



UNIVERSITÀ DI PARMA

UNIVERSITÀ DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN

Scienze del Farmaco delle Biomolecole e dei Prodotti per la Salute

CICLO XXXIV

*Innovative strategies
for non-invasive vaccines administration*

Coordinatore:

Chiar.mo Prof. Marco Mor

Tutore:

Chiar.ma Prof.ssa Patrizia Santi

Dottorando: Adriana Fantini

Anni Accademici 2018/2019 – 2020/2021

Table of contents

<u>1.</u>	<u>INTRODUCTION</u>	<u>1</u>
	1.1 Vaccines	2
	1.2 Immunity	6
	1.3 Currently approved vaccination routes	11
	1.4 Buccal mucosa	17
	1.5 Permeation enhancer strategies in mucosal drug delivery	23
	1.5.1 Chemical enhancers	24
	1.5.2 Microneedles	29
	1.6 Polymers for buccal delivery systems	36
<u>2.</u>	<u>AIM OF THE THESIS</u>	<u>41</u>
<u>3.</u>	<u>MATERIALS AND METHODS</u>	<u>44</u>
	3.1 Materials	45
	3.2 Methods	46
	3.2.1. Tissue preparation	46

3.2.2	<i>Preparation of chitosan/HPMC gels and films</i>	46
3.2.3	<i>Preparation of anionic nanofibrillar cellulose film</i>	48
3.2.4	<i>Preparation of PVP dissolving microneedles</i>	48
3.2.5	<i>Release experiments</i>	49
3.2.6	<i>In vitro permeation studies</i>	49
3.2.7	<i>Analytical method</i>	50
3.2.8	<i>Data elaboration</i>	50
3.2.9	<i>Two-photon microscopy</i>	51
4.	<u>RESULTS AND DISCUSSION</u>	52
4.1	Analytical method validation	53
4.2	Buccal permeation of FITC-dextran from solutions	55
4.2.1	<i>Effect of molecular weight on dextran permeation</i>	56
4.2.2	<i>Effect of sodium taurocholate</i>	57
4.2.3	<i>Effect of fatty acids</i>	60
4.2.4	<i>Two-photon microscopy</i>	64
4.3	Polymeric systems for buccal delivery	73
4.3.1	<i>Chitosan/HPMC gels and films</i>	73
4.3.2	<i>Anionic nanofibrillar cellulose film</i>	77
4.4	Dissolving microneedles for buccal delivery	83
4.4.1	<i>Effect of microneedles length</i>	83
4.4.2	<i>Two-photon microscopy</i>	87

<u>5.</u>	<u>GENERAL CONCLUSION</u>	<u>97</u>
<u>6.</u>	<u>REFERENCES</u>	<u>100</u>
<u>7.</u>	<u>ACKNOWLEDGEMENTS</u>	<u>120</u>

Abstract

The development of vaccines administered in a non-invasive way is considered a great goal to improve their safety and ease of administration, especially for children vaccination and during pandemic situations. Recently, mucosal tissues have received great attention as target sites for vaccination due to their ability to elicit both local and systemic immune responses. With this in mind, in the present work, fluorescent dextrans of different molecular weight were used as model polysaccharide antigens to investigate the possibility to administer vaccines through the buccal mucosal tissue; chemical enhancers, such as fatty acids and bile salts, and physical methods, i.e. microneedles arrays, were used to improve tissue penetration. Among chemicals enhancers tested, caprylic acid pre-treatment and sodium taurocholate co-administration resulted the best absorption strategies: dextrans of molecular weight higher than 40 kDa were able to cross the tissue only in the presence of enhancers. The use of two-photon microscopy, an innovative technique allowing three-dimensional and deep tissue imaging, enabled dextrans visualization in the tissue.

Chitosan, hydroxypropyl methyl cellulose (HPMC) and anionic type nanofibrillar cellulose (ANFC) were used to produce and test suitable buccal delivery systems in the form of gel or film. The chitosan/HPMC gel formulation in association with chemical enhancers can deliver dextran with 20 kDa m.w.

but the film with the same composition allows dextran transport exclusively after tissue pre-treatment. ANFC film instead is surprisingly able to deliver dextrans with m.w. smaller than 20 kDa without any treatment.

Finally, a minimally invasive approach was applied: fluorescent dextrans were loaded in dissolving microneedles, of different lengths, and applied to the buccal mucosa. Two photon microscopy imaging revealed that only microneedles of appropriate length (500 and 800 μm) were able to penetrate the tissue; in these cases, dextran permeation was very high and quick.

The data reported in this work show that it is possible to increase buccal permeation of high molecular weight dextrans by applying certain enhancing strategies, such as the pre-treatment of tissue, co-administration of absorption enhancers and/or production of particular formulations and devices suitable for buccal delivery. Besides this non-invasive approach, a minimally invasive strategy based on the use of dissolving microneedles guaranteed, with a very short application time (10 minutes), a considerable permeation of dextran.

1. INTRODUCTION

1.1 Vaccines

Vaccination is one of the most successful applications of immunology and for a long time has depended on injective administration protocols. However, recent studies have pointed to the promise of mucosal vaccination because of its ease, economy and efficiency in inducing an immune response not only systemically, but also in the mucosal compartment where many pathogenic infections start [1].

Vaccinology dates back as far as 1796 when Edward Jenner illustrated that inoculation of human skin with cowpox virus was protective against subsequent smallpox infection. This phenomenon gave rise to the term “vaccine”, which stems from the Latin root *vacca* meaning cow. A vaccine is defined as a biological preparation that is used to establish or improve immunity to a particular disease [2]. Vaccination is considered one of the greatest public health achievements of the 20th century by significantly decreasing global morbidity and mortality [3].

Vaccines are of different types: traditional vaccines (i) vaccines containing live attenuated virus/bacteria; (ii) vaccines containing killed microorganisms; (iii) subunit-based vaccines; (iv) toxoid-based vaccines. Several innovative vaccines have been recently developed such as conjugate vaccines, recombinant vaccines (see (iii)), (v) DNA vaccines and (vi) mRNA vaccines [4].

1. Introduction

(i) Live-attenuated vaccines/viral vectors

Live vaccines use a weakened (or attenuated) form of the pathogen that causes a disease. Because these vaccines are so similar to the natural infection they create a strong and long-lasting immune response. Just 1 or 2 doses of most live vaccines can give lifetime protection against a specific pathogen and the disease it causes. Live vaccines are used to protect against Measles, Mumps, Rubella (MMR combined vaccine), Rotavirus, Smallpox, Chickenpox, Yellow fever. Viral vector vaccines use a modified version of a different virus as a vector to deliver protection. Some vaccines recently used for Ebola outbreaks have used viral vector technology, and many studies have focused on viral vector vaccines against other infectious diseases such as Zika, flu, and HIV.

Several different viruses have been used as vectors, including influenza, vesicular stomatitis virus (VSV), measles virus, and adenovirus, which causes the common cold. Adenovirus is one of the viral vectors used in some COVID-19 vaccines, such as Oxford-AstraZeneca, Sputnik V, Johnson & Johnson.

(ii) Inactivated vaccines

Inactivated vaccines use the killed version of the pathogen that causes a disease. Inactivated vaccines usually do not provide a level of protection as high as live vaccines, but several doses may need over time (booster) in order to get ongoing immunity against diseases.

1. Introduction

Inactivated vaccines are used to protect against Hepatitis A, Flu, Polio, Rabies.

(iii) Subunit, recombinant, polysaccharide, and conjugate vaccines

Subunit, recombinant, polysaccharide and conjugate vaccines use specific parts of the germ like its protein, sugar or capsid. Because these vaccines use only specific pieces of the pathogen, they give a very strong immune response that is targeted to key parts of the germ. They can also be used on almost everyone who needs them, including people with weak immune systems and long-term health problems. One limitation of these vaccines is that booster shots may be needed to get ongoing protection against diseases.

These vaccines are used to protect against Hib (Haemophilus influenzae type b) disease, Hepatitis B, HPV (Human papillomavirus), Whooping cough (part of the DTaP combined vaccine), Pneumococcal disease, Meningococcal disease, Shingles.

(iv) Toxoid vaccines

Toxoid vaccines use a toxin made by the germ that causes a disease. They create immunity to the parts of the pathogen that cause disease instead of to the germ itself. That means the immune response is targeted to the toxin instead of the whole germ. Like some other types of vaccines, booster shots may need to get ongoing protection against diseases.

Toxoid vaccines are used to protect against Diphtheria and Tetanus.

1. Introduction

(v) DNA vaccines

DNA vaccines use small, circular DNA molecules, called plasmids, to introduce a gene from a bacterium or virus to trigger an immune response. After entering a human cell, the plasmid must make its way through the cytoplasm, cross the nucleus membrane, and enter the cell nucleus. Enzymes in the nucleus convert the viral or bacterial gene that the plasmid carries into messenger RNA (mRNA). The mRNA must then travel to the cytoplasm, where enzymes convert into a bacterial or viral protein. The immune system identifies the bacterial or viral protein as a foreign body and elicits an immune response.

Plasmid DNA degrades within a few weeks, but these memory immune cells provide continuous immunity against the pathogen.

In August 2021, Indian authorities gave emergency approval to ZyCoV-D against Covid-19 disease. Developed by Cadila Healthcare, it is the first DNA vaccine¹ approved for humans [5].

¹ ZyCov-D is also India's first needle-free Covid-19 jab. It is administered with a disposable needle-free injector, which uses a narrow stream of the fluid to penetrate the skin and deliver the jab to the proper tissue (Biswas, 2021).

1. Introduction

(vi) Messenger RNA vaccines—also called mRNA vaccines

mRNA vaccines have several benefits compared to other types of vaccines, including shorter manufacturing times and, because they do not contain a live virus, no risk of causing disease in the person getting vaccinated.

Researchers have been studying and working with mRNA vaccines for decades and this technology was used to make some of the COVID-19 vaccines (Pfizer-BioNTech, Moderna).

1.2 Immunity

Immunization derives from the Greek word *immune* meaning protected. Acquired immunity is the immunity that follows exposure to an antigen or a subject receives exogenous antibodies. Immunization can be of two different types, active immunization, when antigen exposure induces the immune response and passive immunization, when exogenous antibodies are given. During primary infections, pathogens cause a disease which is followed by host immune system response, inducing immunity. Immunity includes the production of humoral immunity and cell-mediated immunity (CMI). Humoral and CMI immunity can be concurrent providing protection when the same disease comes back. On the other hand, vaccination introduces antigens of the pathogen, which induces a similar immune response except the disease development [2].

1. Introduction

Humoral immunity is also called antibody-mediated immunity. With assistance from helper T cells, B cells will differentiate into plasma B cells that can produce antibodies against a specific antigen. The humoral immune system deals with antigens from pathogens that are freely circulating, or outside the infected cells. Antibodies produced by the B cells will bind to antigens, neutralizing them, or causing lysis or phagocytosis [6]. Cellular immunity occurs inside infected cells and is mediated by T lymphocytes. The pathogen's antigens are expressed on the cell surface or on an antigen-presenting cell. Helper T cells release cytokines that help activated T cells bind to the infected cells' MHC-antigen complex and differentiate the T cell into a cytotoxic T cell. The infected cell then undergoes lysis [6].

Antibodies produced by humoral immunity mechanisms can perform their protective functions reaching any district in the body, including mucosae. The surface barriers of the organism separate the self from the external environment (non-self). These physical barriers include the skin and the mucosal barriers of the mouth, respiratory, gastrointestinal, and urogenital tracts. They are selectively permeable, allowing the transport of water, ions, nutrients, and gases while preventing the entry of pathogens and toxins [7]. The host must maintain a dynamic and flexible immunologic barrier at the mucosal surface to protect against invasion by pathogens. At the same time, the mucosal tissue of mouth and respiratory tract, for example, has essential roles in the physiologic functions of ingestion and inhalation respectively.

1. Introduction

These concomitant roles (immunologic barrier and physiologic machinery) are unique characteristic of the mucosa [8]. To provide a protective barrier at mucosal surfaces, the innate and acquired immune systems must operate simultaneously. Physical barriers, consisting of tight junctions and a dense layer of mucins [9], and biochemical barriers, provided by secreted antimicrobial peptides [10], are crucial elements in the first line of defence, together with components of innate immunity, including Toll-like receptors [11] and innate immune cells (e.g., natural killer cells, natural killer T cells, mast cells, and eosinophils) [12] [13]. For acquired immunity at the mucosal epithelium, antigen-specific secretory IgA (SIgA) immune responses are key players in preventing pathogen invasion. SIgA directly neutralizes the infectivity of pathogens and their toxins [14] and contributes to the creation and maintenance of mucosal homeostasis [15]. For the induction of antigen-specific SIgA antibodies, antigens must be presented directly to the mucosal surface, especially its mucosa-associated lymphoid tissue (MALT), antigen-sampling cells (microfold or membranous cells [M cells]) [9] and antigen-presenting cells (e.g., dendritic cells) [16]. Therefore, oral and nasal deposition of antigens that target gut-associated lymphoid tissue (GALT) and nasopharynx-associated lymphoid tissue (NALT) leads to the generation of antigen-specific SIgA antibodies in the secretions of the gastrointestinal and upper respiratory tracts. In contrast, although very effective in the induction of systemic immunity (e.g., serum IgG antibody), the systemic presentation of antigens, such as through

1. Introduction

their injection, apparently does not induce effective mucosal antigen-specific SIgA antibody responses [17].

In fact, subcutaneous vaccination is often ineffective for the induction of antigen-specific mucosal immune responses [18] [19]. These facts indicate that new-generation vaccines must be rationally designed to efficiently elicit antigen-specific mucosal immunity at the entry sites of aero-digestive infectious agents.

The buccal region of the oral cavity is an attractive target for the administration of several drugs and therapeutic agents, including vaccines [20]. Mucosal delivery in the mouth can occur *via* sublingual or buccal routes. In particular, the buccal sites for vaccine delivery are inner lips, gingivae and cheeks. A large number of studies showed that buccal immunization can induce both systemic and local immune response; overall the immune response after mucosal immunization results stronger than after parenteral one [21] [22] [23]. In addition, there are other advantages associated with mucosal vaccination, such as the avoidance of a needle injection, which not only causes pain but also requires the assistance of a professional, the suitability for mass vaccination [24] and fewer systemic adverse events compared with parenteral administration [25].

Mucosal immunocompetence is due to the presence of Langerhans cells (LC), within epithelial cells, myeloid dendritic cells (MDC), along the lamina propria and plasmacytoid cells (PDC) located in the submucosal tissue (**Figure 1**).

1. Introduction

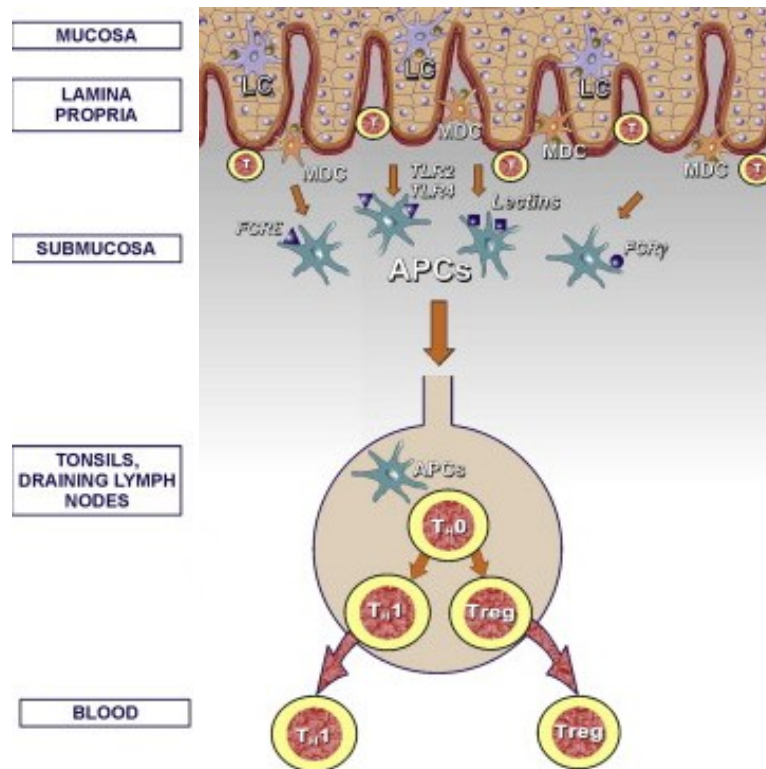


Figure 1: Immune mechanism associated with oral mucosa. Source Moingeon, 2013 [26].

To induce an immune response, an antigen must be captured by antigen-presenting cells (APC), namely Langerhans cells and myeloid dendritic cells, which then migrate to lymph nodes where they interact with naïve CD4 and CD8 T cells (indicated with letter T in **Figure 1**). The activation of CD4 T lymphocytes leads to the induction of antigen-specific helper T cells (Th) and/or regulatory T cell (Treg) mediated immune responses, whereas CD8 T cells induce cytotoxic T lymphocyte (CTL) responses. The differentiated effector T cells enter the circulation and migrate to selected mucosal site. Vaccine delivery to the sublingual and buccal mucosa can generate mucosal immune responses at distal mucosal sites including the urogenital tract, the respiratory tract, and the intestinal tract [27].

1.3 Currently approved vaccination routes

Antigens are often large molecules with high molecular weight and due to this feature, most of them are generally administered as injection, to overcome the problem of absorption through a biological membrane and reach the systemic circulation.

Each vaccine has a recommended administration route and site [28]. This is a critical factor for the success of the immunization. There are five routes used to administer vaccines; deviation from the recommended route may reduce vaccine efficacy or increase local adverse reactions [29].

- **Oral Route (PO):**

The oral vaccine is administered through drops to the mouth. Rotavirus vaccine (RV1 [Rotarix], RV5 [RotaTeq]) is the only routinely recommended vaccine administered orally. Rotavirus vaccine should never be injected [29].

- **Intranasal Route (NAS):** Live, attenuated influenza vaccine (LAIV² [FluMist]) is the only vaccine administered by the intranasal route. The intranasal vaccine is administered into each nostril using a manufacturer-

² LAIV vaccine was first introduced in Italy during the 2020-2021 flu campaign. It has been approved for administration to children aged between 2 and 6 years and, subsequently, also to children and adolescents from 6 to 18 years old [140].

1. Introduction

filled nasal sprayer. A dose-divider clip, located on the plunger, separates the total vaccine dose of 0.2 ml into two equal parts of 0.1 ml each. LAIV should never be injected [29].

- **Subcutaneous Route (SC):**

Subcutaneous injections are administered into the fatty tissue found below the dermis and above muscle tissue. Approved vaccines administered by subcutaneous injection include varicella and measles vaccines. For infants under the age of 12 months, a subcutaneous injection is usually administered into the fatty tissue of the thigh, although the upper outer triceps area of the arm may be used if necessary. For subjects aged over 1 year, subcutaneous injections are given in the fatty tissue above the upper outer triceps of the arm [29].

- **Intradermal (ID):**

Injection administers the vaccine in the deeper layer of the skin. Bacille Calmette-Guerin (BCG-against tuberculosis) is the only vaccine approved for this route. Intradermal injection of BCG vaccine reduces the risk of neurovascular injury compared to the subcutaneous one. [30].

- **Intramuscular Route (IM) [29]:**

Intramuscular injections are administered into the muscle through the skin and subcutaneous tissue. Vaccines containing adjuvants should be injected IM to reduce adverse local effects. There are only two recommended sites for administering vaccines by IM injection:

1. Introduction

→ Vastus lateralis muscle in the anterolateral thigh

→ Deltoid muscle in the upper arm

Injection at these sites reduces the chance of involving neural or vascular structures. The preferred site depends on the patient's age, weight, gender, and degree of muscle development. For both sites, an IM injection ideally should be administered into the middle of the muscle where the muscle tissue is thickest.

Routinely recommended vaccines administered by IM injection include:

- HepA (Havrix, Vaqta)
- HepB (Engerix B, Recombivax HB)
- HepB-CpG (Heplisav-B)
- HepA-HepB (Twinrix)
- HPV (Gardasil 9)
- MenACWY (Menactra, MenQuadfi, Menveo)
- MenB (Bexsero, Trumenba)

The main way of vaccines administration is by injection, as summarized in **Figure 2**.

1. Introduction

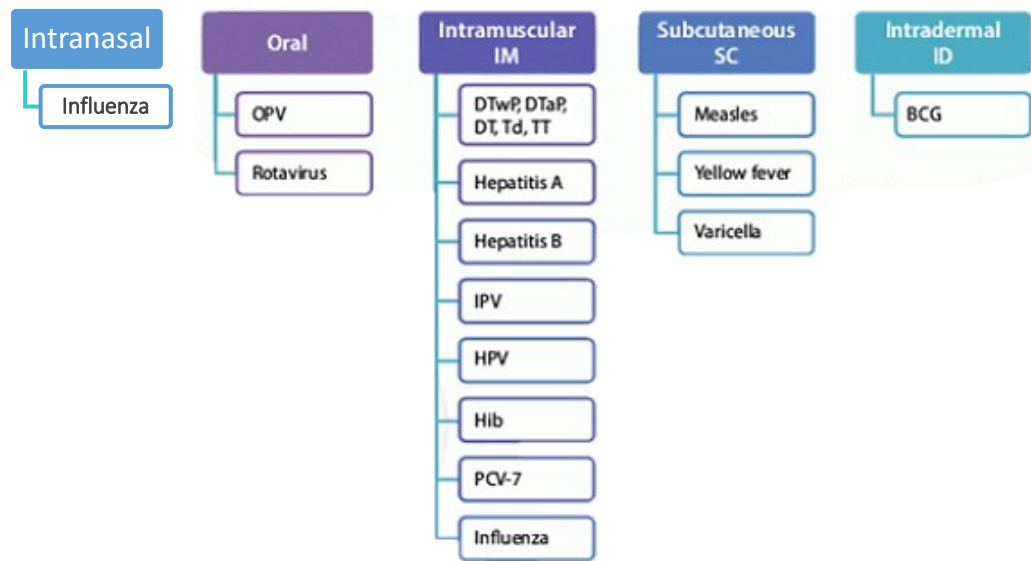


Figure 2: overview diagram of main vaccines and routes of administration. Source: World Health Organization

Vaccine injections are often cited as a common source of pain in children, but it can be a stressful experience for persons of any age. It has been estimated that up to 25% of adults have a fear of needles, with most needle fears developing during childhood. If not addressed, these fears can have long-term effects such as preprocedural anxiety and avoidance of needed health care throughout a person's lifetime. Fear of injections and needlestick pain are often cited as reasons why children and adults refuse vaccines [29].

Vaccines are often formulated as solutions to be delivered through a syringe, solutions often require cold storage. One of the main obstacles of vaccines production, distribution and storage is the need for cold storage; the vaccine must be maintained in either frozen storage, defrosted, and kept cold until injection into a patient's arm. Other disadvantages with IM and SC

1. Introduction

injections include the need for medical personnel to perform the injection, risk of accidental needle stick, the needle must be properly discarded in biohazard sharps and must never be reused [31].

The SARS-CoV-2 pandemic has highlighted the logistical challenges in the access and distribution of COVID-19 vaccines. There are areas of the world where there is a scarcity of medical personnel, syringes and needles, freezers (also electricity), even ice and ice containers to maintain the stability of the vaccines [31]. For these reasons, there is an urgent need to develop alternative methods to deliver the vaccine, which may require a different formulation to guarantee a safe and effective immune response. Alternative delivery options include oral delivery, intranasal delivery, buccal delivery or microneedle patches. Mucosal vaccine administration is a good option to protect against viruses or bacteria that enter through the mucosa, such as the coronavirus. Examples of mucosal vaccines that have a proven record of safety and protection are the oral polio vaccine (OPV) and the FluMist® Quadrivalent intranasal vaccine, both consisting of a live attenuated virus. Traditional needle vaccination does not generally elicit a strong mucosal response; mucosal delivery, instead, may elicit both a systemic and mucosal response with the production of IgA antibodies which are important in mucosal immunity [25]. Most mucosal vaccines consist of live attenuated viruses; therefore, a mucosal COVID-19 vaccine may require a completely different formulation from the current mRNA vaccines.

1. Introduction

Intradermal (ID) vaccine delivery via microneedle patches does not require medical personnel to administer, or syringes and hypodermic needles. Microneedle (MNs) patches deliver the vaccine antigen into the dermis and epidermis and to the APCs within. Since the skin is densely packed with APCs, a smaller amount of vaccine may be sufficient to get a comparable response as seen in IM or SC administration. ID vaccination using microneedles is typically manufactured as a patch with micro projections ranging in height from 25–2000 μm and attached to a micro platform [32]. The patches can deliver, via a solution, micromolecules or macromolecules, without bleeding, therefore, eliminating the potential for any type of cross-contamination. There are several types of patches and techniques which have the potential to deliver a lot of antigens, also COVID-19 vaccine [33]. In the “poke and patch” approach, the tissue is poked with a small patch containing microneedles, once removed the vaccine is then topically applied to the “damaged” tissue. In the “coat and poke” method microneedles are loaded with the vaccine, placed on the tissue, creating micro holes in which the vaccine can be delivered.

MNs have been widely investigated for transdermal drug delivery but the oral or buccal mucosa has recently been identified as a potential site for MN-based delivery. While the skin remains the main target for MN application due to accessibility, the buccal cavity, despite its curved nature and moist surface, is gaining focus for MN delivery [34]. The advantages of this innovative vaccine

1. Introduction

technology include no need for cold storage, no need for sharps disposal and very little vaccine waste [31] [35].

1.4 Buccal mucosa

The oral cavity extends from the lips to the posterior oropharynx (**Figure 3**) and consists of lips, cheeks, gingiva, anterior two-thirds of the tongue, the floor of the mouth, and the hard palate.

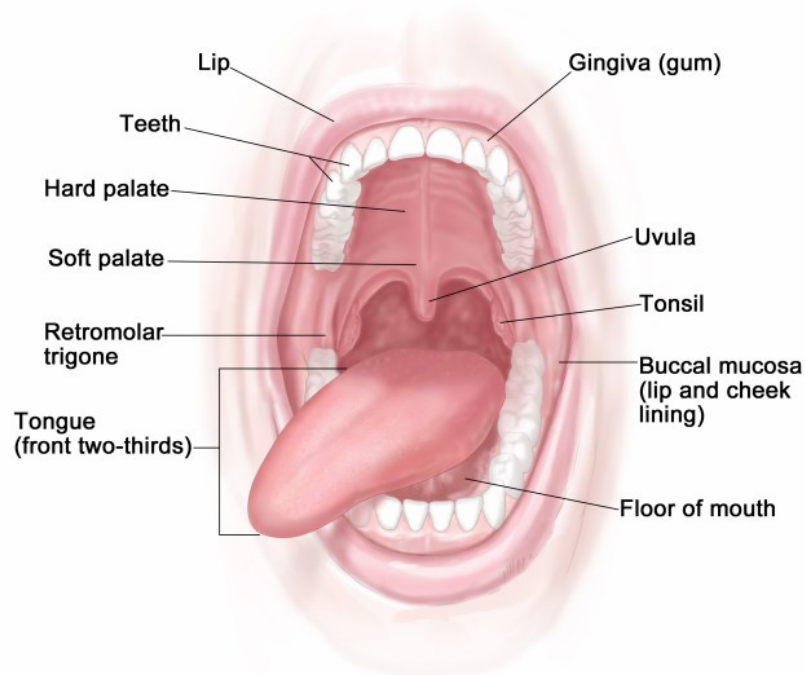


Figure 3: Anatomy of the oral cavity. Source: PDQ Adult Treatment Editorial [36]

The mucosal lining of the oral cavity is a stratified squamous epithelium (40-50 cell layers; thickness 0.5-0.8 mm) with histologic variations that adapt

1. Introduction

to specific functionality [36] and it covers an estimated surface area of $50.2 \pm 2.9 \text{ cm}^2$.

The epithelium serves as a barrier and consists of, from top to bottom, a superficial layer, an intermediate layer and a basement membrane tightly attached to the underlying *lamina propria* (**Figure 4**).

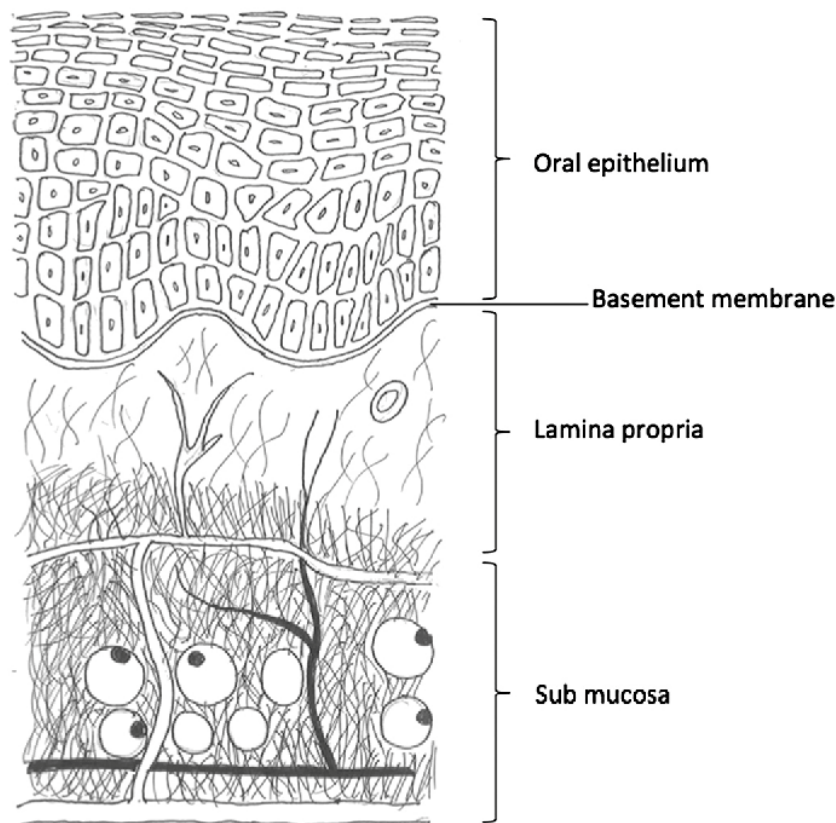


Figure 4: The oral mucosa. Source: Muthukumaran & Sekhar 2013.

Within the oral cavity, the regions that are subject to mechanical force of mastication, gingivae and hard palate, are lined by a keratinized epithelium (**Figure 5 A**) [37] as well the outer lips.

1. Introduction

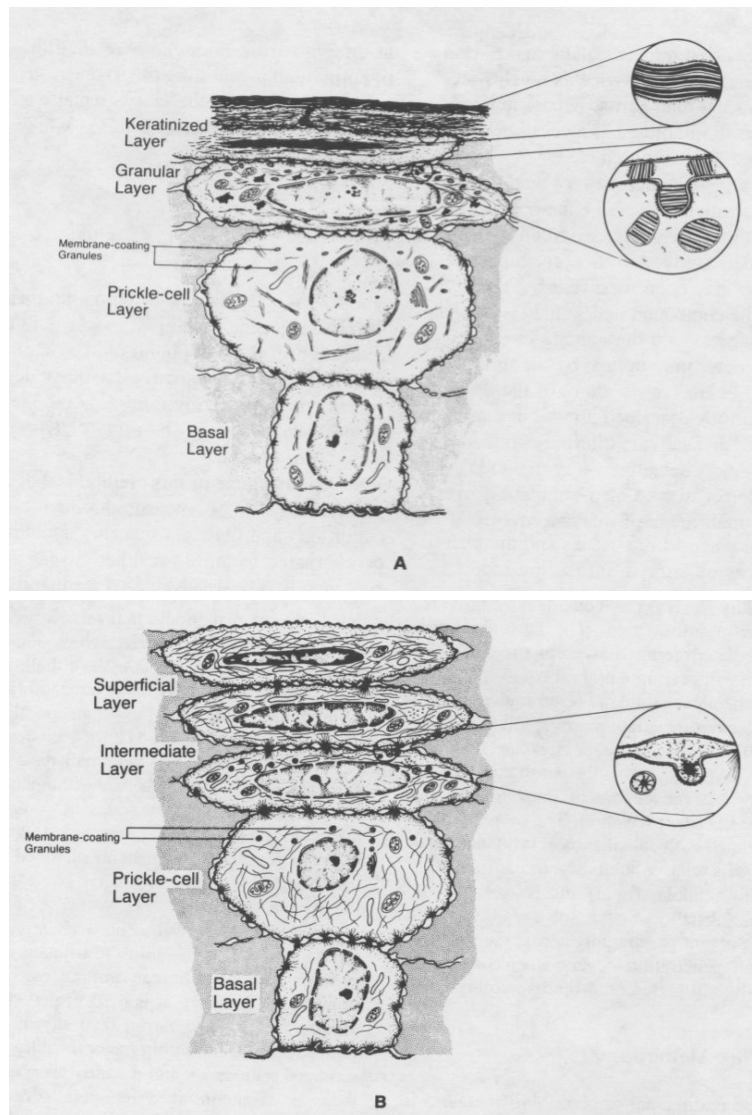


Figure 5: A diagram of keratinized stratified epithelium. B diagram of a nonkeratinized stratified epithelium. Source: Squier, 1991.

The floor of the mouth, the inner surface of the lips and cheeks and the ventral side of the tongue are covered by a nonkeratinized epithelium rendering

1. Introduction

these regions more elastic and potentially more permeable than keratinized mucosa (**Figure 5 B**) [38].

A gel-like secretion known as mucus covers the entire oral cavity. Mucus acts as a protective layer to the cells below and it is a viscoelastic hydrogel. It mostly consists of 1-5% of water insoluble glycoproteins, 95-99% water, and other components in small quantities, such as proteins, enzymes, electrolytes, and nucleic acids [39].

It is believed that the barrier of the nonkeratinized oral epithelium is composed of lipid material, since treatment of oral mucosa with chloroform/methanol mixtures has resulted in a reduced barrier function [40]. Several analyses through thin-layer chromatography revealed the chemical nature of these lipids [41][40][42]. In common with porcine epidermis, keratinized palatal and gingival mucosae contained high quantities of ceramides and cholesterol, and a low proportion of cholesterol esters and glycosylceramides. In contrast, the buccal and sublingual mucosae, which are nonkeratinized, contained higher quantities of more polar phospholipids, cholesterol esters, glycosylceramides, and minimal amounts of ceramides. Histochemical staining indicated that the polar lipids were localized in the intercellular spaces of the nonkeratinized oral epithelium [43]. Therefore, the intercellular lipids of the nonkeratinized regions of the oral cavity are of a more polar nature than the lipids of the epidermis, palate, and gingiva, and this difference in the chemical nature of the lipids may contribute to the differences

1. Introduction

in permeability observed between these tissues [40]. Therefore, it appears that it is not only the amount of intercellular lipid packing in keratinized epithelia that creates a more effective barrier, but also the chemical nature of the lipids present within that barrier [44].

The buccal mucosa is less permeable compared to the intestinal epithelium; the usage of permeation enhancers has been extensively investigated in buccal drug delivery dosage forms [45]. The permeability of buccal mucosa finds midway to that of the intestinal mucosa and epidermis. It is estimated that the buccal mucosa permeability is 4-4000 times greater than that of the skin [46] [47]. The permeability order of the oral cavity is

sublingual >buccal>palatal

The rank order is based on the relative thickness and degree of keratinization.

The cell membranes are relatively lipophilic and may create a barrier to polar hydrophilic permeants, and therefore, hydrophilic molecules perhaps permeate the buccal mucosa via the paracellular route [48]. However tight junctions are rare in oral mucosa and their existence among intestinal epithelial cells is the key barrier to paracellular drug transport through the intestine; consequentially, the passage of drugs through the intercellular domain of the buccal epithelium is more favourable than intestine [44].

1. Introduction

There are different modes of permeations:

- Passive diffusion
 - Transcellular or intracellular route (crossing the cell membrane and entering the cell)
 - Paracellular or intercellular route (between the cells)

Passive diffusion pathways are shown in **Figure 6**.

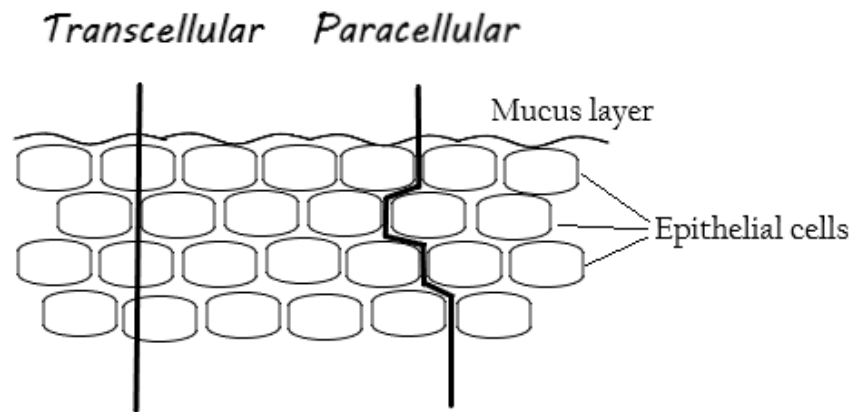


Figure 6: Passive diffusion pathways.

- Carrier mediated transport
- Endocytosis

Permeants can use these two routes simultaneously, but one route is usually chosen over the other based upon the physicochemical properties of the diffusant. Since the cytoplasm and intercellular spaces are hydrophilic, lipophilic compounds would have low solubilities in this environment [39].

Usually, both pig buccal mucosa and skin are used to simulate the respective human tissues due to their high similarities in terms of permeability, structure and composition [49].

1.5 Permeation enhancer strategies in mucosal drug delivery

Membrane permeation of many drugs administered via the buccal route can be often limited due to the epithelium that lines the oral mucosa is a very effective barrier. In order to deliver broader classes of drugs across the buccal mucosa, several methods to overcome the tissue barrier must be employed. This requisite has supported the study of penetration enhancers that will safely alter the permeability of the buccal mucosa. It has been shown that buccal penetration can be improved by using various classes of penetration enhancers such as bile salts, surfactants, fatty acids and derivatives, chitosan and so on.

Drug absorption can also be enhanced mechanically, by removing the outermost layers from epithelium to decrease the barrier thickness or using microneedles technology to pierce the tissue and overcome the barrier, or, finally, electrically, by application of electric fields or by sonophoresis.

1.5.1 Chemical enhancers

A chemical penetration enhancer (CPE), or absorption promoter, is a substance added to a formulation in order to increase membrane permeation or absorption rate of a drug, without damaging the membrane [39]. Chemical permeation enhancers facilitate molecules' penetration through biological tissues by altering the structure of the cellular membrane (transcellular route) and/or the tight junctions between cells (paracellular route) of the epithelium [50]. Unfortunately, many reports indicate that enhancer efficacy is often linked to toxicity [51] [52]. However, the full potential of CPEs for oral and buccal delivery remains unclear since there is no fundamental understanding of the principles that govern enhancer behaviour [53].

The main categories of chemical enhancers are anionic surfactants, cationic surfactants, zwitterionic surfactants, non-ionic surfactants, bile salts, fatty acids, fatty esters, fatty amines, sodium salts of fatty acids, nitrogen-containing rings. Since the purpose of this thesis was the buccal administration, substances as far as possible safe were used, sodium taurocholate (a bile salt) and several fatty acids. Both bile salts and fatty acids are also endogenous molecules and for this reason, are deemed quite safe.

Bile salts

Bile salts are made of bile acids that are conjugated with amino acids or their derivatives such as glycine or taurine. Glyco-bile and tauro-bile acids are also referred to as conjugated bile acids; examples of bile acids and conjugates

1. Introduction

are shown in **Figure 7**. Bile salts have a large, rigid, and planar hydrophobic moiety of a steroid nucleus with two or three hydroxyl groups [54]; They exhibit a great surface activity and can self-associate in water forming micelles if their concentrations are above the so-called critical micellar concentration (CMC). Each micelle contains 4–50 molecules, depending on the type and structure, which can solubilize lipids and other hydrophobic molecules [55]. The hydrophobicity of bile salts and thus their CMC is determined by the number of hydroxyl groups and their stereospecific position in the molecule [56].

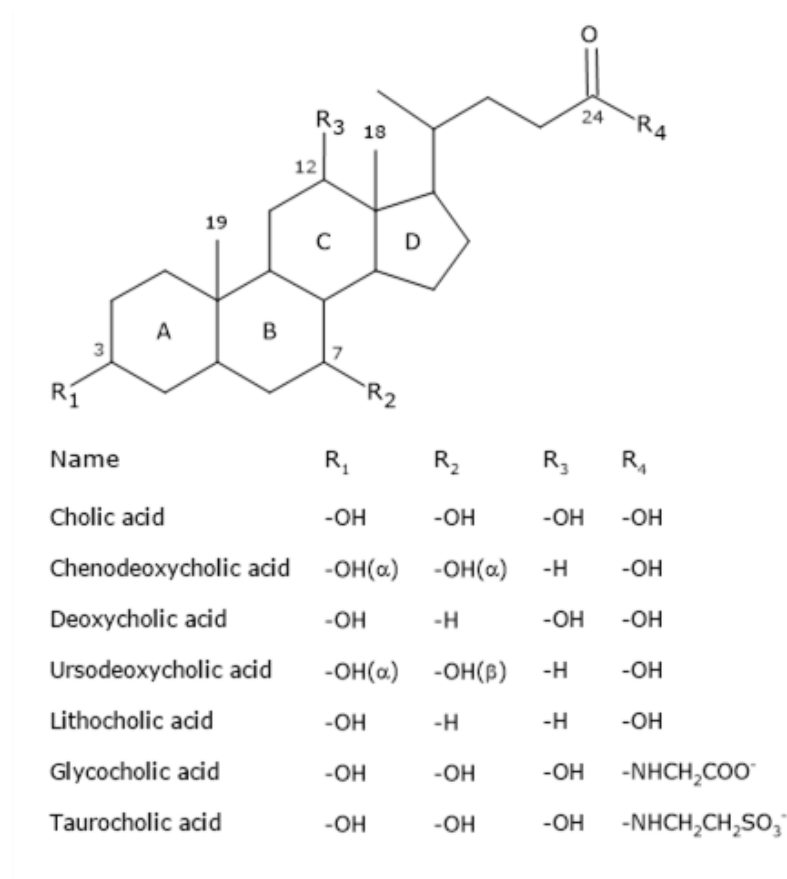


Figure 7: Bile acids and their conjugates.

1. Introduction

It has been demonstrated that bile salts enhance the epithelial transport of hydrophilic drugs through the paracellular route and that of hydrophobic compounds through both paracellular and transcellular routes [57]. The possible mechanism of promotion effect could be the incorporation of bile acids into the cell membrane where, at a certain concentration, they could form reverse micelles that included water molecules creating hydrophilic pores in the cell membrane [58] [59]. Additionally, bile salts may increase the penetration of drugs via the paracellular route by binding calcium ions, in that way triggering tight junctions between the cells to open, and by producing a mucolytic effect [60]. The mechanisms for improvement of transcellular absorption include increasing the solubility and dissolution rate of otherwise slightly soluble drugs by micelle formation [61]. Solubilization of lipophilic drugs within bile salt mixed micelles may facilitate diffusion through the aqueous diffusion layer, improving absorption [62]. Micelles increase the permeability of the mucosal membrane by overcoming resistance at the aqueous diffusion layer [60]. Solubilization by bile acids reduces particulate size and consequently may allow the drug to enter the cell [63].

A possible mechanism for the capability of bile salts to change the permeability of various cell membranes to drugs could be the interaction of bile salt micelles with membrane lipids, such as phospholipids [64]. It has been demonstrated that some bile salts inhibit the activity of efflux of P-glycoprotein

(Pgp) [65], probably by changing the lipid environment of the Pgp transporter or by interaction with Pgp itself [66].

Fatty acids

Fatty acids consist of an aliphatic hydrocarbon chain and a terminal carboxyl group [67]:

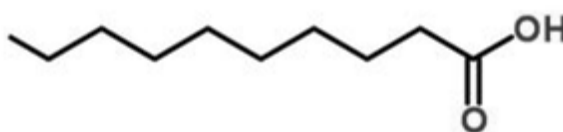


Figure 8: Capric acid/decanoic acid, an example of fatty acid structure.

Most fatty acids were first isolated and identified from a particular source, and the names given to some fatty acids tended to reflect this (e.g. Palmitic acid, from palm oil). Gradually, the basic relationship to the aliphatic linear hydrocarbons became clearer and systematic nomenclature was adopted [68]. The most common fatty acids found in nature are saturated and unsaturated compounds with a chain length of C16 and C18. If there are only single bonds between carbons in the hydrocarbon chain, a fatty acid is said to be saturated (such as the fatty acid in **Figure 8**).

1. Introduction

Table 1: Systematic name, common name and source of main fatty acids used.

Number of carbons	Systematic name	Common name	Source
6	Hexanoic acid	Butyric acid	Butter
7	Heptanoic acid	Caproic acid	Butter, palm oil, coconut oil
8	Octanoic acid	Caprylic acid	Palm oil, coconut oil
10	Decanoic acid	Capric acid	Palm oil, coconut oil
12	Dodecanoic acid	Lauric acid	Plants of lauraceae
18	11-octadecenoic acid	Oleic acid	Olive oil, peanut oil, linseed oil
18	<i>Cis,cis</i> -9,12-octadecadienoic acid	Linoleic acid	Olive oil, peanut oil, linseed oil, soybean oil

When the hydrocarbon chain has a double bond, the fatty acid is said to be unsaturated. If there is just one double bond in fatty acid, it is monounsaturated, while if there are multiple double bonds, it's polyunsaturated. The double bonds in unsaturated fatty acids can exist in either a *cis* or a *trans* configuration (**Figure 9**).

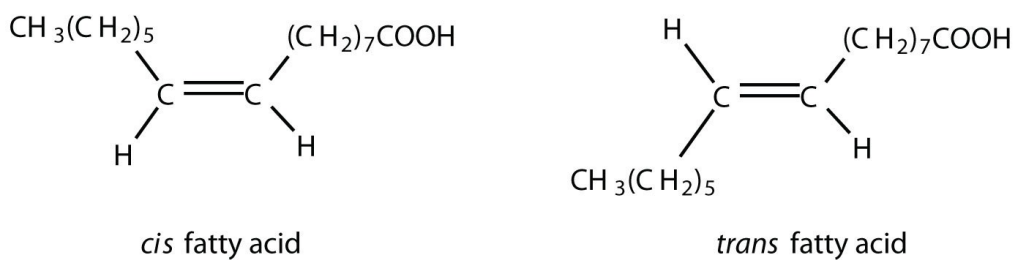


Figure 9: *cis* and *trans* fatty acids configuration.

Fat molecules with fully saturated tails can pack tightly against one another. This tight packing results in fats that are solid at room temperature.

1. Introduction

For instance, most of the fat in butter is saturated. In contrast, *cis*-unsaturated fatty acid tails are curved due to the *cis* double bond. This makes it hard for fat molecules with one or more *cis*-unsaturated fatty acid tails to pack tightly. Hence, fats with unsaturated tails tend to be liquid at room temperature and they are commonly called oils. *Trans* fats are rare in nature but are easily produced in an industrial procedure called partial hydrogenation.

Generally, C6 to C14 saturated fatty acids have been widely reported as skin penetration enhancers. Also oleic acid, a C18 unsaturated fatty acid, is one of the most extensively studied penetration enhancers among the fatty acids [69] [70] .

1.5.2 Microneedles

Despite the higher permeability of the oral mucosa in comparison to the skin one [37] [49], its outermost layer, the stratified squamous epithelium, represents a significant obstacle in drug delivery because it acts as an important barrier to drug penetration [71]. Microneedles (MNs) represent a new approach for topical drug delivery for either local or systemic effects. This system consists of micron-scaled needles of different shapes having a height in the range of 25–2,000 μm , supported on a base, designed to penetrate the barrier and enhance drug delivery in a minimally invasive and painless manner [72].

1. Introduction

Delivery *via* mucosal route is an important substitute to hypodermic and oral administration for drugs. It can solve the problem of gastrointestinal or liver metabolism and consequent drugs degradation. Moreover, it is non-invasive, painless, cheaper and may be self-administered. It can provide prolonged release of drugs to improve patient compliance [73] [74].

Merits and demerits of microneedles arrays are summarized in **Table 2**.

Table 2: Merits and demerits of MNs

Merits	Demerits
The administration of large molecules is possible.	Dosage accuracy may be less.
Non-invasive and painless application.	Administration of only a small dose of a drug is possible.
Avoid the first-pass metabolism.	Possibility of tissue irritation.
Enhanced drug efficacy results in dose reduction.	Upon removal of the patch, the needle may break and remain intact in the tissue.
Ease of administration.	The external environment may affect delivery (i.e. hydration of the skin)
Controlled drug delivery.	Compressed dermal tissues can block hollow MNs.
Faster recovery at the injection site achieved.	
Good tolerability without irritation.	
Reduction of production and distribution costs.	
Targeted drug delivery achieved at a specific area of skin.	

1. Introduction

Nowadays, most microneedles patches were developed to deliver high-molecular-weight drugs without using injections. Caffarel-Salvador, et al. [34] described a novel fabrication technique to produce clinically relevant highly concentrated MNs able to deliver human insulin (HI) and human growth hormone (hGH) through the buccal mucosa. These MNs patches can deliver relevant macromolecular drug doses after buccal mucosa administration systemically, quickly, and painlessly. Moreover, the painless sensation reported by some study participants suggests that their MN patch would potentially facilitate drug delivery to the pediatric population. During the last decade, MNs arrays for vaccines administration received more interest, being this field very challenging. One of the first studies regarding the application of microneedles for vaccine delivery to the oral cavity was by Ma, et al. in 2014 [75]. Systemic and mucosal immune responses were measured by delivering three different antigen types: ovalbumin as a model soluble protein antigen; E2V3, an HIV virus-like particle vaccine and a gp160 HIV DNA vaccine. Antigen-specific IgG antibodies against all three antigens ($p < 0.05$) were generated in blood demonstrating the potential of systemic immunization using the oral cavity. In this work, both microneedle approach and injective method were investigated for comparison purposes and sIgA specific to all three antigens was significantly ($p < 0.05$) increased in saliva when the three antigens were delivered using microneedles delivery system on mucosal tissue than when delivered intramuscularly. Therefore, while the coated microneedle approach of

1. Introduction

delivering vaccines to the oral cavity could activate both systemic and mucosal immunity, the intramuscular method could only activate systemic immunity.

In the literature, five main categories of microneedles have been described: solid, dissolving, hollow, coated and hydrogel-forming microneedles (**Figure 10**).

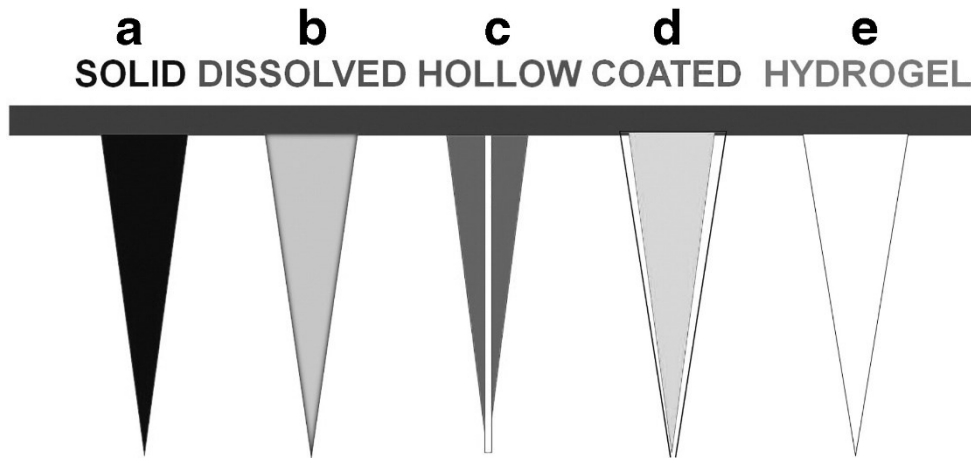


Figure 10: Types of MN: solid MNs (a); dissolving MNs (b); hollow MNs (c); coated MNs (d); and hydrogel forming MNs (e) [74].

Solid MNs

Solid MNs are developed to deliver drugs into the skin based on the mechanism of “poke-and-patch” [76].

The delivery mechanism is passive diffusion [77]: solid MNs are applied onto the tissue creating transient microchannels and then a patch with the drug formulations is applied onto the skin so that the drug can diffuse into the skin through the transient microchannels [76]. Solid MNs were first developed in 1998 and used for pre-treatment of the skin by creating pores through pointed

1. Introduction

needle tips [78]. The fabrication of solid MNs is easy; they are composed of glass, metals, silicon, and other non-degradable polymers [79].

Advantages: simple technique, provide extended release.

Disadvantages: no precise dosing, two step administration process, reformulation of the drug is needed, a low fraction of drug may be delivered.

Dissolving MNs

Dissolving MNs deliver drugs into the skin based on the mechanism of “poke-and-release” [76]. The dissolving MNs are entirely composed of sugars or soluble polymers in which the drug is dissolved or dispersed [80]. Biodegradable polymers are used to fabricate these MNs. The mechanism consists in dissolution after insertion into the skin, followed by the release of the drug [77]. These MNs are used for long-term therapy and provide better patient compliance [81]. These MNs have been also developed for vaccines administration (e.g., influenza) [82].

Advantages: no sharp waste, pump or patch is not required, dosing is precise.

Disadvantages: reformulation of drug needed, small dose, often fewer sharp MNs.

Hollow MNs

Hollow MNs deliver drugs via the “poke-and-flow” approach. Similar to the hypodermic injection, the liquid drug formulation can continuously flow into the skin via the holes in the hollow MNs driven by a pressure [76]. Hollow MNs are generally used for diagnosis and disease monitoring [77]. There are various mechanisms through which a drug is delivered: diffusion, osmosis, capillary action, or external applied negative pressure [74]. These are efficient in delivering bolus insulin for the treatment of type 1 diabetes, and rapid absorption of insulin is observed [83].

Advantages: delivery of high volumes, drug reformulation is not needed, rate of delivery can be regulated, precise dosing.

Disadvantages: more complex device, increased risk of arrays leakage, risk of blocking compromised MNs.

Coated MNs

Coated MNs consist of needles coated with drug solution which release the drug based on the “coat-and-poke” method, after insertion into the tissue [76]. In particular, MNs coated with drug formulation are inserted into the tissue, then the drug dissolves from the MNs and finally, MNs are pulled out of the tissue [74].

Advantages: simple approach, one single step to deliver the drug [76].

1. Introduction

Disadvantages: the MNs can only be coated with a small amount of drugs [84], efficient coating procedure is required, drug reformulation is needed, sharpness of MN is reduced, reducing penetration ability [74].

Hydrogel forming MNs

Hydrogel forming type MNs (HFMs) utilize super-swelling polymers that can absorb a high quantity of water. They are also known as *in situ* MNs: when inserted into the tissue, the polymer swells by absorbing water, then the swollen network further creates conduits and acts as a reservoir, from this reservoir, the drug is released and enters the circulation [74]. It also controls the delivery and maintains the solidity of MNs in the skin. The materials used for the fabrication include polysaccharides, polymers like poly(ethylene glycol) diacrylate (PEGDA) and polyvinyl alcohol (PVA), or poly(acrylic acid-co-maleic) acid. The delivery of a high dose of therapeutic agents through hydrogel MNs depends on their swelling ability [85] [86].

Advantages: the polymers used in HFMs are highly biocompatible, have excellent degradability, and are nontoxic. HFMs can be used to intrinsically control drug release due to their hydrogel structure [87].

Disadvantages: the degree and rate of drug delivery from hydrogels are limited. The initial release of a drug from a hydrogel is often in high concentrations, known as a burst-release, followed by a steady rate of release. If the polymers used to produce the hydrogel are not sufficiently crosslinked, they may dissolve when in contact with the interstitial fluid. If the polymers

possess cytotoxic properties, an accumulation of toxic polymers in the body can occur [87].

Microneedles can be fabricated from a variety of materials into different geometries and different sizes to reach specific depths in various tissues. They have also been used to deliver a broad range of antigen and adjuvant combinations [22].

Many different vaccines can be formulated into microneedles due to the abundance of materials and designs that are available. Vaccine compositions have included virus-like particles, DNA polyplexes, protein subunits, and viral vectors [88][89]. Vaccine components can be formulated onto solid microneedles as coatings through a variety of dry and wet coating techniques, and these coatings can be further layered to incorporate both antigen and adjuvant [90]. Dissolving microneedles may contain antigen distributed throughout the polymer matrix forming the needle or encapsulated within nano- or microparticles in the needle [22].

1.6 Polymers for buccal delivery systems

The main limiting factor for using the buccal route for drug delivery is the limited retention at the site of absorption as the dosage form can be washed away by the salivary flow. With the use of mucoadhesive polymers in the dosage form, the drug is localized in a particular region and the retention time

1. Introduction

is prolonged thereby increasing bioavailability [91]. Several dosage forms containing mucoadhesive polymers have been developed for buccal delivery [92]. Buccal films are the most recently developed dosage form for buccal administration. Buccal films have more flexibility and comfort compared, for example, to buccal tablets. In addition, they are more resistant to saliva wash out in comparison to oral gels [20]. Many polymers have good bioadhesive properties and could be potential excipients for use in buccal films [93]. The ideal polymer should have good mucoadhesive properties, form a flexible continuous film of appropriate tensile strength; after hydration the degree of swelling should be limited otherwise it could lead to a decrease in adhesive strength and possibly a loss of adhesion. The main polymers used as buccal films for drug delivery are summarized in **Table 3**.

Table 3: Various categories of mucoadhesive polymers.

<i>Type</i>	<i>Example Of Mucoadhesive Polymers</i>
NON-IONIC POLYMERS	Hydroxy ethyl cellulose, hydroxypropyl cellulose, polyvinyl pyrrolidone, polyvinyl alcohol, polyethylene oxide, eudragit analogs,
ANIONIC POLYMERS	Sodium alginate, sodium carboxy methylcellulose, carbopol
CATIONIC POLYMER	Chitosan

1. Introduction

Solvent evaporation method is widely used for the preparation of buccal films. Drug and polymer are dissolved in the solvent mixture, then the solution obtained is laminates and finally dried. This technique is time consuming because of the long processing and poses some concerns with the environment due to the use of different types of organic solvents. Hot-melt extrusion could also be used to overcome some drawbacks; hot-melt extrusion consists in applying heat and pressure to melt polymer and drug and forcing it through an orifice in a continuous process [20].

Chitosan is a cationic polymer widely used for mucoadhesive formulations. It is produced from deacetylation of chitin, the second-most abundant polysaccharide in the world [94]. Chitosan exhibits several favorable properties such as biodegradability, biocompatibility, bioadhesion, and antifungal/antimicrobial properties. Chitosan was also studied to have a significant enhancing effect on permeation of drugs across the buccal mucosa [95].

Hydroxypropyl methylcellulose (HPMC) is widely implemented in pharmaceutical manufacturing as a binder, thickening agent, hydrophilic matrix material and film-forming material. It is classified into several grades based on viscosity, degree of hydroxypropyl substitution and degree of methoxy substitution [96]. HPMC is nowadays the most common cellulose ether used to form swellable-soluble matrices. It is a water-soluble hydrophilic, non-ionic cellulose whose gel is stable over the pH range 3.0–11.0 and is enzyme resistant.

1. Introduction

HPMC is used to provide the release of a drug in a controlled manner. Its classification as GRAS by the FDA is one reason why the polymer is widely used [97].

Cellulose and its derivatives have a long history in pharmaceutical application. Nanofibrillar cellulose (NFC) (also cellulose nanofibers, nanocellulose, microfibrillated or nanofibrillated cellulose) has been investigated as a renewable bio-based polymer for various applications, as well as in the pharmaceutical field [98]. NFC has been generally regarded as a safe, non-toxic and biodegradable material. The material consists of simple cellulose fibrils or their bundles, whose diameter is typically in the range of 24-40 nm and the length of the fibers can exceed 1 μm [99]. Cellulose nanofibers are usually produced from never-dried cellulose pulp fibers because the drying of cellulose is known to have a negative effect on nanofibril fabrication [100]. The effects of drying on cellulose fiber structure are usually referred to as hornification. As a consequence of this hornification, the swelling capacity of the fibers decreases and they become stiffer, with a poorer water holding capability and reduced strength properties [101] [102] [103]. These phenomena are reflected in changes in the pore size distribution of the fibers due to the collapsing and closure of pores [104], decreases in water retention value and the fiber saturation point and an increase in crystallinity [105] [106] [107]. Hornification is an irreversible phenomenon and rewetting does not restore the properties of the initial material. Chemical pretreatments can be used to

1. Introduction

facilitate microfibrillation, promote swelling of the fiber cell wall and reduce the formation of hydrogen bonds between the microfibrils. Thus, they are also possible to reduce the effects of hornification and enhance the microfibrillation of dried or recycled cellulose fibers [100].

Garcia et al. [108] have described previous that enzymatic pretreatment of the cellulose could compensate for the hornification effect, it has also been shown that once-dried and never-dried cellulose will produce similar nanofibrils in TEMPO [(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl]-mediated oxidation if the charge density is high enough (1.5 mmol/g); on the other hand, never-dried fibers disperse into water more easily than once-dried fibers with a lower COOH content [109]. TEMPO-mediated oxidation, which converts the primary hydroxyl groups of cellulose into carboxyl groups, is one of the most efficient methods for facilitating the microfibrillation of cellulose [110] [111] [112]. In this way, it is possible to produce anionic NFC (ANFC) fibers; in fact, the surface of native NFC fibers can be chemically modified with e.g. TEMPO [(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl] oxidation to contain negatively charged carboxyl groups directly on the fiber surface [109]. Further, native as well as anionic TEMPO oxidized NFC grades are biocompatible and nontoxic in various in vitro cell models making them appealing for pharmaceutical applications [113] [114].

2. AIM OF THE THESIS

2. Aim of the thesis

The aim of the project was to test the possibility to administer model polysaccharide antigens by non-invasive administration routes, such as across the buccal mucosa, using a suitable formulation and strategies of enhancing absorption.

The buccal region of the oral cavity is an attractive target for the administration of several drugs and therapeutic agents including vaccines [20] since buccal immunization can induce both systemic and local immune responses. However, one of the main challenges of non-invasive antigen administration is to overcome the barrier, considering also that antigens are characterized by high molecular weight. Moreover, saliva washout limits the timespan of the drug in the oral cavity, as it removes the drug from the application site further into the gastrointestinal tract. These disadvantages can be partially overcome by enhancement of absorption using mucoadhesive drug formulation [115] or chemical/physical enhancers [116]. For this reason, fluorescently labelled dextrans (FITC-dextran) of different molecular weights (4 to 150 kDa) were used as polysaccharide model antigens and porcine esophageal mucosa, an accepted model of human buccal mucosa [117], was used as barrier. The optimization of a suitable formulation and the efficacy of an enhancing approach, such as a chemical penetration enhancer or microneedles arrays, were studied. In particular chitosan, hydroxypropyl methyl cellulose (HPMC) and anionic type nanofibrillar cellulose (ANFC) were used to produce and test suitable buccal delivery systems in the form of gel or film. Moreover, a

2. Aim of the thesis

minimally invasive approach was applied: fluorescent dextrans were loaded in dissolving microneedles, of different length, and applied to buccal mucosa. To supporting permeation data and to try to understand dextrans permeation mechanism, two photon microscopy technique was used an innovative technique allowing three-dimensional and deep tissue imaging, enabled dextrans visualization in the tissue.

3. MATERIALS AND METHODS

3.1 Materials

Fluorescein isothiocyanate labelled dextrans of 4 (FD-4), 20 (FD-20), 40 (FD-40), 70 (FD-70) and 150 (FD-150) kDa molecular weight (m.w.) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium taurocholate (NaTC) was from Sigma-Aldrich (St. Louis, MO, USA). Caprylic acid (C8) and capric acid (C10) were from Sigma-Aldrich (St. Louis, MO, USA), lauric acid (C12) from Merck (Darmstadt, D). Polyvinylpyrrolidone 10 000 m.w. were from Sigma-Aldrich (St. Louis, MO, USA). Chitosan 70 000 Da m.w. 85 % deacetylation was obtained from ACEF (Fiorenzuola D'Arda, PC, Italy). Hydroxypropyl methyl cellulose (HPMC) K4 was from Colorcon. Glycerin and Acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2.7% Anionic nanofibrillar cellulose (ANFC) hydrogel was provided by UPM-Kymmene Corporation (Finland). Dulbecco's Phosphate Buffered Saline (10x) concentrate without calcium and magnesium was purchased from Gibco (UK).

3.2 Methods

3.2.1 Tissue preparation

Pig esophageal epithelium was prepared according to the protocol described by Diaz Del Consuelo et al [117]. Fresh esophagi were obtained from a local slaughterhouse and transported to the laboratory in isotonic buffer (PBS, pH: 7.4). The esophageal mucosa was separated from the outside muscular layer with a scalpel. The mucosa was cut longitudinally and then excised in pieces of adequate dimensions. The epithelium was peeled off from the connective tissue after immersion in ultrapure water for 120 seconds at 60-65 °C. The isolated samples were frozen at -20°C until use. Before use, the tissue was thawed by soaking in PBS at room temperature.

3.2.2 Preparation of chitosan/HPMC gels and films

The composition of gels, used as such and for films preparation are given in **Table 4**.

3. Materials and methods

Table 4: Chitosan/HPMC and chitosan/HPMC containing NaTC gels composition.

Excipient	Gel chit/HPMC	Gel chit/HPMC/NaTC
<i>Chitosan</i>	1.2 g	1.2 g
<i>HPMC</i>	300 mg	300 mg
<i>NaTC</i>	-	102 mg
<i>Glycerin</i>	2 ml	2 ml
<i>Water</i>	28 ml	28 ml
<i>Acetic acid 1%</i>	0.56 ml	0.56 ml

HPMC=Hydroxypropyl methyl cellulose; NaTC= Sodium taurocholate

The polymers were accurately weighed and dispersed in water. Acetic acid was dropped on the polymers dispersion in water and kept under weak magnetic stirring for two days. After 48 hours glycerine was added and, in order to eliminate air bubbles, the gel obtained was left still at room temperature for 24 hours. For the chit/HPMC/NaTC gel, NaTC was added when the entire gel was formed, previously dissolved in a little amount of water (i.e. 100 μ l). Finally, FD-20 was added as the last component with a final concentration of 2 mg/ml concerning gels used as such and 0.66 mg/ml in the initial gel used to prepare a dry film.

For film production, a layer of 1.5 mm of gel was placed on a polyester sheet and dried at room temperature for 24 hours to obtain a buccal film.

3.2.3 Preparation of anionic nanofibrillar cellulose film

1% ANFC hydrogel formulation used for film production was prepared in 10 ml syringes. The hydrogel was homogenized with fluorescein isothiocyanate labelled dextrans water solution, by mixing for 10 min inside two attached syringes. 2 ml of the hydrogel formulation was placed in a 3D printed (Ultimaker 2+ 3D printer, Netherlands) round PLA mold with a diameter of 4 cm on top of a flat Teflon surface and oven-dried at 45 °C. After 1 hour, additional 2 ml of the hydrogel formulation was placed on top of the first layer hydrogel and oven-dried for 1 h at 45 °C. Finally, the last step of the drying process was made at room temperature for 48 h. Fluorescent dextrans concentrations used were 0.5-1-5 mg/ml on the initial hydrogel.

3.2.4 Preparation of PVP dissolving microneedles

The preparation of biodegradable microneedles has been performed in different steps by Professor Lai and Doctor Cardia from the University of Cagliari, Italy. Firstly, a water solution of FD 70 or FD 150 (25 mg/ml) was mixed with a dispersion of PVP (0.8 g/ml). The obtained dispersion was dropped in silicon templates and centrifugated at 4000 rpm for 15 minutes to allow the penetration and homogeneous distribution of the suspension in the microneedles tips. Finally, after 24 hours of drying at room temperature, the template was filled with a dispersion of PVP (0.8 g/ml) in order to create a solid base for microneedles patch. Last drying was done at room temperature

for 48-72 hours. Microneedles patches obtained were observed with two-photon microscopy technique, as such and after application onto tissue; they were also used to perform permeation experiments.

3.2.5 Release experiments

The release of FITC-dextran from gel and films was tested with an incubator shaker Titramax 1000/Incubator 1000 (Heidolph, Germany) at 37 °C 300 rpm. The film dosage forms were cut into squares with an area of 1 cm² or discs with an area of 0.6 cm² and placed inside glass vials. The glass vials were filled with 10 ml of phosphate buffer saline (10 x DPBS) and the film pieces remained immersed in the release buffer throughout the release study. 0.2 ml samples were collected at predeterminate time points up to 60 min or 180 min and replaced with the same volume of fresh buffer.

3.2.6 *In vitro* permeation studies

In vitro permeation studies were conducted across the porcine esophageal epithelium. The tissue was mounted, using a regenerated cellulose filter (0.45 µm, pore size) as inert support, in Franz's type diffusion cells (DISA, Milan, Italy), with a diffusion area of 0.6 cm² and a receptor volume of about 4 ml filled with degassed pH 7.4 PBS, kept under magnetic stirring during the

3. Materials and methods

experiment. The donor compartment was filled with the appropriate formulation (see single sections).

Samples of 300 μ l were collected from the receptor side at 60 min intervals up to 6 or 7 h and replaced with the same amount of fresh PBS.

3.2.7 Analytical method

The concentration of FDs in samples was determined using a Spark multimode microplate reader (TECAN, Mannendorf, CH) or automated multimode plate reader VarioSkan Flash (Thermo Fisher Scientific, Finland), using 96-multiwell plates flat bottom, black polystyrene (Corning Life Sciences, Corning, NY, USA). The excitation and emission λ were 490 and 520 nm, respectively. A standard curve was developed by plotting different concentrations of FDs (ng/ml) versus their fluorescence values. The analytical method was validated for specificity (absence of interference from tissue), linearity and limit of quantification for each dextran.

3.2.8 Data elaboration

From the linear portion of the permeation profiles the flux was calculated, as the slope of the regression line. Then, the Permeability coefficient was obtained using the following relationship:

3. Materials and methods

$$P=J/CD \quad (1)$$

where CD ($\mu\text{g ml}^{-1}$) is donor concentration and J ($\mu\text{g cm}^{-2} \text{ h}^{-1}$) is dextrans flux across the membrane. The enhancement factor (EF) was calculated as the ratio of the permeability coefficient value in the presence and absence of the penetration enhancer.

The significance of the differences among the results was assessed using one-way ANOVA followed by Duttet's test.

3.2.9 Two-photon microscopy

Specimens were analyzed with a Two-Photon Microscope Nikon A1R MP+ Upright equipped with a femtosecond pulsed laser Coherent Chameleon Discovery (80 MHz repetition rate, tunable wavelength output 660-1320 nm). A 25x water dipping objective with numerical aperture 1.1 and 2-mm working distance was employed for focusing the excitation beam and for collecting the two-photon excited fluorescence (TPEF) signal, which is detected by non-descanned high sensitivity GaAsP detectors. TPEF imaging of both ex-vivo porcine esophagus and micro-needles are collected with excitation light at 850 nm. To prevent their structural deformation, the micro-needles were analyzed in absence of water.

4. RESULTS AND DISCUSSION

4.1 Analytical method validation

Calibration parameters and detection (LOD) and quantitation (LOQ) limits for the determination of fluorescent dextrans by the proposed method are shown in **Table 5**. Calibration curves were run using five standard solutions for each dextran.

Table 5: Data for calibration curves, precision and accuracy, LOQ and LOD of the analytical method.

Concentration range 0.05-0.2 (µg/ml)								
Dextran	Regression equation	R ²	Nominal concentration (µg/ml)	Fitted value (µg/ml)	RE %	RSD %	LOQ* (µg/ml)	LOD* (µg/ml)
FD-4	y=56.569x-434.49	1.00	0.05	0.051±0.001	2.08	3.32	0.038	0.012
			0.1	0.0098±0.002	1.65	2.81		
			0.2	0.205±0.00	2.76	0.00		
FD-20	y=59.203x+174.07	1.00	0.05	0.049±0.003	1.31	5.56	0.026	0.009
			0.1	0.101±0.0015	1.10	1.51		
			0.2	0.193±0.003	3.34	1.86		
FD-40	y=51.717x-624	1.00	0.05	0.049±0.0008	0.30	2.10	0.043	0.014
			0.1	0.0091±0.000	8.12	0.00		
			0.2	0.202±0.000	1.14	0.00		
FD-70	y=52.576x-597.17	1.00	0.05	0.051±0.0022	2.26	5.51	0.026	0.009
			0.1	0.098±0.0007	1.69	0.99		
			0.2	0.204±0.003	2.02	0.32		
FD-150	y=58.663x-304.53	1.00	0.05	0.049±0.0008	2.91	1.77	0.015	0.005
			0.1	0.102±0.0005	1.51	0.5		
			0.2	0.202±0.001	1.26	1.37		

4. Results and discussion

Table 2: Data for calibration curves, precision and accuracy, LOQ and LOD of the analytical method (continued).

Concentration range 0.2-0.8 (µg/ml)

Dextran	Regression equation	R ²	Nominal concentration (µg/ml)	Fitted value (µg/ml)	RE %	RSD %	LOQ* (µg/ml)	LOD* (µg/ml)
FD-4	y=56.114x-482.75	1.00	0.2	0.198±0.011	1.14	5.63	0.132	0.043
			0.4	0.382±0.0007	4.50	0.20		
			0.8	0.799±0.008	0.14	1.08		
FD-20	y=67.054x-743.44	1.00	0.2	0.193±0.003	3.34	1.86	0.192	0.019
			0.4	0.395±0.000	1.44	0.00		
			0.8	0.775±0.006	3.26	0.87		
FD-40	y=61.518x-725.6	1.00	0.2	0.206±0.006	3.35	3.88	0.117	0.039
			0.4	0.407±0.012	1.66	3.36		
			0.8	0.806±0.016	0.79	2.12		
FD-70	y = 54,64x - 1201	1.00	0.2	0.204±0.003	2.02	0.32	0.070	0.023
			0.4	0.394±0.005	1.51	1.42		
			0.4	0.805±0.007	0.25	0.86		
FD-150	y=68.412x-2152	1.00	0.2	0.202±0.001	1.26	1.37	0.038	0.013
			0.4	0.404±0.006	0.91	1.68		
			0.8	0.803±0.004	0.37	0.49		

4.2 Buccal permeation of FITC-dextrans from solutions

Dextrans are branched polysaccharides, naturally produced by bacteria, made of many glucose molecules linked by α -1,6 glycosidic linkages while branches begin from α -1,3 linkages. Dextrans have high water solubility and are considered very safe molecules.

Fluorescein isothiocyanate (FITC)-dextrans are synthesized by coupling fluorescein isothiocyanate (5-isomer) to dextrans molecules. FITC-dextran has an excitation maximum of 493 nm and an emission maximum of 518 nm. Maximal fluorescent intensity is observed at $\text{pH} \geq 7.5$.

FITC-dextran is mainly used for studying permeability and microcirculation. It can also be used as a molecular size marker, for studies of drug delivery and more.

In this work, several fluorescent dextrans were used as molecular model for antigens, because they are high molecular weight molecules. FITC-dextrans from 4 to 150 KDa molecular weight were tested across porcine esophageal mucosa with and without chemical absorption enhancers.

4. Results and discussion

4.2.1 Effect of molecular weight on dextrans permeation

In vitro permeation studies were conducted as described in section 3.2.6.

The donor compartment was filled with 400 μ L of FD solution in PBS pH 7.4 (2 mg/mL) without any treatment. Samples of 300 μ L were collected from the receptor side at 60 min intervals up to 7 h and replaced with the same amount of fresh PBS.

Figure 11 (a) shows the diffusion profiles of dextrans without any treatment, i.e. in passive condition, from a 2 mg/ml solution in pH 7.4 PBS.

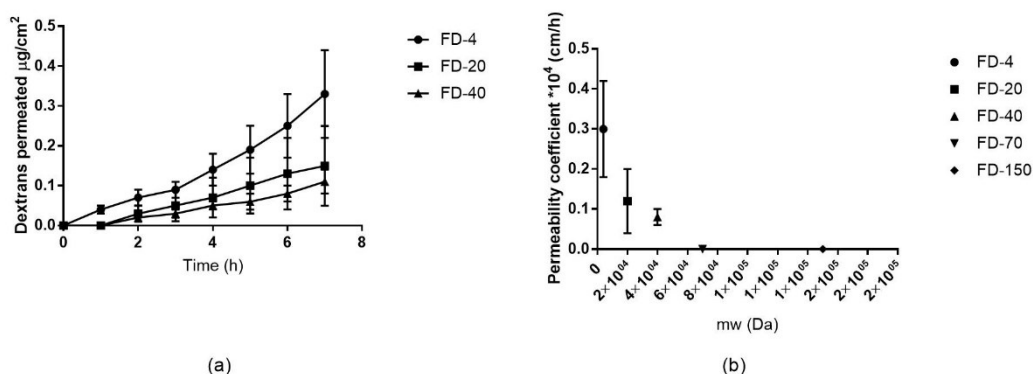


Figure 11: (a) Passive permeation profiles of dextrans from 2 mg/ml solutions in PBS pH 7.4. The amounts of FD-70 and FD-150 found in the receptor compartment were below the limit of quantification of the analytical method. (b) Permeability coefficient of dextrans as a function of molecular weight. Mean values $\pm$ sem.

Although the total amount of FDs recovered in the receptor phases was very low, it is possible to observe that dextran diffusion is roughly in inverse relation to its molecular weight; the higher molecular weight dextrans, FD 70 and FD 150, did not cross the membrane in detectable amounts. The permeation parameters obtained are reported in **Table 6**.

4. Results and discussion

Table 6: Permeation parameters of dextran across pig esophageal epithelium. Mean values $\pm$ sem.

Dextran	Enhancer	Flux ($\mu\text{g cm}^{-2} \text{ h}^{-1}$)	Permeability coefficient * 10^{-4} (cm h^{-1})	Number of replicates
FD-4	None	0.06 ± 0.02	0.30 ± 0.12	7
FD-20	None	0.02 ± 0.01	0.12 ± 0.09	6
FD-40	None	0.016 ± 0.003	0.08 ± 0.03	7
FD-70	None	n.d.	-	4
FD-150	None	n.d.	-	6

Concerning the effect of molecular weight on dextran permeation, the value of permeability coefficient decreases from 4 000 to 40 000 Da, as shown in **Figure (b)**. Because of the low passive permeability of high molecular weight dextrans, the following experiments were focused on the identification of the effect of penetration enhancers.

4.2.2 Effect of sodium taurocholate

Buccal drug delivery has high patient acceptability due to the elimination of pain associated with injections and nausea and vomiting induced through oral administration; it is more accessible for the application and removal of a dosage form. Unfortunately, buccal mucosa is poorly permeable and so unable to give rapid absorption [118]. Bile salts have been extensively investigated for their ability to enhance buccal penetration of drugs, therapeutic agents and/or tracer molecules in general. Their effect is supposed to be reversible and dependent on their concentration.

4. Results and discussion

The aim of this part of work was to investigate the possibility to improve the permeation across buccal mucosa of fluorescent dextrans with different molecular weights co-administrating sodium taurocholate, a bile salt.

In vitro permeation studies were conducted as explained in section 3.2.6. The donor compartment was filled with 400 μ L of FD solution in PBS pH 7.4 (2 mg/mL) and NaTC 100 mM. Samples of 300 μ l were collected from the receptor side at 60 min intervals up to 7 h and replaced with the same amount of fresh PBS.

Sodium taurocholate has been demonstrated to increase buccal absorption of peptides as well as the *in vitro* and *in vivo* permeability of porcine buccal mucosa to fluorescein isothiocyanate-dextran (m.w. 4, 10, 20 and 40 kDa) [119] [120]. The result obtained in the present work confirms the ability of 100 mM NaTC to increase the transport of the lower m.w. dextrans and promote the transport of FD-70 and FD-150. **Figure 12** reports the permeation profiles of the different dextrans in the presence of 0.1 M NaTC, the respective permeation parameters are reported in **Table 7**. NaTC can increase FD-4 transport across the esophageal epithelium by almost two orders of magnitude, whereas the EF obtained for FD-20 and FD-40 is 48 and 49 respectively. Interestingly, NaTC promotes FD-70 and FD-150 transport, unable to cross the mucosa in detectable amounts by passive diffusion.

4. Results and discussion

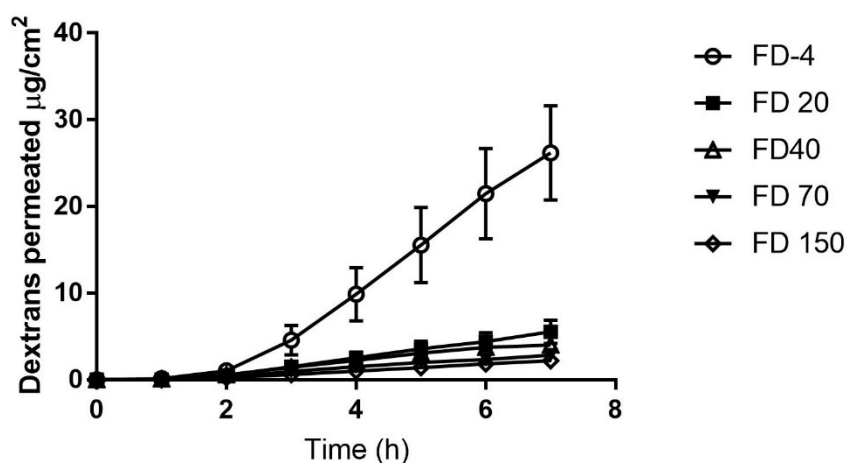


Figure 12: Permeation profiles of dextrans in the presence of 0.1 M sodium taurocholate. Mean values $\pm$ sem.

The high increment observed on FD-4 with NaTC (much higher effect than that obtained on the other dextrans) can be explained by the increased permeability across the cellular membrane produced by a high concentration of bile salts, which can be more relevant for the lower molecular weight dextran. Another hypothesis can be the formation of NaTC micelles that can host – in their hydrophilic portion - a small molecular weight (radius) molecule such as FD-4 but not the other dextrans.

4. Results and discussion

Table 7: Permeation parameters of dextran across pig esophageal epithelium (mean values $\pm$ sem) using bile salts as penetration enhancers.

Dextran	Enhancer	Administration mode ^a	Flux ($\mu\text{g cm}^{-2} \text{ h}^{-1}$)	Permeability coefficient *10 ⁻⁴ (cm h^{-1})	EF	Number of replicates
FD-4	None	-	0.06 $\pm$ 0.02	0.30 $\pm$ 0.12	-	7
	NaTC	Co-administration	5.84 $\pm$ 1.01 **	29.21 $\pm$ 5.06	96	7
FD-20	None	-	0.02 $\pm$ 0.01	0.12 $\pm$ 0.09	-	6
	NaTC	Co-administration	1.20 $\pm$ 0.21 **	5.99 $\pm$ 1.34	48	6
FD-40	None	-	0.016 $\pm$ 0.003	0.08 $\pm$ 0.03	-	7
	NaTC	Co-administration	0.8 $\pm$ 0.05	4.00 $\pm$ 0.26	49	11
FD-70	None	-	n.d. ^a	-	-	4
	NaTC	Co-administration	0.46 $\pm$ 0.04	2.29 $\pm$ 0.22	-	9
FD-150	None	-	n.d. ^a	-	-	6
	NaTC	Co-administration	0.40 $\pm$ 0.03	2.01 $\pm$ 0.15	-	8

^a The concentrations found were below the limit of quantification of the analytical method.

**p<0.001 significantly different from the respective passive condition.

4.2.3 Effect of fatty acids

Fatty acids have been extensively studied as skin penetration enhancers for the development of successful topical and transdermal delivery systems of different classes of drugs. Despite the interest obtained in transdermal delivery, they have received limited attention for buccal delivery, focused mainly on oleic acid; oleic acid was successfully used to increase the permeation of small molecules such as propranolol [121], buspirone [122] and lidocaine [123], whereas it was ineffective in the case of 5-FU [124] and didanosine [125].

4. Results and discussion

Regarding molecules with higher molecular weight, the literature is even more limited [126].

A recent study by Padula et al. [127] investigated the effect of a series of medium and long chain fatty acids on the permeation of high molecular weight and hydrophilic model molecule, fluorescein isothiocyanate labeled dextran (FD-4, m.w. 4 kDa), across porcine esophageal mucosa. This work reports the first attempt to find a structure-activity relationship on the effect of fatty acids on the mucosa permeation considering chain length, presence of double bonds and their configuration. For the purpose of this thesis caprylic acid (C8), capric acid (C10), and lauric acid (C12) were used.

In vitro permeation studies were basically conducted as reported in section 3.2.6. The different techniques applied using fatty acids are explained below:

Co-administration: these experiments were performed using C12. A donor solution containing lauric acid 4% (w/v) and FD-20 2 mg/ml dissolved in ethanol 60% were prepared and 400 µl of this solution were applied to the mucosa. The duration of the experiments was 6 h.

Pre-treatment: 20 µl of fatty acid - either 5% (lauric acid, C12) or 10% (w/v) (capric and caprylic acid, C8 and C10) - in ethanol solution were applied, in non-occluded condition, to the epithelium; after 1 h pre-treatment, the donor compartment was filled with 400 µl of FD solution (2 mg/ml), as described above. The diffusion of the permeant was monitored up to 7 h.

4. Results and discussion

Firstly, lauric acid was tested in co-administration with FD-20, in 60% water:ethanol solution. The enhancing effect of the co-administration of lauric acid resulted lower compared to NaTC, as shown in **Table 7**, so fatty acids were then tested as pre-treatment.

Among the fatty acids studied, C8 (applied as 10% solution) resulted as the best performer. Pre-treatment consisted in the application of a small volume (20 μ l) of fatty acid in ethanol solution in non-occluded condition for 1 h before the donor compartment was filled with dextran solution.

The results obtained with FD-20 are reported in **Figure 13**, whereas the flux values are in where the enhancement factor values (EF), calculated as the ratio of permeability coefficient with and without the enhancer, are reported as well.

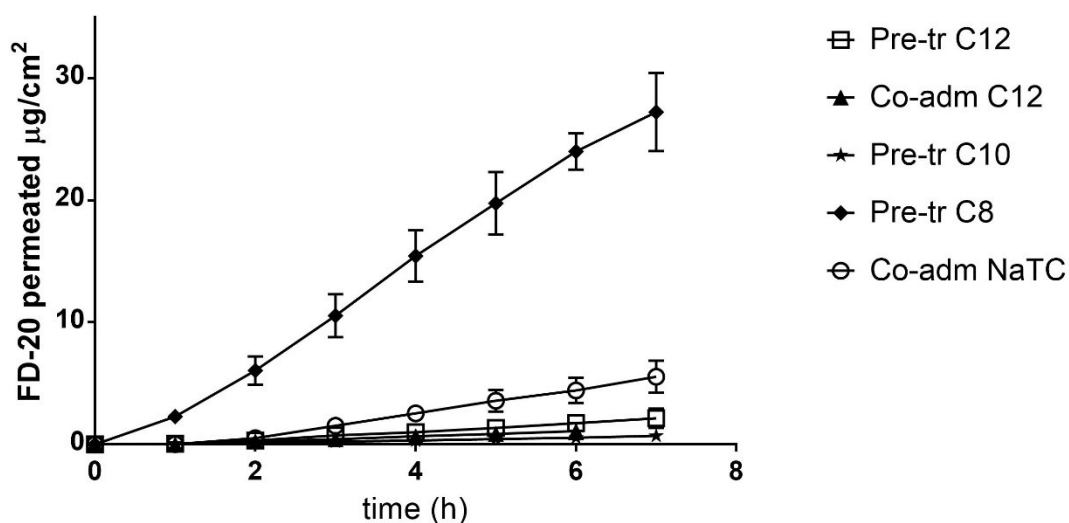


Figure 13: FD-20 permeation profile from a solution 2 mg/ml applied in different conditions of pre-treatment or co-administration. Mean values $\pm$ sem.

4. Results and discussion

Among the fatty acids tested, C8 appears as the best performing, followed by C12 and C10. Due to these results, only C8 was tested on the higher molecular weight dextrans. The respective results are reported in **Figure 14**.

Table 8: Permeation parameters of dextran across pig esophageal epithelium (mean values $\pm$ SEM) using fatty acids and bile salts as penetration enhancers.

Dextran	Enhancer	Administration mode ^a	Flux ($\mu\text{g cm}^{-2} \text{h}^{-1}$)	Permeability coefficient *10 ⁻⁴ (cm h^{-1})	EF	Number of replicates
FD-4	None	-	0.06 $\pm$ 0.02	0.30 $\pm$ 0.12	-	7
	C8	Pre-treatment ^a	13.36 $\pm$ 1.96	66.78 $\pm$ 9.78	219	5
	NaTC	Co-administration	5.84 $\pm$ 1.01 ***	29.21 $\pm$ 5.06	96	7
FD-20	None	-	0.02 $\pm$ 0.01	0.12 $\pm$ 0.09	-	6
	C8	Pre-treatment	4.24 $\pm$ 0.47 **	21.22 $\pm$ 2.37	171	6
	C10	Pre-treatment	0.13 $\pm$ 0.06	0.64 $\pm$ 0.29	5	3
	C12	Pre-treatment	0.39 $\pm$ 0.14	1.93 $\pm$ 0.69	16	11
	C12	Co-administration	0.22 $\pm$ 0.11	1.12 $\pm$ 0.53	9	3
	NaTC	Co-administration	1.20 $\pm$ 0.21 ***	5.99 $\pm$ 1.34	48	6
FD-40	None	-	0.016 $\pm$ 0.003	0.08 $\pm$ 0.03	-	7
	C8	Pre-treatment	1.10 $\pm$ 0.07	5.49 $\pm$ 0.35	68	6
	NaTC	Co-administration	0.8 $\pm$ 0.05	4.00 $\pm$ 0.26	49	11
FD-70	None	-	n.d. ^b	-	-	4
	C8	Pre-treatment	0.80 $\pm$ 0.03	3.99 $\pm$ 0.13	-	4
	NaTC	Co-administration	0.46 $\pm$ 0.04	2.29 $\pm$ 0.22	-	9
FD-150	None	-	n.d. ^b	-	-	6
	C8	Pre-treatment	0.59 $\pm$ 0.06	2.95 $\pm$ 0.29	-	4
	NaTC	Co-administration	0.40 $\pm$ 0.03	2.01 $\pm$ 0.15	-	8

^a Pre-treatment was performed by applying 20 μl of fatty acid - either 5% (lauric acid, C12) or 10% (w/v) (caprylic and capric acid, C8 and C10), in ethanol solution for 1 h in non-occlusive conditions. In the case of co-administration lauric acid 4% (w/v) and FD-20 2 mg/ml were dissolved in ethanol 60%.

^b The concentrations found were below the limit of quantification of the analytical method. ** $p < 0.01$, *** $p < 0.001$ significantly different from the respective passive condition.

4. Results and discussion

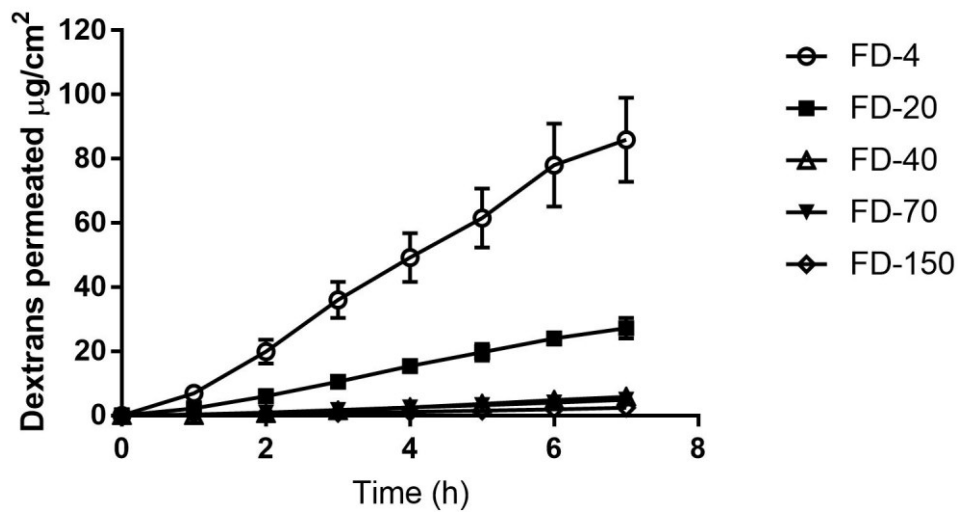


Figure 14: Permeation profiles of FD-4, FD-20, FD-40, FD-70 and FD-150 after pre-treatment with caprylic acid (C8). Mean values $\pm$ sem.

C8 enhances the transport of FD-4, FD-20 and FD-40, with an EF ranging from 68 to 219, and promotes the transport of FD-70 and FD-150, which in passive condition do not cross the mucosa in significant amounts. The permeability coefficients of FD-70 and FD-150 were very similar, indicating that the enhancing effect was comparable, regardless of the molecular weight of the permeant.

4.2.4 Two-photon microscopy

Biological tissues usually scatter light and this phenomenon makes it impossible to obtain high-resolution deep images using traditional fluorescence microscopy. Most widely used in biological imaging is fluorescence excitation by two-photon absorption. In this way even multiply scattered signal photons

4. Results and discussion

can be transferred to their origin as the result of localized non linear signal generation [128].

In order to understand the mechanisms of fluorescent dextrans permeation across tissue with and without enhancing strategies, several samples were observed with two-photon microscopy. Samples were analyzed with a Two-Photon Microscope as described in section 3.2.9.

Non treated tissue

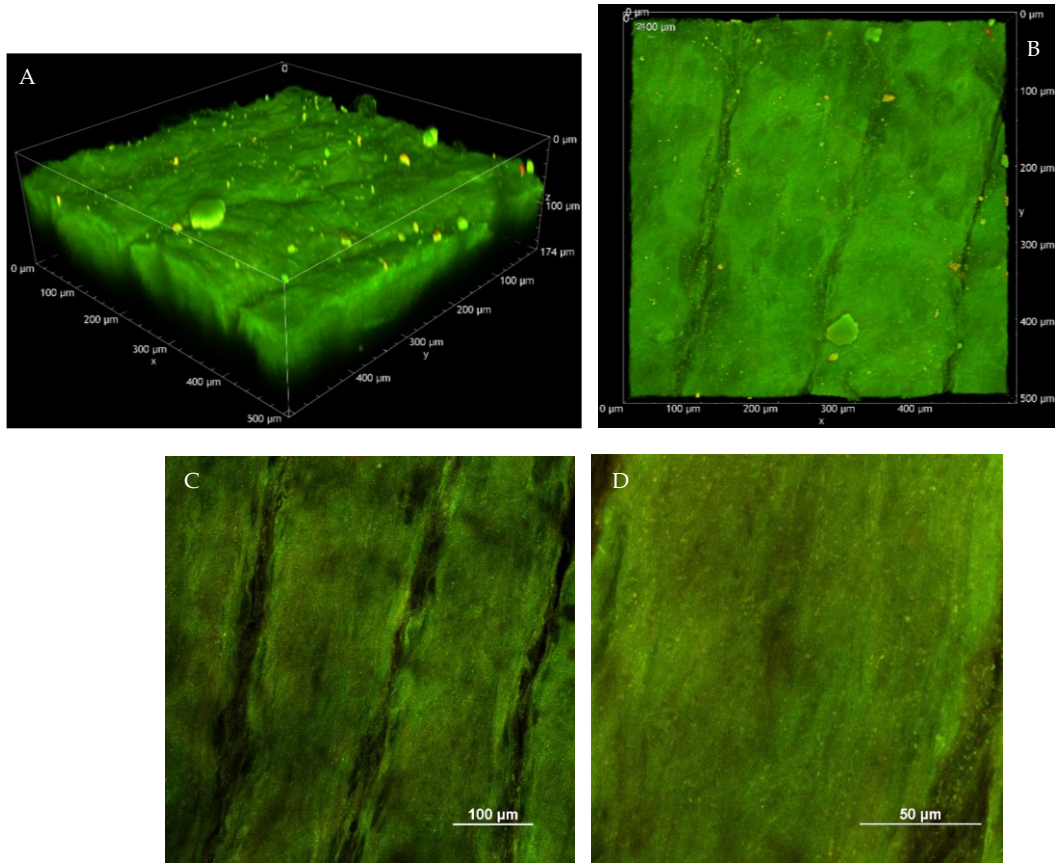


Figure 15: (A) Volume rendering overview of the untreated porcine esophagus (fibrous tissue region) reconstructed from the Z-stack, acquired with excitation wavelength of 850 nm; (B) XY view; (C) Autofluorescence image acquired 40 μm from the tissue surface (image size 500 μm x 500 μm); (D) Autofluorescence 3x zoomed image acquired 40 μm from the tissue surface (image size 165 μm x 165 μm).

4. Results and discussion

Non treated esophagus has been imaged (**Figure 15** and **Figure 16**), obtaining different structures: some of them are characterized by a fairly fibrous structure with regular grooves on the surface and only a few visible cells (**Figure 15**) while others by numerous elongated cells with an irregular shape (**Figure 17**).

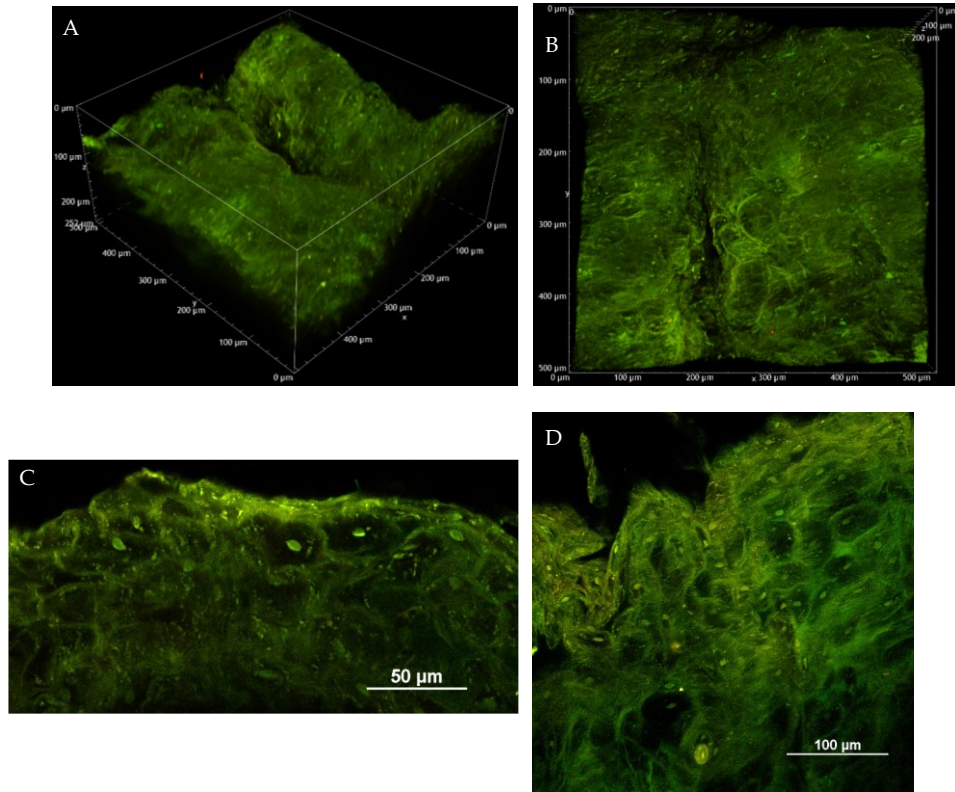


Figure 16: (A) Volume rendering overview of the untreated porcine esophagus (cellularized tissue region) reconstructed from the Z-stack, acquired with excitation wavelength of 850 nm; (B) XY view; (C) Autofluorescence image (image size 375 μm x 190 μm); (D) Autofluorescence image (image size 500 μm x 500 μm).

At higher excitation wavelength (1100 nm), it was obtained similar images (**Figure 17**). The autofluorescence spectra of tissues is collected exciting both at 850 nm and at 1100 nm (**Figure 18**): as expected, the autofluorescence signal is red-shifted when the tissue is excited at higher wavelengths.

4. Results and discussion

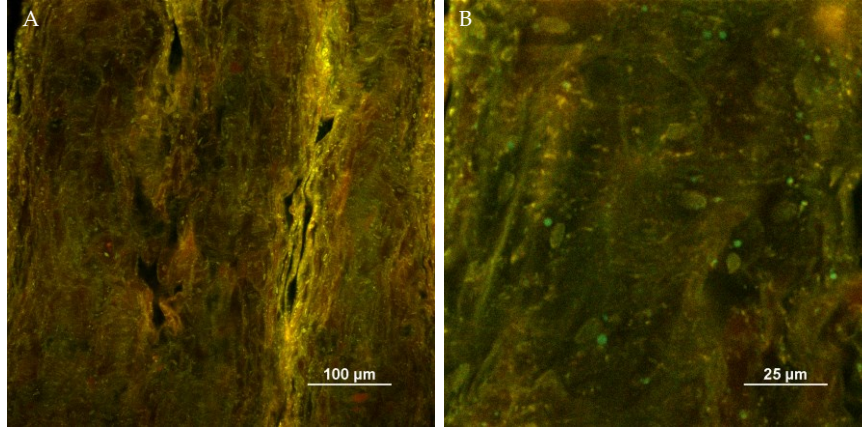


Figure 17: Autofluorescence images acquired at 1100 nm: non zoomed (A, image size: 500 μm x 500 μm) and zoomed (B, image size: 125 μm x 125 μm).

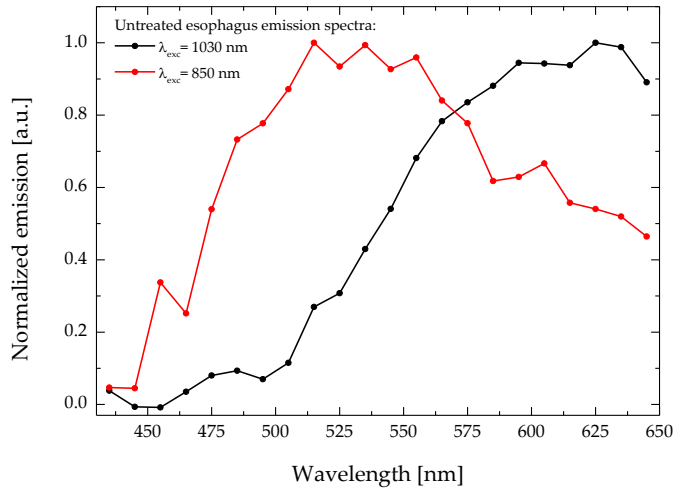


Figure 18: Normalized emission spectra of untreated esophagus recorded exciting the tissue at 850 and 1030 nm.

4. Results and discussion

Pre-treated tissue

As explained in section 4.2.3 (*Pre-treatment*), a mucosa sample was pre-treated with ethanolic solution of C8 10 %, after 1 hour, 400 μ l of a FD-70 solution 2 mg/ml were left in contact with tissue for 2 hours or 4 hours. Finally, the tissue was washed with water and visualized.

Figure 19 shows the images of the pre-treated sample with caprylic acid acquired using the excitation wavelength of 1030 nm, which allows enhancing the contrast between the tissue and fluorescein emissions.

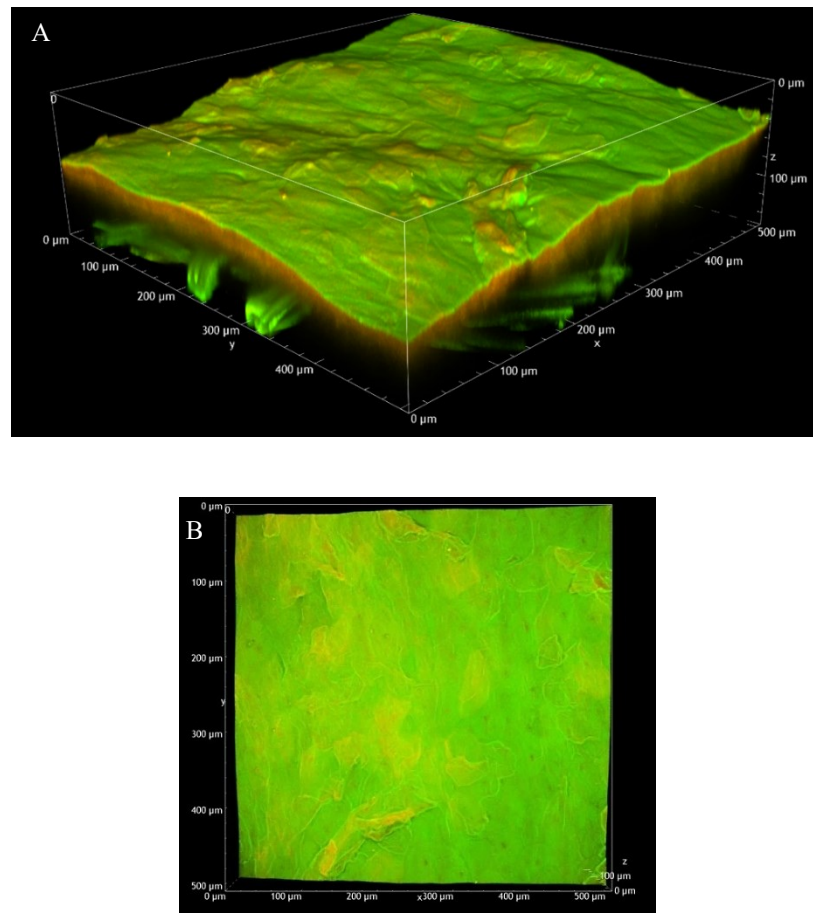


Figure 19: (A) Volume rendering overview of the pre-treated porcine esophageal epithelium, reconstructed from the Z-stack; (B) XY view. All the images have been acquired with excitation wavelength of 1030 nm.

4. Results and discussion

The Z-scan of **Figure 19 (A)** after application of FITC-dextran solution in pre-treatment condition shows that fluorescein is mainly accumulated in the superficial layers of the tissue, probably in the cytoplasm of the epithelial cells. In **Figure 19 (B)**, it is possible to distinguish some epithelial cells characterized by darker membranes and nuclei.

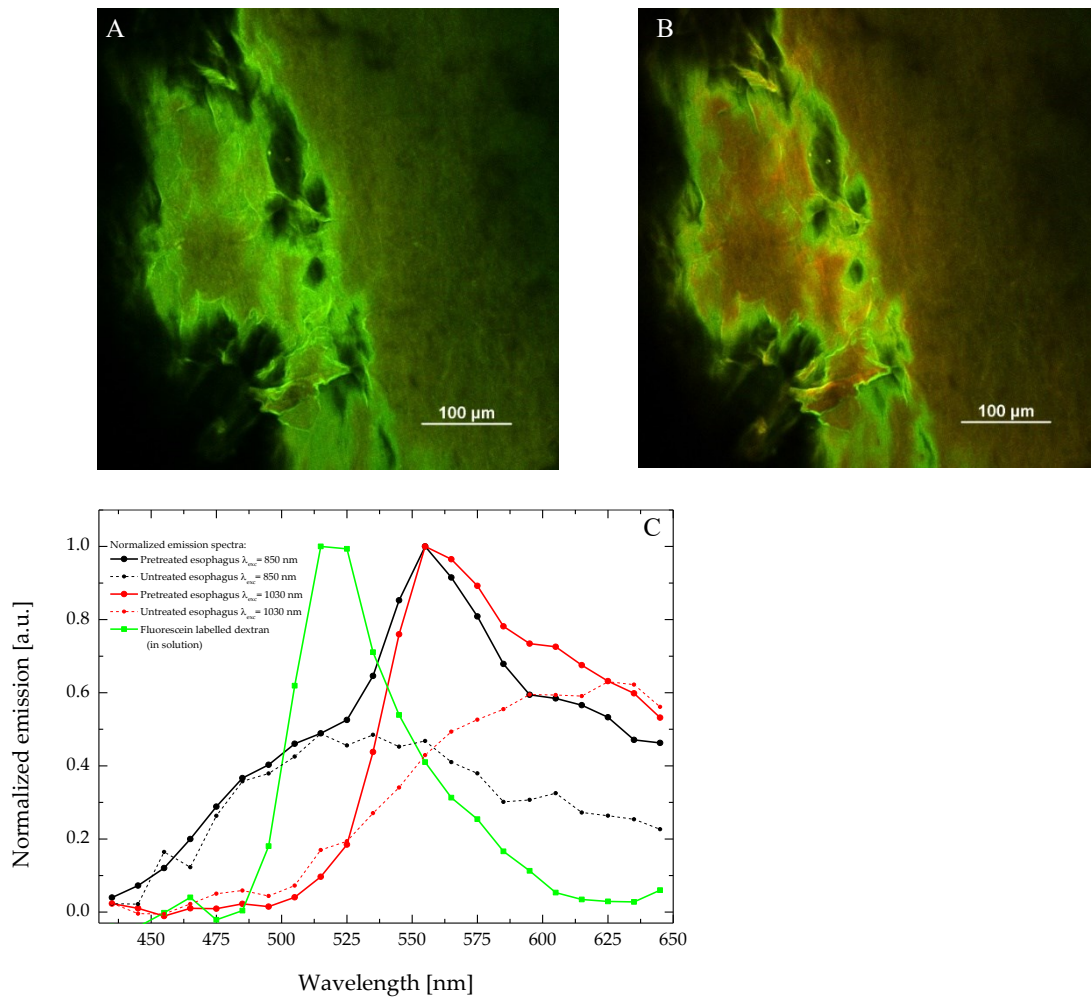


Figure 20: (A) and (B): TPEF image acquired at an intermediate section of the pretreated tissue (image size: 500 μm x 500 μm) with an excitation wavelength of 850 nm and 1030 nm, respectively; (C) comparison between normalized emission spectra of the pretreated tissue (recorded in correspondence of the (A) and (B) focal plane), untreated tissue and fluorescein labelled dextran solution.

4. Results and discussion

Figure 20 (A) and (B) show the images collected from a layer of the pre-treated tissue below the surface, obtained exciting the sample at two different wavelengths. In Figure (C), emission spectra obtained from images reported in panels A and B (black and red continuous lines) are compared with emission detected in untreated tissues (collected exciting at the same wavelength, dashed black and red lines) and with the emission of the fluorescein solution. Emission of fluorescein is red shifted of ~ 30 nm when it's loaded in the tissue. The peak at 555 nm can be safely attributed to fluorescein because it's located in the same position at both the excitation wavelengths and it's absent in the tissue autofluorescence profiles.

To verify if the fluorescent label is permeated in the inner layers of the tissue, emission spectra have been acquired from two different zoomed regions, reported in **Figure 21**.

4. Results and discussion

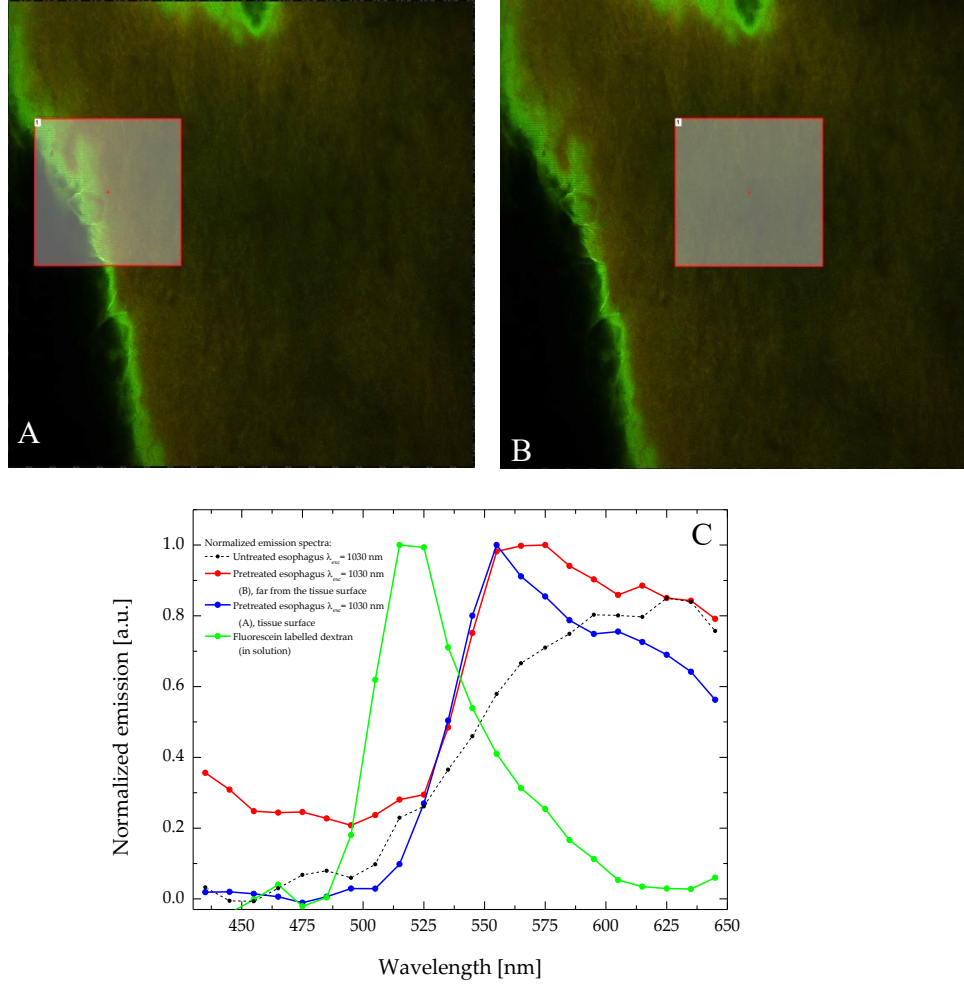


Figure 21: (A) and (B) TPEF image acquired with an excitation wavelength of 1030 nm with highlighted the regions exploited to acquire the emission spectra reported in (C); (C) comparison between normalized emission spectra of the pretreated tissue (recorded in correspondence of the two different zoomed regions reported in (A) and (B)), untreated tissue and fluorescein labelled dextran solution.

From the emission spectra reported in **Figure 21** (C), the presence of fluorescein labelled dextran is evident also in the inner layers of the tissue (peak at 555 nm).

As expected, after 2 hours the FD-70 signal is present exclusively in the most superficial layer of the mucosa. In fact, as discuss before (**Figure 14**), no FD-70 permeation was observed after 2 hours.

4. Results and discussion

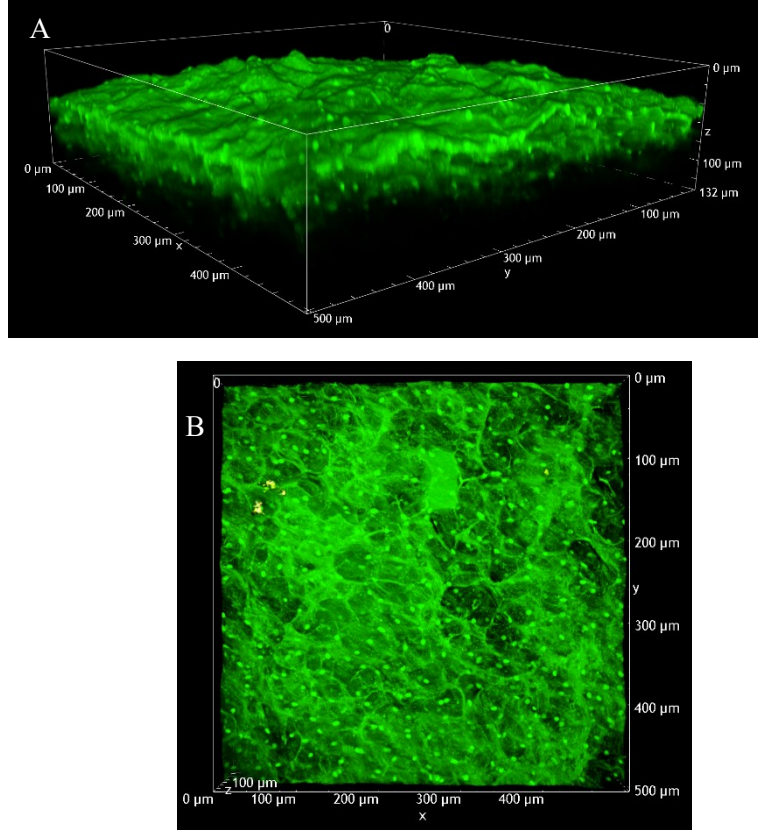


Figure 22: (A) Volume rendering overview of the pre-treated porcine esophageal epithelium, reconstructed from the Z-stack; (B) XY view. All the images have been acquired with excitation wavelength of 1030 nm.

From the volume rendering reported in **Figure 22** after 4 hours of contact with the solution, it is possible to assess that FD-70 has fully stained the first 130 μm of esophageal tissue. Probably also the underlying part of the tissue retained the fluorescent dextran, but due to the high scattering it was not possible to acquire the entire full-thickness tissue in a single Z-stack. This is also supported by the permeation data analyzed above (see **Figure 14**).

4.3 Polymeric systems for buccal delivery

4.3.1 Chitosan/HPMC gels and films

The formulations introduced in section 3.2.2 were only produced with FD-20 dextran in order to have just preliminary information.

Chitosan/HPMC gel prepared as described in **Table 4**, looks yellow and completely transparent. *In vitro* permeation experiments were performed applying 400 μ l of gel as donor in three different conditions: chitosan/HPMC gel loaded with FD-20 2 mg/ml without any treatment, chitosan/HPMC gel loaded with FD-20 2 mg/ml containing NaTC and chitosan/HPMC gel loaded with FD-20 2 mg/ml after 1 hour pre-treatment with caprylic acid. Data are plotted in **Figure 22**.

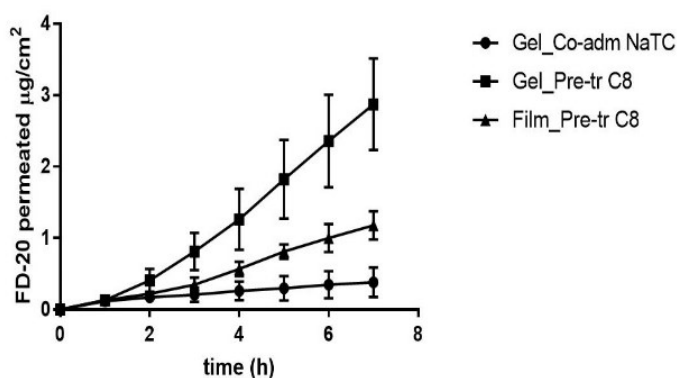


Figure 22: Permeation profile of FD-20 across porcine esophageal epithelium after application of chitosan/HPMC gels and film in different conditions. No permeation of FD-20 was observed without treatments. Mean values $\pm$ sd.

When chitosan/HPMC gel was applied as donor without further treatments, no permeation of FD-20 was observed. Otherwise, the association

4. Results and discussion

with chemical enhancers both in co-administration and in pre-treatment condition allows to observe a relevant FD-20 permeation, especially as regards tissue pre-treatment that is the better enhancing strategy.

According to these promising results, the same gels formulations were used to produce dry films for buccal delivery. The final film appears yellow, transparent, homogeneous and free from bubbles. Film thickness was 182 ± 15 μm , measured with a digital multimeter (Mitutoyo, Japan).

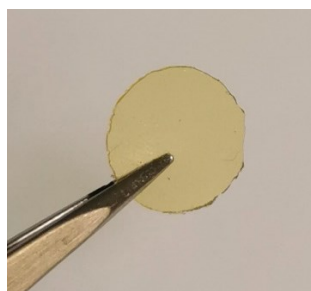


Figure 23: 0.6 cm^2 disc of chitosan/HPMC containing FD-20 used for release experiments.

A piece of chitosan/HPMC film was cut with an area of 0.6 cm^2 (see **Figure 23**) then release experiment was performed as described previously in section 3.2.6. The release profile, amount of FD-20 detected versus time, is shown in **Figure 24**.

4. Results and discussion

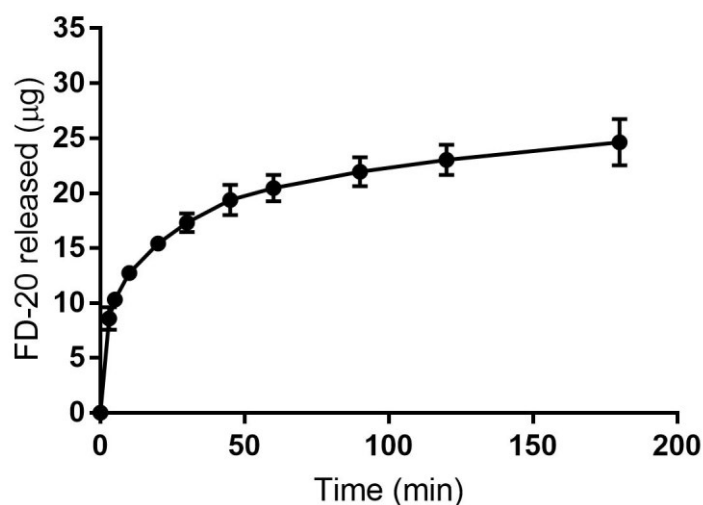


Figure 24: FD-20 release profile from chitosan/HPMC film. Amount released in μg vs time. Mean values $\pm$ sd.

The total amount loaded in each piece of tested film was 32.18 μg . FD-20 release was quite fast during the first half an hour; after 60 minutes the curve seems to reach a plateau and the last data point could lead to the interpretation that the total amount loaded was released.

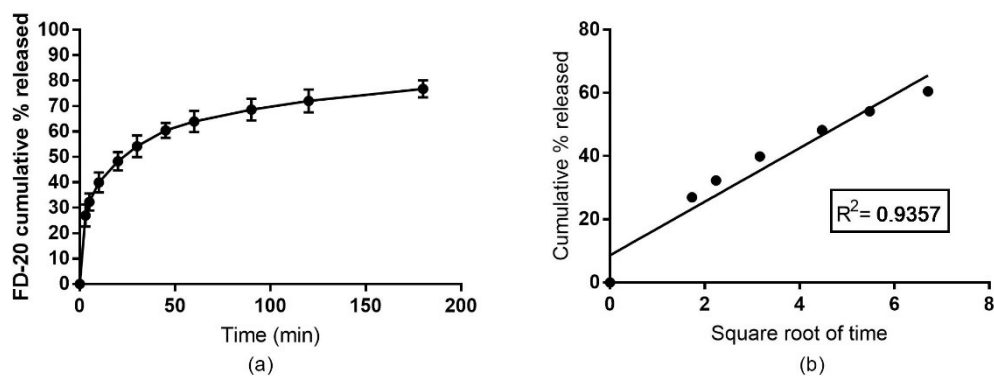


Figure 25: Panel (a) Cumulative percentage of FD-20 released from chitosan/HPMC film. Mean values $\pm$ sd. Panel (b) Higuchi kinetic model fitting.

4. Results and discussion

Actually, at the end of the experiment (180 min), not all FD-20 loaded inside the film was released. Data plotted as cumulative percentage released versus time are presented in **Figure 25** (a).

This percentage of release (approx. 77 %) indicates that the film probably entrapped the molecule, preventing the total release. Dextran can probably interact with HPMC or with chitosan.

Finally, simple theoretical fits (**Figure 25** (b)) confirmed diffusion limited release ($R^2 > 0.9$). In fact, since the film is flat and does not dissolve, release from chitosan/HPMC film can be modelled with Higuchi equation (2)

$$Q = A\sqrt{D\varepsilon/\tau}(2C - \varepsilon C_s)C_s t \quad (2)$$

where Q is the amount of drug released at each time point t, A is the surface area of the film, D is the diffusion coefficient of the molecule loaded into the film, ε is the porosity of the film, τ is film tortuosity, C is the drug initial concentration and C_s is the saturated solubility of molecule into the film [129].

Despite the application of gel containing NaTC as promoter enhancer in co-administration condition allowed FD-20 permeation, after application of chitosan/HPMC/NaTC film no dextran permeation has been observed.

Otherwise, as shown in **Figure 22**, tissue pre-treatment with C8 10% permitted to observe a relevant transport of dextran from chitosan/HPMC film as well. It may be asserted that pre-treatment with caprylic acid is the best

4. Results and discussion

enhancing strategy compared to bile salt co-administration both for gel and for film formulations; moreover, gel formulation allows higher permeation than dry film, being the gel a semisolid formulation when molecules can migrate faster and better than in a dry formulation.

4.3.2 Anionic nanofibrillar cellulose film

ANFC film, prepared according to the method reported in section 3.2.3, looks very thin and quite fragile, transparent and yellow due to the presence of fluorescent dextrans.

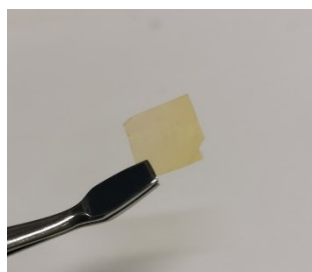


Figure 26: square 1 cm x 1 cm of ANFC film containing FITC-dextrans used for release experiments.

ANFC film thickness was $35 \pm 4 \mu\text{m}$, measured with a digital multimeter (Mitutoyo, Japan).

The drug release studies were performed in order to evaluate the effect of molecular weight of different dextrans on release capabilities of ANFC films containing 1mg/ml of fluorescent dextrans before drying.

4. Results and discussion

Films loaded with FD-4 and FD-20, released the loaded compounds completely; in fact, at the end of the experiment, the film was uncoloured. Film loaded with FD-40 released around 70% of dextran previously loaded; finally, FD-70 film released only 20% of FD-70 total amount loaded in the film. Data showed in **Figure 27**.

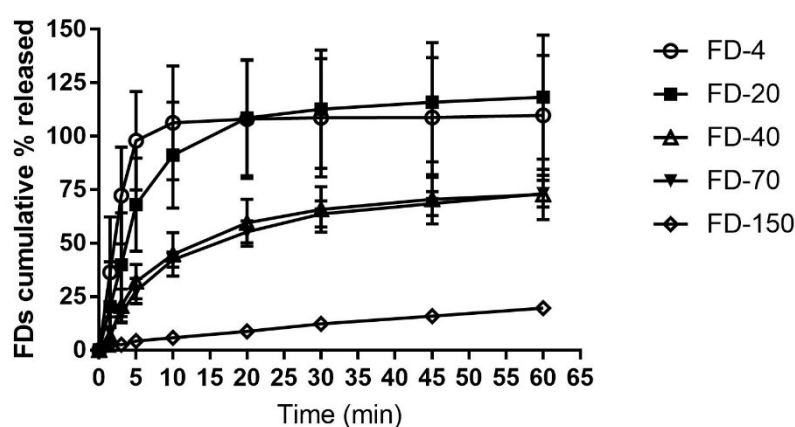


Figure 27: Cumulative percentage of dextrans release from ANFC film. Mean values $\pm$ sd.

The release kinetics were evaluated by using of the above mentioned Higuchi equation (Equation (2)). Since the ANFC films tested are considered homogeneous planar matrix which does not dissolve, data fitted well to the Higuchi model as expected (R^2 value greater than 0.9 for the release of all dextrans) indicating that the dextrans release from ANFC films follows Higuchi diffusion kinetics.

4. Results and discussion

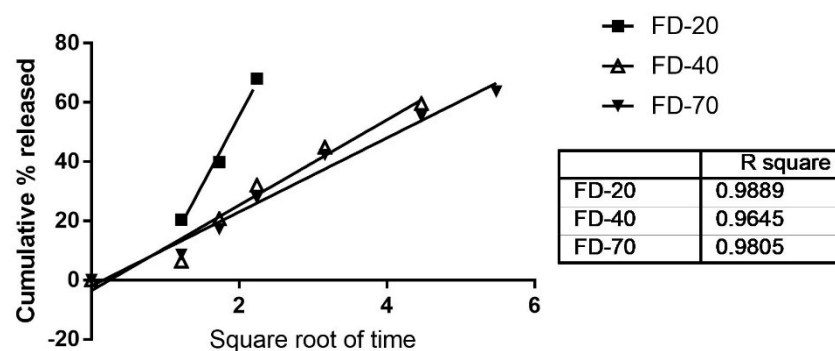


Figure 28: Higuchi kinetic model fitting and R square values calculated for FD-20, FD-40 and FD-70.

The effect of dextran loading on the release efficiency was also evaluated. Different films were prepared with different concentrations of FD-20 such as 0.5 mg/ml and 5 mg/ml. 0.5mg/ml film released 100 % of dextran content, as well as the 1 mg/ml concentrated film (already seen on previous release experiments). Regarding 5 mg/ml film, the release percentage was not much more than 50 %. Results are plotted in **Figure 29**.

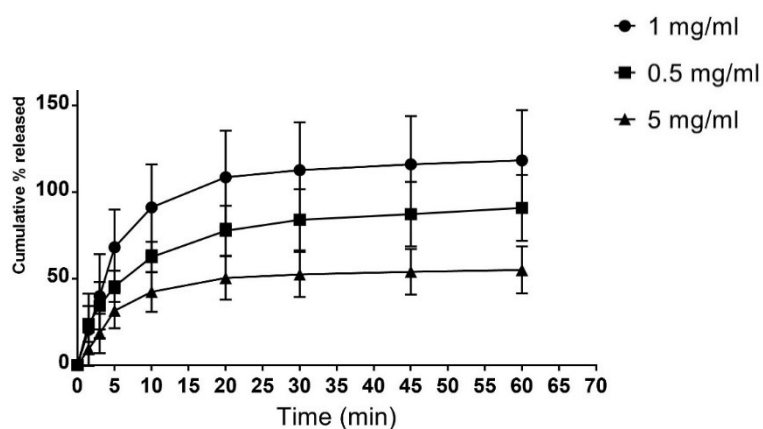


Figure 29: release of FD-20 from different concentrations loading ANFC film. Mean values $\pm$ sd.

4. Results and discussion

For *in vitro* permeation studies pieces of ANFC film were cut with a circular shape with the same dimension of Franz's cells permeation area (see paragraph 0); each piece of film was placed on top of mucosa and a little square of transparent polyethylene sheet was positioned onto ANFC film to fix it; finally, the entire system was set between donor and receptor compartments of Franz's type diffusion cells. FITC-dextran permeation across mucosa was monitored up to 7 hours.

The same setup was used for permeation experiments after mucosa pre-treatment with caprylic acid; before applying the film, an ethanolic solution of caprylic acid 10% was dropped (20 μ l) onto mucosa and left 10 min to air dry.

4. Results and discussion

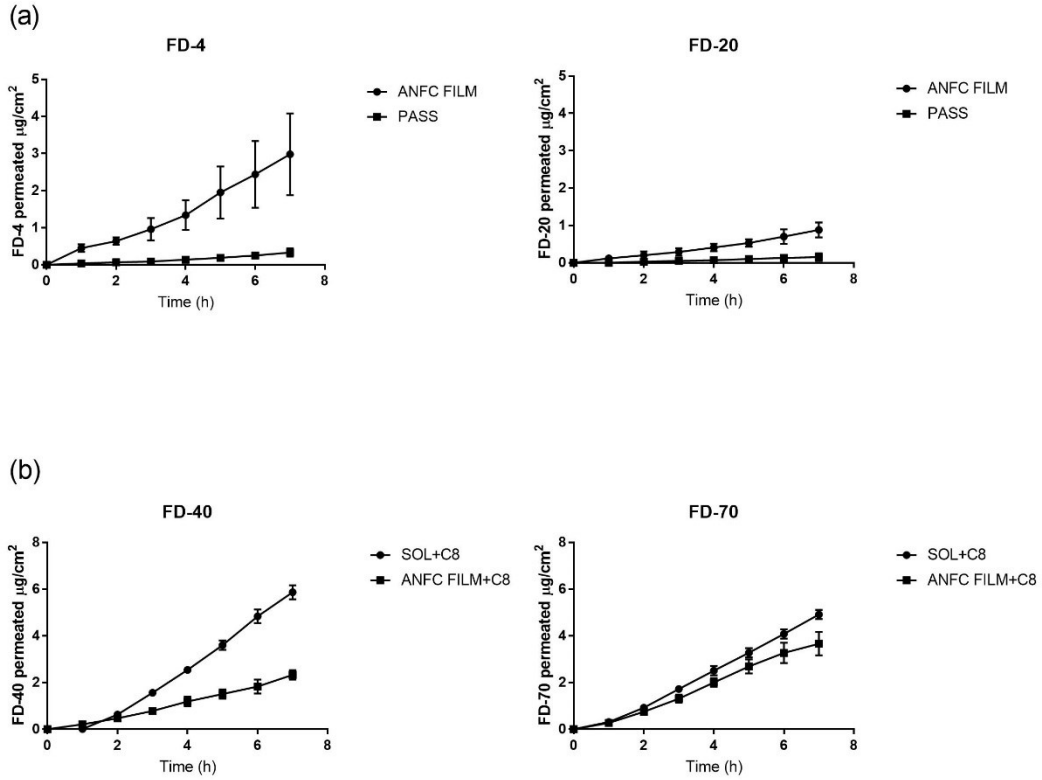


Figure 30: Dextran permeations comparison charts. Panel (a): permeation profiles of FD-4 and FD-20 in passive condition (PASS) and after application of ANFC films without tissue pre-treatment (ANF FILM). Panel (b): permeation profiles of FD-40 and FD-70 in pre-treatment condition after application of a solution 2 mg/ml (SOL+C8) or ANFC film (ANFC FILM+C8). Mean values $\pm$ sem ($n=6-9$).

For comparison purposes, **Figure 30** reports the permeation profiles for each dextran, both from solution and from ANFC film, with or without enhancer treatments. FD-4 and FD-20 permeation from ANFC film (applied without any pre-treatment) was slightly but significantly higher compared to permeation from the respective solutions (**Figure 30** panel (a)). Some authors demonstrated the possibility of interaction between ANFC and mucin [99] and this can explain the improvement of dextran permeation observed.

Dextran with m.w. higher than 20 000 Da did not cross the tissue from film without treatment (fluorescence signal of samples from FD-40 and FD-70

4. Results and discussion

were below the LOQ), therefore films containing FD-40 and FD-70 were tested in pre-treatment condition. The permeation profiles after film application (ANFC FILM+C8) are plotted in panel (b) (**Figure 30**), in comparison with the respective solution application (SOL+C8). From the comparison of the data, it appears that in this case the permeation from solution is more effective than the application of ANFC film, although for FD-70 solution and film are almost superimposed.

In conclusion, ANFC films allow dextrans permeation across esophageal mucosa even without tissue pre-treatment for smaller molecules, such as FD-4 and FD-20; for higher m.w. dextrans, ANFC film pre-treatment with fatty acids is required.

Chitosan/HPMC gel and film require the presence of chemical enhancers also in the case of 20 kDa dextran. This difference is probably related also to the release ability of the two films: ANFC releases 100 % of the contained dextrans whereas chitosan/HPMC film releases only 77 %. As assumed above, dextran probably interacts with chitosan or HPMC, preventing its complete release from the film. Therefore, ANFC proves to be more inert material, suitable for use as delivery system.

4.4 Dissolving microneedles for buccal delivery

Microneedles technology represents a promising approach to overcome different kind of tissue barriers, offering a minimally invasive and painless route of drug administration, as explained before. The dissolving microneedles arrays allow the delivery of a broad range of drug and therapeutic high molecular weight molecules after the needles dissolving in the tissue.

The following work aimed to investigate the effect of needles length of dissolving microneedles patches on fluorescent dextrans permeation through porcine esophageal epithelium. Fluorescent dextrans with 70 k Da and 150 kDa molecular weight were used as a model for antigens or, most in general, therapeutic high molecular weight molecules. Microneedles patches were also observed with two-photon microscopy technique, as such and after application onto tissue.

4.4.1 Effect of microneedles length

Microneedle patch was applied onto mucosa pressing with the operator's thumb. After 10 min, the patch was removed and the mucosa, supported by regenerated cellulose filter, was mounted between donor and receptor compartments of Franz's type diffusion cells (DISA, Milan, Italy). FITC-dextran permeation across mucosa was monitored up to 2 hours from the MN patch removal. Samples of receptor phase were collected at predeterminate

4. Results and discussion

time. Collected samples from 800 and 500 μm microneedles patches were diluted 1:5 before the analysis; collected samples from 150 μm microneedles patches were analysed without dilution.

Figure 31, Figure 32 and Table 9, Table 10 report the amount of fluorescent dextrans found in the receptor compartments for each type of microneedles (length).

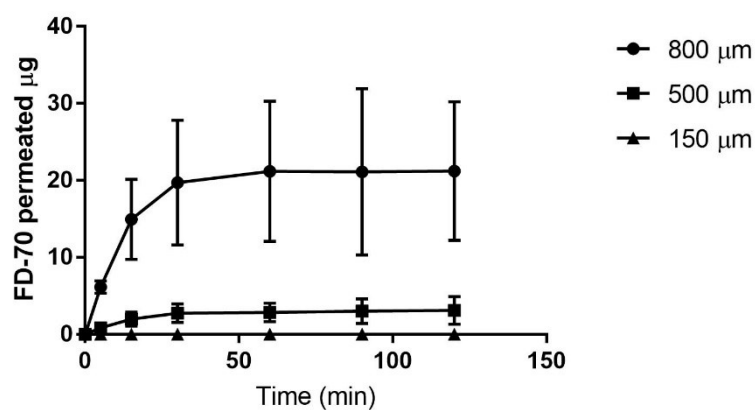


Figure 31: FD-70 permeation curves after application of different microneedles patches for 10 min. Mean values $\pm$ sem ($n=6$).

4. Results and discussion

Table 9: amount of FD-70 (μg) collected at established time. Mean values $\pm$ sd

<i>Time (min)</i>	<i>800 μm</i>	<i>500 μm</i>	<i>150 μm</i>
0	0.00 $\pm$ 0	0.00 $\pm$ 0	0
5	6.14 $\pm$ 0.8	0.85 $\pm$ 0.2	0
15	14.96 $\pm$ 5.2	1.97 $\pm$ 0.9	0
30	19.72 $\pm$ 8.1	2.76 $\pm$ 1.2	0
60	21.19 $\pm$ 9.1	2.85 $\pm$ 1.2	0
90	21.12 $\pm$ 10.8	3.01 $\pm$ 1.6	0
120	21.21 $\pm$ 9.0	3.11 $\pm$ 1.8	0

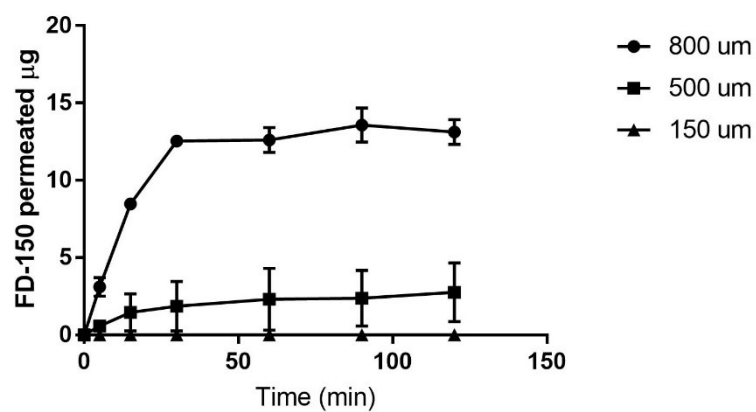


Figure 32: FD-150 permeation curves after application of different microneedles patches for 10 min. Mean values $\pm$ sem (n=8)

4. Results and discussion

Table 10: amount of FD-150 (μg) collected at established time. Mean values $\pm$ sd

<i>Time (min)</i>	<i>800 μm</i>	<i>500 μm</i>	<i>150 μm</i>
0	0.00 $\pm$ 0.0	0.00 $\pm$ 0.0	0
5	3.11 $\pm$ 0.6	0.58 $\pm$ 0.2	0
15	8.47 $\pm$ 0.1	1.45 $\pm$ 1.2	0
30	12.54 $\pm$ 0.0	1.86 $\pm$ 1.6	0
60	12.60 $\pm$ 0.8	2.30 $\pm$ 2.0	0
90	13.58 $\pm$ 1.1	2.37 $\pm$ 1.8	0
120	13.12 $\pm$ 1.9	2.75 $\pm$ 1.9	0

The amount of dextrans permeated across mucosa seems to be dependent on the length of the needles, needles of 800 and 500 μm are able to completely cross the membrane from side to side allowing a rapid and effective passage of molecules. After application of 150 μm microneedles, no dextrans permeation was observed. Probably the needles are too small and/or fragile (they could break and therefore do not penetrate the tissue) or should penetrate partially the membrane, only in the most superficial layer, depositing dextrans within the tissue, but not allowing the passage in the receptor compartment.

4.4.2 Two-photon microscopy

Tissue samples after application of MNs patches were observed with two photon microscope as described in paragraph 0 Both “blank” and fluorescent dextrans loaded microneedles have been studied, collecting 3D images (Z-stacks, **Figure 33**) and emission spectra (**Figure 34**).

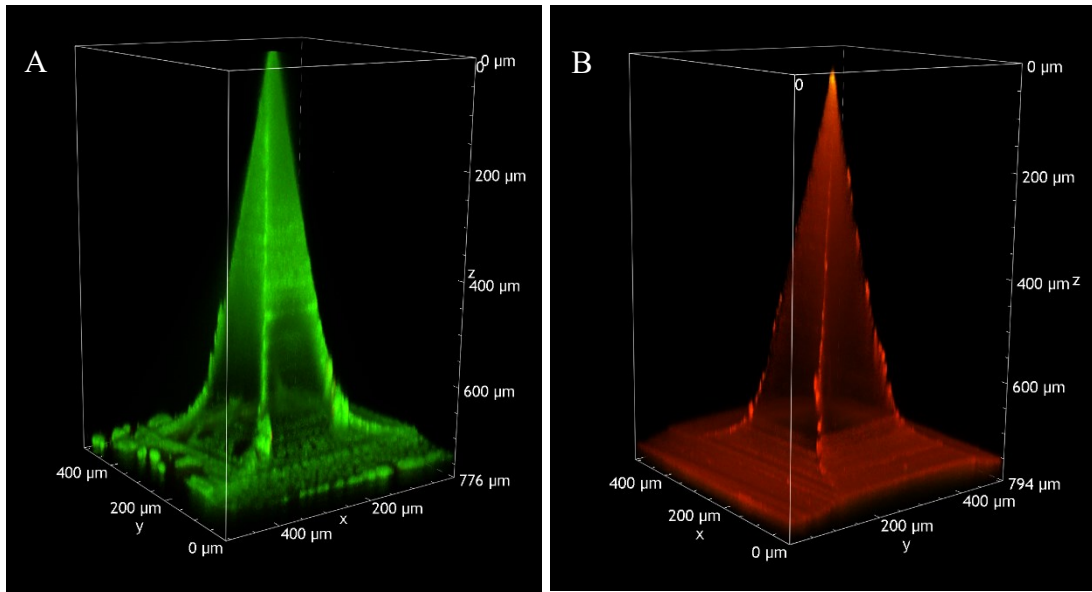


Figure33: Volume rendering of a fluorescent dextran loaded microneedle (A) and of a blank microneedle (B) reconstructed from the Z-stacks, acquired with excitation wavelength of 850 nm.

The dimensions of microneedles are in line with the data from the producer, approximately 800 μm. Emission spectra reported in **Figure** suggest that the green emission of FD-MNs comes from fluorescein, while the red fluorescence detected from the “blank” MN probably comes from the polyvinylpyrrolidone (PVP) scaffold itself (or from some impurities).

4. Results and discussion

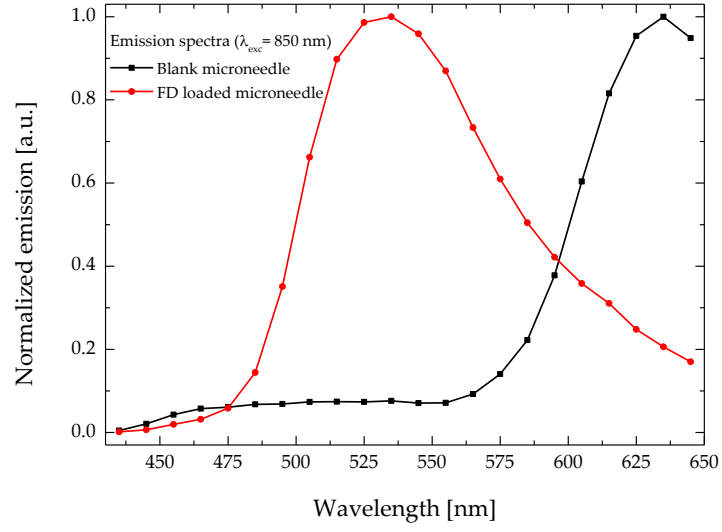


Figure 34: Normalized emission spectra of blank and FD loaded microneedles.

150 μ m MNs loaded with FD-70

No significant information has been obtained from this type of MNs.

Possibly, the microneedles did not penetrate the tissue.

4. Results and discussion

500 μm MNs loaded with FD-70

In order to demonstrate the presence of fluorescent dextran (coming from MNs) in the tissue, we acquired images exciting at 1030 nm (**Figure 35** (A) e (B)), detecting the emission from fluorescein labelled dextran in the green channel (506–593 nm) and the tissue autofluorescence in red one (604–679 nm). The detector gain of the red channel has been set at a higher value than the green one, to enhance the contrast between the fluorescein and the tissue emission. In **Figure 35** (C) and (D) a volume rendering of the untreated porcine esophagus, acquired in the same experimental condition, is shown for comparison.

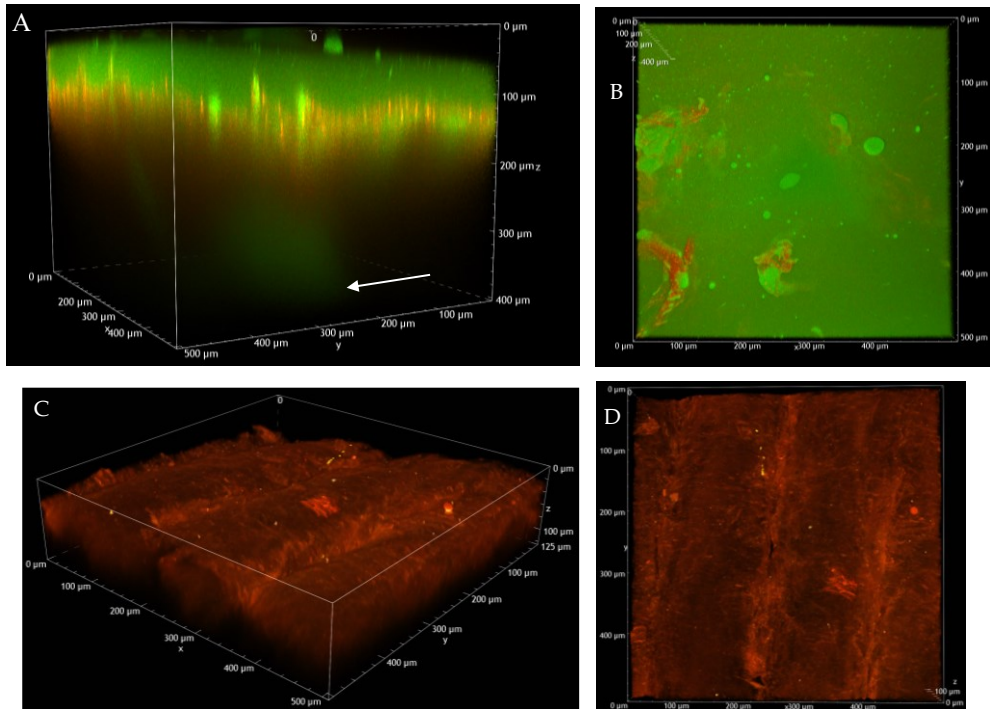


Figure 35: (A) Volume rendering overview of a fluorescent 500 μm MN applied to porcine esophagus reconstructed from the Z-stack; (B) XY view. (D) Volume rendering overview of untreated porcine esophagus reconstructed from the Z-stack; (C) XY view. All the images have been acquired with excitation wavelength of 1030 nm and the same detector gains.

4. Results and discussion

To minimize the dissolution of the polymer (PVP), all the images of samples treated with MNs have been acquired without using water to dip the objective: this procedure affects the quality of the images both in terms of resolution and brightness. Despite this precaution, the water contained in the tissue and the humidity in the air led to the complete or partial dissolution of microneedles, as can be seen from the green color in Figure 35 (A) and (B). The white arrow in **Figure 35** (A) indicates the presence of a partially dissolved MN in the tissue, at a depth of 300-400 μm . The surface of the sample is covered by fluorescein present in the completely dissolved PVP patch, as can be seen in **Figure 35** (B).

Figure 36 shows the image obtained collecting the autofluorescence of the untreated tissue (A) and the image of a “hole” in the tissue caused by a MN (B). Emission spectra collected from blank tissue (A) (blue line), microneedle “hole” in the tissue (B) (black line) and of loaded microneedles (red line) are reported in the graph of **Figure 36** (C): the comparison confirms the presence of fluorescein (peak at 525 nm in both black and red lines) in the emission spectrum collected from the “hole” (B) and the presence of tissue autofluorescence (600-650 nm) in the untreated sample (A).

4. Results and discussion

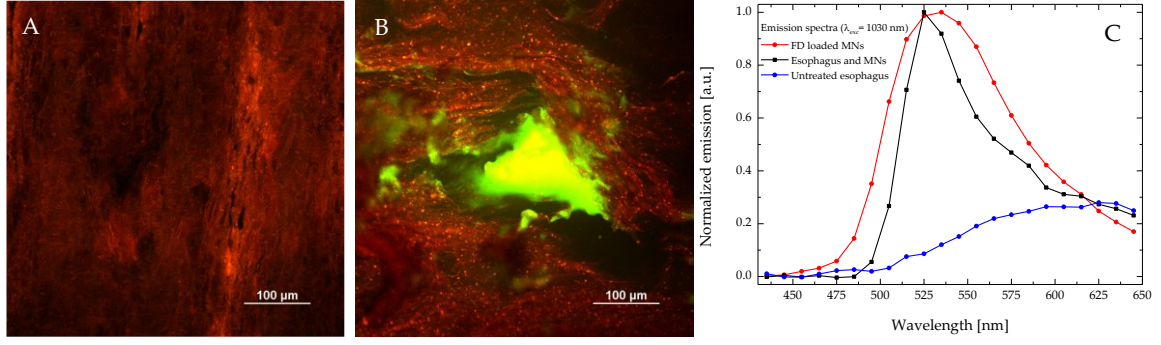


Figure36: (A) Autofluorescence image of untreated porcine esophagus (image size: 500 μm x 500 μm); (B) TPEF image of porcine esophagus punctured by a fluorescent microneedle (image size: 500 μm x 500 μm). Both images have been acquired with an excitation wavelength of 1030 nm and the exact same detector gains. (C) Comparison between normalized emission spectra of FD loaded MNs alone, untreated tissue alone and esophagus with applied MNs (spectrum recorded in correspondence of (A) and (B) focal planes).

4. Results and discussion

800 μm microneedles loaded with FD-70

In **Figure 37**, it is possible to observe 4 partially dissolved individual microneedles inside the esophageal tissue, as indicated by the white arrows in panel (B). Due to the length of needles, the area of fluorescence is more evident compared to 500 μm needles (See **Figure 35(A)**).

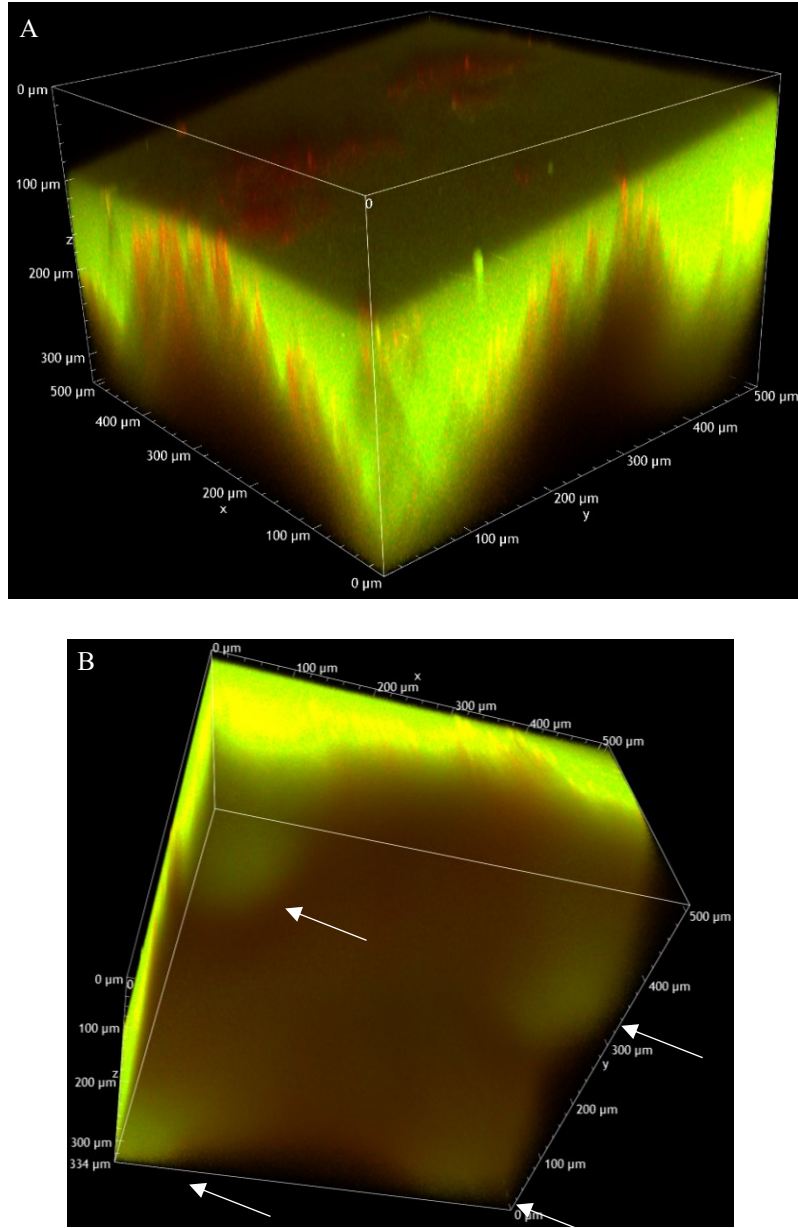


Figure 37: (A) and (B): Volume rendering overview of 4 fluorescent 800 μm MNs applied to porcine esophagus, reconstructed from the Z-stack. All the images have been acquired with excitation wavelength of 1030 nm.

4. Results and discussion

In **Figure 38**, a volume rendering of a different region of the same sample is reported, with a single MN penetrated in the tissue.

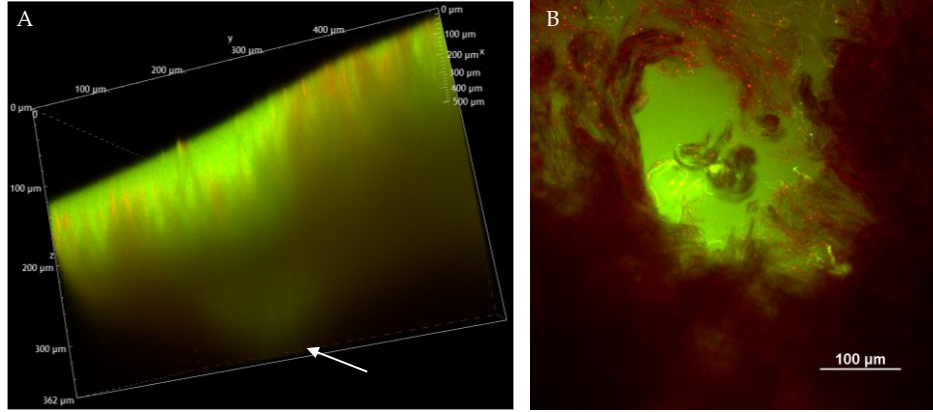


Figure 38: (A) Volume rendering overview of a fluorescent 800 μm MN applied to porcine esophagus, reconstructed from the Z-stack; (B) TPEF image acquired at an intermediate height in the Z-stack (image size: 500 μm x 500 μm). All the images have been acquired with excitation wavelength of 1030 nm.

4. Results and discussion

800 μm microneedles loaded with FD-150

Then, 800 μm MNs were loaded with a high molecular weight dextran, FD-150.

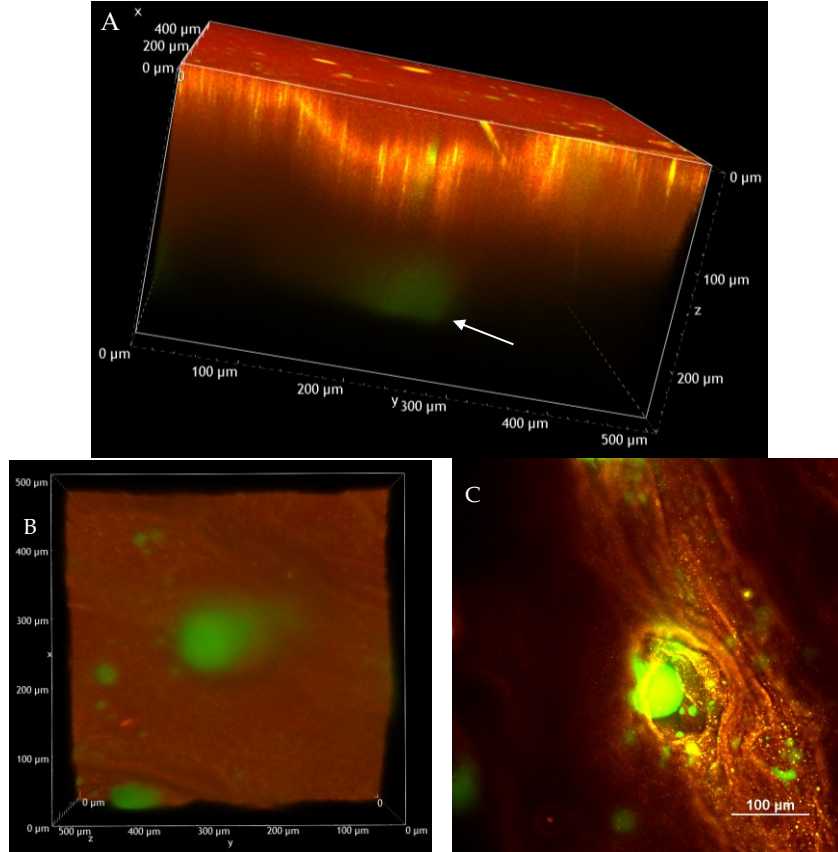


Figure 39: (A) Volume rendering overview of a fluorescent 800 μm MN (loaded with FD-150) applied to porcine esophagus, reconstructed from the Z-stack; (B) XY view. (C) TPEF image acquired at an intermediate height in the Z-stack (image size: 500 μm x 500 μm). All the images have been acquired with excitation wavelength of 1030 nm.

Surprisingly, the images collected from these samples are quite different from the previous ones since in the presence of FD-150 loaded MNs, an intense red fluorescence is observed on the surface of the sample. This red fluorescence peaks at 630 nm (**Figure 40**, black line), and could be attributed to the emission

4. Results and discussion

of the PVP matrix (**Figure 40**, green line). The fluorescein signal can be distinguished clearly only deeper in the tissue (**Figure 39**, white arrow and emission peak at 525 nm in **Figure 40**), but as can be seen from the spectra of **Figure 40**, a strong contribution from the PVP emission remains.

Also in this case (as for FD-70 loaded MNs) MNs patch was completely dissolved, although it remains to be explained why fluorescein was not present on the surface of the tissue.

4. Results and discussion

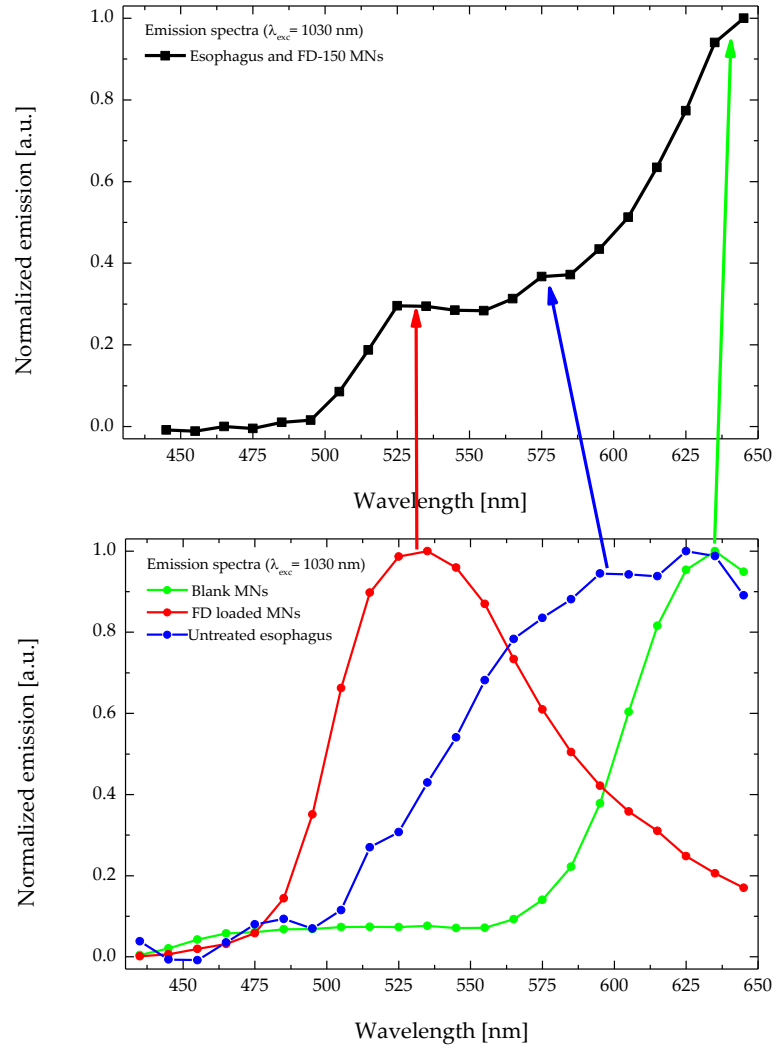


Figure 40: Comparison between normalized emission spectra of FD-70 loaded MNs, blank MNs, untreated tissue alone and esophagus with applicated MNs loaded with FD-150 (spectrum recorded in correspondence of Figure 38 (C) focal plane).

Summarizing the results of these experiments, MNs with a length of at least 500 μm can penetrate the mucosal tissue to a considerable extent; being formed of a soluble polymer (PVP), they dissolve in contact with water contained in the tissue, releasing dextran. On the contrary, shorter MNs (150 μm) are not able to “pierce” the tissue.

5. GENERAL CONCLUSION

5. General conclusion

Mucosal vaccine delivery, in particular through the buccal mucosa, could be an advantageous option to carry out mass vaccination quickly and safely avoiding patients' pain and stress and the involvement of specialized personnel. The aim of this project was to investigate the possibility to administer antigen via the buccal route, studying enhancing strategies and suitable formulations.

The data reported in the present work show that it is possible to increase or promote mucosa permeation of high molecular weight dextrans, used as model of polysaccharide antigens, by using caprylic acid or sodium taurocholate as chemical enhancers. With these enhancers, used in pre-treatment or co-administration conditions respectively, dextrans with molecular weight of 70 and 150 kDa could cross the mucosa in significant amounts, although the passive permeation was below the limit of detection of the analytical method.

Several polymeric formulations were produced and tested. Chitosan/HPMC gel, in association with caprylic acid or sodium taurocholate as chemical enhancers, can improve FD-20 transport through mucosal tissue, in particular after pre-treatment with caprylic acid. When the gel was dried to form a film, more suitable for buccal administration, the efficiency decreased and only small amounts of FD-20 penetrated the tissue after pre-treatment with caprylic acid. As an alternative, anionic nano fibrillar cellulose (ANFC) recently widely investigate for buccal delivery, was used as film forming material. Very promising results were observed after permeation experiments.

5. General conclusion

FD-4 and FD-20 loaded inside ANFC film could cross the tissue in measurable amount without any further chemical enhancer use and also FD-40 and FD-70 were found after tissue pre-treatment with caprylic acid, although the passive permeation was below the limit of detection.

Finally, microneedle devices with different needles length, were also tested for the delivery of high molecular weight dextrans. The results showed that microneedle arrays with 800 and 500 μm needles length permitted 70 kDa and 150 kDa dextrans to cross the tissue in a minimally invasive way. Permeations data were confirmed by two-photon microscopy technique.

The results obtained in the present work suggest that buccal administration of high molecular weight polysaccharides is feasible, using enhancing strategies associated with a suitable formulation.

6. REFERENCES

-
- [1] S.-H. Kim e Y.-S. Jang, «Antigen targeting to M cells for enhancing the efficacy of mucosal vaccines.,» *Experimental & Molecular Medicine*, 2014.
 - [2] V. V. Shukla e R. C. Shah, «Vaccinations in Primary Care,» *The Indian Journal of Pediatrics*, p. 1118–1127, 2018.
 - [3] B. L. Bartlett, A. J. Pellicane e S. K. Tying, «Vaccines immunology,» *Dermatologic therapy*, 2009.
 - [4] O. o. I. D. a. H. P. (OIDP), «HHS.gov,» 29 Apr 2021. [Online]. Available: <https://www.hhs.gov/immunization/basics/types/index.html>. [Consultato il giorno 6 Oct 2021].
 - [5] A. Guildford, «medicalnewstoday,» 28 Sep 2021. [Online]. Available: <https://www.medicalnewstoday.com/articles/dna-vs-mrna-vaccines-similarities-and-differences>. [Consultato il giorno 6 Oct 2021].
 - [6] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts e P. Walter, *Molecular Biology of the Cell*, 4th edition, New York: Garland Science, 2002.
 - [7] I. Nel, L. Bertrand, A. Toubal e A. Lehuen, «MAIT cells, guardians of skin and mucosa?,» *Mucosal immunology*, pp. 803-814, 2021.
 - [8] H. Kiyono e T. Azegami, «The mucosal immune system: From dentistry to vaccine development.,» *Proceedings of the Japan Academy*, pp. 423-439, 2015.
 - [9] T. Kato e R. L. Owen, «Structure and functional intestinal mucosal epithelium.,» in *Mucosal immunology*, Amsterdam, Elsevier Academic Press, 2005, pp. 131-153.

6. References

- [10] B. M. Peters, M. E. Shirtliff e M. A. Jabra-Rizk, «Antimicrobial peptides: primeval molecules or future drugs?,» *PLoS Pathog*, p. e1001067, 2010.
- [11] R. W. DePaolo, K. Kamdar, S. Khakpour, Y. Sugiura, W. Wang e B. Jabri, «A specific role for TLR1 in protective T(H)17 immunity during mucosal infection.,» *J. esp. med.*, pp. 1437-1444, 2012.
- [12] M. Cella, A. Fuchs, W. Vermi, F. Facchetti, K. Otero, J. K. Lennerz, J. M. Doherty, J. C. Mills e M. Colonna, «A Human natural killer cell subset provides an innate source of IL-22 for mucosal immunity.,» *Nature*, pp. 722-725, 2009.
- [13] S. Yousefi, J. A. Gold, N. Andina, J. J. Lee, A. M. Kelly, E. Kozlowski, I. Schmid, A. Straumann, J. Reichenbach, G. J. Gleich e H. U. Simon, «Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense.,» *Nat. med.*, pp. 949-953, 2008.
- [14] N. Alvarez, O. Otero, F. Camacho, R. Borrero, Y. Tirado, A. Puig, A. Aquilar, C. Rivas, A. Cervantes, G. Falero-Díaz, c, A. Cádiz, M. E. Sarmiento, M. N. Morazmi, R. Hernández-Pando e A. Acosta, «Passive administration of purified secretory IgA from human colostrum induces protection against *Mycobacterium tuberculosis* in a murine model of progressive pulmonary infection.,» *BMC immunol.*, p. 14 (Suppl 1), 2013.
- [15] F. E. Johansen, M. Pekna, I. N. Norderhaug, B. Haneberg, M. A. Hietala, P. Krajci, C. Betsholtz e P. Brandtzaeg, «Absence of epithelial immunoglobulin A transport with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice.,» *J. exp. med.*, pp. 195-222, 1999.

6. References

- [16] K. Hirota, J. E. Turner, M. Villa, J. H. Duarte, J. Demengeot, O. M. Steinmetz e B. Stockinger, «Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependant IgA responses.,» *Nat. Immunol.*, pp. 247-257, 2013.
- [17] S. Babiuk, D. J. Asper, D. Rogan, G. K. Mutwiri e A. A. Potter, «Subcutaneous and intranasal immunization with type III secreted proteins can prevent colonization and shedding of Escherichia coli O157:H7 in mice.,» *Microb. Pathog.*, pp. 7-11, 2008.
- [18] M. Ohmura, M. Yamamoto, C. Tomiyama-Miyaji, Y. Yuki, Y. Takeda e H. Kiyono, «Nontoxic Shiga toxin derivatives from Escherichia coli possess adjuvant activity for the augmentation of antigen-specific immune responses via dendritic cell activation.,» *Infect. Immun.* , pp. 4088-4097, 2005.
- [19] D. Bumann, C. Behre, K. Behre, S. Herz, B. Gewecke, E. J. Gessner, B. U. von Specht e U. Baumann, «Systemic, nasal and oral live vaccines against Pseudomonas aeruginosa: a clinical trial of immunogenicity in lower airways of human volunteers,» *Vaccine*, pp. 707-713, 2010.
- [20] D. C. Sharma, P. Tangri, S. Jawla e R. Mishra, «Different variable and recent development in novel buccal drug delivery system.,» *International journal of trend in scientific research and development*, pp. 345-351, 2019.
- [21] B. Corthésy e G. Bioley, «Lipid-based particles: versatile delivery system for mucosal vaccination against infection.,» *Frontiers in immunology*, 2018.
- [22] R. L. Creighton e K. A. Woodrow, «Microneedle-mediated vaccine delivery to the oral mucosa,» vol. 8, n. 4, 2019.

6. References

- [23] L. Aayam, A. Tatsuhiko e H. Kiyono, «The mucosal immune system for vaccine development.,» *Vaccine* , p. 6711–6723, 2014.
- [24] S. Hazebrouck, L. Przybylski-Nicaise, S. Ah-Leung, K. Adel-Patient, G. Corthier, P. Langella e J.-M. Wal, «Influence of the route of administration on immunomodulatory properties of bovine beta-lactoglobulin-producing *Lactobacillus casei*.,» *Vaccine*, pp. 5800-5805, 2009.
- [25] L. Zhang, W. Wang e S. Wang, «Effect of vaccine administration modality on immunogenicity and efficacy.,» *Expert Review of Vaccines*, pp. 1509-1523, 2015.
- [26] P. Moingeon, «Update on immune mechanisms associated with sublingual immunotherapy: practical implications for the clinician.,» *The journal of allergy and Clinical Immunology: in practice.*, pp. 228-241, 2013.
- [27] H.-J. Lee, H. Cho, M.-G. Kim, Y.-K. Heo, Y. Cho, Y.-D. Gwon, K. H. Park, J. Jin, Y.-K. Oh e Y. B. Kim, «Sublingual Immunization of Trivalent Human Papillomavirus DNA Vaccine in Baculovirus Nanovector for Protection against Vaginal Challenge,» 19 March 2015. [Online]. Available: <https://doi.org/10.1371/journal.pone.0119408>. [Consultato il giorno 29 Sep 2021].
- [28] C. f. D. C. a. Prevention, «Centers for Disease Control and Prevention,» 8 Sep 2021. [Online]. Available: <https://www.cdc.gov/vaccines/hcp/admin/administer-vaccines.html>. [Consultato il giorno 14 Oct 2021].

6. References

- [29] J. Wolicki e E. Miller, «Chapter 6: vaccine administration,» in *Epidemiology and prevention of vaccine-preventable diseases*, Washington, D.C., Public Health Foundation, 2021, pp. 80-87.
- [30] WHO, «vaccine safety basics. e-learning course,» 2021. [Online]. Available: <https://vaccine-safety-training.org/route-of-administration.html>. [Consultato il giorno 14 Oct 2021].
- [31] A. Gutierrez, «Technology Networks biopharma,» 1 June 2021. [Online]. Available: <https://www.technologynetworks.com/biopharma/articles/changing-up-the-route-of-vaccine-administration-349321>. [Consultato il giorno 14 Oct 2021].
- [32] M. Giese, «Delivery Technologies,» in *Introduction to Molecular Vaccinology*, Springer, Cham, 2016, pp. 233-258.
- [33] E. Kim, G. Erdos, S. Huang, T. W. Kenniston, S. C. Balmert, D. C. Carey, S. V. Raj, M. W. Epperly, W. B. Klimstra, B. L. Haagmans, E. Korkmaz, L. Falo Jr, L. D. Falo Jr e A. Gambotto, «Microneedle array delivered recombinant coronavirus vaccines: immunogenicity and rapid translational development.,» *EBioMedicine*, vol. 55, 2020.
- [34] E. Caffarel-Salvador, S. Kim, V. Soares, R. Y. Tian, S. R. Stern, D. Minahan, R. Yona, X. Lu, F. R. Zakaria, J. Collins, J. Wainer, J. Wong, R. McManus, S. Tamang, S. McDonnell, K. Ishida, A. Hayward, X. Liu, F. Hubalek, J. Fels, A. Vegge, M. R. Frederiksen, U. Rahbek, T. Yoshitake, J. Fujimoto, N. Roxhed, R. Langer e G. Traverso, «A microneedle platform for buccal macromolecule delivery,» *Science advances*, 2021.

6. References

- [35] J. O'Shea, M. R. Prausnitz e N. Rouphael, «Dissolvable Microneedle Patches to Enable Increased Access to Vaccines against SARS-CoV-2 and Future Pandemic Outbreaks,» *Vaccines*, p. 320, 2021.
- [36] M. B. Maymone, R. O. Greer, L. K. Burdine, A. Dao-Cheng, S. Venkatesh, P. C. Sahitya, A. C. Maymone, J. Kesecker e N. A. Vashi, «Benign oral mucosal lesions: Clinical and pathological findings,» *Journal of the American Academy of Dermatology*, pp. 43-56, 2018.
- [37] C. A. Squier, «The permeability of oral Mucosa,» *Critical reviews in oral biology and medicine*, pp. 13-32, 1991.
- [38] H. Kraan, H. Vrieling, C. Czerkinsky, W. Jiskoot, G. Kersten e J.-P. Amorij, «Buccal and sublingual vaccine delivery,» *Journal of Controlled Release*, pp. 580-592, 2014.
- [39] S. Dodla e S. Velmurugan, «Buccal penetration enhancers-an overview,» *Asian journal of pharmaceutical and clinical research*, pp. 39-47, 2013.
- [40] R. B. Stoughton, «Enhanced percutaneous absorption with 1-dodecylazacycloheptan-2-one (Azone),» *Arch. Dermatol.*, pp. 474-477, 1982.
- [41] W. Schurmann e P. Turner, «Membrane model of the human oral mucosa as derived from buccal absorption performance and physicochemical properties of the beta-blocking drugs atenolol and propranolol,» *J. Pharm. Pharmacol.*, pp. 137-147, 1978.
- [42] J. Fang, T. Hwang, C. Fang e H. Chiu, «In vitro and in vivo evaluations of the efficacy and safety of skin permeation enhancers using flurbiprofen as a model drug,» *Int. J. Pharm.*, pp. 153-166, 2003.

6. References

- [43] U. T. Lashmar, J. Hadgraft e N. Thomas, «Topical application of penetration enhancers to the skin of nude mice: a histopathological study.,» *J. Pharm. Pharmacol.*, pp. 118-121, 1989.
- [44] J. Nicolazzo, B. Reed e B. Finnin, «Buccal penetration enhancers - How do they really work?,» *Journal of Controlled Release*, n. 105, pp. 1-15, 2005.
- [45] A. H. Shojaei, «Buccal mucosa as a route for systemic drug delivery: a review,» *J. Pharm. Pharm. Sci.*, pp. 15-30, 1998.
- [46] W. R. Galey, H. K. Lonsdale e S. Nacht, «The in vitro permeability of skin and buccal mucosa to selected drugs and tritiated water,» *J Invest Dermatol*, pp. 713-717, 1976.
- [47] A. Flo, A. C. Calpena, L. Halbaut, E. I. Araya, F. Fernandez e B. Clares, «Melatonin Delivery: Transdermal and Transbuccal Evaluation in Different Vehicles.,» *Pharmaceutical Research*, pp. 1615-1627, 2016.
- [48] V. Hearnden, V. Sankar, K. Hull, D. V. Juras e M. Greenberg, «A New developments and opportunities in oral mucosal drug delivery for local and systemic disease.,» *Advanced Drug Delivery Reviews*, pp. 16-28, 2012.
- [49] C. A. Lesch, C. A. Squier, A. Cruchley, D. M. Williams e P. Speight, «The permeability of human oral mucosa and skin to water,» *J Dent Res*, pp. 1345-1349, 1989.
- [50] S. Buckley, «Oral Semaglutide: Delivering new possibilities in the treatment of diabetes.,» New York, 2018.

6. References

- [51] M. Kondoh, T. Yoshida, H. Kakutani e K. Yagi, «Targeting tight junction proteins-significance for drug development.,» *Drug Discov. Today.*, pp. 180-186, 2008.
- [52] S. Maher, R. J. Mersny e D. J. Brayden, «Intestinal Permeation Enhancers for Oral Peptide Delivery.,» *Adv. Drug Deliv. Rev.*, pp. 277-319, 2016.
- [53] K. Whitehead, N. Karr e S. Mitragotri, «Safe and effective Permeation enhancers for oral drug delivery,» *Pharmaceutical research*, pp. 1782-1788, 2008.
- [54] H. Sugioka e Y. Moroi, «Micelle formation of sodium cholate and solubilization into the micelle,» *Biochimica et Biophysica Acta (BBA)-lipids and lipid metabolism*, pp. 99-110, 1998.
- [55] M. Stojancevic, N. Pavlovic, S. Golocorbin-Kon e M. Mikov, «Application of bile acids in drug formulation and delivery,» *Frontiers in life science*, pp. 112-122, 2014.
- [56] D. Wustner, A. Herrmann e p. Muller, «Head group-independent interaction of phospholipids with bile salts. A fluorescence and EPR study.,» *J Lipid Res.*, pp. 395-404, 2000.
- [57] K. Kim, I. Yoon, I. Chun, N. Lee, T. Kim e H. S. Gwak, «Effects of bile salts on the lovastatin pharmacokinetics following oral administration to rats.,» *Drug Delivery*, pp. 78-83, 2011.
- [58] M. C. Carey e D. M. Small, «Micelle formation by bile salts. Physical-chemical and thermodynamic considerations,» *Arch Intern Med.*, pp. 506-527, 1972.
- [59] M. Posa, S. Kevresan, M. Mikov, V. Cirin-Novta e K. Kuhajda, «Effect of cholic acid and its keto derivatives on the analgesic action of lidocaine

6. References

- and associated biochemical parameters in rats.,» *Eur J Drug Metab Pharmacokinet.*, pp. 109-117, 2007.
- [60] M. Mikov, A. Raskovic, E. Jakovljevic, D. Dudvarski e J. P. Fawcett, «Influence of the bile salt sodium dihydroxy-12-oxo-5 β -cholanate on ampicillin pharmacokinetics in rats.,» *Asian Journal of Drug Metabolism and Pharmacokinetics.*, pp. 197-200, 2005.
- [61] S. D. Mithani, V. Bakatselou, C. N. TenHoor e J. B. Dressman, «Estimation of the increase in solubility of drugs as a function of bile salt concentration.,» *Pharm. Res.*, pp. 163-167, 1996.
- [62] B. K. Nanjwade, D. J. Patel, R. A. Udhani e F. V. Manvi, «Functions of lipids for enhancement of oral bioavailability of poorly water-soluble drugs.,» *Sci Pharm*, pp. 705-727, 2011.
- [63] C. Darkoh, L. M. Lichtenberger, N. Ajami, E. J. Dial, Z. D. Jiang e H. L. DuPont, «Bile acids improve the antimicrobial effect of rifaximin.,» *Antimicrob Agents Chemother*, pp. 3618-3624, 2010.
- [64] T. Murakami, Y. Sasaki, R. Yamajo e N. Yata, «Effect of bile salts on the rectal absorption of sodium ampicillin in rats.,» *Chem Pharm Bull*, pp. 1948-1955, 1984.
- [65] F. Ingels, B. Beck, M. Oth e P. Augustijns, «Effect of simulated intestinal fluid on drug permeability estimation across Caco-2 monolayers.,» *Int J Pharm.*, pp. 221-232, 2004.
- [66] G. Chen, L. Yang, H. Zhang, I. G. Tucker e J. P. Fawcett, «Effect of ketocholate derivatives on methotrexate uptake in Caco-2 cell monolayers.,» *Int J Pharm*, pp. 89-93, 2012.
- [67] R. J. Babu, L. Chen e N. Kanikkannan, «Fatty Alcohols, Fatty Acids, and Fatty Acid Esters as Penetration Enhancers.,» *Percutaneous*

6. References

- Penetration Enhancers Chemical Methods in Penetration Enhancement*, pp. 133-150, 2015.
- [68] R. G. Ackman, *Marine biogenic lipids, fat & oils*, Boca Raton: CRC press, 1989.
- [69] B. Yu, C. Y. Dong, P. T. So, D. Blankschtein e R. Langer, « In vitro visualization and quantification of oleic acid induced changes in transdermal transport using two-photon fluorescence microscopy.,» *J Invest Dermatol*, pp. 16-25, 2001.
- [70] R. M. Hathout, T. J. Woodman, S. Mansour, N. D. Mortada, A. S. Geneidi e R. H. Guy, «Microemulsion formulations for the transdermal delivery of testosterone.,» *Eur J Pharm*, pp. 188-196, 2010.
- [71] L. Serpe, A. Jain, C. G. De Macedo, M. C. Volpato, F. C. Groppo, H. S. Gill e M. Franz-Montan, «Influence of salivary washout on drug delivery to the oral cavity using coated microneedles: An in vitro evaluation.,» *European Journal of Pharmaceutical Sciences*, pp. 215-223, 2016.
- [72] H. S. Gill, D. D. Denson, B. A. Burris e M. R. Prausnitz, «Effect of microneedle design on pain in human volunteers.,» *Clin J Pain*, pp. 585-594, 2008.
- [73] Y. Ito, N. Hamasaki, H. Higashino, Y. Murakami, N. Miyamoto e K. Takada, «Method to increase the systemically delivered amount of drug from dissolving microneedles.,» *Chem Pharm Bull*, pp. 8-15, 2013.
- [74] J. Gupta, R. Gupta e Vanshita, «Method to increase the systemically delivered amount of drug from dissolving microneedles.,» *ASSAY and drug development technologies*, 2021.

6. References

- [75] Y. Ma, W. Tao, S. J. Krebs, W. F. Sutton, N. L. Haigwood e H. S. Gill, «Vaccine delivery to the oral cavity using coated microneedles induces systemic and mucosal immunity.,» *Pharm Res*, pp. 2393-2403, 2014.
- [76] X. He, J. Sun, J. Zhuang, H. Xu, Y. Liu e D. Wu, «Microneedle System for Transdermal Drug and Vaccine Delivery: Devices, Safety, and Prospects.,» *Dose-response*, pp. 1-18, 2019.
- [77] S. H. Barya, M. C. Gohel, T. A. Mehta e O. P. Sharma, «Microneedles: an emerging transdermal drug delivery.,» *J Pharm Pharmacol*, pp. 11-29, 2012.
- [78] R. Ali, P. Mehta, M. S. Archad, I. Kucuk, M. W. Chang e Z. Ahmad, «Transdermal microneedles-a materials perspective.,» *AAPS PharmSciTech*, pp. 1-14, 2019.
- [79] V. Sachdeva e A. K. Banga, «Microneedles and their applications.,» *Recent Pat Drug Deliv Formul*, pp. 95-132, 2011.
- [80] M. S. Giri Nandagopal, R. Antony, S. Rangabhashiyam, N. Sreekumar e N. Selvaraju, «Overview of microneedle system: a third generation transdermal drug delivery approach.,» *Microsyst Technol*, pp. 1249-1272, 2014.
- [81] R. R. Hartman e U. B. Kompella, «Intravitreal, subretinal, and suprachoroidal injections: evolution of microneedles for drug delivery.,» *J Ocul Pharmacol Ther*, pp. 141-153, 2018.
- [82] S. P. Sullivan, D. G. Koutsonanos, M. Del Pilar Martin e at al., «Dissolving polymer microneedle patches for influenza vaccination.,» *Nat Med*, pp. 915-920, 2010.
- [83] D. P. Wermeling, S. L. Banks, D. A. Hudson, H. S. Gill, J. Gupta, M. R. Prausnitz e A. L. Stinchcomb, «Microneedles permit transdermal

6. References

- delivery of a skin-impermeant medication to humans.,» *Proc Natl Acad Sci U S A*, pp. 2058-2063, 2008.
- [84] K. Van der Maaden, W. Jiskoot e J. Bouwstra, «Microneedle technologies for (trans)dermal drug and vaccine delivery.,» *Journal of control release*, pp. 645-655, 2012.
- [85] R. Nagarkar, M. Singh, H. X. Nguyen e S. Jonnalagadda, «A review of recent advances in microneedle technology for transdermal drug delivery.,» *J Drug Deliv Sci Tech*, pp. 1-18, 2020.
- [86] S. Sharma, K. Hatware, P. Bhadane, R. Sindhikar e D. K. Mishra, «Recent advances in microneedle composites for biomedical applications: advanced drug delivery technologies.,» *Mater Sci Eng C*, pp. 1-14, 2019.
- [87] J. G. Turner, L. R. White, P. Estrela e H. S. Leese, «Hydrogel-Forming Microneedles: Current Advancements and Future Trends,» *Macromolecular bioscience*, 2020.
- [88] M. C. Chen, K. Y. Lai, M. H. Ling e C. W. Lin, «Enhancing immunogenicity of antigens through sustained intradermal delivery using chitosan microneedles with a patch-dissolvable design,» *Acta Biomater*, pp. 66-75, 2018.
- [89] P. D. Becker, C. Hervouet, G. M. Mason, S. Y. Kwon e L. S. Klavinskis, «Skin vaccination with live virus vectored microneedle arrays induce long lived CD8(+) T cell memory.,» *Vaccines*, pp. 4691-4698, 2015.
- [90] P. C. DeMuth, Y. Min, B. Huang, J. A. Kramer, A. D. Miller, D. H. Barouch, P. T. Hammond e D. J. Irvine, «Polymer multilayer tattooing for enhanced DNA vaccination,» *Nat mater.*, pp. 367-376, 2013.

6. References

- [91] L. M. Bruschiand e O. de Freitas, «Oral bioadhesive drug delivery systems,» *Drug Dev Ind Pharm*, pp. 293-310, 2005.
- [92] S. Skulason, M. S. Asgeirsdottir, J. P. Magnusson e T. Kristmundsdottir, «Evaluation of polymeric films for buccal drug delivery,» *Pharmazie*, pp. 197-201, 2009.
- [93] N. Salamat-Miller, M. Chittehang e T. P. Johnston, «The use of mucoadhesive polymers in buccal drug delivery,» *Adv Drug Deliv Rev*, pp. 1666-91, 2005.
- [94] L. Kumar , S. Verma, B. Vaidya e V. Gupta, «Bioadhesive polymers used for delivery of therapeutic molecules,» in *Nanotechnology-Based Approaches for Targeting and Delivery of Drugs and Genes*, Academic Press, 2017, pp. 329-330.
- [95] J. Hao e P. W. S. Heng, «Buccal Delivery Systems,» *Drug Development and Industrial Pharmacy*, pp. 821-832, 2003.
- [96] P. Panraksa, S. Udomsom, P. Rachtanapun, C. Chittasupho, W. Ruksiriwanich e P. Janatrawut, «Hydroxypropyl Methylcellulose E15: A Hydrophilic polymer for fabrication of orodispersible film using syringe extrusion 3D printer,» *Polymers*, p. 2666, 12 November 2020.
- [97] C. L. Li, L. G. Martini, J. E. Ford e M. Roberts, «The use of hypromellose in oral drug delivery,» *Journal of pharmacy and pharmacology*, pp. 533-546, 21 January 2005.
- [98] M. Jorfi e E. J. Foster, «Recent advances in nanocellulose for biomedical applications.,» *Journal of Appl Polym Sci*, p. 132, 2015.
- [99] P. Laurén, H. Paukkonen, T. Lipiainen, Y. Dong, T. Oksanen, H. Raikkonen, H. Ehlers, P. Laaksonen, M. Yliperttula e T. Laaksonen,

6. References

- «Pectin and mucin enhance the bioadhesion of drug loaded nanofibrillated cellulose films,» *Pharm res*, 2018.
- [100] K. Kekalainen, H. Liimatainen, M. Illikainen, T. C. Maloney e J. Niinimäki, «The role of hornification in the disintegration behaviour of TEMPO-oxidized bleached hardwood fibres in a high-shear homogenizer,» *Cellulose*, pp. 1163-1174, 2014.
- [101] O. Eriksen, K. Syveryd e O. Gregersen, «The use of microfibrillated cellulose produced from kraft pulp as strength enhancer in TMP paper.,» *Nord pulp pap res j*, pp. 299-304, 2008.
- [102] E. Letkova, M. Letko e M. Vrska, «Influence of recycling and temperature on the swelling ability of paper.,» *Chem pap*, pp. 822-828, 2011.
- [103] M. A. Hubbe, R. A. Venditti, R. L. Barbour e M. Zhang, «Changes to unbleached kraft fibers due to drying and recycling.,» *Prog pap recyc*, pp. 11-20, 2003.
- [104] T. C. Maloney e H. Paulapuro, «The formation of pores in the cell wall.,» *J pulp pap sci*, pp. 430-436, 1999.
- [105] Y. Chen, J. Wan, M. Huang, Y. Ma, H. Wang e J. Yang, «Influence of drying temperature and duration on fiber properties of unbleached wheat straw pulp.,» *Carbohydr polym*, pp. 759-764, 2011.
- [106] M. A. Hubbe, R. A. Venditti e O. J. Rojas, «What happens to cellulosic fibers during papermaking and recycling? A review.,» *BioResources*, pp. 739-788, 2007.

6. References

- [107] A. Idstrom, H. Brelid, M. Nydén e L. Nordstierna, «CP/MAS ^{13}C NMR study of pulp hornification using nanocrystalline cellulose as a model system.,» *Carbohydr polym*, pp. 881-884, 2013.
- [108] O. Garcia, A. L. Torres, J. F. Colom, F. I. J. Pastor, P. Diaz e T. Vidal, «Effect of cellulase-assisted refining on the properties of dried and never-dried eucalyptus pulp.,» *Cellulose*, pp. 115-125, 2002.
- [109] T. Saito, S. Kimura, Y. Nishiyama e A. Isogai, «Cellulose nanofibers prepared by TEMPO-mediated oxidation of native cellulose.,» *Biomacromolecules*, pp. 2485-2491, 2007.
- [110] I. Besbes, S. Alila e S. Boufi, «Nanofibrillated cellulose from TEMPO-oxidized eucalyptus fibres: effect of the carboxyl content.,» *Carbohydr polym*, pp. 975-983, 2011.
- [111] A. Isogai, T. Saito e H. Fukuzumi, «Review: TEMPO-oxidized cellulose nanofibers.,» *Nanoscale*, pp. 71-85, 2011.
- [112] G. Rodionova, T. Saito, M. Lenes, O. Eriksen, O. Gregersen, R. Kuramae e A. Isogai, «TEMPO-mediated oxidation of Norway spruce and Eucalyptus pulps: preparation and characterization of nanofibers and nanofiber dispersions.,» *J polym environ*, pp. 207-212, 2013.
- [113] L. Alexandrescu, K. Syverud, A. Gatti e G. Chinga-Carrasco, «Cytotoxicity tests of cellulose nanofibril-based structures.,» *Cellulose*, pp. 1765-1775, 2013.
- [114] M. Bhattacharya, M. M. Malinem, P. Lauren, Y. Lou, S. W. Kuisma, L. Kanninen, M. Lille, A. Corlu, C. GuGuen-Guillouzo, O. Ikkala, A. Laukkanen, A. Urtti e M. Yliperttula, «Nanofibrillar cellulose hydrogel promotes three-dimensional liver cell culture.,» *Journal of control release*, pp. 291-298, 2012.

6. References

- [115] M. Montenegro-Nicolini e J. O. Morales, «Overview and future potential of buccal mucoadhesive films as drug delivery systems for biologics,» *AAPS PharmSciTech*, pp. 2-14, 2017.
- [116] P. Berka, D. Stranska, V. Semecky, K. Berka e P. Dolezal, «Testing the limits of in vitro sublingual macromolecular drug delivery: dextrans and albumin with nanofiber mats,» *International Journal of Pharmaceutics*, 2019.
- [117] I. Diaz Del Consuelo, G. P. Pizzolato, F. Falson, R. H. Guy e Y. Jacques, «Evaluation of pig esophageal mucosa as a permeability barrier model for buccal tissue.,» *Journal of pharmaceutical sciences*, pp. 2777-2788, 2005.
- [118] V. Hearnden, V. Sankar, K. Hull, D. Juras Vidovic, M. Greenberg, A. R. Kerr, P. B. Lockhart, L. L. Patton, S. Porter e M. H. Thornhill, «New developments and opportunities in oral mucosal drug delivery for local and systemic disease.,» *Advanced drug delivery reviews*, pp. 16-28, January 2012.
- [119] H. E. Junginger, J. A. Hoogstraate e J. C. Verhoef, «recent advances in buccal drug delivery and absorption-in vitro and in vivo studies,» *journal of controlled release*, vol. 62, n. 1-2, pp. 149-159, 1999.
- [120] M. Artusi, P. Santi, P. Colombo e H. E. Junginger, «Buccal delivery of thiocolchicoside: in vitro and in vivo permeation studies.,» *Int. J. Pharm.*, vol. 250, n. 1, pp. 203-213, 2003.
- [121] A. M. Manganaro e P. W. Wertz, «The effects of permeabilizers on the in vitro penetration of propranolol through porcine buccal epithelium.,» *Mil. Med.*, pp. 669-672, 1996.

6. References

- [122] R. Birudaraj, B. Berner, S. Shen e X. Li, «Buccal permeation of buspirone: Mechanistic studies on transport pathways.,» *J. Pharm. Sci*, pp. 70-78, 2005.
- [123] R. Abu-Huwaij, S. Assaf, M. Salem e A. Sallam, «Potential mucoadhesive dosage form of lidocaine hydrochloride: II. In vitro and in vivo evaluation.,» *Drug Dev. Ind. Pharm.*, pp. 437-438, 2007.
- [124] M. K. Dhiman, A. Dhiman e K. K. Sawant, «Transbuccal delivery of 5-fluorouracil: Permeation enhancement and pharmacokinetic study.,» *AAPS PharmSciTech* , pp. 258-265, 2009.
- [125] E. Ojewole, R. Kalhapure, K. Akamanchi e T. Govender, «Novel oleic acid derivatives enhance buccal permeation of didanosine.,» *Drug Dev. Ind. Pharm.* , pp. 657-668, 2014.
- [126] J. O. Morales e J. T. McConville, «Novel strategies for the buccal delivery of macromolecules.,» *Drug Dev. Ind. Pharm.*, pp. 579-590, 2014.
- [127] C. Padula, S. Pescina, S. Nicoli e P. Santi, «New insights on the mechanism of fatty acids as buccal permeation enhancers.,» *Pharmaceutics*, vol. 10, n. 4, p. 201, 2018.
- [128] F. Helmchen e W. Denk, «Deep tissue two-photon microscopy.,» vol. 2, n. 12, 2005.
- [129] S. Dash, P. N. Muthy, L. Nath e P. Chowdhury, «KINETIC MODELING ON DRUG RELEASE FROM CONTROLLED.,» *Acta Poloniae Pharmaceutica ñ Drug Research*, pp. 217-223, 2010.
- [130] O. Lieleg e K. Ribbeck, «Biological hydrogels as selective diffusion barriers.,» *Trends in Cell Biology*, pp. 543-551, 2011.

6. References

- [131] T. Caon e C. M. O. Simoes, «effect of freezing and type of mucosa on ex vivo drug permeability parameters,» *AAPS PharmSciTech*, vol. 12, n. 2, pp. 587-592, 2011.
- [132] A. J. Hoogstraate, S. Senel, C. Cullander, J. Verhoef, H. E. Junginger e H. E. Boddé, «Effects of bile salts on transport rates and routes of FITC-labelled compounds across porcine buccal epithelium in vitro.,» *Journal of control. release*, pp. 211-221, 1996.
- [133] I. D. Del Consuelo, F. Falson, R. H. Guy e Y. Jacques, «Transport of fentanyl through pig buccal and esophageal epithelia in vitro. Influence of concentration and vehicle pH.,» *Pharm. Res.*, pp. 1525-1529, 2005.
- [134] A. Deevan Paul, P. Samantha, S. Manasa, R. Munemma e D. Supriya, «Modeling the Oral Cavity with Mucoadhesive Drug Delivery Systems - A Potential Alternative to Conventional Therapy,» *International Journal of Pharmaceutical Sciences and Drug Research*, pp. 299-307, 2017.
- [135] C. J. Batty, M. T. Heise, E. M. Bachelder e K. M. Ainslie, «Vaccine formulations in clinical development for the prevention of severe acute respiratory syndrome coronavirus 2 infection,» *Advanced Drug Delivery Reviews*, pp. 168-189, 2021.
- [136] M. Tiboni, L. Casettari e L. Illum, «Nasal vaccination against SARS-CoV-2: Synergistic or alternative to intramuscular vaccines?,» *International Journal of Pharmaceutics*, 2021.
- [137] J. A. Matriano, M. Cormier, J. Johnson, W. A. Young, M. Buttery, K. Nyam e P. E. Daddona, «Macroflux microprojection array patch technology: a new and intracutaneous immunization,» *Pharmaceutical research*, pp. 63-70, 2002.

6. References

- [138] M. H. Ling e M. C. Chen, «Dissolving polymer microneedle patches for rapid and efficient transdermal delivery of insulin to diabetic rats.,» *Act Biomater.*, pp. 8952-8961, 2013.
- [139] S. Biswas, «BBC,» 20 August 2021. [Online]. Available: <https://www.bbc.com/news/world-asia-india-57774294>.
- [140] Min. Salute, «© FIMP – Federazione Italiana Medici Pediatri,» 8 April 2021. [Online]. Available: https://fimp.pro/wp-content/uploads/2021/04/Circolare_Prevenzione_controllo_influenza_2021_22_DEF.pdf.
- [141] PDQ Adult Treatment Editorial, «PDQ Cancer Information Summaries [Internet],» Bethesda (MD): National Cancer Institute (US), 22 luglio 2021.[Online].Available: https://www.ncbi.nlm.nih.gov/books/NBK65887/figure/CDR0000258017__308/.

7. ACKNOWLEDGEMENTS

7. Acknowledgements

I thank the PhD coordinator Professor Marco Mor for the rigor and seriousness with which he follows, supports and encourages the students of the doctoral course.

I wish to extend my special thanks to my esteemed supervisor Professor Patrizia Santi, who assisted and guided me throughout this project. I'm grateful to her for having been my mentor over the years.

I would thank Professor Francesco Lai and Doctor Cristina Cardia from the University of Cagliari for kindly providing microneedles patches.

Professor Cristina Sissa and Andrea Delledonne from the University of Parma are also gratefully acknowledged for providing two photon microscopy images.

Additionally, my gratitude extends to Professor Timo Laaksonen and Doctor Patrick Lauren for hosting me during my secondment at the Faculty of Pharmacy of the University of Helsinki.

Lastly, I would like to thank Doctor Silvia Pescina for encouraging and supporting me throughout my studies.