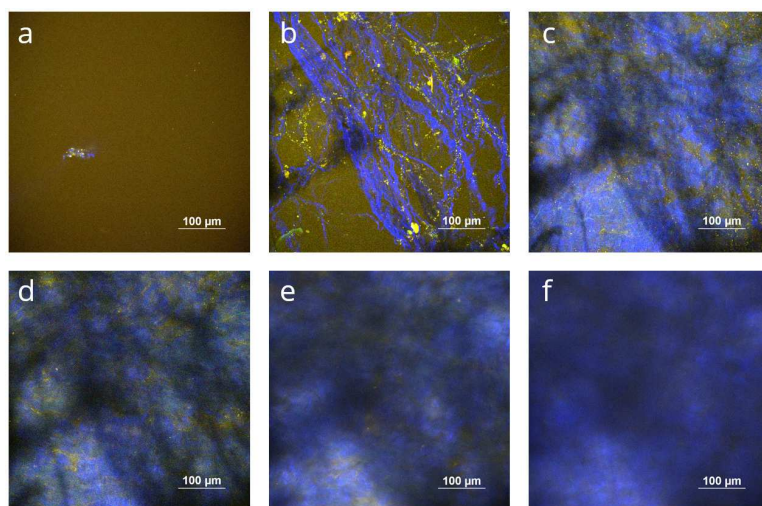
Figure 2.33: Volume renderings of the scleral tissue treated with T-m@D reconstructed from Z-stacks (z step: $1.5\ \mu\text{m}$, total depth: $228\ \mu\text{m}$): Panel(a): 3D overview of channels overlay; Panel(b): XY view of channels overlay; Panel(c): 3D overview of the green channel; panel(d): XY view of the green channel. Excitation wavelength: $1150\ \text{nm}$.

2.5.2 FRET micelles

Finally, sclera was treated with CTAB and TPGS micelles simultaneously loaded with DiI and DiD (C-m@I,D and T-m@I,D).

In order to observe FRET we selected 950 nm as excitation wavelength, which is good to excite the energy donor (DiI, as discussed in the previous section) and at the same time to prevent direct excitation of the energy acceptor (DiD). The direct excitation of DiD was ruled out by recording the Two-Photon Absorption (TPA) excitation spectra of both dyes in ethanol (Figure 2.34, technical details on these measurements are reported in Appendix B), which show a negligible absorption at this wavelength for DiD. Additionally, we acquired images of the tissue treated with DiD-loaded TPGS micelles exciting at both 950 nm and 1150 nm (Figure 2.35). At the former wavelength, almost no signal in the red channel was detected (Figure 2.35 bottom panel), while exciting the same region of the tissue at 1150 nm lead to a strong and diffuse DiD emission.

In Figure 2.36 are shown TPM images acquired at different depths inside the tissue after the permeation of T-m@I,D. The overall behavior of the nanocarriers is similar to what we described in the previous section for single-dye loaded TPGS micelles, the signal in the green and red channels is homogenously distributed in between the interfibrillar spaces. From Figure 2.36a the backdiffusion of the dyes into the liquid interface above the surface of the tissue is evident. The emission signal collected in the red channel is much stronger than the one observed after the treatment with T-m@I (Figure 2.26), even if we are selectively exciting the very same dye molecule (the donor of energy, DiI), and the resulting images present a yellowish signal in the regions where the fluorescent probes are present. This observation is consistent with the occurrence of FRET, since a significant emission of DiD can be detected when selectively exciting DiI at 950 nm.



In Figure 2.37 are reported emission spectra acquired inside the treated tissue at three different depths: on the surface, 100 and 150 μm below the scleral surface. The spectra collected from the tissue, especially the one from the surface (that is less convoluted to the tissue autofluorescence), clearly show a contribution from the low-wavelength tail of the DiD emission, further confirming the FRET phenomenon.

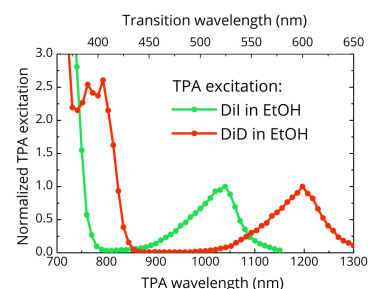


Figure 2.34: Two-photon absorption excitation spectra of DiI and DiD in ethanol, recorded with our microscope.

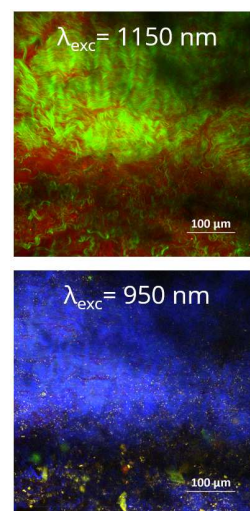


Figure 2.35: Top panel: channels overlay of sclera after treatment with T-m@D (excitation wavelength: 1150 nm). Bottom panel: channels overlay of sclera after treatment with T-m@D (excitation wavelength: 950 nm).

Figure 2.36: TPM images (channels overlay) acquired exciting the sclera treated with TPGS micelles loaded simultaneously with DiI and DiD (T-m@I,D) with an incident wavelength of 950 nm. Panels(a)-(d): starting from the surface of the tissue, each image was acquired 20 μm deeper than the previous one, increasing depth from (a) to (d). Panels (e) and (f): images acquired 100 and 150 μm below the scleral surface, respectively.

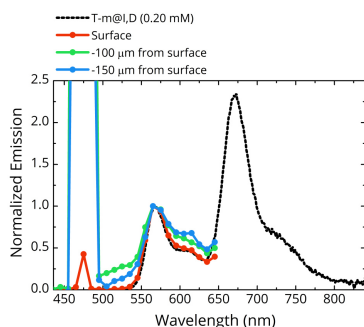


Figure 2.37: Emission spectra acquired at different depths of sclera after treatment with T-m@I,D. The corresponding TPM images are reported in Figure 2.36 and the emission of an aqueous suspension of T-m@I,D (0.20 mM) is shown as a comparison (black line). All the spectra are normalized to the emission peak of DiI.

Figure 2.38: TPM images (channels overlay) acquired exciting the sclera treated with CTAB micelles loaded simultaneously with DiI and DiD (C-m@I,D) with an incident wavelength of 950 nm. Panels(a)-(f): starting from the surface of the tissue, each image was acquired 20 μm deeper than the previous one, increasing depth from (a) to (f).

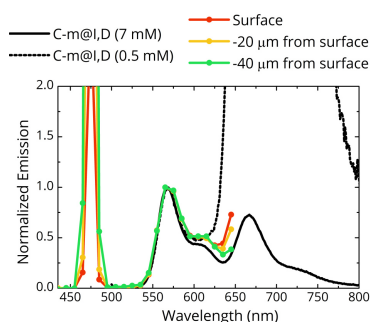
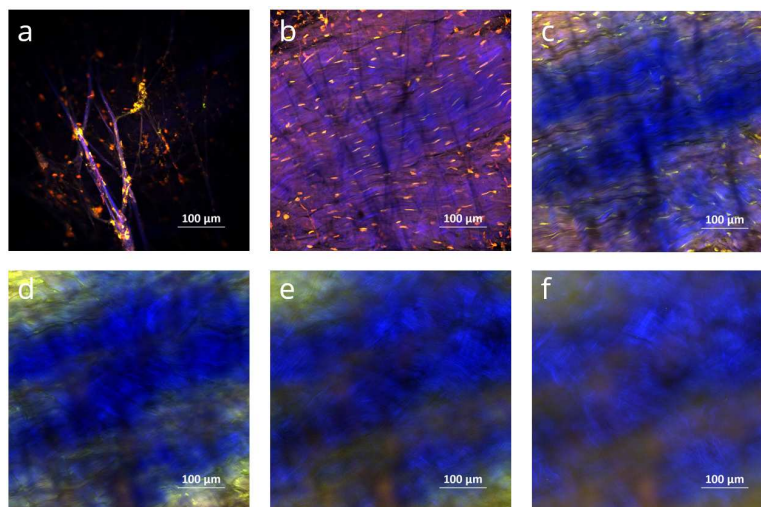


Figure 2.39: Emission spectra acquired at different depths of sclera after treatment with C-m@I,D. The corresponding TPM images are reported in Figure 2.38 and the emissions of C-m@I,D in aqueous suspensions above and below the CMC (7 and 0.5 mM) are shown as a comparison (black line). All the spectra are normalized to the emission peak of DiI.

The same measurements described above have been performed also upon the permeation of C-m@I,D through a scleral sample. In Figure 2.38 six TPM images at different depths in the tissue are reported. From the more superficial images [panels(a)-(c), up to 40 μm below the tissue surface] is possible to observe the same globular structures that we described for single dye-loaded CTAB micelles. Differently from the tissue treated with C-m@I, the globules that were detected in this case presents a much stronger signal in the red and far-red channels, suggesting efficient FRET, at least up to the first tenths of μm below the scleral surface. The globular structures are no longer visible in the TPM images recorded more than 60 μm deep inside the tissue [panels(d)-(f)], and the signal from the dyes resulted more homogeneously distributed when compared to the superficial layers of the sample.

Emission spectra recorded inside the tissue at different depths, corresponding to the TPM images in Figure 2.38, are reported in Figure 2.39. In the most superficial emission profile, a strong contribution from the low-wavelength tail of the DiD emission was detected, which seems even larger than the one observed for C-m@I,D emission in aqueous suspensions (above the CMC). This observation is in line with the disruption of micelles when in contact with the tissue, and the formation of aggregates on the scleral surface that contain both dyes boosting the FRET efficiency to higher values with respect to micelles, similarly to what we demonstrated in aqueous suspension after the dilution below the CMC. Analyzing the spectra acquired deeper inside the tissue, a gradual decrease in DiD emission and consequently, in FRET efficiency, can be observed.

In Figure 2.40 are reported two Z-scans acquired on sclera treated with T-m@I,D or C-m@I,D, in the same regions of the tissues employed to record the TPM images and emission spectra discussed before.

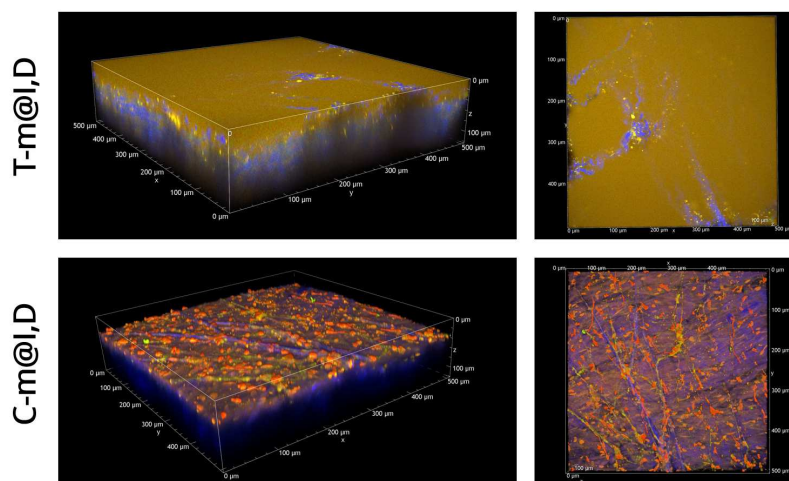


Figure 2.40: Volume renderings of the scleral tissue treated with T-m@I,D (top panels) and C-m@I,D (bottom panels) reconstructed from Z-stacks (Z step: $1.5\ \mu\text{m}$, total depth: $123\ \mu\text{m}$ for T-m@I,D and Z step: $1\ \mu\text{m}$, total depth: $121\ \mu\text{m}$ for C-m@I,D). Both 3D overviews and XY views of channels overlay are shown. Excitation wavelength: $950\ \text{nm}$.

This figure shows the different behaviour of TPGS and CTAB micelles loaded simultaneously with DiI and DiD when exposed to scleral tissue. With our experimental set-up, the analysis of FRET efficiency in biological tissues is not trivial, since the available spectral detector does not allow to collect the whole emission spectrum of the energy acceptor (DiD). However, we can exploit the different emission intensities of DiI and DiD in the green and red channels (Figure 2.24), which are associated to the non-descanned detectors that we employ to collect images: in Figure 2.41 the ratio between the red and green signals (extracted from the Z-scans in Figure 2.40) is reported as a function of the penetration depth, while exciting selectively the energy donor (DiI). In absence of FRET, this ratio is 0.9^8 (dotted black line). An increase of the ratio over 0.9 indicates a stronger emission in the red (emission of DiD, the energy acceptor), and hence the occurrence of FRET.

In the sample treated with TPGS micelles (T-m@I,D, blue line) the signal ratio starts from ~ 1.2 and decrease to 0.9 after $\sim 50\ \mu\text{m}$ from the tissue surface, suggesting a progressive reduction of FRET efficiency at greater depths. Conversely, in the case of CTAB micelles (C-m@I,D, red line), a very high value of 1.6 was detected from the more superficial region, which remained constant for the first $30\ \mu\text{m}$ and then decreased to 0.9 , the same residual value observed for the sample treated with T-m@I,D (at greater depths) and in absence of FRET from aqueous suspensions of micelles.

The higher ratio observed in the superficial tissue layers after the permeation of CTAB micelles suggests higher FRET efficiency when compared to the sample treated with TPGS micelles, even if the efficiency value calculated for the former nanocarriers in aqueous suspension is lower than the latter (see Table 2.6). This phenomenon is in line with the spectroscopic data obtained in aqueous suspension after dilution below CMC, and can be explained considering the presence of aggregates on the surface of tissue treated with CTAB, such as the globular structures observed in TPM images, that can form upon disaggregation of micelles and are characterized by very efficient FRET.

Deep inside the tissue, the ratio between the two signals becomes similar for the samples treated with CTAB or TPGS micelles and FRET is lost

8: In order to obtain the red over green signal ratio in absence of FRET, we measured an aqueous suspensions of mixed C-m@I and C-m@D micelles with our Two-photon microscope, exciting the sample at $950\ \text{nm}$. Moreover, this kind of evaluation is possible only because we kept the red and green detector gains strictly equals for all the measurements we performed. Since the emission profile in absence of FRET is not significantly different, the ratio is assumed to be the same also for TPGS.

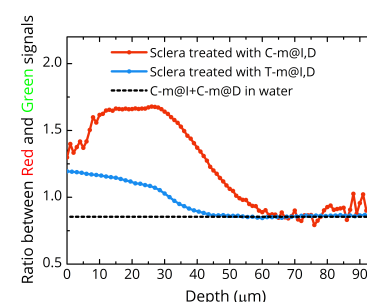


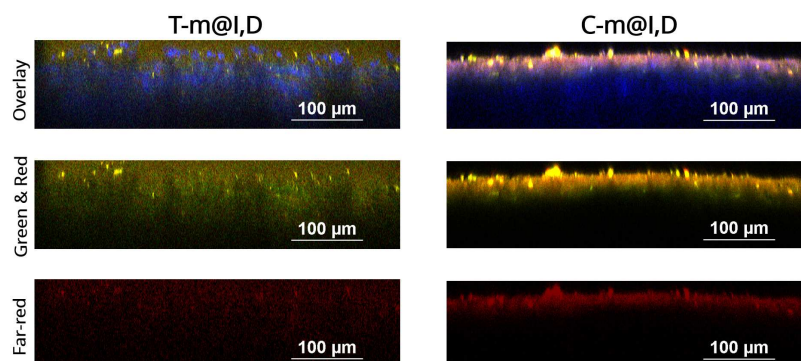
Figure 2.41: Comparison between the ratio of the signals in the red and green channels extracted from the Z-scans of sclera samples treated with T-m@I,D or C-m@I,D. The signal ratio in absence of FRET, taken from a suspension of mixed C-m@I and C-m@D, is shown as a comparison. The greater noise that characterize the orange curve at higher depths is due to very low detector gains required to prevent saturation of the detectors by the very high signal observed in correspondence of the tissue surface.

after ~ 50 or ~ 60 μm below the scleral surface, respectively. From this result, we can infer that both TPGS and CTAB micelles lose their integrity during the permeation, after more than 2 hours of contact with the tissue. In the case of C-m@I,D, following the aggregation on the surface, the residual DiI and DiD molecules are split apart and continue the permeation of the tissue separately, most probably with the aid of the residual surfactant.

In the case of T-m@I,D, the micelles may traverse the sclera intact through its pores but subsequently disassemble, allowing the two dyes to move far apart, possibly influenced by electrostatic interactions between the positively charged dyes and negatively charged groups present in the tissue. The spatial separation of the two dyes inevitably leads to a decrease in FRET efficiency in the tissues treated with both CTAB and TPGS micelles.

To provide a more detailed and visual understanding of signal distribution across different channels according with the depth in the sclera, in Figure 2.42 are reported XZ slices, extracted from the Z-scan shown in Figure 2.40. The loss of FRET over depth inside the tissues can be observed by the decrease of signal in the far-red channel (bottom panels) and from the increased green channel signal in the deeper regions (intermediate panels).

Figure 2.42: XZ views obtained from Z-stacks of the scleral tissue treated with T-m@I,D (left panels) and C-m@I,D (right panels). Z step: 1.5 μm , total depth: 123 μm for T-m@I,D and Z step: 1 μm , total depth: 121 μm for C-m@I,D. Apart from the total channels overlays, also the red and green channels overlays and the far-red channels are shown. Excitation wavelength: 950 nm.



2.6 Conclusions and perspectives

In this chapter, we presented a comprehensive experimental study of DiI and DiD photophysics in two different micellar environments, CTAB and TPGS, above and below the CMC of the corresponding surfactant, and after permeation through *ex vivo* scleral tissue.

Stable micelles were obtained and easily loaded with either DiI or DiD employing CTAB or TPGS suspensions above the CMC. Independently on the surfactant, marginal aggregation signatures in optical spectra of micelles loaded with either of the dyes suggest that a few dyes aggregate before reaching the micelles, so that at least some micelles containing two or more dyes are formed.

Below the CMC, when micelles are no longer stable, aggregation phenomena become important in the case of CTAB. Conversely, TPGS suspensions showed negligible aggregation features even after the dilution below the CMC. The quantum yields calculated for both TPGS and CTAB micelles are suitable for bioimaging purposes, but the former ones are even higher than ethanolic solutions of the two dyes. TPGS micelles seems to grant solubility even below the CMC, a greatly valuable feature for bioimaging applications where the nanocarriers often undergo extensive dilution.

A straightforward strategy to obtain both CTAB and TPGS micelles simultaneously loaded with DiI and DiD (C-m@I,D and T-m@I,D, respectively) is also presented. These nanocarriers show efficient FRET and offer an interesting tool to tune the emission of the fluorescent labels and increase the Stokes shift for reduced noise in microscopy applications. The stability of the dye-loaded micelles above the CMC is confirmed upon mixing two solutions containing micelles loaded with either DiI or DiD: signatures of FRET are not observed over days from the preparation. As expected, when T-m@I,D is diluted below the CMC, the dyes that were loaded in the same micelle above the CMC tend to move far apart, decreasing the FRET efficiency progressively with the extent of the dilution. Surprisingly, in the case of C-m@I,D the FRET efficiency considerably increases when the suspension is brought below the CMC. A possible explanation for this counterintuitive behavior is that other clusters, different from micelles, composed of DiI, DiD, and CTAB are formed below the CMC, where FRET is promoted by an increased number of dyes in the same aggregate and/or short distances among acceptors and donors. In the case of the TPGS, after the disruption of the nanocarriers, the interactions of the dyes with the TPGS surfactant prevent any form of aggregation, and the FRET phenomenon is gradually lost with dilution.

The distribution and the integrity of the two different nanocarriers were then probed by two-photon microscopy after permeation in the scleral tissue. By monitoring the variations of FRET efficiency at different depths in the biological sample, guided by the work done on aqueous suspensions.

After permeation of TPGS micelles through sclera, a gradual decrease in FRET efficiency was observed. This phenomenon is compatible with an increased distance between DiI and DiD, which could be due to the progressive disassembling of the micelles during the permeation through the tissue or to the dye leaking promoted by electrostatic interactions, such as those between the negatively charged scleral proteoglycan matrix and the two positively charged fluorophores. In any case, DiI and DiD

remained well solubilized throughout the entire volume of all the analyzed tissues, in line with the results obtained from the TPGS micelles in aqueous suspension, that showed minor aggregation effects and FRET decrease when diluted below the CMC. We assessed the integrity of the micelles after a contact period of more than two hours, hence, they might have traversed the tissue while still intact, breaking down only at a later stage.

In the case of CTAB micelles, permeation through sclera revealed significantly increased FRET efficiency in the superficial layers and a decrease in deeper regions inside the tissue, making the rationalization of the results in terms of nanocarriers integrity more difficult. However, thanks to our careful preliminary spectroscopic study, we were able to conclude that the micelles collapsed on the surface of the tissue, forming globular aggregates characterized by higher FRET efficiency when compared to the micelles in water. The disaggregation of the CTAB micelles is most probably driven by electrostatic interactions with the scleral proteoglycan matrix, causing all the components of the nanoprobe (CTAB, DiI and DiD) to be stuck on the superficial collagen fibers, forming aggregates similar to those observed when diluting C-m@I,D below the CMC.

The behavior of the two surfactants when labelled with the same FRET pair (DiI and DiD), resulted drastically different both in aqueous suspensions and during the permeation of an *ex vivo* tissue, raising an important warning for the widespread use of FRET to assess the stability of nanoparticles. Indeed, these studies can reach reliable conclusions only if a preliminary and extensive spectroscopic characterization is performed on FRET efficiency also upon controlled disaggregation.

Part of the results presented in this chapter have been published in Refs. [76, 98].

2.7 Materials and Methods

1,10-Dioctadecyl-3,3,30,30-tetramethyl-indocarbocyanine perchlorate (DiI) and 1,10-dioctadecyl-3,3,30,30-tetramethyl-indocarbocyanine perchlorate (DiD) were purchased from ThermoFisher (Invitrogen). Hexadecyltrimethylammonium bromide (CTAB, AnalaR NORMAPUR $\geq 99.0\%$) was obtained from VWR Chemicals. TPGS was purchased from PMC Isochem, (Vert-le-Petit, France). All the purchased chemicals have been employed without further purification.

2.7.1 Preparation of DiI and DiD aggregates

DiI aggregates

65 mg of a previously sonicated 1220 μM stock solution of DiI in ethanol was put into a dark vial. Ethanol was added until reaching 1.5 g of mass, followed by bidistilled water being rapidly added at room temperature until the mixture was 5 g in weight, in order to obtain a 70/30 (m/m) mixture of water/ethanol. The resulting mixture was homogenized at the vortex for 40 s.

DiD aggregates

149 mg of a previously sonicated 530 μM stock solution of DiD in ethanol was put into a dark vial. Ethanol was added until reaching 0.5 g of mass, and then bidistilled water was rapidly added until the mixture was 5 g in weight, in order to obtain a 90/10 (m/m) solution of water/ethanol. The resulting mixture was homogenized at the vortex for 40 s.

2.7.2 Aggregates in the gel

0.5 g of agarose powder was weighed in a 50 mL beaker, and 25 mL of bidistilled water was added, forming a white suspension. The suspension was heated up to its boiling point. After boiling for 20 min, the agarose powder was completely dissolved, and a water-clear solution was obtained. The agarose solution was cooled down until it reached 40 °C, then 1 mL of aggregates suspension in water/ethanol mixture was added. The resulting suspension, containing the agarose and the aggregates, was poured into a plastic cuvette to register UV-vis absorption and in a small circular plastic holder for linear fluorescence and two-photon excited microspectroscopy (shown in Figure 2.43). We point out that at 40 °C the suspension is still liquid, and the hydrogel is then obtained after cooling down at room temperature. The temperature at which aggregates are added is crucial: if it is too high, then they could break or modify; if it is too low, then they would not diffuse homogeneously into the bulk due to its high viscosity.

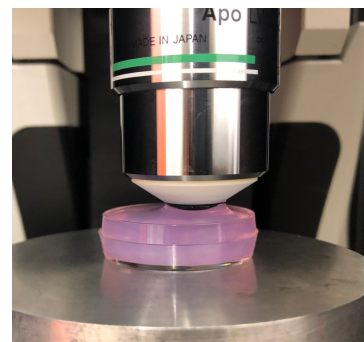


Figure 2.43: DiI aggregates in agarose hydrogel placed under the two-photon microscope inside a small circular plastic holder.

2.7.3 Preparation of dye-Loaded CTAB and TPGS micelles

Preparation of CTAB Micelles

A 7.8 mM suspension of CTAB micelles was prepared by dissolving CTAB in bi-distilled water. The suspension was heated at 38 °C for 30 minutes before use.

Preparation of CTAB micelles loaded with DiI or DiD

3 mL of a solution with 100 μ M DiI or DiD in ethanol was prepared. The solution was heated at 38 °C for 10 minutes, and then it was slowly added to 25 mL of the CTAB micelles suspension (7.8 mM CTAB concentration). In the resulting suspension (11% V/V ethanol/water), the CTAB concentration is 7 mM, and the dye concentration is 11 μ M. All diluted suspensions (named CTAB 2 mM, CTAB 1 mM, CTAB 0.5 mM, and CTAB 0.25 mM) were obtained by diluting the original 7 mM suspension with bi-distilled water.

Preparation of CTAB micelles loaded with DiI and DiD

3 mL of a solution with 50 μ M DiD and 50 μ M DiI was prepared in ethanol. The solution was heated at 38 °C for 10 minutes and then slowly added to 25 mL of the CTAB micelles suspension at 7.8 mM CTAB concentration. The resulting suspension (11% V/V ethanol/water) contains CTAB 7 mM and the two dyes (DiI + DiD) with a total concentration of 11 μ M. All subsequent suspensions (CTAB 2 mM, CTAB 1 mM, CTAB 0.5 mM, and CTAB 0.25 mM) were obtained by diluting the original 7 mM CTAB/11 μ M dye suspension with bi-distilled water.

Preparation of TPGS micelles loaded with DiI and DiD

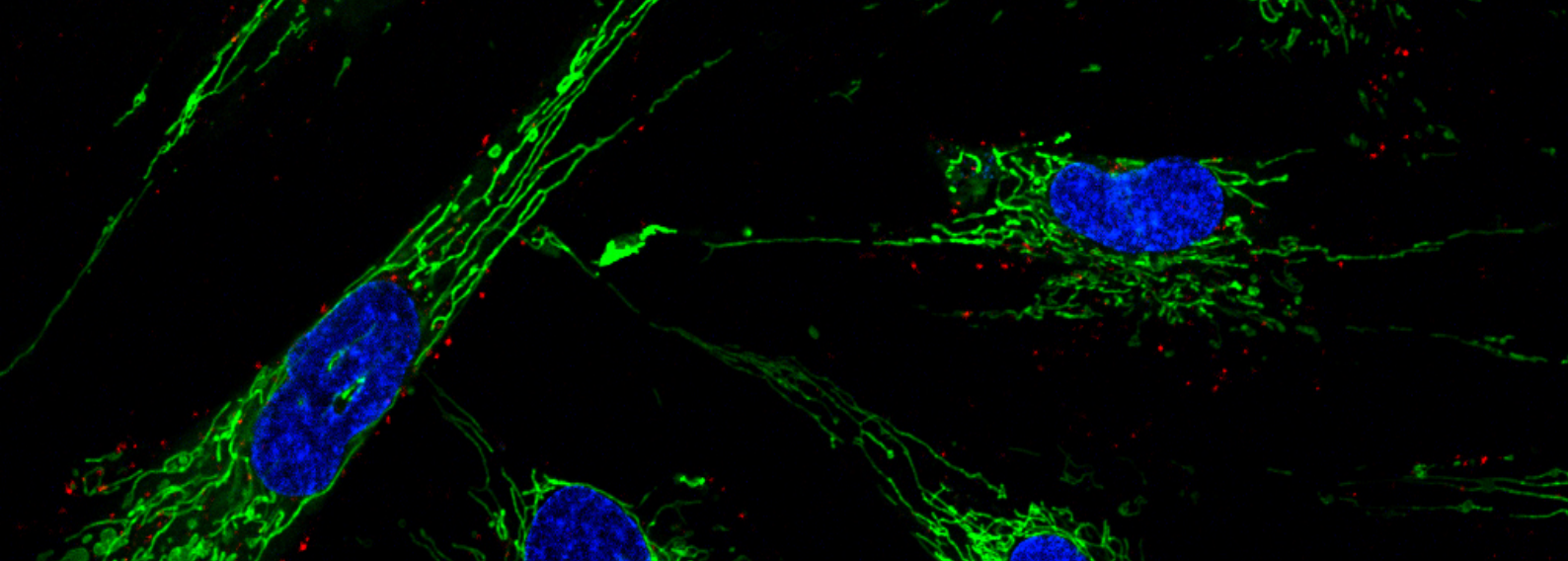
720 μ L of a solution with 280 μ M DiD and 280 μ M DiI was prepared in ethanol. The solution was heated at 38 °C for 10 minutes and then slowly added to 6 mL of the TPGS micelles suspension at 22 mM TPGS concentration. The resulting suspension (11% V/V ethanol/water) contains TPGS 20 mM and the two dyes (DiI + DiD) with a total concentration of 60 μ M. All subsequent suspensions (TPGS 0.2 mM, TPGS 0.05 mM, TPGS 0.01 mM, and TPGS 0.002 mM) were obtained by diluting the original 20 mM TPGS/60 μ M dye suspension with bi-distilled water.

Preparation of TPGS micelles loaded with DiI or DiD

720 μ L of a solution with 560 μ M DiI or DiD in ethanol was prepared. The solution was heated at 38 °C for 10 minutes, and then it was slowly added to 6 mL of the TPGS micelles suspension (22 mM TPGS concentration). In the resulting suspension (11% V/V ethanol/water), the TPGS concentration is 20 mM, and the dye concentration is 60 μ M. All diluted suspensions (TPGS 0.2 mM, TPGS 0.05 mM and TPGS 0.01 mM) were obtained by diluting the original 20 mM suspension with bi-distilled water.

Preparation of Mixture of Micelles Loaded with DiI and Micelles Loaded with DiD

The mixtures of DiI and DiD loaded micelles were obtained by mixing equal volumes of the solutions containing micelles loaded with either DiI or DiD.



3 Fluorescent Quatsomes for bioimaging applications

3.1 Introduction

Nanovesicles (NVs) are self-assembled organic nanoparticles, characterized by a double-membrane structure resembling cellular lipid bilayers and encapsulating a water-filled compartment known as lumen.[102] Liposomes, considered the earliest form of nanovesicles (NVs), have been widely explored as potential candidates for drug delivery, diagnostic and/or theranostic applications and some liposome-based drug products successfully transitioned from the lab-bench to the commercial market.[103–105] Their success is attributed to the ability of encapsulating both hydrophobic and hydrophilic molecules, providing efficient transport and protection within the body, facilitating targeted delivery. However, liposomes still face some intrinsic challenges, including limited shelf-life, poor colloidal stability because of their fluid nature, and restricted and expensive conditions of preparation due to their fundamental constituents (phospholipids). The advent of innovative self-assembly tools has significantly advanced the field of NVs design and synthesis, and non-liposomal NVs have been recently developed.[106] This new generation of nanovesicles may complement liposomes, addressing many of their limitations.

In the wide domain of non-liposomal NVs formulations, Quatsomes (QS) stand out for their remarkable homogeneity, stability, and cost-effectiveness.[107] Developed by Nanomol-Bio group at the Institut de Ciència de Materials de Barcelona (ICMAB) of CSIC, these thermodynamically stable nanostructures are composed by ionic surfactants (originally quaternary ammonium salts, hence the name) and sterol derivatives. Quatsomes are classified as small unilamellar nanovesicles (SUVs), since they are characterized by a diameter less than 200 nm and formed by a single lipid bilayer. They appear as highly uniform vesicles, that maintain their structure over time, even under varying conditions such as pH,

3.1 Introduction	53
3.2 Quatsomes decorated with a hydrophilic donor and a hydrophobic acceptor . . .	57
3.3 DiR-loaded MKC-Quatsomes for specific targeting	72
3.4 Conclusions and perspectives	92
3.5 Materials and Methods . .	94

1: These multi-step and time-consuming procedures have also a high risk of damaging the functionality of bioactive molecules, if present, and of oxidizing the vesicle membrane components.

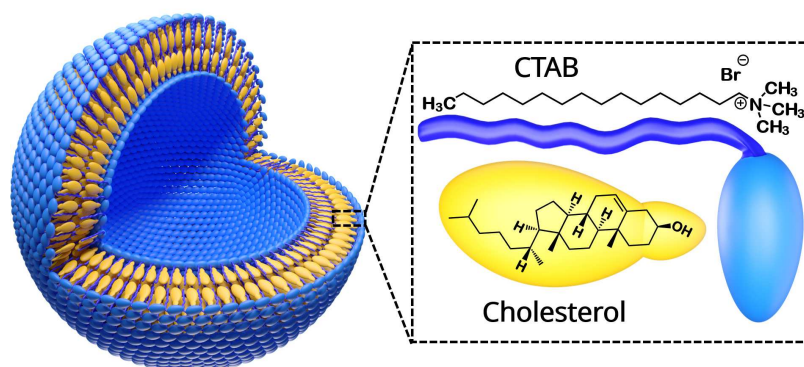
2: Interestingly, differently from the conventional methodologies previously mentioned, this technique allows efficient incorporation of cholesterol, due to the absence of a free-solvent step.[117] However, this novel process still present some limitations, such as the elevated pressures required.

temperature, salt concentrations, and dilution (even going below the critical micellar concentration of the surfactant component).[94, 108, 109] Quatsomes and NVs in general can be prepared using several different techniques, such as thin-film hydration[110, 111], reverse-phase evaporation,[112] and ethanol injection.[113] Most of these procedures involve a solvent-free step at some point, which can be problematic in the case of Quatsomes due to the formation of sterol-rich domains able to hinder the self-assembly process. Furthermore, post-formation steps like sonication[114] and extrusion[115] are often required in order to precisely control the size distribution of the resulting NVs and to reduce the vesicle to vesicle structural heterogeneity¹. To overcome these drawbacks, the Nanomol-Bio group of ICMA-B-CSIC developed and patented a new procedure based on the use of compressed CO₂ for the preparation of colloidal suspensions: the depressurization of an expanded organic solution-suspension (DELOS-SUSP) process.[116]

The DELOS-SUSP methodology (described in detail in Section 3.5.1) is a simple, robust, scalable, and one-step process to prepare QSs and a wide variety of SUVs with different functionalities and high structural homogeneity²[116, 117]

The most studied Quatsomes are composed of CTAB, a quaternary ammonium surfactant, and cholesterol (Figure 3.1). It is worth mentioning that none of the individual components of these nanostructures spontaneously aggregate into vesicles in water, since CTAB forms micelles (as demonstrated in Chapter 2) and the insoluble cholesterol forms crystals. But when they are placed together in aqueous environment in an equimolar ratio, they form stable nanovesicles called CTAB-Quatsomes (average size around 60 nm).[107, 117, 118]

Figure 3.1: Schematic representation of CTAB-Quatsomes composed by the association of cetyltrimethylammonium bromide (CTAB) and cholesterol. The molecular structures of the components are also shown.



As shown in Figure 3.1, CTAB and cholesterol are self-assembled in a quasi-cylindrical bimolecular synthon, that works as a single entity, analogously to a double-tailed amphiphilic. In this organization, studied by molecular dynamics simulations (MD), the hydroxyl group of cholesterol establishes ion-dipole interactions with the hydrophilic positively charged head of CTAB, while both components point towards the aqueous media. Due to the cationic ammonium groups, both the inner and outer surfaces of CTAB-Quatsomes have positive charges. Meanwhile, the hydrophobic tail of the CTAB and the cholesterol's backbone compose the hydrophobic region of the membrane³. [118, 119]

Similarly to liposomes, CTAB-Quatsomes represent ideal platforms to encapsulate a wide variety of both hydrophobic and hydrophilic substances. They have been employed for successfully entrapping hydrophobic

3: The self-assembly of sterol/quaternary ammonium surfactant mixtures into exceptionally homogeneous and stable bilayer vesicles has to be attributed to a strong synergy between the two molecular entities.[107]

fluorescent dyes like fluorene derivatives[108] and diketopyrrolopyrroles,[120] or larger compounds like silicon nanocrystals.[121] Moreover, CTAB-Quatsomes have been decorated also with both small hydrophilic molecules like fluorescein,[122] and larger ones, such as epidermal growth factor[123] and bovine serum albumin[124] proteins.

In our research lab, fluorescent Quatsomes are investigated since many years thanks to a fruitful collaboration with ICMAB-CSIC and Nanomol Technologies S.L. in Barcelona, Spain. In 2018, a new family of fluorescent quatsomes loaded with three commercial indocarbocyanines (DiI, DiD and DiR) were proposed as stable nanovesicles for bioimaging.[94] The detailed investigations of these nanoparticles, including molecular dynamics simulations, showed that the alkyl chains of the indocarbocyanines are located within the membrane, while the chromophoric core is exposed to water. In a recent paper,[125] it was demonstrated that quatsomes are efficient platforms for FRET: DiI and DiD were loaded simultaneously in the same nanoparticle, and FRET efficiency depends on the local concentration of dyes, with sizeable effects attributed to dye aggregation. Quatsomes were also incubated with fluorescein, with the aim to produce fluorescent nanoparticles with the fluorophore covering the external membrane: these systems showed good emissive properties, but poor stability. [122]

In Section 3.2, the preparation and characterization of new fluorescent CTAB-Quatsomes loaded with a FRET pair are addressed. The acceptor molecule consists in a cyanine residing in the membrane (DiD) while the donor is a hydrophilic dye (Rhodamine B isothiocyanate or Eosin Y). Samples were tested to determine whether it is possible to obtain Qs in which the hydrophilic energy donor is loaded in the lumen and/or attached to the membrane. Two different preparation procedures were tested: directed loading during the preparation of Qs, and post-processing incubation. From the spectroscopic characterization results and the comparison between the two preparation methodologies, we concluded that the two dyes are mostly interacting with the QS membrane. In the case of the samples decorated with Eosin Y, high fluorescence quantum yields and efficient FRET were observed, independently on the preparation procedure, making these nanovesicles promising tools for bioimaging applications.

The work discussed in Section 3.2 has been performed in collaboration with Dr. Guillem Vargas Nadal, post-doc researcher at the University of Parma at the time and expert in the preparation and fluorescent labelling of nanovesicles. The preparation and preliminary physicochemical characterization of the Qs were carried out under the supervision of Prof. Nora Ventosa and Dr. Santi Sala during two secondments in the laboratories of Nanomol Technologies S.L., in Barcelona (Spain). The preparation and the study of the dye-incubated samples was performed together with Giacomo Cotelli, in the context of his master thesis.

Section 3.3 concerns multifunctional myristalkonium chloride (MKC) Qs loaded with a near infrared fluorophore (DiR) for *in vitro* specific targeting. The external surface of the NVs was functionalized with two different ligands to achieve preferential internalization into specific cells. MKC-Quatsomes have been extensively characterized spectroscopically and proved capable of bringing DiR in aqueous media without degrading its spectroscopic properties, obtaining fluorescence quantum yields suit-

able for microscopy applications. Confocal microscopy measurements were then performed after incubation of two different cell lines with the MKC-Quatsomes. The preferential internalization (in one specific cell line) of the Qs decorated with the two ligands has been confirmed through imaging of both live and fixed cells, by quantification of the integrated DiR intensity per cell, and by comparing the results with images obtained with not functionalized MKC-Quatsomes.

The development, synthesis and preliminary physicochemical characterization of the MKC-Qs were done by Sara Garrido from ICMAB/CSIC. The spectroscopic characterization of the Qs, cell cultures, *in vitro* experiments and confocal microscopy measurements were performed in collaboration with Sara Garrido, during a secondment in the laboratory of Prof. Kevin Belfield (New Jersey Institute of Technology), in Newark (U.S.).

All the work described in this chapter was done in the context of and received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 101007804 (Micro4Nano).

3.2 Quatsomes decorated with a hydrophilic donor and a hydrophobic acceptor

As extensively demonstrated in Chapter 2 and Ref.[98], in order to employ the FRET phenomenon as integrity marker for nanoparticles, a detailed spectroscopic investigation is required in controlled conditions, also upon disaggregation of the nanoparticles. Indeed, if a lipophilic D-A pair is selected, the dyes may remain in close contact after disaggregation of the carriers in aqueous environment, leading to significant FRET even when the integrity of the initial system is compromised.

In the case of Quatsomes, the disassembling of nanovesicles may result in the formation of micelles or other water-soluble aggregates containing the loaded lipophilic dyes, surfactant, and possibly cholesterol. A strategy to overcome these issues could be the loading in the nanovesicles of at least a water-soluble FRET moiety, ideally localized in the lumen. This approach should ensure the dispersion of the water-soluble dye in the solvent upon vesicle destruction, rapidly preventing FRET in the disaggregated system. Moreover, the simultaneous loading of QSs with dyes strategically positioned in distinct compartments of the nanovesicles, represents a novel achievement that has not been previously demonstrated.

For this purpose, we selected two distinct hydrophilic energy donor molecules, Eosin Y (referred to as EoY) and Rhodamine B isothiocyanate (referred to as RhB), to be loaded inside CTAB/cholesterol QSs as part of a FRET pair along with DiD, the energy acceptor. EoY and RhB belonging to the xanthene dye family, stand out not only for their water solubility but also for their good photostability and fluorescence quantum yields[126, 127]. These dyes have found widespread applications in various research fields, including bioimaging[128, 129], and as reliable spectroscopic probes.[130, 131] The hydrophobic cyanine DiD is already known for its capability of nanostructuring itself inside the Quatsomes membranes and is a good acceptor of energy for both the donor molecules that we selected, as highlighted in Figure 3.2. The choice of CTAB/cholesterol QSs as a platform for this study is due to their high stability over time, robustness in morphology and vesicle homogeneity in terms of size, lamellarity and membrane supramolecular organization. [107, 116, 117] The molecular structures of the dyes selected for being loaded in Quatsomes nanovesicles are reported in Figure 3.3.

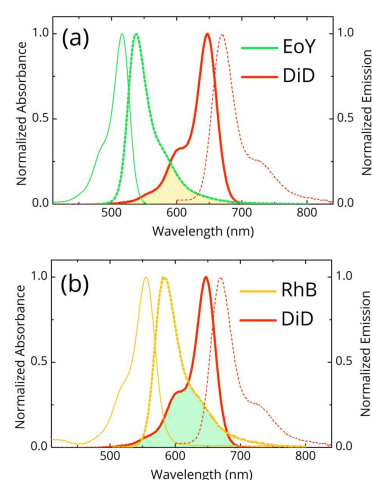


Figure 3.2: Absorption (continuous lines) and emission (dotted lines) spectra of EoY [Panel(a)] and RhB [Panel(b)] in milliQ water (pH ~6) and DiD in ethanol. The spectral overlap between the emission of EoY or RhB (energy donor) and the absorption of DiD (energy acceptor) is highlighted.

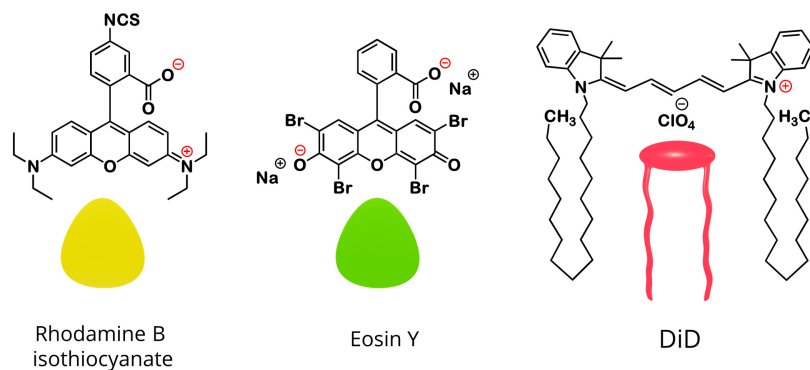


Figure 3.3: Molecular structures and schematic representation of the dyes discussed in this chapter: Eosin Y (EoY) and Rhodamine B isothiocyanate (RhB) as donors of energy and DiD as acceptor.

The preparation of QSs suspensions was accomplished through the DELOS-susp compressed-fluid technique, as detailed in Section 3.5. However, two different strategies have been employed to decorate the

nanovesicles with the the donor dyes: direct loading during the DELOS-susp or incubation.

The samples obtained with the two procedures will be referred to as dye-loaded QSs and dye-incubated QSs, respectively. During the direct loading procedure, EoY or RhB were added to the organic phase employed in the DELOS-susp methodology, while the incubation involved the addition of EoY or RhB to an aqueous suspensions of pre-formed QSs. Further details on the two preparation procedures are reported in Section 3.5.1. All the samples were then purified via diafiltration (Section 3.5) and characterized via DLS, ELS, cryogenic Transmission Electron Microscopy (cryoTEM), UV-visible and fluorescence spectroscopy (details about these techniques are reported in Section T.1 and Section T.2).

The prepared samples and the corresponding theoretical concentrations, based on the addition of the dyes stock solution, are listed below.

Samples containing only the donor dye:

- ▶ **QS@EoY-L** – QSs with EoY at a low concentration (138 μM)
- ▶ **QS@EoY-H** – QSs with EoY at a high concentration (320 μM)
- ▶ **QS@RhB-L** – QSs with RhB at a low concentration (215 μM)
- ▶ **QS@RhB-H** – QSs with RhB at a high concentration (520 μM)

Samples containing both the donor and acceptor dyes:

- ▶ **QS@DiD-EoY-L** – QSs with DiD (100 μM) and EoY at a low concentration (138 μM)
- ▶ **QS@DiD-EoY-H** – QSs with DiD (100 μM) and EoY at a high concentration (320 μM)
- ▶ **QS@DiD-RhB-L** – QSs with DiD (100 μM) and RhB at a low concentration (215 μM)
- ▶ **QS@DiD-RhB-H** – QSs with DiD (100 μM) and RhB at a high concentration (520 μM)

All the samples listed above have been prepared following both the direct addition and the incubation procedures, with the exception of QS@DiD-RhB-L, that was prepared only by incubation. The higher concentrations chosen for RhB containing samples are due to the lower encapsulation efficiency associated with those samples, as demonstrated in Section 3.2.3. By increasing the initial amount of RhB in the suspensions, we aimed to achieve concentrations comparable to those observed for samples containing EoY. The dyes concentrations measured after the preparation and purification of the samples are reported in Table 3.2.

In addition to their water solubility, EoY and RhB bear ionizable fragments (negatively charged after deprotonation) and can interact electrostatically with the inner and outer surfaces of QSs, which are positively charged because of CTAB and, if present, DiD. Moreover, the lipophilic xanthene core that characterizes both EoY and RhB, may lead these dyes to enter the membrane and interact with it due to hydrophobic effect⁴. Also, MD studies[119] showed a similar behavior for dianionic fluorescein (non-brominated structural analogue of EoY), which was able to be adsorbed in the polar region of the CTAB/cholesterol QS membrane.

Thanks to their physico-chemical properties, EoY and RhB can be reasonably loaded in QSs in different regions (Figure 3.4): they can be dispersed in the aqueous lumen, electrostatically attached to the membrane surface,

⁴: It has been demonstrated that xanthene dyes are able to penetrate the polar region of bilayers composed of cationic derivatives of phosphocholine.[132]

or inside the bilayer. Moreover, due to their solubility in water, they could also be found as free dye in the outer solution.

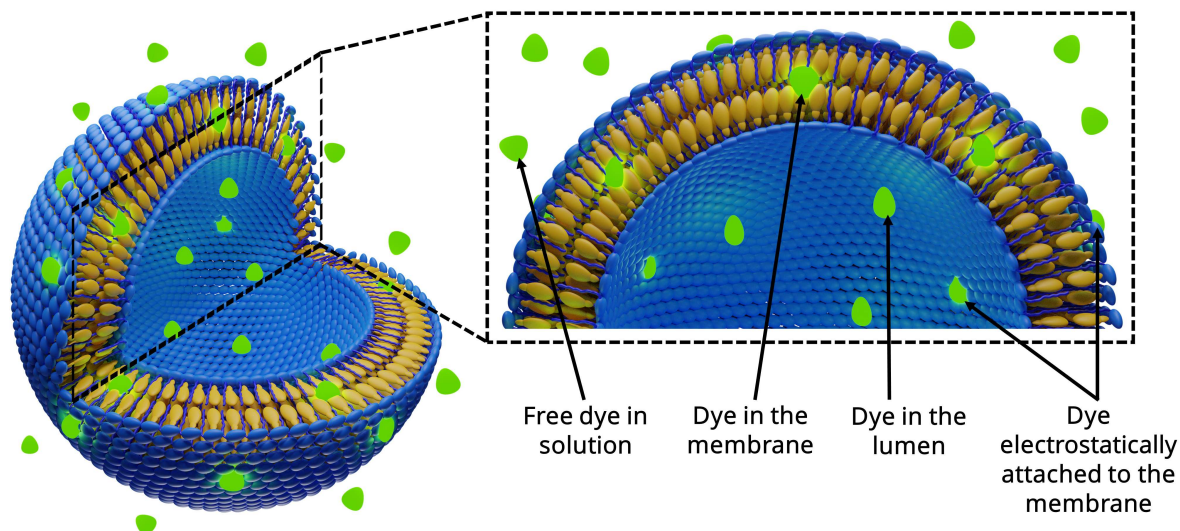


Figure 3.4: Summary of the possible dye locations in dye-loaded QS samples prepared with EoY and RhB through the DELOS methodology.

For samples obtained through the incubation procedure, the inner lumen of the QSs should not be accessible to the dyes, that can either attach to the external surface of the nanovesicles and/or enter the bilayer membrane.

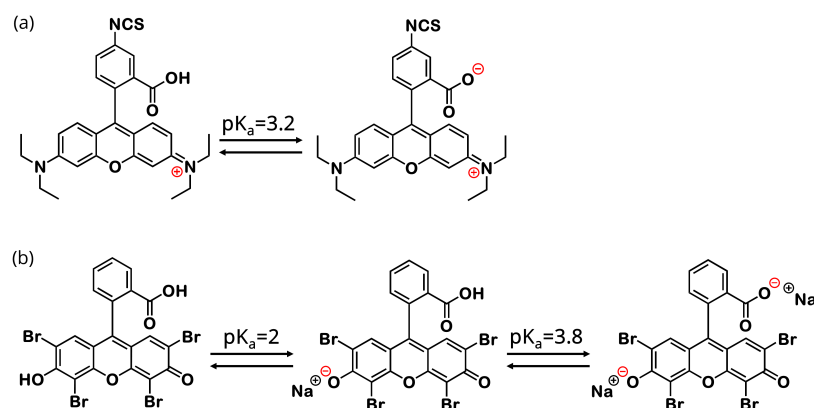
3.2.1 Spectroscopic properties of EoY and RhB at varying pH

Before discussing the dye-loaded Quatsomes suspensions, it is important to highlight that the two xanthene-based donors EoY and RhB differ in their charge state and acid-base properties:

- EoY possesses two acidic groups with pK_a of 2.0 and 3.8[133];
- RhB possesses one acidic group with pK_a of 3.2[134].

From these pK_a values, we can infer that at pH $\approx$ 5-6, roughly the pH of diafiltrated QSs samples, EoY is mostly in the di-anionic form, while RhB is in the zwitterionic, globally-neutral form. The charge state of the EoY and RhB can influence the electrostatic interactions between the dyes and the QS surface: specifically, electrostatic interactions with the cationic QS surface are expected to be stronger with di-anionic EoY than with overall-neutral RhB.

Figure 3.5: Deprotonation/protonation equilibria for RhB (a) and EoY (b) in water. The corresponding pK_a are shown.



Moreover, the protonated and deprotonated forms of EoY and RhB (Figure 3.5) may potentially exhibit different spectroscopic properties. In order to study the pH-dependence of the spectroscopic properties, two different buffers solutions were selected:

- acetic acid/sodium acetate buffer solution at pH 4.8;
- PBS buffer solution at pH 7.4

The resulting fluorescence quantum yields (Φ), extinction coefficients (ϵ) and fluorescence lifetimes (τ) were measured for the two dyes after solubilization in the buffer solutions and are reported in Table 3.1.

Table 3.1: Spectroscopic properties of EoY and RhB at different pH.

	Eosin Y			Rhodamine B		
	ϵ (M ⁻¹ cm ⁻¹)	ϕ (%)	τ (ns)	ϵ (M ⁻¹ cm ⁻¹)	ϕ (%)	τ (ns)
AcOH/ AcONa (pH 4.8)	67 600	14	1.18	41 900	15	1.26
PBS (pH 7.4)	66 500	20	1.17	35 600	25	1.32
Distilled water (pH $\approx$ 6)	-	25	1.13	-	17	1.58

Consistently with literature[135], negligible differences in the molar extinction coefficient are observed for EoY between pH 4.8 and 7.4, possibly because EoY is already fully deprotonated at pH 4.8. The extinction coefficient of RhB slightly decreases at increasing pH. An increase in

the fluorescence quantum yields (Φ) at increasing pH is observed for both dyes, while no significant variations are detected for fluorescence lifetimes depending on pH. The bandshapes and peak positions of the absorption, emission and excitation spectra are unaffected by pH for both dyes (data not shown).

3.2.2 Physicochemical characterization of Quatsomes

In order to verify whether the interactions between the two donor dyes (RhB and EoY) and the Quatsomes affects their colloidal stability, size distribution and ζ -potential, we monitored the produced batches of dye-loaded and dye-incubated nanovesicles, in the presence and absence of DiD, through DLS and ELS measurements. Nanovesicles were studied both before and after the diafiltration process, without dilution, and the results obtained from the diafiltered suspensions are reported in Figure 3.6.

Figure 3.6: Hydrodynamic diameter, Pdl, and apparent ζ -potential of dye-loaded (yellow columns) and dye-incubated (blue columns) samples after diafiltration. The error bars represent the corresponding standard error of the measurement mean (SEM). Measurements were performed at 25°C as described in Section T.1.

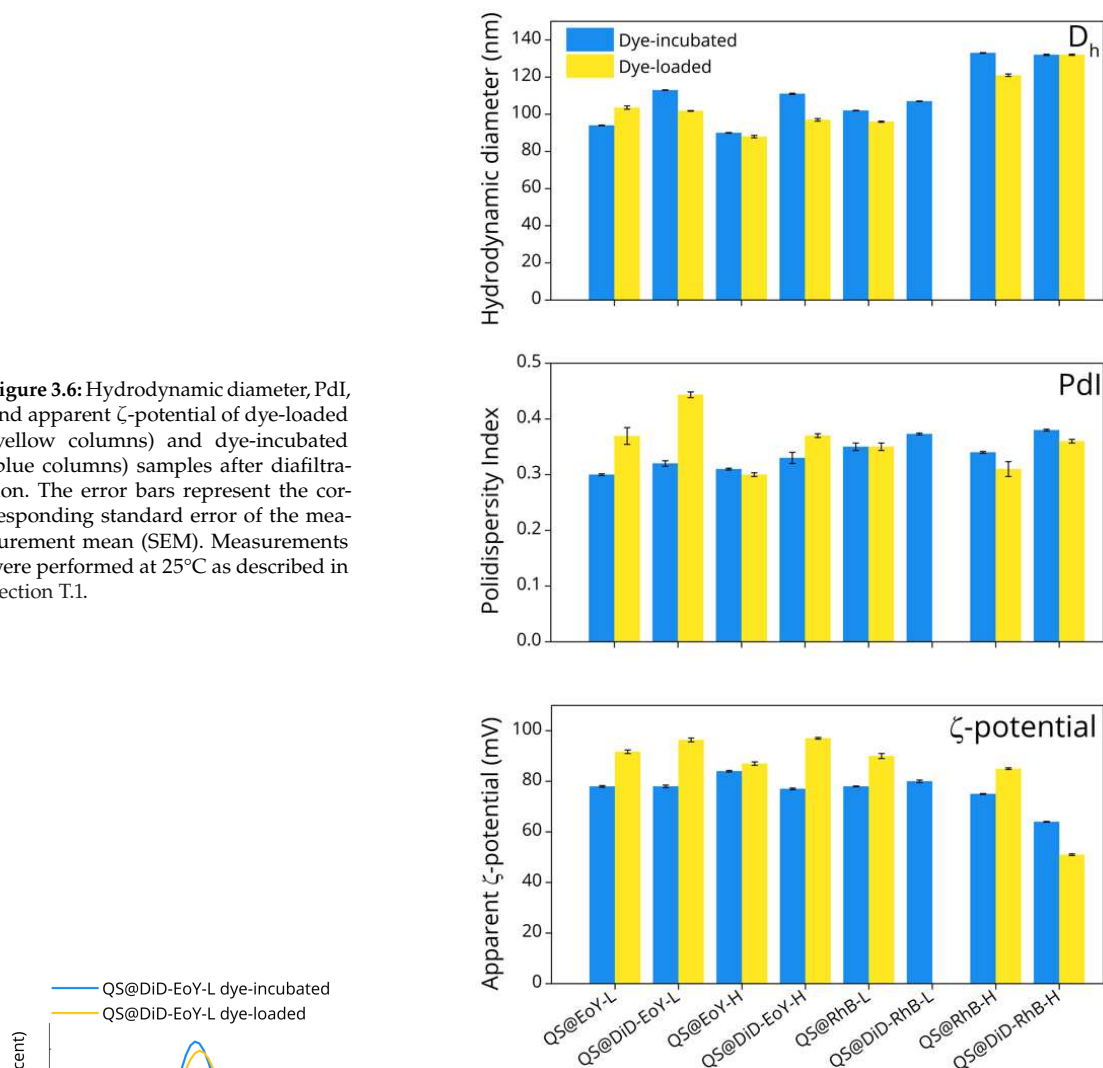


Figure 3.7: Comparison between particle size distributions by intensity of QS@DiD-EoY-L prepared through direct loading and incubation procedures. The results are the average of three independent measurements.

The measured hydrodynamic diameters (expressed as Z-average) and Pdl of all the samples fall within the usual range for CTAB/cholesterol QSs (~90 nm and < 0.4, respectively), with the exceptions of the ones containing Rhodamine B at high concentrations: QS@RhB-H and QS@DiD-RhB-H, that showed a D_h of about 130 nm. The size of the NPs is not affected by the preparation method (see Figure 3.7 for a comparison between two particle size distributions as an example).

The colloidal stability of all the prepared suspensions is ensured by the

quite high ζ -potentials (~ 80 - 90 mV), typical of the positively charged CTAB/Cholesterol QSs, even if in the case of QS@DiD-RhB-H a value below the average was detected (~ 50 - 60 mV). The measured ζ -potentials resulted systematically lower in the case of the suspensions prepared through incubation with the donor dyes (yellow columns, bottom panel in Figure 3.6), with the exception of QS@DiD-RhB-H, that showed the opposite behavior. This result is in line with the shielding effect exerted by the negatively charged EoY over the cationic CTAB, as observed in the case of fluorescein-decorated CTAB/cholesterol QSs.^[122] Overall, any significant difference in the colloidal properties of the suspensions was detected after diafiltration.

More data on the actual morphology of individual Quatsomes were obtained through cryogenic Transmission Electron Microscopy (cryoTEM). This technique enables the direct observation of nanoscale objects, whether synthetic or biological, without the need for fixation or staining.⁵

Only the dye-loaded samples were analyzed by cryoTEM and some representative micrographs are reported in Figure 3.8.

⁵: Prior to cryoTEM, specimens were rapidly frozen to temperature of 94 K (liquid ethane), preserving their natural state in a thin layer of vitreous ice. The cryoTEM images can indeed provide detailed insights on supramolecular structures and aggregation in their native environment, with nanometric resolution.

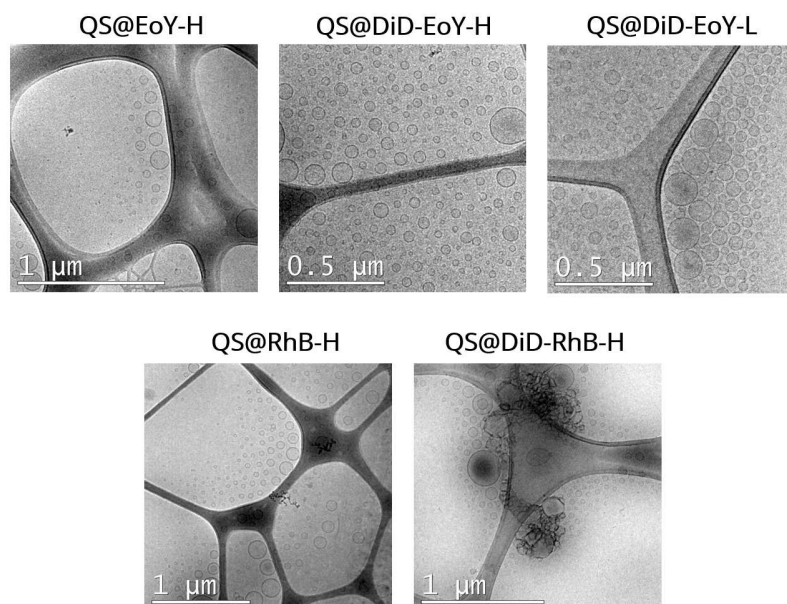


Figure 3.8: CryoTEM micrographs of Quatsomes obtained directly loading the donor dyes during the DELOS-SUSP procedure. All the samples have been diafiltered before measuring. Images acquisition and sample preparation were performed with the help of the Servei de Microscòpia at the Universitat Autònoma de Barcelona.

Independently on the donor dye and on the presence of DiD, QSs observed in the cryoTEM images appeared as spherical and closed unilamellar vesicles. No patches formed by the dyes were observed on the surface of the nanovesicles. Even if the morphology of the majority of the QSs was not affected by the dyes nanostructuration, in the case of QS@DiD-RhB-H and QS@DiD-RhB-H we observed many distorted and collapsed vesicles, as if RhB acted as a "glue" between them.

3.2.3 Encapsulation efficiency estimation

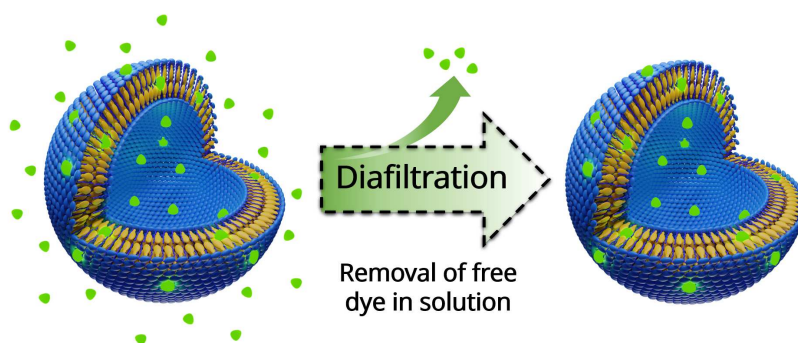
⁶: The absorption spectra recorded in ethanol (not shown) correspond to those of the isolated dyes in ethanol.

The actual concentration of the dyes in suspension was measured using the Lambert-Beer law (Section 3.5), after breaking down the QSs by diluting the samples in ethanol, which grants the full solubilization of the components.⁶ These data were used to obtain the Encapsulation Efficiency (E.E.), defined as the fraction of RhB, EoY and DiD that is retained in the suspensions after the diafiltration process, calculated with the following equation:

$$E.E. = \frac{[dye]_{after}}{[dye]_{before}} \times 100 \quad (3.1)$$

where *before* and *after* refer to the diafiltration process that followed the incubation or direct loading procedures. The diafiltration should indeed completely remove the free dyes in solution, as schematically represented in Figure 3.9, and may at least partially eliminate the dyes electrostatically attached to the outer surface of the nanovesicles.

Figure 3.9: Schematic representation of the diafiltration process, that results in the removal of the free dye in solution.



Dyes concentrations calculated from all the diafiltered QSs suspensions are reported in Table 3.2, while the corresponding E.E. of donor dyes are showed in Figure 3.10. Upon diafiltration, RhB is eliminated more efficiently than EoY, which on the contrary is not removed by a relevant amount. Independently on the preparation procedure, for both DiD and EoY we observed E.E. in the range of 80-90%, which is comparable to the results achieved in previous studies on CTAB/cholesterol QSs loaded with DiI and DiD[122, 125]. In the case of RhB, E.E. lower than 60% were observed for all the samples, with particularly low values for the ones loaded with high amount of donor dye (30-40 %). RhB-decorated QSs showed scarce reproducibility in the dye-loading results, but no significant difference was observed between the direct dye-loading and dye-incubation methodologies.

The reduced encapsulation of RhB is probably related to its permanent positive charge, which is responsible for electrostatic repulsion with the cationic surfactant of the vesicles. The same effect was not observed for the positively charged DiD molecules, most likely due to the presence of the long alkyl chains that favor the nanostructuration of the dye into the membrane. On the other hand, the anionic EoY can interact strongly with the cationic CTAB contained in QSs, increasing the efficiency of encapsulation. The similarity between the E.E. obtained from samples prepared through incubation and direct dye-loading suggests that the

majority of the donor dyes molecules are actually attached to the membrane of the nanovesicles or located within it. Indeed, the QSs lumen should not be accessible during the incubation protocol.

Both EoY and RhB-decorated QSs prepared in the context of this thesis presented higher E.E. with respect to fluorescein-incubated QSs obtained in previous studies[122]. In the case of fluorescein-incubated QSs, no separation of the free dye in solution was possible through diafiltration without completely removing all the dye molecules attached to the nanovesicles surface.

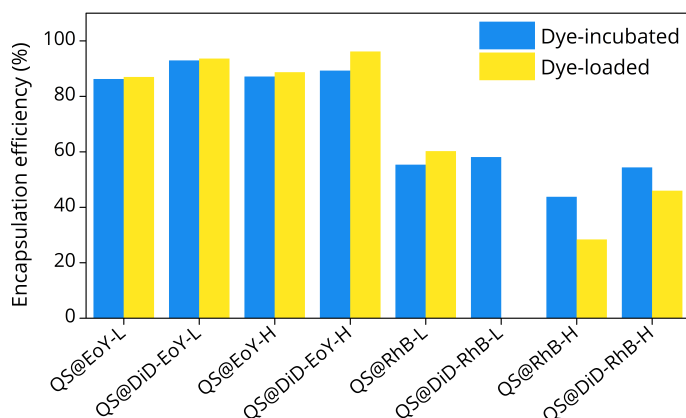


Figure 3.10: Encapsulation efficiencies of donor dyes, expressed as percentage, calculated for donor-incubated (blue columns) and donor-loaded (yellow columns) samples. The E.E. has been calculated as reported in Equation 3.1.

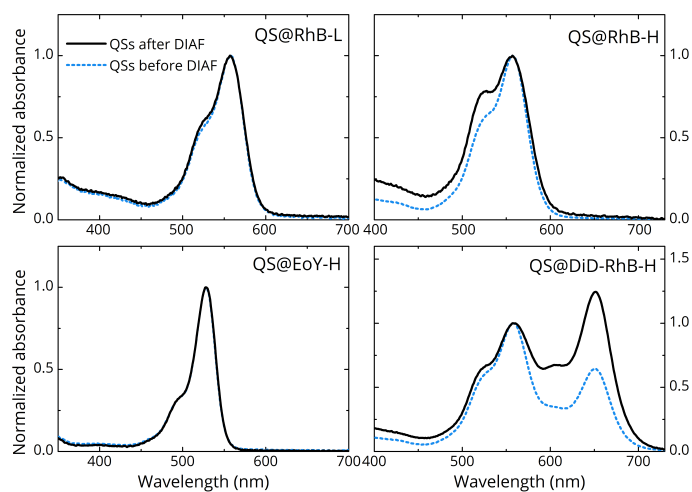
Sample label	Dye-loaded			Dye-incubated		
	Donor (μM)	DiD (μM)	Donor/DiD ratio	Donor (μM)	DiD (μM)	Donor/DiD ratio
QS@-EoY-L	113	-	-	103	-	-
QS@-EoY-H	281	-	-	290	-	-
QS@-RhB-L	125	-	-	138	-	-
QS@-RhB-H	163	-	-	189	-	-
QS@-DiD-EoY-L	129	77	1.7	123	66	1.9
QS@-DiD-EoY-H	308	77	4	298	60	5
QS@-DiD-RhB-L	-	-	-	153	64	2.4
QS@-DiD-RhB-H	234	83	2.8	240	54	4.4

Table 3.2: Dyes concentrations in all the prepared suspensions, estimated via UV-visible absorption after diafiltration. Extinction coefficients: $246\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 648 nm for DiD, $88\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 525 nm for EoY and $55\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 545 nm for RhB (in ethanol). The preparation procedure is described in Section 3.5.1.

Differently from ethanol, QSs maintain their structural integrity when diluted in water. Absorption spectra of dye-loaded QSs diluted in water are reported in Figure 3.11 (both before and after diafiltration). In case of EoY-loaded samples, the absorption profiles before and after diafiltration are almost perfectly overlapping independently of the presence of DiD and of the dye concentration. For this reason, only the spectra of QS@EoY-H are showed in Figure 3.11 as an example. In RhB-loaded samples, conversely, the absorption intensity is greatly reduced after diafiltration, as is evident from the decrease in relative intensity compared to the DiD peak in QS@DiD-RhB-H (bottom right panel in Figure 3.11). This observation is in agreement with the E.E. results (Figure 3.10). The spectra of RhB at high concentration (label H in the legend) show an increase of the shoulder at 515 nm after diafiltration, that can reasonably be assigned to the formation of H-aggregates. Diafiltration removes the free dye in

solution, making the effects of aggregation more noticeable.

Figure 3.11: UV–visible absorption spectra recorded in water for donor-loaded samples. Spectra before and after diafiltration are showed. L/H labels refer to high/low concentration, respectively. All spectra have been normalized to the energy donor maximum.



The very same observations also hold for dye-incubated QSs, and the corresponding absorption spectra are not shown for clarity.

3.2.4 Optical spectroscopy and FRET

The absorption and emission spectra recorded from all QSs suspensions in water (after diafiltration) are reported in Figure 3.13. The position of emission and absorption maxima resulted independent of the preparation method (dye-loading or dye-incubation), of the amount of dye and of the presence of DiD. Spectra of EoY-decorated QSs are red-shifted with respect to the ones of the free dye in distilled water and superimposable with those in ethanol (Figure 3.12a). The opposite behavior was observed for RhB, that exhibited absorption and emission slightly shifted with respect to RhB in ethanol and more similar to those of the free dye in water (Figure 3.12b).

Fluorescence quantum yields (Φ) and fluorescence lifetimes ($\langle\tau\rangle$) of all the prepared samples are listed in Table 3.3. In the absence of DiD, Φ and $\langle\tau\rangle$ of both dye-loaded and dye incubated QS@EoY-L are higher than those measured for the free EoY in distilled water. At high concentration of EoY (QS@EoY-H, both dye-loaded and dye-incubated), the Φ and $\langle\tau\rangle$ decreased by approximately two thirds, indicating substantial aggregation caused quenching, even without significant effects on the absorption spectral bandshapes. In QS@RhB-L and QS@RhB-H, independently of the preparation methodology, Φ decreases sharply when compared to the free dye in water. These observation suggests the presence of aggregation phenomena for all the samples containing RhB, despite we detected important variations of the absorption profile only for QS@RhB-H.

Concerning the samples containing both EoY or RhB and DiD, the emission of the latter has been observed in all the suspensions, even when selectively exciting EoY and RhB at 475 nm, confirming the occurrence of FRET. For all donor-incubated QSs (Figure 3.13b), DiD emission is less intense than in their dye-loaded counterparts (Figure 3.13a).

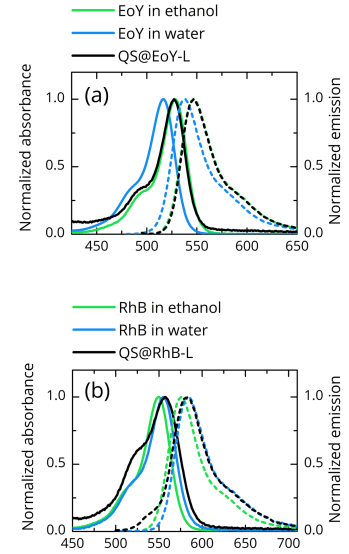


Figure 3.12: Comparison between absorption and emission spectra of EoY [Panel(a), $\lambda_{exc} = 475$ nm] and RhB [Panel(b), $\lambda_{exc} = 490$ nm] in water, ethanol and in CTAB/cholesterol QSs.

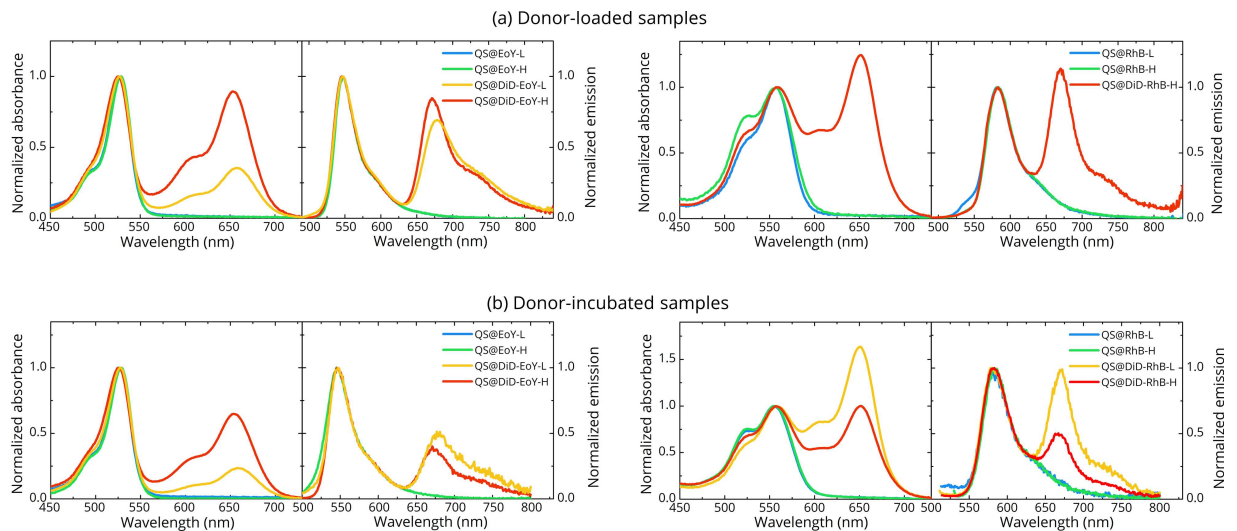


Figure 3.13: Absorption and emission spectra recorded in water for donor-loaded [Panels(a)] and donor-incubated [Panels(b)] samples, containing EoY (left) and RhB (right), in the presence and in absence of DiD. For recording emission spectra in the presence of DiD, the donor of energy has been selectively excited at 475 nm. All spectra are normalised to the energy donor maximum.

Table 3.3: Spectroscopic properties of dye-loaded and dye-incubated QSs.

Sample	Donor quantum yield ϕ^a (%)		Donor fluorescence lifetime $\langle\tau\rangle^b$ (ns)	
	Dye-loaded	Dye-incubated	Dye-loaded	Dye-incubated
QS@-EoY-L	32	33	1.71	1.62
QS@-EoY-H	9	8	0.64	0.58
QS@-DiD-EoY-L	2	5	1.01	1.29
QS@-DiD-EoY-H	2	3	0.63	0.44
EoY in water		25		1.13
QS@-RhB-L	5	2	1.88	1.38
QS@-RhB-H	2	2	1.68	1.56
QS@-DiD-RhB-L	-	1	-	1.25
QS@-DiD-RhB-H	< 1	1	1.46	1.40
RhB in water		17		1.58

^a ϕ have been obtained exciting selectively the energy donor at 490 nm and considering only the integrated area of donor bandshapes in the emission spectra.

^b Fluorescence lifetimes have been acquired exciting the samples at 405 nm and collecting the emission at 550 nm or 580 for EoY and RhB, respectively. The τ have been obtained by bi-exponential fitting and reported as the average lifetime: $\langle\tau\rangle = \frac{B_1\tau_1^2 + B_2\tau_2^2}{B_1\tau_1 + B_2\tau_2}$

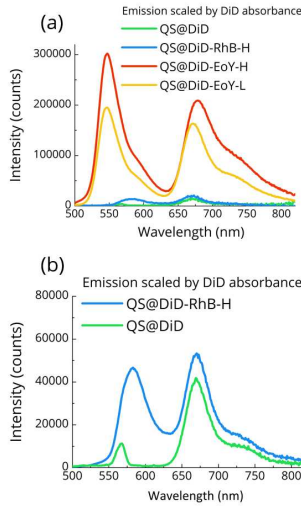


Figure 3.14: Panel(a): comparison between emission spectra of dye-loaded samples: QS@DiD-RhB-H, QS@DiD-EoY-L, QS@DiD-EoY-H with a batch of nanovesicles loaded only with the acceptor dye (QS@DiD), all scaled by the corresponding DiD absorbance. Panel(b): close up on the emission spectra of QS@DiD-RhB-H and QS@DiD. All the emissions reported were acquired in the very same experimental conditions exciting the sample at 475 nm.

In the presence of DiD, the Φ of EoY-decorated samples decreases with respect to the suspensions containing only the donor, with a stronger effect for QSs containing lower amount of EoY. This decrease, reasonably due to FRET, is slightly more important for dye-loaded samples, in agreement with their emission bandshapes that exhibit higher DiD emission. The decrease of $\langle\tau\rangle$ is sizable in the presence of DiD, consistently with FRET, only at low donor concentrations.

Having established the occurrence of the FRET in EoY-containing samples, we can estimate the efficiency of the process according to Equation 2.1. The calculated FRET efficiencies for dye-loaded samples amount to 94% and 78% for QS@DiD-EoY-L and QS@DiD-EoY-H, respectively. In the case of dye-incubated samples, the slightly lower values of 85% and 63% were obtained for QS@DiD-EoY-L and QS@DiD-EoY-H, respectively. The higher FRET efficiency observed for dye-loaded samples can be attributed to the presence of EoY on both sides of the QSs bilayer, while only the external leaflet is available for dye-incubated ones. For this reason, in dye-loaded QSs the number of potential energy transfer events is higher, since they involve also the DiD molecules nanostructured in the inner leaflet of the membrane.

For both RhB-loaded and incubated samples, Φ is much lower than in water, essentially unaffected by concentration and only weakly reduced by the presence of DiD, suggesting that FRET is not significant in this case, despite the DiD bandshape was observed in the emission spectra. In Figure 3.14, the emission of dye-loaded QS@DiD-RhB-H, QS@DiD-EoY-H and QS@DiD-EoY-L are compared to a batch of QSs only loaded with DiD (QS@DiD), in order to verify if a contribution from the direct excitation of the acceptor dye was present. The emission spectra were divided by the absorbance of DiD (considered at the absorption maximum, ~ 648 nm) and were recorded in the same experimental conditions. The comparison suggests that direct excitation of DiD at 475 nm is marginal and only affects the spectra of RhB-decorated QSs. In QS@DiD-EoY-H and QS@DiD-EoY-L, the DiD emission mainly stems from the FRET process, while in QS@DiD-RhB-H, the major contribution to the DiD

emission profile comes from direct excitation and FRET is negligible. The same conclusions also hold true in the case of dye-incubated samples.

Excitation spectra (Figure 3.15) confirm that FRET occurs only in EoY-DiD decorated samples. Emission of the energy acceptor, DiD, is selectively detected at 730nm: the excitation band of the energy donor is observed in samples containing EoY, while its relative intensity is negligible for RhB-decorated ones. Interestingly, the contribution of the EoY bandshape to the excitation spectra is always higher than in the corresponding absorption spectra. This phenomenon is probably due to the formation of DiD non emissive H-aggregates in the QSs membrane, that are able to contribute to the DiD bandshape in absorption but not in excitation. The excitation spectra of EoY-loaded and incubated QSs without DiD overlap the corresponding absorption spectrum and are not shown. Conversely, for RhB-loaded and incubated ones, the low-wavelength shoulder detected in the absorption spectrum is almost absent in the excitation spectrum, independently on the concentration, possibly due to the formation of non-fluorescent H-aggregates. The aggregation of RhB in both QS@RhB-H and QS@RhB-L justify also the lower Φ of RhB-decorated samples (Table 3.3).

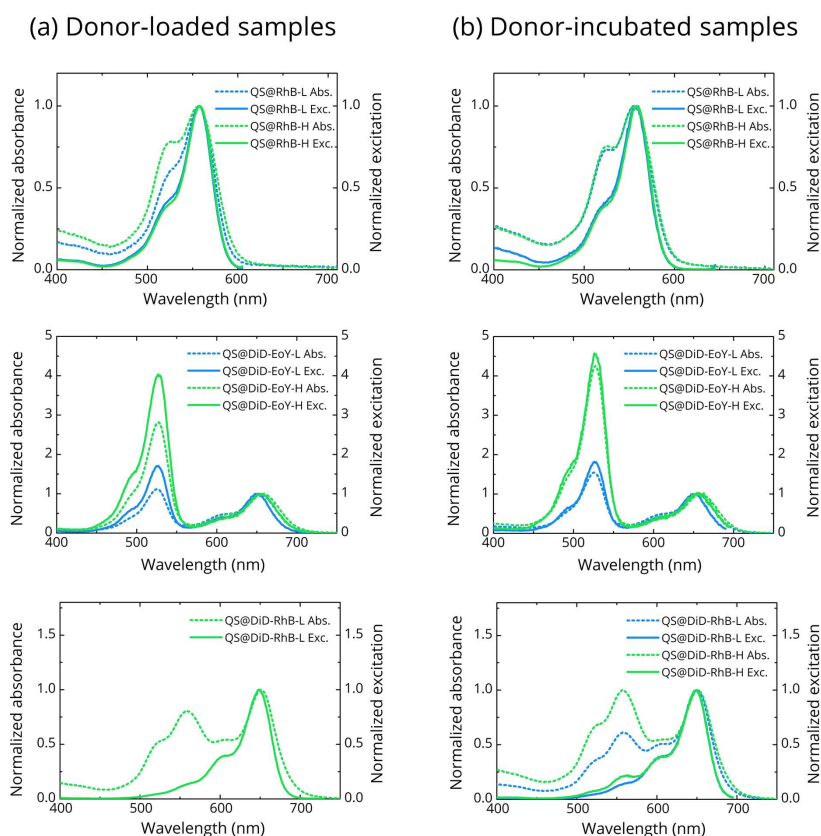


Figure 3.15: Fluorescence excitation and absorption spectra recorded in water for diafiltrated dye-loaded [Panels(a)] and dye-incubated [Panels(b)] QSs. In the presence of DiD, the spectra are normalised to DiD emission intensity. For suspensions containing only RhB, we collected the emission at 610 nm. Excitation spectra of QSs decorated with both DiD and one of the energy donor were acquired detecting the emission at 730 or 750 nm, in order to minimize contributions from the donors. When DiD is present, the spectra have been normalized to its peak.

The lack of FRET in RhB-containing QSs may be attributed to the formation of non-fluorescent H-aggregates of the donor dye, that being characterized by weak emission transition dipole moments, hinder the energy transfer process. A second possibility is that the RhB has been at least partially loaded in the nanovesicles lumen, and the donor-acceptor distances in these conditions are no longer suitable for the FRET process to occur.

3.2.5 Two-Photon Microscopy of Quatsomes suspensions

To confirm the encapsulation of the donor dyes within the nanovesicles or their presence as free molecules in solution, TPM images were acquired for both donor-loaded and donor-incubated QS samples before and after diafiltration, and the results are collected in Figure 3.16. If present, the free dissolved dye in solution should relevantly contribute to the background signal in the sample. However, since other sources of background signal are always present (e.g out-of-focus Qs), the amount of background signal can be only be evaluated by comparison between images taken in the same experimental conditions. In the case of both donor-loaded and donor-incubated EoY-containing suspensions, remarkably low background signal is present, with emission mainly stemming from the NPs. EoY exhibited a low tendency to re-dissolve in water, even before the diafiltration and after months from the preparation of the nanovesicles. RhB-decorated Qs showed a different behavior: all the non-diafiltered suspensions are characterized by strong background signal, suggesting the presence of free dye in solution. This observation is in line with the low encapsulation efficiencies of these samples and with the variations detected in the absorption spectra upon diafiltration (Figure 3.11). The diafiltered QS@RhB-H suspensions exhibited lower background signal, although some residual signal was still observed.

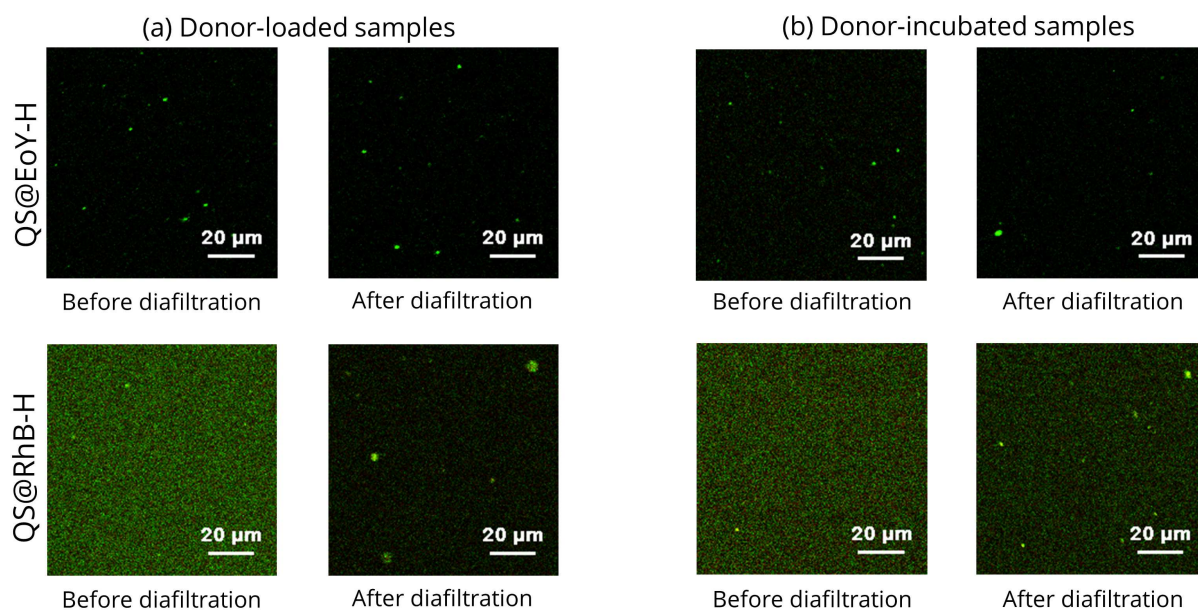


Figure 3.16: TPM images of donor-loaded [Panel(a)] and donor-incubated [Panel(b)] samples: QS@EoY-H and QS@RhB-H, both before and after diafiltration. The measurements were performed irradiating at 1000 nm with 3% laser power for EoY containing samples, irradiating at 1050 nm with 10% laser power in the case of RhB containing samples. The decrease in background signal upon diafiltration is much greater for RhB than for EoY.

3.2.6 Effect of the preparation procedure on EosinY-decorated Quatsomes

To assess the impact of the preparation procedure on the resulting QSs suspension, two additional batches of QS@EoY-L and QS@DiD-EoY-L samples were prepared.

In the first approach, EoY was added to the organic phase along with cholesterol and, if present, DiD. In the second approach, CTAB was added to the organic phase, while EoY was kept alone in the aqueous phase. More details on all the employed preparation protocols are reported in Section 3.5.1. These procedures were investigated to determine whether altering the location of the donor dye during the preparation protocol could affect its interactions with the components of the QSs. The absorption and emission spectra (data not shown) of QS@EoY-L were superimposable to those shown in Figure 3.13. The Φ is 32% for both the two new samples, similarly to the dye-loaded and dye-incubated samples (Table 3.3).

Absorption and emission spectra of QS@DiD-EoY-L prepared with the four distinct procedures are shown in Figure 3.17. In the absorption profile we can detect some differences in the relative intensity of donor and acceptor bandshapes, primarily attributed to experimental variability in the actual dye loading of the suspensions. Nonetheless, all the emission spectra acquired selectively exciting the energy donor, features efficient FRET, with comparable relative intensity of the DiD band.

No significant effects were observed in the physico-chemical properties of the suspensions, both the ζ -potentials and hydrodynamic diameter are in line with the standard dye-loading and incubation procedures (see Figure 3.18). The only parameter falling outside the expected range is the ζ -potential of the sample prepared with CTAB in the organic phase, which exhibited an unusually low value only when prepared in the presence of DiD (Figure 3.18b).

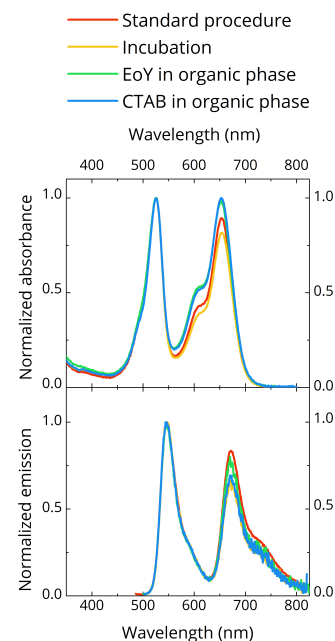


Figure 3.17: Comparison between absorption and emission spectra acquired on QS@DiD-EoY-L prepared following four different procedures. The emission spectra were recorded exciting the sample at 490 nm.

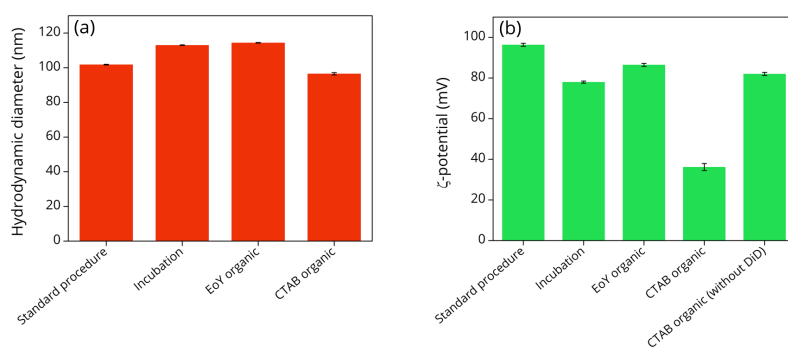


Figure 3.18: Hydrodynamic diameter [Panel(a)] and apparent ζ -potential [Panel(b)] of QS@DiD-EoY-L samples prepared with the four different protocols described in the text. In the case of the suspension obtained with CTAB in the organic phase, the ζ -potential of the corresponding sample without DiD is shown as a comparison. The error bars represent the corresponding standard error of the measurement mean (SEM). Measurements were performed at 25°C as described in Section T.1.

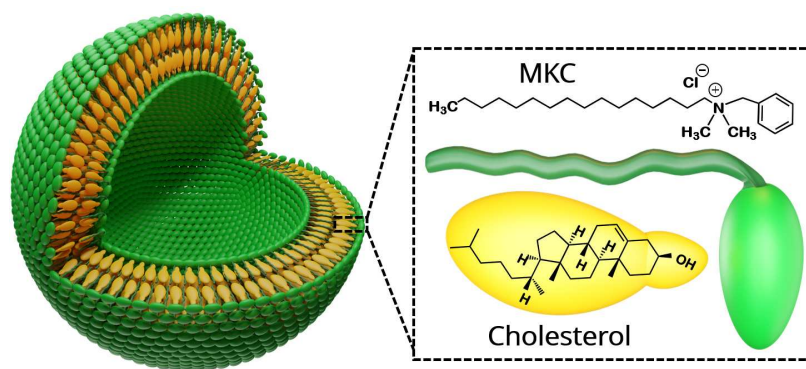
3.3 DiR-loaded MKC-Quatsomes for specific targeting

As described in Section 3.1, Quatsomes are composed of a sterol molecule and an ionic surfactant, and the most studied ones present CTAB and cholesterol as building blocks (CTAB-Quatsomes).^[107, 118] However, the inherent nature of the CTAB surfactant raises safety concerns, particularly for *in vivo* applications besides topical administration. The most common use of CTAB concerns indeed non-parenteral pharmaceutical formulations, already approved by medical regulatory agencies such as the EMA (European Medicines Agency) or the FDA (Food and Drug Administration).^[136] On the contrary, the other main component of CTAB-QSs, cholesterol, is generally considered as a nontoxic and nonirritant material, and is frequently employed as excipient⁷.^[136]

⁷: Being the major sterol of the higher animals, this biocompatible lipid is approved by European and U.S. regulatory authorities as an inactive ingredient in intravenous injections^[137] (e.g. Ambisome®,^[138] a liposomal formulation containing cholesterol).

To address these limitations and broaden the applicability of QSs in formulations for parenteral administration, the Nanomol-Bio group recently modified the membrane composition by replacing CTAB with myristalkonium chloride (MKC, also known as tetradecyldimethylbenzylammonium chloride, as shown in Figure 3.19), a different quaternary ammonium surfactant. MKC is the C14 homolog of benzalkonium chloride (BAK), characterized by a phenyl group that hides the positive charge of the polar head and chloride anion as a counterion. BAK is a mixture of different homologs with alkyl chain lengths ranging from 8 to 18 carbon atoms,^[136] widely employed as an antimicrobial preservative in many ophthalmic preparations^[139] and also parenteral formulations, suggesting very high safety of usage.^[140]

Figure 3.19: Schematic representation of MKC-Quatsomes composed by the association of Myristalkonium chloride (MKC) and cholesterol. The molecular structures of the components are also shown.



MKC-Quatsomes already demonstrated good cell viability in experiments involving an epithelial colorectal carcinoma cell line, displaying a certain degree of selectivity toward lysosomes.^[108] Their colloidal stability and morphology are in line with those of conventional QSs.^[141] Since their former applications, MKC-QSs have emerged as highly promising nanocarriers and versatile platforms for drug delivery, sensing, and bioimaging. Thanks to the positively charged surface, these vesicles demonstrated to efficiently entrap negatively-charged small RNA molecules, like miRNA or siRNA.^[142] The resulting complexes of MKC-Quatsomes and small RNA are also able to halt the growth of cancer cells, particularly in cellular models of high-risk neuroblastoma, an extremely aggressive pediatric tumor that requires novel therapeutic approaches.^[143, 144]

Similarly to CTAB-QSs, these nanostructures can be loaded with a wide

range of fluorescent probes, and already proved to be effective in *in vitro* and *in vivo* bioimaging studies employing non-water-soluble dyes. As an example of *in vitro* application, these nanovesicles successfully encapsulated fluorene-based probes, yielding nanostructures with a distinct "patchy" vesicular morphology and resulting suitable for cellular imaging via confocal microscopy.[108] In parallel, MKC-QSs have been harnessed for *in vivo* bioimaging applications upon loading the near-infrared fluorescent carbocyanine DiR. The intravenous injection of DiR-labeled MKC-QSs into mice bearing xenografted colorectal tumors has allowed for biodistribution assays, revealing their accumulation in specific organs, notably the tumor, liver, spleen, and kidneys.[145] These findings collectively highlight the efficacy of MKC-QSs for diverse bioimaging scenarios, ranging from cellular studies to *in vivo* assessments.

In this section, a series of MKC-QSs loaded with DiR and functionalized in the external surface with two different ligands for specific targeting (namely ligand 1, L1 and ligand 2, L2) has been spectroscopically characterized and then employed for *in vitro* bioimaging experiments to study cells internalization.

The samples were prepared by Sara Garrido, from the Nanomol-Bio group (ICMAB-CSIC), under the supervision of Prof. Nora Ventosa and Dr. Mariana Köber.

DiR (1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide) dye is a near-infrared fluorophore, belonging to the same indocarbocyanine family of DiI and DiD, and capable of nanostructuring in the membrane of MKC-QSs, as previously demonstrated.[141, 145] Moreover, the near-infrared light emitted from this probe falls within the transparency window of biological tissues (700-1500 nm), allowing for deep imaging in the case of future *in vivo* applications.[10] The molecular structure of DiR, characterized by a polymethinic bridge with two more carbons than DiD, is shown in Figure 3.20, together with a schematic representation of a DiR-loaded MKC-Quatosome.

The functionalization with L1 and L2 (schematically reported in Figure 3.21) aim at achieving preferential internalization into distinct cellular targets. Two cellular models have been employed as negative control and as target for this investigation, respectively denoted as cell lines A (control) and B (target cell line).

The cells were visualized through laser scanning confocal fluorescence microscopy after incubation with the nanoparticles, to verify the selectivity of the L1 and L2 decorated QSs towards the cell line B, employing the cell line A and co-culture experiments as negative controls.

This work represent a preliminary assessment of the potentialities offered by multifunctional QSs for specific targeting. Some details of the work (ligands molecular structures, their interaction with QSs and the target cell lines) cannot be disclosed to guarantee intellectual property protection.

The confocal microscopy experiments have been performed on both live and fixed cells, in order to assess if the used fixation protocol can alter the outcome in terms of DiR signal and specificity. The results obtained with the ligand-targeted MKC-QSs were also compared with non functionalized MKC-Quatsomes, to evaluate the effect of L1 and L2

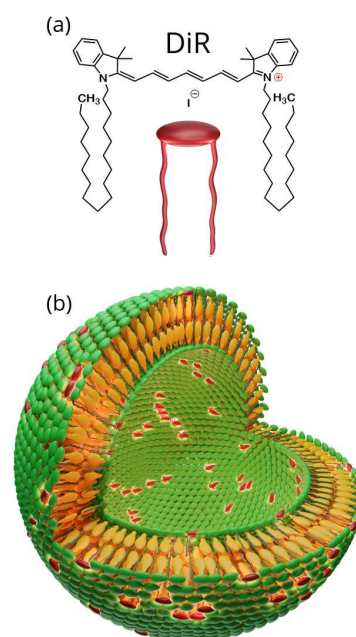
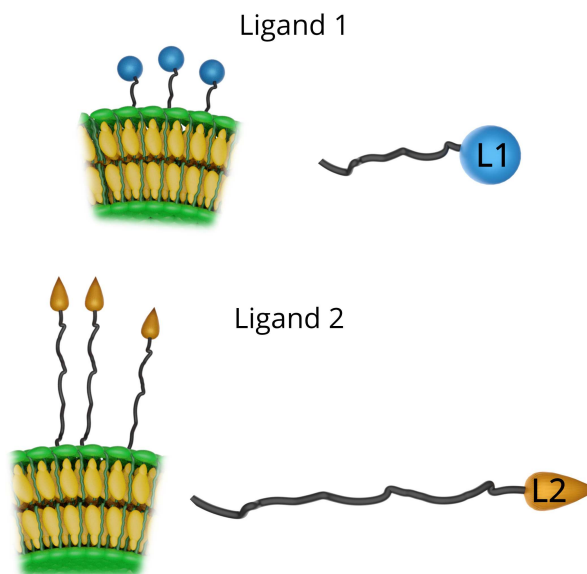


Figure 3.20: Panel(a): Molecular structure and schematic representation of DiR. Panel(b): Schematic representation of a DiR-loaded MKC Quatosome.

Figure 3.21: Schematic representation of the two ligands employed for the nanoparticles surface functionalization, also shown upon nanostructurization inside the MKC-QS membrane.



units in promoting the localization of the nanovesicles into the cell line B. Hence, a grand total of 14 different DiR-labelled samples were prepared, changing the amount of loaded DiR and also the surface functionalization densities of both L1 and L2 (2 or 4 mol %), to study how these modifications can impact on the biological interaction with cells. The best candidates for *in vitro* experiments have been selected after a careful spectroscopic characterization (reported in Section 3.3.1).

The non functionalized QSs have been prepared both in water and in a saline buffer, while the functionalized ones resulted poorly stable when prepared in a saline buffer (the formation of a precipitate was observed) and were studied only in milliQ water.

A complete list of all the prepared and studied samples is presented below.

Not functionalized samples, loaded with DiR:

- ▶ **QS@DiR-25 μ M-W** – DiR-loaded MKC-QSs (25 μ M), dispersed in milliQ water.
- ▶ **QS@DiR-50 μ M-PBS** – DiR-loaded MKC-QSs (50 μ M), dispersed in Phosphate-Buffered Saline (PBS).
- ▶ **QS@DiR-60 μ M-PBS** – DiR-loaded MKC-QSs (60 μ M), dispersed in PBS.
- ▶ **QS@DiR-80 μ M-PBS** – DiR-loaded MKC-QSs (80 μ M), dispersed in PBS.
- ▶ **QS@DiR-50 μ M-RL** – DiR-loaded MKC-QSs (50 μ M), dispersed in Ringer's lactate (RL).

Samples functionalized only with ligand 1:

- ▶ **QS@L1-2%-DiR-1 μ M-W** – DiR-loaded MKC-QSs (1 μ M) functionalized with 2 mol % of L1, dispersed in milliQ water.
- ▶ **QS@L1-2%-DiR-25 μ M-W** – DiR-loaded MKC-QSs (25 μ M) functionalized with 2 mol % of L1, dispersed in milliQ water.
- ▶ **QS@L1-4%-DiR-1 μ M-W** – DiR-loaded MKC-QSs (1 μ M) functionalized with 4 mol % of L1, dispersed in milliQ water.
- ▶ **QS@L1-4%-DiR-25 μ M-W** – DiR-loaded MKC-QSs (25 μ M) functionalized with 4 mol % of L1, dispersed in milliQ water.

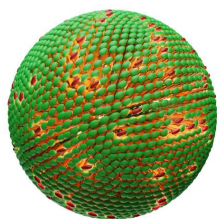


Figure 3.22: Schematic representation of a DiR-loaded MKC Quatsome, not functionalized.

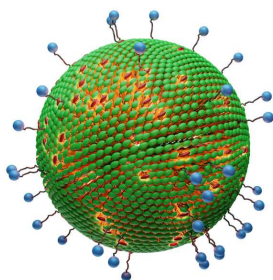


Figure 3.23: Schematic representation of a DiR-loaded MKC Quatsome functionalized with ligand 1.

- **QS@L1-4%-DiR-50 μ M-W** – DiR-loaded MKC-QSs (25 μ M) functionalized with 4 mol % of L1, dispersed in milliQ water.

Samples functionalized only with ligand 2:

- **QS@L2-2%-DiR-1 μ M-W** – DiR-loaded MKC-QSs (1 μ M) functionalized with 2 mol % of L2, dispersed in milliQ water.
- **QS@L2-4%-DiR-25 μ M-W** – DiR-loaded MKC-QSs (25 μ M) functionalized with 4 mol % of L2, dispersed in milliQ water.

Samples functionalized with both ligand 1 and ligand 2 (dual targeting):

- **QS@L2-2%-L1-2%-DiR-1 μ M-W** – DiR-loaded MKC-QSs (1 μ M) functionalized with 2 mol % of L1 and 2 mol % of L2, dispersed in milliQ water.
- **QS@L2-2%-L1-2%-DiR-25 μ M-W** – DiR-loaded MKC-QSs (25 μ M) functionalized with 2 mol % of L1 and 2 mol % of L2, dispersed in milliQ water.

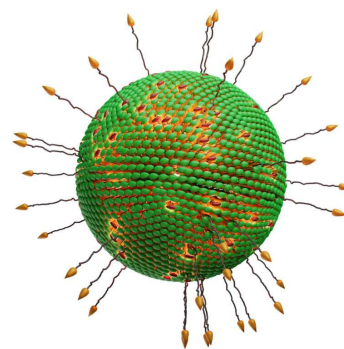


Figure 3.24: Schematic representation of a DiR-loaded MKC Quatsome functionalized with ligand 2.

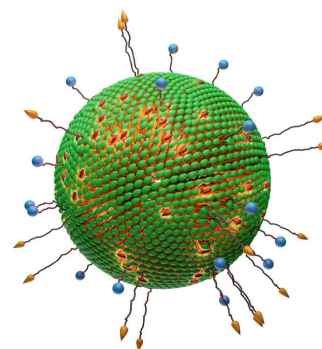


Figure 3.25: Schematic representation of a DiR-loaded MKC Quatsome simultaneously functionalized with ligand 1 and ligand 2.

Physicochemical characterization

Apart from the development of the MKC-QSs, Sara Garrido from ICMAB/CSIC also performed an initial physicochemical characterization of the nanovesicles, reported in Figure 3.26. The nanovesicles suspended in water are characterized by a hydrodynamic diameter (D_h) of ~ 70 nm, a lower value with respect to those prepared in Phosphate-Buffered Saline (PBS) or Ringer's lactate (RL), that present D_h in the range 140-180 nm (Figure 3.26, left panel). The only outlier is QS@DiR-25 μ M-W, with an average D_h of about 250 nm, associated to a large error. The apparent ζ -potential showed the opposite behaviour (Figure 3.26c): higher values are observed for the surface functionalized QSs suspensions in distilled water (60-80 mV) than the plain ones in PBS or RL (~ 40 mV), in agreement with literature data [141, 145]. The two samples QS@L2-2%-L1-2%-DiR-25 μ M-W and QS@L2-2%-DiR-25 μ M-W are characterized by lower ζ -potential values (~ 40 nm).

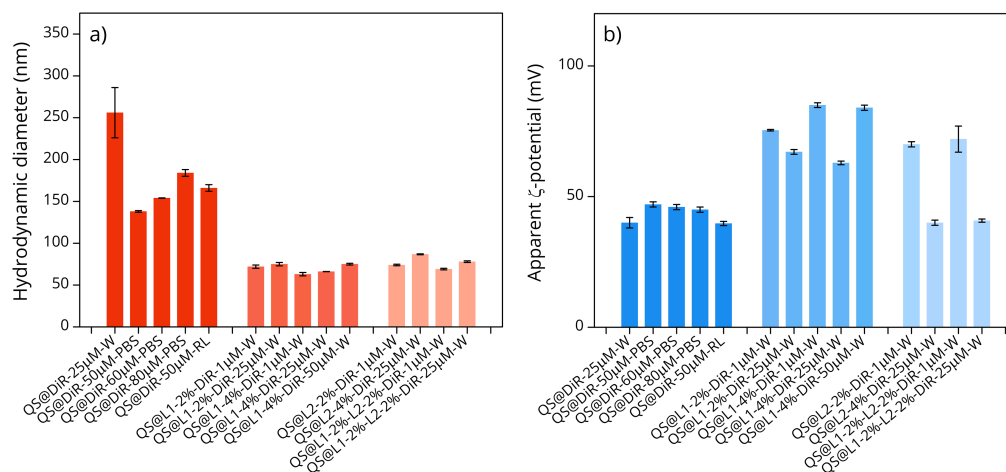


Figure 3.26: DLS and ELS data of the 14 DiR-loaded MKC-QSs suspensions, in the absence and in the presence of surface functionalization.

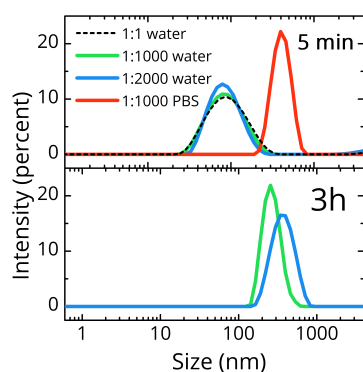


Figure 3.27: Size distributions by intensity of QS@L1-4%-DiR-25 μ M-W after dilution (by various extent) in water or PBS. The results obtained 5 minutes or 3 hours after the dilution are reported in the top and bottom panel, respectively.

The stability over dilution of the functionalized nanovesicles has also been tested. The main objective of the study was to verify if these QSs suspensions are stable at the dilution level employed for *in vitro* experiments (1:1000), where the concentration of the nanoparticles is drastically reduced and a mixture of salts and proteins are present in the medium. The size distributions by intensity obtained after dilution of a representative sample (QS@L1-4%-DiR-25 μ M-W) in different environment are reported in Figure 3.27. Right after diluting, the nanovesicles are stable up to a 1:2000 dilution (concentration of membrane components: ~ 0.4 - 0.8 μ g/mL according to the sample) in distilled water, and no differences were observed in the intensity distributions. After 3 hours from the dilution, the Z-average (mean D_h) of the nanovesicles diluted 1:1000 and 1:2000 increases to 350 and 400 nm, respectively, suggesting aggregation phenomena.

Diluting the same suspensions with a 1:1000 ratio in PBS leads to an increased hydrodynamic diameter right after the dilution, with a calculated Z-average of about 600 nm (Figure 3.27). After three hour in PBS at this dilution level, the aggregation is extensive, and the quality of the data is too poor for fitting the correlogram: no size distributions or Z-average values could be extracted.

In order to achieve more stable nanovesicles upon dilution and in the presence of dissolved salts, PEG chains could be employed to further decorate the surface of the functionalized samples, without changing the density of the active ligand.

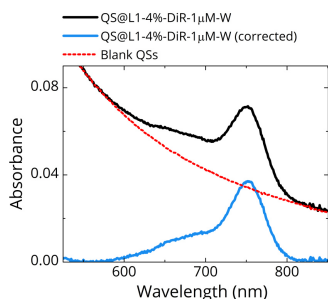


Figure 3.28: Comparison between the absorption spectra of a QSs suspension (QS@L1-4%-DiR-1 μ M-W) before and after the scattering correction. The adjusted scattering profile, obtained from a blank QSs suspension, is also shown.

8: The ideal measurements would have required an integrating sphere, but unfortunately, it was not available at either University of Parma and NJIT.[146]

9: Ringer's lactate (RL) is a buffer solution employed for intravenous injections and it has different salts: NaCl (6 g/L), KCl (0.4 g/L), CaCl₂ dihydrate (0.27 g/L) and sodium lactate (3.12 g/L). It has a pH between 5.0 and 7.0 and an osmolarity of 277 mOsm/L.

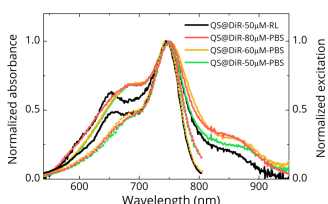


Figure 3.29: Comparison between normalized absorption (continuous lines) and excitation (dashed lines) spectra of non functionalized samples prepared in PBS or RL. To record excitation spectra, the emitted light has been monitored at 810 nm.

3.3.1 Spectroscopic characterization

All samples listed in Section 3.3 were studied measuring absorption spectra, emission spectra, fluorescence quantum yields and lifetimes. The main purpose of this spectroscopic characterization was to evaluate the best candidates to be employed for the confocal microscopy experiments. Since some of the MKC-QSs suspensions were characterized by quite low DiR concentration (1 μ M), and the small size of the nanoparticles causes them to strongly scatter visible and UV-light, we observed that scattering phenomena contribute strongly to their absorption bandshapes. Therefore, in order to estimate the real absorbance of the samples loaded with DiR 1 μ M it was necessary to remove the scattering contribution to the measured absorption profiles. The real absorbance of the samples is indeed necessary for the calculation of the Φ ⁸. [10]

A very common way to get rid of scattering contributions concerns the use of a blank suspension, that scatters exactly like the sample, as a reference during the absorption measurements. In the case of QSs, this approach is not easy to apply, since it is not possible to control the features of the scattering signal of each nanovesicles batch, even if they are prepared through the DELOS-SUSP methodology. For this reason, we recorded the absorption spectra of blank MKC-QSs (not loaded with DiR), with and without surface functionalization, and subtracted them from the ones obtained with DiR-loaded samples (as shown in Figure 3.28). Before subtracting, the profiles obtained from blank suspensions were adjusted for each sample, making them overlapping to the sample bandshapes in correspondence of two wavelengths at the edges of the main absorption peak of DiR (500 and 850 nm), where the main contribution to the measured absorbance is due to scattering phenomena.

The normalized absorption (after scattering correction) and emission spectra of all the samples are reported in Figure 3.30. The absorption profiles of all the not functionalized QSs suspensions prepared in PBS or Ringer's lactate⁹ are characterized by significant aggregation features, showing an increase of the relative intensity of the low-wavelength absorption shoulder and the occurrence of a red-shifted shoulder above 800 nm. The enhancement of the lower-wavelength shoulder is in line with the formation of weakly fluorescent H-aggregates, as suggested also by the very low quantum yields of these samples. The origin of the red-shifted shoulder above 800 nm is not fully understood and requires more detailed investigation. The excitation profiles of the suspensions in PBS reported in Figure 3.29 are similar to the absorption of the monomer (e.g. DiR in ethanol), further confirming H-aggregation in the system (H-aggregates are usually non-fluorescent). In the case of the sample in RL, a slight increase in the low-wavelength shoulder is observed also in the excitation spectrum.

Absorption and emission bandshapes of all the suspensions prepared in water resulted similar, without discernible aggregation features observed across the various DiR concentrations (Figure 3.30). The formation of aggregates is more prominent for QSs suspensions prepared in PBS or Ringer's lactate most probably due to the higher ionic force of the solution during the formation of the nanovesicles, that could have promoted the DiR aggregation.

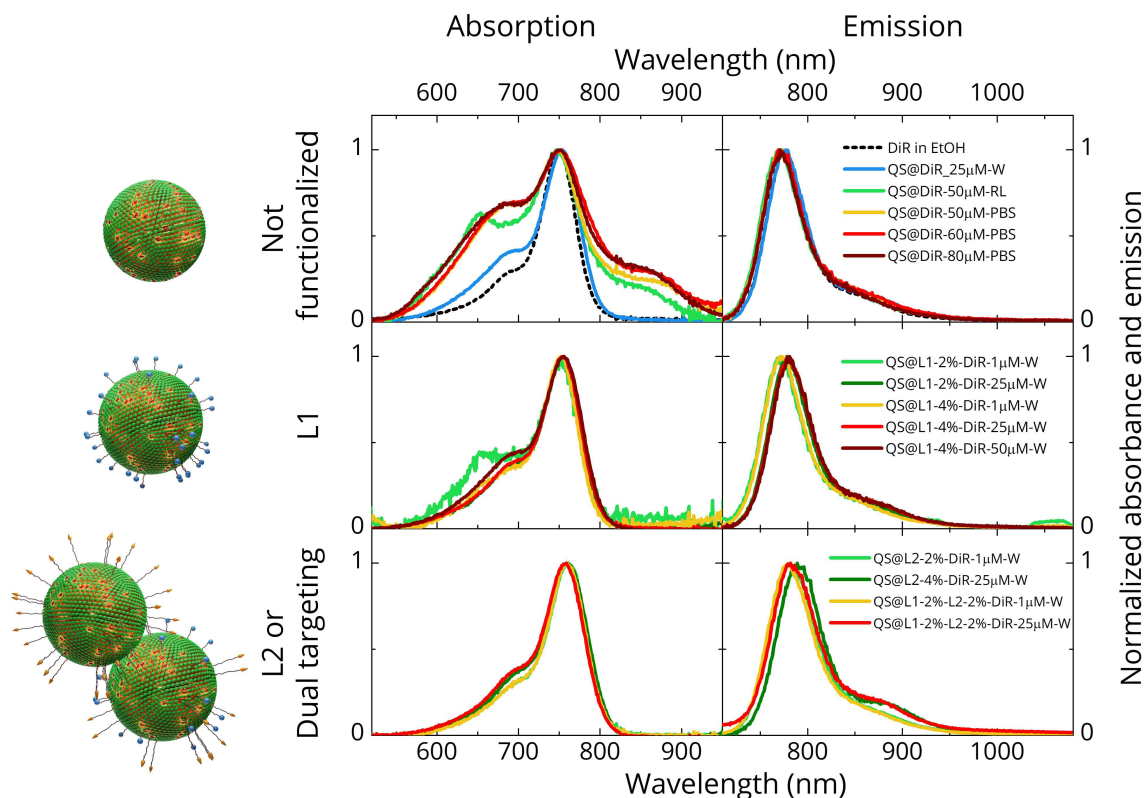


Figure 3.30: Comparison between normalized absorption and emission spectra of all the DiR-loaded QSs prepared during this work. Prior to measuring emission spectra, all suspensions were diluted with milliQ water in order to reach absorbance values lower than 0.1. The emission spectra were recorded with an excitation wavelength of 700 nm.

Fluorescence quantum yields were measured employing HITC (1,1',3,3,3',3'-Hexamethylindotricarbocyanine iodide) as a reference compound (as detailed in Section T.2), and are reported in Figure 3.31 and Table 3.4. The samples with DiR concentration of 50 μM or higher showed quite low ϕ values ($\sim 2\text{-}3\%$), suggesting significant H-aggregation also for QS@L1-4%-DiR-50 μM -W, a suspension that do not exhibit any substantial increase in the low-wavelength shoulder of the absorption profile. In the case of samples loaded with DiR 25 μM , the measured ϕ resulted in the range 5-8%. The best outcomes in terms of fluorescence quantum yield were obtained for the suspensions loaded with a DiR concentration of 1 μM , with values varying between 18 and 22% that suggest marginal aggregation. All the prepared QSs suspensions exhibited lower ϕ when compared to DiR in ethanol (31%).

The formation of aggregates in the samples characterized by a DiR concentration equal or higher than 25 μM can be detected also from the fluorescence lifetimes (reported in Table 3.4). For these suspensions, values lower than 0.9 ns were obtained, while the samples with DiR concentration of 1 μM present lifetimes of about 1.2 ns, similar to the free dye in ethanol.

Spectroscopic data are summarized in Table 3.4.

Figure 3.31: Fluorescence quantum yields of all the studied DiR-loaded QSs suspensions. The value for DiR in ethanol is also shown as a comparison.

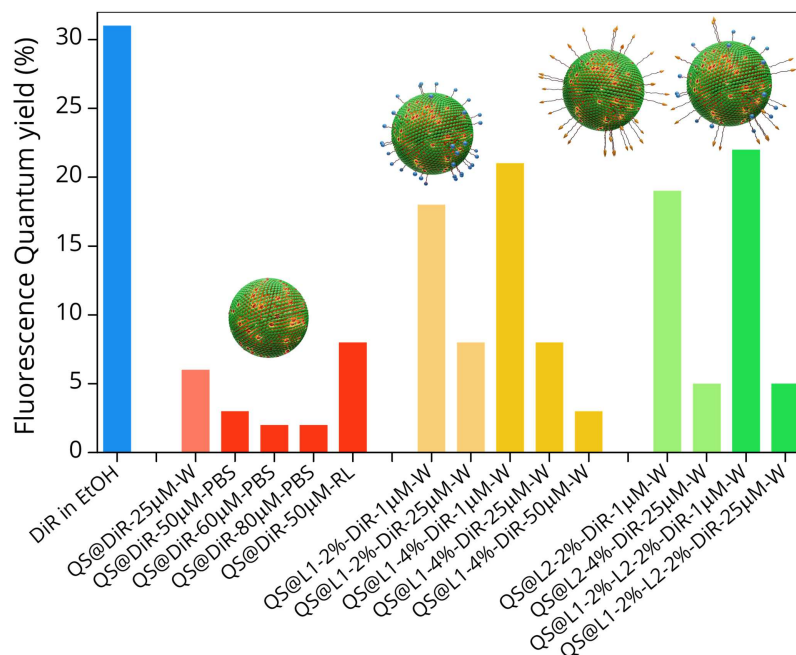


Table 3.4: Spectroscopic properties of DiR-loaded MKC-QSs.

Sample	Fluorescence quantum yield ϕ (%)	fluorescence lifetime $\langle\tau\rangle^a$ (ns)	Molar extinction coefficient ^b at $\lambda_{\max}$ ($M^{-1} cm^{-1}$)	Absorption peak (nm)	Emission peak (nm)
DiR in EtOH	31	1.24	305 000	751	775
QS@DiR-25μM-W	6	0.78	227 000	752	777
QS@DiR-50μM-PBS	3	0.88	-	750	771
QS@DiR-60μM-PBS	2	0.79	-	753	769
QS@DiR-80μM-PBS	2	0.83	-	750	772
QS@DiR-50μM-RL	8	0.98	-	746	773
QS@L1-2%-DiR-1μM-W	18	1.21	-	752	773
QS@L1-2%-DiR-25μM-W	8	0.82	237 000	752	778
QS@L1-4%-DiR-1μM-W	21	1.14	-	754	771
QS@L1-4%-DiR-25μM-W	8	0.77	239 000	754	779
QS@L1-4%-DiR-50μM-W	3	0.74	221 000	756	781
QS@L2-2%-DiR-1μM-W	19	1.15	-	760	781
QS@L2-4%-DiR-25μM-W	5	0.72	222 000	762	789
QS@L2-2%-L1-2%-DiR-1μM-W	22	1.18	-	761	780
QS@L2-2%-L1-2%-DiR-25μM-W	5	0.75	224 000	759	782

^a The corresponding lifetime decays are reported in . The τ have been obtained by bi-exponential fitting and reported as the average lifetime: $\langle\tau\rangle = \frac{B_1\tau_1^2 + B_2\tau_2^2}{B_1\tau_1 + B_2\tau_2}$

^b Estimated considering the concentration values obtained from the absorbance of the samples diluted in ethanol by using the Lambert-Beer equation.

Over-time stability

The absorption of all the samples prepared in water have been monitored over-time, up to 1 month from the first measurement. The results are reported in Figure 3.32.

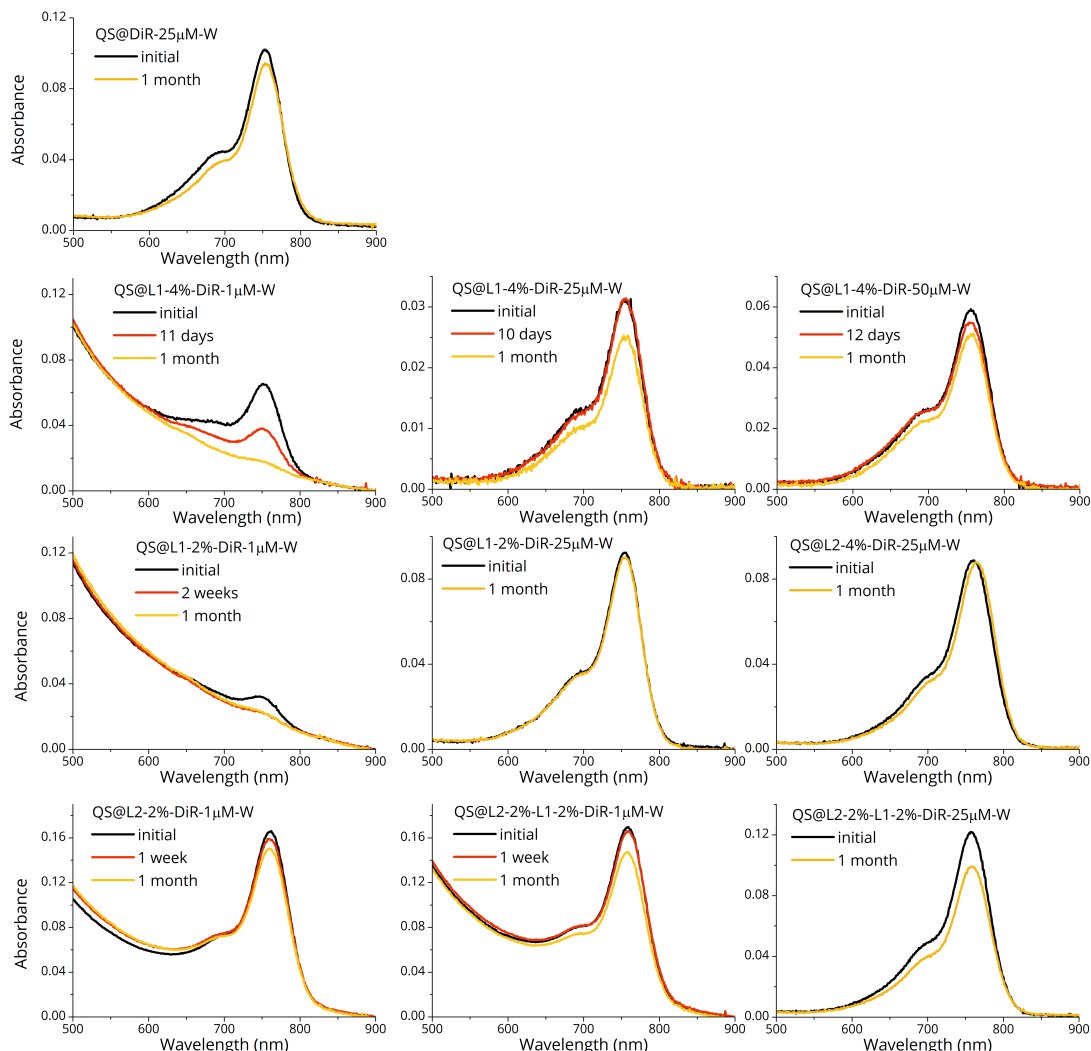


Figure 3.32: Absorption spectra of all the DiR-loaded MKC-QSs, monitored over-time. Each sample was diluted by the same extent prior measuring the absorption profile.

For all samples, absorbance decreases over time. However, the rate of decrease varies a lot, and it is not easy to find a trend to correlate the over-time stability with the concentration of different components. For sure, sample having $1\mu\text{M}$ DiR are less stable, and for this reason we decided to choose the series of suspensions with DiR $25\mu\text{M}$ for the *in vitro* experiments, even if their ϕ is lower. Also, the higher amount of loaded dye can be beneficial, enabling to work with more diluted suspensions.

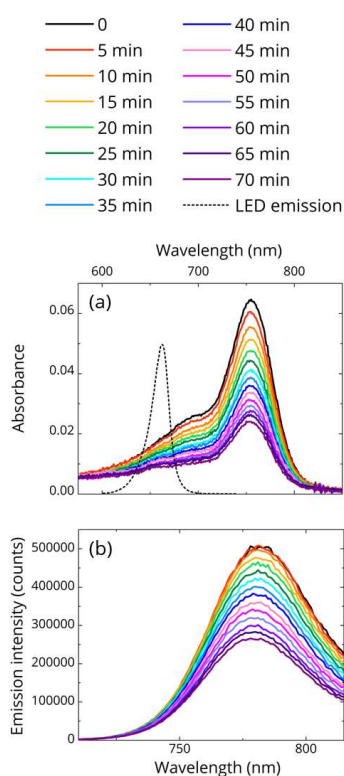


Figure 3.33: Over-time variations of absorption [Panel(a)] and emission spectra [Panel(b)] of QS@L1-4%-DiR-25 μ M-W upon continuous LED irradiation (λ_{exc} = 660 nm, bandwidth: 20 nm, I = 21 mW/cm²). In Panel(a), the emission profile of the LED, arbitrarily normalized, is shown as a comparison.

Photodegradation experiments

The photostability of fluorophores is an important parameter to assess their applicability as imaging probes. Cyanine dyes, especially those absorbing in the far-red or near-infrared spectral region are particularly prone to photodegradation phenomena. For this reason, their mechanism of photodegradation has been widely studied in the literature, evaluating also the influence of different counterions, substituents in the polymethine chain and formation of inclusion complexes.[147–149]

Even if the encapsulation inside QSs can boost the photostability of a dye, as reported for fluorescein in CTAB-QSs[122], the opposite effect was detected for DiI and DiD, two carbocyanines structurally related to DiR.[94]

In order to evaluate the photostability of DiR in the prepared MKC-QSs suspensions, we monitored the absorption and the emission spectra under continuous-wave excitation, over a time period of 70 minutes. The samples were placed in a macro cuvette (optical path: 1 cm) and irradiated with a LED source (λ_{exc} = 660 nm, bandwidth: 20 nm, I = 21 mW/cm²) illuminating the whole volume of the suspension. More technical details on these experiments are reported in Section 3.5.2. The absorption and emission measurements were carried out subsequently at each time point. An example of the variations observed in the spectra upon irradiation is shown in Figure 3.33 for QS@L1-4%-DiR-25 μ M-W.

In Figure 3.34, variations in the absorption and emission maxima of 7 different samples are shown as a function of the irradiation time. DiR resulted more photostable when dissolved in ethanol than when embedded in all the MKC-QSs membrane, similarly to what was observed in the case of DiI and DiD after loading in CTAB-QSs.[94] The photobleaching properties are often solvent dependent, and water, with respect to organic solvents, can lead to higher intersystem crossing rates, making photodegradation by oxidative mechanism more favorable.[150] The absorbance (Figure 3.34a) of all the samples with surface functionalization decreases slower than QS@DiR-25 μ M-W, suggesting that the presence of the ligands (in particular ligand 2) increases the photostability of the encapsulated dye.

Surprisingly, a different behaviour was observed when comparing the evolution of the absorption and emission maxima for all the studied samples. The emission intensity decreases more slowly than the absorbance and, for three suspensions: QS@L2-2%-L1-2%-DiR-25 μ M-W, QS@L2-4%-DiR-25 μ M-W and QS@L1-4%-DiR-50 μ M-W, the detected emission resulted increased by $\sim 10\%$ after 30 minutes of irradiation and started to decrease only after more than 40 minutes. This phenomenon can be beneficial for bioimaging applications, since the property of interest, i.e. the overall emission intensity, is quenched only after prolonged irradiation times.

Notably, the emission increase is more pronounced in QS@L1-4%-DiR-50 μ M-W than in QS@L1-4%-DiR-25 μ M-W. This suggests a potential disaggregation of the H-aggregates during irradiation, that could increase the quantum yield of the suspensions exhibiting more significant aggregation. However, the mechanism behind the evolution of the emission maxima remains unclear and requires further investigation.

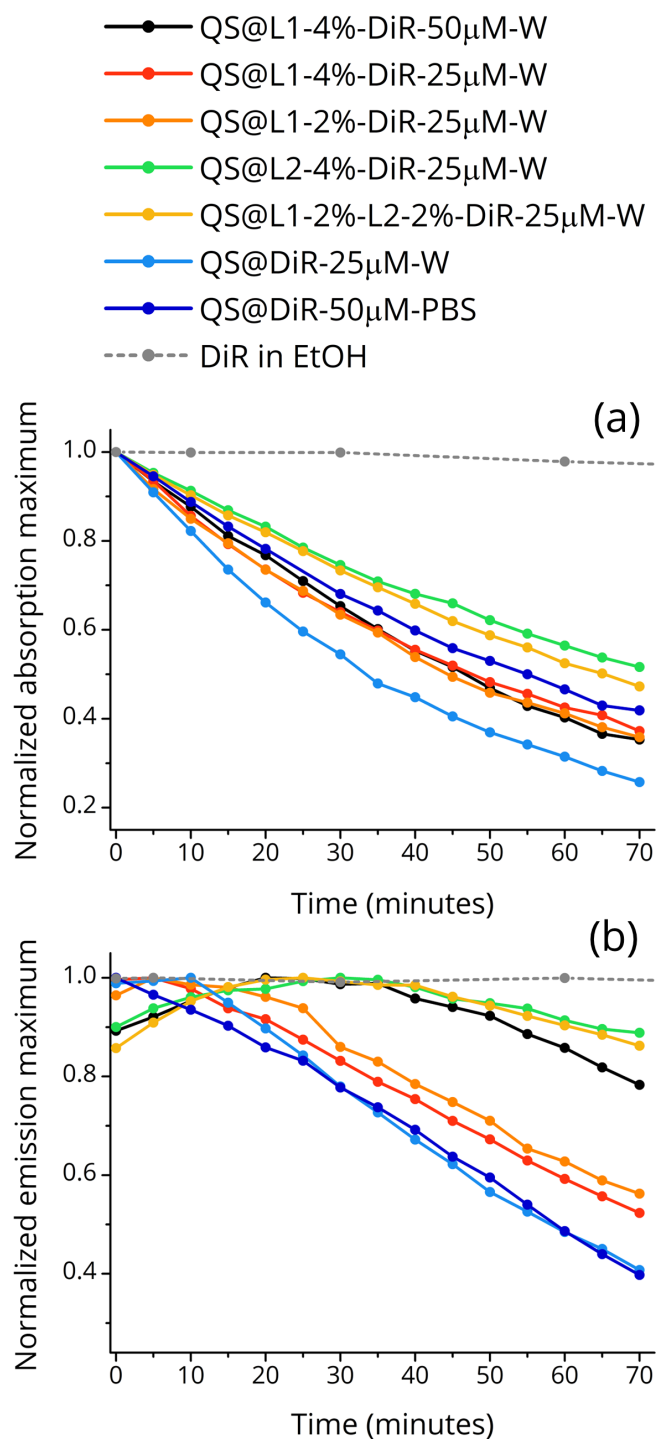


Figure 3.34: Over-time variation of the absorption and emission maxima of 7 different samples upon continuous LED irradiation ($\lambda_{exc} = 660$ nm, bandwidth: 20 nm, $I = 21$ mW/cm²). The measured absorbances and emission intensities were normalized to the maximum value obtained for each sample.

3.3.2 *In vitro* confocal imaging experiments

The extensive spectroscopic characterization discussed in Section 3.3.1 lead us to select only the 5 samples labelled with a DiR concentration of 25 μ M for *in vitro* studies:

- ▶ QS@DiR-25 μ M-W
- ▶ QS@L1-4%-DiR-25 μ M-W
- ▶ QS@L1-2%-DiR-25 μ M-W
- ▶ QS@L2-4%-DiR-25 μ M-W
- ▶ QS@L2-2%-L1-2%-DiR-25 μ M-W

These samples represent the best compromise in terms of stability and optical properties.

The cellular uptake of MKC-QSs has been previously demonstrated in healthy NHDF cells as well as HCT116 and SW1417 colon tumor cell lines, employing DiR-loaded nanovesicles suspended in Ringer's Lactate and confocal microscopy as characterization technique.[145] Thanks to co-localization studies with a lysosomal marker, it was concluded that the internalization occurs via endosomal pathway in all three cell lines.

The main objective of the current investigation was to study if the presence of L1 and L2 targeting units located on the surface of the particles can improve the cellular uptake towards the B cell line. The internalization of the selected MKC-QSs and the effect of the different surface functionalization was evaluated by laser scanning confocal fluorescence microscopy after incubation with cells from cell line B and, as a negative control, cell line A-derived cells. Each sample was diluted by a slightly different extent before the incubation procedures, in order to expose the cells to QSs suspensions having the same total absorbance. Further details on the QSs concentrations are reported in Table 3.5 (Section 3.5.3). After the incubation with the nanoparticles suspensions, cells derived from both A and B cell lines were visualized immediately as live cells and after the fixation with paraformaldehyde, to verify if consistent results can be retrieved with the two different approaches.

The fixation protocol and the quantification analysis employed on all the confocal images were performed following similar methodologies to those previously developed and implemented by the Nanomol-Bio group in Ref. [151].

Live cell imaging

Fluorescence microscopy of live cells allows to observe and analyze their dynamic behavior in real-time. Unlike traditional fixed-cell imaging, where cells are chemically fixed and observed in a static state, live cell imaging provides information about cellular processes over time. For our purposes, live cell imaging present the key advantage of being non-invasive, minimizing perturbations to cellular physiology and especially to the fluorescent nanocarriers eventually employed to treat cell cultures. Fixation procedures can indeed introduce artifacts in the cellular distribution of fluorescent probes, as demonstrated in the case of cell-penetrating peptides[152] and liposomes.[153]

The experimental protocol for live cell imaging experiments is schematically summarized in Figure 3.36.

Cells derived by cell lines A and B were seeded into disposable plastic petri dishes with optical glass bottom coated with poly-d-lysine and incubated for 3 hours (37 °C, 5 % CO₂) with the 5 different MKC-QSs diluted in culture media. Before confocal imaging, mitotracker green and Hoechst dye were employed to label, respectively, mitochondria and nuclei, allowing for a better visualization of the cells and their location. The emission profile of DiR-loaded QSs, mitotracker green and Hoechst are reported in Figure 3.35, together with the spectral ranges of the detectors used to acquire the images. Further details on the experimental methodology, such as sample preparation and fluorescent labelling are reported in Section 3.5.3.

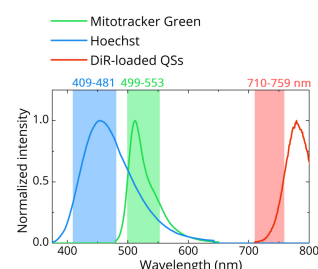


Figure 3.35: Setup employed for the confocal microscopy measurements on live cells. The spectral ranges of the three detectors and the emissions of the dyes are reported.

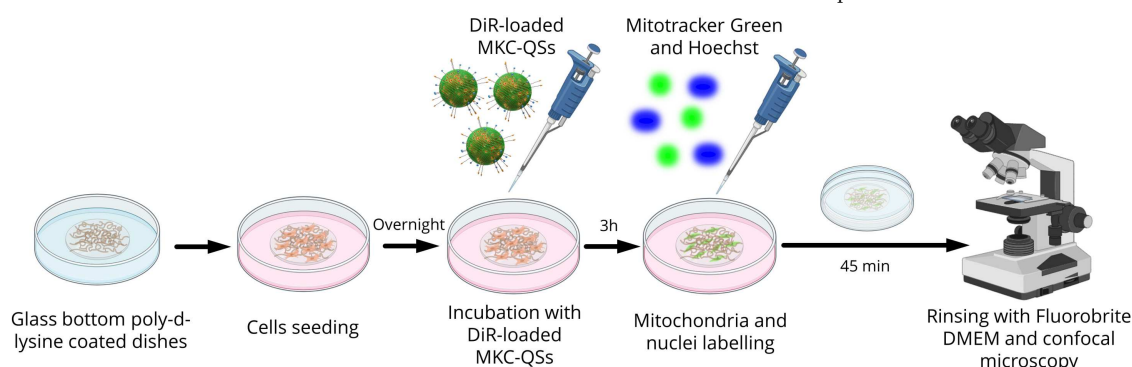


Figure 3.36: Schematic representation of the experimental protocol employed for *in vitro* studies on live cells by confocal imaging. Further technical details are reported in Section 3.5.3.

Several captures of the two cell lines treated with the 5 nanovesicles suspensions were taken from samples prepared in at least two independent experiments per suspension. In Figure 3.38, a representative image of each system is shown. The nuclei and mitochondria of all the analyzed cells were successfully labelled, as is evident from the blue and green signal, respectively. The signal in the red channel is due to the DiR-loaded QSs emission, and it was present in all samples apart from the control ones (cells not treated with the nanovesicles). The DiR-QSs localized in correspondence of cells derived by both cell lines, indicating that the nanovesicles either were uptaken by the cells or were interacting with the cell membranes. This result is in agreement with literature data on the cellular internalization of MKC-QSs.[145] No significant variations in the distribution of the DiR signal were detected, independently on the cell line and surface functionalization of the particles. As can be observed from the close up in Figure 3.37, the DiR emission mainly stems from round particles accumulated in the region of space occupied by the cells, that probably correspond to aggregates of Quatsomes.

Since the amount of DiR signal can vary drastically among the images acquired from each sample, general trends in intensity and signal area were determined analyzing large numbers of cells using Fiji, an open-source image processing package based on ImageJ. In the statistical analysis discussed below, a preliminary data analysis has been conducted, based on the methodology employed by the Nanomol-Bio group in Ref. [151]. The area of each cell has been contoured and measured, exploiting the signal in the green channel. Then, DiR emission inside each contoured

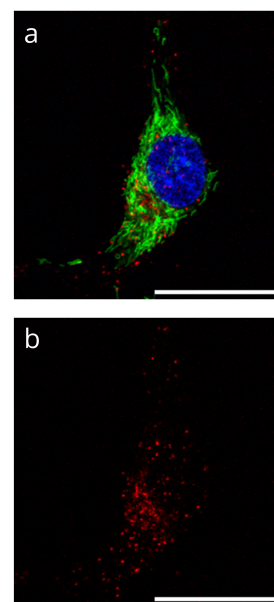


Figure 3.37: Confocal image of a single cell (cell line B) after treatment with QS@L2-2%-L1-%-DiR-25μm-W (dual targeting). In panel(a) is reported the merge of the three channels, while in panel(b) the red channel employed to detect the DiR emission is shown. Scale bar represent 25 μm.

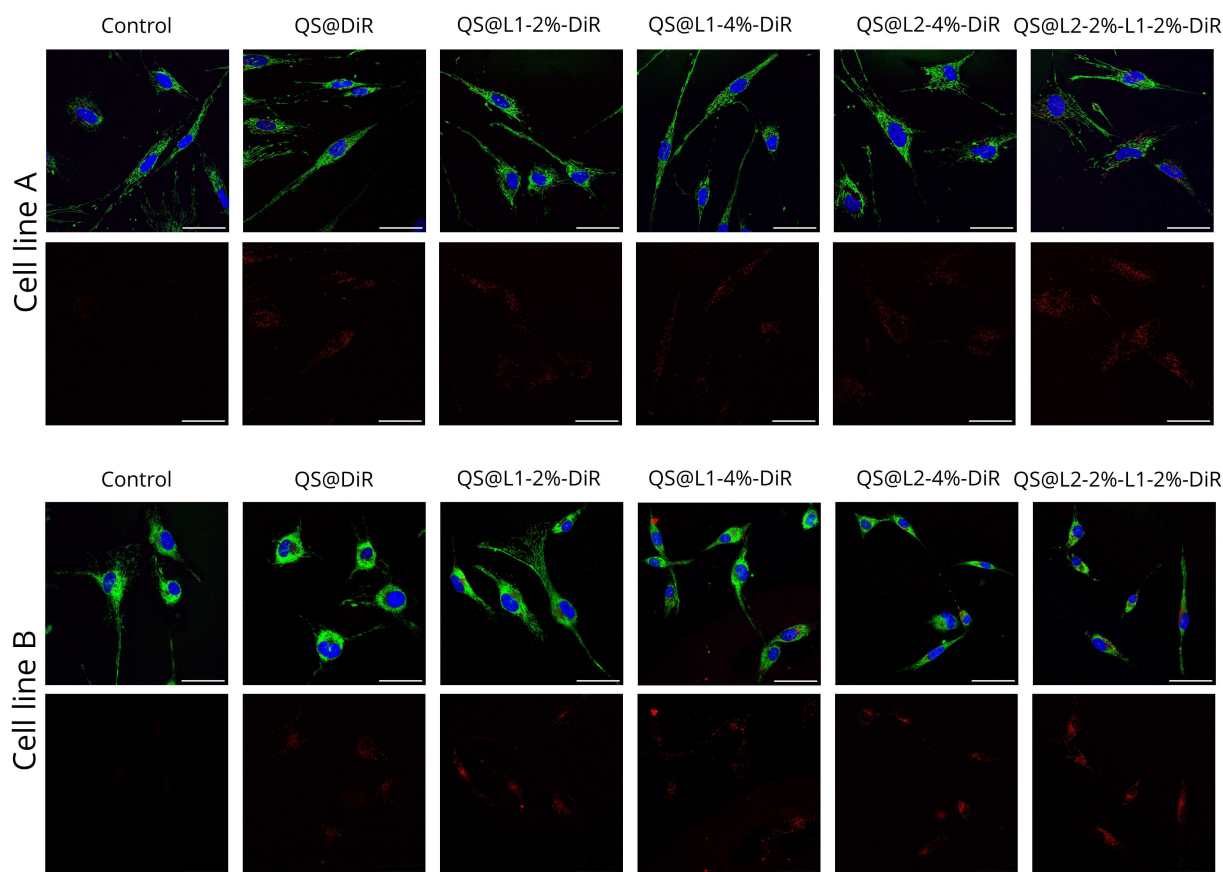


Figure 3.38: Representative confocal images of live cells derived by cell lines A (top panels) and B (bottom panels) after treatment with the 5 QSs suspensions. Control images acquired on cells not exposed to the QSs are also reported. Both the merge of the three channels and the red channel employed to detect the DiR emission are shown. Scale bar represent 50 μm .

10: The mean fluorescence intensity per cell corresponds to the integrated signal density, i.e., the sum of each pixel intensity in the selected region, divided by the cell area.

cell has been quantified considering both the mean intensity and the total surface of the red signal. In order to remove the background noise, the same threshold was applied to all the images.

After iterating the operations described above for a large number of images for both cell lines treated with the QSs suspensions, two main parameters were analysed: the area corresponding to DiR signal normalized by the cell area (expressed as a percentage) and the mean fluorescent intensity per cell¹⁰. Results are reported in Figure 3.39.

The two different analysis correlate quite well between them. In the case of cell line B, the target of the two ligands L1 and L2, higher signal from the QSs was detected after treatment with all the functionalized nanovesicles, apart from QS@L1-2%-DiR-25 μM -W, when compared to the non functionalized QS@DiR-25 μM -W. The highest DiR fluorescence signal per cell, as well as the highest percent area were detected for the dual targeting suspension (QS@L2-2%-L1-2%-DiR-25 μM -W), that showed averaged values (black solid squares in Figure 3.39) about 3 times higher than the non functionalized QSs. The two suspensions decorated with 4% of ligand 1 or ligand 2 showed comparable accumulation into cell line B, with an almost two-fold increase of averaged DiR intensity and area percent when compared to QS@DiR-25 μM -W. On the other hand, QS@L1-2%-DiR-25 μM -W performed slightly worse than the non functionalized QSs, in terms of both mean intensity and area percent. In the case of cell line A, the control one, all the QSs suspensions internal-

ized in the cells with a similar efficiency, apart from the dual targeting suspension, QS@L2-2%-L1-2%-DiR-25 μ M-W, that exhibited statistically higher mean DiR fluorescence intensity and area percent.

When comparing the accumulation of each QSs suspension in the two different cell lines, better results were obtained for cell line B with all the samples, even the non functionalized nanovesicles. These results suggests that these cells internalize more efficiently all the nanoparticles, also in absence of the surface functionalization, but its presence boosts even more the labelling performances of QSs.

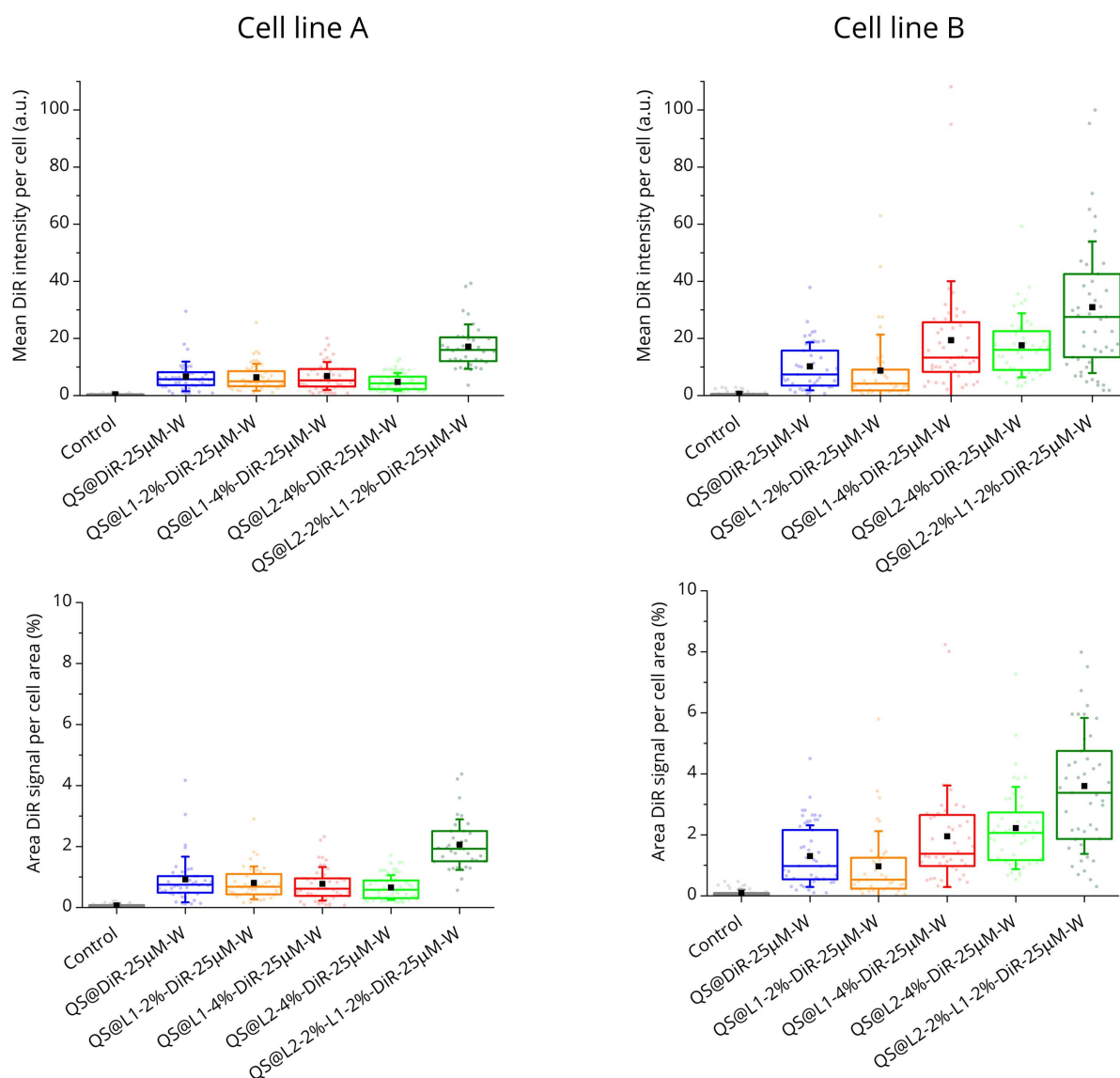


Figure 3.39: Quantification of DiR signal during live cell imaging experiments. Top panels: mean fluorescence intensity per cell (i.e., integrated signal density divided by cell area), bottom panels: area of DiR signal compared to the whole cell area (expressed as percentage). Data for cell line A are shown in the left panels, while the results for cell line B are reported in the right ones. Control experiments were performed without incubating the cells with DiR-loaded QSs. Individual cells are represented by solid dots (number of cells per group variable between 33 and 46); boxes show the 25–75 % percentile, central line the median, whiskers 1-fold standard deviation, and the solid black square the average.

Co-culturing experiments

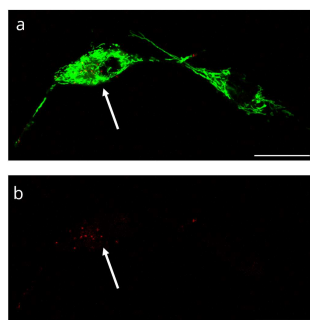


Figure 3.40: Confocal image of co-cultured A and B cell lines after treatment with QS@L1-4%-DiR-25 μ m-W. In panel(a) is reported the merge of the green and red channels, while in panel(b) the red channel employed to detect the DiR emission is shown. Cells derived from cell line B, characterized by a higher signal from the mitochondria labelling, is highlighted by a white arrow. Scale bar represent 25 μ m.

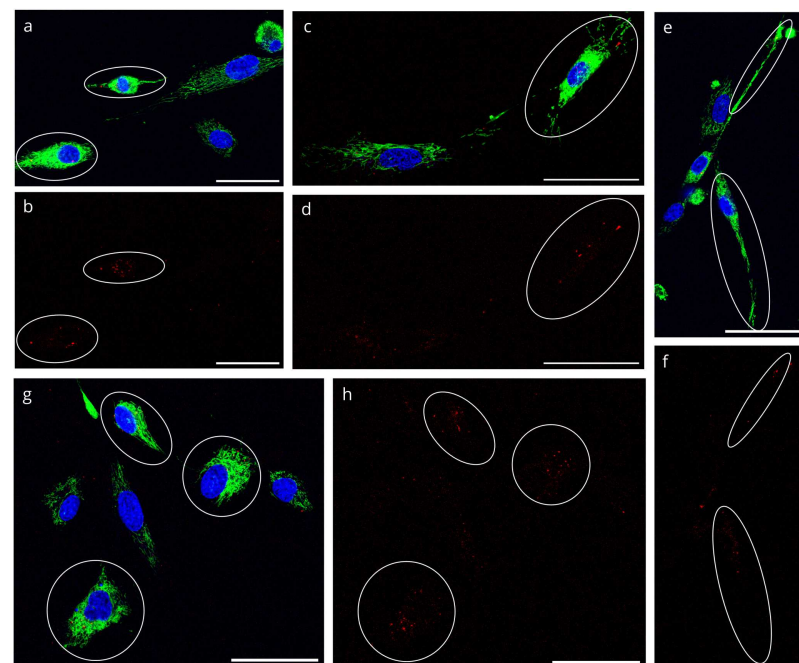


Figure 3.41: Confocal images of co-cultured A and B cell lines, after treatment with QS@L1-4%-DiR-25 μ m-W. Panels a, c, g and e show the three channel-merge while the corresponding red channels are reported in panels b, d, f and h, respectively. The cells derived from cell line B are highlighted by white circles. Scale bar represent 50 μ m.

To further confirm the preferential accumulation of the functionalized nanovesicles in the target cell line B, co-culture experiments were performed, exposing both cell lines (A and B) to the same QSs suspension simultaneously. Only the QS@L1-4%-DiR-25 μ m-W suspension has been employed for the co-culture experiments. The cells derived from B cell line can be distinguished from the others due to their apparent higher density of mitochondria and stronger labelling with mitotracker green, that results in a much brighter green signal. A and B cell lines were seeded simultaneously into the same petri dish with optical glass bottom coated with poly-d-lysine and incubated for 1 hours (37 °C, 5 % CO₂) with QS@L1-4%-DiR-25 μ m-W diluted in culture media. Before confocal imaging, mitotracker green and Hoechst dye were employed to label, respectively, mitochondria and nuclei. The outcome is shown in Figure 3.40: the DiR-signal is mainly localized in the cells exhibiting stronger mitochondrial labelling, i.e. those derived from cell line B. Other representative images showing the same preferential internalization of QSs in cell line B are reported in Figure 3.41.

Fixed cell imaging

The same confocal microscopy study was repeated also fixing the cells after treatment with QSs suspensions, in order to verify if the fixation protocol can introduce artifacts. The experimental procedure employed for preparing and visualizing fixed cells is schematically summarized in Figure 3.43. Briefly, A and B cell lines were seeded into fibronectin-coated glass substrates and incubated for 3 hours (37 °C, 5 % CO₂) with the 5 different MKC-QSs diluted in culture media. After the exposure to QSs, the cells were fixed, permeabilized and labelled with fluorescent dyes. Specifically, phalloidin-conjugated Alexa fluor 488 and Hoechst dyes were employed to label, respectively, cytoskeleton and nuclei of the cells. Phalloidin can indeed bind to actin filaments, allowing for the visualization of the cell cytoskeleton in the green channel. The emission profile of DiR-loaded QSs, Alexa488 and Hoechst are reported in Figure 3.42, together with the spectral ranges of the detectors used to acquire the images. Further details on the experimental methodology, such as sample preparation and fluorescent labelling are reported in Section 3.5.3.

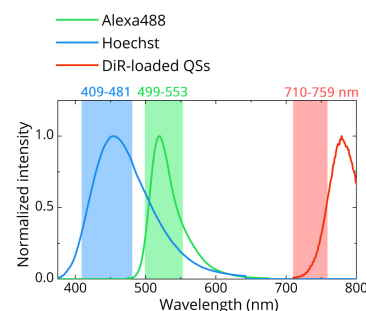


Figure 3.42: Setup employed for the confocal microscopy measurements on fixed cells. The spectral ranges of the three detectors and the emissions of the dyes are reported.

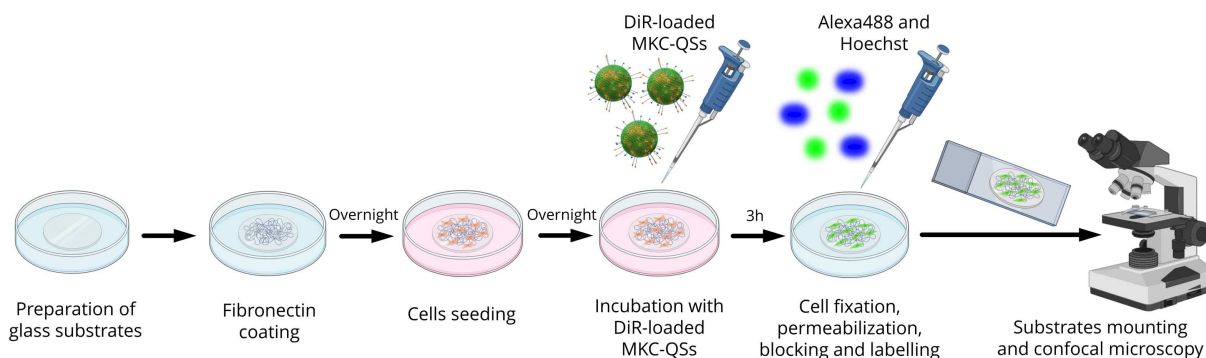


Figure 3.43: Schematic representation of the experimental protocol employed for the *in vitro* studies on fixed cells by confocal imaging. Further technical details are reported in Section 3.5.3.

The substrates containing fixed and fluorescently labelled cells were then mounted onto glass slides, and confocal imaging was performed for internalization analysis. Also in this case, different representative captures of each system were taken from glass substrates prepared in two independent experiments for each QSs suspension, and the results are reported in Figure 3.45. The nuclei and cytoskeleton of both cell lines were successfully labelled, as is evident from the blue and green signal, respectively. Even if much weaker than for live cells, the red signal, due to the DiR-loaded QSs emission, was present in all samples apart from the control ones (not treated with QSs). However, quite large amount of cells presented an almost null DiR fluorescence signal after the treatment with the 5 QSs suspensions. When the red signal was detected, as shown in Figure 3.44, its distribution resulted similar to live cells: the dye mainly accumulated in round structures localized in correspondence of the cells.

In order to evaluate the internalization of the different QSs suspensions into fixed cells we performed the same preliminary statistical analysis described in the previous section for live cells. The area corresponding to DiR signal normalized by the cell area and the mean fluorescence intensity per cell were compared for the two cell lines after the treatment with

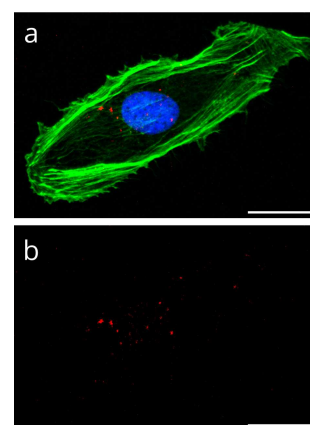


Figure 3.44: Confocal image of a single cell (cell line B) after treatment with QS@L1-4%-DiR-25μm-W and fixation. In panel(a) is reported the merge of the three channels, while in panel(b) the red channel employed to detect the DiR emission is shown. Scale bar represent 25 μm.

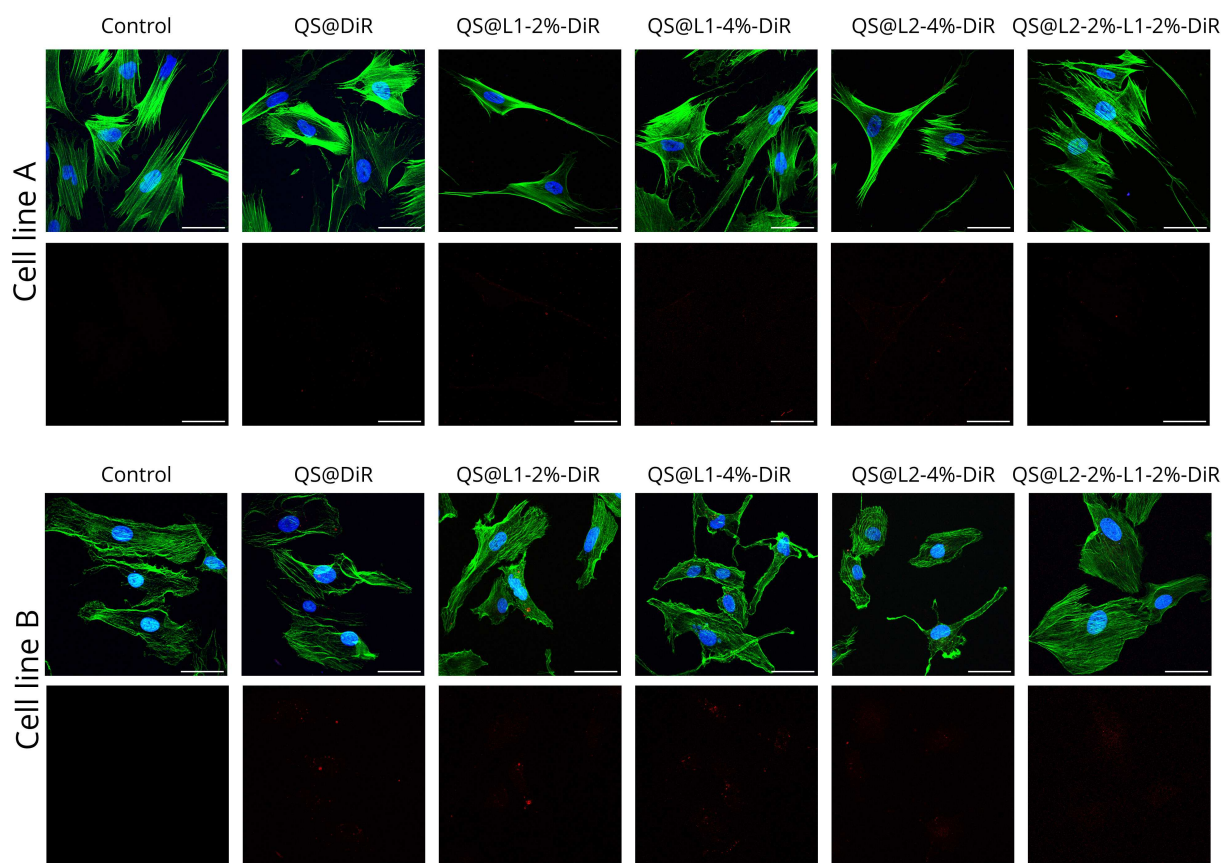


Figure 3.45: Representative confocal images of fixed cells derived by cell lines A (top panels) and B (bottom panels) after treatment with the 5 QSs suspensions. Control images acquired on cells not exposed to the QSs are also reported. Both the merge of the three channels and the red channel employed to detect the DiR emission are shown. Scale bar represent 50 μm .

QSs, and the results are presented in Figure 3.46. For what concern cell line B, QS@L1-2%-DiR-25 μM -W and QS@L1-4%-DiR-25 μM -W showed an enhanced accumulation of DiR signal when compared to the non functionalized QSs, with mean intensity and area percent increased by $\sim 30\%$ and 100% , respectively. The two samples decorated with ligand 2 exhibited worse results than in the case of live cells, especially the dual targeting one (QS@L2-2%-L1-2%-DiR-25 μM -W), characterized by a strongly suppressed signal in the red channel.

In the case of cell line A, all the QSs suspensions internalized in the cells with a similar efficiency, a part from the dual targeting suspension, that showed almost null DiR fluorescence intensity and area percent.

It is important to highlight that the fixation protocol lead to an overall decrease in the detected signal intensity (and signal area) of DiR-loaded QSs accumulated in the cells, when compared to live cells experiments.

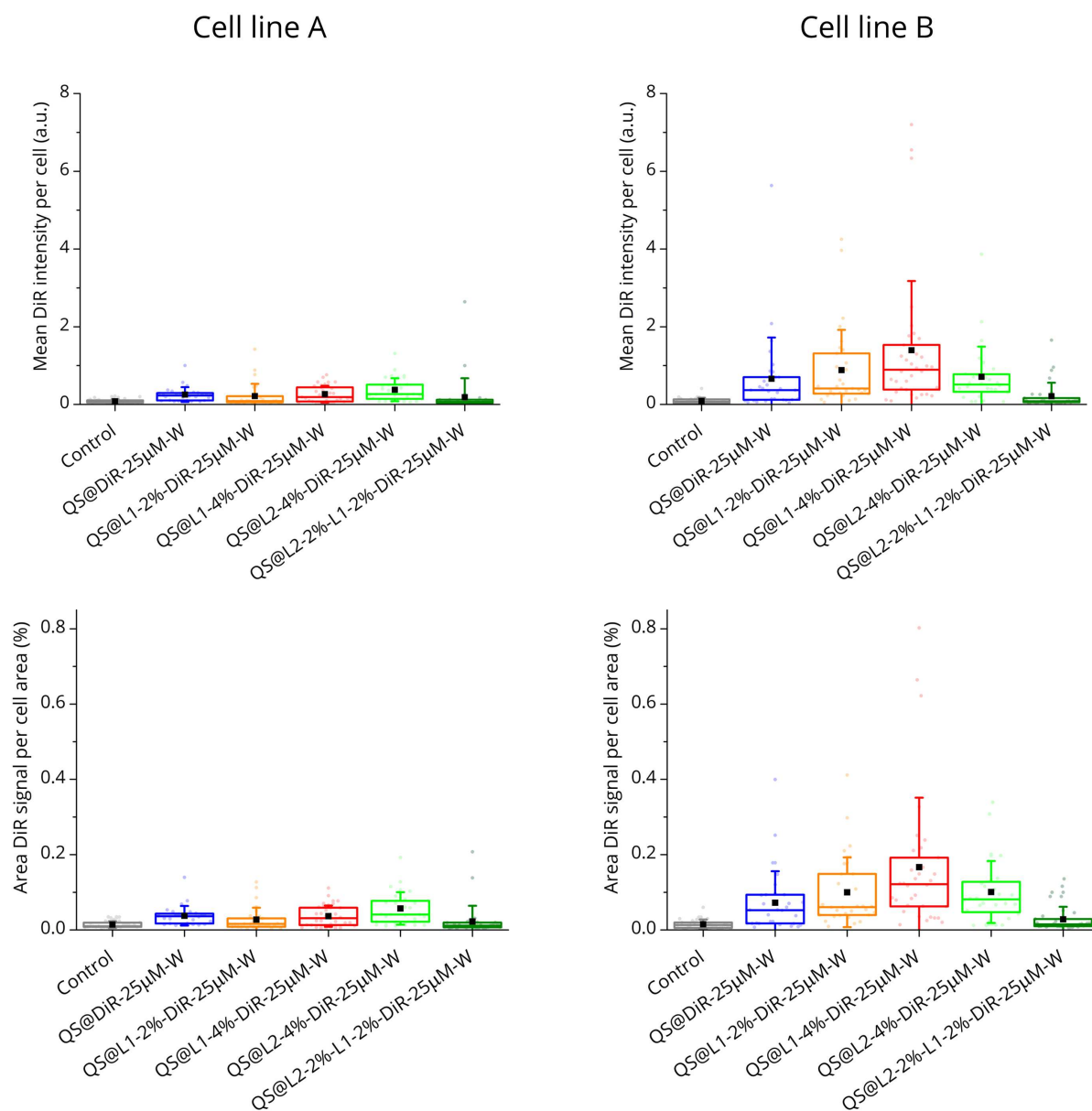


Figure 3.46: Quantification of DiR signal during fixed cell imaging experiments. Top panels: mean fluorescence intensity per cell (i.e., integrated signal density divided by cell area), bottom panels: area of DiR signal compared to the whole cell area (expressed as percentage). Data for cell line A are shown in the left panels, while the results for cell line B are reported in the right ones. Control experiments were performed without incubating the cells with DiR-loaded Qs. Individual cells are represented by solid dots (number of cells per group variable between 28 and 34); boxes show the 25–75 % percentile, central line the median, whiskers 1-fold standard deviation, and the solid black square the average.

3.4 Conclusions and perspectives

Qs serve as highly versatile platforms capable of accommodating multiple (macro)molecular objects, each conferring distinct functionalities. In this Chapter, two families of new fluorescent Qs have been discussed: CTAB-Qs loaded with two dyes and MKC-QS loaded with a NIR-emitter and two different ligands for specific targeting.

CTAB-Qs were prepared to explore the possibility of dye loading in different positions of the nanovesicle, also opening the possibility of dye-loading after preparation of the Qs by incubation. Successful encapsulation of EoY was achieved, most probably thanks to strong electrostatic interactions between the negatively charged EoY and the positively charged CTAB. On the opposite, the same approach failed for RhB, and a very large amount of the dye was lost after diafiltration process. In order to verify if the dyes are mainly localized on the Qs surface or inside their membrane, some additional experiments, such as competitive assays for the electrostatic interactions (with e.g. SDS or heparin), should be performed. The novel EoY-loaded FONs possess good optical properties (quantum yield, stability, molar extinction coefficient, etc.) for their application in bioimaging. For this purpose, FRET Qs loaded with EoY and DiD were successfully prepared and characterized. The dye-loaded samples exhibited higher FRET efficiencies than the dye-incubated ones. Testing different preparation procedures, we verified that moving the CTAB or the hydrophilic dye to the organic phase during the DELOS-SUSP does not affect significantly the spectroscopic properties of the resulting nanovesicles, including the FRET efficiency. These results indicate that the interactions between the hydrophilic donor and the Qs membrane are of the same kind for all the preparation procedures, including incubation. Moreover, the incubation protocol emerges from this work as a promising approach for functionalizing Qs in post-production. The DELOS-SUSP procedure can in principle be employed to firstly prepare plain or acceptor-loaded Qs, that can be subsequently incubated with the desired hydrophilic dye. As demonstrated in this study, this process can lead to bright nanoparticles, also showing efficient FRET if a second dye is present in the system.

Ensuring a stable binding of the fluorophores to the nanovesicles, both over time and over dilution is crucial for potential applications. Samples intended for bioimaging purposes are frequently subjected to substantial dilution and prolonged exposure to biological media. Indeed, fluorophores leaching in these conditions would give serious problems such as loss of brightness of the nanoparticles and high background signal coming from the free dye in solution, that could also cause unwanted labelling of structures during image acquisition. For these reasons, also the over time stability of the EoY-loaded samples should be addressed, and all the samples should be tested to verify if they are stable upon dilution.

In the second part of this work, MKC-Qs were functionalized with DiR, a NIR-emitter, that can be used for *in vivo* applications, also for in-depth imaging (TPM). The loading of two ligands (L1 and L2) on the FONs surface was also tested, with the aim to prepare multifunctional Qs for specific targeting. The spectroscopic characterization highlighted the effect of the DiR concentration on the fluorescence quantum yield,

fluorescence lifetime, spectral bandshape and photostability of the resulting Qs. Significant aggregation features were observed for all the samples characterized by a DiR concentration equal or higher than 25 μM , mainly due to their diminished emissivity and fluorescence lifetime compared to the samples loaded with DiR 1 μM . No important differences were detected among the samples with the same amount of DiR, even with a different surface functionalization. The samples loaded with a DiR concentration of 25 μM proved to be the best compromise between fluorescence quantum yield, dye concentration and stability over-time, hence they were selected for *in vitro* cellular internalization studies. From a preliminary analysis of the confocal microscopy measurements on live cells, a sizable enhancement of the L1 and L2 decorated Qs uptake was detected in cell line B, when compared to non-functionalized ones. The best results were achieved with the dual targeting formulation, which presents both L1 and L2 motifs and gave rise to a three-fold increase of DiR signal. The negative controls experiments with cell line A and co-culture further corroborated the results.

These findings confirm the positive role of the surface functionalization with the two ligands in boosting the internalization of the nanovesicles into the cells target of the study. Confocal microscopy experiments and the preliminary data analysis were then repeated also on fixed cells, to verify if consistent information could be retrieved. However, the fixation protocol lead to a significant overall decrease in the detected DiR-loaded Qs signal in both cell lines, with respect with live cells experiments. In order to improve the results, higher amounts of Qs should be employed in the incubations experiments before fixing the cells, to compensate for the signal reduction.

All the findings and analyses outlined in this chapter are pending submission to a peer-reviewed scientific journal for publication.

3.5 Materials and Methods

Eosin Y (for microscopy) and Rhodamine B isothiocyanate (BioReagent, mixed isomers) were obtained from Carlo Erba reagents S.A.S. and Sigma-Aldrich, respectively. All the purchased chemicals have been employed without further purification.

3.5.1 Preparation of dye-loaded Quatsomes

11: Scalability, high reproducibility, high vesicle-to-vesicle homogeneity, and the use of low cost, easy-to-get, nontoxic components make this technique suitable for both laboratory use and industrial scale-up.

The DELOS-SUSP method (Depressurisation of an Expanded Liquid Organic Solution-suspension) is a compressed fluid technique which enables the straightforward synthesis of nanovesicles in aqueous media from their membrane components.¹¹ Specifically, in the standard DELOS-SUSP protocol, QSs can be prepared using a solution in ethanol of membrane components and any hydrophobic substance to be encapsulated. As schematised in Figure 3.47, the ethanolic solution (organic phase) is expanded with supercritical carbon dioxide and then depressurised at constant pressure (while purging with dry nitrogen) over an aqueous phase containing the surfactant and any hydrophilic substance to be encapsulated. In the last step, the expanded organic solution experiences a large, abrupt, and extremely homogeneous decrease in temperature, due to CO₂ evaporation. This might explain the resulting homogeneous vesicles in terms of size, morphology and lamellarity.

This standard procedure was employed for the preparation of all dye-loaded suspensions. The dye-incubated QSs were obtained by adding the hydrophilic dyes through incubation of plain or DiD loaded QSs, previously prepared through DELOS-SUSP performed in the presence of only the CTAB surfactant in the aqueous phase.

All samples listed in Section 3.2 were prepared according to the standard procedure (dye-loaded QSs) or incubation (dye-incubated QSs), and then purified by diafiltration. More technical details on the employed procedures are reported in the following sections.

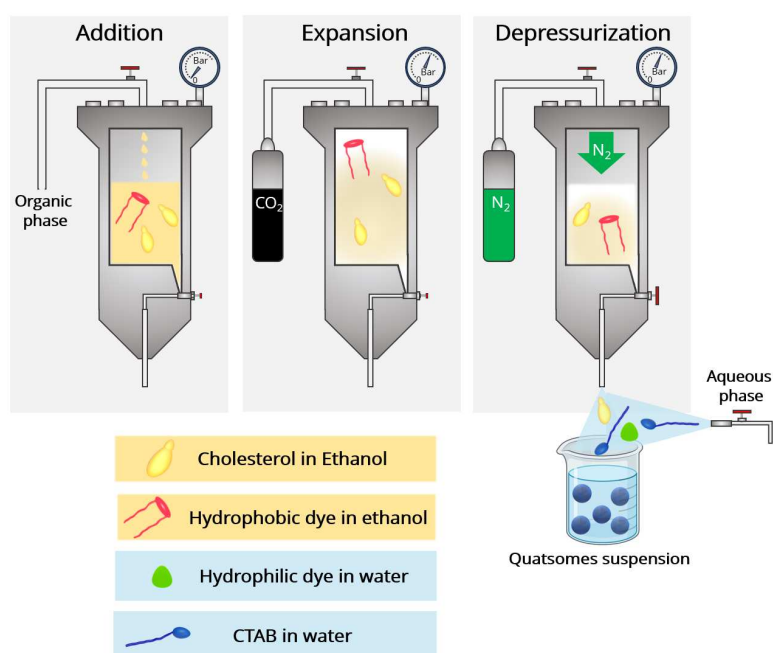


Figure 3.47: Scheme of the DELOS-SUSP process: an ethanol solution of QS membrane components (cholesterol and hydrophobic dyes) is expanded with supercritical CO₂ and subsequently depressurized over an aqueous phase, containing CTAB and hydrophilic dyes, to obtain Quatsomes.

Standard DELOS-SUSP procedure for EoY/RhB-decorated Qs

79.88 mg of cholesterol were dissolved in a vial with 2.91 mL of ethanol HPLC grade or 2.91 mL of a 970 μM DiD ethanolic solution, in the case of samples decorated with DiD. The vial was closed, sealed with parafilm and heated in a bath (probe temperature: 40 °C) under stirring (750 rpm) for 20 minutes. When the cholesterol was completely dissolved, the solution was loaded in a high pressure vessel, washing the vial with 0.2 mL of ethanol. The vessel was covered with a heating mantle set at the temperature of 35 °C. After 10 minutes of heating, CO₂ was introduced at a pressure of 115 bar and the vessel was left to equilibrate for one hour, in order to homogenize the system. Meanwhile, 72.48 mg of CTAB were solubilized in 25.10 mL of MilliQ water or EoY/RhB solution in MilliQ water with the desired concentration (calculated considering that the final volume at the of the preparation is 28.22 mL). The CTAB solution was sealed with parafilm and heated in a bath (40 °C) under stirring (750 rpm) for 20 minutes. Finally, the vessel was then slowly depressurised in the aqueous solution while purging with dry nitrogen at 115 bar, to keep constant the pressure inside the reactor. Samples were stored at 4 °C. After several days to allow for stabilization, the samples were purified through diafiltration, and stored at 4 °C.

Alternative DELOS-SUSP procedures for EoY/RhB-decorated Qs

For what concern the results reported in Section 3.2.6, two batches of Qs were prepared following alternative procedures. In both cases, the only difference from the standard procedure described above consists in the location of EoY and CTAB.

- **EoY in the organic phase:** EoY was added to the organic phase instead of the aqueous one, obtaining a 1.338 mM ethanolic solution of EoY. After solubilizing 79.88 mg of cholesterol and possibly adding DiD (970 μM), the organic phase was expanded and then depressurized over an aqueous phase containing 72.48 mg of CTAB, as previously described.
- **CTAB in the organic phase:** CTAB was added to the organic phase instead of the aqueous one, solubilizing 72.48 mg of surfactant in ethanol. After adding 79.88 mg of cholesterol and possibly DiD (970 μM), the organic phase was expanded and then depressurized over an aqueous phase containing EoY at a concentration of 155 μM , as previously described.

Samples were stored at 4 °C. Also in this case, after several days to allow for stabilization, the samples were purified through diafiltration, and stored at 4 °C.

Incubation procedure

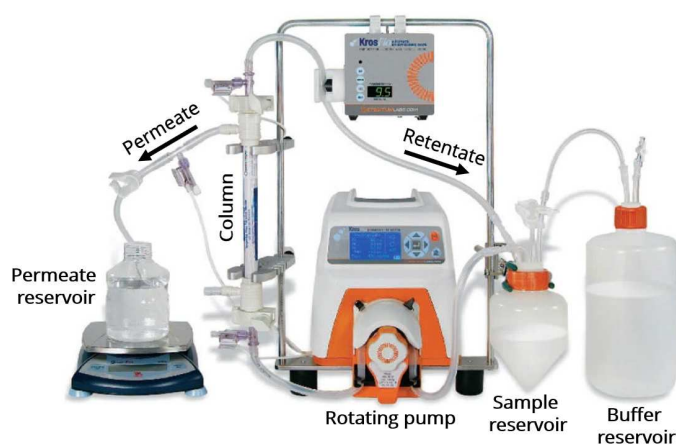
A suitable amount of EoY or RhB aqueous solution (in the range 70-275 μL , according to the concentration of the stock solution) was added to a previously prepared suspension of plain or DiD-loaded Qs, with a total volume of 6 mL. The suspensions were left to incubate for 30 minutes at room temperature under stirring (750 rpm) and then, 5 mL of

each incubated sample were diafiltrated. In the case of the re-prepared QS@DiD-EoY-L described in Section 3.2.6, 552 μL of EoY 2 mM in water were added to 7448 μL of DiD-loaded Qs. The sample was left to incubate for 30 minutes at room temperature under stirring (750 rpm) and then 6 mL were diafiltered.

Diafiltration

Diafiltration is a purification technique employed to separate water-soluble components from solution or suspension based on their dimensions, exploiting a tangential flow along a membrane with controlled pore size. The principle of the procedure is similar to dialysis, but diafiltration is faster and lead to lower amounts of wastes. In our case, the purpose of this purification was to remove not only the ethanol present in the raw suspensions ($\sim 10\%$), but also cholesterol, CTAB and dye in excess, residuals from the DELOS-SUSP protocol. The selection of an appropriate membrane and of a suitable trans-membrane pressure are crucial parameters to ensure the removal of excess components from a Qs sample, while preserving nanovesicles integrity. For all the samples diafiltered in the context of this thesis we employed C04 columns with 100 kDa pore size and 5 psi as trans-membrane pressure (flow speed: 27 mL/min). The setup used for diafiltration was KrosFlow Research Ili TFF system (Spectrum Labs, USA), illustrated in Figure 3.48.

Figure 3.48: Illustration of the diafiltration setup employed in this thesis for the purifications of all the prepared Quatsomes suspensions. Adapted from the product information and operating instructions manual from the producer (Spectrum Labs).



The aqueous medium in which QS were dispersed was thus progressively substituted with a buffer solution (milliQ water): the purified sample exiting the column, known as retentate, was repeatedly cycled through the instrument through a rotating pump, while the liquid phase filtered by the column (permeate) was discarded. The permeate contains all the low molecular weight compounds (ethanol, excess CTAB, cholesterol etc.) that can be eliminated by passing through the membrane pores. For every drop of permeate, a drop of fresh milliQ water (from the buffer reservoir) was added to the sample. Also, by definition one diafiltration volume corresponds to the initial volume of the sample: a sample is considered to be properly diafiltrated when at least six diafiltration volumes of permeate have been collected. For the suspensions prepared within this thesis, the diafiltration process was stopped after a minimum of six diafiltration volumes or until no colour was observed in the permeate.

Estimation of dye-loading in Quatsomes

The amount of dye present in a QS sample can be measured via UV–visible spectrophotometry. Specifically, as the extinction coefficients of the dyes loaded inside QSs are usually unknown, and in order to avoid aggregation effects, the dye is quantified after dissolving the sample in ethanol. Aqueous suspensions of QSs were diluted at least 1/50 (V/V) in pure ethanol before measuring the absorbance, assuming that the extinction coefficient of the dyes is not affected by the presence of the other QSs components and corresponds to the value in ethanol. The extinction coefficients employed for the calculations amount to $246\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 648 nm for DiD, $88\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 525 nm for EoY and $55\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 545 nm for RhB (in ethanol). A Jasco-780 spectrophotometer was employed to measure the absorbances of the samples diluted in ethanol. After the estimation of the dyes concentrations from the absorbances, through the Lambert-Beer law ($A = \epsilon cl$), on both raw (before diafiltration) and diafiltered suspensions, the encapsulation efficiency (E.E.) of the dyes enclosed in QSs were obtained using Equation 3.1.

The same procedure, based on UV–visible spectrophotometry, was employed also to check the concentration of all the dyes stock solutions.

3.5.2 Photobleaching experiments

The photostability of DiR in the prepared MKC-QSs suspensions was evaluated monitoring absorption and emission spectra under continuous-wave excitation, over a time period of 70 minutes. The samples were placed in a macro cuvette (optical path: 1 cm) and irradiated with a ThorLabs M660L4 mounted LED ($\lambda_{exc} = 660\text{ nm}$, bandwidth: 20 nm, $I = 21\text{ mW/cm}^2$) illuminating the whole volume of the suspension (see Figure 3.49). The average irradiance of the LED at 6 cm (i.e., the distance between the sample and the LED source) was measured using a powermeter (FieldMaster with LM-1 detector head from Coherent). Samples have not been degassed before performing the measurements. After every 5 minutes of irradiation, we recorded absorption and emission spectra and then re-positioned the cuvette in front of the LED source.

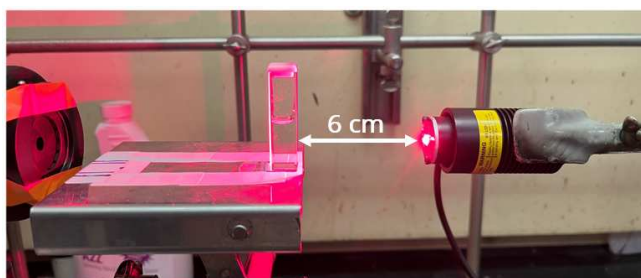


Figure 3.49: Setup employed for the photodegradation experiments.

3.5.3 *In vitro* experiments

Cells (purchased from ATCC, America Type Culture Collection, Manassas, VA, U.S.A.) were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM 1X + 4.5 g/L glucose and L-glutamine, Gibco, Thermo Fisher Scientific, USA) or McCoy's 5A (Modified) medium (Gibco, Thermo Fisher Scientific, USA). Both culture media were supplemented with 10 % FBS (Fetal Bovine Serum) and 1 % of penicillin/streptomycin (100x, Gibco). Cells were incubated in a humidified atmosphere at 37 °C and 5 % CO₂.

Fixed cell imaging

First, glass substrates (round borosilicate glass, 10 mm diameter) were cleaned with ethanol (70%), extensively rinsed with distilled water and then dried. A fibronectin 10 µg/mL solution (Fibronectin bovine plasma, F1141, Sigma Aldrich, stock 1 mg/mL) was prepared in PBS (1:10 volume). Substrates were placed into a 6-well plate and incubated with fibronectin (100 µL per substrate) overnight at 37 °C and 5 % CO₂ in humidified atmosphere.

Before cell seeding, substrates were rinsed three times with sterile PBS (PBS 1X, pH 7.2, Phosphate Buffered Saline, Gibco). Thereafter, 45,000 cells were seeded onto each substrate and wells were filled with cell medium (3 mL final volume). Substrates were incubated overnight at 37 °C and 5 % CO₂ allowing for cell adhesion and spreading out. Next, each well was incubated with 2 mL of medium containing DiR-loaded MKC-QSs for 3 h (37 °C and 5 % CO₂), before cell fixation and fluorescent labelling. MKC-QSs Quatsomes were placed into the culture medium adjusting the dilution according to their absorbance, as shown in Table 3.5:

Table 3.5: Dilutions of DiR-loaded MKC-QSs employed for *in vitro* experiments.

Sample	Absorbance after dilution 1:30	Dilution in culture media	Concentration of membrane components in culture media
QS@DiR-25µM-W	0.092	1:1400	~0.69 µg/mL
QS@L1-4%-DiR-25µM-W	0.066	1:1000	~0.99 µg/mL
QS@L1-2%-DiR-25µM-W	0.073	1:1110	~1.43 µg/mL
QS@L2-4%-DiR-25µM-W	0.087	1:1315	~0.71 µg/mL
QS@L2-2%-L1-2%-DiR-25µM-W	0.1141	1:1724	~0.75 µg/mL

Absorption measurements on MKC-QSs were performed before every incubation, in order to account for over-time variations. The concentrations of the membrane components listed in Table 3.5 are obtained after lyophilization for removing water and organic solvents and mass concentration, measuring the resulting solid by gravimetric analysis (mass balance).

Cells were then rinsed with FluoroBrite DMEM (Gibco, Thermo Fisher Scientific, USA) supplemented with HEPES 20 mM, to prevent cell detachment. Subsequently, 4 % paraformaldehyde aqueous solution (Thermo Fisher Scientific, USA, 1.5 mL/sample, 20 min at room temperature) was added to each well, fixing the cells. Then, substrates were rinsed 3 times with PBS and cells were permeabilized by adding 0.1% v/v Triton (Triton™ X-100, Sigma Aldrich) prepared in PBS (3 mL/sample, 5 min at room temperature). After fixation and permeabilization, samples were treated with a blocking solution (1 % w/v bovine serum albumin in PBS, Sigma Aldrich, filtered by 0.22 μ m nylon syringe filter before use) to prevent non-specific binding (1.5 mL/sample, 30 minutes at room temperature).

After blocking, actin and nuclei were stained employing appropriate fluorescent labelling agents. First, a labelling solution containing Alexa Fluor™ 488 Phalloidin (A12379, Invitrogen, Thermo Fisher) and Hoechst (Hoechst 33342, Invitrogen, Fisher Scientific, stock at 10 mg/mL) was prepared in 1% w/v BSA in PBS, with concentrations 165 nM and 4.05 μ M (1.25 μ g/mL), respectively. Samples were then covered with the labelling solution (100 μ L per sample) for 45 min at 37 °C and 5 % CO₂ in humidified atmosphere inside the incubator (hence, in the dark).

Finally, substrates were placed in a clean 6-well plate and rinsed 3 times with fresh PBS. They were transferred into a standard glass microscope slide and mounted in antifading oil (ProLong™ Gold antifade reagent, P36930, Invitrogen, Thermo Fisher, a drop per sample). Samples were left to dry (at least 24 h) and stored at room temperature in the dark until the image acquisition.

Live cell imaging

Cells were seeded on confocal disposable plastic petri dishes (MatTek) with optical glass bottom coated with poly-d-lysine, ensuring cell adherence (35 mm and 10 mm glass diameter). 45,000 cells were seeded onto each confocal dish, that was then filled with culture medium (3 mL final volume) and incubated overnight at 37 °C and 5 % CO₂.

Next, each sample was incubated with 2 mL of medium containing DiR-loaded MKC-QSs for 3 h (37 °C and 5 % CO₂). MKC-QSs Quatsomes were placed into the culture medium adjusting the dilution according to their absorbance, as described in the fixed cell protocol and shown in Table 3.5.

Cells were then rinsed three times with fresh culture media, to prevent cell detachment. Subsequently, a labelling solution containing Mitotracker Green (M7514, Invitrogen, Thermo Fisher Scientific) and Hoechst was prepared in fresh culture media, with concentrations 200 nM and 810 nM (0.5 μ g/mL), respectively. Samples were then incubated with the labelling solution (2 mL per dish) for 45 min at 37 °C and 5 % CO₂ in humidified atmosphere inside the incubator (hence, in the dark).

Finally, each dish was washed three times with FluoroBrite DMEM (supplemented with HEPES 20 mM) and an additional mL of FluoroBrite DMEM was added before confocal imaging. Each sample was visualized

for no longer than 40 minutes since the labelling with mitotracker green and Hoechst.

Confocal fluorescence microscopy

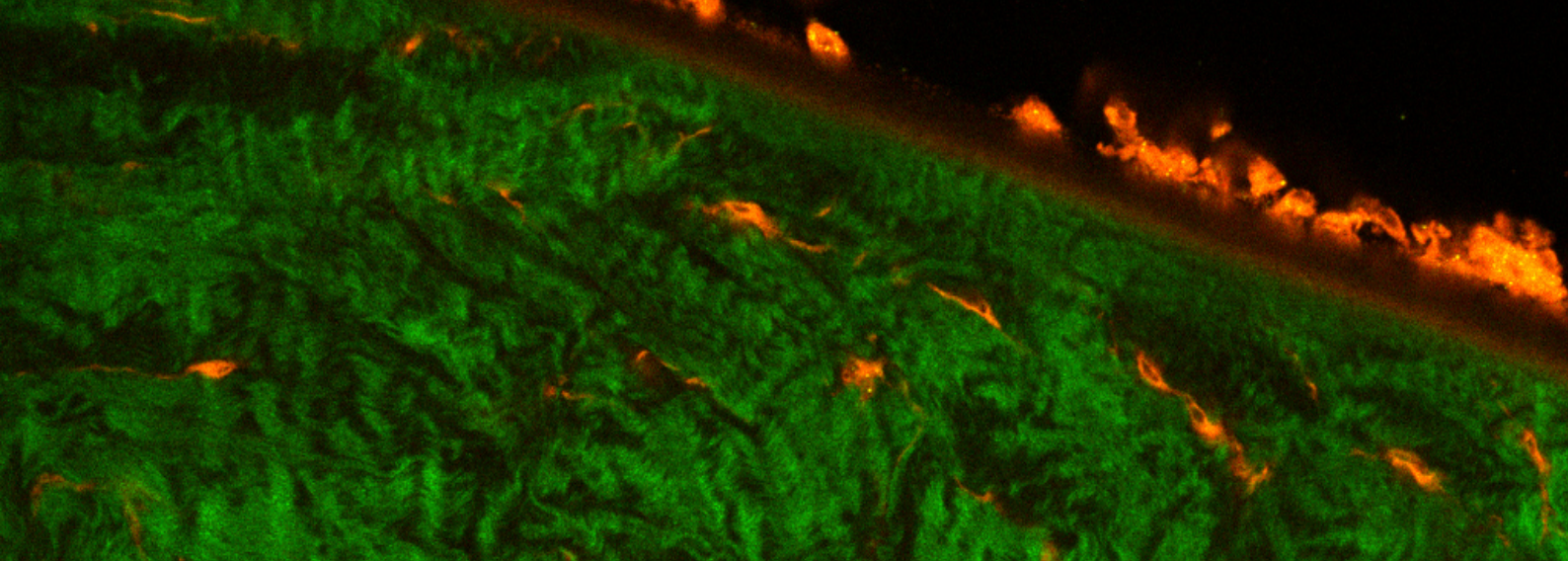
Cell images were recorded with a Zeiss LSM 780 confocal microscope equipped with a C-Apochromat 40x/1.20 W Korr FCS M27 objective (pinhole 42.5 μm , airy 1 with respect to the blue channel; numerical aperture 1.20), available in the laboratory of Prof. K. Belfield at New Jersey Institute of Technology.

Simultaneous excitation of Hoechst33342 (nuclei), phalloidin-Alexa488 (actin, for fixed cells imaging) or Mitotracker Green (mitochondria, for live cells imaging), and DiR (Quatsomes) was carried out using 405, 488 and 633 nm laser lines respectively. Detection was performed with PMTs at specific ranges as follows: Hoechst was detected within the range 409–481 nm, phalloidin-Alexa-488 and Mitotracker Green within 499–553 nm and DiR within 710–759 nm range.

Laser power and PMT settings for DiD were fixed (60 % and 1200 V gain/0 offset, respectively) and kept throughout all measurements, allowing for subsequent comparison and quantification of the signal in the images.

In order to avoid cross talking between the channels dedicated to nuclei staining and to actin/mitochondria labelling, the 405 nm laser source was maintained at a much lower intensity in comparison to the 488 nm one (0.2% vs 3%), while the detector gain of the channel for nuclei detection was enhanced (1000 V vs 650 V).

Captures were taken at 40X magnifications (no zoom, pixel dwell 1.58 μs) to obtain detailed pictures of few cells with a field of view of 212.5 μm x 212.5 μm (1024 x 1024 pixels resolution, with a pixel size of 0.12 μm). The images were processed using Fiji, an open source image processing software.



4 Two-photon microscopy applied to drug delivery systems

4.1 Introduction

Drug delivery, a dynamic field at the intersection of pharmaceuticals and material sciences, seeks to optimize the administration of therapeutic agents to achieve targeted and controlled effects within the body. Over the years, drug delivery strategies have evolved, encompassing numerous innovative approaches ranging from traditional oral medications to sophisticated nanocarriers and advanced delivery systems.[154] Indeed, nanotechnology could help overcome the limitations of conventional drug delivery, addressing both large-scale issues such as biodistribution, and smaller-scale barriers such as intracellular trafficking.[155] Nanocarriers play a key role in optimizing drug formulation through the improvement of hydrophobic drug water-solubility[156, 157] and the stabilization of easily degradable compounds.[158, 159] The great potentialities of nanosystems concern the promotion of drug retention in tissues, the protection from enzymatic degradation, the enhancement of cellular uptake, and the highly targeted delivery,[160] strongly impacting on the treatments efficacy.[161] Depending on the material used for their preparation, nanosized systems can be classified as lipidic, polymeric, inorganic, peptide-based and virus-like nanocarriers.[156] Lipid-based carriers involve nanovesicles, micelles and solid lipid nanoparticles. One of the most recent applications of lipid nanoparticles can be found in the delivery of mRNA, as demonstrated by their use for the administration of the mRNA-based vaccine against SARS-CoV-19.[162–164] Among polymeric nanosystems, formulated by using natural or synthetic polymers, several structures (such as nanoparticles, nanocapsules, micelles and dendrimers) with various properties can be found. In this thesis, polymeric micelles and lipid-based nanocarriers are exploited as drug nanocarriers for different topical applications.

The behavior of these nanocarriers strictly depends upon their composition and general characteristics, such as size, superficial charge,

4.1 Introduction	101
4.2 TPGS micelles for drug delivery applications . . .	104
4.3 Nanostructured lipid carriers for enhanced transscleral delivery . . .	116
4.4 Buccal permeation of polysaccharide high molecular weight compounds .	121
4.5 Conclusions and perspectives	124
4.6 Materials and Methods .	125

shape, thermodynamic and kinetic stability. All these properties are essential to determine their fate in the organism and the consequent drug bioavailability. Moreover, following the fate of the nanocarrier is extremely important under a toxicological point of view, in particular when a contact with delicate tissues (such as retina) can be envisaged.[60] In this context, imaging techniques are fundamental for the preclinical evaluation of nanomedicine-based drug delivery systems and can provide important insights into their mechanism of action and therapeutic effect.[165] Among them, optical microscopy techniques are widely employed to study biological systems, making use of linear (one-photon) absorption processes for image generation. However, the high-resolution visualization of thick tissues is limited to their surface (a few tenths of μm) because at greater depths strong and multiple light scattering overly decreases the signal intensity and/or blurs the images.[58]

During the last two decades of the past century, new optical microscopy techniques have been developed that employ non-linear light-matter interactions to generate signal contrast.[58, 166–168] The non-linear phenomena exploited by these techniques grant them with intrinsic 3D resolution and reduced scattering, making them more suited for high-resolution imaging in thick tissues. In particular, two-photon microscopy (TPM) is an advanced non-linear optical technique, employed for in-depth 3D visualization of biological tissues with only minimal sample handling. TPM exploits low-energy photons (in the red and near-infrared region) to excite fluorophores, which then can proceed along de-excitation pathways involving fluorescence-emission.¹ Apart from two-photon excited fluorescence, also other non-linear processes can lead to a detectable signal, such as second harmonic generation, that occurs only in certain ordered non-centrosymmetric molecular structures such as collagen, myosin or tubulin.[169, 170] A more detailed description of the two-photon absorption and SHG phenomena is reported in Appendix A.

TPM has opened a rapidly expanding field of imaging studies in intact tissues and living animals. Thanks to this technique, specimens as diverse as lymphatic organs,[171] kidney,[172] heart,[173] skin[174], eyes,[175] and brain[176] can now be examined in detail at depths of up to one millimeter,[176] while leaving the tissue intact. Moreover, TPM is already used as a tool to study the development, progression and potential treatment of pathological conditions such as tumors[177] and Alzheimer disease[178].

In this chapter we address TPM as a support tool for various drug delivery systems such as polymeric micelles, chemical permeation enhancers and nanostructured lipid carriers designed for topical applications in different thick porcine *ex vivo* tissues. To enable tracking and monitoring of nanocarriers or model molecules within biological tissues, labelling with fluorophores has been implemented in the studied systems.

In Section 4.2, the penetration of Nile Red (NR) loaded TPGS micelles through ocular tissues (sclera and cornea, Section 4.2.1) and skin (Section 4.2.2) has been studied by TPM, shedding light on the penetration mechanisms. The fate of the nanocarriers resulted to be strictly dependent on the tissue: micelles disruption and dye release occurs in contact with the skin and cornea, while micelles diffuse intact in the interfibrillar pores of the sclera.

The same experimental approach proposed for TPGS micelles has been extended in Section 4.3 for the study of NR-loaded nanostructured

¹: Even if the emitted light can be scattered during a TPM measurement, all the fluorescence photons are known to originate from near the focus and thus can provide useful signal. Therefore, the best detection strategy becomes to collect as many photons as possible, wherever they come from.

lipid nanocarriers (NLCs), after the application onto porcine sclera. In the last section (Section 4.4), TPM was employed to analyze the administration of fluorescently labeled dextrans (molecular weight 70 kDa) across the buccal mucosa, exploiting the effect of caprylic acid as a permeation enhancer. The strong signal detected inside the tissue from the fluorescent probe, a fluorescein derivative (Fluorescein isothiocyanate, FITC), highlighted the distribution of the dextrans in the mucosa and confirmed their capability to permeate through it.

The research works presented in this chapter are the result of a close collaboration with Prof. Sara Nicoli and Prof. Silvia Pescina from the ADDRes Lab (Advanced Drug Delivery Research Lab) of the Food and Drug Department at the University of Parma. They were responsible for the handling of the *ex vivo* tissues, the permeation experiments and the drug delivery studies, including the quantification of drug permeation and retention.

4.2 TPGS micelles for drug delivery applications

As introduced in Chapter 2, TPGS is a non-ionic polymeric surfactant soluble in water, derivative of vitamin E, made up by a PEG 1000 chain linked to vitamin E through a succinate bond (Figure 4.1). In addition to its high biocompatibility and biodegradability, TPGS has gained widespread recognition in various fields of drug delivery[179–181] because of solubilization capacity and permeation enhancing properties.[74]

The following sections concern the use of this polymeric amphiphilic for permeation experiments across porcine ocular tissues and skin tissues, mainly with the scope of improving drug solubilization and retention. The corresponding permeation and retention experiments have been performed by Martina Ghezzi from ADDRes Lab. In order to follow the behavior of the nanocarrier in the tissues by TPM, the non fluorescent active drug has been substituted with the organic fluorophore Nile Red (NR, Figure 4.1). NR exhibits several appealing features, including high fluorescence quantum yield and satisfactory photostability[182], that combined to its high affinity for nonpolar environments, made this dye widely employed for bioimaging applications.[42, 183, 184] Moreover, similarly to C153 described in Chapter 1, NR is a solvatochromic dye, i.e., its absorption and emission spectra are sensitive to the polarity of the surroundings, and it can be useful to probe the polarity of the local environment.[182, 185]

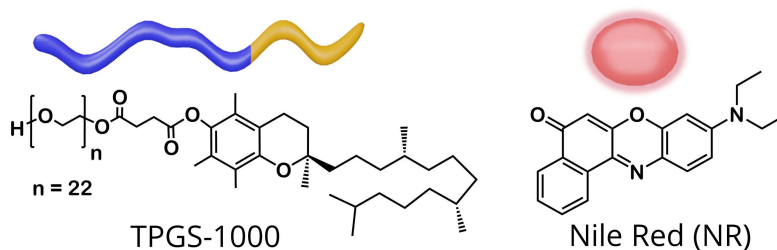
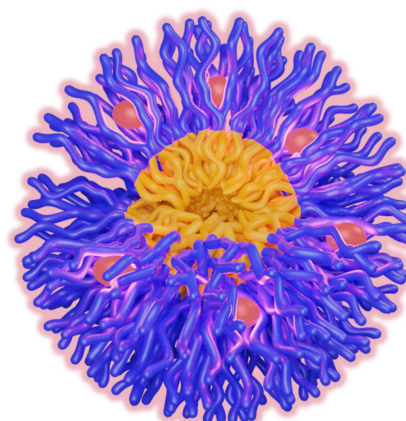


Figure 4.1: Molecular structures and schematic representation of TPGS-1000 and Nile Red (NR). A schematized NR-loaded TPGS micelle is also shown.



NR-loaded TPGS micelle

4.2.1 TPGS micelles for ocular delivery

In a recent work,[60] our collaborators from the Food and Drug Department employed TPGS micelles to solubilize a relevant amount of Cyclosporine A and promote its transport across ocular barriers (cornea and sclera). Cyclosporine A, a neutral cyclic peptide, is an immunomodulatory drug² that functions by inhibiting T cells' activation, blocking the transcription of cytokine genes, including those encoding for IL-2 and IL-4.[191] The formulation and delivery of Cyclosporine A to ocular tissues face challenges due to its relatively high molecular weight (1202.6 g/mol), poor water solubility,[192] and pronounced lipophilicity.

The loading of the drug into TPGS micelles drastically improved its water solubility (from $<5 \mu\text{g/L}$ to 5 mg/mL) enhancing at the same time permeation and retention within cornea and sclera.[60]

To further investigate micelles penetration, Nile Red (NR)-loaded TPGS micelles were prepared and, before being used for permeation experiments, characterized from a physicochemical and spectroscopic point of view.

Characterization of NR-loaded TPGS micelles

In order to obtain NR-loaded TPGS micelles suitable for spectroscopic measurements (absorbance lower than 0.1), a 0.5 mM TPGS suspension has been loaded with a NR concentration of $2.7 \mu\text{M}$. The DLS measurements performed on the NR-loaded TPGS suspensions showed a slightly higher mean D_h (Table 4.1) compared to the value in absence of the dye. The corresponding size distribution (Figure 4.2) resulted broadened for NR-loaded TPGS micelles. Despite the low intercept value observed due to NR fluorescence, the quality of the fitting analysis was good, and the obtained size data were reliable and reproducible.

Sample	Hydrodynamic diameter ^a (nm)	PdI	Intercept
Plain TPGS	13.2 ± 0.1	0.18 ± 0.01	0.94 ± 0.01
NR-loaded TPGS	15.1 ± 0.5	0.25 ± 0.01	0.11 ± 0.01

In Figure 4.3, normalized absorption and emission spectra of NR in TPGS micelles, PEG600 and water are presented. NR is poorly soluble in water, and its absorption in this environment (not shown for clarity) results in a very broad band, unstable over-time. The corresponding emission is sizably red-shifted and its intensity is low. The successful encapsulation of NR inside the TPGS micelles is evident from the comparison with the spectra in water: absorption and emission spectra of NR in the presence of TPGS are blue-shifted and similar to those collected in PEG600, the main constituent of the micelles external shell. This result suggests that NR is most probably entrapped in the hydrated polyoxyethylene corona of the TPGS micelles, that is expected to have similar local polarity compared to that of PEG600.

The estimated fluorescence quantum yield (ϕ) of NR-loaded TPGS

2: Beyond treating mild-to-severe dry eye syndrome and other corneal conditions,[186, 187] this drug has demonstrated efficacy in treating blepharitis,[188] as well as diseases of the intermediate and posterior segments of the eye, including uveitis[189] and Behçet disease.[190]

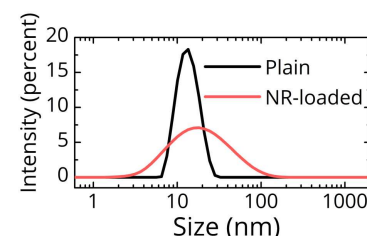
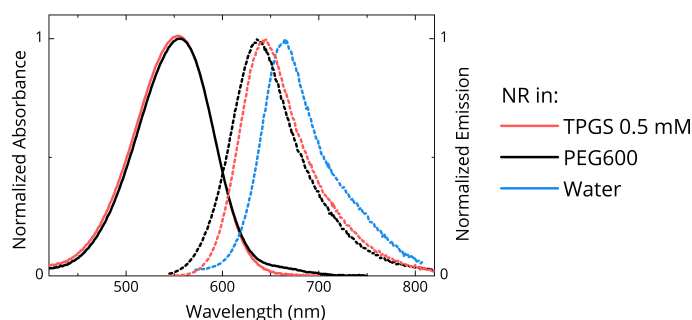


Figure 4.2: Size distributions by intensity of TPGS micelles (0.5 mM) before and after the addition of NR.

Table 4.1: DLS results of TPGS micelles suspensions in the presence and absence of NR as the average of three measurements with the corresponding standard error of the measurement mean (SEM). Measurements were performed at 25°C as described in Section T.1.

^a Z-average extracted from cumulant fit.

Figure 4.3: Normalized emission and absorption spectra of NR in TPGS 0.5 mM, PEG600 and water. The absorption profile in water was characterized by high noise and clear aggregation features, hence is not shown for clarity. The emission spectra have been acquired exciting the sample at 540 nm.



NR-saturated TPGS micelles (technical details on the preparation procedure are reported in Section 4.6.1) were employed to treat cornea and sclera, according to the procedure reported in Section T.3. After 2h of permeation, the *ex vivo* tissues were then imaged with TPM with an excitation wavelength of 860 or 1080 nm. As reference, a saturated aqueous solution of NR was used. NR, despite being a lipophilic compound, has notable differences in structure and MW if compared to cyclosporine. This means that we cannot claim that the behavior of NR-loaded micelles will be exactly the same as the drug-loaded micelles. However, the data obtained with NR nicely support the drug/TPGS retention experiments performed by our collaborators at the Food and Drug department.

Cornea treated with NR-loaded TPGS

Cornea is a transparent tissue that covers the iris and the pupil in the anterior segment of the eye. It is constituted mainly by three layers: epithelium, stroma and endothelium. Epithelium is the outermost layer, composed of several layers of cells. Endothelium is the innermost layer, and is made up of a single layer of cells. Stroma is located in the middle, consisting mainly of collagen fibers and fibroblasts. More details on corneal structure, and a complete overview of its characterization with the multiphoton microscope is reported in Appendix A.

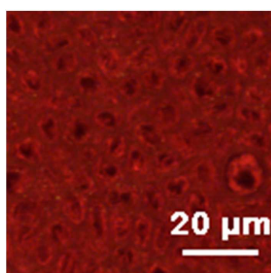


Figure 4.4: TPM image of corneal epithelium cells after treatment with NR-loaded TPGS micelles. The sample has been excited at 860 nm

Figure 4.5 collects TPM images and Z-scan of the corneal epithelium and the upper stroma after a 2h treatment with NR-loaded micelles and with the reference NR aqueous solution, when excited at 860 nm. In these conditions, NR is mainly detected in the red channel, while the corneal epithelium gives an autofluorescence signal which falls primarily in the green spectral region. The stroma is blue, due to the second harmonic generation signal (that falls at 430 nm at this excitation wavelength) typical of collagen fibers. The intensity of NR signal obtained in the presence of TPGS is much higher compared to NR in water (reference solution), mainly due to the higher fluorescent probe concentration. The epithelium cells have been completely stained by the dye, with the exception of the nuclei, as shown in the zoomed image in Figure 4.4. The NR that reached the stroma clearly accumulated inside the corneal fibroblasts (also known as keratocytes, Figure 4.5b). Apart from the striking difference in signal intensity, the presence of TPGS did not affect the distribution of the fluorophore in the tissue.

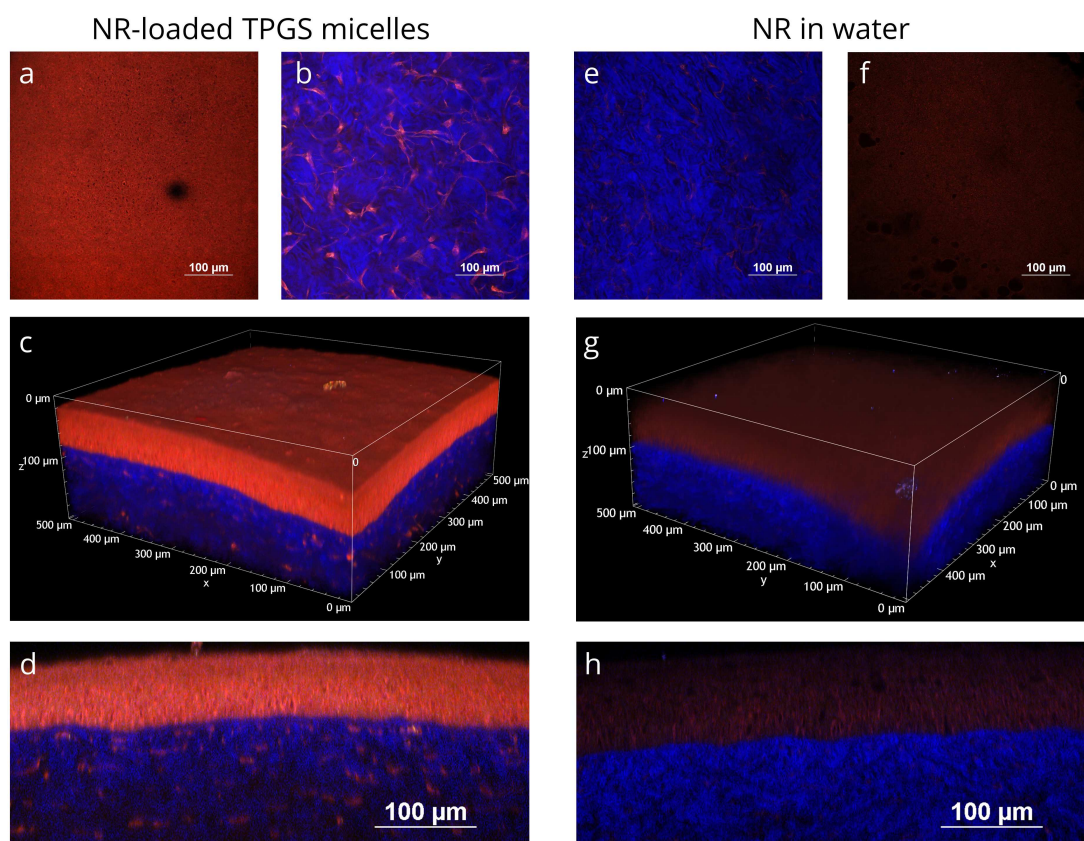


Figure 4.5: TPM images and volume renderings of corneal tissue (epithelium side) reconstructed from Z-stack, acquired with an excitation wavelength of 860 nm (SHG from collagen in blue, NR fluorescence detected in both red and green channels). Panels(a-d): sample treated with TPGS micelles loaded with NR (Z-step: 1 μm , total depth: 202 μm). Panels(a,b): XY scans collected at 40 and 180 μm depth, respectively; panel(c): 3D overview; panel(d): XZ slice (512 μm x 202 μm). Panels(e-h): reference treated with NR saturated aqueous solution (Z- step: 1 μm , total depth: 202 μm); panel(e,f): XY scans collected at 40 and 180 μm depth, respectively, panel(g): 3D overview; panel(h): XZ slice (512 μm x 202 μm). All the images reported in this figure were acquired with the exact same detector gains and laser power.

In order to obtain more information on micelles-tissue interaction, we acquired emission spectra from the tissue at specific focal planes. Thanks to the solvatochromic properties of the probe, the spectra allow to differentiate between NR encapsulated in the micellar structure and released, consequently located in cells and/or in the intercellular matrix. The emissions were collected exciting the sample at 1080 nm, in order to minimize the tissue autofluorescence and maximize at the same time the NR signal. In Figure 4.6 (top panel), the emission spectrum of an aqueous suspension of NR-loaded TPGS micelles (blue dashed line) is compared with those collected from cornea treated with NR-loaded micelles or the NR aqueous solution at a depth of 40 μm (i.e. in the epithelium, corresponding to the images in Figure 4.7, top panels) and 300 μm (i.e. in the corneal stroma, corresponding to the image in Figure 4.7, bottom panels) from the sample surface. All the emission spectra collected from the tissue are broadened and shifted toward shorter wavelengths compared to those of NR in water/TPGS suspension, and the shift increases with tissue depth.

The emissions collected after the treatment with the NR aqueous solution present higher contributions from the tissue autofluorescence (in the epithelium, Figure 4.6 top panel) and SHG (in the stroma, Figure 4.6 bottom panel).

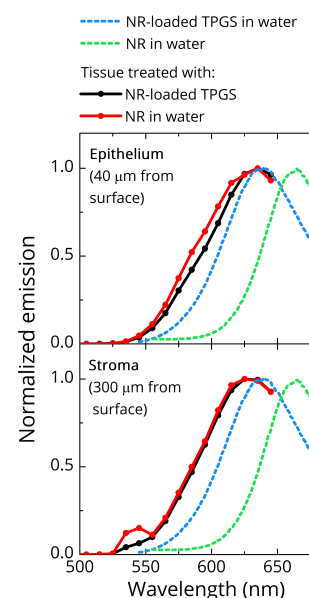


Figure 4.6: Emission spectra acquired inside cornea at different depths after treatment with NR-loaded TPGS micelles and NR in water, corresponding to the images reported in Figure 4.7. The sample has been excited at 1080 nm. The emission profiles of NR-loaded TPGS micelles and NR in water are reported as a comparison (blue and green dashed lines, respectively).

The blue-shift observed after the treatment with NR-loaded micelles and NR in water suggests that the probe is located in same environment in both cases, and the local polarity is different with respect to the micelles, indicating NR release from the nanocarrier.

This conclusion supports the results of TPGS and cyclosporine corneal retention experiments, which show that the drug is probably released as the micelles come in contact with the epithelial cells. Indeed, the TPGS:cyclosporine weight ratio in the TPGS micelles (in water) is approximately 6:1 while a ratio lower than 3:1 was found inside the tissue after extraction and quantification. Both the TPM study and the quantification analysis show that the micelles do not penetrate intact in the cornea and they disassemble once in contact with the epithelium.

Figure 4.7: TPM images of cornea after treatment with NR-loaded TPGS micelles (left panels) and NR in water (right panels) acquired at two different depths: 40 μm and 300 μm below the surface, for epithelium and stroma, respectively. The sample has been excited at 1080 nm.

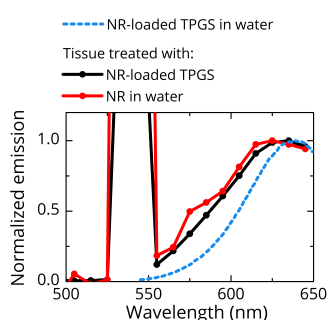
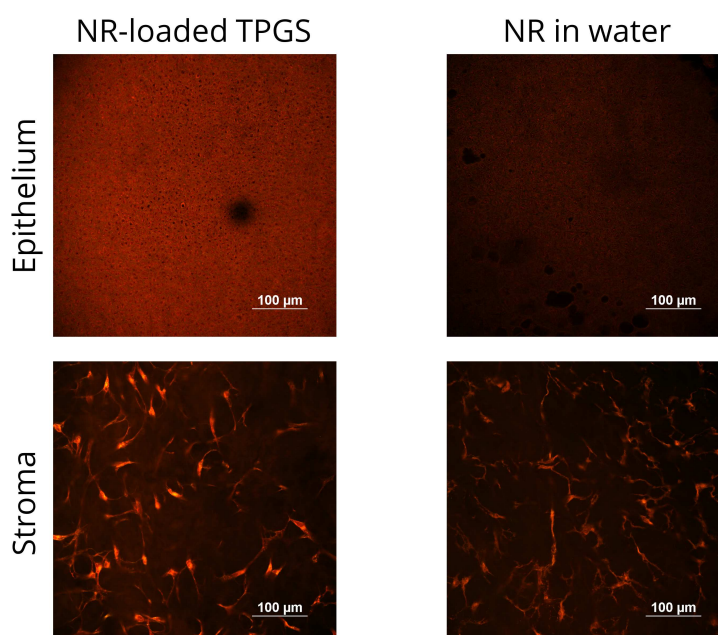


Figure 4.8: Emission spectra acquired inside the endothelial side of cornea (150 μm below the surface) after treatment with NR-loaded TPGS micelles and NR in water, corresponding to the images reported in Figure 4.7. The sample has been excited at 1080 nm. The emission profile of NR-loaded TPGS micelles in water is reported as a comparison (blue dashed line).

The presence of NR on the endothelial side of the tissues has been investigated as well, acquiring Z-scans on the opposite side of the samples treated with NR-loaded micelles and NR in water. As can be observed from the images reported in Figure 4.9, the dye permeated through the tissue up to the endothelial side of the cornea, even in absence of the TPGS micelles, highlighting its high permeation capability. However, the signal in the red channel is significantly enhanced in the tissue treated with NR-loaded TPGS micelles, further confirming the positive role of the surfactant in increasing the amount of dye permeated in the cornea. The thin, superficial acellular structure visualized in the green channel (mainly due to autofluorescence) is the so-called Descemet's membrane, situated between the stroma and the endothelium. The endothelium cells layer is visible only in the sample treated with NR in water (even if partially damaged), while in the one treated with NR-loaded TPGS only a few cells are present. Indeed, the endothelium is a very fragile structure, and the cells can be easily lost during the preparation of the tissue. The residual cells of the endothelium, where they are present, have been clearly stained by NR.

Emission spectra were acquired 150 μm below the endothelium surface, and are reported in Figure 4.8. The emission bandshapes observed after the permeation of NR-loaded micelles and NR in water are superimpos-

able, providing further evidence that the dye has been released from the micelles.

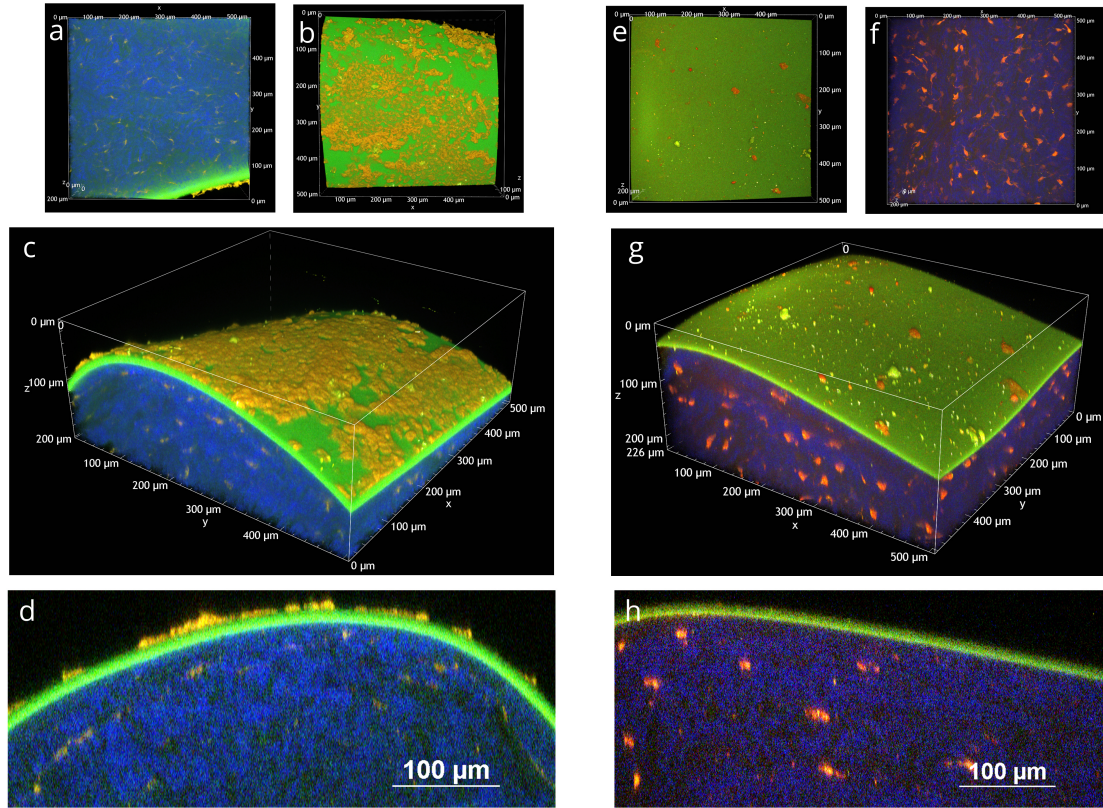


Figure 4.9: Volume renderings of corneal tissue (endothelium side) reconstructed from Z-stack, acquired with an excitation wavelength of 860 nm (SHG from collagen in blue, NR fluorescence detected in both red and green channels). Panels(a-d): sample treated with TPGS micelles loaded with NR (Z-step: 1 μm , total depth: 226 μm). Panel(a): -XY view, Panel(b): XY view, Panel(c): 3D overview, Panel(d): XZ slice (512 μm x 226 μm). Panels(e-h): reference treated with NR aqueous suspension (Z-step: 1.5 μm , total depth: 203 μm). Panel(e): XY view, Panel(f): -XY view, Panel(g): 3D overview, Panel(h): XZ slice (512 μm x 203 μm). All the images reported in this figure were acquired with the exact same detector gains and laser power.

Sclera treated with NR-loaded TPGS

Figure 4.10 reports images of sclera after 2h treatment with NR-loaded TPGS micelles, upon excitation at 860 nm. Collagen fibers appear in blue as a result of their SHG signal, while the red signal is once again given by NR fluorescence. As shown in Figure 4.10 [panels(b-c)] there is almost no overlap between the blue and red signals, demonstrating that the fluorescent dye arranges in the interfibrillar spaces of the scleral collagen matrix.

Figure 4.10: Panel(a) represents the scleral structure (XY plane) after treatment with NR-loaded micelles when irradiated at 860 nm (SHG from collagen in blue, NR fluorescence in red). Panel(b) (blue and red channels overlay), panel(c) (blue channel) and panel(d) (red channel) represent XZ views of scleral collagen fibers extracted from a Z- stack (image size: $77\ \mu\text{m} \times 39\ \mu\text{m}$, Z step: $0.42\ \mu\text{m}$).

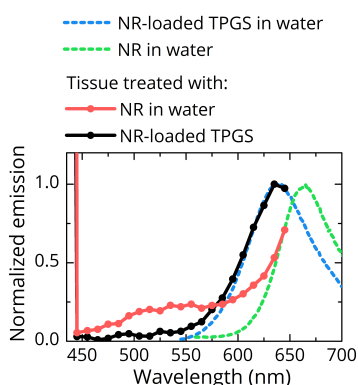
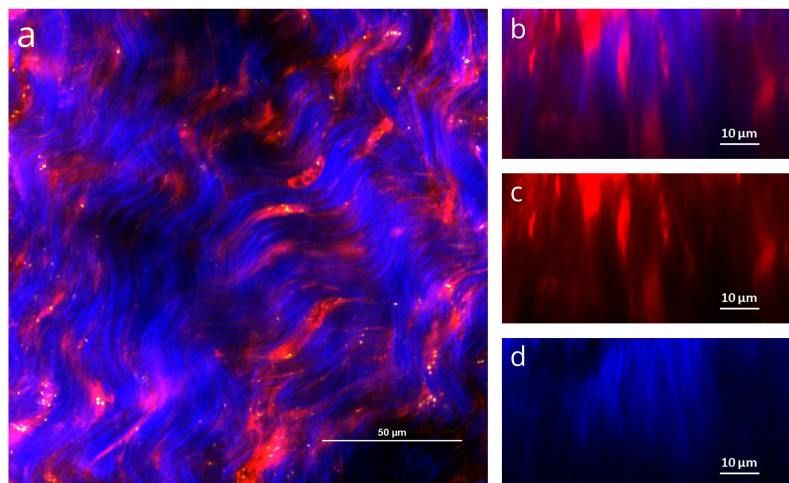


Figure 4.11: Emission spectra (excitation at 860 nm) recorded in the sclera ($100\ \mu\text{m}$ from surface) after treatment with NR-loaded TPGS micelles (black line) or the NR saturated aqueous solution (red line); emission spectrum of NR-loaded TPGS micelles (blue dashed line) and NR in water (green dashed line) are also reported. The last two spectra have been acquired using a fluorimeter exciting at 540 nm. The NR saturated aqueous solution emission has been arbitrarily normalized in order to make the point at 650 nm coincident with the spectrum recorded at the fluorimeter (green line).

As previously described for cornea, to get further insight into micelles penetration mechanism, emission spectra were recorded from the tissue and are reported in Figure 4.11. Differently from cornea, the emission profile recorded from the sclera treated with NR-loaded TPGS micelles is superimposable with the one of micelles in solution, indicating that the probe is still interacting with the surfactant and the polarity of its local environment is not affected during the permeation. Indeed, the interaction with a cellular tissue such as the cornea affects micelles integrity much more than a collagen-based structure such as the sclera. Further insights on the different structures of sclera and cornea are discussed in Appendix A.

When the aqueous NR solution was applied to the sclera, the spectrum obtained is clearly shifted toward higher wavelengths (Figure 4.11), indicating a more hydrophilic environment, in agreement with the presence of hydrated glycosaminoglycans in the interfibrillar spaces. Indeed, the emission profile is very similar to the one obtained with the fluorimeter from an aqueous solution of the dye.

From the quantification of drug and surfactant concentrations inside the sclera, our collaborators found that the TPGS/cyclosporine ratio after 6h of contact corresponds to the one present in the micellar formulation (6:1), suggesting permeation of intact micelles. Micelles diffusion through sclera occurs in the interfibrillar matrix made of negatively charged hydrated proteoglycans consisting of a protein core with glycosaminoglycan sidechains of repeating disaccharide units.[99, 100] Indeed, previous data have demonstrated the ability of TPGS micelles loaded with NR to diffuse intact through a hyaluronic acid gel,[101] possibly due to the presence of the pegylated corona.[193] Even if the micelles can most probably diffuse intact across the hydrated interfibrillar matrix of the

tissue, the FRET study in DiI and DiD-loaded TPGS micelles discussed in Chapter 2 showed a loss of integrity for the nanocarriers after more than 2h of permeation. In conclusion, even if the micelles are partially disassembled after penetrating into sclera, NR continues to interact with the surfactant inside the tissue pores for a long time.

In Figure 4.12 are reported Z-scans for both tissues (treated with NR-loaded TPGS and NR in water), acquired by exciting them at 860 nm and employing the same experimental conditions. The red signal coming from NR emission is much more intense in the presence of TPGS, further proving the capability of these micelles to increase the amount of dye permeated in the tissue. The sample treated with NR-loaded TPGS micelles shows also signs of backdiffusion of the nanocarriers, as can be observed from the diffuse red signal in the region above the surface of collagen (Panel c of Figure 4.12). Accordingly, the same phenomenon has been observed also after the permeation, in the same tissue, of TPGS micelles loaded with DiI/DiD (Chapter 2).

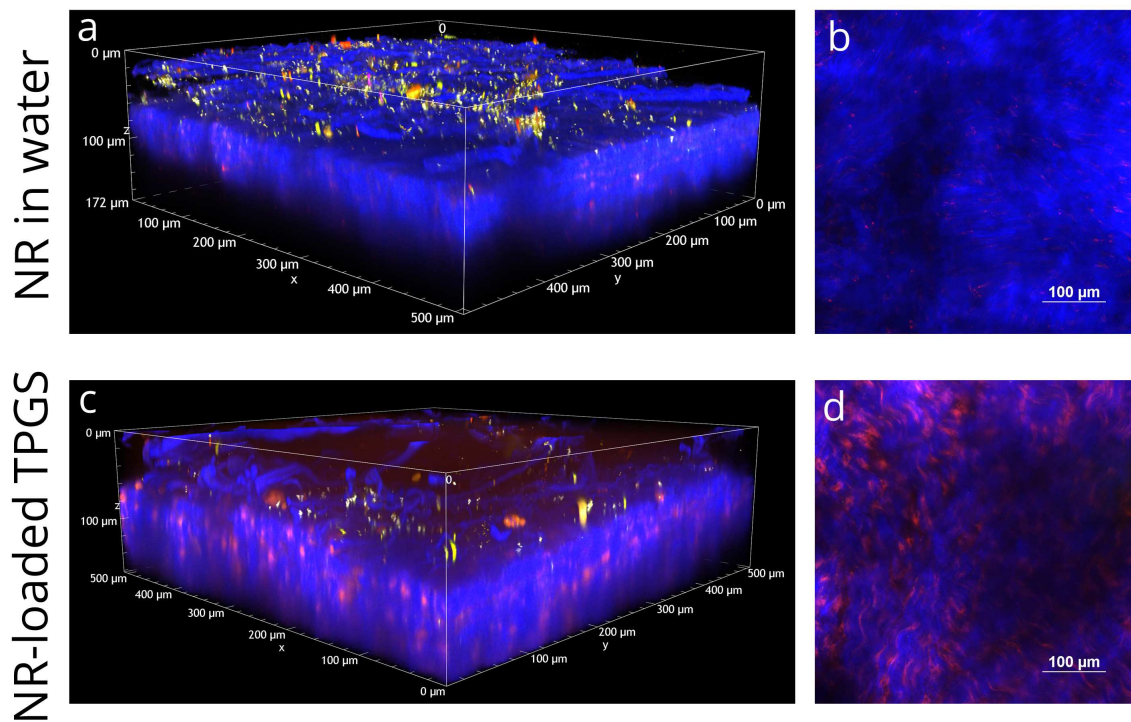


Figure 4.12: Comparison between TPM images and 3D renderings reconstructed from the Z-scans acquired exciting at 860 nm the sclera treated with the NR aqueous suspension [panels(a,b)] and the sclera treated with the NR-loaded TPGS formulation [panels(c,d)]. Panel(a): 3D overview (Z-step: 1 μm , total depth: 172 μm), panel(b): TPM image (acquired 100 μ from tissue surface). Panel(c): 3D overview (Z-step: 1 μm , total depth: 162 μm), panel(d): TPM image (acquired 100 μ from tissue surface). All the images presented in this figure have been recorded with the same detector gains and laser power.

3: Other uses of the drug have been approved by the U.S. Food and Drug Administration (FDA) including the treatment of external genital and perianal warts and superficial basal cell carcinoma[194] but also several off-labels indications towards skin infections and skin cancers have been registered.[101]

4: Furthermore, considering the amphiphilic structure of the polymers, an augment of drug retention in the skin could be favored by their capacity to alter the spatial structure of lipids and keratin in the SC reducing its resistance to drug penetration,[199] in addition to a modification of barrier lipophilicity.[200]

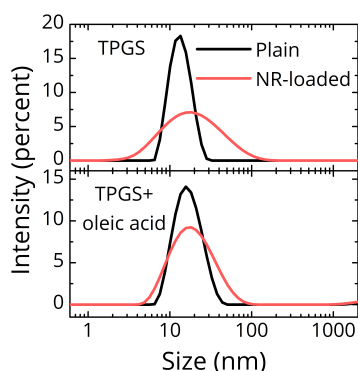


Figure 4.13: Top panel: size distributions by intensity of TPGS micelles (0.5 mM) before and after the addition of NR. Bottom panel: size distributions by intensity of TPGS micelles (0.5 mM) loaded with oleic acid before and after the addition of NR.

Table 4.2: DLS results of TPGS micelles+oleic acid suspensions in the presence and absence of NR as the average of three measurements with the corresponding standard error of the measurement mean (SEM). Measurements were performed at 25°C as described in Section T.1.

^a Z-average extracted from cumulant fit.

4.2.2 Skin retention of TPGS micelles

Besides ocular delivery, our collaborators from the Food and Drug Department recently employed TPGS micelles also to load Imiquimod,[101] a synthetic drug belonging to the class of imidazoquinolones, for topical administration on the skin. This drug has widely demonstrated its usefulness in the treatment of skin precancerous lesions (Actinic keratosis) thanks to its immunostimulant activity³ mediated by the binding to the toll-like receptors 7 and 8. These receptors play a pivotal role in controlling innate immune system response as they induce the secretion of pro-inflammatory cytokines such as interferons (IFN- α , IFN- γ), tumor necrosis factor-alpha (TNF- α) and interleukin 12 (IL-12).[195, 196] Despite the effectiveness of imiquimod for skin disorder treatments is well established, its topical administration has proved to be quite challenging due to the very low water-solubility and the limited penetration capacity,[197] presumably related to its very low affinity for stratum corneum and underlying tissues. The encapsulation in polymeric micelles, similar to TPGS, has already demonstrated to facilitate drugs penetration into the skin by several pathways such as the transcellular route,[198] the intercluster penetration and the follicular way, thus promoting a slow and sustained drug release by creating drug depots in the tissue⁴. [201]

In order to increase the affinity of the drug for the micellar core, our collaborators co-encapsulated a lipophilic compound, oleic acid, into TPGS micelles (with a final fatty acid concentration of 3 mg/mL). The results showed strong improvements in the imiquimod solubility (from few μ g for plain TPGS micelles to $\sim$ 1.1 mg/mL) and delivery efficiency into *ex vivo* porcine skin (42-folds increase with respect to commercial creams).

For the purpose of visualizing the behavior of this formulation into the skin tissue through TPM, NR was co-encapsulated in the TPGS micelles (in place of imiquimod) together with oleic acid (see Section 4.6.1 for detail on the preparation procedure) before employing them for permeation experiments.

In the presence of oleic acid, a slight increase in the mean D_h of the micelles has been observed, both before and after the loading of NR (Figure 4.13). The calculated Z-averages for plain and NR-loaded TPGS/oleic acid suspensions resulted 16.5 nm and 17.3, respectively (as reported in Table 4.2). The variations with respect to plain TPGS micelles (Table 4.1) can be attributed to the cis double bond contained in the structure of oleic acid, that can hinder the packing of the surfactant molecules in the lipophilic core of the micelles.

Sample	Hydrodynamic diameter ^a (nm)	PdI	Intercept
TPGS + oleic acid	16.5 $\pm$ 0.1	0.21 $\pm$ 0.01	0.93 $\pm$ 0.01
NR-loaded TPGS + oleic acid	17.3 $\pm$ 0.3	0.25 $\pm$ 0.02	0.14 $\pm$ 0.01

The absorption and emission spectra of NR in TPGS, TPGS+oleic acid

and pure oleic acid are reported in Figure 4.14. No differences have been observed between the spectra acquired from micellar suspensions in the presence and absence of oleic acid, in terms of spectral position, shape and quantum yield (amounting to 43 and 44%, respectively). The blue-shifted emission and absorption measured in pure oleic acid further confirm that the dye does not reach the hydrophobic core of the micelles.

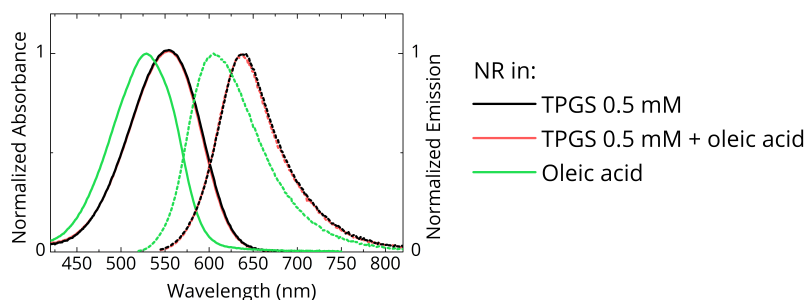


Figure 4.14: Normalized emission and absorption spectra of NR in TPGS 0.5 mM (in the presence or absence of oleic acid) and pure oleic acid. The emission spectra have been acquired exciting the sample at 540 nm.

Only TPGS micelles loaded with both NR and oleic acid have been then employed for permeation experiments through porcine skin, and from here on will be simply referred to as NR-loaded TPGS micelles. As a reference, a blank experiment was also done, by using saline solution to treat the tissue. Further information of the permeation experiments are reported in Section T.3. The skin epidermis and dermis have been visualized by TPM. The former is the outermost layer, primarily composed of stratified squamous epithelium, whose most superficial part is called stratum corneum. The dermis is a thicker layer situated under the epidermis composed of connective tissue (such as collagen). Additional details on the structure of skin and a complete overview of the blank tissue autofluorescence are reported in Appendix A.

Skin treated with NR-loaded TPGS micelles and saline solution have been analyzed with both 890 and 1070 nm excitation wavelengths, and the corresponding TPM images are reported in Figure 4.16a-d. When exciting at 890 nm, the collagen SHG detected from the dermis falls within the blue channel, while the autofluorescence of the tissue and NR emission are visualized mainly in the green and red ones, respectively. The corresponding emission profiles are reported in Figure 4.15 (top panel), which shows a clear convolution between NR emission and the tissue autofluorescence in the emission bandshape of the sample treated with NR-loaded TPGS micelles. When exciting at 1070 nm, the SHG is detected by the green channel, while both NR emission and the autofluorescence from the tissue fall mainly in the red one. Even if the emission profiles (Figure 4.15 bottom panel) become more similar at this excitation wavelength, the autofluorescence intensity is drastically reduced and stems almost exclusively from the stratum corneum (Figure 4.16c).

The emission bandshape collected in the tissue after the permeation of the NR-loaded TPGS micelles has also been compared to the one measured from an aqueous suspensions of the same micelles (Figure 4.15) and resulted shifted towards shorter wavelengths. This blue-shift indicates a variation in the probe environment polarity, suggesting that NR has been released from the micelles, similarly to what we observed in Section 4.2.1 after the treatment of cornea with NR-loaded TPGS micelles.

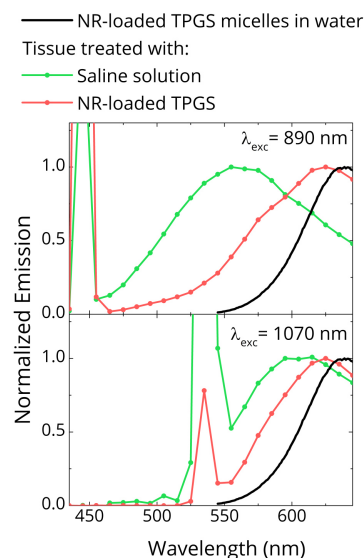


Figure 4.15: Comparison between the normalized emission spectra acquired in skin samples after the treatment with saline solution (green lines) or NR-loaded TPGS micelles (red lines). The emissions were collected exciting the samples at 890 nm (top panel) or 1070 nm (bottom panel). The emission profile of an aqueous suspension of NR-loaded TPGS micelles (black line) is also shown as a comparison.

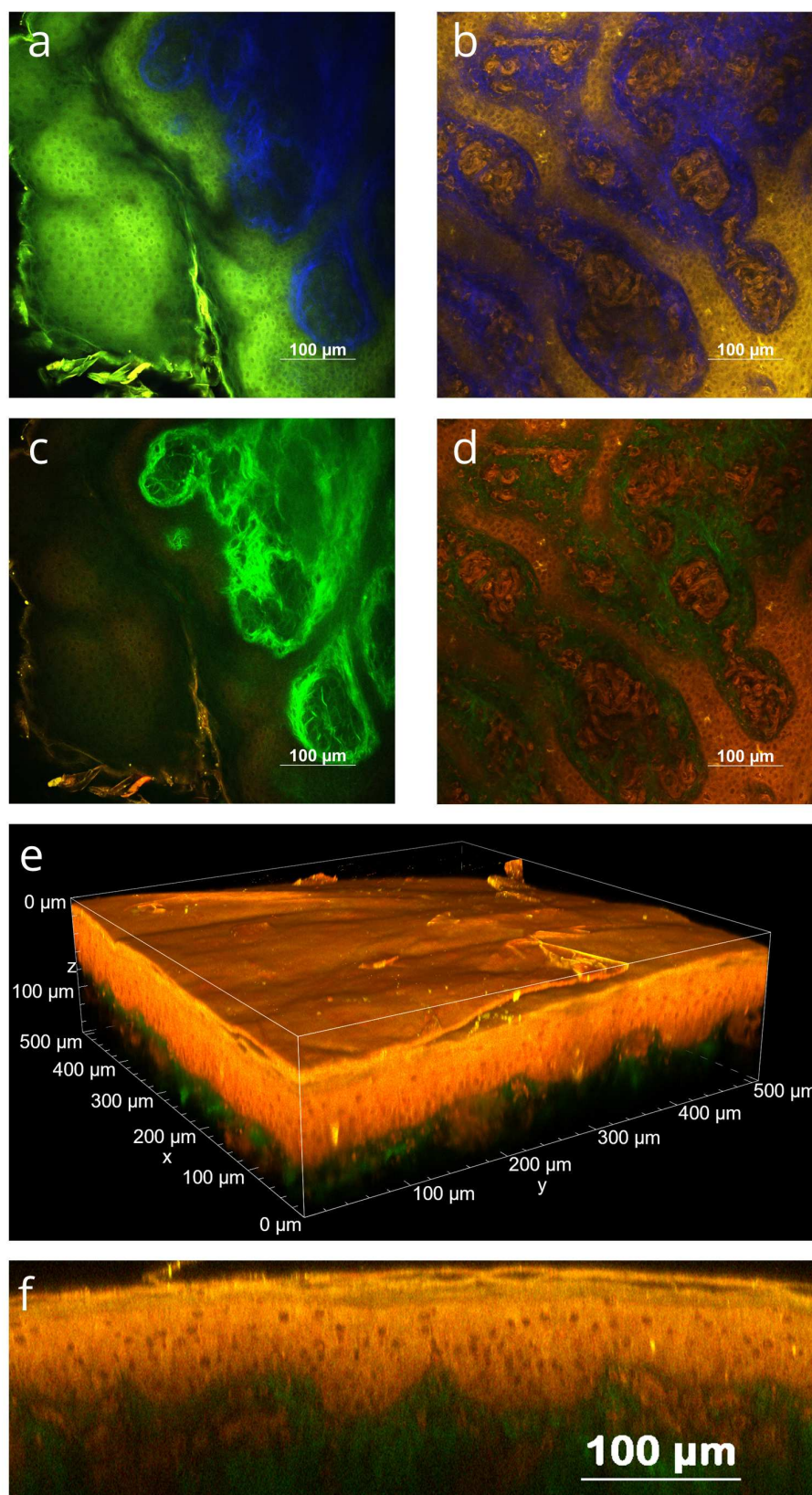


Figure 4.16: TPM images of porcine skin. Panels(a-d): TPM images of the intermediate region between epidermis and dermis, acquired about 80 μm from the tissue surface of a sample treated with NR-loaded micelles [panel(b,d)] or saline solution [panel(a,c)]. Panels(a,b) were acquired exciting at 1070 nm, while panels(c,d) at 890 nm. Panel(e,f): Volume rendering of flat porcine skin reconstructed from the Z-stack (Z-step: 0.8 μm , total depth: 151 μm). Panel(e): 3D overview, panel(f): XZ slice (512 μm x 151 μm). The Z-stack has been acquired exciting the sample at 1070 nm.

Figure 4.16e,f illustrates the volume rendering of a flat skin region after permeation of NR-loaded TPGS micelles obtained exciting the sample at 1080 nm. From all the images reported in Figure 4.16, the different skin layers are clearly visible as well as NR accumulation in the stratum corneum and viable epidermis. The fluorescent probe labelled all the cellular elements of the skin, i.e., epidermis and also vascular and corpuscular elements in the dermis. Analogously to cornea (Section 4.2.1), the epithelium cells have been completely stained by the NR, with the exception of the nuclei, as shown in the zoomed image in Figure 4.17. Skin sections with hair follicles, presented in Figure 4.18, were also analyzed. From the comparison between Figure 4.18a, skin treated with NR-loaded TPGS micelles, and Figure 4.18b, skin treated with saline solution (both excited at 1100 nm), the solubilization of a large amount of NR and its accumulation in the tissue due to the presence of TPGS are evident. The strong signal coming from the epidermis of the tissue treated with the fluorescent micelles clearly indicates the retention of the probe.

Figure 4.18c,d demonstrate the NR accumulation in the hair shaft and its penetration into the hair follicle. Especially from Figure 4.18d, obtained saturating the signal in the red channel, the deep penetration of the probe into the hair follicle is highlighted.

Overall, both the TPM images and emission spectra support the capability of the TPGS micelles loaded with oleic acid to efficiently deliver the hydrophobic probe, NR, into the skin tissue, providing a reliable support to permeation and retention studies obtained by our collaborators with imiquimod.

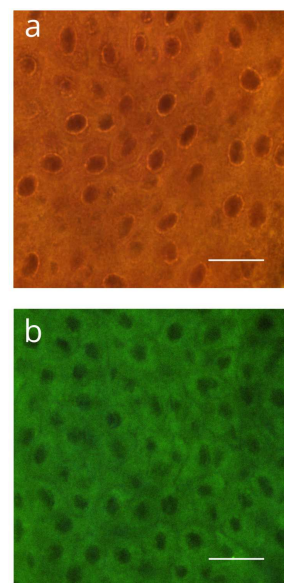


Figure 4.17: Zoomed TPM images acquired from the epithelial cells of the epidermis of a skin sample treated with NR-loaded TPGS micelles [panel(a)] and saline solution [panel(b)] exciting at 890 nm. Bar length: 20 μm .

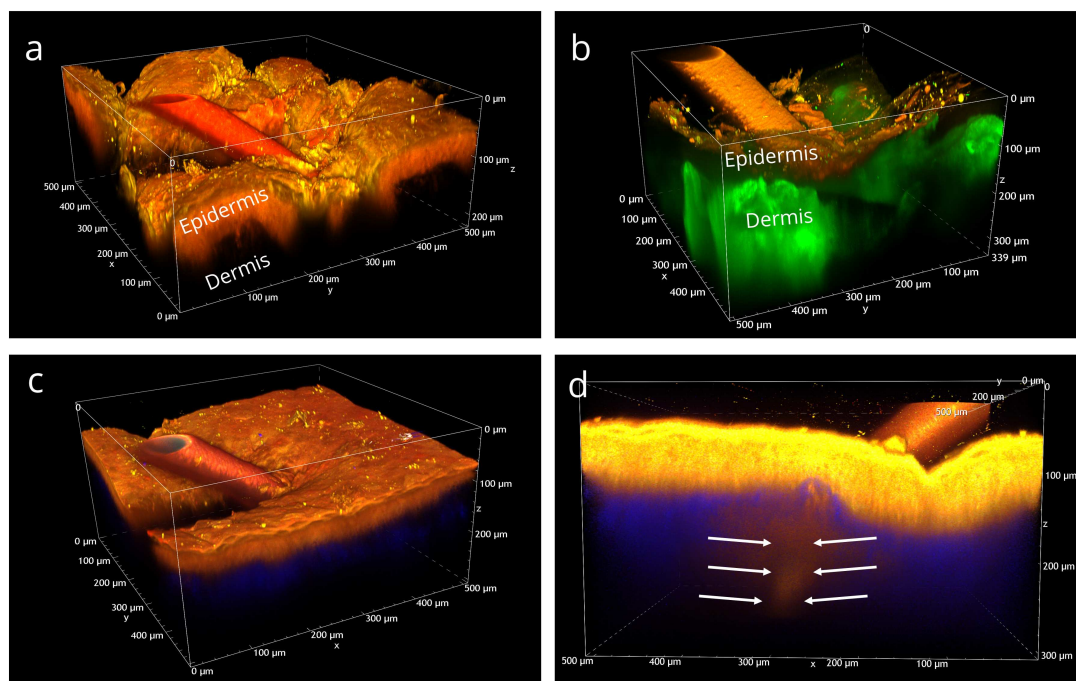


Figure 4.18: Volume renderings of porcine hair follicles reconstructed from Z-stacks. Panel(a): Skin treated with NR-loaded TPGS micelles (Z-step: 1 μm , total depth: 222 μm); laser power: 19.2 mW; Panel(b): skin treated with saline solution (Z-step: 1.5 μm , total depth: 222 μm); laser power: 102.4 mW. The detector gain and the laser power were lower for panel(b) compared to panel(a) to avoid saturation; for this reason, the SHG of dermis collagen is not visible in panel(a). Panels(c,d): volume rendering of the same hair follicle reconstructed from the Z-stack (Z-step: 0.8 μm , total depth: 307 μm). Panel(c) 3D rendering overview; Panel(d): XZ view with red channel saturated using LUTs, in order to highlight the red signal in the innermost layers of the tissue (arrows). Panels(a,b) were acquired exciting at 1100 nm, while panels(c,d) at 850 nm.

4.3 Nanostructured lipid carriers for enhanced transscleral delivery

During the end of the last century, several nanoparticles delivery systems based on lipid matrices (also termed lipid-based nanocarriers) have emerged, such as nanoemulsions, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLCs).[202] Among the different nanosystems currently available, lipid-based carriers are known for efficiently solubilizing and protecting poorly water-soluble drugs within biocompatible lipid matrices while improving bioavailability due to a high specific surface area.[203, 204]

Nanostructured lipid carriers (NLCs) are second-generation lipid-based nanocarriers, consisting of a core matrix of solid and liquid lipids stabilized by a surfactant-cosurfactant mixture, with improved drug loading and stability in comparison to early SLN, i.e., nanoparticles composed of solid lipid cores surrounded by surfactants. The addition of a liquid lipid to the nanoparticle core avoids rearrangement into densely packed lattice structures, which has been identified as a limitation of SLN since it might lead to premature drug expulsion.[205] Effective retinal delivery of lipophilic agents can benefit from this approach to overcome ocular barriers, thus achieving therapeutic doses.[206]

Based on these promising features, our collaborators from the Food and Drug department of the University of Parma recently developed dexamethasone acetate-loaded nanostructured lipid carriers intended for periocular administration to the posterior segment of the eye.[207]

Dexamethasone, a potent glucocorticoid, not only downregulates the expression of multiple proinflammatory chemokines and cytokines, but also inhibits retinal neovascularization.[208] Nonetheless, its high lipophilicity hinders effective therapeutic doses when applied topically and several approaches have been proposed over the years.[209] Water soluble derivatives, such as dexamethasone sodium phosphate, have been developed to obtain more concentrated eye drops, while more lipophilic prodrugs, such as dexamethasone acetate have been selected to promote drug permeation across corneal epithelium.[210, 211]

The development of the NLCs and the associated permeation/retention studies in sclera have been performed by Felipe Gonz  les from the AD-DRes Lab. In order to encapsulate dexamethasone acetate into NLCs, pre-formulation studies were performed to find solid and liquid lipid mixtures for dexamethasone acetate solubilization. Pseudoternary diagrams at 65  C were constructed to select the best surfactant based on the macroscopic transparency and microscopic isotropy of the systems. The resulting NLCs, obtained following an organic solvent-free methodology, were composed of triacetin, Imwitor  491 (glycerol monostearate >90%) and tyloxapol with Z-average = 106.9 ± 1.2 nm, PdI = 0.104 ± 0.019 and apparent ζ -potential = -6.51 ± 0.575 mV.[207] The molecular structures of the selected components are reported in Figure 4.19.

Ex vivo studies carried out with porcine ocular tissues demonstrated good permeation and retention of the DexAc within scleral tissue following application of the NLCs.[207] Permeation experiments revealed a considerable metabolism (due to the presence of ocular esterase enzymes) in the sclera of dexamethasone acetate into free dexamethasone, which showed higher permeation capabilities.

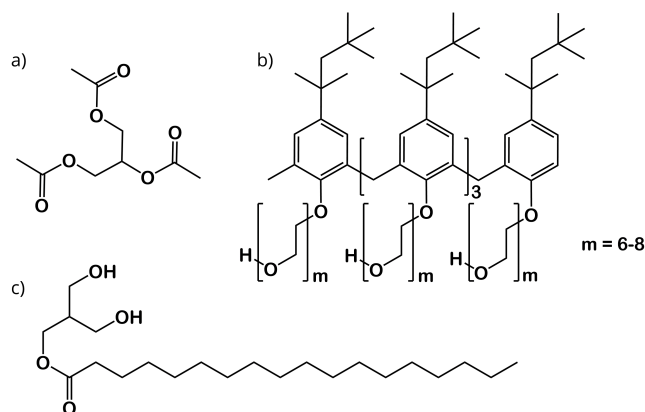


Figure 4.19: Molecular structures of the components employed for the preparation of NLCs. a): glycerin triacetate (triacetin), b): Tyloxapol, c): glycerol monostearate.

Blank NLCs resulted fluorescent in the UV region, as reported in Figure 4.20. This weak fluorescence is attributed to the tyloxapol surfactant, which has the same emission profile. However, the emission of tyloxapol cannot be exploited in the TPM measurements, since it falls in a spectral region that cannot be detected with our experimental setup.

For this reason, NR was employed also in this case as fluorescent and solvatochromic probe, in order to elucidate the behavior of the NLCs once applied on excised porcine sclera.

NR-loaded NLCs were firstly spectroscopically characterized to investigate Nile Red encapsulation. The produced NR-loaded NLCs were purified from unencapsulated NR via size exclusion chromatography, with the help of desalting columns from which several fractions were collected. No differences have been observed between the emission (and excitation) spectra of the unseparated sample and of the filtered fractions, suggesting that NR is probably embedded in a similar environment (Figure 4.22a). The successful encapsulation of NR inside the NLCs is evident from the comparison between emission spectra in different media reported in Figure 4.22b and the TPM images reported in Figure 4.21.

The emission spectrum of the NR-loaded NLCs is very similar to the one collected from NR at a concentration of $1 \mu\text{M}$ in a $\sim 2.7 \text{ mg/mL}$ tyloxapol aqueous solution (corresponding to the concentration of tyloxapol in the NLCs formulation). The spectral position of the emissions suggests that NR is entrapped between the polyoxyethylene chains of the surfactant, i.e., in the surface layer of the NLCs. The similarity of the emission spectra of NR collected in tyloxapol and NLCs does not exclude the presence of some NR-loaded tyloxapol micelles in the suspension, which could be in thermodynamic equilibrium with the NLCs and thus, not completely removed by the size exclusion chromatography. This assumption is further supported by the presence of a non-negligible background emission signal in the TPM images reported in Figure 4.21, that could stem from the presence of NR-loaded tyloxapol micelles.

To investigate the behavior of the NLCs once in contact with porcine sclera, NR distribution inside the tissue was studied by TPM, following the same experimental approach that we proposed in the previous sections. Figure 4.23 reports images acquired at 40 and $150 \mu\text{m}$ depth from the scleral surface after the permeation of NR-loaded NLCs (NR concentration in triacetin: $0.1 \mu\text{g/mg}$). The green signal is mainly attributed to the SHG of collagen fibers; the black spaces among green fibers are aqueous pores

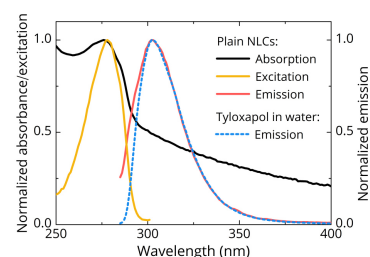


Figure 4.20: Normalized absorption, excitation, and emission spectra of a diluted plain NLCs aqueous suspension. The emission of Tyloxapol surfactant is shown as a comparison (dashed blue line). The emission have been recorded by exciting at 280 nm, while the excitation spectrum of the NLCs was acquired collecting the emission at 300 nm.

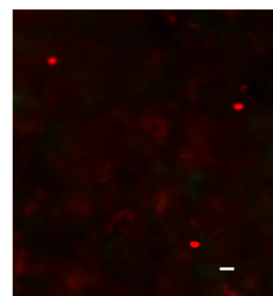
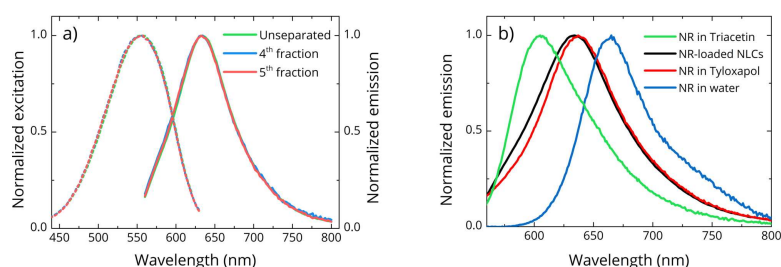


Figure 4.21: TPM images obtained from an aqueous suspension of NR-loaded NLCs with an excitation wavelength of 1100 nm. Images size: $101 \mu\text{m} \times 94 \mu\text{m}$, bar length: $5 \mu\text{m}$

Figure 4.22: Panel(a): Comparison between the excitation and emission spectra of the unseparated sample and the two separated fractions of NR-loaded NLCs. Panel(b): Comparison between emission spectra of NR in triacetin, NLCs (aqueous suspension), tyloxapol (2.7 mg/mL in water), and water. The emission spectra have been acquired exciting the sample at 500 nm, while the excitation ones were recorded by collecting the light emitted at 650 nm.



(Figure 4.23a,d), since sclera is composed of 70% water.[59] Collagen fibers appear thicker in the outermost scleral layers (Figure 4.23a) than in the innermost ones (Figure 4.23d), as previously observed with a different technique.[212] NR emission, collected in the red channel (Figure 4.23b,e), was detected between the collagen fibers, as clearly observed in panels c and f of Figure 4.23. The distribution of the NR signal is similar to what we described after the permeation of dye-loaded TPGS micelles (Section 4.2.1 and Chapter 2) and unimer micelles (Chapter 1) in the same tissue.

Figure 4.23: TPM images of porcine sclera (excitation wavelength: 1100 nm) after 2 h of contact with NR-loaded NLCs (0.1 $\mu\text{g}/\text{mg}$ triacetin), at a depth of 40 μm [panels(a,b,c)] and 150 μm [panels(d,e,f)] from the tissue surface. Panels(a,d): signal detected in the green channel, mainly due to the SHG of collagen fibers; panels(b,e): signal collected in the red channel, which is attributed to NR emission; panels(c,f): corresponding channels overlay, showing NR distribution (red) between collagen fibers (green). Images size: 170 $\mu\text{m} \times 170 \mu\text{m}$.

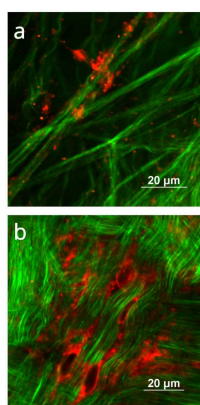
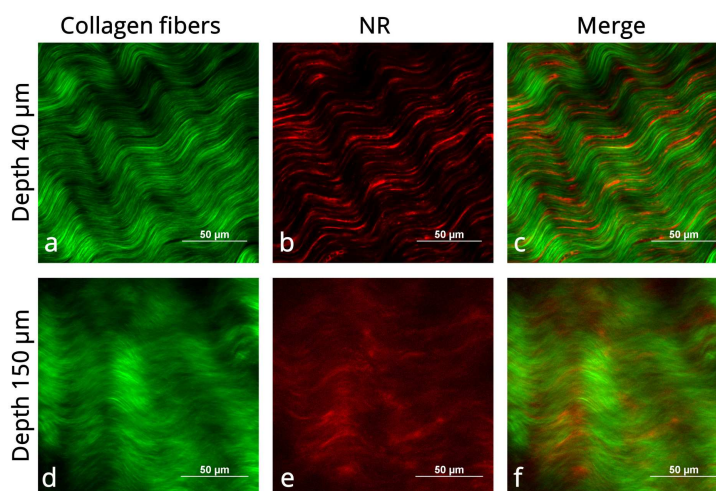


Figure 4.24: TPM images of sclera treated with NR-loaded NLC (0.1 $\mu\text{g}/\text{mg}$ triacetin). Panel(a): collagen fibers on the tissue surface with big aggregates emitting in the red channel (size: 82 $\mu\text{m} \times 82 \mu\text{m}$). Panel(b): TPM image of NR labelled fibroblasts, acquired 30 μm below the surface (size: 92 $\mu\text{m} \times 92 \mu\text{m}$). The excitation wavelength was set to 1100 nm for all the collected images.

Figure 4.25 reports both volume renderings and bidimensional images of scleral samples permeated with NLCs loaded with NR (starting from two different concentrations of NR in triacetin, namely 0.1 $\mu\text{g}/\text{mg}$ and 0.5 $\mu\text{g}/\text{mg}$) and without dye. In the presence of NR, some fluorescent particles and big aggregates were present on the surface of the tissue, adhered to the most external collagen fibers (see Figure 4.24a for a zoomed view). The emission signal of NR was detected from scleral porosities up to the innermost tissue layers that have been visualized, 192 μm from the surface for the sample treated with higher concentration of the dye. Since NR is a hydrophobic probe characterized by very weak emission in aqueous environments, the bright emission observed in the red channel suggests that it is well solubilized even after the permeation in the tissue. As shown in Figure 4.24b, the NR also stained scleral fibroblasts, located in between the collagen fibers.

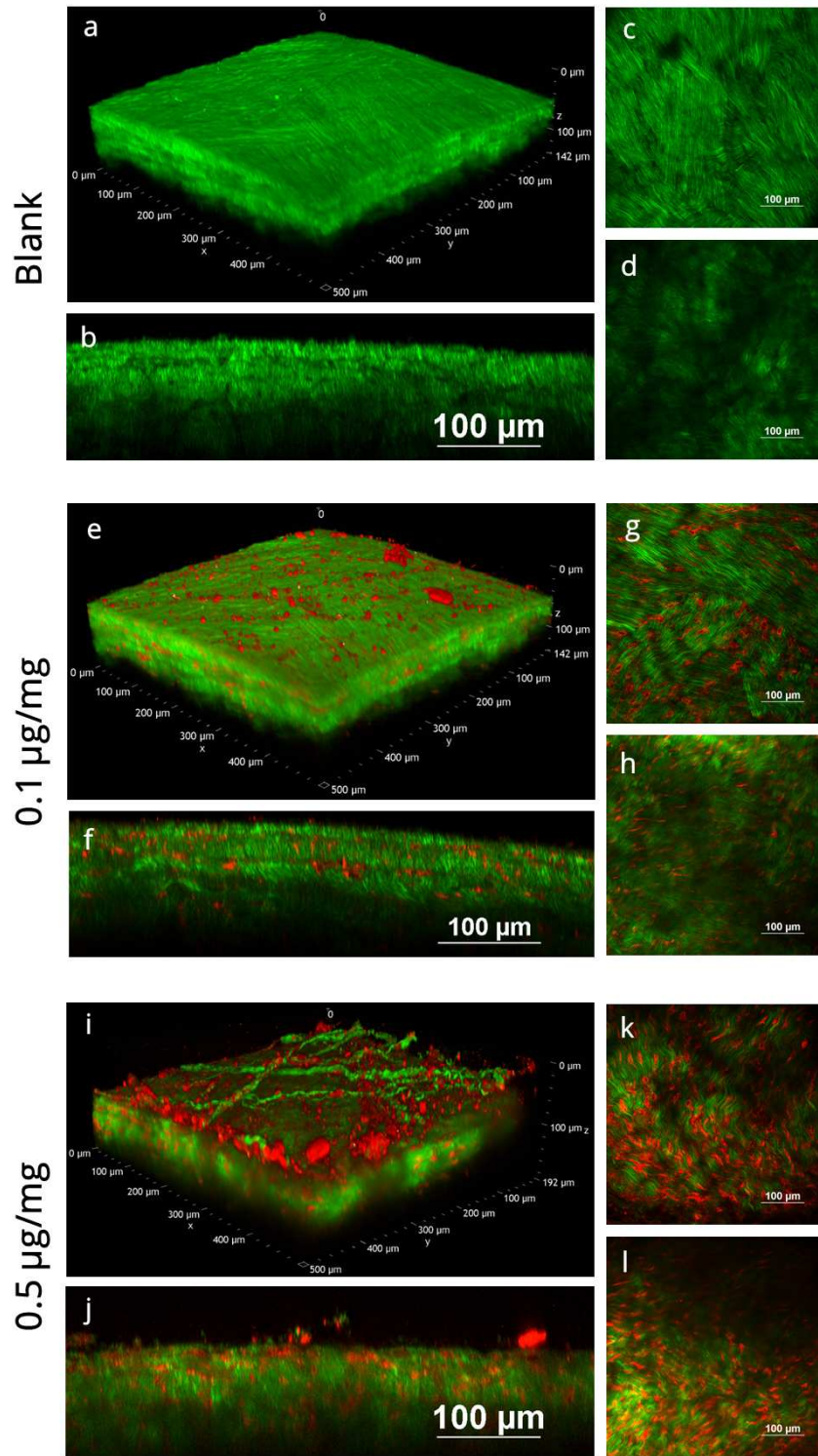


Figure 4.25: TPM images and volume renderings reconstructed from the Z-stacks of the scleral tissues treated with blank NLCs (Z-step: 1 μm , total depth: 142 μm , panels(a-d)), 0.1 $\mu\text{g}/\text{mg}$ NR-loaded NLCs (Z-step: 1 μm , total depth: 142 μm , panels(e-h)), 0.5 $\mu\text{g}/\text{mg}$ NR-loaded NLCs (Z-step: 1 μm , total depth: 192 μm , panels(i-l)). Panels(a,e,i) report the volume overviews, panels(b,f,j) XZ slice extracted from the Z-stack, panels(c,g,k): TPM images collected 40 μm from tissue surface, panels(d,h,l): TPM images collected 150 μm from tissue surface. The same experimental conditions were employed to acquire the images and the three Z-stacks: laser power, detectors gain and excitation wavelength (1100 nm).

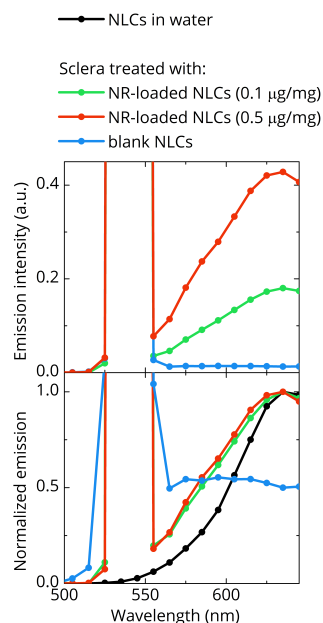


Figure 4.26: TPEF (broad band) / SHG (intense sharp peak; peak maximum is not shown) profiles obtained with the spectral detector in correspondence of images Figure 4.25c,g and k focal planes ($40\ \mu\text{m}$ below the surface), exciting at $1080\ \text{nm}$. The spectra in the bottom panel have been normalized and compared to the emission signal collected with the microscope from a NR-loaded NLCs aqueous suspension. Spectra were acquired in the same experimental conditions (detector gains and laser power). The spectrum of the tissue treated with blank NLCs has been arbitrarily normalized to facilitate a clearer comparison with other spectra.

The emission spectra of the tissue after the permeation of NLCs both loaded with NR ($0.1\ \mu\text{g}/\text{mg}$ and $0.5\ \mu\text{g}/\text{mg}$ dye concentrations) and without dye were also recorded. The three emission profiles, obtained $40\ \mu\text{m}$ below the scleral surface, are reported in Figure 4.26, while the TPM images collected in correspondence of the same focal planes are shown in Figure 4.25c,g and k. All the emission profiles were characterized by a strong and sharp peak centered at $540\ \text{nm}$, due to the SHG process promoted by the collagen fibers. The spectrum collected from the tissue permeated with blank NLCs (blue line in Figure 4.26) has a very intense SHG signal, and very weak autofluorescence. The spectra obtained from the samples treated with NR-NLCs were broadened and slightly blue shifted when compared to the emission recorded from NR-NLCs in aqueous suspension (bottom panel of Figure 4.26). The broadening of spectra is often associated with an increase of the disorder in the local environment.[213] As can be hypothesized from the very large size of the particles (roughly 10 times higher than TPGS micelles), it is likely that the NLCs do not permeate intact within the tissue: in this case, a redistribution of the NR molecules would be expected, exposing the dye to the lipophilic components of the nanocarriers, as well as to the hydrophilic surfactant, the scleral pores and fibroblasts. The resulting emission spectrum is the superposition of emission spectra of NR in different environments, resulting in an overall broadening of the bandshape.

4.4 Buccal permeation of polysaccharide high molecular weight compounds

Macromolecules represent, nowadays, exceptionally promising tools for the treatment of several diseases. The number of macromolecular drugs on the market and in clinical trials is increasing year by year, and the discovery of pathologies molecular basis is expected to bring new high molecular weight (MW) compounds onto the pharmaceutical market⁵.[\[215\]](#)

However, macromolecules often suffer from bioavailability problems linked to low stability and to low permeability across biological membranes. For this reason, at the moment, the preferred administration route is the parenteral one.[\[216\]](#) A possible alternative is represented by the systemic administration of these compounds via buccal delivery. Indeed, the buccal mucosa has been investigated as a potential site for the delivery of macromolecules because of its accessibility[\[217\]](#) and low enzymatic activity compared to the gastro-intestinal tract.[\[218\]](#) Despite the potentiality of this administration route, macromolecules are poorly permeable across the buccal mucosa, characterized by a stratified squamous epithelium keratinized or nonkeratinized, depending on the location inside the oral cavity. Previous studies have highlighted that the human buccal epithelium has a permeability limit of 20–40 kDa.[\[218\]](#)

Physical[\[219, 220\]](#) and chemical[\[221\]](#) penetration enhancers can represent a valuable tool for promoting the transmucosal flux of macromolecules, thus increasing the delivered amounts of the drug, and the molecular weight cut-off of the membrane, allowing for the permeation of bigger molecules. Not all substances that alter the barrier integrity can be considered good candidates; in fact, only a small number of chemical enhancers have progressed to clinical testing, having a history of safe use in humans.[\[222\]](#)

Among them, our collaborators from the Food and Drug department focused their attention on bile salts, the compounds present in our intestine as surfactants, and fatty acids, widely used as chemical penetration enhancers in the formulation of the mucosal, dermal, and transdermal dosage forms, to improve drug diffusion. Their aim was to explore the possibility of administering high molecular weight molecules across the buccal mucosa using fluorescently-labeled dextrans (FITC-dextran, functionalized with Fluorescein isothiocyanate) of different molecular weights (4 to 150 kDa) as model molecules. Medium- and long-chain fatty acids and the bile salt sodium taurocholate were tested as penetration enhancers either in pre-treatment or co-administered with FITC dextrans. The porcine esophageal mucosa, an accepted model of the human buccal mucosa,[\[223\]](#) was used as a barrier. With a permeability limit of 40 kDa, the structure and lipid composition of the porcine esophageal mucosa are similar to that of the buccal tissue,[\[224\]](#) its isolation from the underlying tissues is simpler, its surface is larger, and its integrity is guaranteed.

Permeation studies performed by Adriana Fantini (from ADDRes Lab) showed that it is possible to promote the mucosa permeation of high molecular weight dextrans by using sodium taurocholate (a bile salt) or, better, caprylic acid as chemical enhancers.[\[225\]](#) It was demonstrated, for the first time, that with these enhancers, used in co-administration or as a pre-treatment, respectively, dextrans with a molecular weight of 70

5: In this context, proteins and antibodies are dominant; but polysaccharidic compounds are also emerging as bioactive compounds for the treatment of various diseases. For instance, together with the well-known activity of heparin (2–40 kDa) as an anticoagulant, mesoglycan, a mixture of antithrombotic and pro-fibrinolytic glycosaminoglycans, and pentosane sulphate (2–9 kDa) for the treatment of interstitial cystitis, other more recent examples are given by plant-derived polysaccharides with anti-oxidant, immunomodulatory, anti-tumor, anti-inflammatory, anticoagulant, and hypoglycemic activity.[\[214\]](#)

6: The mechanism of action proposed for the fatty acids includes increased partitioning, the extraction of the intercellular lipids, membrane destabilization due to cholesterol dissolution, a change of membrane fluidity, and a disturbance of the lipid packing.[221, 226]

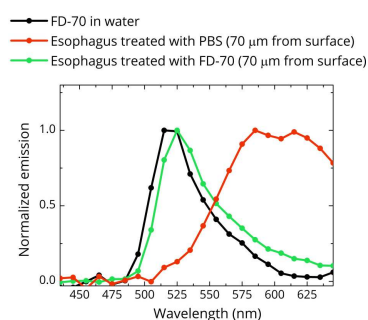


Figure 4.27: Comparison between the normalized emission spectra of an FD-70 aqueous solution and the blank and treated tissues (excited at 1030 nm and recorded in correspondence with panels(a,b) focal planes).

and 150 kDa (FD-70 and FD-150) could cross the mucosa in significant amounts. When applied in passive conditions, these two dextrans do not cross the mucosa in significant amounts. The permeability coefficients of FD-70 and FD-150 in presence of caprylic acid resulted very similar (~ 4 and $3 \cdot 10^{-4}$ cm/h), indicating that the enhancing effect was comparable despite the large difference in the molecular weight of the permeant.⁶ These permeation results suggest that higher MW dextrans can enter the tissue only by the paracellular route following the tight junction opening.

To further investigate the mechanism of the penetration of FD-70 across the epithelium and the effect of the caprylic acid pre-treatment, two-photon microscopy was employed. According to available information, the literature does not report any description of the porcine esophageal mucosa obtained by two-photon microscopy.

The tissue was pre-treated for 1 h with 100 mg/mL of caprylic acid[227] and left in contact with the FD-70 solution for 4 h, as described in Section 4.6.2. The sample was then washed with water and visualized by TPM with an excitation wavelength of 1030 nm. A blank reference tissue was also prepared, treated only with caprylic acid. Figure 4.28 compares the treated and blank samples, highlighting the substantial penetration of FD-70 in the tissue depth. The treated sample is characterized by a strong green emission, which is ascribable to the emission of FITC, as confirmed by the corresponding emission spectrum (Figure 4.27). On the contrary, the autofluorescence of the reference tissue is detected both in the green and red channels, giving rise to a yellowish image (Figure 4.28a). In both tissues, some epithelial cells can be recognized. The treatment with FD-70 uniformly stains the tissue and facilitates the identification of the superficial cell nuclei (the white arrows in Figure 4.28b), which are less visible in the reference sample (the white arrows in Figure 4.28a). Such behavior is compatible with a combination of the paracellular and intracellular routes for the penetration of FD-70, at least in the most superficial region of the buccal mucosa. The emission spectra recorded from the blank sample (Figure 4.27) is clearly different and strongly red-shifted when compared to the one recorded from the treated tissue, which is characterized by a peak (525 nm) superimposable with the FD-70 in solution.

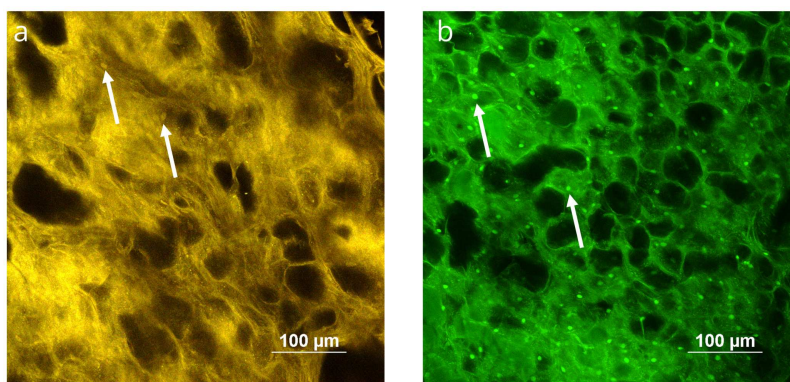


Figure 4.28: TPEF images (image size: $500 \times 500 \mu\text{m}$) acquired at an intermediate depth ($70 \mu\text{m}$ from the surface) with an excitation wavelength of 1030 nm from the blank and treated tissues. Arrows in panels a and b display epithelial cell nuclei.

The apparent presence of “holes” in the tissue showed in Figure 4.28 can be attributed to the corrugated surface of the mucosa, as it is revealed from the volume renderings reported in Figure 4.29. From these measurements, it is possible to safely assess that, after 4 h of permeation, FD-70 has fully stained the first 130 μm of the esophageal tissue. Most probably, the underlying part of the tissue also retained the fluorescent dextran, but due to the high scattering of the samples, it was not possible to acquire images in the deeper regions without modifying the experimental conditions (laser power and detector gain).

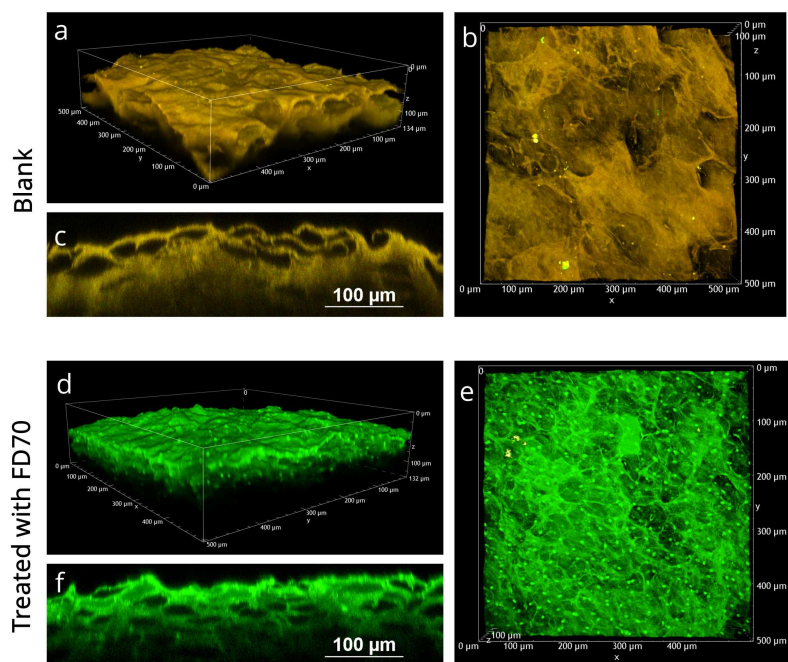


Figure 4.29: Volume renderings of the blank [panels(a-c)] (Z-step: 1 μm , total depth: 134 μm) and treated [panels(d-f)] (Z-step: 1 μm , total depth: 132 μm) porcine esophageal epithelium, reconstructed from the Z-stacks. Panels(a,d) 3D overviews, panels(b,e) XY views, panels(c,f): XZ slices. All the images were acquired with an excitation wavelength of 1030 nm and equal detector gains in the green and red channels.

4.5 Conclusions and perspectives

In this chapter, the potentialities of TPM as a tool for supporting drug-delivery studies for topical applications has been demonstrated. The measurements involved a combination of bidimensional images, volume renderings (Z-stacks) and emission spectra acquired directly inside thick porcine *ex vivo* tissues, after the treatment with fluorescently labelled nanocarriers (TPGS and NLCs) or model molecules (high MW dextrans). The fate of the drug delivery systems has been assessed, elucidating their behaviour and studying the distribution and emission properties of the exogenous fluorophore in the sample:

- After the permeation through cornea and sclera of TPGS micelles loaded with NR, we observed that the dye emission is drastically enhanced when the polymeric surfactant is present, proving the capability of these micelles to increase the amount of dye accumulated in the tissues. Moreover, the emission profiles recorded inside the samples proved the complete disaggregation of the nanocarriers only in contact with the corneal tissue, in good agreement with the quantification data collected by our collaborators. These results have been published in Ref. [60].
- In the case of TPGS micelles for skin administration, NR has been co-loaded with oleic acid, that can increase the solubilization of hydrophobic compounds in the micellar core. The NR signal has been detected in all the cellular elements of the skin, such as the epidermis and also vascular and corpuscular structures in the dermis. The emission spectra acquired inside porcine skin revealed the disaggregation of micelles during the permeation in the sample, similarly to what we observed for cornea, another tissue characterized by high density of cells. The accumulation of the fluorescent probe in hair follicles has also been addressed. These findings are reported in Ref. [101].
- NR-loaded NLCs have been studied after the permeation through sclera, employing two different dye concentrations. The NR signal was distributed in the interfibrillar spaces and scleral fibroblasts, and resulted more intense for the more concentrated system. Independently on NR concentration, the emission spectra collected inside the tissue were broadened and slightly blue shifted when compared to the intact nanocarriers in aqueous suspension, suggesting the exposure of the solvatochromic probe to environments with different local polarity and thus the disruption of the NLCs. These data are described in Ref. [207].
- The administration of FITC-labeled dextrans (MW 70 kDa) across the buccal mucosa, exploiting the effect of caprylic acid as permeation enhancer, has also been studied with TPM. The FITC emission signal was detected throughout the entire analyzed mucosa volume, confirming the capability of caprylic acid to enhance the permeation and retention of large MW molecules in this tissue. The uniform staining of superficial epithelial cells suggests a combination of the paracellular and intracellular routes for the penetration of the 70 kDa dextran. The aforementioned results have been published in Ref. [225].

4.6 Materials and Methods

Nile red (NR; IUPAC 9-(diethylamino)benzo[a]phenoxazin-5-one; MW 318.37 g/mol) was purchased from TCI Europe N.V. (Zwijndrecht, Belgium) or Sigma-Aldrich (Saint Louis, MO, USA). The vitamin E-derived surfactant tocopherol polyethylene glycolsuccinate (TPGS; MW approx. 1513 g/mol) was a kind gift of PMC ISOICHEM (Vert-Le-Petit, France). Caprylic acid $\geq 99\%$ (C8), fluorescein isothiocyanate-labeled dextrans were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the purchased chemicals have been employed without further purification.

4.6.1 Preparation of NR-loaded TPGS micelles

Polymeric micelles were prepared by direct dissolution of TPGS in high purity water, with a final concentration of 20 mM. For the two-photon microscopy analysis, NR-loaded TPGS 20 mM micelles were prepared. Briefly, 10 μL of a 10 mg/mL NR solution in DMSO were added to 1.5 mL of blank TPGS 20 mM micelles. The sample was then centrifuged at 13,000 rpm for 5 min to precipitate the exceeding NR and to collect the supernatant.

In the case of the micelles employed for permeation through porcine skin, before dye-loading the micelles were saturated with oleic acid. Briefly, 20 mM TPGS suspension was added with oleic acid at 4:1 volumetric ratio and the obtained mixture was subjected to 100 revolutions (made by hand) to ensure complete saturation of the aqueous phase. After saturation, the oily phase was removed and a 2-folds filtration (regenerated cellulose, Minisart RC 0.2 μm , Sartorius, Gottingen, Germany) of the aqueous phase was performed in order to get a clear micellar solution.

TPGS micelles employed for spectroscopic analysis have been loaded with NR adding 20 μL of a 403 μM stock solution in DMSO to 3 mL of micellar suspension (ratio DMSO/water 1:150, 0.7% w/w). During this process, the micelles suspension has been heated up to 35°C. The same procedure has been applied both in the presence and absence of oleic acid, and the resulting suspensions contain NR with a total concentration of 2.7 μM .

4.6.2 Caprylic acid pre-treatment of buccal mucosa

The esophageal mucosa was separated from the outer muscle layer with a scalpel, and the epithelium was peeled off from the connective tissue after immersion in distilled water at 60 °C for 60 s.^[227] Samples obtained were frozen until use, which occurred within 3 months. The tissue was mounted using a regenerated cellulose filter (0.45 μm , pore size) as the inert support in Franz's type diffusion cells (DISA, Milan, Italy), with a diffusion area of 0.6 cm^2 and a receptor volume of about 4 mL filled with a degassed pH 7.4 PBS and was kept under magnetic stirring during the experiment. The sample was pre-treated with 20 μL of fatty acid 100 mg/mL (caprylic acid C8, 690 mM) in ethanol, by application in a non-occluded condition to the epithelium; after 1 h of pre-treatment, the tissue was left in contact for 4 h with the FD-70 solution and then analyzed with the Two-Photon Microscope.

General Conclusions

In the context of this thesis, several advanced optical spectroscopic/microscopic techniques have been applied to the investigation of fluorescent organic nanoparticles as a whole and the encapsulated dyes, assessing their interactions in the native environment (typically aqueous suspensions) as well as when in contact with biological samples. The research work presented in this thesis marks a significant advancement in the exploration of phenomena and nanomaterials for bioimaging applications.

This work has demonstrated how fluorescence spectroscopy and microscopy techniques can provide information of fundamental importance for the study of fluorescent organic NPs, not only regarding color and emissivity but also concerning the local environment of the fluorescent probe and, therefore, its localization within the NP. Additionally, the fate of the fluorescent carrier within complex systems, such as biological systems, has been successfully monitored. Furthermore, it has been confirmed that multiphoton microscopy, widely used in the medical field for diagnostics, is a highly valuable technique for drug delivery studies. Several formulations for topical applications were analyzed using *ex vivo* porcine models of eye, skin and buccal mucosa: the results obtained are in agreement with observations in permeation and retention studies.

More in detail, by comparing the data obtained from absorption and emission spectra, fluorescence lifetimes and quantum yields, the encapsulation of hydrophobic dyes (C153, NR, DiI, DiD and DiR) has been demonstrated into various systems: single-chain NPs (unimer micelles), TPGS micelles, CTAB micelles, NLCs and CTAB/MKC-Quatsomes. The good emissive properties of the dyes solubilized in organic solvents, largely impaired when placed in water, are preserved when they are loaded into aqueous suspensions of the NPs.

Solvatochromic studies for C153 and NR encapsulated in the PEGMA-co-FA unimer micelles and TPGS micelles or NLCs, respectively, indicated that the two dyes are located in an environment less polar than water, similar to non-hydrated PEG.

Further insights into the encapsulation of dyes have been derived from fluorescence anisotropy measurements, as detailed in both Chapter 1 and Chapter 2. Steady-state excitation anisotropy offers a straightforward way to resolve absorption spectra, verifying the number of excited states contributing to the transition. If the anisotropy profile is flat within the excitation band, as we observed for the dye-loaded unimer micelles and TPGS/CTAB micelles, only a single excited state is involved. Moreover, the anisotropy values measured from aqueous suspensions of FONs resulted higher when compared to dye solutions in poorly viscous organic solvents such as ethanol or acetone, further confirming the embedding of the dyes within the NPs. The analysis of the anisotropy decay obtained with time-resolved measurements allowed to study the mobility of the encapsulated dyes. In the case of C153-loaded unimer micelles (Chapter 1) the motion of the dye (restricted wobbling and translational) has been

disentangled from the orientational diffusion of the nanocarrier as a whole, by fitting with an appropriate model. In the case of CTAB micelles loaded with DiD, the qualitative behavior of the anisotropy decay was employed to demonstrate both the encapsulation of the dye and its aggregation after dilution below the CMC.

The FRET process is an extremely attractive feature for fluorescence microscopy applications, and in this thesis the preparation of both micelles and Quatsomes showing efficient FRET has been demonstrated. However, as it was shown in Chapter 2 with the comparison between CTAB and TPGS micelles, a comprehensive spectroscopic characterization should always be performed in order to withdraw reliable conclusions on the NPs integrity. Indeed, a preliminary investigation of FRET efficiency is required also upon disaggregation of the NPs in controlled conditions.

Confocal and two-photon microscopy have been successfully applied to the visualization of many different FONs during *in vitro* cell cultures experiments and after the permeation through various *ex vivo* thick tissues, respectively. The statistical analysis on high number of confocal images highlighted the selective accumulation of surface functionalized MKC-Quatsomes into the target cell line. In this context, the comparison between live and fixed cells underlined the importance of minimizing the perturbations to the treated cell prior the imaging experiments.

Two-photon microscopy allowed for the deep imaging of ocular (cornea and sclera), skin and buccal tissues in the presence or absence of dye-loaded nanocarriers. The combination of images, three-dimensional renderings reconstructed from Z-stacks and emission spectra enabled a detailed analysis of the samples and revealed subtle features not visible with standard imaging techniques. The distribution of different FONs inside *ex vivo* tissues has been discussed, studying their integrity based on solvatochromic shifts of the emission spectra (Chapter 1 and Chapter 4) or variations of FRET efficiency (Chapter 2) at different depths. In the case of FONs with application as drug delivery systems, TPM proved to be an extremely powerful tool to study the behavior of the nanocarriers in contact with thick tissues, especially when coupled to permeation and retention quantification data.

T.1 Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS)

Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS) are advanced analytical techniques employed in the characterization of colloidal systems and particles, with sizes ranging from a few nanometers to several micrometers.

DLS, also known as photon correlation spectroscopy, is based on the analysis of time-dependent fluctuations of light scattered by particles undergoing Brownian motion, that stems from collisions between the suspended particles and solvent molecules. Autocorrelation analysis is performed on the collected data, and the diffusion coefficient (D) of the particles is determined by fitting the autocorrelation function to a mathematical model (such as the cumulant fit model). Subsequently, the size of the particles can be calculated as hydrodynamic diameter through the Stokes-Einstein equation:[228]

$$D = \frac{k_b T}{3\pi d_h \eta}$$

where T is the temperature, k_b is the Boltzmann constant, η is the medium viscosity, and d_h is the hydrodynamic diameter. d_h corresponds to the diameter of the sphere characterized by the same translational diffusion coefficient as the particle. It refers to an effective size parameter that characterizes the particles diffusion in a fluid medium, taking into account its translational motion and interactions with the surrounding solvent molecules. As a consequence, the sizes measured by DLS also accounts for the hydration shell of the particle, and depends not only on the actual particle size but also on the composition of medium, including the presence of ions and surface structures.

DLS measurements were performed at 25 °C with a Malvern Zetasizer Nano ZSP apparatus equipped with a 633 nm HeNe laser (Malvern Instruments, Malvern, UK). The backscattering mode (scattered light is collected at an angle of 173°) was employed for all the analyzed samples.

ELS is a technique used for the characterization of colloidal particles based on their electrophoretic mobility. This method provides insights into the surface charge of the charged particles in suspension. During an ELS measurement, an electric field is applied to the colloidal suspension, inducing the charged particles to move in response to the applied force. The electrophoretic motion of particles through the solution leads to the displacement of surrounding solvent molecules inducing fluctuations in the intensity of the light scattered by the sample, which are then analyzed using correlation techniques. The correlation analysis provides information about the speed of particle motion, allowing the estimation of electrophoretic mobility (u_e) expressed as particle velocity in a unit electric field.

The derived electrophoretic mobility is then used to calculate the ζ -potential, a parameter related to the surface charge and stability of colloidal particles. ζ -potential is defined as the potential difference between the bulk dispersion medium and the stationary layer of fluid attached to the particle's surface within the so-called slipping plane. The slipping plane refers to the hypothetical boundary within the electrical double layer where the fluid velocity equals the velocity of the particle.

The most general way to estimate the ζ -potential from the measured electrophoretic mobility involves the O'Brien and White equations,[229] which are valid under all experimental conditions. Since the solution of the O'Brien and White equations is not trivial, approximations can be applied, such as the Helmholtz-Smoluchowski equation employed in the context of this thesis:

$$u_e = \frac{\epsilon_{rs}\epsilon_0\zeta}{\eta}$$

where ζ is the ζ -potential, ϵ_{rs} is the relative permittivity of the electrolyte solution, ϵ_0 is the electric permittivity of vacuum and η the viscosity of the dispersant.

The determination of electrophoretic mobility through ELS was done using the Malvern Zetasizer Nano ZSP equipment, equipped with a 633 nm HeNe laser. To converse from the electrophoretic mobility to ζ -potential, the Helmholtz-Smoluchowsky approximation was employed, thus the corresponding values reported in this thesis consist in the apparent ζ -potential of the sample. The measurements were performed collecting the scattered light at 13° (forward detection).

Both DLS and ELS measurements were conducted in triplicate at a temperature of 25°C.

In the case of samples that absorb light at 633 nm the Malvern Zetasizer Nano ZSP equipment cannot be employed, especially if they are fluorescent.

The data reported in Chapter 3, i.e., hydrodynamic diameter, PdI, and ζ -potential measurements of Quatsomes loaded with EoY/RhB/DiD/DiR, were collected on undiluted samples in 1 mL polystyrene cuvettes using a Zetasizer Ultra (Malvern, UK), equipped with a fluorescence filter, that was available at ICMA/CSIC. The filter allows to perform measurements on samples containing fluorescent dyes that absorb the laser light.

T.2 Linear spectroscopic measurements

Solutions for spectroscopic measurements were prepared using spectrophotometric or HPLC-grade solvents. UV–vis absorption measurements were performed with a PerkinElmer Lambda650 double beam spectrophotometer, while both emission/excitation spectra and fluorescence anisotropies were recorded with an FLS1000 Edinburgh fluorometer, equipped with automatic polarizers (along emission and excitation pathways).

UV–Vis absorption spectroscopy

All absorption spectra presented in this thesis are absorbance spectra, employing a cuvette filled with the solvent as the reference during acquisition. Absorbance is directly related to the transmittance of the sample T and that of the reference T_{ref} as defined by the following equations:

$$T = \frac{I_{trans}}{I_{inc}}$$

$$absorbance = -\log\left(\frac{T}{T_{ref}}\right)$$

where I_{trans} and I_{inc} represent the light transmitted by the sample (or by the reference in the case of T_{ref}) and the incident one, respectively. Generally, the absorbance is not an absolute measure of the optical properties of the sample and depends on the arbitrarily chosen reference. Indeed, any non-vanishing reflectance, scattering, or absorption signal will give rise to a finite 'absorbance'.^[230] All absorbance measurements reported in this thesis are collected from solutions/suspensions, choosing as reference the cuvette filled with the solvent/dispersion medium. For this reason, the reflection can be considered practically absent and the measured absorbance is only related to optical losses (sum of absorption and scattering phenomena). However, most of the colloidal suspensions studied in this thesis were characterized by NPs with sufficiently small size (d_h less than 10 nm) and/or sufficiently high local concentration of the loaded dyes, so that even the scattering contribution was negligible in the spectral region of interest. The only exceptions were some of

the MKC-QSs suspensions discussed in Section 3.3, due to the quite low DiR concentration employed ($1 \mu\text{M}$) and large size of the dispersed NPs ($d_h \sim 100 \text{ nm}$). In this specific case, a method to remove the scattering contribution from the measured absorbance has been addressed, as described in Section 3.3.1.

Fluorescence spectroscopy

All fluorescence spectra were obtained from diluted solutions (absorbance < 0.1) and duly corrected for the excitation intensity and detector sensitivity.

Fluorescence quantum yields (ϕ) were measured employing quartz cuvettes (1 cm optical path) and fluorescein in NaOH_{aq} 0.1 M ($\phi = 0.90$) as reference, with the exception of DiR-loaded MKC-Quatsomes studied in Chapter 3, whose quantum yields were obtained by comparison with HITC in ethanol ($\phi = 0.28$, from Ref. [231]), using the following equation:

$$\phi = \phi_{\text{ref}} \frac{A_{\text{ref}}}{A} \frac{E}{E_{\text{ref}}} \frac{\eta}{\eta_{\text{ref}}}$$

where A is the absorbance, E is the integrated corrected emission intensity, and η is the refractive index of the solvent. The subscript “ref” is related to the properties of the reference, while all the others terms refer to the sample. In the case of strongly scattering samples, appropriate longpass filters have been inserted in the emission path to reduce the contributions from the scattered excitation light. For fluorescence quantum yield, an error of $\pm 10\%$ is assumed.[232]

Fluorescence anisotropy measurements at 77 K were collected using an Oxford Instrument OptistatDN cryostat by rapidly cooling down 2-MeTHF (stored over molecular sieves for one night, and filtered before use) to obtain a glassy matrix. Fluorescence anisotropy (r) is measured by exciting the sample with linearly polarized light and collecting the emission with parallel (I_{VV}) and orthogonal (I_{VH}) polarization with respect to the exciting beam. Fluorescence anisotropy is defined as follows:

$$r = \frac{I_{\text{VV}} - GI_{\text{VH}}}{I_{\text{VV}} + 2GI_{\text{VH}}}$$

where I is the emission intensity, and the subscripts V/H represent the orientation of the excitation and emission polarizers, respectively. The G factor correction accounts for the different sensitivities of the detector to the vertical and horizontal polarization of the emitted light and it is defined as:

$$G = \frac{I_{\text{HV}}}{I_{\text{HH}}}$$

Steady-state excitation anisotropy spectra are recorded as a function of the excitation wavelength and detecting the emission at a fixed wavelength.

Lifetime decays and time-resolved anisotropy measurements were acquired by exciting the sample with a pulsed diode laser ($\sim 200 \text{ ps}$ pulse duration and 405 or 375 nm as excitation wavelength) at a repetition rate of 1 MHz. The fitting of lifetime decays was repeated multiple times to ensure that the result did not change significantly ($< 10\%$) when varying the fitting parameters. In the case of the time-resolved anisotropy spectra reported in Chapter 2, we employed a Fluoromax-3 Horiba Jobin-Yvon instrument, exciting the sample with a pulsed nanoLED at 591 nm (pulse duration 1.5 ns), i.e. within the first absorption band of DiD, and detecting the emission at 675 nm.

The spectroscopic measurements reported in Section 3.3 of Chapter 3 have been performed in the laboratory of Prof. Kevin D. Belfield at the New Jersey Institute of Technology, employing a Tecan infinite 200 pro for absorptions and FLS980 Edinburgh fluorometer for fluorescence measurements, equipped with a 670 nm pulsed diode laser (with $\sim 200 \text{ ps}$ pulse duration) for time-resolved measurements.

Normalized spectra are obtained by dividing the raw spectra by a positive number, typically the maximum value in the spectrum, to ensure that the peak of the spectrum corresponds to a unit value, unless otherwise

specified. This normalization process allows for easier comparison between spectra, especially when comparing bandshapes collected under different conditions or from different samples.

T.3 *Ex-Vivo* permeation experiments

The permeation experiments have been performed in collaboration with Prof. Silvia Pescina, from the ADDRes Lab (Advanced Drug Delivery Research Lab) of the Food and Drug Department at the University of Parma. Porcine tissues (breed: Landrace and Large White; sex: female and male animals; weight: 145–190 kg; age: 10–11 months; provided by a local slaughterhouse) were isolated from fresh pig eyes (for ocular tissues), ears (for skin tissues) and esophagi (in the case of esophageal mucosa) within 2 h from the animals' sacrifice. The tissues were mounted on a glass Franz-type diffusion cell (DISA, Milano, Italy) with a permeation area of 0.6 cm² (setup schematized in Figure T1). The donor chamber was filled with 100–400 µL of the formulation of interest, which has been usually applied without dilution at infinite dose.

In the case of the ocular tissues, visualized with Two-Photon Microscopy (TPM) after 2 h of treatment, the receiving chamber was filled with saline solution (NaCl 9 g/L) or pH 7.4 PBS previously degassed. For sclera, the episclera (i.e., the outer side) faced the donor chamber, and the choroidal side (i.e., the inner surface) was in contact with the receiving chamber. When preparing the corneal samples, only bulbs with macroscopically intact corneas were used, whereas eyes showing opaque or damaged corneas were discarded.

Skin tissues were defrosted and mounted on Franz-type cells with the stratum corneum facing the donor compartment. The receptor compartment was filled with 1% w/v bovine serum albumin solution in PBS pH 7.4 and the donor solution was applied for 6 h prior to TPM visualization.

Esophageal samples were mounted using a regenerated cellulose filter (0.45 µm, pore size) as the inert support in Franz's cells and the receptor volume filled with a degassed pH 7.4 PBS. They were visualized via TPM after 4 h of contact with the donor solution.

The receiving solutions were kept at 37 °C and magnetically stirred during the experiments to guarantee sink conditions. Each permeation experiment has been performed at least in duplicate. All replicas were visualized under the microscope collecting several measurements in different regions of the sample, to ensure the reproducibility of the discussed results.

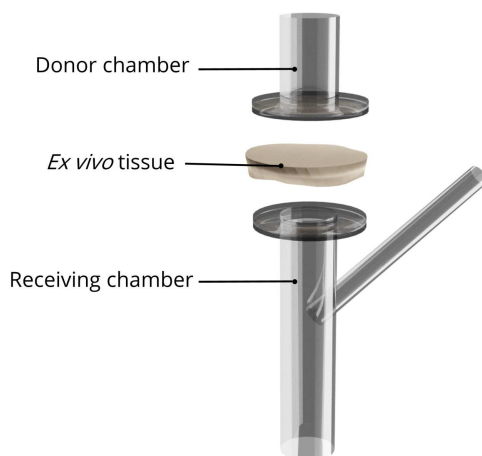


Figure T1: Scheme of a Franz-type diffusion cells. The setup can be thermostated at the desired temperature and different diffusional areas can be employed according to the sample. The receiving chamber can also be kept under magnetic stirring.

T.4 Two-Photon Microscopy setup

Ex vivo tissues were punched into discs and placed in a dedicated plexiglass holder, right after dismantling them from the Franz-type diffusion cell. Saline solution was employed to dip the microscope objective and to

avoid dehydration, as shown in Figure T2. In the case of frozen and/or partially de-hydrated tissues, the samples were soaked with sterilized saline solution for 20 min at room temperature before measurements, to allow complete thawing and/or re-hydration.

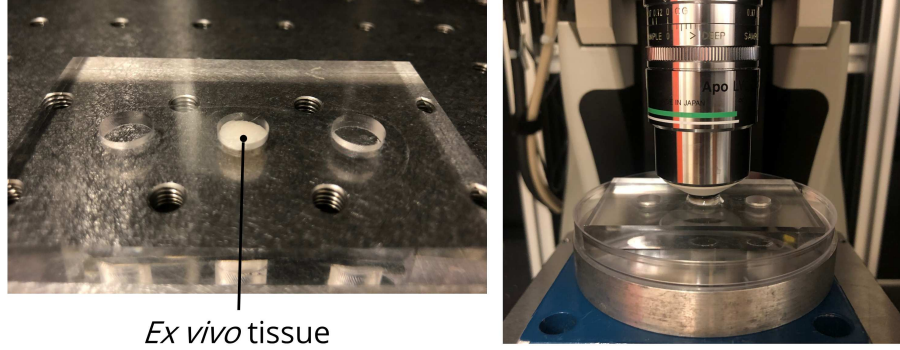


Figure T2: Example of an *Ex vivo* tissue disc (porcine skin) placed in the dedicated plexiglass sample holder under the two-photon microscope.

Ex vivo porcine tissues were analyzed with a Two-Photon Microscope Nikon A1R MP+ Upright equipped with a femtosecond pulsed laser Coherent Chameleon Discovery (~100 fs pulse duration with 80 MHz repetition rate, tunable excitation range 700–1300 nm). A 25× water dipping objective with numerical aperture (NA) 1.2 and 2 mm working distance was employed for focusing the excitation beam and for collecting the two-photon excited fluorescence (TPEF) and the second harmonic generation (SHG) signals.

TPEF/SHG signal was detected by four non-descanned detectors (NDDs) and a spectral detector (descanned, DD) connected to the microscope through an optical fiber. The series of NDDs is composed of three high sensitivity Gallium Arsenide Phosphide (GaAsP) and a Multi- Alkali photomultiplier tubes (PMTs), each one preceded by a specific filter cube in order to detect different spectral regions simultaneously: blue channel (415–485 nm), green channel (506–593 nm), red channel (604–679 nm) for the GaAsP's PMTs and far-red (698–750 nm) for the Multi-Alkali PMT. The spectral detector consists of a GaAsP PMT preceded by a dispersive element that allows to collect emission spectra in the 400–650 nm region with a 10 nm resolution. When using the NDDs, the associated dichroic filter allows for excitation in between 820 and 1300 nm, while the descanned spectral detector is associated with a dichroic mirror allowing for excitation in the 700–1080 nm region. A simplified scheme of the two-photon microscope setup is reported in Figure T3. The TPM images were recorded using the NDDs, merging the simultaneously acquired channels through the operation software of the microscope (NIS-Elements). According to the emission features of the sample, only some of the available detection channels have been employed. The data analysis of the two-photon measurements has been performed with the NIS-Elements software.

Images were acquired with a typical field of view of 500 $\mu\text{m} \times 500 \mu\text{m}$ (except where explicitly reported) with 1024 $\times$ 1024 pixels definition and an average acquisition time per image between 2 and 8 s in order to achieve a suitable signal-to-noise ratio.

Laser pulse power density

In diffraction limited techniques (such as two-photon microscopy), the minimum laser beam area can be defined as the distance between the first minima of the diffraction pattern, known as airy disc. The diameter of the airy disc central spot, which contains 84% of the total light intensity, is given by the following expression:^[233]

$$d = \frac{1.22\lambda}{NA}$$

where NA is the objective numerical aperture, and λ the excitation wavelength. In our set-up, NA = 1.2 and λ is tunable from 700 nm to 1300 nm. The resulting diameter range is ~710–1320 nm. Considering the airy disc

area (calculated from the diameter assuming a circular shape) and the excitation wavelength-dependent output power (1.2-1.8 W for the range 1300-700 nm) of the laser, an average power density in the range 73-380 MW/cm² can be estimated. The pulse duration of the Coherent Chameleon Discovery laser is around 100 fs, with a repetition rate of 80 MHz, leading to a pulse power density in the range of 9-46 TW/cm² according to the excitation wavelength (as shown in Figure T3). The calculated pulse power density for our excitation source is in reasonable agreement with previously reported two-photon fluorescence excitation measurements.[234, 235] The calculated values refer to the total output power of the laser, during each individual experiment the excitation power was be attenuated by a suitable percentage in order to completely avoid bleaching and saturation phenomena.

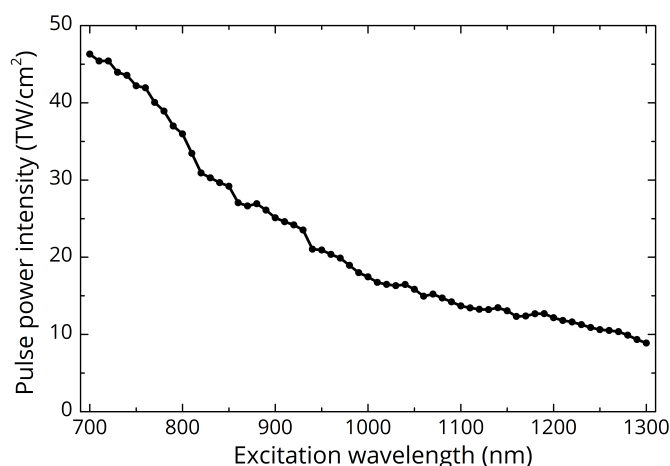


Figure T3: Pulse power intensity of the pulsed laser employed for TPM measurements, as a function of the excitation wavelength.

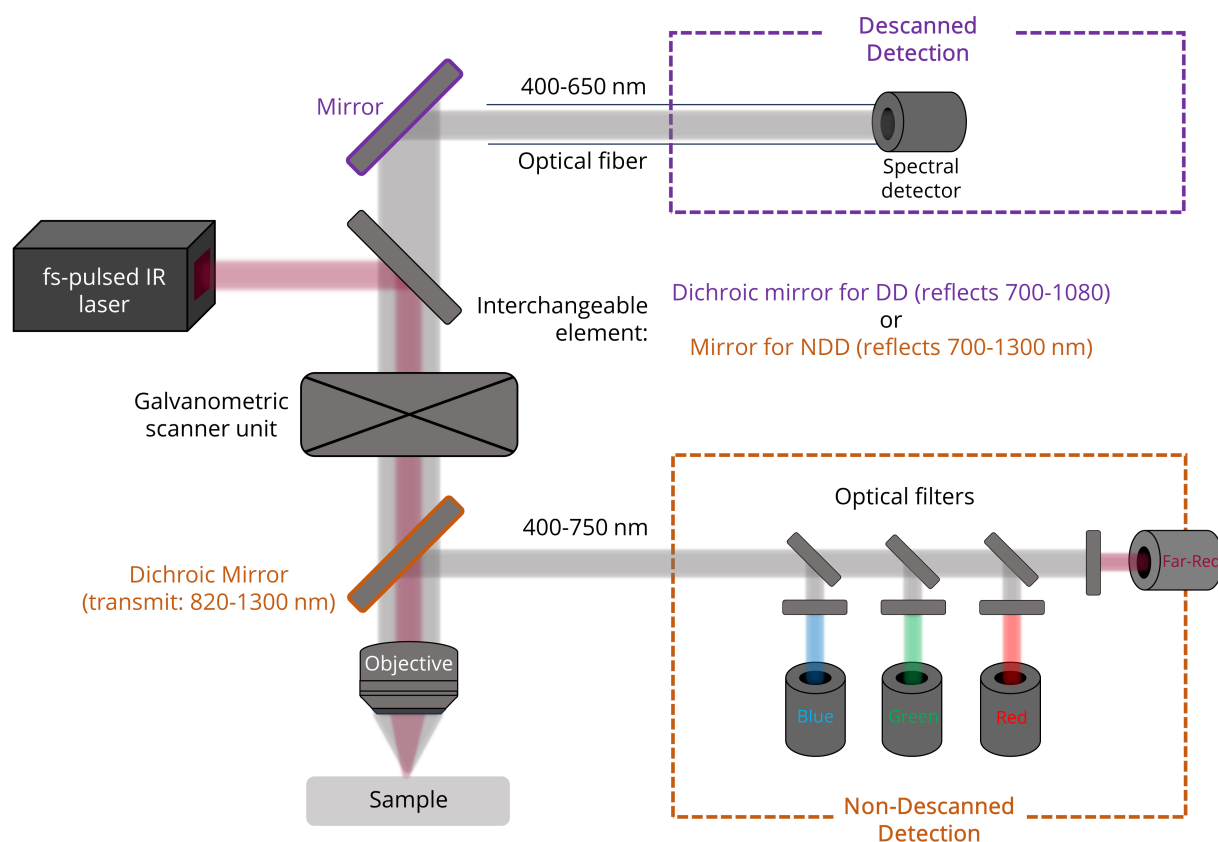


Figure T3: Simplified scheme of the two-photon microscope employed in this research work.

APPENDICES

Two-Photon Microscopy of blank tissues

A

A.1 TPA and SHG

As outlined in Chapter 4, TPM is an imaging technique which exploits nonlinear optical phenomena, mainly two-photon excited fluorescence, TPEF (promoted by two-photon absorption, TPA) and SHG, to acquire in-depth 3D images of the samples.

TPA is a third-order¹ nonlinear optical process involving the transition from the ground state to excited states through simultaneous absorption of 2 photons, characterized by the same or different frequencies, provided that their sum coincides with the sample transition frequency (Figure A.1). In fluorescence microscopy applications, the one-color (same frequency of the two absorbed photons) TPA process is more commonly exploited (since it requires a single excitation source) and it is characterized by a rate proportional to $\sigma_2 I^2$, where σ_2 correspond to the two-photon absorption cross section of the sample and I^2 to the squared excitation intensity. The quadratic dependence of the TPA probability on the incident radiation intensity allows to distinguish this nonlinear process from linear absorption, as demonstrated in Appendix B. TPA does not directly originate a resulting new electromagnetic field, but once the sample is excited, it can relax back to the ground state by emitting radiation, giving rise to two-photon excited fluorescence (TPEF).[236, 237]

Second Harmonic Generation (SHG) is a second-order nonlinear optical process that occurs when two photons of the same frequency combine due to the interaction with the medium to generate a new photon with twice the frequency of the original photons (as shown in the bottom panel of Figure A.1). It is a specific case of sum frequency generation, where the sum of the frequencies of two input photons results in the generation of a photon with a frequency equal to the sum. For each photon of the newly generated electromagnetic field, 2 photons of the incident field are destroyed, hence, the process do not involve any actual absorption². Differently from TPA, due to symmetry constraints specific to even-order nonlinear processes, SHG can be observed only in non-centrosymmetric systems from both a microscopic and macroscopic point of view. For this reason, solutions or isotropic dispersions are not suitable for observing this phenomenon, even if the microscopic constituents are non-centrosymmetric and possess even-order nonlinear responses.

The majority of nonlinear processes are characterized by extremely low transition probabilities at the conventional light intensities employed for promoting linear processes. To generate a nonlinear signal, at least 2 photons must arrive simultaneously at the active material, hence the excitation light needs to be significantly concentrated in space and time. High spatial densities can be easily achieved by focused laser beams, while the concentration in the time domain requires expensive pulsed lasers, emitting ultrashort pulses (< 1 ps) with high peak intensities. Indeed, historically, the use of nonlinear optical processes in a microscope only became possible after the development of ultrafast pulsed lasers.

1: The order of a nonlinear optical process is defined on the basis of the dependence of the induced polarization of the material on the excitation light intensity. For a third-order process, such as TPA, the polarization is proportional to the cube of the incident electric field.

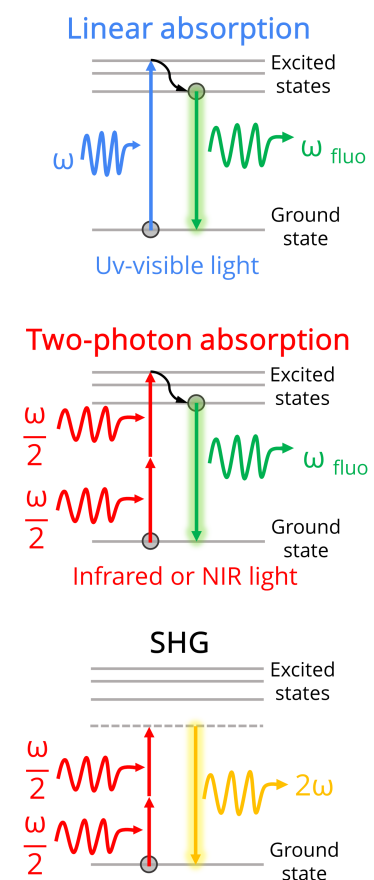
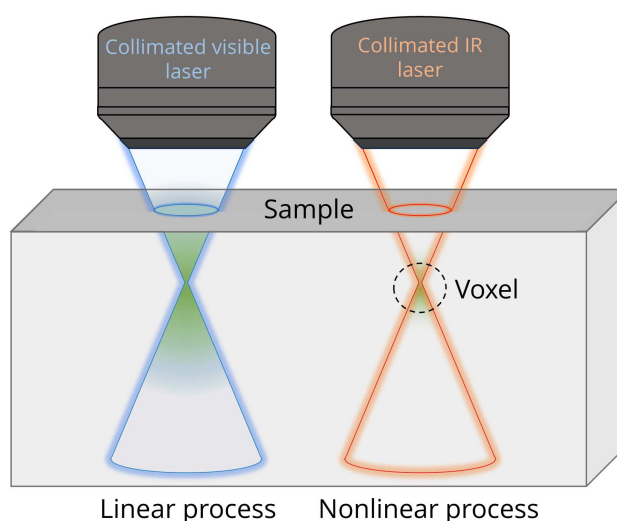


Figure A.1: Jablonski energy level diagrams describing the linear absorption, two-photon absorption and second-harmonic generation processes (from top to bottom). Note that for SHG no actual electronic excitation takes place. The subsequent spontaneous emission after linear or two-photon absorption is also showed.

2: The processes that occur without energy exchange between the material system and the electromagnetic field are classified as parametric. It can be demonstrated that even-order nonlinear processes such as SHG can be non-parametric (i.e. with energy exchange) only if one of the input fields is static.

Moreover, nonlinear optical phenomena efficiently occur only in the region of maximum intensity of a laser beam, i.e. at the focal point, where the probability of having 2 (or more) photons in the same place at the same time is high enough to promote the process. In analogy with the two-dimensional pixel, the small volume around the focal point of a laser beam, where the probability of nonlinear phenomena is non-negligible is often called voxel (see Figure A.2 for a schematic representation). The fact that nonlinear processes only occur in an very small volume (about a femtoliter), whose position can be modified simply by moving the laser beam focus, confers to these processes an intrinsic 3D resolution making them extremely appealing for applications in microscopy.

Figure A.2: Simplified scheme depicting the different spatial features of the fluorescence promoted by linear and two-photon absorption processes through suitable excitation sources. An entire cone of fluorescence light (green) is generated through linear absorption, whereas nonlinear signal production is localized to the proximity of the focal point.



The inherent 3D resolution in nonlinear optical processes allows to avoid confocal equipment, such as the pinhole, which is instead necessary to achieve 3D resolution with conventional fluorescence microscopy techniques (based on fluorescence promoted by linear absorption).

The excitation laser sources commonly employed in TPM fall within the far-red/Near-Infrared (NIR) spectral region, in order to excite, through TPA, fluorophores linearly absorbing in the UV-visible region. The same excitation sources can be useful to simultaneously promote SHG phenomena in the same spectral region. The use of far-red/NIR radiation, falling within the "biological transparency window" (between 700 nm and 1500 nm approximately), also allows deep penetration into tissues. Indeed, these photons are less absorbed and scattered (the Rayleigh scattering probability is proportional to the fourth power of the incident frequency) by biological tissues, leading at the same time to low photodamage and better signal/noise ratio when compared to the visible excitation sources available for confocal microscopy.^[238]

TPEF and SHG signals can provide complementary information: the former is associated to the presence of endogenous fluorophores (autofluorescence) or to the addition of exogenous dyes³, the latter allows for structural imaging, i.e. imaging of specific organized structures, like collagen and other structural proteins.

In the following sections, a study of blank porcine *ex vivo* skin and ocular (sclera and cornea) tissues with our TPM setup (described in Section T.4) is addressed. The analysis of the blank skin and corneal tissues has been carried out in the context of Ilaria Ferraboschi's master thesis.

³: The addition of exogenous dyes can be useful for many purposes, such as the labelling of specific structures of interest, the visualization of their distribution in the tissue and probing the polarity of the local environment in the case of solvatochromic probes.

A.2 Ocular tissues

The eye is a complex and isolated sensory organ, exhibiting a well-organized structure that is commonly divided into an anterior and posterior segment, each housing specific components vital for the process of vision (as schematically showed in Figure A.3).[239]

The anterior segment is located at the front of the eye, and it is composed by 3 main elements, involved in focusing the incoming light:

- ▶ Cornea, situated at the very front of the organ, is a transparent layer responsible for the initial light refraction. It is constituted mainly by three layers: epithelium, stroma and endothelium;
- ▶ Iris, adjacent to the cornea, regulates the amount of light entering the eye by adjusting the size of the pupil.
- ▶ Crystalline lens, located behind the iris, is a tissue dedicated to fine-tuning the focus of light onto the retina, contributing to the clarity of visual perception.

The posterior segment is located at the back of the eye and it is composed by components crucial for light detection and signal processing, such as:

- ▶ Sclera, the white external part of the eye, is a tough protective outer layer, responsible for maintaining the structural integrity of the eye providing resistance from both internal and external stimuli;
- ▶ Choroid, positioned in between sclera and retina, is a thin and highly vascularized layer that regulates the light absorption and provides nutrients to the retina;
- ▶ Retina, the innermost layer, contains specialized cells organized in three sub-layers (pigmented cells, photoreceptors rods and cones and neuronal cells), which convert light into electrical signals for transmission to the brain.

In the context of this thesis, we focused on the TPM characterization of cornea and sclera.

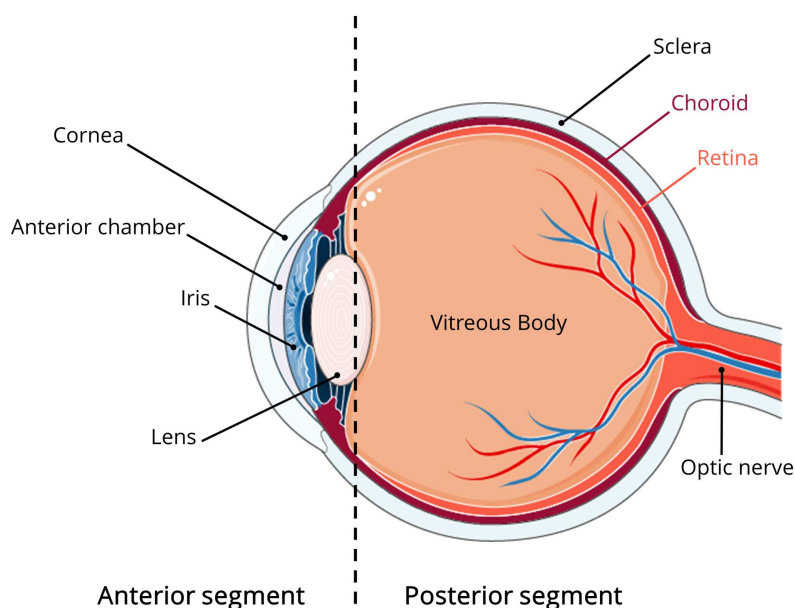


Figure A.3: Structure of human eye. Figure modified with text and arrows after adaptation of "Eye" from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License (<https://smart.servier.com>).

A.2.1 Sclera

The sclera is primarily composed of collagen fibers, which are arranged in a dense and interwoven network. The main cellular components in this tissue are the scleral fibroblasts, specialized cells responsible for the production of collagen and other extracellular constituents of the proteoglycan matrix. Elastin fibers and capillaries can also be found.[240] The total thickness of porcine sclera amounts to 830-1250 μm .[241]

Collagen, being a fibrous protein with noncentrosymmetric arrangement, can be mainly visualized due to the SHG phenomenon.[242] The SHG signal can be easily distinguished from the TPEF since it always falls at half the wavelength of the excitation light. A proof of the SHG signal coming from collagen fibers in the episclera (the outer face of the sclera) is reported in Figure A.4.

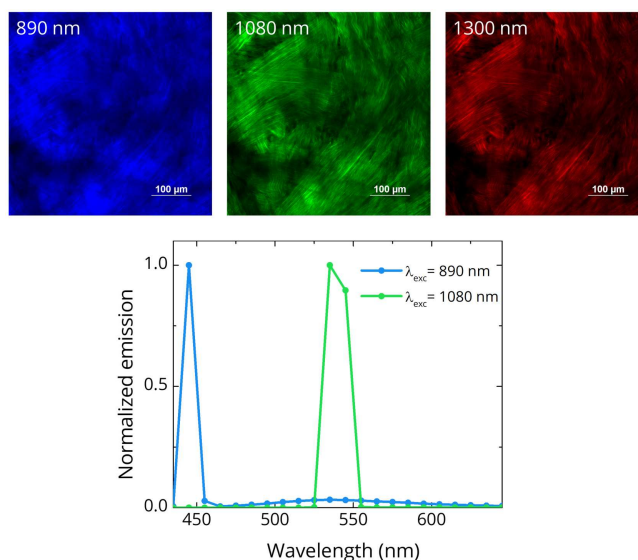


Figure A.4: SHG images of scleral collagen acquired with different excitation wavelengths. In the bottom panel are reported the spectrum profiles corresponding to the images collected at 890 and 1080 nm. The employed TPM setup (Section T.4) does not allow for excitation wavelengths higher than 1080 nm when measuring emission spectra.

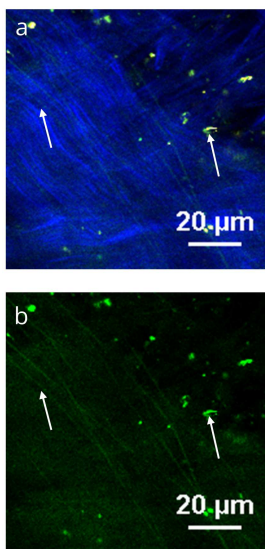


Figure A.5: Zoomed images of episclera excited at 830 nm showing elastic fibers and autofluorescent aggregates (highlighted by white arrows). Both the merged image [panel(a)] and the green channel are showed [panel(b)].

In between the collagen fibers of episclera, elastin fibers and autofluorescent globules were detected, as shown in the zoomed image reported in Figure A.5. When excited at 830 nm, the collagen fibers are visible in the blue channel, while both the fibers and aggregate structures are characterized by a TPEF signal mainly localized in the green channel with some contributions to the red one. The autofluorescent spots are probably due to aggregates of endogenous fluorophores, such as lipofuscins (pigment granules produced by lipids and proteins digestion) or melanin.

In Figure A.6 are reported images from both sides of the scleral tissue, the episclera [panels(a-c)] and the choroidal side [panels(e-g)], acquired about 50 μm below the tissue surface with an excitation wavelength of 830 nm. The signal detected in green channel from the choroidal side of the sclera clearly suggests the presence of fibroblasts. The corresponding emission spectra, reported in Figure A.6 (panels d and h), do not present any significant difference among them and most probably present contributions by a mix of different species, such as collagen, elastin, flavins, melanin and lipofuscins.[243]

The TPEF signal detected from the choroidal side of the tissue resulted more intense, especially on the surface, presumably due to the presence of residuals from the highly pigmented choroid. This difference is also clear

from the 3D renderings of the two sides of the sclera reported in Figure A.7. However, as demonstrated in Chapter 1, Chapter 2 and Chapter 4, when an external fluorophore is loaded into sclera at a sufficient amount, the contribution of these autofluorescence signals is practically negligible.

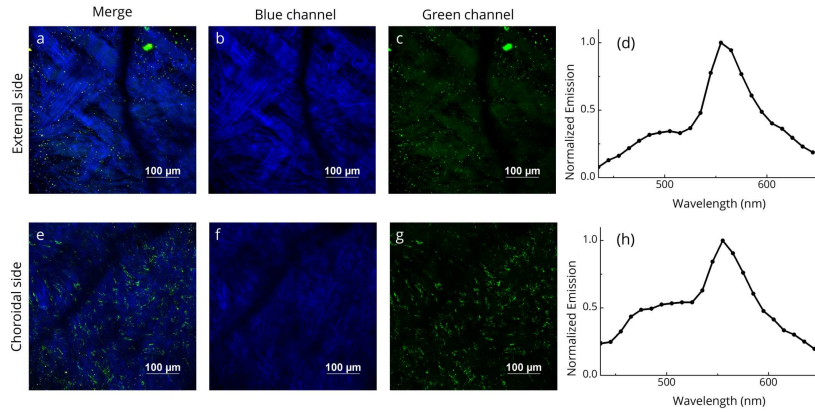


Figure A.6: TPM images and emission spectra of episclera [panels(a-d)] and the choroidal side of sclera [panels(e-h)], acquired 50 μm from the surface exciting the samples at 830 nm. Apart from the merged images, blue and green channels are also showed.

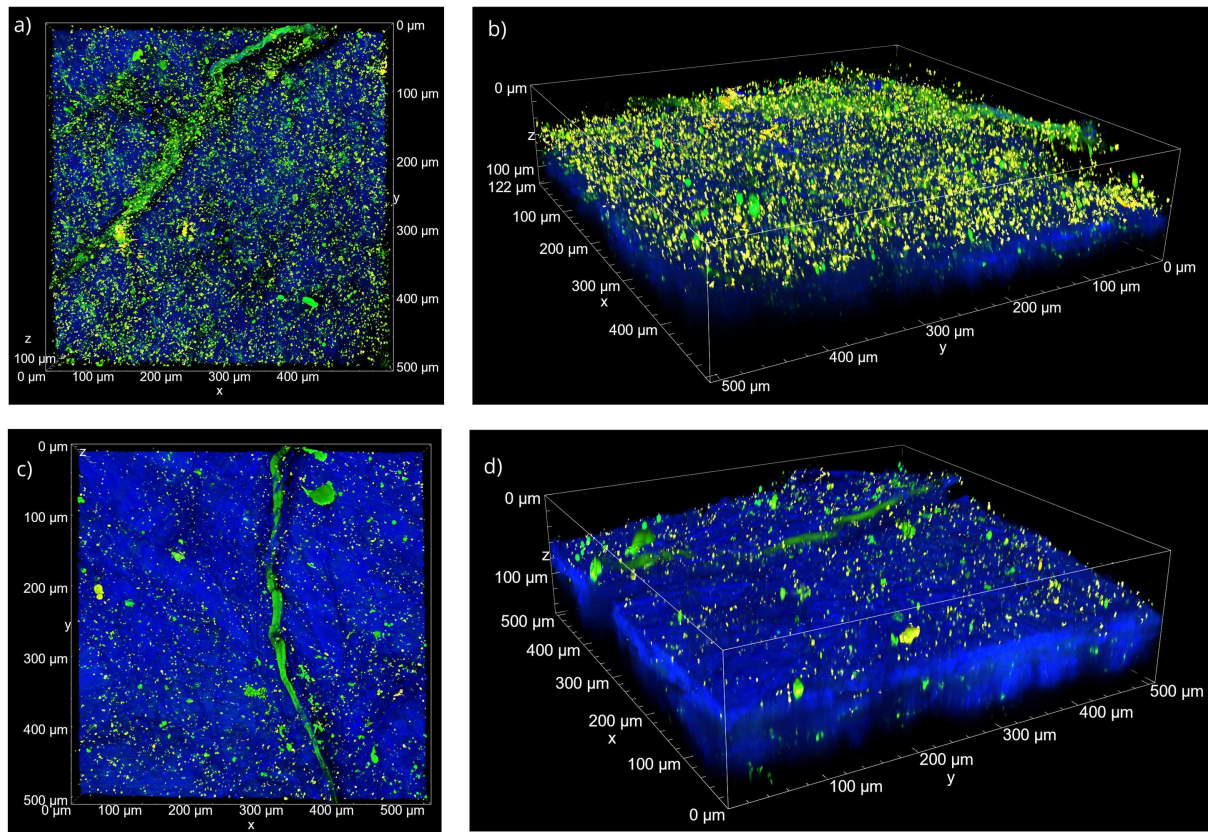


Figure A.7: Volume renderings of episclera and the choroidal side of sclera, reconstructed from Z-stacks acquired exciting the samples at 830 nm. Panels(a-c): choroidal side (Z-step: 1 μm, total depth: 122 μm): (a) XY view, (b) 3D overview. Panels(c-d): episclera (Z-step: 1 μm, total depth: 151 μm): (c) XY view, (d) 3D overview.

A.2.2 Cornea

Cornea is composed of three different layers:

- Epithelium (thickness: 55-105 μm [241]), the outermost one, composed by several layers of stratified squamous epithelial cells;
- Stroma (thickness: 900 μm [241]), composed by regularly arranged collagen lamellar fibers and corneal fibroblasts, called keratocytes. In the human eye the stroma is separated from the epithelium by the Bowman's membrane, which is absent for pigs;
- Endothelium (thickness: 30 μm , considering also the Descemet's membrane[241]), the innermost layer, composed by a single layer of flattened endothelial cells. This tissue is separated from the stroma by the thin and acellular Descemet's membrane.

The collagen fibers in the stroma, along with the precise arrangement of cells, contribute to the cornea's ability to refract light accurately onto the retina for visual perception. The highly organized structure of the cornea is essential for its transparency and optical clarity and allows for the acquisition of TPM Z-scans over a depth of about 1 mm without changing the laser power. A 3D rendering reconstructed from one of these Z-scans, acquired at 850 nm, is reported in Figure A.8. The tissue can be almost completely visualized within a single Z-stack, only 100 μm of stroma and the endothelium layer are missing. The endothelium and the stroma can be easily distinguished: the former corresponds to the superficial green layer, visible due to the autofluorescence of the epithelial cells, while the latter can be visualized below the epithelium thanks to the collagen SHG (blue channel) and the green autofluorescence of the keratocytes.

Bi-dimensional TPM images of the corneal epithelium are reported in Figure A.9. From this figure, is clear how the epithelium cells on the tissue surface are bigger than the deeper ones, as expected in this kind of tissue.[240] These cells are autofluorescent at this excitation wavelength (850 nm) mainly due to the presence of flavoproteins, and the TPEF signal is mostly localized into the cytoplasm.

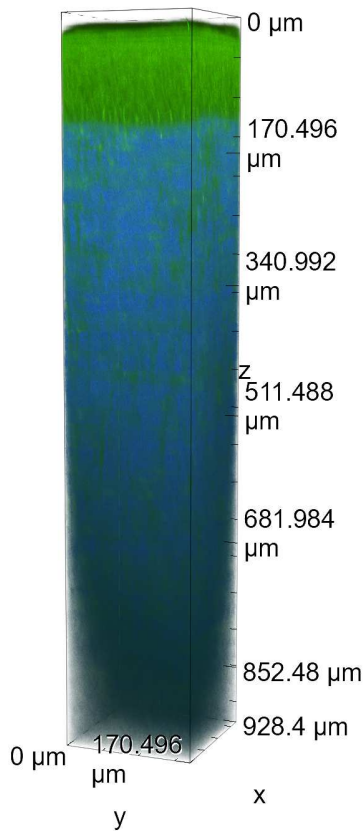


Figure A.8: 3D rendering of porcine cornea reconstructed from Z-stack (Z-step: 1 μm , total depth: 928 μm), obtained by exciting the sample at 850 nm.

Figure A.9: TPM images of the epithelium layer acquired with an excitation wavelength of 850 nm. From panel(a) to panel(e): every image is 10 μm deeper than the previous one (size: 96 μm x 96 μm). Panel(f) corresponds to a non flat region of the tissue, showing all the stratified cells layers and the initial region of the stroma.

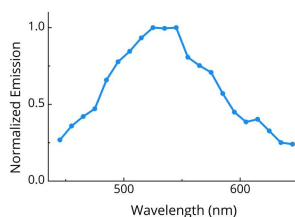
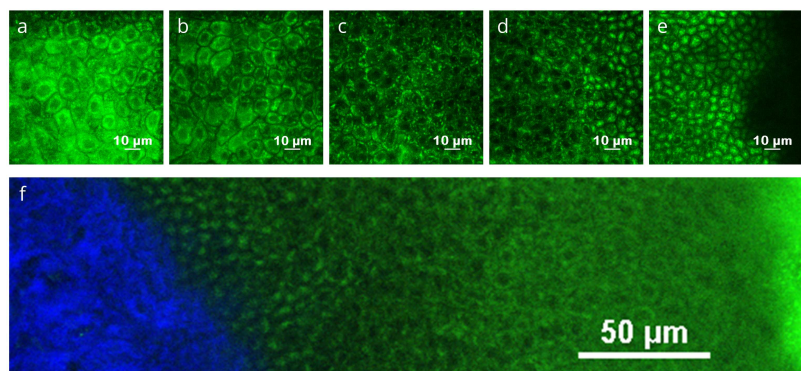


Figure A.10: Emission spectrum of corneal epithelium collected 80 μm below the surface of the tissue by exciting at 850 nm.

The source of autofluorescence in the corneal epithelium has been confirmed also by collecting an emission spectrum inside the tissue (Figure A.10), which resulted in line with the emission of flavins reported in the literature.[243]

In Figure A.11 is reported an image acquired from the corneal stroma about 200 μm below the surface of the tissue. Analyzing the collagen SHG detected in the blue channel (Figure A.11b), is possible to observe

the different architecture of the corneal collagen with respect to sclera (Section A.2.1), in this case the fibers are organized into lamellae, i.e. sheet-like layers of collagen fibers. These lamellae are arranged in a crisscross pattern, creating an organized lattice-like structure, which is completely different from the irregular arrangement of collagen fibers observed in scleral tissues. The presence of keratocytes is evident in the green channel (Figure A.11c), where they are clearly distinguished from the autofluorescence of the extracellular matrix.

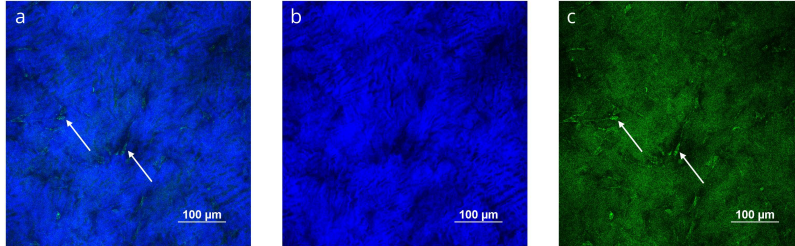


Figure A.11: TPM image of corneal stroma acquired about 200 μm below the surface, exciting the sample at 850 nm. Apart from the merged image [panel(a)], also the blue [panel(b)] and green [panel(c)] channels are shown. Keratocytes are highlighted by white arrows.

In Figure A.12 a TPM image of the endothelium layer is shown, acquired from a full-thickness corneal sample. The endothelium cells are visible due to autofluorescence, similarly to the epithelium ones.

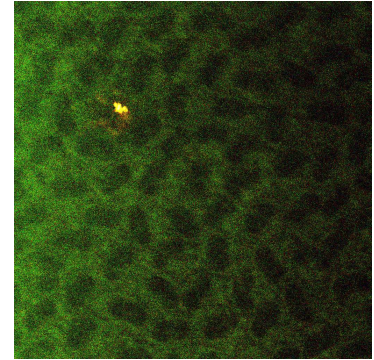


Figure A.12: TPM image of endothelium cells (size: 170 μm $\times$ 170 μm), obtained by exciting the sample at 850 nm.

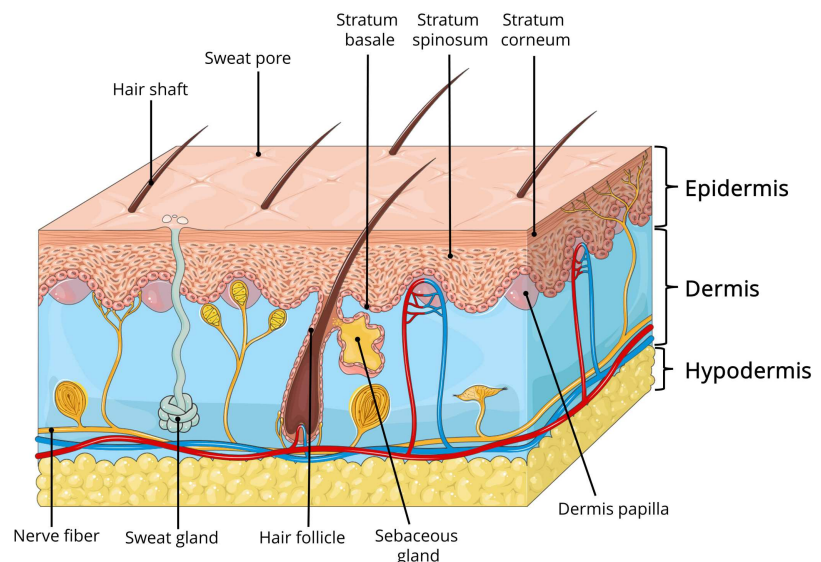
A.3 Skin

The skin is the largest organ in the human body and serves as a physical barrier from the external environment. It has various functions, including protection against physical damage, pathogens, and dehydration, as well as temperature regulation and sensory perception.[244] The skin is composed of three primary layers (see Figure A.13 for a schematic representation):

- ▶ Epidermis, the outermost layer, serves as a protective barrier and consists of a stratified squamous epithelium composed of multiple cells layers. The most superficial cell layer of the epidermis is the stratum corneum, formed by multiple sheets of flattened, fully keratinized, and dead cells. These cells are continuously shed and replaced by the underlying layers.
- ▶ Dermis, located under the epidermis, is a thicker layer that contains connective tissues (mostly collagen), blood vessels, nerves, and accessory structures such as hair follicles and sweat glands. It provides structural support to the skin and houses important sensory receptors for touch, pressure, temperature, and pain;
- ▶ Hypodermis, located under the dermis, consists of adipose (fat) tissue and connective tissue. It acts as an insulator, helping to regulate body temperature, and serves as a cushion that protects underlying structures from trauma.

In the context of this thesis, *ex vivo* porcine skin taken from pig ears has been studied, since it presents comparable structure and thickness to the human one and it is considered as a good preliminary model.[245]

Figure A.13: Structure of human skin. Figure modified with text and arrows after adaptation of “Integumentary system” from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License (<https://smart.servier.com>).



TPM images obtained at different depths from epidermis and the superficial part of dermis exciting at 850 nm are reported in Figure A.14. Similarly to corneal epithelium, also the cells in the skin epidermis can be visualized due to their green autofluorescence, mainly stemming from the endogenous pigments contained in the cytoplasm. The membranes and nuclei result darker, apart from in the stratum corneum, which contains anucleated cells. Furthermore, skin cells are keratin-filled and the keratin amount increases from the deeper strata to the outermost one,

which explains why the fluorescence intensity of the stratum corneum is higher. The main endogenous fluorophores contributing to the epidermis autofluorescence are: keratin, melanin and flavins.[246] The dermis collagen becomes visible starting from $\sim 50 \mu\text{m}$ below the surface of the skin, thanks to the SHG signal detected within the blue channel. Initially, the collagen is organized in circular structures, corresponding to the ridge-like structures present in the “papillary region” between dermis and epidermis (known as the dermo-epidermal junction). More deep in the tissue, the collagen matrix becomes more homogeneous. Emission spectra acquired from the epidermis at two different excitation wavelengths (890 and 1070 nm) have been reported in Figure 4.15.

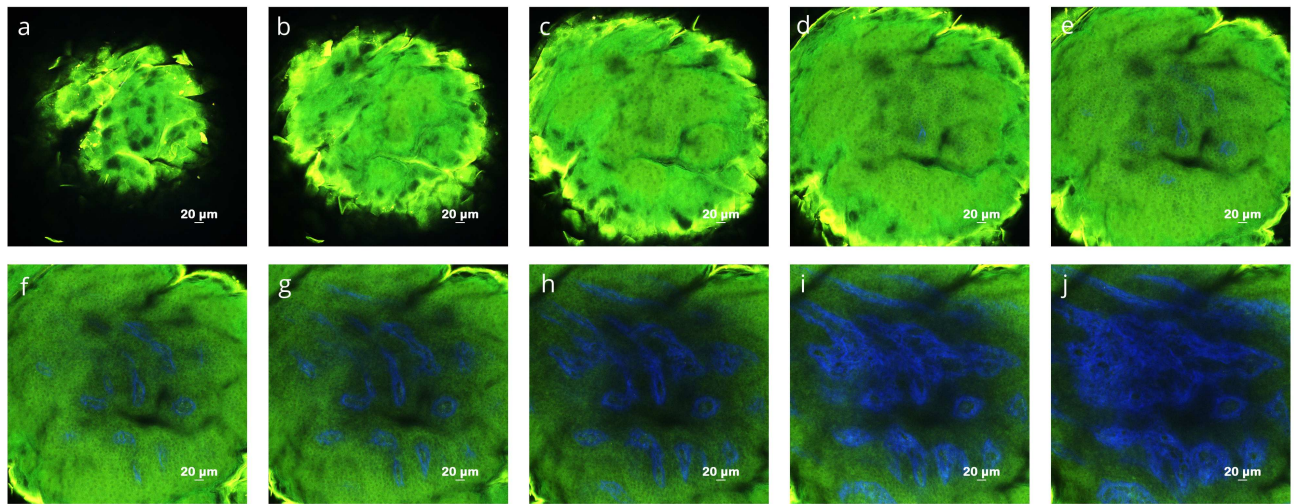


Figure A.14: TPM images of blank porcine skin acquired at 850 nm. Starting from the surface, each image was acquired $10 \mu\text{m}$ deeper than the previous one, increasing depth from panel(a) to panel(j).

In Figure A.15 are reported Z-scans acquired from two different regions of a porcine skin sample, a flat region and a hair follicle. In both cases, the epidermis and the superficial part of the dermis are clearly visible. A porcine hair can be observed stemming from the follicle. Similarly to the epithelial cells, the hair autofluorescence falls within the green channel and is mainly due to keratin and melanin.

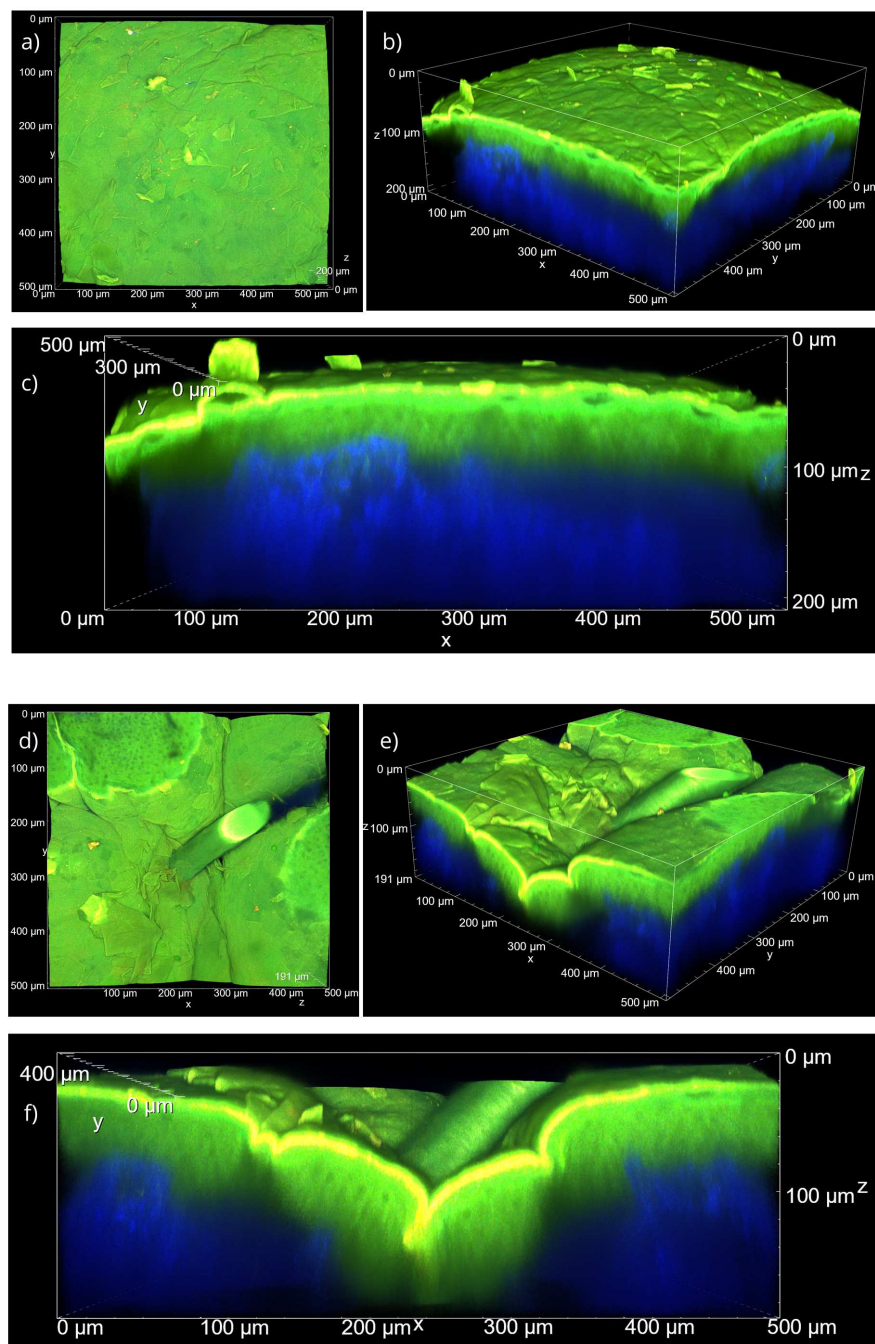


Figure A.15: Volume renderings of porcine skin samples reconstructed from the Z-stacks (Z-step: 1 μm) acquired with excitation wavelength of 850 nm. A flat skin region (total depth: 209 μm) can be observed in panels(a-c), with XY view (a) 3D overview (b) and XZ view (c). A hair follicle (total depth: 191 μm) can be observed in panels(d-f): XY view (d) 3D overview (e) and XZ view (f). The blue signal is due to the SHG of the collagen present in the dermis. The green signal is due to stratum corneum and epidermis autofluorescence.

TPA spectroscopic measurements

B

In addition to the acquisition of images and emission spectra from tissues and NPs suspensions, the two-photon microscope has been also employed to carry out nonlinear spectroscopic analysis, measuring TPA spectra and, when possible, the TPA cross sections by collecting the corresponding TPEF signal at different excitation wavelengths.[76, 247] Part of these measurements have been carried out in the context of Ilaria Ferraboschi and Francesco Bertocchi master theses. The TPA cross section (σ_2), as introduced in Appendix A, corresponds to the intrinsic ability of a molecule to undergo two-photon absorption, and it is typically measured in Goeppert-Mayer (GM) units ($1 \text{ GM} = 10^{-50} \cdot \text{cm}^4 \cdot \text{s} \cdot \text{photon}^{-1}$).

In order to use the two-photon microscope for spectroscopic purposes, a correction curve for excitation spectra, accounting for the different efficiency of the excitation system (mainly laser and microscope optics) as a function of the wavelength was needed to retrieve reliable TPA bandshapes. This excitation correction has been obtained by using 4 reference fluorophores (whose absolute two-photon absorption cross sections were available in the literature[234, 248, 249]), absorbing in the whole spectral range covered by the tunable laser (700-1300 nm):

- ▶ Fluorescein in NaOH_{aq} 0.1 M;
- ▶ Styryl-9M in chloroform;
- ▶ C153 in toluene;
- ▶ C153 in DMSO.

The excitation correction curve has been obtained as the ratio between the reference spectra available in the literature and the "raw" ones obtained with our setup.

The sensitivity of the spectral detector as a function of the emission wavelength has also been taken into account, calibrating the instrument with a commercial kit. The validity of the spectral detector correction curve was tested by comparing the corrected TPEF spectra of a few fluorophores (acquired with the microscope) with the corrected emission spectra obtained with a reliably corrected Edinburgh FLS1000 fluorometer.

Liquid samples (solutions and suspensions) were analyzed in quartz cuvettes placed horizontally under the microscope objective. Each cuvette was completely filled with the liquid sample to avoid the presence of air between the upper cuvette wall and the solution (as shown in Figure B.1). Distilled water was employed to ensure the contact between the objective and the cuvette. Each measurement has been conducted focusing the excitation beam as near as possible to the cuvette upper wall, to avoid artifacts due to the refractive index of the solvent and inner-filter effects.

B.1 TPA cross section estimation

The two-photon absorption (TPA) cross section σ_2 , which expresses the probability of the TPA process, can be measured using a relative method. Fluorescein in NaOH 0.1 M was used as a reference ($\Phi_{f,\text{ref}} =$

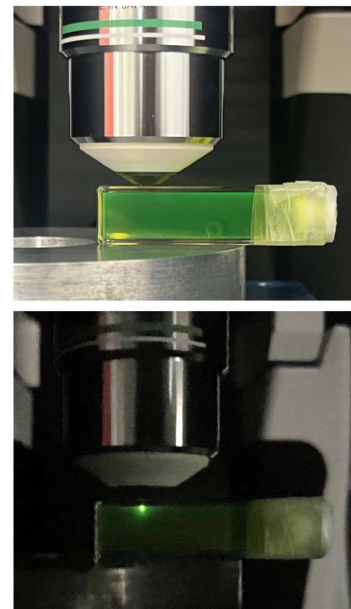


Figure B.1: Pictures of the experimental setup employed for nonlinear spectroscopic measurements on liquid samples with the two-photon microscope. In the figure, a cuvette, completely filled with a solution of fluorescein in NaOH_{aq} 0.1 M, is placed horizontally under the objective. When the laser excitation is turned on (bottom panel), a luminous spot is observed, and corresponds to light stemming from the "voxel" where the TPEF process occurs.

0.9), considering the absolute values of cross section reported in the literature.[234] The following equation was used to estimate the sample's cross section as a function of the incoming wavelength λ :[250]

$$\sigma_2(\lambda) = \sigma_{2,\text{ref}}(\lambda) \frac{\Phi_{f,\text{ref}}}{\Phi_f} \frac{C_{\text{ref}}}{C} \frac{P_{\text{ref}}^2(\lambda)}{P^2(\lambda)} \frac{F(\lambda)}{F_{\text{ref}}(\lambda)} \frac{\eta_{\text{ref}}}{\eta} \quad (\text{B.1})$$

where Φ_f is the fluorescence quantum yield, C is the solution concentration, F is the integrated TPEF spectrum, and P is the laser power and η the refractive index of the solvent. The subscript "ref" indicates the properties of the reference, while all the others terms refer to the sample. The reliable comparison of the integrated TPEF spectra of sample (F) and reference (F_{ref}) requires to collect the major part of the TPEF spectra and correct them for the wavelength-dependent sensitivity of the employed detector. This is possible only for the spectral detector, so only for samples whose emission band mainly falls inside its sensitivity region (400-650 nm). Unfortunately, the Non-Descanned detectors (NDDs, described in Section T.4) do not allow to correct the TPEF intensity (the signal collected by the NDDs is relevant to the whole bandwidth of the corresponding bandpass filter and no sensitivity curve is available).

The procedure for determining the TPA cross section has been validated by measuring the σ_2 values for a solution of C153 in toluene and comparing the results with those published in Ref. [234]. The σ_2 has been measured at 4 different excitation wavelengths (using fluorescein in NaOH 0.1 M as reference) reported in Table B.1.

Table B.1: Cross section values of C153 in toluene measured at 4 different wavelengths.

Wavelength	Our σ^2	Rebane's σ^2	% Error
920	0.725316	0.76052	4.6%
870	11.17585	11.66169	4.2%
810	17.05083	16.83443	1.3%
780	13.00155	12.78087	1.7%

The percentage errors with respect to the literature data are much lower than 20%, that is the experimental error usually considered in the determination of the cross section.

B.2 Examples of TPA spectroscopic measurements

In order to record the TPA spectra of DiI and DiD in ethanol, discussed in Section 2.5.2, the TPEF signal was measured with NDDs and/or with the spectral detector, according to the excitation and fluorescence spectral ranges of the sample. More specifically, in the case of DiI and DiD, for excitation between 700 and 1080 nm, the TPEF is detected using the spectral detector (for DiD only a tiny portion of the emission spectrum falls inside the 400-650 region, so that a TPEF signal could be measured with the spectral detector but a scale factor accounting for the missing part of the emission spectrum, needed to estimate the cross section, could

not be reliably guessed). For excitation above 820 nm, TPEF is detected using the green (for DiI) and the red (for DiD) NDDs. The overlapping excitation region (820- 1080 nm) was used to merge the two parts of the excitation spectra acquired with different detectors. For the explained reasons, the TPA cross section could not be quantified for DiD, whose fluorescence (or most of it) falls outside the region covered by the spectral detector: for this compound, only the bandshape of the corresponding TPA spectrum is available (reported in Figure 2.34). TPEF of DiI, instead, could be (almost entirely) measured with the spectral detector, so that the TPA cross section could be retrieved for this sample (reported in Figure B.2). As TPA involves the simultaneous absorption of two photons, its probability should be quadratic with the excitation power. For TPA spectra collected in solution, deviations from quadraticity resulted below $\pm 15\%$ repeating the measurements with three different laser powers.

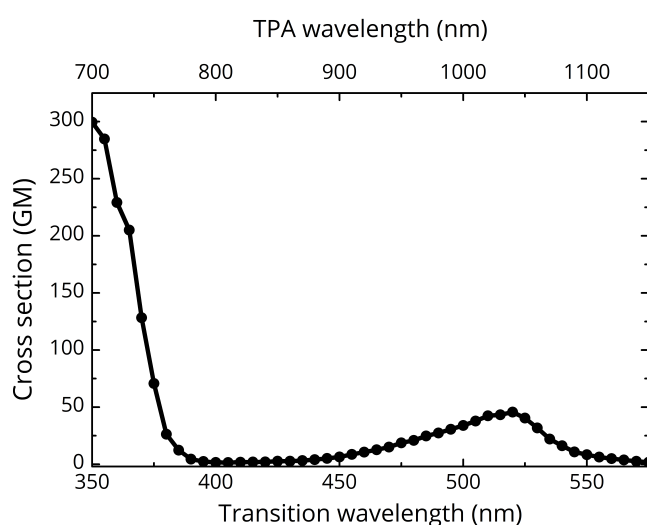


Figure B.2: TPA cross section of DiI in ethanol as a function of wavelength, expressed in Goeppert-Mayer (GM) units.

Apart from the TPA cross section of DiI and the TPA bandshape of DiD, other systems have been studied in the context of this thesis:

- Bandshape of DiR in ethanol, measured with the far-red NDD (the only suitable detector for collecting its TPEF signal) exciting the sample in the 900-1300 nm spectral region;[76]
- Bandshape of DiI aggregates in liquid suspension and in agarose hydrogel;[76]
- Cross section of two molecules belonging to the class of phenyleneethynylenes.[247]

Bibliography

- [1] Nikzamir, M.; Akbarzadeh, A.; Panahi, Y. 'An overview on nanoparticles used in biomedicine and their cytotoxicity', *Journal of Drug Delivery Science and Technology*, 61, 102316, **2021** (cited on page 1).
- [2] He, Z.; Zhang, Z.; Bi, S. 'Nanoparticles for organic electronics applications', *Materials Research Express*, 7 (1), 012004, **2020** (cited on page 1).
- [3] Mohajerani, A.; Burnett, L.; Smith, J. V.; Kurmus, H.; Milas, J.; Arulrajah, A.; Horpibulsuk, S.; Abdul Kadir, A. 'Nanoparticles in construction materials and other applications, and implications of nanoparticle use', *Materials*, 12 (19), 3052, **2019** (cited on page 1).
- [4] Astruc, D. Introduction: nanoparticles in catalysis, 2020 (cited on page 1).
- [5] Khodabandehloo, H.; Zahednasab, H.; Hafez, A. A. 'Nanocarriers usage for drug delivery in cancer therapy', *Iranian journal of cancer prevention*, 9 (2), **2016** (cited on page 1).
- [6] Nune, S. K.; Gunda, P.; Thallapally, P. K.; Lin, Y.-Y.; Laird Forrest, M.; Berkland, C. J. 'Nanoparticles for biomedical imaging', *Expert opinion on drug delivery*, 6 (11), 1175–1194, **2009** (cited on page 1).
- [7] Baetke, S. C.; Lammers, T.; Kiessling, F. 'Applications of nanoparticles for diagnosis and therapy of cancer', *The British journal of radiology*, 88 (1054), 20150207, **2015** (cited on page 1).
- [8] Xie, J.; Lee, S.; Chen, X. 'Nanoparticle-based theranostic agents', *Advanced drug delivery reviews*, 62 (11), 1064–1079, **2010** (cited on page 1).
- [9] Ghezzi, M.; Pescina, S.; Padula, C.; Santi, P.; Del Favero, E.; Cantù, L.; Nicoli, S. 'Polymeric micelles in drug delivery: An insight of the techniques for their characterization and assessment in biorelevant conditions', *Journal of Controlled Release*, 332, 312–336, **2021** (cited on page 1).
- [10] Vargas-Nadal, G.; Köber, M.; Nsamela, A.; Terenziani, F.; Sissa, C.; Pescina, S.; Sonvico, F.; Gazzali, A. M.; Wahab, H. A.; Grisanti, L., et al. 'Fluorescent Multifunctional Organic Nanoparticles for Drug Delivery and Bioimaging: A Tutorial Review', *Pharmaceutics*, 14 (11), 2498, **2022** (cited on pages 1, 5, 73, 78).
- [11] Cheng, M. H.; Harmatys, K. M.; Charron, D. M.; Chen, J.; Zheng, G. 'Stable J-aggregation of an aza-BODIPY-lipid in a liposome for optical cancer imaging', *Angewandte Chemie*, 131 (38), 13528–13533, **2019** (cited on page 1).
- [12] Asahi, T.; Sugiyama, T.; Masuhara, H. 'Laser fabrication and spectroscopy of organic nanoparticles', *Accounts of chemical research*, 41 (12), 1790–1798, **2008** (cited on page 1).
- [13] Agranovich, V. M., *Excitations in organic solids*; OUP Oxford: 2009; Vol. 142 (cited on page 2).
- [14] Ma, S.; Du, S.; Pan, G.; Dai, S.; Xu, B.; Tian, W. 'Organic molecular aggregates: From aggregation structure to emission property', *Aggregate*, 2 (4), e96, **2021** (cited on page 2).
- [15] Diniz, A. M.; Cruz, R. P.; Avó, J. 'Probing the Local Polarity in Biocompatible Nanocarriers with Solvatofluorochromism of a 4-Carbazole-1, 8-naphthalimide Dye', *Photochem*, 2 (3), 489–502, **2022** (cited on page 2).
- [16] Kobayashi, H.; Ogawa, M.; Alford, R.; Choyke, P. L.; Urano, Y. 'New strategies for fluorescent probe design in medical diagnostic imaging', *Chemical reviews*, 110 (5), 2620–2640, **2010** (cited on page 5).
- [17] Tian, X.; Murfin, L. C.; Wu, L.; Lewis, S. E.; James, T. D. 'Fluorescent small organic probes for biosensing', *Chemical Science*, 12 (10), 3406–3426, **2021** (cited on page 5).
- [18] De Silva, A. P.; Gunaratne, H. N.; Gunnlaugsson, T.; Huxley, A. J.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. 'Signaling recognition events with fluorescent sensors and switches', *Chemical reviews*, 97 (5), 1515–1566, **1997** (cited on page 5).
- [19] Hong, Y.; Lam, J. W.; Tang, B. Z. 'Aggregation-induced emission', *Chemical Society Reviews*, 40 (11), 5361–5388, **2011** (cited on page 5).

- [20] Van Der Velde, J. H.; Oelerich, J.; Huang, J.; Smit, J. H.; Aminian Jazi, A.; Galiani, S.; Kolmakov, K.; Gouridis, G.; Eggeling, C.; Herrmann, A., et al. 'A simple and versatile design concept for fluorophore derivatives with intramolecular photostabilization', *Nature communications*, 7 (1), 10144, **2016** (cited on page 5).
- [21] Luty, G. A. 'The acute intravenous toxicity of biological stains, dyes, and other fluorescent substances', *Toxicology and applied pharmacology*, 44 (2), 225–249, **1978** (cited on page 5).
- [22] Robin, M. P.; O'Reilly, R. K. 'Strategies for preparing fluorescently labelled polymer nanoparticles', *Polymer International*, 64 (2), 174–182, **2015** (cited on page 5).
- [23] Bou, S.; Klymchenko, A. S.; Collot, M. 'Fluorescent labeling of biocompatible block copolymers: Synthetic strategies and applications in bioimaging', *Materials advances*, 2 (10), 3213–3233, **2021** (cited on page 5).
- [24] Banik, B. L.; Fattahi, P.; Brown, J. L. 'Polymeric nanoparticles: the future of nanomedicine', *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, 8 (2), 271–299, **2016** (cited on page 5).
- [25] Kulkarni, S. A.; Feng, S.-S. 'Effects of particle size and surface modification on cellular uptake and biodistribution of polymeric nanoparticles for drug delivery', *Pharmaceutical research*, 30, 2512–2522, **2013** (cited on page 5).
- [26] Elsabahy, M.; Wooley, K. L. 'Design of polymeric nanoparticles for biomedical delivery applications', *Chemical Society Reviews*, 41 (7), 2545–2561, **2012** (cited on page 5).
- [27] Lyon, C. K.; Prasher, A.; Hanlon, A. M.; Tuten, B. T.; Tooley, C. A.; Frank, P. G.; Berda, E. B. 'A brief user's guide to single-chain nanoparticles', *Polymer Chemistry*, 6 (2), 181–197, **2015** (cited on page 6).
- [28] Pomposo, J. A., *Single-Chain Polymer Nanoparticles: Synthesis, Characterization, Simulations, and Applications*; John Wiley & Sons: 2017 (cited on page 6).
- [29] Morishima, Y.; Nomura, S.; Ikeda, T.; Seki, M.; Kamachi, M. 'Characterization of unimolecular micelles of random copolymers of sodium 2-(acrylamido)-2-methylpropanesulfonate and methacrylamides bearing bulky hydrophobic substituents', *Macromolecules*, 28 (8), 2874–2881, **1995** (cited on page 6).
- [30] Terashima, T.; Sugita, T.; Fukae, K.; Sawamoto, M. 'Synthesis and single-chain folding of amphiphilic random copolymers in water', *Macromolecules*, 47 (2), 589–600, **2014** (cited on page 6).
- [31] Domenici, F.; Guazzelli, E.; Masotti, E.; Mahmoudi, N.; Gabrielli, S.; Telling, M. T.; Martinelli, E.; Galli, G.; Paradossi, G. 'Understanding the Temperature-Responsive Self-Assemblies of Amphiphilic Random Copolymers by SANS in D2O Solution', *Macromolecular Chemistry and Physics*, 222 (5), 2000447, **2021** (cited on pages 6–9).
- [32] Irshadeen, I. M.; Truong, V. X.; Frisch, H.; Barner-Kowollik, C. 'Red light induced folding of single polymer chains', *Chemical Communications*, 58 (93), 12975–12978, **2022** (cited on page 6).
- [33] Pelosi, C.; Guazzelli, E.; Calosi, M.; Bernazzani, L.; Tiné, M. R.; Duce, C.; Martinelli, E. 'Investigation of the LCST-thermoresponse behavior of novel oligo (ethylene glycol)-modified pentafluorostyrene homopolymers', *Applied Sciences*, 11 (6), 2711, **2021** (cited on page 6).
- [34] Ko, J. H.; Bhattacharya, A.; Terashima, T.; Sawamoto, M.; Maynard, H. D. 'Amphiphilic fluororous random copolymer self-assembly for encapsulation of a fluorinated agrochemical', *Journal of Polymer Science Part A: Polymer Chemistry*, 57 (3), 352–359, **2019** (cited on pages 6, 9).
- [35] Bartolini, A.; Tempesti, P.; Resta, C.; Berti, D.; Smets, J.; Aouad, Y. G.; Baglioni, P. 'Poly (ethylene glycol)-graft-poly (vinyl acetate) single-chain nanoparticles for the encapsulation of small molecules', *Physical Chemistry Chemical Physics*, 19 (6), 4553–4559, **2017** (cited on page 6).
- [36] Liu, S.; Rong, J.; Liu, R.; Lindsey, J. S. 'Single-fluorophore single-chain nanoparticle undergoes fluorophore-driven assembly with fluorescence features retained in physiological milieu', *ACS Applied Polymer Materials*, 3 (4), 1767–1776, **2021** (cited on page 6).
- [37] Pomposo, J. A.; Perez-Baena, I.; Lo Verso, F.; Moreno, A. J.; Arbe, A.; Colmenero, J. How far are single-chain polymer nanoparticles in solution from the globular state?, 2014 (cited on page 6).

- [38] Lutz, J.-F. 'Polymerization of oligo (ethylene glycol)(meth) acrylates: Toward new generations of smart biocompatible materials', *Journal of Polymer Science Part A: Polymer Chemistry*, 46 (11), 3459–3470, **2008** (cited on page 6).
- [39] Ghosh, C.; Nandi, S.; Bhattacharyya, K. 'Probing micro-environment of lipid droplets in a live breast cell: MCF7 and MCF10A', *Chemical Physics Letters*, 670, 27–31, **2017** (cited on page 7).
- [40] 'Temperature Effect on the Fluorescence Anisotropy Decay Dynamics of Coumarin-153 Dye in Triton-X-100 and Brij-35 Miscellar Solutions' (cited on page 7).
- [41] De-La-Cuesta, J.; González, E.; Pomposo, J. A. 'Advances in fluorescent single-chain nanoparticles', *Molecules*, 22 (11), 1819, **2017** (cited on page 7).
- [42] Maiti, N. C.; Krishna, M. M.; Britto, P. J.; Periasamy, N. 'Fluorescence dynamics of dye probes in micelles', *The Journal of Physical Chemistry B*, 101 (51), 11051–11060, **1997** (cited on pages 7, 12, 13, 104).
- [43] Koley, S.; Ghosh, S. 'Encapsulation and residency of a hydrophobic dye within the water-filled interior of a PAMAM dendrimer molecule', *The Journal of Physical Chemistry B*, 121 (8), 1930–1940, **2017** (cited on pages 7, 10).
- [44] Singh Malik, D.; Mital, N.; Kaur, G. 'Topical drug delivery systems: a patent review', *Expert opinion on therapeutic patents*, 26 (2), 213–228, **2016** (cited on page 7).
- [45] Guazzelli, E.; Martinelli, E.; Galli, G.; Cupellini, L.; Jurinovich, S.; Mennucci, B. 'Single-chain self-folding in an amphiphilic copolymer: An integrated experimental and computational study', *Polymer*, 161, 33–40, **2019** (cited on pages 8, 9, 15).
- [46] Martinelli, E.; Guazzelli, E.; Galli, G.; Telling, M. T.; Poggetto, G. D.; Immirzi, B.; Domenici, F.; Paradossi, G. 'Prolate and Temperature-Responsive Self-Assemblies of Amphiphilic Random Copolymers with Perfluoroalkyl and Polyoxyethylene Side Chains in Solution', *Macromolecular Chemistry and Physics*, 219 (15), 1800210, **2018** (cited on pages 8, 9).
- [47] Guazzelli, E.; Masotti, E.; Biver, T.; Pucci, A.; Martinelli, E.; Galli, G. 'The self-assembly over nano-to submicro-length scales in water of a fluorescent julolidine-labeled amphiphilic random terpolymer', *Journal of Polymer Science Part A: Polymer Chemistry*, 56 (7), 797–804, **2018** (cited on page 8).
- [48] Guazzelli, E.; Masotti, E.; Kriechbaum, M.; Uhlig, F.; Galli, G.; Martinelli, E. 'Thermoresponsive Reversible Unimer Micelles of Amphiphilic Fluorinated Copolymers', *Macromolecular Chemistry and Physics*, 224 (3), 2200360, **2023** (cited on pages 8, 9, 13).
- [49] Calosi, M.; Guazzelli, E.; Braccini, S.; Lessi, M.; Bellina, F.; Galli, G.; Martinelli, E. 'Self-Assembled Amphiphilic Fluorinated Random Copolymers for the Encapsulation and Release of the Hydrophobic Combretastatin A-4 Drug', *Polymers*, 14 (4), 774, **2022** (cited on pages 8, 9, 19).
- [50] Jones, G.; Jackson, W. R.; Choi, C. Y.; Bergmark, W. R. 'Solvent effects on emission yield and lifetime for coumarin laser dyes. Requirements for a rotatory decay mechanism', *The Journal of Physical Chemistry*, 89 (2), 294–300, **1985** (cited on pages 10, 11).
- [51] Lewis, J.; Maroncelli, M. 'On the (uninteresting) dependence of the absorption and emission transition moments of coumarin 153 on solvent', *Chemical Physics Letters*, 282 (2), 197–203, **1998** (cited on page 10).
- [52] Lakowicz, J. R., *Principles of fluorescence spectroscopy*; Springer: 2006 (cited on pages 11, 12, 23, 32, 35).
- [53] Horng, M.-L.; Gardecki, J.; Maroncelli, M. 'Rotational dynamics of coumarin 153: Time-dependent friction, dielectric friction, and other nonhydrodynamic effects', *The Journal of Physical Chemistry A*, 101 (6), 1030–1047, **1997** (cited on page 11).
- [54] Birch, D. J. S.; Yip, P. In *Fluorescence Spectroscopy and Microscopy: Methods and Protocols*, Engelborghs, Y., Visser, A. J., Eds.; Humana Press: 2014 (cited on page 12).
- [55] Prazeres, T. J.; Beija, M.; Fernandes, F. V.; Marcelino, P. G.; Farinha, J. P. S.; Martinho, J. 'Determination of the critical micelle concentration of surfactants and amphiphilic block copolymers using coumarin 153', *Inorganica Chimica Acta*, 381, 181–187, **2012** (cited on page 14).
- [56] McCarthy, P.; Blanchard, G. 'AM1 study of the electronic structure of coumarins', *The Journal of Physical Chemistry*, 97 (47), 12205–12209, **1993** (cited on page 14).

- [57] Rajulu, A. V.; Sab, P. M. 'Refractometric studies in water/polyethylene glycol-300 mixtures', *European polymer journal*, 34 (1), 31–32, **1998** (cited on page 14).
- [58] Helmchen, F.; Denk, W. 'Deep tissue two-photon microscopy', *Nature methods*, 2 (12), 932–940, **2005** (cited on pages 15, 102).
- [59] Nicoli, S.; Ferrari, G.; Quarta, M.; Macaluso, C.; Govoni, P.; Dallatana, D.; Santi, P. 'Porcine sclera as a model of human sclera for in vitro transport experiments: histology, SEM, and comparative permeability', *Molecular vision*, 15, 259, **2009** (cited on pages 15, 118).
- [60] Ghezzi, M.; Ferraboschi, I.; Delledonne, A.; Pescina, S.; Padula, C.; Santi, P.; Sissa, C.; Terenziani, F.; Nicoli, S. 'Cyclosporine-loaded micelles for ocular delivery: Investigating the penetration mechanisms', *Journal of Controlled Release*, 349, 744–755, **2022** (cited on pages 16, 102, 105, 124).
- [61] Koda, Y.; Terashima, T.; Sawamoto, M.; Maynard, H. D. 'Amphiphilic/fluorous random copolymers as a new class of non-cytotoxic polymeric materials for protein conjugation', *Polymer Chemistry*, 6 (2), 240–247, **2015** (cited on page 19).
- [62] Delledonne, A.; Guazzelli, E.; Pescina, S.; Bianchera, A.; Galli, G.; Martinelli, E.; Sissa, C. 'Amphiphilic Fluorinated Unimer Micelles as Nanocarriers of Fluorescent Probes for Bioimaging', *ACS Applied Nano Materials*, 6 (17), 15551–15562, **2023** (cited on pages 20, 41).
- [63] Fassiolli, F.; Dinshaw, R.; Arpin, P. C.; Scholes, G. D. 'Photosynthetic light harvesting: excitons and coherence', *Journal of The Royal Society Interface*, 11 (92), 20130901, **2014** (cited on page 23).
- [64] Wu, E. C.; Arsenault, E. A.; Bhattacharyya, P.; Lewis, N. H.; Fleming, G. R. 'Two-dimensional electronic vibrational spectroscopy and ultrafast excitonic and vibronic photosynthetic energy transfer', *Faraday discussions*, 216, 116–132, **2019** (cited on page 23).
- [65] Stryer, L. 'Fluorescence energy transfer as a spectroscopic ruler', *Annual review of biochemistry*, 47 (1), 819–846, **1978** (cited on page 23).
- [66] Sun, X.; Wang, G.; Zhang, H.; Hu, S.; Liu, X.; Tang, J.; Shen, Y. 'The blood clearance kinetics and pathway of polymeric micelles in cancer drug delivery', *ACS nano*, 12 (6), 6179–6192, **2018** (cited on page 23).
- [67] Morla-Folch, J.; Vargas-Nadal, G.; Zhao, T.; Sissa, C.; Ardizzone, A.; Kurhuzenkau, S.; Köber, M.; Uddin, M.; Painelli, A.; Veciana, J., et al. 'Dye-loaded quatsomes exhibiting FRET as nanoprobe for bioimaging', *ACS applied materials & interfaces*, 12 (18), 20253–20262, **2020** (cited on pages 23, 30, 32, 35).
- [68] Efimova, S.; Sorokin, A.; Lebedenko, A.; Malyukin, Y. V.; Obukhova, E. 'Nonradiative energy transfer in carbocyanine dye compositions inside surfactant micelles', *Journal of Applied Spectroscopy*, 73, 164–170, **2006** (cited on page 23).
- [69] Zou, P.; Chen, H.; Paholak, H. J.; Sun, D. 'Noninvasive fluorescence resonance energy transfer imaging of in vivo premature drug release from polymeric nanoparticles', *Molecular pharmaceutics*, 10 (11), 4185–4194, **2013** (cited on page 23).
- [70] Iqbal, A.; Arslan, S.; Okumus, B.; Wilson, T. J.; Giraud, G.; Norman, D. G.; Ha, T.; Lilley, D. M. 'Orientation dependence in fluorescent energy transfer between Cy3 and Cy5 terminally attached to double-stranded nucleic acids', *Proceedings of the National Academy of Sciences*, 105 (32), 11176–11181, **2008** (cited on page 23).
- [71] Yang, L.; Chen, Q.; Wang, Z.; Zhang, H.; Sun, H. 'Small-molecule fluorescent probes for plasma membrane staining: Design, mechanisms and biological applications', *Coordination Chemistry Reviews*, 474, 214862, **2023** (cited on page 24).
- [72] Rosen, M. J.; Kunjappu, J. T., *Surfactants and interfacial phenomena*; John Wiley & Sons: 2012 (cited on page 24).
- [73] Yang, C.; Wu, T.; Qi, Y.; Zhang, Z. 'Recent advances in the application of vitamin E TPGS for drug delivery', *Theranostics*, 8 (2), 464, **2018** (cited on page 24).

- [74] Luiz, M. T.; Di Filippo, L. D.; Alves, R. C.; Araújo, V. H. S.; Duarte, J. L.; Marchetti, J. M.; Chorilli, M. 'The use of TPGS in drug delivery systems to overcome biological barriers', *European Polymer Journal*, 142, 110129, **2021** (cited on pages 24, 104).
- [75] Porter, M. R., *Handbook of surfactants*; Springer: 2013 (cited on page 24).
- [76] Bertocchi, F.; Delledonne, A.; Vargas-Nadal, G.; Terenziani, F.; Painelli, A.; Sissa, C. 'Aggregates of Cyanine Dyes: When Molecular Vibrations and Electrostatic Screening Make the Difference', *The Journal of Physical Chemistry C*, **2023** (cited on pages 24, 27, 48, 147, 149).
- [77] Cravcenco, A.; Yu, Y.; Edhborg, F.; Goebel, J. F.; Takacs, Z.; Yang, Y.; Albinsson, B.; Borjesson, K. 'Exciton delocalization counteracts the energy gap: a new pathway toward NIR-emissive dyes', *Journal of the American Chemical Society*, 143 (45), 19232–19239, **2021** (cited on page 26).
- [78] Spano, F. C. 'The spectral signatures of Frenkel polarons in H-and J-aggregates', *Accounts of chemical research*, 43 (3), 429–439, **2010** (cited on page 26).
- [79] Hestand, N. J.; Spano, F. C. 'Expanded theory of H-and J-molecular aggregates: the effects of vibronic coupling and intermolecular charge transfer', *Chemical reviews*, 118 (15), 7069–7163, **2018** (cited on page 26).
- [80] V. Berlepsch, H.; Böttcher, C. 'H-aggregates of an indocyanine Cy5 dye: transition from strong to weak molecular coupling', *The Journal of Physical Chemistry B*, 119 (35), 11900–11909, **2015** (cited on pages 26, 30).
- [81] Debnath, P.; Chakraborty, S.; Deb, S.; Nath, J.; Dey, B.; Bhattacharjee, D.; Hussain, S. A. 'Stability of J-aggregated species in an indocarbocyanine dye in Langmuir–Blodgett Films', *Journal of Luminescence*, 179, 287–296, **2016** (cited on page 26).
- [82] Anzola, M.; Di Maiolo, F.; Painelli, A. 'Optical spectra of molecular aggregates and crystals: testing approximation schemes', *Physical Chemistry Chemical Physics*, 21 (36), 19816–19824, **2019** (cited on page 27).
- [83] Moulik, S. P.; Haque, M. E.; Jana, P. K.; Das, A. R. 'Micellar properties of cationic surfactants in pure and mixed states', *The Journal of physical chemistry*, 100 (2), 701–708, **1996** (cited on page 28).
- [84] Bahri, M. A.; Hoebeke, M.; Grammenos, A.; Delanaye, L.; Vandewalle, N.; Seret, A. 'Investigation of SDS, DTAB and CTAB micelle microviscosities by electron spin resonance', *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 290 (1-3), 206–212, **2006** (cited on page 28).
- [85] Sheu, M.-T.; Chen, S.-Y.; Chen, L.-C.; Ho, H.-O. 'Influence of micelle solubilization by tocopheryl polyethylene glycol succinate (TPGS) on solubility enhancement and percutaneous penetration of estradiol', *Journal of controlled release*, 88 (3), 355–368, **2003** (cited on page 28).
- [86] Hasanuddin, H. A. B.; MMR, M. M. A.; Salama, M.; Tripathy, M. 'Micellization of D- α -tocopheryl polyethylene Glycol 1000 succinate (TPGS 1000): thermodynamics and related solute solvent interactions', *Orient J Chem*, 30 (3), 1119–1123, **2014** (cited on page 28).
- [87] Yu, L.; Bridgers, A.; Polli, J.; Vickers, A.; Long, S.; Roy, A.; Winnike, R.; Coffin, M. 'Vitamin E-TPGS increases absorption flux of an HIV protease inhibitor by enhancing its solubility and permeability¹', *Pharmaceutical research*, 16, 1812–1817, **1999** (cited on page 28).
- [88] Jain, S.; Pandey, S.; Sola, P.; Pathan, H.; Patil, R.; Ray, D.; Aswal, V. K.; Bahadur, P.; Tiwari, S. 'Solubilization of carbamazepine in TPGS micelles: Effect of temperature and electrolyte addition', *Aaps Pharmscitech*, 20, 1–8, **2019** (cited on page 28).
- [89] Sadoqi, M.; Lau-Cam, C.; Wu, S. 'Investigation of the micellar properties of the tocopheryl polyethylene glycol succinate surfactants TPGS 400 and TPGS 1000 by steady state fluorometry', *Journal of colloid and interface science*, 333 (2), 585–589, **2009** (cited on page 28).
- [90] Puig-Rigall, J.; Grillo, I.; Dreiss, C. A.; González-Gaitano, G. 'Structural and spectroscopic characterization of TPGS micelles: disruptive role of cyclodextrins and kinetic pathways', *Langmuir*, 33 (19), 4737–4747, **2017** (cited on pages 28, 37).

- [91] Cieřla, J.; Koczańska, M.; Narkiewicz-Michalek, J.; Szymula, M.; Bieganski, A. 'The physicochemical properties of CTAB solutions in the presence of α -tocopherol', *Journal of Molecular Liquids*, 222, 463–470, **2016** (cited on page 29).
- [92] Zuccari, G.; Alfei, S.; Zorzoli, A.; Marimpietri, D.; Turrini, F.; Baldassari, S.; Marchitto, L.; Caviglioli, G. 'Increased water-solubility and maintained antioxidant power of resveratrol by its encapsulation in vitamin E TPGS micelles: A potential nutritional supplement for chronic liver disease', *Pharmaceutics*, 13 (8), 1128, **2021** (cited on page 29).
- [93] Kawabe, Y.; Kato, S. 'Spectroscopic study of cyanine dyes interacting with the biopolymer, DNA', *Dyes and Pigments*, 95 (3), 614–618, **2012** (cited on page 30).
- [94] Ardizzone, A.; Kurhuzenkau, S.; Illa-Tuset, S.; Faraudo, J.; Bondar, M.; Hagan, D.; Van Stryland, E. W.; Painelli, A.; Sissa, C.; Feiner, N., et al. 'Nanostructuring lipophilic dyes in water using stable vesicles, quatsomes, as scaffolds and their use as probes for bioimaging', *Small*, 14 (16), 1703851, **2018** (cited on pages 30, 54, 55, 82).
- [95] Morton, S. W.; Zhao, X.; Quadir, M. A.; Hammond, P. T. 'FRET-enabled biological characterization of polymeric micelles', *Biomaterials*, 35 (11), 3489–3496, **2014** (cited on page 35).
- [96] Cookey, G. A.; Obunwo, C. C. 'Effects of sodium bromide salt and temperature on the behaviour of aqueous solution of cetyltrimethylammonium bromide', *IOSR J. Appl. Chem*, 7, 34–38, **2014** (cited on page 37).
- [97] Illa-Tuset, S.; Malaspina, D. C.; Faraudo, J. 'Coarse-grained molecular dynamics simulation of the interface behaviour and self-assembly of CTAB cationic surfactants', *Physical Chemistry Chemical Physics*, 20 (41), 26422–26430, **2018** (cited on page 37).
- [98] Delledonne, A.; Morla-Folch, J.; Anzola, M.; Bertocchi, F.; Vargas-Nadal, G.; Köber, M.; Sissa, C.; Ventosa, N.; Painelli, A. 'Increasing resonance energy transfer upon dilution: a counterintuitive observation in CTAB micelles', *Journal of Materials Chemistry C*, 9 (33), 10952–10964, **2021** (cited on pages 37, 48, 57).
- [99] HOLLYFIELD, J. G.; RAYBORN, M. E.; TAMMI, M.; TAMMI, R. 'Hyaluronan in the interphotoreceptor matrix of the eye: species differences in content, distribution, ligand binding and degradation', *Experimental eye research*, 66 (2), 241–248, **1998** (cited on pages 40, 110).
- [100] Watson, P. G.; Young, R. D. 'Scleral structure, organisation and disease. A review', *Experimental eye research*, 78 (3), 609–623, **2004** (cited on pages 40, 110).
- [101] Ghezzi, M.; Pescina, S.; Delledonne, A.; Ferraboschi, I.; Sissa, C.; Terenziani, F.; Remiro, P. D. F. R.; Santi, P.; Nicoli, S. 'Improvement of imiquimod solubilization and skin retention via TPGS micelles: exploiting the co-solubilizing effect of oleic acid', *Pharmaceutics*, 13 (9), 1476, **2021** (cited on pages 41, 110, 112, 124).
- [102] Apolinario, A. C.; Hauschke, L.; Nunes, J. R.; Lopes, L. B. 'Lipid nanovesicles for biomedical applications: 'What is in a name'?', *Progress in lipid research*, 82, 101096, **2021** (cited on page 53).
- [103] Muthu, M. S.; Feng, S.-S. 'Theranostic liposomes for cancer diagnosis and treatment: current development and pre-clinical success, 2013 (cited on page 53).
- [104] Chang, H.-I.; Yeh, M.-K. 'Clinical development of liposome-based drugs: formulation, characterization, and therapeutic efficacy', *International journal of nanomedicine*, 49–60, **2012** (cited on page 53).
- [105] Deissler, V.; Rüger, R.; Frank, W.; Fahr, A.; Kaiser, W. A.; Hilger, I. 'Fluorescent liposomes as contrast agents for in vivo optical imaging of edemas in mice', *Small*, 4 (8), 1240–1246, **2008** (cited on page 53).
- [106] Grimaldi, N.; Andrade, F.; Segovia, N.; Ferrer-Tasies, L.; Sala, S.; Veciana, J.; Ventosa, N. 'Lipid-based nanovesicles for nanomedicine', *Chemical society reviews*, 45 (23), 6520–6545, **2016** (cited on page 53).
- [107] Ferrer-Tasies, L.; Moreno-Calvo, E.; Cano-Sarabia, M.; Aguilera-Arzo, M.; Angelova, A.; Lesieur, S.; Ricart, S.; Faraudo, J.; Ventosa, N.; Veciana, J. 'Quatsomes: vesicles formed by self-assembly of sterols and quaternary ammonium surfactants', *Langmuir*, 29 (22), 6519–6528, **2013** (cited on pages 53, 54, 57, 72).

- [108] Liu, X.; Ardizzone, A.; Sui, B.; Anzola, M.; Ventosa, N.; Liu, T.; Veciana, J.; Belfield, K. D. 'Fluorenyl-loaded Quatsome nanostructured fluorescent probes', *ACS omega*, 2 (8), 4112–4122, **2017** (cited on pages 54, 55, 72, 73).
- [109] Cano-Sarabia, M.; Angelova, A.; Ventosa, N.; Lesieur, S.; Veciana, J. 'Cholesterol induced CTAB micelle-to-vesicle phase transitions', *Journal of colloid and interface science*, 350 (1), 10–15, **2010** (cited on page 54).
- [110] Bangham, A. D.; Horne, R. 'Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope', *Journal of molecular biology*, 8 (5), 660–IN10, **1964** (cited on page 54).
- [111] Torchilin, V. P. 'Recent advances with liposomes as pharmaceutical carriers', *Nature reviews Drug discovery*, 4 (2), 145–160, **2005** (cited on page 54).
- [112] Smirnov, A. 'Preparation of liposomes by reverse-phase evaporation and by freezing and thawing', *Bulletin of Experimental Biology and Medicine*, 98 (2), 1146–1149, **1984** (cited on page 54).
- [113] Weissig, V., *Liposomes*; Springer: 2010 (cited on page 54).
- [114] Woodbury, D. J.; Richardson, E. S.; Grigg, A. W.; Welling, R. D.; Knudson, B. H. 'Reducing liposome size with ultrasound: bimodal size distributions', *Journal of liposome research*, 16 (1), 57–80, **2006** (cited on page 54).
- [115] Hope, M.; Bally, M.; Webb, G.; Cullis, P. 'Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential', *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 812 (1), 55–65, **1985** (cited on page 54).
- [116] Cano-Sarabia, M.; Ventosa, N.; Sala, S.; Patino, C.; Arranz, R.; Veciana, J. 'Preparation of uniform rich cholesterol unilamellar nanovesicles using CO₂-expanded solvents', *Langmuir*, 24 (6), 2433–2437, **2008** (cited on pages 54, 57).
- [117] Elizondo, E.; Larsen, J.; Hatzakis, N. S.; Cabrera, I.; Bjørnholm, T.; Veciana, J.; Stamou, D.; Ventosa, N. 'Influence of the preparation route on the supramolecular organization of lipids in a vesicular system', *Journal of the American Chemical Society*, 134 (4), 1918–1921, **2012** (cited on pages 54, 57).
- [118] Gumí-Audenis, B.; Illa-Tuset, S.; Grimaldi, N.; Pasquina-Lemonche, L.; Ferrer-Tasies, L.; Sanz, F.; Veciana, J.; Ratera, I.; Faraudo, J.; Ventosa, N., et al. 'Insights into the structure and nanomechanics of a quatsome membrane by force spectroscopy measurements and molecular simulations', *Nanoscale*, 10 (48), 23001–23011, **2018** (cited on pages 54, 72).
- [119] Illa Tuset, S., *Molecular modelling of quatsome nanovesicles*, 2019 (cited on pages 54, 58).
- [120] Ardizzone, A.; Blasi, D.; Vona, D.; Rosspeintner, A.; Punzi, A.; Altamura, E.; Grimaldi, N.; Sala, S.; Vauthey, E.; Farinola, G. M., et al. 'Highly Stable and Red-Emitting Nanovesicles Incorporating Lipophilic Diketopyrrolopyrroles for Cell Imaging', *Chemistry–A European Journal*, 24 (44), 11386–11392, **2018** (cited on page 55).
- [121] Silbaugh, D. A.; Ferrer-Tasies, L.; Faraudo, J.; Veciana, J.; Ventosa, N.; Korgel, B. A. 'Highly fluorescent silicon nanocrystals stabilized in water using quatsomes', *Langmuir*, 33 (50), 14366–14377, **2017** (cited on page 55).
- [122] Ardizzone, A., *New fluorescent nanovesicles, by self-assembly of organic fluorophores, sterols and surfactants, as probes for bioimaging*, 2017 (cited on pages 55, 63–65, 82).
- [123] Cabrera Puig, I., *Nanovesicle-bioactive conjugates to be used as nanomedicines, prepared by a one-step scalable method using CO₂-expanded solvents*; Universitat Autònoma de Barcelona, 2014 (cited on page 55).
- [124] Cabrera, I.; Elizondo, E.; Esteban, O.; Corchero, J. L.; Melgarejo, M.; Pulido, D.; Cordoba, A.; Moreno, E.; Unzueta, U.; Vazquez, E., et al. 'Multifunctional nanovesicle-bioactive conjugates prepared by a one-step scalable method using CO₂-expanded solvents', *Nano letters*, 13 (8), 3766–3774, **2013** (cited on page 55).
- [125] Morla-Folch, J.; Vargas-Nadal, G.; Fuentes, E.; Illa-Tuset, S.; Köber, M.; Sissa, C.; Pujals, S.; Painelli, A.; Veciana, J.; Faraudo, J., et al. 'Ultrabright Förster Resonance Energy Transfer Nanovesicles: The Role of Dye Diffusion', *Chemistry of Materials*, 34 (19), 8517–8527, **2022** (cited on pages 55, 64).

- [126] Zhang, X.-F.; Zhang, J.; Liu, L. 'Fluorescence properties of twenty fluorescein derivatives: lifetime, quantum yield, absorption and emission spectra', *Journal of fluorescence*, 24, 819–826, **2014** (cited on page 57).
- [127] Zhang, X.-F.; Zhang, Y.; Liu, L. 'Fluorescence lifetimes and quantum yields of ten rhodamine derivatives: Structural effect on emission mechanism in different solvents', *Journal of luminescence*, 145, 448–453, **2014** (cited on page 57).
- [128] Ali, H.; Ali, S.; Mazhar, M.; Ali, A.; Jahan, A.; Ali, A. 'Eosin fluorescence: A diagnostic tool for quantification of liver injury', *Photodiagnosis and Photodynamic Therapy*, 19, 37–44, **2017** (cited on page 57).
- [129] Chen, X.; Pradhan, T.; Wang, F.; Kim, J. S.; Yoon, J. 'Fluorescent chemosensors based on spiroring-opening of xanthenes and related derivatives', *Chemical reviews*, 112 (3), 1910–1956, **2012** (cited on page 57).
- [130] Derayea, S. M.; Nagy, D. M. 'Application of a xanthene dye, eosin Y, as spectroscopic probe in chemical and pharmaceutical analysis; a review', *Reviews in Analytical Chemistry*, 37 (3), 20170020, **2018** (cited on page 57).
- [131] Zhu, J.; Li, J.-J.; Zhao, J.-W. 'Spectral characters of Rhodamine B as a multi-information fluorescence probe for bovine serum albumins', *Sensors and Actuators B: Chemical*, 138 (1), 9–13, **2009** (cited on page 57).
- [132] Kachel, K.; Asuncion-Punzalan, E.; London, E. 'The location of fluorescence probes with charged groups in model membranes', *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1374 (1-2), 63–76, **1998** (cited on page 58).
- [133] Batistela, V. R.; Pellosi, D. S.; de Souza, F. D.; da Costa, W. F.; de Oliveira Santin, S. M.; de Souza, V. R.; Caetano, W.; de Oliveira, H. P. M.; Scarminio, I. S.; Hioka, N. 'pKa determinations of xanthene derivatives in aqueous solutions by multivariate analysis applied to UV-Vis spectrophotometric data', *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 79 (5), 889–897, **2011** (cited on page 60).
- [134] Mchedlov-Petrosyan, N. O.; Kukhtik, V. I.; Alekseeva, V. I. 'Ionization and tautomerism of fluorescein, rhodamine B, N, N-diethylrhodol and related dyes in mixed and nonaqueous solvents', *Dyes and pigments*, 24 (1), 11–35, **1994** (cited on page 60).
- [135] Gao, D.; Tian, Y.; Liang, F.; Jin, D.; Chen, Y.; Zhang, H.; Yu, A. 'Investigation on the pH-dependent binding of Eosin Y and bovine serum albumin by spectral methods', *Journal of Luminescence*, 127 (2), 515–522, **2007** (cited on page 60).
- [136] Sheskey, R. C. R. P. J.; Quinn, M. E., *Handbook of pharmaceutical excipients*, 2009 (cited on page 72).
- [137] Pottel, J.; Armstrong, D.; Zou, L.; Fekete, A.; Huang, X.-P.; Torosyan, H.; Bednarczyk, D.; Whitebread, S.; Bhatarai, B.; Liang, G., et al. 'The activities of drug inactive ingredients on biological targets', *Science*, 369 (6502), 403–413, **2020** (cited on page 72).
- [138] Pramanick, S.; Singodia, D.; Chandel, V. 'Excipient selection in parenteral formulation development', *Pharma Times*, 45 (3), 65–77, **2013** (cited on page 72).
- [139] 'Benzalkonium chloride used as an excipient', *European Medicines Agency, EMA/352187/2012*, **2012** (cited on page 72).
- [140] Okahara, A.; Tanioka, H.; Takada, K.; Kawazu, K. 'Ocular toxicity of benzalkonium chloride homologs compared with their mixtures', *Journal of Toxicologic Pathology*, 26 (4), 343–349, **2013** (cited on page 72).
- [141] Vargas Nadal, G. 'Novel Quatsome nanovesicles, prepared using CO₂, for the development of advanced nanomedicines', **2020** (cited on pages 72, 73, 76).
- [142] Rossetti, M.; Stella, L.; Morlà-Folch, J.; Bobone, S.; Boloix, A.; Baranda, L.; Moscone, D.; Roldán, M.; Veciana, J.; Segura, M. F., et al. 'Engineering DNA-Grafted Quatsomes as Stable Nucleic Acid-Responsive Fluorescent Nanovesicles', *Advanced Functional Materials*, 31 (46), 2103511, **2021** (cited on page 72).

- [143] Segura, M. F.; Gallego, S.; Sánchez de Toledo, J.; Soriano, A.; Ventosa, N.; Veciana, J.; Boloix, A.; Segovia Ramos, N. V. 'Nanovesicles and its use for nucleic acid delivery', **2020** (cited on page 72).
- [144] Boloix, A., *Quatsomes as a novel nanocarrier for clinical delivery of small RNA*, 2019 (cited on page 72).
- [145] Vargas-Nadal, G.; Muñoz-Úbeda, M.; Álamo, P.; Mitjans, M.; Céspedes, V.; Köber, M.; González-Mira, E.; Ferrer-Tasies, L.; Vinardell, M. P.; Mangues, R., et al. 'MKC-Quatsomes: A stable nanovesicle platform for bio-imaging and drug-delivery applications', *Nanomedicine: Nanotechnology, Biology and Medicine*, 24, 102136, **2020** (cited on pages 73, 76, 84, 85).
- [146] Mori, A.; Yamashita, K.; Tabata, Y.; Seto, K.; Tokunaga, E. 'Absorbance spectroscopy of light scattering samples placed inside an integrating sphere for wide dynamic range absorbance measurement', *Review of Scientific Instruments*, 92 (12), **2021** (cited on page 78).
- [147] Tatikolov, A. S.; Costa, S. M. 'Photophysics and photochemistry of hydrophilic cyanine dyes in normal and reverse micelles', *Photochemical & Photobiological Sciences*, 1 (3), 211–218, **2002** (cited on page 82).
- [148] Lepaja, S.; Strub, H.; Lougnot, D.-J. 'Photophysical Study of a Series of Cyanines Part III. The direct photooxidation reaction', *Zeitschrift für Naturforschung A*, 38 (1), 56–60, **1983** (cited on page 82).
- [149] Muddana, H. S.; Sengupta, S.; Sen, A.; Butler, P. J. 'Enhanced brightness and photostability of cyanine dyes by supramolecular containment', *arXiv preprint arXiv:1410.0844*, **2014** (cited on page 82).
- [150] Rettig, W.; Strehmel, B.; Schrader, S.; Seifert, H.; Eggeling, C.; Widengren, J.; Rigler, R.; Seidel, C. 'Photostability of fluorescent dyes for single-molecule spectroscopy: mechanisms and experimental methods for estimating photobleaching in aqueous solution', *Applied fluorescence in chemistry, biology and medicine*, 193–240, **1999** (cited on page 82).
- [151] Tomsen Melero, J. 'Study of New Liposomes for the Delivery of Enzymes through Biological Membranes', **2021** (cited on pages 84, 85).
- [152] Lundberg, M.; Johansson, M. 'Positively charged DNA-binding proteins cause apparent cell membrane translocation', *Biochemical and biophysical research communications*, 291 (2), 367–371, **2002** (cited on page 84).
- [153] Fretz, M. M.; Koning, G. A.; Mastrobattista, E.; Jiskoot, W.; Storm, G. 'OVCAR-3 cells internalize TAT-peptide modified liposomes by endocytosis', *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1665 (1-2), 48–56, **2004** (cited on page 84).
- [154] Wang, B.; Hu, L.; Siahaan, T. J., *Drug delivery: principles and applications*; John Wiley & Sons: 2016 (cited on page 101).
- [155] Mitchell, M. J.; Billingsley, M. M.; Haley, R. M.; Wechsler, M. E.; Peppas, N. A.; Langer, R. 'Engineering precision nanoparticles for drug delivery', *Nature reviews drug discovery*, 20 (2), 101–124, **2021** (cited on page 101).
- [156] Chen, Z. 'Small-molecule delivery by nanoparticles for anticancer therapy', *Trends in molecular medicine*, 16 (12), 594–602, **2010** (cited on page 101).
- [157] Salehi, B.; Calina, D.; Docea, A. O.; Koirala, N.; Aryal, S.; Lombardo, D.; Pasqua, L.; Taheri, Y.; Marina Salgado Castillo, C.; Martorell, M., et al. 'Curcumin's nanomedicine formulations for therapeutic application in neurological diseases', *Journal of Clinical Medicine*, 9 (2), 430, **2020** (cited on page 101).
- [158] Caddeo, C.; Manconi, M.; Fadda, A. M.; Lai, F.; Lampis, S.; Diez-Sales, O.; Sinico, C. 'Nanocarriers for antioxidant resveratrol: formulation approach, vesicle self-assembly and stability evaluation', *Colloids and surfaces B: Biointerfaces*, 111, 327–332, **2013** (cited on page 101).
- [159] Kumar, A.; Ahuja, A.; Ali, J.; Baboota, S. 'Curcumin-loaded lipid nanocarrier for improving bioavailability, stability and cytotoxicity against malignant glioma cells', *Drug Delivery*, 23 (1), 214–229, **2016** (cited on page 101).
- [160] Irvine, D. J.; Dane, E. L. 'Enhancing cancer immunotherapy with nanomedicine', *Nature Reviews Immunology*, 20 (5), 321–334, **2020** (cited on page 101).

- [161] Patra, J. K.; Das, G.; Fraceto, L. F.; Campos, E. V. R.; Rodriguez-Torres, M. d. P.; Acosta-Torres, L. S.; Diaz-Torres, L. A.; Grillo, R.; Swamy, M. K.; Sharma, S., et al. 'Nano based drug delivery systems: recent developments and future prospects', *Journal of nanobiotechnology*, 16 (1), 1–33, **2018** (cited on page 101).
- [162] Eygeris, Y.; Patel, S.; Jozic, A.; Sahay, G. 'Deconvoluting lipid nanoparticle structure for messenger RNA delivery', *Nano letters*, 20 (6), 4543–4549, **2020** (cited on page 101).
- [163] CHMP 'COVID-19 Vaccine Moderna', *Assessment Report*, **2021** (cited on page 101).
- [164] CHMP 'Comirnaty', *Assessment Report*, **2020** (cited on page 101).
- [165] Man, F.; Lammers, T.; TM de Rosales, R. 'Imaging nanomedicine-based drug delivery: a review of clinical studies', *Molecular imaging and biology*, 20, 683–695, **2018** (cited on page 102).
- [166] Helmchen, F.; Denk, W. 'New developments in multiphoton microscopy', *Current opinion in neurobiology*, 12 (5), 593–601, **2002** (cited on page 102).
- [167] Mertz, J. 'Nonlinear microscopy: new techniques and applications', *Current opinion in neurobiology*, 14 (5), 610–616, **2004** (cited on page 102).
- [168] Denk, W.; Piston, D. W.; Webb, W. W. In *Handbook of biological confocal microscopy*; Springer: 1995, pp 445–458 (cited on page 102).
- [169] Zipfel, W. R.; Williams, R. M.; Christie, R.; Nikitin, A. Y.; Hyman, B. T.; Webb, W. W. 'Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation', *Proceedings of the National Academy of Sciences*, 100 (12), 7075–7080, **2003** (cited on page 102).
- [170] Zoumi, A.; Yeh, A.; Tromberg, B. J. 'Imaging cells and extracellular matrix in vivo by using second-harmonic generation and two-photon excited fluorescence', *Proceedings of the National Academy of Sciences*, 99 (17), 11014–11019, **2002** (cited on page 102).
- [171] Bousso, P.; Robey, E. A. 'Dynamic behavior of T cells and thymocytes in lymphoid organs as revealed by two-photon microscopy', *Immunity*, 21 (3), 349–355, **2004** (cited on page 102).
- [172] Molitoris, B. A.; Sandoval, R. M. 'Intravital multiphoton microscopy of dynamic renal processes', *American Journal of Physiology-Renal Physiology*, 288 (6), F1084–F1089, **2005** (cited on page 102).
- [173] Rubart, M. 'Two-photon microscopy of cells and tissue', *Circulation research*, 95 (12), 1154–1166, **2004** (cited on page 102).
- [174] Laiho, L. H.; Pelet, S.; Hancewicz, T. M.; Kaplan, P. D.; So, P. T. 'Two-photon 3-D mapping of ex vivo human skin endogenous fluorescence species based on fluorescence emission spectra', *Journal of Biomedical Optics*, 10 (2), 024016–024016, **2005** (cited on page 102).
- [175] Wang, B.-G.; Eitner, A.; Lindenau, J.; Halhuber, K.-J. 'High-resolution two-photon excitation microscopy of ocular tissues in porcine eye', *Lasers in Surgery and Medicine: The Official Journal of the American Society for Laser Medicine and Surgery*, 40 (4), 247–256, **2008** (cited on page 102).
- [176] Theer, P.; Hasan, M. T.; Denk, W. 'Two-photon imaging to a depth of 1000 μm in living brains by use of a Ti: Al₂O₃ regenerative amplifier', *Optics letters*, 28 (12), 1022–1024, **2003** (cited on page 102).
- [177] Jain, R. K.; Munn, L. L.; Fukumura, D. 'Dissecting tumour pathophysiology using intravital microscopy', *Nature Reviews Cancer*, 2 (4), 266–276, **2002** (cited on page 102).
- [178] Skoch, J.; Hickey, G. A.; Kajdasz, S. T.; Hyman, B. T.; Bacskaï, B. J. 'In vivo imaging of amyloid- β deposits in mouse brain with multiphoton microscopy', *Amyloid proteins: methods and protocols*, 349–363, **2005** (cited on page 102).
- [179] Ahmed, T. A.; El-Say, K. M.; Ahmed, O. A.; Aljaeid, B. M. 'Superiority of TPGS-loaded micelles in the brain delivery of vinpocetine via administration of thermosensitive intranasal gel', *International journal of nanomedicine*, 5555–5567, **2019** (cited on page 104).
- [180] Patil, P. H.; Mahajan, H. S. 'Mixed micelles for bioavailability enhancement of nelfinavir mesylate: In vitro characterisation and In vivo pharmacokinetic study', *Materials Technology*, 33 (12), 793–802, **2018** (cited on page 104).

- [181] Grimaudo, M. A.; Pescina, S.; Padula, C.; Santi, P.; Concheiro, A.; Alvarez-Lorenzo, C.; Nicoli, S. 'Poloxamer 407/TPGS mixed micelles as promising carriers for cyclosporine ocular delivery', *Molecular pharmaceutics*, 15 (2), 571–584, **2018** (cited on page 104).
- [182] Greenspan, P.; Fowler, S. D. 'Spectrofluorometric studies of the lipid probe, Nile red.', *Journal of lipid research*, 26 (7), 781–789, **1985** (cited on pages 104, 106).
- [183] Kucherak, O. A.; Oncul, S.; Darwich, Z.; Yushchenko, D. A.; Arntz, Y.; Didier, P.; Mély, Y.; Klymchenko, A. S. 'Switchable Nile red-based probe for cholesterol and lipid order at the outer leaflet of biomembranes', *Journal of the American Chemical Society*, 132 (13), 4907–4916, **2010** (cited on page 104).
- [184] Lampe, J. N.; Fernandez, C.; Nath, A.; Atkins, W. M. 'Nile Red is a fluorescent allosteric substrate of cytochrome P450 3A4', *Biochemistry*, 47 (2), 509–516, **2008** (cited on page 104).
- [185] Boldrini, B.; Cavalli, E.; Painelli, A.; Terenziani, F. 'Polar dyes in solution: A joint experimental and theoretical study of absorption and emission band shapes', *The Journal of Physical Chemistry A*, 106 (26), 6286–6294, **2002** (cited on page 104).
- [186] Gupta, V.; Sahu, P. K. 'Topical cyclosporin A in the management of vernal keratoconjunctivitis', *Eye*, 15 (1), 39–41, **2001** (cited on page 105).
- [187] Tatlipinar, S.; Akpek, E. K. 'Topical cyclosporin in the treatment of ocular surface disorders', *The British journal of ophthalmology*, 89 (10), 1363, **2005** (cited on page 105).
- [188] Rubin, M.; Rao, S. N. 'Efficacy of topical cyclosporin 0.05% in the treatment of posterior blepharitis', *Journal of Ocular Pharmacology & Therapeutics*, 22 (1), 47–53, **2006** (cited on page 105).
- [189] Vitale, A. T.; Rodriguez, A.; Foster, C. S. 'Low-dose cyclosporin A therapy in treating chronic, noninfectious uveitis', *Ophthalmology*, 103 (3), 365–374, **1996** (cited on page 105).
- [190] Saleh, Z.; Arayssi, T. 'Update on the therapy of Behçet disease', *Therapeutic advances in chronic disease*, 5 (3), 112–134, **2014** (cited on page 105).
- [191] Krönke, M.; Leonard, W. J.; Depper, J. M.; Arya, S. K.; Wong-Staal, F.; Gallo, R. C.; Waldmann, T. A.; Greene, W. C. 'Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription.', *Proceedings of the National Academy of Sciences*, 81 (16), 5214–5218, **1984** (cited on page 105).
- [192] Lallemand, F.; Felt-Baeyens, O.; Besseghir, K.; Behar-Cohen, F.; Gurny, R. 'Cyclosporine A delivery to the eye: a pharmaceutical challenge', *European Journal of Pharmaceutics and Biopharmaceutics*, 56 (3), 307–318, **2003** (cited on page 105).
- [193] Puig-Rigall, J.; Fernández-Rubio, C.; González-Benito, J.; Houston, J. E.; Radulescu, A.; Nguewa, P.; González-Gaitano, G. 'Structural characterization by scattering and spectroscopic methods and biological evaluation of polymeric micelles of poloxamines and TPGS as nanocarriers for miltefosine delivery', *International journal of pharmaceutics*, 578, 119057, **2020** (cited on page 110).
- [194] Bubna, A. K. 'Imiquimod-Its role in the treatment of cutaneous malignancies', *Indian journal of pharmacology*, 47 (4), 354, **2015** (cited on page 112).
- [195] Vidal, D. 'Topical imiquimod: mechanism of action and clinical applications.', *Mini reviews in medicinal chemistry*, 6 (5), 499–503, **2006** (cited on page 112).
- [196] Stanley, M. 'Imiquimod and the imidazoquinolones: mechanism of action and therapeutic potential', *Clinical and experimental dermatology*, 27 (7), 571–577, **2002** (cited on page 112).
- [197] Telò, I.; Pescina, S.; Padula, C.; Santi, P.; Nicoli, S. 'Mechanisms of imiquimod skin penetration', *International journal of pharmaceutics*, 511 (1), 516–523, **2016** (cited on page 112).
- [198] Šmejkalová, D.; Muthný, T.; Nešporová, K.; Hermannová, M.; Achbergerová, E.; Huerta-Angeles, G.; Svoboda, M.; Čepa, M.; Machalová, V.; Luptáková, D., et al. 'Hyaluronan polymeric micelles for topical drug delivery', *Carbohydrate Polymers*, 156, 86–96, **2017** (cited on page 112).
- [199] Moser, K.; Kriwet, K.; Naik, A.; Kalia, Y. N.; Guy, R. H. 'Passive skin penetration enhancement and its quantification in vitro', *European journal of pharmaceutics and Biopharmaceutics*, 52 (2), 103–112, **2001** (cited on page 112).

- [200] Ghosh, I.; Michniak-Kohn, B. 'Design and characterization of submicron formulation for a poorly soluble drug: The effect of Vitamin E TPGS and other solubilizers on skin permeability enhancement', *International journal of pharmaceutics*, 434 (1-2), 90–98, **2012** (cited on page 112).
- [201] Makhmalzade, B. S.; Chavoshy, F. 'Polymeric micelles as cutaneous drug delivery system in normal skin and dermatological disorders', *Journal of advanced pharmaceutical technology & research*, 9 (1), 2, **2018** (cited on page 112).
- [202] González-Fernández, F. M.; Bianchera, A.; Gasco, P.; Nicoli, S.; Pescina, S. 'Lipid-based nanocarriers for ophthalmic administration: towards experimental design implementation', *Pharmaceutics*, 13 (4), 447, **2021** (cited on page 116).
- [203] Alvarez-Trabado, J.; Diebold, Y.; Sanchez, A. 'Designing lipid nanoparticles for topical ocular drug delivery', *International Journal of Pharmaceutics*, 532 (1), 204–217, **2017** (cited on page 116).
- [204] Bonilla, L.; Espina, M.; Severino, P.; Cano, A.; Ettcheto, M.; Camins, A.; García, M. L.; Souto, E. B.; Sánchez-López, E. 'Lipid nanoparticles for the posterior eye segment', *Pharmaceutics*, 14 (1), 90, **2021** (cited on page 116).
- [205] Müller, R. H.; Radtke, M.; Wissing, S. A. 'Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations', *Advanced drug delivery reviews*, 54, S131–S155, **2002** (cited on page 116).
- [206] Sánchez-López, E.; Espina, M.; Doktorovova, S.; Souto, E.; García, M. 'Lipid nanoparticles (SLN, NLC): Overcoming the anatomical and physiological barriers of the eye–Part II–Ocular drug-loaded lipid nanoparticles', *European Journal of Pharmaceutics and Biopharmaceutics*, 110, 58–69, **2017** (cited on page 116).
- [207] González-Fernández, F. M.; Delledonne, A.; Nicoli, S.; Gasco, P.; Padula, C.; Santi, P.; Sissa, C.; Pescina, S. 'Nanostructured Lipid Carriers for Enhanced Transscleral Delivery of Dexamethasone Acetate: Development, Ex Vivo Characterization and Multiphoton Microscopy Studies', *Pharmaceutics*, 15 (2), 407, **2023** (cited on pages 116, 124).
- [208] Gaballa, S. A.; Kompella, U. B.; Elgarhy, O.; Alqahtani, A. M.; Pierscionek, B.; Alany, R. G.; Abdelkader, H. 'Corticosteroids in ophthalmology: Drug delivery innovations, pharmacology, clinical applications, and future perspectives', *Drug Delivery and Translational Research*, 11, 866–893, **2021** (cited on page 116).
- [209] Villanueva, J. R.; Villanueva, L. R.; Navarro, M. G. 'Pharmaceutical technology can turn a traditional drug, dexamethasone into a first-line ocular medicine. A global perspective and future trends', *International journal of pharmaceutics*, 516 (1-2), 342–351, **2017** (cited on page 116).
- [210] Usayapant, A.; Karara, A. H.; Narurkar, M. M. 'Effect of 2-hydroxypropyl- β -cyclodextrin on the ocular absorption of dexamethasone and dexamethasone acetate', *Pharmaceutical research*, 8, 1495–1499, **1991** (cited on page 116).
- [211] Leibowitz, H. M.; Kupferman, A.; Stewart, R. H.; Kimbrough, R. L. 'Evaluation of dexamethasone acetate as a topical ophthalmic formulation.', *American Journal of Ophthalmology*, 86 (3), 418–423, **1978** (cited on page 116).
- [212] Murtomäki, L.; Vainikka, T.; Pescina, S.; Nicoli, S. 'Drug adsorption on bovine and porcine sclera studied with streaming potential', *Journal of pharmaceutical sciences*, 102 (7), 2264–2272, **2013** (cited on page 118).
- [213] Terenziani, F.; Painelli, A.; Comoretto, D. 'Solvation effects and inhomogeneous broadening in optical spectra of phenol blue', *The Journal of Physical Chemistry A*, 104 (47), 11049–11054, **2000** (cited on page 120).
- [214] Guo, R.; Chen, M.; Ding, Y.; Yang, P.; Wang, M.; Zhang, H.; He, Y.; Ma, H. 'Polysaccharides as potential anti-tumor biomacromolecules—a review', *Frontiers in Nutrition*, 9, 838179, **2022** (cited on page 121).
- [215] Montenegro-Nicolini, M.; Morales, J. O. 'Overview and future potential of buccal mucoadhesive films as drug delivery systems for biologics', *AAPS PharmSciTech*, 18, 3–14, **2017** (cited on page 121).
- [216] Iyire, A.; Alaayedi, M.; Mohammed, A. R. 'Pre-formulation and systematic evaluation of amino acid assisted permeability of insulin across in vitro buccal cell layers', *Scientific reports*, 6 (1), 32498, **2016** (cited on page 121).

- [217] Veillard, M. M.; Longer, M. A.; Martens, T. W.; Robinson, J. R. 'Preliminary studies of oral mucosal delivery of peptide drugs', *Journal of Controlled Release*, 6 (1), 123–131, **1987** (cited on page 121).
- [218] Junginger, H. E.; Hoogstraate, J. A.; Verhoef, J. C. 'Recent advances in buccal drug delivery and absorption—in vitro and in vivo studies', *Journal of controlled release*, 62 (1-2), 149–159, **1999** (cited on page 121).
- [219] Patel, M. P.; Churchman, S. T.; Cruchley, A. T.; Braden, M.; Williams, D. M. 'Delivery of macromolecules across oral mucosa from polymeric hydrogels is enhanced by electrophoresis (iontophoresis)', *Dental Materials*, 29 (11), e299–e307, **2013** (cited on page 121).
- [220] Telò, I.; Tratta, E.; Guasconi, B.; Nicoli, S.; Pescina, S.; Govoni, P.; Santi, P.; Padula, C. 'In-vitro characterization of buccal iontophoresis: The case of sumatriptan succinate', *International journal of pharmaceutics*, 506 (1-2), 420–428, **2016** (cited on page 121).
- [221] Nicolazzo, J. A.; Reed, B. L.; Finnin, B. C. 'Buccal penetration enhancers—how do they really work?', *Journal of controlled release*, 105 (1-2), 1–15, **2005** (cited on pages 121, 122).
- [222] Maher, S.; Casettari, L.; Illum, L. Transmucosal absorption enhancers in the drug delivery field, 2019 (cited on page 121).
- [223] Del Consuelo, I. D.; Pizzolato, G.-P.; Falson, F.; Guy, R. H.; Jacques, Y. 'Evaluation of pig esophageal mucosa as a permeability barrier model for buccal tissue', *Journal of pharmaceutical sciences*, 94 (12), 2777–2788, **2005** (cited on page 121).
- [224] Diaz-del Consuelo, I.; Jacques, Y.; Pizzolato, G.-P.; Guy, R. H.; Falson, F. 'Comparison of the lipid composition of porcine buccal and esophageal permeability barriers', *Archives of oral biology*, 50 (12), 981–987, **2005** (cited on page 121).
- [225] Fantini, A.; Giulio, L.; Delledonne, A.; Pescina, S.; Sissa, C.; Nicoli, S.; Santi, P.; Padula, C. 'Buccal Permeation of Polysaccharide High Molecular Weight Compounds: Effect of Chemical Permeation Enhancers', *Pharmaceutics*, 15 (1), 129, **2022** (cited on pages 121, 124).
- [226] Morales, J. O.; McConville, J. T. 'Novel strategies for the buccal delivery of macromolecules', *Drug development and industrial pharmacy*, 40 (5), 579–590, **2014** (cited on page 122).
- [227] Padula, C.; Pescina, S.; Nicoli, S.; Santi, P. 'New insights on the mechanism of fatty acids as buccal permeation enhancers', *Pharmaceutics*, 10 (4), 201, **2018** (cited on pages 122, 125).
- [228] Pusey, P. 'Photon correlation and light beating spectroscopy', *Plenum Press, New York*, **1974** (cited on page 129).
- [229] O'Brien, R. W.; White, L. R. 'Electrophoretic mobility of a spherical colloidal particle', *Journal of the Chemical Society, Faraday Transactions 2: Molecular and Chemical Physics*, 74, 1607–1626, **1978** (cited on page 129).
- [230] Stenzel, O. et al., *The physics of thin film optical spectra*; Springer: 2015; Vol. 366 (cited on page 130).
- [231] Rurack, K.; Spieles, M. 'Fluorescence quantum yields of a series of red and near-infrared dyes emitting at 600–1000 nm', *Analytical chemistry*, 83 (4), 1232–1242, **2011** (cited on page 131).
- [232] https://static.horiba.com/fileadmin/Horiba/Application/Materials/Material_Research/Quantum_Dots/quantumyieldstrad.pdf (cited on page 131).
- [233] <https://www.edinst.com/blog/laser-spot-size-in-a-microscope> (cited on page 133).
- [234] De Reguardati, S.; Pahapill, J.; Mikhailov, A.; Stepanenko, Y.; Rebane, A. 'High-accuracy reference standards for two-photon absorption in the 680–1050 nm wavelength range', *Optics Express*, 24 (8), 9053–9066, **2016** (cited on pages 134, 147, 148).
- [235] Mütze, J.; Iyer, V.; Macklin, J. J.; Colonell, J.; Karsh, B.; Petrášek, Z.; Schwille, P.; Looger, L. L.; Lavis, L. D.; Harris, T. D. 'Excitation spectra and brightness optimization of two-photon excited probes', *Biophysical journal*, 102 (4), 934–944, **2012** (cited on page 134).
- [236] Boyd, R. W.; Gaeta, A. L.; Giese, E. In *Springer Handbook of Atomic, Molecular, and Optical Physics*; Springer: 2008, pp 1097–1110 (cited on page 137).
- [237] Shen, Y.-R. 'Principles of nonlinear optics', **1984** (cited on page 137).

- [238] Mertz, J., *Introduction to optical microscopy*; Cambridge University Press: 2019 (cited on page 138).
- [239] Remington, L. A.; Goodwin, D., *Clinical Anatomy of the Visual System E-Book: Clinical Anatomy of the Visual System E-Book*; Elsevier Health Sciences: 2004 (cited on page 139).
- [240] Wang, B.-G.; König, K.; Halbhauer, K.-J. 'Two-photon microscopy of deep intravital tissues and its merits in clinical research', *Journal of microscopy*, 238 (1), 1–20, **2010** (cited on pages 140, 142).
- [241] Sanchez, I.; Martin, R.; Ussa, F.; Fernandez-Bueno, I. 'The parameters of the porcine eyeball', *Graefe's Archive for Clinical and Experimental Ophthalmology*, 249, 475–482, **2011** (cited on pages 140, 142).
- [242] Han, M.; Giese, G.; Bille, J. F. 'Second harmonic generation imaging of collagen fibrils in cornea and sclera', *Optics express*, 13 (15), 5791–5797, **2005** (cited on page 140).
- [243] Croce, A. C.; Bottiroli, G. 'Autofluorescence spectroscopy and imaging: a tool for biomedical research and diagnosis', *European journal of histochemistry: EJH*, 58 (4), **2014** (cited on pages 140, 142).
- [244] Montagna, W., *The structure and function of skin*; Elsevier: 2012 (cited on page 144).
- [245] Jacobi, U.; Kaiser, M.; Toll, R.; Mangelsdorf, S.; Audring, H.; Otberg, N.; Sterry, W.; Lademann, J. 'Porcine ear skin: an in vitro model for human skin', *Skin Research and Technology*, 13 (1), 19–24, **2007** (cited on page 144).
- [246] Carrer, D. C.; Vermehren, C.; Bagatolli, L. A. 'Pig skin structure and transdermal delivery of liposomes: a two photon microscopy study', *Journal of controlled release*, 132 (1), 12–20, **2008** (cited on page 145).
- [247] Swathi, K.; Sujith, M.; Divya, P.; Delledonne, A.; Huu, D. A. P.; Di Maiolo, F.; Terenziani, F.; Lapini, A.; Painelli, A.; Sissa, C., et al. 'From symmetry breaking to symmetry swapping: is Kasha's rule violated in multibranched phenyleneethynylenes?', *Chemical Science*, 14 (8), 1986–1996, **2023** (cited on pages 147, 149).
- [248] Makarov, N. S.; Drobizhev, M.; Rebane, A. 'Two-photon absorption standards in the 550–1600 nm excitation wavelength range', *Optics express*, 16 (6), 4029–4047, **2008** (cited on page 147).
- [249] Drobizhev, M.; Molina, R. S.; Hughes, T. E. 'Characterizing the two-photon absorption properties of fluorescent molecules in the 680–1300 nm spectral range', *Bio-protocol*, 10 (2), e3498–e3498, **2020** (cited on page 147).
- [250] Albota, M. A.; Xu, C.; Webb, W. W. 'Two-photon fluorescence excitation cross sections of biomolecular probes from 690 to 960 nm', *Applied optics*, 37 (31), 7352–7356, **1998** (cited on page 148).