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Selective adsorption of functional fibronectin through aptamer functionalization: a new concept for tailoring materials bioactivity

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"La stessa emozione, la stessa meraviglia e lo stesso mistero, nascono continuamente ogni volta che guardiamo ad un problema in modo sufficientemente profondo. A una maggiore conoscenza si accompagna un più insondabile e meraviglioso mistero, che spinge a penetrare ancora più in profondità. Mai preoccupati che la risposta ci possa deludere, con piacere e fiducia solleviamo ogni nuova pietra per trovare stranezze inimmaginabili che ci conducono verso domande e misteri ancora più meravigliosi – certamente una grande avventura!"

Richard Feynman
New York, 11 maggio 1918 – Los Angeles, 15 febbraio 1988
Premio Nobel per la Fisica 1965

A chi, come me, non ha mai smesso di crederci.

Abstract

Protein adsorption on biomaterials occurs shortly after scaffold insertion and it is of pivotal importance to address cell-material interaction and to achieve therapeutic success in tissue engineering. Therefore, its control may represent a crucial node in the design of implantable devices. In this thesis, aptamers, which are short oligonucleotides able to recognize, bind and retain target proteins with high affinity, have been identified as an innovative cue to address the selective adsorption of fibronectin from plasma at chitosan interface.

Selective fibronectin chitosan surfaces were prepared and characterized for the amelioration of fibronectin adsorption both quantitatively and qualitatively. Furthermore, cell response was studied against murine osteoblastic-like MC3T3-E1 cells and against human epithelial-like HeLa cells.

The results we obtained supports the use of aptamers as a new viable modification to improve the adsorption of specific proteins at scaffold interface and to promote the response of cells in term of adhesion, spreading, migration and proliferation.

To conclude, we believe to be at the eve of a new promising way to tailor scaffold biocompatibility and to design new targeted and customized therapies in tissue engineering.

Contents

Preface

Annually, millions of people are affected by tissue or organ loss from trauma, congenital defects or disease, and the global shortage of tissues and organs for transplantation has been known to be a major public health challenge. The World Health Organization (WHO) has recently estimated that only the 10% of the worldwide need for organ transplantation is being met. Therefore, the research of more easily accessible solutions is desirable and regenerative medicine represent a viable field of study to supply this problem.

Today, most regenerative studies rely on tissue engineering (TE). Particularly, biological substitutes, namely scaffold, are used to mimic the organization of a provisional extracellular matrix (ECM) and to support the ingrowth and the response of progenitors cells from neighboring tissues, which will then deposit and organize new ECM components. To achieve this goal, biomaterials should provide a favorable microenvironment for cells to leave them to adhere, spread, migrate, proliferate and differentiate. Obviously, exogenous biomaterials do not possess the exact structural complexity of ECM and as such, they have to be finely tailored. In particular, protein adsorption at the interface is of utmost importance in addressing cell response to biomaterials. It occurs shortly after scaffold positioning, rendering the direct recognition between cells and materials virtually impossible and affecting subsequent biological outcomes.

The present thesis focuses the attention on the development of a new method to accurately control selective protein adsorption on biomaterials by means of aptamers, small oligonucleotides with protein-binding capacity. The new insight of this idea is represented by the possibility of enriching materials with desired cues selected from a great pool and to overcome the random deposition/adsorption of unspecific stimuli on material surface. On a clinical level, aptamer-grafting materials could be envisaged as devices able to capture bioactive cues from patients' blood and to concentrate them where they are needed. We believe that many areas of medicine will potentially benefit from these new class of materials.

Chitosan films have been chosen as a substrate for aptamer functionalization. To the best of our knowledge, chitosan is one of the most explored materials for

scaffold design. The main appeal of chitosan is that it possesses the capacity to be mold in a vast array of shapes, which allow it to find application in a wide spectrum of different tissues: from hard and load-bearing tissues, like bone, to the softer ones such as brain or neural tissue. On the other hand, even though chitosan has great affinity for proteins, it has been largely described to support scarce cell response against numerous type of cells. This is the reason for why chitosan was selected as a viable candidate for our aims. In **chapter 1** "*Tailoring the interface of biomaterials to design effective scaffolds*", after a deep discussion on scaffold requirements for being considered biocompatible, an overview on chitosan and on its application in bone, skin, cartilage and cornea TE has been reported. Thus, our first attempt to ameliorate chitosan surface bioactivity through a coating with porcine thiolated gelatin has been reported in **chapter 2** "*Chitosan scaffold modified with D-(+) Raffinose and enriched with thiol-modified gelatin for improved osteoblast adhesion*". The results we obtained in this first paper were promising. The improvement of chitosan with thiol-modified gelatin dramatically enhanced the response of osteoblastic cells in term of adhesion, spreading and proliferation, thus suggesting the potentiality of further ameliorating protein adsorption at the interface more precisely. Considering this, fibronectin was selected as a protein target to be bound and retained on chitosan surface. Fibronectin is an ECM main component, which is widely available both in injured and in regenerating tissues. Its main role is that of providing docking points for cells and it has been demonstrated to be involved in the regulation of cell adhesion, spreading, migration, proliferation and differentiation. For all these reasons, fibronectin was an excellent target for our purposes. Fibronectin structure as well as function and ways that have been explored in the late years to enrich biomaterials are reported in **chapter 3** "*Fibronectin as a crucial step to control cell response at biomaterial interface*". Subsequently, in **chapter 4** "*Aptamers to improve the bioactivity of biomaterials*", aptamers, their potential and their applicability in regenerative medicine are deeply discussed and presented. Thus, in **chapter 5** "*Anti-fibronectin aptamers improve the colonization of chitosan films modified with D-(+) Raffinose by murine osteoblastic cells*", we reported our first attempt to exploit anti-fibronectin aptamer to promote cell response on chitosan substrates. Fascinatingly, the size of cell

response measured in term of adhesion and proliferation was extremely dependent on the amount of aptamer used for the functionalization. However, even though the response of the cells was greatly enhanced, no differences were detected in the amount of fibronectin adsorbed on chitosan in the presence or in the absence of aptamer functionalization. As such, we decided to deeply investigate this aspect to justify the mismatch of the results we obtained. To this purpose, we hypothesized that aptamers lead to the amelioration of fibronectin adsorption qualitatively, rather than quantitatively, thus preserving its biological activity. In **chapter 6** "*Surface modification of chitosan with anti-fibronectin aptamers: a mass spectrometry approach probing evidence of fibronectin behavior*", we proposed to investigate the conformational adsorption of fibronectin through different type of mass spectrometry analysis. The data we obtained confirmed our hypothesis and were phenotypically confirmed in **chapter 7** "*Functional fibronectin adsorption on aptamer-doped chitosan modulates cell morphology by integrin-mediated pathway*". In this last chapter, by inhibiting cytoskeleton components, i.e. actin and myosin II, we wanted to confirm at a biological level that aptamers ameliorated the biological activity of fibronectin adsorbed on chitosan surface, thus leading to the activation of integrin-mediated transduction pathway.

Chapter 1

Tailoring the interface of biomaterials to design effective scaffolds

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Abstract

Tissue engineering (TE) is a multidisciplinary science, which including principles from material science, biology and medicine aims to develop biological substitutes to restore damaged tissues and organs. A major challenge in TE is the choice of the suitable biomaterial to fabricate a scaffold that mimic native extracellular matrix guiding resident stem cells to regenerate the functional tissue. Ideally, the biomaterial should be tailored in order that the final scaffold would be i) biodegradable to be gradually replaced by regenerating new tissue, ii) mechanistically similar to the tissue to regenerate, iii) porous to allow cell growth as nutrient, oxygen and waste transport and iv) bioactive to promote cell adhesion and differentiation.

With this perspective, this review discusses the options and the challenges to face in biomaterial selection when a scaffold has to be designed. We highlight the possibilities in the final mold the material should assume and the most effective technique for its fabrication depending on the target tissue, including the alternatives to ameliorate its bioactivity. Furthermore, particular regard has been addressed to the influence that all these aspects have on resident cells considering the frontiers of materiobiology. In addition, a focus on chitosan as a versatile biomaterial for TE scaffold fabrication has been done, highlighting its latest advances in the literature on bone, skin, cartilage and cornea TE.

1. Introduction

Biomaterials are discernable from any other material for their ability to co-exist with biological tissues without causing adverse effects. In the last century, this unique property has led to a growing interest of scientists for the development of medical devices. However, since the manner in which biomaterials and tissues could interact are almost countless, the concept of biocompatibility, often superficially defined as "*biological inertia*", requires a deeper discussion on its significance.

To the first generation of biomaterial scientists, who developed and introduced the use of implantable medical devices (1940-1980), it was quite obvious how the optimal performance was obtained when no adverse effects were triggered in the host[1]. Consistently, the definition of biocompatibility became soon a list of negative issues that should be avoided, such as non-toxicity, non-immunogenicity, non-carcinogenicity, non-irritancy and so on. The evidences that: i) the response of specific material varied from the site of its application; ii) in an increasing number of clinical needs material should evoke appropriate responses of the surrounding tissues; and iii) that sometimes materials should be degraded after having carried out their function; moved to a re-evaluation of the concept of biocompatibility, which was defined in 1987 as follows:

"*Biocompatibility refers to the ability of a material to perform with an appropriate host response in a specific situation*"[1].

However, this definition was still in its infancy and accordingly to Williams it should be revised. Particularly, biocompatibility is accomplished when a stable equilibrium between the biomaterial and the host is obtained. As such, we have to consider that both the clinical intervention and the unique characteristics of each individual are of considerable significance when defining the concept[2]. Thus, the concept of biocompatibility should be adapted on the clinical need we have[3].

Within the set of long-term implantable medical devices, we challenge a wide spectrum of conditions ranging from hard to soft tissue. In this specific case, we should talk about clinical biocompatibility, which requires the use of mechanically stable non-degradable materials and functionally suitable to definitively support or replace the damaged organ. Examples of these applications may be titanium and its alloys for dental and orthopedic applications, silicones for ophthalmological

devices and breast implants, or polytetrafluoroethylene (PTFE) for heart valves and vascular grafts reconstruction[4-8].

The concept of biocompatibility needs then to be further modified to include the set of degradable implantable materials: devices that have to perform a function for a restricted time frame. A clear example of this type of materials are synthetic polymers, i.e. that commonly used to produce suture materials. With this type of materials, degradability should be included in their biocompatibility definition[9-11].

The paradigm of biocompatibility could be still enhanced to include transient invasive intravascular/intraparenchymal devices, such as catheter for the delivery of substances for nutritional, diagnostic and therapeutic purposes. Clearly, the choice of material must relapse on that with good blood compatibility, in order to avoid unsought effects as thrombosis[12].

All the biomaterials applications mentioned above, as the relative concept of biocompatibility, have been largely studied and have well-known clinical success, but a fundamental shift on the mechanism of biocompatibility has now to be done to approach the vast field of scaffold development for tissue engineering applications.

2. Tissue Engineering

In the last decades, a fundamental shift in the design and use of biomedical materials has been witnessed when the era of regenerative medicine (RM) began. RM is an interdisciplinary field that combines the principles of engineering and life sciences to the goal of building tissues and organs damaged by age, disease, trauma or congenital defects. All conditions that without intervention would spontaneously remain unrepaired because the body possesses only limited ability to regenerate. RM encompasses different strategies, including pharmaceutical targeting of bioactive factors for *in situ* stimulating of stem cells, genetic engineering of autologous cells followed by their grafting or the combination of cells, soluble factors and biomaterials to promote tissue regeneration; this latter approach is know as tissue engineering (TE)[1,13].

In particular, TE paradigm may follow an *in vivo* or an *in vitro* approach (**Fig.1**), but it always relies on the use of a biomaterial, which has to be mold in a 3D scaffold. The scaffold acts as a template to accommodate cells, which adhere, proliferate, differentiate and start to depose new extracellular matrix (ECM), which progressively coat the structure, leading to the regeneration of the new tissue, while the biomaterial is progressively resorbed[1].

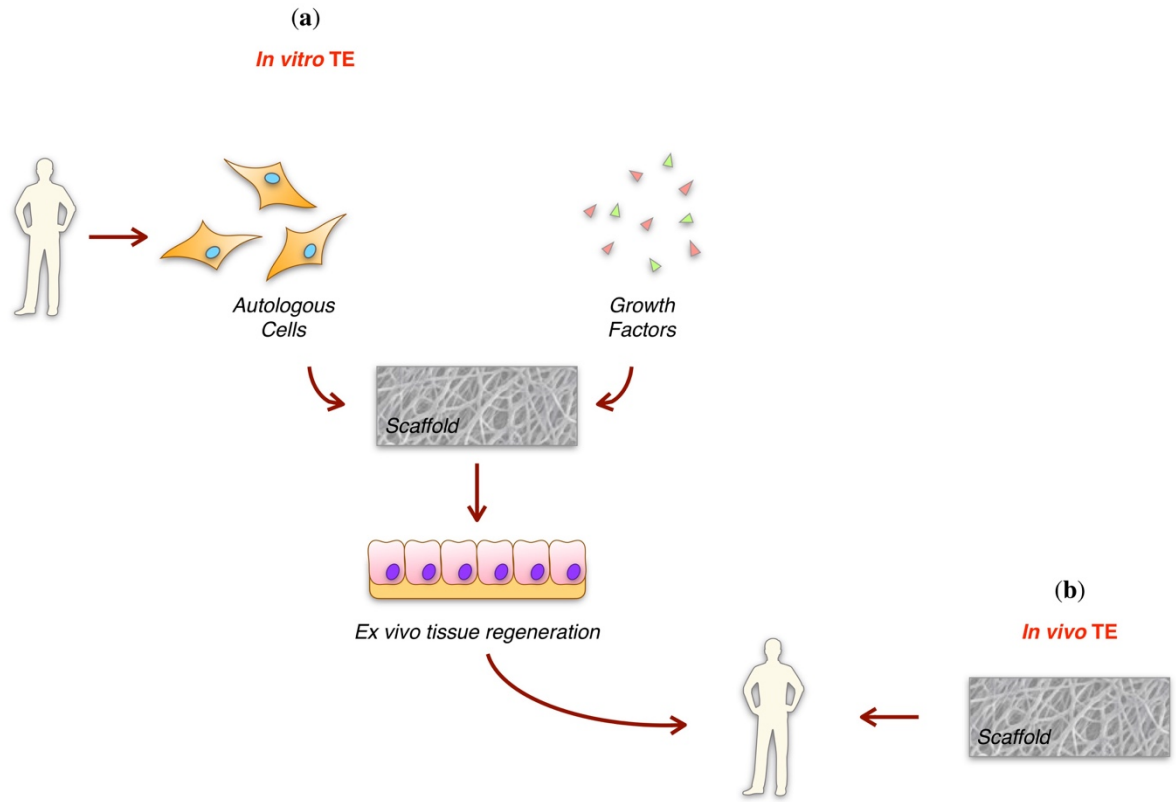

Figure 1. *Tissue Engineering.* (**a**) *In vitro* TE. Autologous cells and growth factors are co-seeded on the biomaterial scaffold and maintain in culture until tissue neo-formation. Tissue regeneration occurs *ex vivo* and once formed, the tissue is grafted. (**b**) *In vivo* TE. Biomaterial scaffold is directly implanted in the damaged anatomical site.

In a such complex scenery, it becomes obvious that the concept of biocompatibility need to be dramatically revised, since the scaffold should be thought as a high dynamic structure able to elicit different responses, in different tissues or organs. The goal is the induction of regeneration where the naturally occurring reparation is scar, with consequent impairment of tissue/organ function. In this arena, the scaffold should encompass different features: it should determine the shape of the defect and promote an appropriate cell response through the control of their phenotype and gene expression profile. Not less important, great significance is assumed by scaffold biodegradability.

3. Scaffold requirements

Simple observation of biological tissues shows that they are extremely complex systems and that their structural organization relies on their functions[13]. The first step towards scaffold fabrication is to identify a biomaterial that could be mold in order to match specific tissue properties allowing the creation of a milieu suitable for cell growth, proliferation, differentiation and ECM deposition for a successful regeneration. Criteria for the choice of a suitable biomaterial include the possibility i) to be degraded without any systemic adverse effects on the host; ii) to assume proper mechanical properties to mirror that of the host tissue; iii) to assume a suitable internal architecture; and iv) to elicit surrounding tissues biological response[14].

3.1 Biodegradability requirements

TE scaffolds are not considered as permanent implants. Therefore, they should be reabsorbed with an opportune rate of degradation hand to hand colonizing cells regenerate the tissue. Furthermore, if the scaffold does not degrade, undesired effects may occur, such as internal pores obstruction by *in vivo* events with consequent tissue necrosis[15]. The choice of a biodegradable biomaterial is thus of great importance with regard of TE and must be accurately done since both the bulk material and its degradation products have not to be inflammatory, mutagens, toxic, carcinogens and so on. For example, materials that may be used in small volumes, with short degradation profile and with apparently physiologically acceptable degradation products, e.g. synthetic materials for sutures, can not be adequate for the realization of complex scaffolds, because they may trigger adverse effects in the host[3].

Biodegradability, otherwise bioresorbability, consists in the capacity of a material to decompose over the time as a result of biological processes, i.e. hydrolysis or enzymatic activity, and it is often a peculiar property of a wide range of polymers. Biodegradable polymers may be of synthetic or natural origin. Here, we propose a brief review of that commonly used in TE[16].

3.1.1 Synthetic biodegradable polymers

Synthetic polymers represent the largest group of biodegradable polymers. They are widely used in biomedical applications, since their properties (e.g. biodegradability, mechanical aspects, porosity) can be predicted and tailored for the specific application during their synthesis and many are also suitable for printing[17,18].

Among the synthetic polymers, aliphatic polyesters (-O-CHR-CO-)$_n$-) are the most attractive group, since their esteric group is easily accessible by human enzymes. Their degradation rate can be controlled by monitoring their synthesis, polymerization conditions or introducing specific functional groups. Furthermore, their degradation products are recognized by the human body and can be removed by common metabolic pathways[16].

The most common alpha polyesters are polyglycolic and polylactic acids (PGA and PLA), which can be combined to form their copolymers. Both PGA and PLA have shown to possess great capacity to support the attachment and proliferation of cells, especially those of bone and chondrocytes. Furthermore, the products of their degradation are glycolic and lactic acid, which are non-toxic and easily removable from the body by physiological metabolic pathways[17,19]. Poly-ε-caprolactone (PCL) is a viable alternative to the use of PGA/PLA polymers. PCL was one of the first synthetic polymer synthetized. Even though it has been forgotten for a long time because of its poor mechanical properties, it has been recently rediscovered thank to its superior viscoelasticity and to its malleable rheological properties that allow its manufacturing into large-scale scaffolds[20].

3.1.2 Natural biodegradable polymers

Natural polymers that include proteins or polysaccharides could be preferred to synthetic ones both because their repeating units are more similar to those commonly metabolized by human body (amino acids and monosaccharides), and because they possess appropriate binding motifs for cells within their molecular structure[3].

Collagen, which is the most abundant structural protein of the human body, is probably the most used biopolymer in medicine. It is normally derived from animals (porcine, equine or bovine) and it has shown great potential in bone and cartilage

TE. Collagen possesses extremely good degradability properties: its biodegradation could be accomplished by natural lysosomal enzymes and collagenase, and most important it may be modulated by controlling its cross-linking during fabrication[21-23]. Another protein, which has recently shown great applicability in RM, is silk fibroin, which is isolated from the cocoon of *Bombyx mori* silkworm and is easily degraded by human hydrolases[24,25].

On the other hand, polysaccharides are gaining interest among biomaterial scientists because of their capacity to trigger specific cell signaling[3]. Hyaluronan is one of ECM main component and thus it presents excellent biocompatibility against cells and tissues. Furthermore, hyaluronan possess excellent solubility in water, which contributes to short residence time after its implantation and rapid resorption[26,27].

Alginate is a polysaccharide isolated from vegetal organisms (brown algae). It contains inflammatory components, but its purification contributes to limit this issue making alginate a suitable material for TE scaffold, which do not elicit any host response within 1 year[28].

Chitosan which is derived from N-deacetylation of chitin, one of the main component of crustacean exoskeleton, is often used in scaffold manufacturing, and will be discussed later in this work.

3.2 Mechanical requirements

Mechanical properties resembling as close as possible those of the native tissue are between the first requirements an engineered scaffold should have. To be considered mechanistically biocompatible, scaffold should maintain the integrity of the defect until complete regeneration of the target tissue, meanwhile opportunely responding to external forces. At the same time, it has to possess fatigue property to avoid its failure when undergoing to cyclic loading.

Rheological parameters for proper scaffold design include i) elastic modulus, that measured strain in response to a given tensile or compressive stress along the plane of the applied force; ii) flexural modulus, that measured the relationship between a bending stress and the resulting strain after a compressive stress applied perpendicularly; ii) tensile strength, that is the maximum stress a material can

withstand before its break and iv) maximum strain, that is the ductility exhibited by the material before a fracture.

These properties, with particular regards to elastic modulus, in turn affect interstitial fluid flow, including nutrient and waste transport, which are of great importance for cell metabolism[29-32]. On their counterpart, tissue cells sense via mechanotransduction the stiffness and the mechanics of the surrounding milieu, that in human body range from hundreds of Pa (skin/subcutaneous tissue – 57Pa[33]) to GPa (trabecular bone – 100GPa[34]), to regulate their growth (adhesion, migration and spreading), proliferation and differentiation[35]. According to mechanobiology theories, cells prefer the adhesion to substrates with stiffness similar to the tissue they belong to, as their way of migration along the material depends on its stiffness[36]. If the rigidity of the substrate is not in compliance with that of the native tissue, cells may switch their way of migration. This often occurs in pathological conditions, e.g. cancer metastasis, where physical properties of the tissue change and cells switch their way of migration from lamellipodia/filopodia to amoeboid mode[37]. In **Figure 2**, the substrates stiffness related to different cell phenotypes are reported.

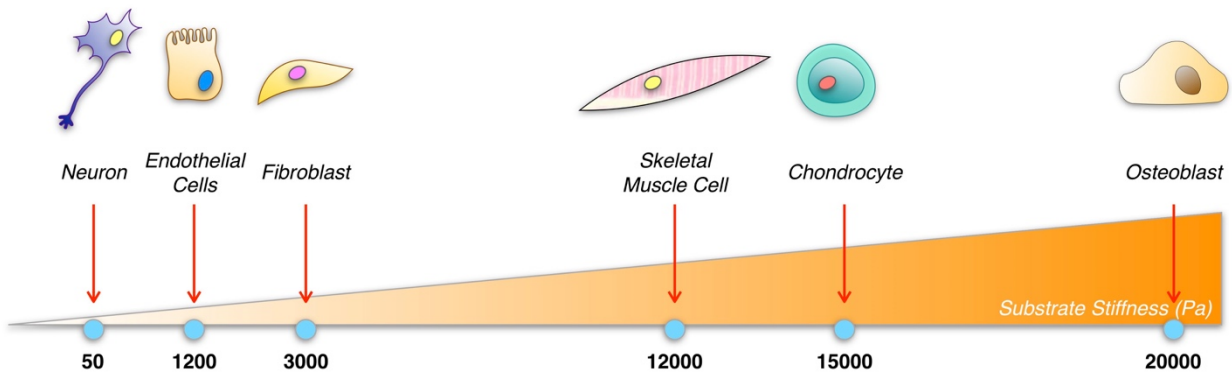

Figure 2. ***Relationship between substrate stiffness and cell phenotype.*** Cell phenotype is shaped by the stiffness of the substrate.

The way of cell adhesion and migration are of pivotal importance for cell differentiation and proliferation within the scaffold. Both these parameters have to be set also considering the function of the cells within the tissue they have to regenerate. Eventually, cell-material interaction, including adhesion, migration and spreading, defines cell morphology, which is a key factor in triggering progenitor

cells commitment[38,39]. It has been extensively shown that stiff substrates (30-35kPa) promote osteogenic differentiation of mesenchymal stem cells, while softer ones (~10kPa) allow myogenic or adipogenic commitment[40,41].

The options for TE include a wide range of materials, whose bulk mechanical properties contribute to the definition of their applicability in different applications. For instance, polysaccharides possess low strength and rigidity, that limit their use to soft tissue regeneration, while fibrous proteins, i.e. collagen, which normally provides stability and mechanical integrity to biological structures, may be exploited for hard tissues. Additionally, it has to be considered that the final mechanical properties of the scaffolds strongly depend on the manufacturing process, that often alters the mechanics of the bulk material. Considering this aspect, TE scaffold of the very same material may be molded into porous, hydrogel and fibrous scaffolds.

3.2.1 Porous scaffolds

The first type of TE scaffold that have been developed were 3D porous scaffolds, such as sponges and foams. Sponges and foams present a highly porous network, which is induced and controlled during the manufacture process. A limit for their application is often the low mechanical properties of the resulting substrates[17].

Event though, to the best of our knowledge, the literature on this topic is still lacking, a recent work by Li et al. investigated a fish collagen-based sponge as dura mater substitute after surgical resection in rabbits. Animal were monitored during the postoperative period. All the animals recovered well and their wound healed properly. Histological analysis revealed that peripheral cells migrated into the outer layer of the implant, but no infiltration of brain cells was observed in the inner layer, indicating a good tolerance of the implant. Furthermore, neovascularization was observed after 30 days[42].

3.2.2 Hydrogels

Hydrogels are networks of cross-linked polymers capable to swell with water. As such, they possess a biochemical similarity with highly hydrated glycosaminoglycan (GAG) organizing ECMs. This aspect has dressed hydrogels to an emerging part in the field of TE[43]. Methods for hydrogel fabrication are generally based either on

chemical gelation, which include the formation of strong covalent bonds between the polymeric chains by secondary molecules interposition (chemical crosslinking), or on physical gelation, as a result of non-covalent interactions among the functional groups of polymer chains (physical crosslinking)[44,45]. It is clear that in the case of chemical gelation, it is more than ever important to control the cytocompatibility of the chemistry exploited to design the scaffold, in order to avoid the introduction of potentially toxic components.

Moreover, recent advances in nano-fabrication techniques achieved the creation of hydrogels with a highly controlled structure. Regarding hydrogels mechanical characteristics, it is easily understood that they are materials with limited application in regenerative procedures of load-bearing tissues. However, these challenge may be at least partly addressed by incorporating various materials, even in the form of nanoparticles, i.e. calcium phosphates or silica nanoparticles, to modulate their stiffness[46-49]. On the other hand, thank to their soft mechanical properties, polymeric hydrogels have been exploited for cell encapsulation and delivery, collecting great success especially for cardiac TE[50,51].

Considering hydrogels limited capacity of diffusion, they have been widely employed as materials for cartilage TE. For example, Zhang et al. prepared a copolymer hydrogel of PLGA and PEG by physical thermal crosslinking and loaded it with bone marrow mesenchymal stem cells for full-thickness articular cartilage defect repair. Specimens were *in vivo* tested in rabbits, which 12 weeks post-surgery showed enhanced fibrosis in control group (no treatment), partial restoration of the defect in gel group and great regeneration in gel/cells group[52].

Another suitable site for injectable hydrogel application is neural tissue. Chedly et al. prepared a chitosan-based hydrogel for spinal cord injury recovering in rats. They observed a reduction in astrocytes activation, which is typical during glial scar formation, and robust axon regrowth already 4 weeks after the injury. Furthermore, the activation of inflammatory macrophages (M1) was up-regulated in the presence of chitosan 4 days after surgery, progressively decreasing in time until reaching maximum down-regulation with significant differences from control at 8 weeks of healing. According to this, reparative macrophages (M2) were statistically up-regulated in the presence of chitosan from 4 to 8 weeks[53].

3.2.3 Fibrous scaffolds

Developing structures to mimic tissue anatomy at a microscopic level is one of the major challenges in TE. The introduction of nanofibers has enhanced the chance to produce scaffold that potentially overcome this issue. Furthermore, nanofibers possess the advantage to supply a high surface/volume ratio, which, combined to their micro-porous structure, promotes peculiar cell responses. Methods to synthetize nanofibers include electrospinning, self-assembly and phase separation (see paragraph 3.3.1)[54].

Nanofibrous materials are probably the most explored scaffolds for TE and their application range from load-bearing hard tissues, i.e. bone or tendon/ligament, to vascular TE. However, their stiffness slightly limits their applicability to soft tissues.

Through the use of an electrospun nanofiber scaffold of polyhydroxybutyrate, chitosan and bioglass, Khoroushi et al. cultured and promoted the differentiation of human exfoliated deciduous teeth pulp cells into odontoblasts[55]. In another study, Chen et al. designed an electrospun gelatin/PLA scaffold blended with a hyaluronic-acid based hydrogel for articular cartilage repair showing complete tissue regeneration in rabbits 12 weeks after surgeries[56]. For tendon TE, Pauly et al. studied the effects of orientation and geometry of PCL electrospun nanofibers on scaffold mechanical properties by measuring rheological parameters, including elastic modulus, yield stress and strain. Interestingly, oriented nanofibers ameliorate the mechanical properties of the structured scaffold and at the same time boost elongation of adult human adipose-derived stem cells after 7 days of culture[57]. Interesting results on the application of nanofibers as scaffolds for TE come also from skeletal muscle and skin TE. Laminin-coated poly(methyl methacrylate) nanofibers showed to promote primary myoblasts proliferation, while nanofiber silk fibroin scaffold revealed to have great potential in wounds healing of skin *in vivo*[58,59].

3.3 Porosity requirements

Another key characteristic that has to be considered during TE scaffold design is mass transport. Mass transport includes the carrier of nutrients, oxygen and waste to and by cells, as of molecules and signals[60]. *In vivo* these events occur by diffusivity and permeability through blood vessels, while in 3D models for TE mass transport

could be obtained adjusting matrix porosity, which in the first step of tissue regeneration leads to the Brownian motion or fluid flow under applied pressure of substances, while hand to hand tissue is regenerating it should lead space and way for blood vessels and capillaries growth[61].

Furthermore, at a cellular level, porosity is of pivotal importance to allow cell migration and scaffold colonization. As such, it is obvious that pore size should be large enough to supply mass transport and not too large to prevent cell migration[62]. Pore size may range from few nanometers to millimeters and it has been shown how different pore sizes may influence different cell processes[63,64]. Nanometric dimensions of pore (d<100nm) promote cell attachment and functioning, while micrometric pores (100nm-100μm) allow the interaction and communication between cells through the extrusion of cytoplasmic protuberances[65]. Finally, the threshold pore size which allows cell migration across the structure depends on cell type and tissue function, i.e. hepatocytes which have a mean diameter of 20-40μm preferred pore diameter of 20μm, while bone cells (20-30μm) according to the requirements of bone tissue need a pore diameter between 100 and 350μm[66-68].

Scaffold porosity, pore size, geometry, distribution and interconnectivity, which in turn affect the mechanical properties, may be controlled by fabrication techniques, which can be divided in "top-down" approaches when the scaffold is pre-fabricated and in "bottom-up" methods, which consist in fabricating microscale tissue building blocks and assembling them to form a larger construct.

3.3.1 Top-down approaches

Traditional TE methods use "top-down" approach for scaffold construction, which consist in seeding cells onto a pre-fabricated scaffold. Generally, this type of approach has been engineered for thin and simple tissues, such as skin, cartilage or bladder[69-71]. Indeed, even though "top-down" methods allow to partially control scaffold porosity, they often present limitations to provide opportune diffusivity. Thus, considering that cells distance from nutrients can not exceed 200μm, "top-down" approaches often present a limit for the regeneration of high density and high metabolic demanding organs and tissues, such as liver or kidney[72]. In spite of this, this type of approach is widely and successfully used in TE.

Solvent casting is the simplest, easy and inexpensive technique for scaffold preparation and it is totally based on solvent evaporation for the creation of a polymeric membrane (film). Since the main issue of this method is the impossibility to obtain pore formation, films obtained in this way have often been used in association with other porous scaffolds, as barriers[73]. To this purpose, Zonari et al. designed a bilayer skin TE construct combining a solvent casting poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) membrane for keratinocytes culture placed on the top of a 3D porous PHBV scaffold seeded with dermal fibroblasts[74].

To control the porosity, particularly pore size and geometry, porogens have to be used in "top-down" approaches. Porogens are components, such as salt, wax or sugars that blended in polymer solution are used to create pores and channels in 3D structures. Leaching techniques exploit a mold filled with porogens, in which polymer solution is cast and polymerized for solvent evaporation or crosslinking. After polymerization, porogens are thus leached away from the scaffold leaving a structure with the 94-95% of percentage porosity[75]. Porogen choice allow to control the size and the geometry of pores, but the design of a structure with accurate pore interconnectivity is still demanding[76]. Exploiting a combination of compression molding, heating, polymer etching and particle leaching, Baheiraei et al. developed a PCL-based scaffold with open pores up to 150μm diameter for cardiac TE, which possess the capacity to support the cultivation of primary cardiomyocytes isolated from newborn rats[77]. A high porous 3D scaffold may be alternatively obtained through gas foaming technique, which consists in obtaining pores by leaching porogens through high pressure gases[78]. Porous gelatin scaffolds were prepared by using hydrogen carbonate as a foaming agent by Poursamar et al., which were able to control pore diameter (280-550μm) by the modulation of glutaraldehyde concentration as a chemical crosslinking agent[79]. A recent advance in leaching techniques provide the use of soluble polymeric microspheres as an option to the use of porogens (melt molding), which can be used to avoid the use of potentially toxic organic solvents during scaffolds preparation[80].

The main challenge of the above cited techniques is the scarce possibility of controlling interconnectivity between formed pores. The introduction of controlled

fiber deposition and freeze drying methods have greatly boost the research on the design of a scaffold with interconnected pores.

Freeze drying is based upon the principle of solvent (normally water) sublimation, which being a fast occurring transition leads to the formation of porosity and interconnectivity: as faster is the freezing as smaller are the generated pores[81]. Freeze drying may be applied both to produce 3D rigid structures either to preserve hydrogels structure until their hydration[74].

However, the most promising technique to produce porous and interconnected woven or non-woven scaffold is the electrospinning. Electrospinning is a commonly used method that exploits high voltage source to create an electrically charged polymer filament that forms fibers after drying, gelation or solidification. Electrospinning allows to have control both over fibers orientation and over pore geometry, consequently leading to the definition of interconnectivity. In the electrospinning process, fibers diameter range from 50 to 1000nm[82]. In the literature, most of the examples on nanofibrous electrospun scaffolds come from skin TE[83].

3.3.2 Bottom-up approaches

All the methods cited among the "top-down" approaches do not allow to opportunely control scaffold geometrical cues, including internal micro-porosity and external macro-anatomy. As such, the emerging "bottom-up" techniques may hold great potential to address these issues, by fabricating tissue microscopic units, better known as building blocks, and combining them through multiple approaches in order to obtain a macroscopic complex organ or tissue[72].

Self-assembling constructs may be considered as the first generation of scaffolds designed through a "bottom-up" approach. Self-assembly method is based on the spontaneous organization of molecules into well defined and ordered structures, which clearly requires a deep understanding of building blocks and of their assembling dynamics and features[84]. By the adjustment of sample parameters, i.e. pH or temperature, it is possible to control self-assembly process and thus scaffold porosity and interconnectivity.

Even though self-assembly technique is still in its infancy, pioneering efforts for its use in TE have been already proposed in the literature. These initial efforts have been

plagued by poor mechanical properties, difficulties in precisely governing 3D-morphology and high cost of production[85,86].

On the contrary, in the last decade addictive manufacturing (AM) techniques, otherwise known as rapid prototyping (RP) or three-dimensional (3D) printing approaches, have gained more and more attention because of the possibility of automation, good accuracy and reproducibility. The main appeal of these methods is the possibility to faithfully reproduce complex geometries of tissues designed with computer-aided design (CAD) systems, starting from 3D data acquired through imaging devices used for diagnosis, i.e. computer tomography (CT) or magnetic resonance imaging (MRI). This gives the concrete possibility to reach a patient-specific and customized therapy[87].

Basically, RP is based on the controlled deposition of a material in two-dimensional (2D) layers, whose sequential addition leads to the creation of a 3D object[88,89]. Among AM techniques, TE scaffolds may be ideally produced through free form fabrication (FFF), vat photopolymerization, which include stereolithography (SLA) and digital light processing (DLP), selective laser sintering (SLS) and inkjet three-dimensional (3D) printing.

FFF methods are the most commonly used for biocompatible scaffolds realization. In this case, 3D objects are created by guiding the extrusion of a polymeric fiber from a plotter on XYZ axis, which once deposited polymerizes by physical crosslinking, most often by cooling[90,91]. Bettahalli et al. demonstrated the possibility to monitor nutrient and oxygen perfusion and to supply cell viability of C2C12 myoblasts *in vitro* by using a FFF polymeric scaffold with controlled porosity. The main advantage of using FFF is the possibility to avoid the use of organic solvents, even though a remarkable limit includes the low level of resolution due to the dimensions of the extruded filament[91,92]. Alternatively, vat polymerization greatly enhances printing resolution offering an unprecedented possibility to control scaffold internal and external geometry by using a liquid photosensitive monomer resin that polymerize when expose to a light power source, most frequently an UV-source[91,93]. Vat polymerization methods include SLA and DLP: both have been used successfully, presenting significant advantages especially in fabricating vascular networks to promote *in vivo* angiogenesis, but cytotoxicity coming from the use of

organic solvent and of uncured photo-initiators represents a major problem of these techniques[94-96].

SLS is another RP method with good resolution that uses a laser as power source to sinter 3D structures from several materials, including ceramics, metals and polymers[91]. Through SLS, in a ground-breaking experiment, Yingying et al. designed a PCL-hydroxyapatite scaffold with graded composition for osteochondral cartilage injuries. This model was tested in a rabbit model, which showed a complete cartilage-like repair 6 months after healing[97].

Finally, a smart alternative to the above described AM techniques is the 3D inkjet bio-printing, which relies on the use of cytocompatible ink in which cells may be encapsulated and directly printed as building blocks. The challenge in this case is the research of a suitable inkjet, which *in primis* should be compatible and capable to allow nutrients, oxygen and waste perfusion to cells[91]. Alginate, silk, collagen, gelatin and fibrin are currently the most suitable options. This point represent nowadays one of the most hot-topic in TE scaffolds design[98,99].

3.4 Bioactivity requirements

According to Williams, this is the concept of biocompatibility in TE:

"The biocompatibility of a scaffold or matrix for a tissue engineering product refers to the ability to perform as a substrate that will support the appropriate cellular activity"[3].

As such, the concept of bioactivity, that is the capability of the material to establish a dynamic dialogue with its biological surrounding, is intrinsic. In other words, biocompatibility means that within its structure the scaffold should possess opportune stimuli recognizable by relevant cells for scaffold colonization and thus proper regeneration[3].

Scaffold biological activity may be promoted by enriching the scaffold with cues for cell adhesion, spreading, migration, proliferation and differentiation. Regarding cell adhesion and spreading, scaffold based on natural polymers have to be preferred to the synthetic ones, because biopolymers already present protein-related binding motifs suitable for cell adhesion[23,25,100-102]. However, many efforts have been done to confer docking points for cells also to synthetic materials, often

by ameliorating protein adsorption at the interface[103-105]. Compositional gradients of inductive molecules may be created as driving force to promote cell migration, as well as combined to proteolytic components to create dynamic pathways for cell motility[35]. For example, bone morphogenetic proteins (BMP-2) or calcium ions may be used in bone TE to recruit bone cells, while abundances of laminin could be associated with angiogenesis and cancer invasion[106,107]. Furthermore, various kinds of ECM molecules have been demonstrated to have functions in regulating cell proliferation and differentiation. Thus, these molecules can be introduced onto 3D scaffolds to guide tissue commitment. For example, it has been observed that scaffold enrichment with polylysine inhibit neuronal differentiation to promote the glial one, while polyaniline and polypyrrole seem to have a role in osteogenic maturation[108,109].

Thus, a key role in cell response to scaffold seems played by proteins, which are mostly non-specifically adsorbed on biomaterials surface shortly after their implantation. Therefore, the control of the amount, the composition and the conformation of adsorbed proteins may be a viable approach to design highly specific platforms for TE.

Two major aspects have to be considered in this sense: i) the introduction of cues to elicit scaffold colonization and ii) surface enrichment with molecular signals that specifically trigger cell fate and function.

3.4.1 Control of cell adhesion

Cell adhesion on surfaces is mediated by ECM components, i.e. fibronectin, collagens and laminins, which possess binding motifs for the recognition of cell integrins. Integrins are a large family of homologous transmembrane receptors, constituted of two non covalently associated glycoprotein subunits, alpha (α) and beta (ß), which after ligand binding, control cytoskeleton organization and thus cells grip to the matrix. In particular, after ECM recognition, the cytoplasmic tail of the ß subunit binds intracellular proteins that form mature focal adhesions, including talin, vinculin, α-actinin and filamin, which in turn interacts with actin bundles to control cytoskeleton organization and cell adhesion, shape and migration[110].

Considering this essential point, the introduction of ECM-derived molecules to coat biomaterial surfaces may be an effective approach to ameliorate bioactivity requirements of TE scaffolds. Historically, the use of entire ECM-derived proteins, particularly of fibronectin, has been considered the gold standard to coat and improve biomaterial surfaces, demonstrating enhanced cell adhesion and proliferation. However, to dispose of them in large quantities, these molecules are often isolated from other organisms and may elicit host immune responses[111-113]. More recently, the use of *in vitro* synthetized, decellularized and solubilized autologous ECM have shown great promises as coating, even though the set of opportune protocols for ECM dissociation avoiding the disruption of proteins assembled in the matrix structure is often demanding[114,115]. In addition, both for single molecules or for entire ECMs, their adsorption on surface may be influenced by material physic-chemical properties, as well as they are highly susceptible to *in vivo* enzymatic degradation. Therefore, the isolation of small peptides containing the cell binding domains may be a viable alternative. Cell binding domains are small amino acidic sequences that mediate cell attachment and that have been found in numerous ECM proteins[116,117]. The most popular is the Arginine-Glycine-Aspartic Acid (RGD) domain, highly conserved among the species from *Drosophila melanogaster* to humans and discovered by Ruoslahti and Pierschbacher in the early 1980s as the minimal recognition sequence within fibronectin[118].

Scaffold coating may occur directly through interaction between molecules and surface by weak chemical bonding. Alternatively, indirectly connected after surface activation and the interposition of chemical linkers to covalently binding molecules (**Fig.3**).

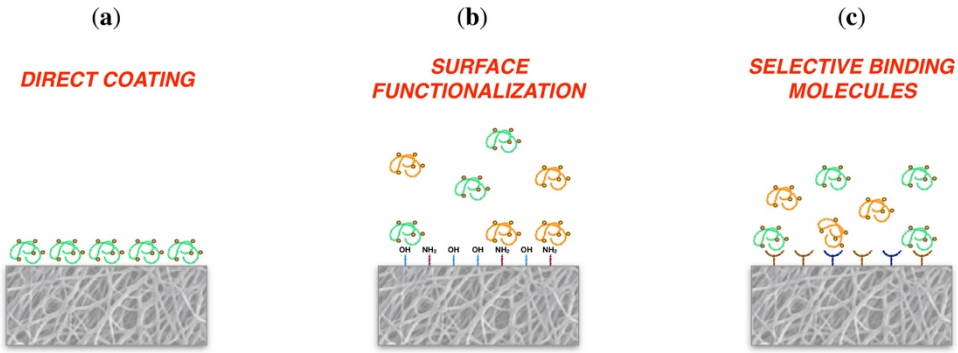

Figure 3. ***Control of cell adhesion through biomaterial coating with ECM-derived molecules.*** (**a**) Scaffold can be directly coated with the ECM-derived molecules. (**b**) Scaffold surface may be activated in order to expose functionalities able to bind ECM circulating molecules, i.e. fibronectin. (**c**) Selective binding molecules may be grafted on scaffold surface to retain ECM circulating molecules.

Biomaterials direct coating – The possibility to coat biomaterials without introducing any surface functionalization has been debated for a long time. The direct adsorption of molecules to biomaterials, otherwise called physical adsorption, occurs thank to the interaction of protein aminoacid lateral chains with the material through weak chemical bonding, i.e. hydrogen bonding and Van der Waals interactions[119-121].

A recent study by Rajabi et al. analyzed the effects of a direct laminin coating directly adsorbed onto electrospun silk fibroin/poly(-ethylene oxide) (SF/PEO) nanofibrous scaffolds on Schwann cells (SCs) proliferation and morphology. After 5 days of culture the number of cells was significantly increased on laminin-enriched SF/PEO substrate. Furthermore, up to 5 days cells seeded on the test scaffolds showed a spindle-like shape and extensions typical of SCs. Together, these results indicate that the coated scaffolds were more favorable for cell adhesion, growth, proliferation and for the maintenance of SCs phenotypic shape[122].

Interestingly, Noh et al. compared the osteogenic potential against human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) of a cell-derived ECM or of a fibronectin coating for a PLGA/PLA mesh scaffold. Noteworthy, hUCB-MSCs proliferation was enhanced both after 2 and 5 days from seeding and the expression level of osteogenic markers, *i.e.* bone sialoprotein, collagen type 1 and alkaline phosphatases, was up-regulated on ECM-derived coating, indicating

a better response of cells in the presence of abundant and different cell-binding motifs[123].

The direct coating of a polyethylene glycol hydrogels with RGD domain was investigated by Kudva et al. against human periosteum-derived cells. Four weeks after seeding the spreading of cells in 3D cultures was significantly ameliorated in the presence of the RGD domain, while in control group cells appeared round and without evident filopodia to adhere to the substrate. These data indicate that materials enrichment with adhesive binding motifs provide docking points for cell adhesion to trigger scaffold colonization[124].

Functionalization of material surface – It has been shown that proteins are capable to preferentially bind specific chemical groups. For instance, fibrinogen binds methyl (-CH_3) enriched surface, but not the carboxyl (-COOH) enriched ones, whereas the introduction of hydroxyl (-OH) groups seems to enhance surface affinity for albumin over fibrinogen[125-127]. Thus, the presence of functionalities on scaffold surfaces may allow the control of protein adsorption. Additionally, some authors retain that the surface stability achieved through a chemical modification is much higher than that obtain by adsorption of molecules at the interface[119,128,129].

The most common way to chemically activate polymer surfaces is photochemistry. This set of techniques involve the irradiation of the surface with high-energy sources, i.e. UV, x or γ-rays, which breaks polymer chemical bond generating functionalities and free-radicals, which eventually lead to the propagation of the reaction[119]. Exploiting UV-light, Ma et al. were able to introduce carboxyl groups onto 3D PLLA scaffolds and to enrich them with collagen type I from bovine tendons for cartilage TE. Subsequently, they cultured primary chondrocytes isolated from rabbit ears in the presence or in the absence of collagen coating and showed that cell proliferation was up-regulated on PLLA-collagen scaffolds up to 6 days. Furthermore, they obtained a desirable way of adhesion and morphology for chondrocytes, since cells were more uniformly distributed in the presence of collagen, as well as more spread[19].

Alternatively, high mobile protons, which are typical of aliphatic polyesters, may be exploited to trigger hydrolysis or aminolysis reactions[130]. To this purpose, Sadeghi et

al. have recently described the possibility of modifying PLGA scaffolds with collagen, for skin TE, by testing the capacity of collagen coating to ameliorate the proliferation of human immortalized keratinocytes (HaCaT) and of human dermal fibroblasts (HDF) up to 14 days. Interestingly, the differences between the groups were significant for HaCaT cells but no for HDS, indicating that the origin of the coating has specific response depending on the site of the application[131].

Plasma treatment is another technique to modify the surface properties of polymers. Plasma is obtained when gases are excited by specific electromagnetic frequencies and small molecules with different energies are created and cause a series of chemical changes of the substrate[119,132]. Li et al. employed the oxygen plasma to activate PLLA substrate and to enrich them with organosilanes as docking points for gelatin functionalization. Human umbilical vein endothelial cells (HUVEC) proliferation and focal adhesions expression were significantly enhances in the presence of gelatin, and were consistent with the efficiency of gelatin immobilization that depends on the organosilane used for the functionalization[133].

Grafting of selective binding molecules – Issues on the the above cited methods are mainly due to the lack of protein mobility and to the potential immunogenicity of proteins, often isolated from other organisms[119].

To overcome these challenges, our group investigated for the first time the possibility of decorating biomaterials surface with selective binding molecules, able to reclaim and retain functional autologous target proteins from the surrounding milieu. Particularly, we exploited aptamers selected against fibronectin by immobilizing them on the surface of a hyaluronic acid-based hydrogel and of a chitosan membrane. We obtained selective binding materials and in both the cases we observed great fibronectin adsorption from culturing medium and an enhanced proliferation, adhesion and migration of osteoblastic cells up to 7 days of culture. Interestingly, cell response was proportional to the amount of aptamers used for the functionalization, suggesting this novel approach as a viable alternative to tailor the surface bioactivity of a scaffold for TE applications[134,135].

3.4.2 Control of cell fate and function

Protein immobilization on biomaterials is important to promote cell adhesion and thus scaffold colonization. However, once cells have colonized the scaffold, opportune biochemical pathways should be triggered to induce cells to accomplish a proper tissue regeneration. In this arena cells commitment is particularly relevant and it could be controlled by enriching scaffolds with target cues. This section will now discuss methods that have been developed to enrich scaffolds with therapeutic molecules and signals, which once delivered in the damaged site may monitor and boost regenerative processes.

Among the molecules that may guide cell fate, growth factors (GFs) are of relevant importance. GFs are proteins secreted by committed cells, which have the ability to control cell proliferation, migration and differentiation by binding specific transmembrane receptors[136]. Numerous GFs have shown great therapeutic potential in preclinical models, including vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), transforming growth factor beta (TGF-ß), platelet-derived growth factors (PDGFs) and bone morphogenetic proteins (BMPs)[137-140]. However, do to their short half-life and consequent rapid deactivation, to date only the BMP-2 and the BMP-7 have been approved from the FDA for lumbar spine fusion and bone fracture treatment. A sophisticated alternative to the use of bare GFs in tissue regeneration may thus be their association with TE scaffolds as bare molecules (direct delivery) or combined to nanocarriers for indirect delivery, i.e. nanoparticles, nanocapsules or liposomes. Three different strategies have been until now studied for biomaterial presentation of GFs in TE: physical, covalent and bioaffinity immobilization (**Fig.4**)[141].

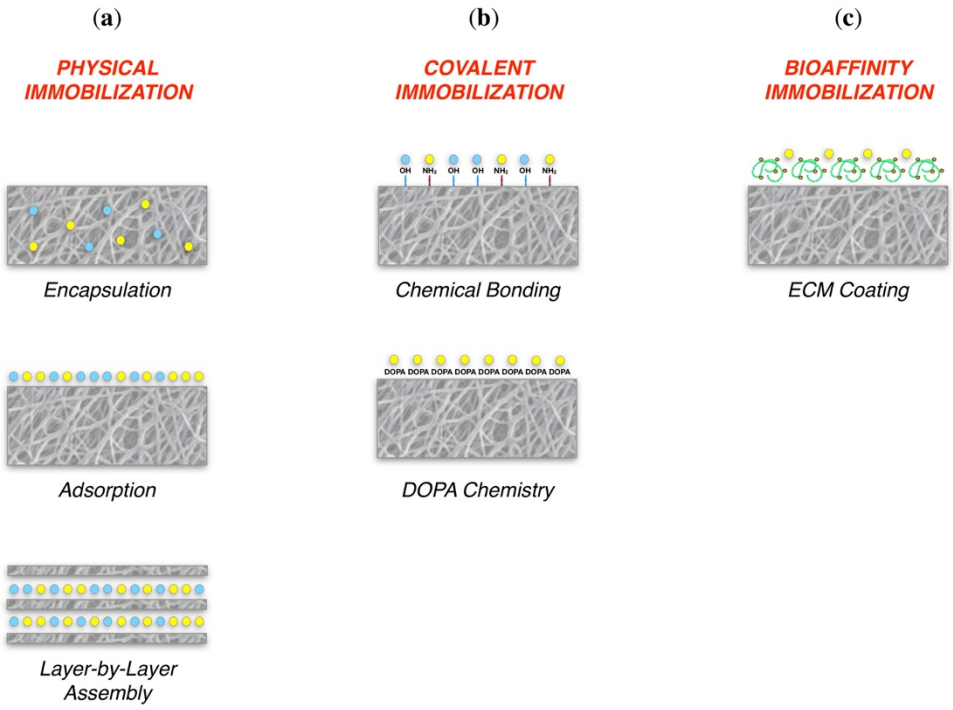

Figure 4: *Control of cell fate and function through bioactive molecules immobilization.* (**a**) Bioactive molecules may be physically immobilized on the scaffold, by encapsulation, simply adsorption or through LbL assembly. (**b**) Scaffold surface may be activated in order to covalently bind bioactive molecules. (**c**) Scaffold direct coating with ECM molecules may be exploited to bind bioactive molecules by affinity.

Physical immobilization – Physical immobilization techniques consists in GFs retention inside scaffold structure without the formation of any chemical bond[141].

GFs encapsulation by their mixing into the scaffold before matrix gelation is the simplest method of physical immobilization and a great amount of works in the literature report this method. To this purpose, Cai et el. have recently shown the possibility to blend BMP-2 into nanometer hydroxyapatite collagen scaffolds. They detected a cumulative release of BMP-2 up to 19 days, significantly faster in the first few days. Furthermore, in the presence of BMP-2 loaded scaffold they observed an enhanced activity of alkaline phosphatase in bone marrow stem cells (BMSCs) from day 4 till day 10, indicating a pivotal role played by BMP-2 grafting in osteoinduction[142]. At this level, it could be of interest the introduction of further external stimuli that regulate scaffold biodegradation and thus bioactive cues release. This could be useful both to further control cell function and to trigger cell fate at the most appropriate moment.

Alternatively, GFs or therapeutic molecules may be simply absorbed on matrix surface. For example, in our previous work, we showed the possibility to adsorb stanozolol on a commercially available bone substitutes and to enhance bone regeneration in a critical size defect in rat calvaria[143]. On the other hand, Wei et al. compared the possibility to mix recombinant BMP-7 (rhBMP-7) with PLGA nanospheres (NS) and to encapsulate them into a PLLA scaffold for bone TE with the possibility of simply adsorbed rhBMP-7 on PLLA. Ectopic bone formation was clearly detectable in a subcutaneous pocket model in rat after 6 weeks of healing when rhBMP-7 was delivered with NS, while control scaffold and passive rhBMP-7 adsorbed scaffold resulted in failure of bone induction[144].

More recently, layer by layer self-assembly (LbL) strategy has been proposed as a novel method of physical immobilization. It consists in the structuration of a multilayered scaffold from the bottom to the top taking advantages from the spontaneous interaction of different charged materials[145,146]. The first LbL membrane capable of releasing BMP-2 was described in 2011 by Macdonald et al., which exploited the combination of the poly(ß-aminoester), a synthetic cationic polymer, and of the cationic chondroitin sulfates. When soaked in cell culturing medium, the LbL film released the 80% of BMP-2 loaded within 2 days inducing early alkaline phosphatase activation in MC3T3-E1 cells (day 6), as well as enhanced calcium deposition and mineralization after 28 days. Furthermore, when implanted in an intramuscular site BMP-2 enriched LbL membranes induced bone formation and calcium deposits visible with the micro-computerized tomography analysis (µCT) from 4 to 9 weeks after implantation[147].

Covalent immobilization – Issues connected to the above described methods, i.e. initial burst release of immobilized molecules, may be overcome by the introduction of GFs chemical bonding to the biomaterials exploiting the reactivity of protein lateral chains functional groups. For more, this type of conjunction lead to the control of GFs desorption rate by enzymatic or hydrolytic cleavage[141]. On this way, a common approach is the creation of a polydopamine (DOPA) film on scaffold surface, by the mimicry of mussel way of adhesion to a variety of substrates[148]. DOPA chemistry has been exploited by Lee et al. to attach recombinant BMP-2 (rhBMP-2)

on a 3D-printed PCL scaffold. The release of rhBMP-2 was monitored for 28 days and revealed a gradually linear release of the molecule. Furthermore, when used as cell culturing substrate the rhBMP-2 grafted scaffold did not affected cell viability, but severely influenced MC3T3-E1 cells alkaline phosphatase activity, calcium deposition and osteocalcin, bone sialoprotein and collagen type I expression, indicating a potential osteoinductive activity[149].

Bioaffinity immobilization – It should be stated that ECM act as a reservoir of GFs, which are able to bind multiple molecules with high affinity. Therefore, biomaterials could be decorated with motifs that are natural component of ECM, i.e. heparin and adhesive proteins such as fibronectin or vitronectin[141].

Tellado et al. have recently projected a biphasic silk fibroin scaffold for tendon/ligament TE functionalized with heparin for transforming growth factor ß2 (TGF-ß2) and growth/differentiation factor 5 (GDF5) delivery. Interestingly, heparin decoration increased the amount of TGF-ß2 and GDF5 retention. Moreover, the combined delivery of these two factors promoted the chondrogenic commitment of adipose-derived mesenchymal stem cells (AdMSCs), which after 14 days showed higher expression of Sox9 and collagen type II (COL2) cartilage markers[150]. Instead, the adhesive properties of fibronectin were exploited to promote the delivery of BMP-2 from a hyaluronic acid hydrogel in an ectopic bone defect model in rat by Kisiel et al. In this case, the authors observed a 2-fold higher formation of ectopic bone and of collagen expression when BMP-2 was delivered in association with fibronectin rather than bare adsorbed on the hyaluronic acid material[151].

3.5 Through a functionally graded scaffold

The organization that tissues, organs, apparatus and organisms have in the bulk nature is largely governed by their function, and most often it is not homogenous, but strictly arranged to combine different cells and tissues[152]. This structured organization is well-known as functional gradient and thus during regenerative procedures, a successful TE engineered scaffold should be functionally graded (FGS)[153].

Gradients may be discrete or continuous, and may ideally be applied to mechanical, porosity and bioactivity scaffold requirements through the employment of RP techniques. For example, the articular cartilage is organized in three zones to accommodate chondrocytes according to tissue biomechanical functions. As such, the fulfillment of a scaffold with collagen fibers oriented according to native cartilage may help a faster and better regeneration of this tissue[154]. Similarly, human bones are a clear example of functional graded porosity with cortical outer layer solid and dense, while a spongy inner compound[152]. Still, it could be extremely useful to create molecular gradients to obtain a driving force that guides cell migration or differentiation[35]. These are only few of the many examples of functional gradation we may report, and it is important to considered that although the view of realizing a FGS construct is still pioneering, the way has to be that. Indeed, only matching all these perspectives it will be really possible to provide a favorable environment for cell growth, proliferation and real tissue regeneration.

4. A case study on chitosan

Among the biological macromolecules, polysaccharides are an important class of polymers found in living organisms with structural and storage-related properties[155]. Chemically, polysaccharides are polymeric carbohydrates (sugars) formed as the result of monosaccharides polymerization, which occurs after the formation of a glycoside bond between the hemiketal group of a saccharide and the hydroxyl group of another one.

Chitin is one of the most naturally abundant polysaccharides. It is the main component of crustacean exoskeleton and of fungi/yeast/green algae cell walls, and from its partial deacetylation it can be easily derived chitosan, which has been known to be a suitable candidate for TE scaffold design[156-159]. Thus, chitosan is a linear and semi-crystalline copolymer made of D-glucosamine [(1-4)-2-amido-2-deoxy-ß-D-glucan] and of N-acetyl D-glucosamine [(1-4)-2-acetamido-2-deoxy-ß-D-glucan], precisely derived from chemical hydrolysis under sever alkaline conditions or from enzymatic hydrolysis mediated by chitin deacetylase (**Fig.5**)[157].

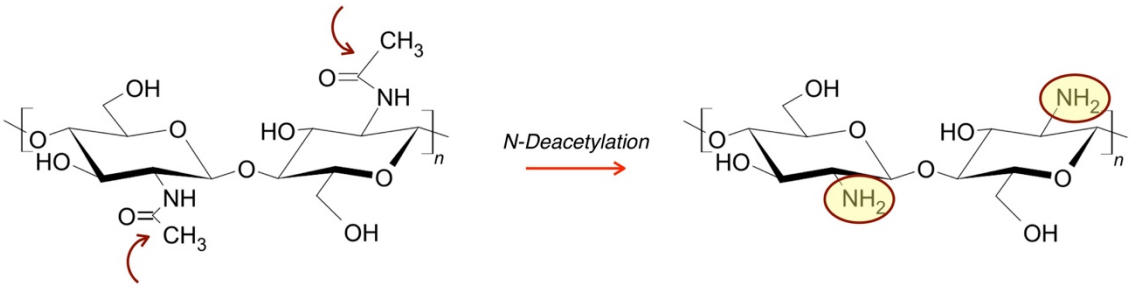

Figure 5. *Chitin and chitosan.* Chemical structure of chitin and of chitosan after chitin N-Deacetylation.

Depending on the chitin source and on the chitosan preparation procedure, the degree of deacetylation (DD - D-glucosamine residues / total residues composing the chain) may range from 30% to 95%. From DD in turn depend chitosan molecular weight (300-1000 kDa) that is inversely proportional to DD, its crystallinity, which on the contrary is directly correlated to DD and chitosan stability to spontaneous degradation, which is mainly performed by lysozyme and that is delayed in high DD chitosan[159]. Furthermore, in contrast with chitin, the presence of amino groups confers to chitosan the capacity to be easily protonated, thus making chitosan the

only naturally-derived polysaccharide positively charged[160]. All together, these properties make chitosan a suitable biocompatible biomaterial for TE scaffold design.

Protonated groups provide solubility in acidic aqueous solution and allow chitosan adaptability under a wide spectrum of conditions, thus endorsing it with a unique structural versatility[161]. Starting from chitosan solution, hydrogels, porous (sponges) or fibrous scaffolds have been prepared for different applications[157].

Chitosan is capable to jellify all by itself and physical reversible hydrogels have been realized by controlling specific parameters (e.g. pH, temperature, etc.) to create interactions between polymer chains[157,162,163]. Alternatively, positively charged D-glucosamine residues may interact with negatively charged molecules (e.g. sulfates, phosphates, etc.) to form hydrogels[157]. The main advantage of this type of gel is that their gelation is easy and may occur upon hydrogel injection offering the possibility to perfectly shape the scaffold to the tissue defect. On the other hand, both the amino and the hydroxyl groups of chitosan lateral chains may be use to form un-reversible hydrogel via covalent bonding (i.e. amide, ester or Schiff base formation) of chemical cross-linkers, thus forming more stable hydrogels[45,164]. Chemical cross-linking agents such as glyoxal or glutaraldehyde have been for a long used, but their high toxicity has led to the introduction of other natural components, i.e. genipin[165].

Porous chitosan sponges could be also obtained, generally by freeze-drying and by gas foaming, or fibrous structures through solvent casting, leaching techniques, electrospinning and bottom-up approaches, e.g. 3D-bioprinting[163,166-169].

As we stated above in this work, the manufacturing technique allow to control scaffold porosity, which is important to ensure proper nutrient, oxygen and waste diffusion to and from cells. Focusing on chitosan, pores play also an important role in defining suitable mechanical properties. In a detailed work, Madihally and Matthew described the correlation between porosity and rheological parameters in chitosan models[161]. They showed that hydrated porous chitosan membranes had a greatly reduced elastic module tensile strength if compared to non-porous ones (0.1-0.5MPa vs 5-7MPa), while maximum strain ranged from 30-40% for non-porous membranes to 30-110% (depending on diameter and orientation) for the porous.

Furthermore, in another work by Chen and Hwa, the effect of chitosan molecular weight on mechanical properties was investigate[170]. Findings revealed that both the tensile strength and the elastic modulus were directly proportional to chitosan molecular weight. As a consequence, chitosan mechanical properties indirectly depend also from DD and crystallinity, while directly from the biodegradation kinetics, as more fast the degradation occur, as more fast the chitosan loose its mechanical stability[159].

The cationic nature of chitosan could be further exploited for the preparation of multilayered films, using LbL technique or for chitosan functionalization with anionic glycosaminoglycans and proteoglycans widely present in natural ECMs. As we stated before (see paragraph 3.4.2) both these methods could be useful for the reception of suitable molecules in guiding cell fate and function[171-173].

One of the important features of chitosan is the presence of three distinct reactive functional groups along its molecular chain. Indeed, the two hydroxyl groups in C3 and C6 positions and the amine group in C2 position makes chitosan flexible for chemical modifications and for the amelioration of its bioactivity through proteins and therapeutic molecules grafting[174].

Fascinatingly, it has been recently described the possibility to introduce suitable chemical groups for chitosan functionalization by enzymatic modifications[175]. Enzymatic modifications are gaining remarkable attention to modify the structure of chitosan because they are selective, specific and they do not require harsh conditions or reactive and toxic compounds. Such procedures have been already successfully used to graft proteins and other suitable molecules on chitosan surface[176,177].

4.1 Chitosan in tissue engineering

As we have widely stated above, the selection of an appropriate material for biocompatible scaffold design is a critical step in TE. To this purpose, thank to its beneficial properties, chitosan has been extensively studied as raw materials for scaffold fabrication.

To the best of our knowledge, more than 2000 works have been published in the last 20 years with an increasing exponential trend from 1997 to the end of 2017. Among

these works an important cut is covered by works on bone TE (43.60%), followed by skin (16.80%) and cartilage (14.50%) TE.

4.1.1 Bone

Differently from other tissues, bone has a great capacity to regenerate itself. Nevertheless, in some situations, such as when important traumas occur or after bone tumor resections, massive bone defects may be created, which need a clinical intervention. Furthermore, possible insufficient blood supply, the presence of infections or of systemic disease, e.g. diabetes, can negatively affects bone healing, resulting in an ineffective regeneration also for contained defects[178,179].

In these cases, the selection of an opportune bone graft has been for a long the gold standard therapeutic approach, including the use of autografts, allografts and xenografts. However, issues related to the used of these substitutes, such as the requirements of a second donor site or the prolonged operative times in the case of autografts and potential immune response in the case of allo- and xenografts, have prompted efforts in bone TE by developing alloplastic and synthetic scaffolding materials[178-180].

As for other tissues, the ideal scaffold for bone TE should fulfill specific requirements and among the most important there are i) osteoinduction, otherwise the capability to recruit and stimulate bone-forming cells, ii) osteoconduction, which is the ability to support cell adhesion, iii) osteogenesis that consists in the formation of new bone and iv) mechanical stability[178,181,182].

In this regard, it has been shown that chitosan similarities with natural biological tissues is useful to trigger the proliferation and differentiation of mesenchymal cells in osteoprogenitors[183]. Thus, 3D chitosan based scaffolds for bone applications have been prepared by solvent casting, gas foaming, freeze-drying and RP, finding good outcomes both *in vitro* and *in vivo* applications[184-186].

One of the latest researches from the literature includes an impressive work by Gan et. al, which prepared a chitosan scaffold whose biocompatibility was implemented at further levels[187]. In particular, biphasic calcium phosphate was used to enhance mechanical properties, RGD-binding motifs to promote scaffold colonization by cells and BMP-2 loaded bovine serum albumin NPs were used to

confer osteoinductive properties to the construct. The scaffold was prepared by freeze-drying and exhibited optimal characteristics for bone TE: a pore size ranging from 10 to 100μm and a compressive strength between 150 and 420GPa. Furthermore, the amelioration of scaffold bioactivity by RGD immobilization via carbodiimide crosslinking chemistry and by BMP-2 loaded BSA NPs by dispersion in chitosan solution, contribute to trigger mesenchymal stem cells proliferation and differentiation *in vitro* and bone regeneration in a femur defect *in vivo*. Noteworthy, Masson's trichromatic staining revealed the presence of organized collagen fibers and of enhanced bone regeneration around the defect region after 12 weeks in chitosan-RGD-BMP-2 scaffold, while no bone, but fibrous tissue was detected in chitosan-RGD scaffold and immature bone in chitosan-BMP-2 scaffold.

4.1.2 Skin

As a consequence of skin lesions the complete regeneration of a functional epithelium is more than ever important to ensure its role of barrier, pigmentory defense against UV irradiation, thermoregulation, as well as mechanical and aesthetic functions[188].

Cutaneous wound healing requires a well-orchestrated coordination between biological and molecular events which start with tissue hemostasis to conclude with epidermis new formation[189,190]. However, while small defects can heal spontaneously, large and important lesions, i.e. burns, need a surgical resection of the necrotic tissue and consequent positioning of a skin grafts. Furthermore, an impairment in skin healing processes progression, normally due to presence of systemic disease, i.e. diabetes, may induce chronic wounds, which lead to an impairment of local cytokines production and to a consequent reduction in angiogenesis and wound vascularization[191].

As for bone, many skin substitutes have been used in the past decades to promote wound healing, but ever for issues connected to antigen susceptibility and limitation of donor sites, synthetic approaches have been developed often in combination with therapeutic cytokines and GFs[13].

Chitosan, which among its properties is capable to trigger hemostasis and platelet activation, is one of the eligible materials for the preparation of wound dressing

substances and numerous materials have been already approved for commercial trade (e.g. Chitoseal® - Abbott, Syvec Patch® - Marine Polymer Technologies, Chitopoly® - Fuji Spinning)[192]. However, even though these products have been shown to promote wound healing, they do not overcome the issue of wound chronicization and in this sense, research efforts to find optimal products for diminished wound healing capacities are still a major goal in skin TE. To this purpose, in a recent work, Wu et al. have successfully applied a chitosan/silk fibroin patch co-seeded with adipose-derived stem cells (ADSCs) as reconstructive support in diabetic wound healing. They showed a great capacity of the patch to enhance wound healing rate, with statistically significant differences up to 21 days, as well as a promotion of angiogenesis with blood vessels growing perpendicular to the wound[193].

4.1.3 Cartilage

Joint degeneration is progressive with aging and its treatment represents a major challenge for clinicians, because articular cartilage self-regeneration is highly limited due to a lack of vascularization and to a limited proliferative capacity of chondrocytes[194,195].

In the past decades, the transplantation of autologous chondrocytes seemed to be a promising procedure, but scarce cell population together with their restricted ability to expand, led in any case to numerous difficulties in repairing large critical defects[196,197]. Thus, the most common option to restore joint surface is nowadays the invasive surgical activation of subchondral bone healing through its injury by drilling, micro fractures realization, arthroplasty or electrical/laser stimulation, to promote the release and the activation of resident mesenchymal stem cells (MSCs)[198]. However, MSCs are an attractive cell source for TE, which can be easily harvested in large amounts from a variety of sources, including adipose tissue, umbilical cord blood, placenta and bone marrow. As such, to overcome drawbacks related to methods for cartilage repair, the way of isolating MSCs from other autologous sources and to seed them on chondrogenic scaffolds has been deeply considered. The structural similarity of chitosan to natural components of cartilage ECMs, including collagen type II and GAGs, which have been known to play a central role

in chondrogenesis orchestration as well as in guiding chondrocytes fate, makes it a suitable scaffolding material for cartilage repair[199-201].

Among the latest advances in the literature on this topic, Deng et al. prepared a chitosan and silk fibroin based scaffold to culture and differentiate primary bone marrow MSCs, prior to construct implant in articular cartilage defects in rabbits[202]. Up to 12 weeks of healing they showed that a chitosan/silk fibroin scaffold may serve as a carrier for MScs to repair cartilage defects and may be further exploited in TE approaches.

4.1.4 Cornea

The cornea is an extremely organized multi-layered structure that allows light to enter in the eye and that constitutes a vital component of the vision system[203]. Corneal blindness is the first cause of vision lost and it is estimated that 4.9 million of people worldwide suffer from bilateral blindness, while from 5 to 23 million from unilateral blindness[204]. To date the golden standard treatment for restoring vision after cornea injury is its transplantation from cadaveric donors (keratoplasty), but in fact there is a growing need for therapeutic options, due to the high risk of graft rejection and to the chronic lack of donors[205]. As such, TE approaches of full or partial portions of the cornea have represent a viable alternative in the last ten years, with particular regard to constructs supporting corneal epithelium, stroma and endothelium regeneration. More often, these approaches have considered chitosan as a suitable biomaterial for scaffold fabrication, which is already a widely used polysaccharide in ophthalmic applications.

The epithelium is the most anterior portion of the cornea, as the most susceptible to accidental damages. In physiological conditions, damages are normally repaired by limbal epithelial stem cells, but in case of their loss, i.e. limbal stem cell deficiency (LSCD), neovascularization and conjunctiva outgrowth occur causing epithelium opacization and vision loss[206]. As TE approaches, the latest work from the literature by Xu et al. proposed the preparation of a carboxymethyl chitosan/gelatin/hyaluronic acid blended membrane to graft a new-forming epithelium from primary rabbit corneal epithelial cell in alkali-induced corneal damages[207]. This study revealed that the membrane was transparent and suitable

to support cells growth and phenotype. Furthermore, the combination of the membrane with cells was a successful achievement for corneal epithelium wound healing, showing a quite complete restoration of the initial structure.

Under the corneal epithelium, the stroma constitutes the 80-90% of cornea thickness. This structure is organized in layers of well aligned collagen bundles and keratocytes, which confer to the epithelium the proper hydration and to the entire cornea proper mechanic and transparency[208]. In case of stroma lesions, the reproduction of a such complexes collagen architecture is a demanding challenge for TE. To this purpose, in a successful work Guan et al. were able to apply a chitosan/silk fibroin scaffold for corneal stroma engineering in rabbits and to obtain a reconstructed lamellar structure comparable to that of native tissue: transparent and expressing vimentin in keratocytes[209].

Eventually, the corneal endothelium is the deeper portion of the cornea. It is a monolayer of cells arrested in G_1 phase of the cell cycle and it is responsible for fluid exchange with the stroma. Due to the incapacity of endothelial cells to proliferate, normal aging causes a gradual decrease in cell number with consequent thickness of the endothelium until blindness[210]. As for stroma the engineering of this portion is nowadays demanding and few works have been proposed to fulfil with an effective endothelium. Among the others, a work by Liang et al. showed the possibility to develop a chitosan-based membrane able to support primary endothelial cells from rabbit growth and morphology[211].

5. Conclusions and future perspectives

The choice of the suitable biomaterial for scaffold fabrication represent a key point in TE. Through the integration of multiple disciplines approaches, tremendous developments have been done in the last decade for the realization of scaffolds more than ever performant both *in vitro* and *in vivo*.

In this review, we highlighted the requirements in term of biodegradability, mechanical properties, porosity and bioactivity that a biomaterial scaffold should have to fulfill a functional tissue regeneration. In particular, we emphasized how these aspects should be tailored in order to reflect the physiological needs of the regenerating tissue and of its surrounding milieu. In addition, the chitosan has been reported as a suitable and versatile biomaterial in order to provide the concrete evidence of the possibility to mold the same material for tissues with far anatomies and physiologies. Based on these advances, we expected that the future directions of TE will continue to combine these aspects in order to obtain functional graded biological substitutes to boost even more tissue regeneration.

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Chapter 2

Chitosan scaffold modified with D-(+) Raffinose and enriched with thiol-modified gelatin for improved osteoblast adhesion

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Abstract

The aim of the present study was to investigate whether chitosan-based scaffolds modified with D-(+) raffinose and enriched with thiol-modified gelatin could selectively improve osteoblast adhesion and proliferation.

Two percent chitosan films were prepared. Chitosan suitability for tissue engineering was confirmed by protein adsorption assay. Scaffolds were incubated with a 2.5 mg/ml BSA solution and the decrease of protein content in the supernatants was measured by spectrophotometry.

Chitosan films were then enriched with thiol-modified gelatin and their ability to bind BSA was also measured.

Then, 2% chitosan discs with or without thiol-modified gelatin were used as culture substrates for MC3T3-E1 cells. After 3 days of culture cells were stained with Trypan blue or with calcein-AM and propidium iodide for morphology, viability and proliferation analysis. Moreover, cell viability was measured after 2, 3, 4 and 7 days to obtain a growth curve.

Chitosan films efficiently bound and retained BSA, but the amount of protein retained was higher on chitosan enriched with thiol-modified gelatin.

Moreover, chitosan discs allowed the adhesion and the viability of cells, but inhibited their proliferation. The functionalization with thiol-modified gelatin enhanced cell spreading and proliferation.

Our data confirm that chitosan is a suitable material for tissue engineering. Moreover, our data show that the enrichment of chitosan with thiol-modified gelatin enhances its biological properties.

1. Introduction

Tissue engineering (TE) is a promising field of regenerative medicine (RM) that relies on the interaction of three main elements: i) a supporting material (scaffold); ii) growth factors and iii) cells; to develop a biological substitute for the replacement, the restoration or the regeneration of damaged tissues and organs[1].

Biomaterials suitable for TE must fulfil with specific properties, among which they should act as a good substrate for cell growth.

Chitosan is one of the most investigated biomaterials for TE applications. It is a polysaccharide derived from the N-deacetylation of chitin, the main structural component of the crustacean exoskeleton[2]. It is suitable for many TE applications because it possesses a good combination of properties such a biocompatibility and bioresorbability, it is not toxic, not allergenic and cheap, and it can be molded into a variety of different shape[3,4]. Chitosan has been used as drug carrier and is reported to participate in wound healing[5]. Moreover, its structural similarity with naturally occurring glycosaminoglycan and its osteogenic capacity suggest that chitosan might be an ideal candidate for the production of scaffolds for bone TE[6]. However, in spite of its numerous appealing properties, chitosan seems to be cytostatic against cells that deposit extracellular matrix (ECM), such as fibroblasts. To this purpose, Bettini et al. have reported how cell responses could be improved by modifying chitosan using sucrose or raffinose-enriched solutions to increase its water content[7]. In the present work we have further enriched these implemented chitosan films with thiol-modified gelatin (**Fig.1**) to observe how this composite material could further enhance osteoblasts adhesion and proliferation.

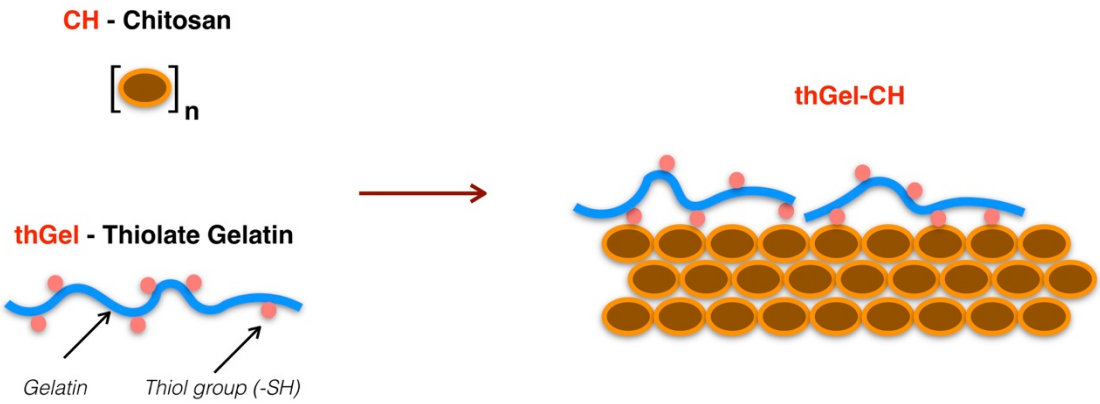

Figure 1. ***Thiol-modified Gelatin enriched Chitosan.*** Diagram representing the structure of a chitosan film functionalized with thiolated gelatin. The thiol functional groups of gelatin bind chitosan by weak bonds, thus coating the entire chitosan film.

2. Materials and Methods

Sample preparation

Chitosan films were prepared accordingly to Bettini et al.[7]. Briefly, a 2% w/v chitosan solution was prepared by dissolving 90% de-acetylated purified chitosan 60-80kDa powder (A.C.E.F., Piacenza, Italy) in a 1% acetic acid aqueous solution. D-(+) raffinose pentahydrate (Sigma-Aldrich, Saint Louis, MO, USA) was then added to the solution at a final concentration of 290nM as viscosity modifying agent. One millimeter of the solution was spread out on a microscope slide (12*25m) to obtain a film with uniform thickness, dried for 1h at 45°C in a ventilated oven and transferred in a 5% potassium hydroxide aqueous solution (NaOH, Sigma-Aldrich) for 24h to jellify. Discs with a diameter of 0.6mm were thus cut off, rinsed in double distilled water and placed in a 96-well plate for further enrichment with thiol-modified gelatin (Gelin-S, ESI-BIO, Alameda, CA, USA) obtained from porcine skin. Briefly, lyophilized gelatin was reconstituted with degased water at the final concentration of 10mg/ml as recommended by the manufacturer and 50µl were added to chitosan and incubated 30min at 37°C and 5% $ppCO_2$ to obtain thiolated gelatin chitosan (thGel-CH). Excess of gelatin was removed through 3 rinses in Phosphate Buffer Saline (PBS, Thermo Fisher Scientific, Carlsbad, CA, USA). Bare 2% chitosan discs were used as control (CH).

Sample characterization

Scanning electron microscopy - Surface micro-topographies of CH and of thGel-CH were analyzed through scanning electron microscopy (SEM) analysis. To this purpose, CH and thGel-CH specimens were lyophilized, sputtered with a thin layer of gold through a SCD 040 coating device (Balzer Union, Wallruf, Germany) and observed with a dual beam Zeiss Auriga Compact system equipped with a GEMINI Field-Effect SEM column (ZEISS, Oberkochen, Germany) at 1kV.

Protein adsorption studies

Spectrophotometry - To measure the capability of CH and of thGel-CH to retain proteins, specimens were soaked in 100µl of a 2.5mg/ml bovine serum albumin (BSA,

Sigma-Aldrich) aqueous solution for 4h at room temperature (RT). Samples were maintained on an orbital shaker to avoid aberration due to protein merely deposition and BSA concentration was quantitated in the supernatants through spectrophotometry (Nanophotometer, ImplenGmbH, München, Germany) after 1, 2, 3 and 4h. The amount of BSA adsorbed by the films was then calculated by subtracting the residual concentration from the initial one.

Cell assays

Cell cultures – In vitro analysis were performed using murine MC3T3-E1 cells obtained from ATCC (LGC Standards srl, Milano, Italy). Cells were grown in complete alpha-MEM (α-MEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 1% glutamine (L-Glu, Thermo Fisher Scientific) and 1% penicillin and streptomycin (PenStrep, Thermo Fisher Scientific). Cells were seeded at a final concentration of 5000 cells/sample and cultured until 7 days, replacing their medium after 2 and 5 days.

Inverted microscopy and cell proliferation assay – After 2, 3, 4 and 7 days photographs were taken with an inverted microscopy (Eclipse TS100, Nikon, Tokyo, Japan). Furthermore, at each experimental point cell viability was measured through chemiluminescence (CellTiterGLO, Promega, Madison, WI, USA) following manufacturer's recommendation. Briefly, culturing medium was removed and replaced with 100µl of a 50:50 solution of complete medium and lysis buffer. Samples were then shaked for 2min, luminescence stabilized for 10min in dark condition and finally assessed with a luminometer (GLOMAX 20/20, Promega).

LIVE/DEAD assay and Trypan Blue staining – To quantify the amount of viable and dead cells, a double staining with calcein-AM (Thermo Fisher Scientific) and propidium iodide (PI, Thermo Fisher Scientific) either a staining with Trypan blue staining were performed at day 3.

For LIVE/DEAD assay, culturing medium was removed and replaced with a 4µM calcein-AM and 7.5µM PI solution in PBS. Samples were then incubated 10min at RT in dark conditions, fixed 20min with a 4% paraformaldehyde solution (PFA, Sigma-

Aldrich) and observed with a microscope equipped for fluorescence (Axio Imager 2, Zeiss). Viable and dead cells were counted through the use of the D3 documentation software (D3, Nikon). Similarly, after Trypan blue (Thermo Fisher Scientific) staining CH and thGel-CH area occupied by viable cells were calculated using an imaging free software (ImageJ, Bethesda, MD, USA).

Statistical analysis

Data were analyzed using Prism6 (GraphPad, La Jolla, CA, USA). All the values are reported as the mean ± standard deviation (SD) of 3 repeated experiments. Differences between groups were evaluated with the two-way ANOVA statistical test and Sidak's multiple comparisons post-test or with the T-test. Differences were considered significant when $p<0.05$.

3. Results

Gelatin did not modify chitosan interface morphological aspect

CH and thGel-CH specimens presented very similar smooth surfaces with no pores (**Fig.2**). This feature determines a high surface area available for the interaction with cells during culture.

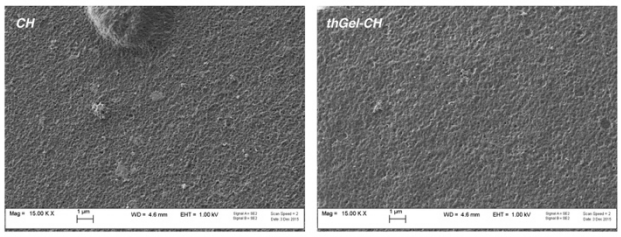

Figure 2. ***Scanning Electron Microscopy analysis.*** Typical SEM images of CH and of thGel-CH scaffold.

Gelatin enhanced chitosan ability to bind proteins

To investigate whether specimens could bind proteins, samples were incubated with a 2.5mg/ml BSA solution and protein presence in the supernatants was quantitated by spectrophotometry. BSA was steadily adsorbed on CH samples within 1h of incubation and subsequently stabilized in a plateau reaching a maximum after 4h (**Fig.3**). When chitosan was implemented with gelatin, protein levels in supernatants decreased faster, but reaching a plateau within 1h as well. From 1 to 4h the thGel-CH bound up to 22% more protein than CH (**Fig.3**), thus showing that thGel-CH enhanced the ability of CH to bind and retain proteins ($p<0.0001$).

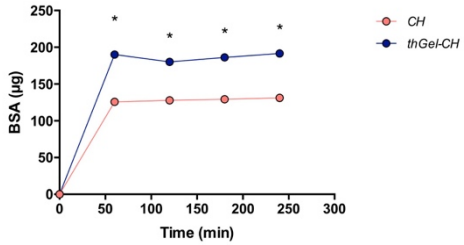

Figure 3. ***Bovine serum albumin adsorption on CH and on thGel-CH.*** BSA concentration in supernatants on thGel-CH decreased significantly more than on CH alone. *=$p<0.0001$.

CH and thGel-CH supported cell viability and proliferation

To observe how cells grew on CH and on thGel-CH, we proceeded by culturing murine osteoblastic MC3T3-E1 cells.

Microphotographs in **Figure 4** show that MC3T3-E1 cells grew faster on thGel-CH, reaching confluence already after 4 days of culture, while, cells on CH did not reach confluence even after 7 days of culture. Chemiluminescence (**Fig.5**) confirmed that MC3T3-E1 viability was higher on thGel-CH at each experimental time point ($p<0.0001$).

Trypan blue staining was retained by the scaffold, thus creating a blue background that clearly showed that cells were more numerous and viable on thGel-CH than on CH sample. Calcein-AM/PI staining confirmed Trypan blue results and highlighted a scarce number of dead cells on both the samples (**Fig.6a**). Additionally, thGel-CH increased the number and spreading of the cells, which appeared bigger and with longer filopodia. However, no statistical differences were detected when the number of viable cells were counted on both CH and thGel-CH sample (**Fig.6b**, $p=0.5235$).

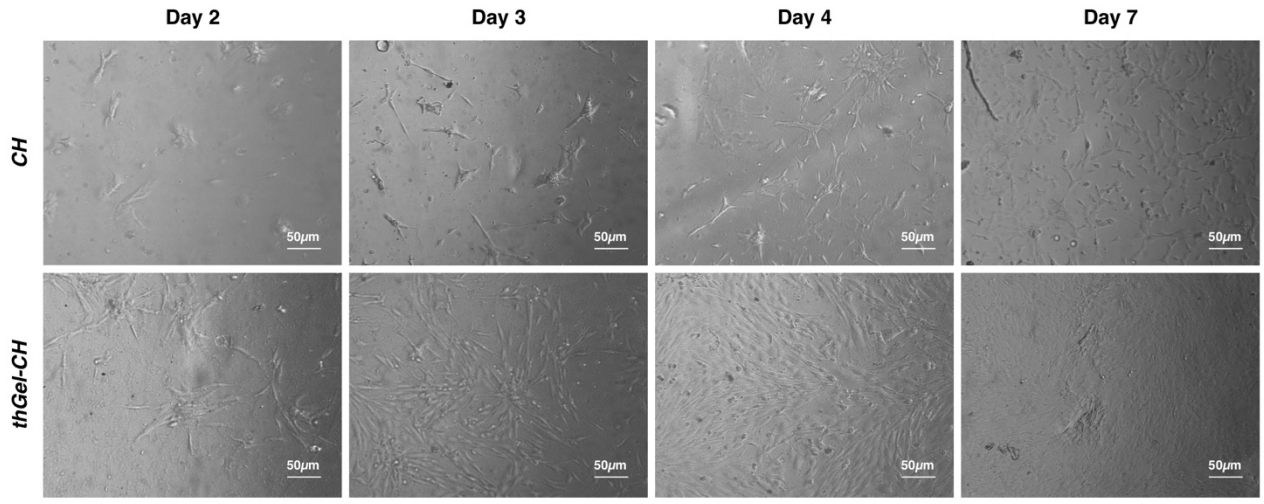

Figure 4. ***Inverted microscopy analysis.*** Typical images showing the progressive growth of murine osteoblastic MC3T3-E1 cells on CH and on thGel-CH films 2, 3, 4 and 7 days after seeding. Cells on thGel-CH were more numerous, more spread and had longer filopodia at each experimental time point.

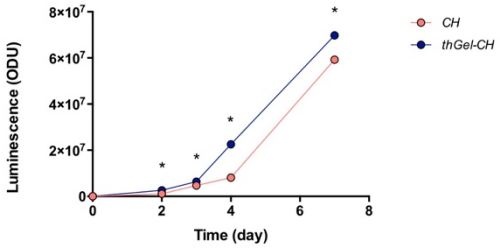

Figure 5. *Cell viability*. Histograms showing murine osteoblastic MC3T3-E1 cells viability on CH and on thGel-CH 2, 3, 4 and 7 days after seeding. Cells on CH proliferated more slowly than cells on thGel-CH with significant differences. *=p<0.0001.

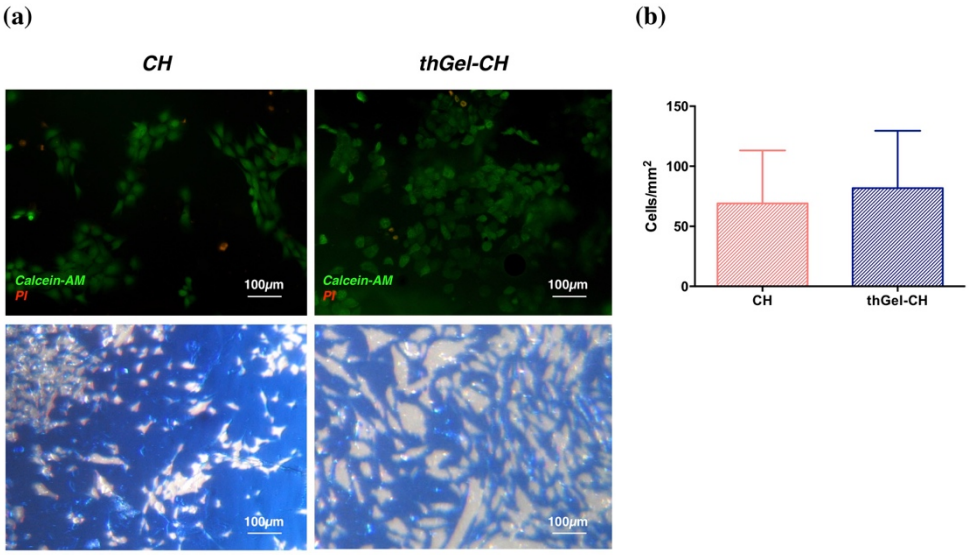

Figure 6. *LIVE/DEAD assay and Trypan blue staining.* (**a**) Typical images showing murine osteoblastic MC3T3-E1 cells on CH and on thGel-CH films after calcein-AM/PI staining (upper panel) and after Trypan blue staining (lower panel). Thiolated gelatin appeared to enhance chitosan cytocompatibility for cell culture, and cells on thGel-CH adhered better to the substrate and were more spread and with longer filopodia. (**b**) Histograms showing murine osteoblastic MC3T3-E1 cells quantitation on CH and on thGel-CH sample. *=p<0.001.

4. Discussion

Tissue regeneration requires the use of biological substitutes that, filling in the damage, provide for a structure able to support a quick ingrowth by regenerative autologous cells. These structures, called scaffolds, should mimic the extracellular environment, both biologically and mechanically, to promote cell colonization[1]. The ECM is composed of several structural and signaling proteins, such as collagenic and non collagenic proteins, which possess molecular domains able to bind cell integrins and to stimulate attachment[8]. Thus, cell adhesion and proliferation are essential parameters that indicate cell colonization and that are used to identify good substrates for TE[9,10].

The purpose of this preliminary study was to investigate whether chitosan scaffold prepared in the presence of D-(+) raffinose, and further enriched with thiol-modified gelatin, could represent a valid substrate for TE, based on the response of osteoblastic cells *in vitro*. Chitosan is a well-known natural chitin-derived biopolymer, which possess interesting biological properties although strongly dependent on preparation protocols. Briefly, it has been reported that fibroblasts do not proliferate on certain chitosan substrates because, according to Izume et al., some chitosan forms may impair cell adhesion and growth[11,12]. However, Bettini et al. showed that chitosan films prepared from D-(+) raffinose or sucrose solutions can dramatically improve *in vitro* cell responses of human lung derived WI-38 and of human umbilical vein endothelial (HUVEC) cells[7]. These sugars, which are completely removed prior to use, possibly act as spacers that reduce chitosan chain packing during gelation, thus modifying its viscosity, increasing its water content and surface wettability[7,13]. These scaffolds appeared as promising for this study, since they were quite stiff, and it has been shown that osteoblastic cells preferentially differentiate on stiff substrates[14]. However, different cell models respond differently to scaffold materials, and osteoblastic MC3T3-E1 cells appeared to grow slowly and with difficulty even on improved chitosan. MC3T3-E1 cells are an established model of osteoblast because they are immortalized calvaria cells and were not derived from a neoplasm. Thus, they retain several features of normal cells, including contact inhibition and are considered a good model to test biomaterials compatibility because their proliferation is not up-regulated regardless of the substrate. To

ameliorate MC3T3-E1 response, chitosan discs were enriched with thiol-modified gelatin. Gelatin is a naturally occurring polymer obtained by collagen hydrolysis, and as such still retains part of the effects of collagen in promoting the assembly of components of the ECM and supporting cell adhesion. Thanks to *in vitro* biocompatibility, gelatin is widely used for cosmetics and pharmaceutical preparation[15]. Recent studies have proposed gelatin modifications with thiol groups to improve its ability on supporting cell attachment and proliferation in a 3D environment[16]. Thiol modification was chosen in the present study because chitosan can avidly bind sulfur-containing compounds. With the regard of this, it may be speculated that thiol groups are strong acids, which, at physiological pH, dissociate to form sulfur ions (S-). Thus, electronic duplets on chitosan deacetylated NH_2 groups may interact with thiol groups by electrostatic attraction[17]. This quick functionalization by thiol-containing compounds could be a useful feature in clinical settings, because it could enable the operator to rapidly attach additional molecules to chitosan prior to use for improved bioactivity. To minimize the amount of gelation required to enrich chitosan, a low concentration of chitosan was chosen. Chitosan scaffold with (thGel-CH) or without (CH) gelatin appeared to avidly bind BSA within a short time frame, which is a useful property for subsequent integration of the scaffold *in vivo*. Furthermore, thGel-CH bound significantly more BSA than CH (**Fig.3**) suggesting that gelatin affected the protein-binding properties of chitosan. This also reflected on cell behavior. When osteoblasts were cultured on the scaffolds, they displayed polygonal morphology, viability and good attachment according to microscopic analysis. The addition of thiol-modified gelatin provided further adhesion sites to cells, which appeared more numerous, more widely spread and bigger. Cells appeared to form bigger cell clusters (**Fig.4**), likely because of enhanced motility on the substrate or proliferation. In fact, osteoblasts cultured on chitosan with thiol-modified gelatin reached confluence on the substrate sooner and showed a higher viability at each experimental time point, similarly to their behavior on tissue culture plate (data not shown). Cellular effects were unlikely to be caused by changes in the architecture of the scaffold, as SEM revealed no differences between CH and thGel-CH (**Fig.2**).

Our results confirmed that chitosan scaffolds produced in the presence of D-(+) raffinose are a good substrate for TE, and demonstrated that its further enrichment with thiol-modified gelatin would benefit cell colonization in TE applications thank to an amelioration in protein adsorption at the interface.

5. Conclusions

Taken together these data suggest that thGel-CH could be a good approach for tissue regeneration. The tested scaffolds displayed excellent protein affinity and good properties for cell proliferation, proving to be a viable approach for the amelioration of implantable scaffold bioactivity.

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Chapter 3

Fibronectin as a crucial step to control cell response at biomaterial interface

Abstract

The bioactivity of biomaterials is very closely related to cell response on contact with them. However, shortly after their insertion, materials are soon covered with proteins that constitute the biological fluids, and which render the direct surface recognition by cells virtually impossible. The control of protein adsorption at the interface is therefore desirable. Extracellular matrix proteins are of particular interest in this sense, due to their well-known ability to modulate cell behavior. Particularly, fibronectin is aware of playing a leading role and of being present in both healthy and injured tissues undergoing regeneration. As such, the aim of the present work is to give a panoramic on fibronectin and on its involvement in the control of cell behavior providing evidences of its ability to control cell adhesion, spreading, migration, proliferation and differentiation. A deep insight into methods to enrich biomaterials surface with fibronectin will be then discussed, as well as open questions on the possibility to design tailored platform able to specifically retain fibronectin from the surrounding extracellular milieu will be expressed.

1. Introduction

Once a biomaterial is inserted in the host anatomical site, protein adsorption from biological fluids, e.g. blood plasma, occurs rapidly, mediating the interaction surface-cells. The composition of the adsorbed protein layer at the interface plays a vital role in determining the nature of the tissue-material reciprocal fate, determining crucial characteristics of cell response, including adhesion, spreading, migration, proliferation and differentiation[1]. Particularly, some proteins can stimulate a constructive cell response, thus promoting wound healing and tissue regeneration, only when correctly presented, while when in different conformation or modified may mask the material as a foreign one, thus triggering a host immune reaction leading to material removal or isolation. Regrettably, protein adsorption on biomaterials is mostly a haphazard process and it is mainly driven by the chemical and physical characteristics of the material, as by protein availability and reciprocal interactions, which may lead to the adsorption of proteins which do not convey useful stimuli to cells because of an impaired conformation[2,3]. Thus, controlling specific protein adsorption at the interface of biomaterials may represent a viable approach in tissue engineering (TE), to design highly performant scaffolds able to address cell activity in detail[4].

Fibronectin (FBN) is an extracellular matrix (ECM) component that through binding integrin receptors of the cell surface, acts as a key player of the communication between the intra and the extracellular environment, thus controlling cell behavior. Therefore, the modulation of integrin-FBN interaction may offer a promising approach to tailor tissue regenerative responses[5].

The aim of the present chapter is to review the research supporting this crucial role of FBN and the methods developed to ameliorate scaffold bioactivity modulating functional FBN availability at the cell-biomaterial interface in TE approaches.

2. Fibronectin as a controller of cell behavior

The ECM is the non-cellular component of tissues and it basically provides physical scaffolding for cells, besides transmitting biochemical and biomechanical stimuli required for tissue morphogenesis and homeostasis. ECM main components are water, proteoglycans and proteins. Proteoglycans fill the major part of the extracellular environment and are the responsible for ECM force-resistance properties[6]. On the other hand, proteins are involved in orchestrating cell adhesion and migration. Among them, FBN is an important cell-adhesive ECM protein, which is present also in injured tissues undergoing regeneration. It exists into two main forms: i) the soluble FBN, which is a major component of blood plasma (300µg/ml) and it is synthetized by hepatocytes and ii) the less-soluble cellular FBN that is synthetized by different types of fibroblasts to be then assembled into the ECMs[7].

FBN plays a key role in cell behaviors, such as adhesion, migration and differentiation, as well as in morphogenesis and wound healing. For all these reasons, FBN is a clear candidate for the amelioration of scaffold bioactivity.

2.1 Fibronectin structure

FBN exists in human in more than 20 alternative splicing isoforms. Structurally, FBN is a dimeric high-molecular weight glycoprotein (~440 kDa), composed by two nearly identical subunits (~250 kDa) covalently bound by disulfide bonds near their C-term portion (**Fig.1**)[7]. Each of these subunits consists of three different types of repeats: type I, II and III.

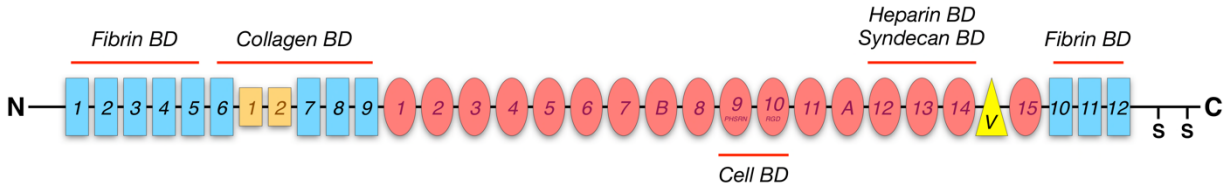

Figure 1. *Fibronectin.* Diagram representing the structure of fibronectin single subunit. Repeats and binding domains (BD) are indicated.

Type I repeats are 12 modules of 40 amino acidic residues, structured as stacked ß-sheets, linked by a disulphide bond, and that present a hydrophobic core made up of aromatic conserved residues. Type II repeats (2 modules) are 60 residues long,

constituted by perpendicular antiparallel ß-sheets linked together by 2 intra-chain disulphide bonds. Eventually, type III repeats, are 90 residues long and between 15 and 17 modules. These repeats do not possess disulfide bonds and the antiparallel ß-sheets are linked together with flexible loops and stabilized through hydrogen bonding[8-10]. As such, type III repeats are the highest sensitive to possible FBN unfolding[11].

2.2 Fibronectin-Integrin recognition

FBN communication with cells occurs through integrin binding. Integrins are the main cell surface receptors that mediate cell-matrix adhesion, some of which are ubiquitously expressed, while others are tissue-specific. Structurally, integrins are heterodimers generated by the coupling of 18-alpha (a) and 8-beta (ß) subunits, which specifically bind different ECM molecules. Each subunit consists of a large extracellular domain with selectivity for ECM ligands, a transmembrane domain and a short cytoplasmic tail. Because integrins lack of intrinsic enzymatic activity, the cytoplasmic tail of the ß subunit is structured to engage intracellular signaling molecules after dimerization and to activate the integrin-mediated transduction pathway[12,13].

Table 1: ***The integrin family of adhesion receptors.***

Cell Integrin	FBN and other ECM ligands	Cell expressing Integrin
α3ß1	Fibronectin, Collagen-I, Epiligrin, Laminin, Nidogen, Entactin	B-lymphocytes, Kidney glomerulus cells
α4ß5	Fibronectin, VCAM-1	Lymphocytes, Monocytes, Eosinophils, NK-cells, Thymocytes
α5ß1	Fibronectin	Bone cells, Memory-T cells, Monocytes, Platelets, Fibroblasts
α8ß1	Fibronectin	Not yet identified
αVß1	Fibronectin, Vitronectin	Not yet identified
αVß3	Fibronectin, Fibrinogen, Von Willebrand's factor, Vitronectin, Thrombospondin	Bone cells, Endothelial cells, B-cells, Platelets, Monocytes
αIIbß3	Fibronectin, Fibrinogen, Von Willebrand's factor, Vitronectin	Platelets
αVß6	Fibronectin	Carcinoma cells

There are many different integrins recognizing FBN (**Tab.1**) and each of them extremely depend on FBN structural conformation and on type III residues sensitiveness to unfolding. For example, the classic receptor for FBN is known to be the a5ß1 integrin (FBN-a5ß1 $Kd=8*10^{-7}M$)[14]. The a5ß1 recognizes and binds FBN through the interaction with an isolated tri-peptide sequence, the arginine-glycine-aspartic acid (RGD), which is contained in the 10th type III repeat of FBN and that synergizes with a further sequence, the proline-histidine-serine-arginine-asparagine (PHSRN), on the adjacent 9th type III repeat of FBN[15]. In bulk conditions, the RGD cell-binding domain and the recognition sequence PHSRN are separated from 32Å. This distance results to be extremely important for specific recognition between FBN and a5ß1 integrin. Indeed, if FBN-10th III domain unfolds as an effect of a 10pN external force application, the RGD loop is pulled away from the PHSRN on the FBN-9th III domain, resulting in a 23Å removal, which greatly diminishes the ability of a5ß1 integrin to recognize FBN, but which enhances that of the aVß3 integrin isoform[11]. As a results, FBN conformational changes may dramatically drive integrin specificity and pathophysiological cell and tissue responses, including the reactions to grafted biomaterials.

2.3 Fibronectin and the control of cell behavior

Precisely, cell-FBN interaction occurs by synergic interplay of proteins at three different level: i) FBN that offers docking points for cells, ii) integrins that allow the recognition of the FBN and iii) intracellular proteins that activate specific transduction pathways to control cell response, including adhesion, spreading, migration, proliferation and differentiation (**Fig.2**).

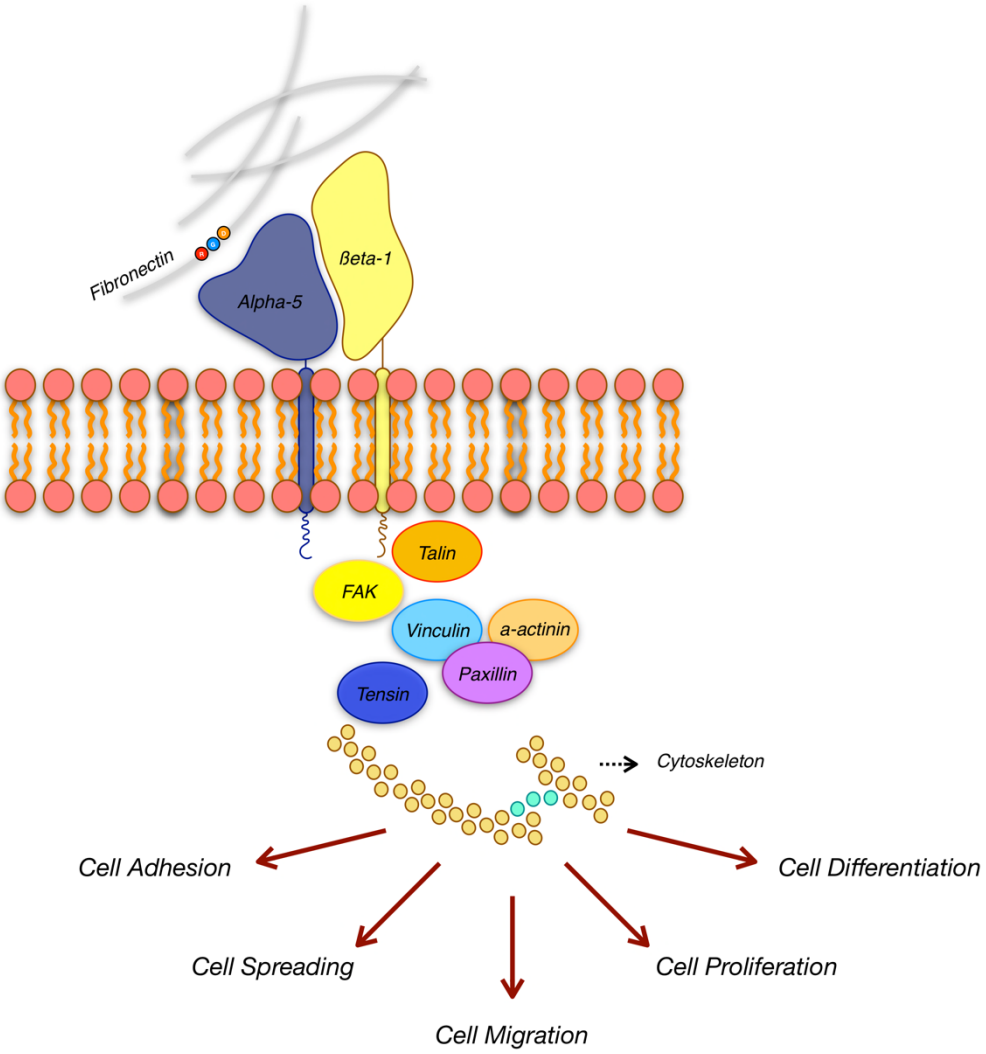

Figure 2. ***Integrin-mediated transduction pathway.*** After the FBN-RGD domain recognition, integrin subunits dimerize, thus recruiting intracellular proteins that trigger cell specific response.

2.3.1 Cell adhesion and spreading

Cell adhesion refers both to the mechanisms by which neighboring cells interact, attach or communicate each other by cell junctions (cell-cell adhesion), as to the

ability of cells to interact with their surrounding ECM or with an artificial substrate through focal contacts (cell-matrix adhesion)[16].

The sites of cell adhesion with the extracellular environment are called focal adhesions. At this level, after integrin dimerization, a network of 156 components and of more than 690 interactions form the adhesome and lead in the end to cytoskeleton proteins rearrangement. This wide spectrum of proteins may be divided in three categories: i) integrin-binding proteins, ii) adaptors or scaffolding proteins and iii) enzymes[17]. Integrin-binding proteins are directly recruited by the cytoplasmic tail of the integrin ß subunit. Among them, the binding of talin have been established to have a key role in integrin activation and it has been demonstrated that competition for talin binding may severely down-regulate integrin transduction pathway activation. Thus, adaptors or scaffolding proteins (e.g. vinculin, paxillin and a-actinin), link integrin-associated proteins with cytoskeleton components, while enzymes, which are mainly tyrosine-associated kinases (e.g. focal adhesion kinase - FAK, Rho family associated GTPases, Src), contribute to molecular signal transmission[17].

Cell spreading is directly connected to cell adhesion and it is fundamental for subsequent proliferation, migration and differentiation. A lack in cell adhesion/spreading may result in consequent cell apoptosis.

As we stated above, FBN and many others ECM components, possess adhesive motifs with the ability to promote cell adhesion and spreading. Thus, these molecules may be introduced on biomaterials to modify cell response. For example, the coating of substrates with increasing amount of FBN, leads to an evident promotion of cell adhesion and spreading in term of degree and speed[18].

2.3.2 Cell migration

Cell migration is another important cellular activity, which directly depend on cell adhesion. After cell spreading, cell migration occurs along the substrates through the formation of new focal adhesions and the release of the old ones. Physiologically, the human body has compositional gradients of soluble signals in tissues, which allow the creation of a natural driving force to direct the migration of

cells (chemotaxis). Alternatively, substrate-attached factors (haptotaxis) or mechanical cues (durotaxis) may govern cell migration[16].

More specifically, during cell migration processes, gradients guide actin assembly to the cell's leading edge, thus determining the direction of cell movement. At the migration front, the activation of cell surface receptors, i.e. integrins, generates the activation of the Rho-family GTPases and of the phosphatidylinositol biphosphate (PIP_2) pathway, which in turn activate the WASp/Scar proteins and eventually the Arp2/3 complex that guide the formation of new actin filaments branching from the preexisting ones. This process leads to the pushing of cell membrane forward. Simultaneously, at the back side of the cells, myosin II interacts with old actin filaments to enhance local rigidity, preventing lateral filopodia extension and allowing the retraction of the trailing end[19].

The abundances of FBN in the clot is closely related to fibroblasts recruitment during wound healing. As such, FBN introduction on biomaterials may allow the creation of dynamic pathway for cells to move along the scaffold[20]. Accordingly, Nuttelman et al. observed that NIH3T3 fibroblasts migrated faster on poly(vinyl alcohol) hydrogels modified with FBN, if compared to tissue culture plate and to control hydrogels.

2.3.3 Cell proliferation

Cell proliferation consists in cell number growth as a consequence of cell division. In adult organisms, cell proliferation is generally restricted to cell that replenish tissues. Cell division occurs as eventual stage of the cell cycle, which is broken up into four moments. The G1 phase is the first moment of a cell life and it is mainly characterized by cell growth and development. G1 phase is followed by the S phase, which consists in DNA replication. Thus, G2 phase, which is the gap before to cell division phase, ensures the proper replication of DNA and its packaging prior to cell division (M phase)[21].

The progression of the cell cycle is positively regulated by a family of protein kinases called cyclin-dependent kinases (Cdks), which turn on specific genes promoting growth (cyclines) and off their inhibitors at specific time. Particularly, the transition from G1 to S phase, which is the most critical point in the cell cycle, is positively regulated by Ckd2 and Ckd4, which induce cyclin E and cyclin D1 respectively, via

the MAPK/ERK pathway[21]. A few studies have correlated the induction of cyclin D1 by integrin signaling, highlighting that the integrin-dependent phosphorylation of FAK plays a key role in the phosphorylation of ERK and in the induction of cyclin D1. Thus, the decoration of substrates with FBN may be involved in increasing cell proliferation[22,23]. Noteworthy, it has been observed that FBN a5ß1 integrin activation mediates cell proliferation through the activation of the MAPK/ERK associated to the epithelial growth factor (EGF) receptor[22].

2.3.4 Cell differentiation

Eventually, cell differentiation is the process that leads to a cell to reach its specialized and mature phenotype, through the signaling of a defined combinations of transcription factors. Besides growth factors, various kind of ECM-derived proteins have function in regulating cell differentiation. In this sense, it has been shown that active integrin signaling is essential for driving differentiation: e.g. genetic removal of ß1 integrin subunits inhibits the differentiation pathway of the epithelial cells. As such, tailoring quantity and activity of fibronectin onto substrates could be used to selectively guide cells fate. Fascinatingly, the capacity of FBN to bind multiple integrins by slightly modulating its conformation, represent a design challenge to control specific cell behavior during their commitment. In a work by Martino et al. it was shown the capacity of a structurally stabilized FBN III9*-10 domain to promote osteogenic differentiation both in 2D and in 3D environment if compared to whole FBN or to the less specific FBN III9-10 or FBN III10 fragment. FBN III9*-10 presented a single mutation that switched the Leucine^{1408} into a Proline on the III9 domain, thus conferring to FBN enhanced affinity for a5ß1 integrin[24]. This example demonstrate how engineered cellular adhesive interaction with the surrounding milieu can lead to cell commitment control.

3. Fibronectin adsorption to biomaterials

The adsorption of proteins, particularly of FBN, is of the utmost relevance for tissue-biomaterial interaction. However, body fluids contain a heterogeneity of biomolecules and their adsorption on biomaterials is a complex process. Blood, for instance, consists of more than 150 proteins further supplemented with lipids, carbohydrates and other molecules that compete for their adsorption at the interface of materials. More specifically, when a surface is exposed to blood plasma, certain molecules are preferentially deposited from the bulk and both the affinity of proteins for the surface (e.g. size, charge, conformational stability) and kinetic factors (e.g. size, concentration) contribute to determine the profile of molecules stably adsorbed on the surface. Simply considering the diffusion, molecules that are present in the bulk solution at high concentration and/or with small size are deposited quicker than low concentrated and/or heavier ones (**Tab.2**).

Table 2: ***Exchange hierarchy of plasma proteins on surfaces.***[25]

Protein	Blood Plasma Concentration (mg/ml)	Molecular Weight (Da)	Adsorption
Albumin	40	66000	
Immunoglobulin-G	15	150000	
Fibrinogen	3	340000	
Fibronectin	0,2	220000	
Factor XII	0,015-0,047	80000	

The strength of the adhesion on the biomaterial plays also a role: molecules presenting greater affinity for the surface may induce the detaching of the previously and less affine adsorbed ones. These exchanges start to occur when all the binding sites of the substrates are occupied and continue until the surface is populated with proteins and molecules having strong affinity and interaction for the

material. This hierarchical tendency has been called the Vroman effect[26]. It is thus evident that to avoid competitive protein exchange on surfaces, desired proteins, i.e. FBN, could be preferentially immobilized, in order to trigger specific responses. **Table 3** summarized the methods that have been proposed to enrich biomaterials interface with FBN highlighting their major issues and anticipates the discussion on the next two paragraphs.

Table 3: ***Methods to enrich biomaterial interface with fibronectin.***

Fibronectin Source	Way to enrich biomaterial interface with Fibronectin	Drawbacks
Heterologous	Direct immobilization through physical adsorption	Possible host immune response; Possible spontaneous desorption; Possible adsorption in an undesired conformation.
	Surface functionalization and consequent covalent immobilization	Possible host immune response; Possible adsorption in an undesired conformation; Possible loss of protein mobility.
Recombinant Fragments	Direct immobilization through physical adsorption	Possible spontaneous desorption; Lack of entire protein availability; High costs of production.
	Surface functionalization and consequent covalent immobilization	Lack of entire protein availability; High costs of production.
Recombinant Cell Binding Domains	Surface functionalization and consequent covalent immobilization	Lack of specific binding sites interaction.
Autologous	Monoclonal Antibody Immobilization	Possible host immune response; High molecular size; High costs of production.
	Aptamer immobilization	High costs of production.

3.1 Immobilization of heterologous or recombinant fibronectin

The *ex vivo* decoration of biomaterials with FBN or with its derived fragment or domains has been for years the gold standard strategy to enrich scaffolds with cues to direct control cell response.

The immobilization of FBN on scaffold surface may occur by physical adsorption or by surface functionalization and consequent covalent immobilization[4]. Even though covalently immobilized FBN is more complex to obtain, it faces and bypasses several

issues connected to the physical adsorption, i.e. FBN spontaneous desorption or undesired conformational changes, which in turn lead to FBN loss of function[27,28]. For instance, in a work by Custodio et al. the biological activity of FBN simply adsorbed on chitosan or covalently immobilized via carbodiimide chemistry was compared employing human osteosarcoma SaOs-2 cells[18]. In opposition to bare chitosan, chitosan with adsorbed or immobilized FBN promoted cell adhesion. In the presence of FBN cells were well spread and presented the typical elongated morphology of mature osteoblasts. No differences were detected among the two methods in guiding cell adhesion and spreading, indicating that they were very similar in ameliorating chitosan adhesive properties. On the other hand, proliferation on FBN-immobilized surfaces was clearly enhanced after 7 days if compared to FBN-adsorbed chitosan and to control, suggesting a competitive adsorption of serum proteins contained in the culturing medium. Accordingly, desorption studies revealed that surfaces with immobilized FBN retained more proteins if compared to that with adsorbed FBN. Eventually, this study highlighted the importance of the covalent immobilization as a more desirable method to retain bioactive moieties at scaffold interface.

A clearly limitation linked to the immobilization of entire FBN is the difficulty in completely controlling protein conformational adsorption, which strongly depends on the underlying substrate. For example, Keselowsky et al. have demonstrated that the enrichment of a surface with different functionalities dramatically modulated FBN conformational adsorption and cell response, because of a shift in cell-binding domain availability during FBN adsorption[29]. As such, the immobilization of FBN recombinant fragment or of binding domains, have represented a sought after alternative in the upcoming years. However, since the interaction of numerous FBN specific domains (i.e. PHSRN, IKVAV and RGD) is required for the correct interaction with cell integrins, the anchorage of FBN fragment is more desirable than the immobilization of single binding domains[20,30-32].

3.2 Selective fibronectin binding biomaterials

The enrichment of biomaterials with FBN or with its fragments is mainly limited by the large molecular weight of FBN, which limits its stability and bioavailability. Therefore,

the creation of selective fibronectin binding materials is desirable. In this sense, an innovative idea could be the introduction of smaller molecules on scaffold surface, which could be exploited to capture FBN from the extracellular space and to retain it on the surface. The advantages of this approach appear evident.

The concept of adding selective binding capabilities to biomaterials was considered to promote the retention of the bone morphogenetic protein 2 (BMP-2) by grafting monoclonal antibodies (mABs) on scaffolds for bone regeneration[33,34]. A similar approach was later attempted by Oliveira et al.[35]. However, the use of mABs is of course limited by compatibility issues, but also by high costs of production, high size and unable to recognize small molecules. As such, to bypass this problem aptamer may be a viable alternative. Hoffmann et al. were the firsts to pioneer this concept in 2011 by using aptamers to retain endothelial cells from blood on vascular grafts[36]. Similarly, Chen et al. decorated synthetic biomaterials with aptamers to promote the colonization by cells, demonstrating an aptamer dose-dependent response of cell behavior[37]. Both these studies showed the potential of aptamers in designing selective binding biomaterials. Thus, considering these premises, in our work we demonstrated for the first time the possibility to promote the adsorption of serum FBN and to ameliorate osteoblasts colonization by using anti-FBN aptamers (**Fig.3**)[38].

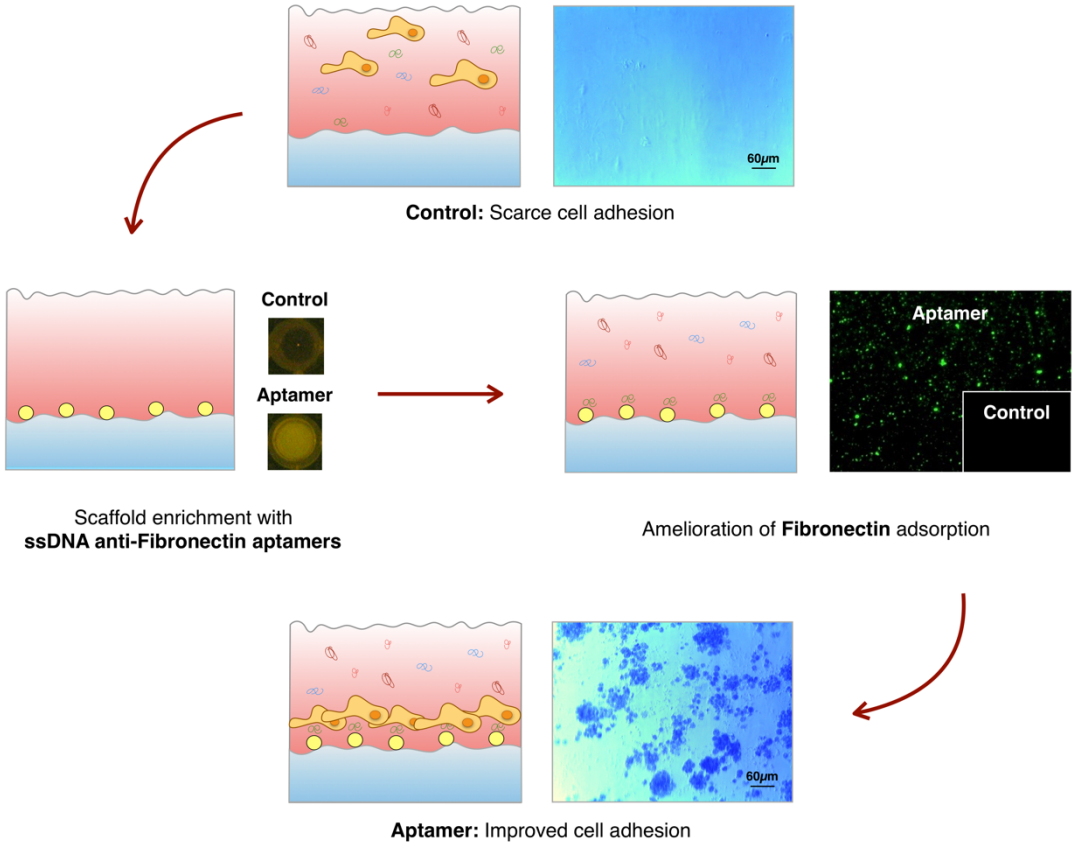

Figure 3. ***Improved scaffold biocompatibility through anti-Fibronectin aptamer functionalization.*** Graphical Abstract[38].

4. Conclusions

The role played by FBN in the control of cell behavior makes it the optimal candidate for the amelioration of materials surface bioactivity. Its adsorption at the interface can be controlled through different methods and sFBN-materials seemed to be the most promising way because of the possibility of exploiting autologous FBN and to avoid the lost of its bioactivity. As such, further investigation in this thesis will aim to describe the potentiality of this unexplored method to ameliorate the bioactivity of materials and device for regenerative medicine.

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Chapter 4

Aptamers to improve the bioactivity of biomaterials

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Abstract

The research in the field of biomaterials has largely been focused on the development of methods to enhance their bioactivity. Adopted strategies include chemical-physical approaches, including the use of monoclonal antibodies, which confer the system target specificity. However, antibodies are marred by numerous issues, such as low physical-chemical stability or the risk of inducing immunological responses in the host, which often make their use difficult. Aptamers, a new class of molecules discovered in the early nineties, are small oligonucleotides, or in some cases peptides, and have been proposed to rival antibodies in biomedicine, countering at least some of the antibody-related drawbacks. The aim of this review is to provide a background to nucleic acid aptamers and to explore their novel applications. In addition, to provide brief overview of their therapeutics applications, here we have assessed the methods that employ aptamers to improve the bioactivity of biomaterials, in particular, those that ameliorate scaffold biocompatibility for tissue engineering.

1. Introduction

The term biomaterial was first defined in 1987 at the Consensus Conference on the Definitions in Biomaterial Science of the European Society for Biomaterials as:

"*Non viable material used in a medical device, intended to interact with biological systems*"[1].

Subsequently, with the advancements in cell and molecular biology, chemistry, material science and engineering, the term has significantly evolved over the past 30 years, and biomaterials are nowadays defined as:

"*Materials intended to interface with biological systems to evaluate, treat, argument or replace any tissue or function of the body*"[2].

As we have largely discussed in the first chapter of this thesis, a concept closely connected to that of biomaterial is biocompatibility. Materials were first considered biomaterials, and therefore biocompatible, if they could be placed in contact with tissues without damaging them, thus being essentially inert. However, research efforts progressively revealed that biological inertia was impossible to achieve and that any material that comes in contact with tissues induce a non-self response from the host immune system. The term biocompatibility was thus revised and for years it has been associated with the lack of toxicity, immunogenicity, carcinogenicity and irritancy against the human body. Subsequently, new evidence in the early eighties led to a further updated definition of biocompatibility as it become clear that all materials react with tissues and are not inert. It was also showed that biological responses to a biomaterial are different among the tissues and that tissues themselves affect material biocompatibility. Furthermore, clinical evidence indicated that some situations require materials to get degraded and removed from the host after accomplishing their function[1]. Taking all these considerations together, at the Consensus Conference in Boston in 1987, the definition of biocompatibility was outlined as follows:

"*Biocompatibility refers to the ability of a material to perform with an appropriate host response in a specific situation*"[1].

As we stated earlier in this thesis, the definition of biocompatibility encompasses a new concept, that of bioactivity, which can be associated to the ability of the material to perform for its function.

Considering what all above, the aim of the present review is to focus on the concept of bioactivity related to biomaterials thought as scaffold for tissue engineering (TE) and on how to improve their performance, thus their function, by means of aptamers.

2. Biomaterials in tissue engineering

Regenerative medicine (RM) is a new therapeutic approach, which aims to restore structure and function of damaged tissue and organs to find a solution to that permanently damaged and untreatable[3]. Tissue regeneration is a complex task and may be achieved with RM through three type of approaches: molecular, cellular and TE.

TE was first defined in 1988 at the first TE symposium in California as:

"*An interdisciplinary field of research that applies the principles of engineering and the life science towards the development of biological substitutes that restore, maintain and improve tissue function*".

TE offers great potentials in the clinical practice an it is mainly centered on the development of a scaffold, which combined with cells and molecules, allows the activation of tissue regenerative mechanisms.

Scaffold is a central concept of TE and consists in a 3D structure designed to promote cell adhesion, proliferation and extracellular matrix (ECM) molecules deposition[4]. Scaffolds can be made of biological or synthetic materials. Biological ones are derived from human, animal and vegetal tissues, while the synthetic are prepared with artificial biomaterials[5].

Biocompatibility is a key concept for TE approaches. A scaffold can be considered for *in vivo* application if it has been proven to be biocompatible *in vitro*, e.g. supporting cell adhesion and proliferation. Cell behavior heavily depends on the quality of protein adsorption at the interface, which is a spontaneous phenomenon that occurs when a material comes in contact with biological fluids[6]. Furthermore, it has been convincingly demonstrated that shortly after the insertion of a material in an anatomical site, it is covered with a macromolecular film of host proteins which are essential for scaffold colonization from autologous cells[7]. In this view, the physic-chemical characteristics of the material play a pivotal role during their adsorption and may conduct to their conformational change, thus totally or partially impairing their function. Similarly, they play an essential role in controlling the amount of adsorbed proteins too. As such, a series of methods have been developed in years to enhance scaffold surface biocompatibility, controlling the amount, the composition and the conformation of adsorbed proteins: these methods include

the immobilization of short peptides or proteins on scaffolds and chemical and physical treatments.

In **Chapter 1,** we have largely discussed that chemical and physical treatments exploit the ability of some proteins to bind certain chemical groups better than others, by enriching surfaces with specific functional groups through the combination of chemical and physical methods[8,9]. Alternatively, the quite recent discovery of integrin-binding sequences opened the possibility of immobilizing them on materials and to enrich scaffolds with docking points for cells[10], able to enhance the adhesion, migration and differentiation of cells *in vitro*. Consistently with this, another interesting method to improve scaffold biocompatibility concerns in their coating with entire proteins that mimic ECM, while novel approaches moved the attention to the use of antibodies as docking molecules capable to retain certain growth factors on scaffold surface[11-15].

All considered, the immobilization of monoclonal antibodies seems to be a valid alternative to enhance the bioactivity of materials. However, the use of monoclonal antibodies faces numerous issues, prompting researchers to develop new methods to enhance scaffold bioactivity. One such approach involves the use of aptamers, a new class of molecules which act in a manner similar to antibodies, but with several less drawbacks.

3. Aptamers

In the 1980s, molecular virology revealed that small structured oligonucleotides could bind proteins with high affinity and specificity. This evidence led to the use of oligonucleotides as selective receptors and to the discovery of aptamers nearly 10 years later[16]. The word "aptamer" was first used in 1990 by Ellington and Szostak to describe small RNA molecules able to bind small organic dyes. It derives from the fusion of the Latin expression "*aptus*", which means "to fit", and the Greek word "*meros*", which means "part"[17]. Since then, aptamers have been defined as short oligonucleotides that by adopting specific 3D conformations are able to bind specific and selected target[18].

Nucleic acid aptamers are short, single or double-stranded DNA or RNA oligonucleotides, 20-80 bp long and 6-30 kDa heavy. Aptamer structure is characterized from a random sequence in the center, which is important for target recognition, and from two flanked constant designed primer binding sites at the 3' and the 5' ends, which are necessary for aptamer amplification during the selection process. After that recognition occurs, aptamer-ligand interaction is stabilized by hydrogen-bonding, van der Waals forces and electrostatic interactions, which render this intimate connection highly specific and able to discriminate ligands from its analogues (i.e. the recognition of L-arginine could be 12.000-fold more affine than that for D-arginine)[19-21]. The process of aptamer-target recognition is reported in **Figure 1**.

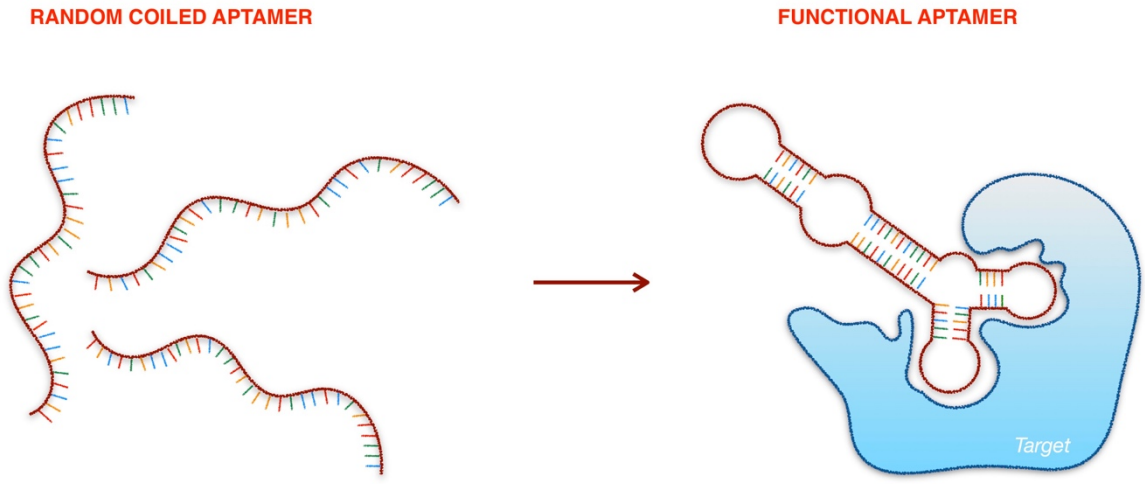

Figure 1. ***Aptamer-target binding.*** Diagram representing aptamer 3D conformational rearrangement in the presence of the target.

The ability to selectively bind molecules makes aptamer a viable alternative to the use of antibodies, since if compared they overcome the issues connected to the use of antibodies and improve their clinical applicability as their suitability for industrialization. First of all, aptamers are low-immunogenic and low-toxic molecules, and they are not directly recognized by the human immune system as foreign agents[22-24]. Unlike antibodies, aptamers have a wider range of targets, they are smaller so that they can easily permeate into tissue barriers and cells[25]. Moreover, aptamers can also bind small ligands, such as ions and small molecules, which cannot be recognized by antibodies. Additionally, aptamers are thermally stable and can face repeated cycles of denaturation/renaturation without damaging their binding efficiency. Finally, aptamer production and eventually their modification is cheaper, easier and faster than that of antibodies[26].

To date, the research interest on aptamers is growing, as it arises by the publication rate on this topic, which has exponentially grown in 25 years, leading to more than 8000 published articles in the PubMed database including the term "aptamer" in August 2018[20].

However, in spite of their popularity, aptamers clinical applications is still limited, and at the present moment only one aptamer-based drug has been approved by the US Food and Drug Administration (FDA). Pfizer/Eyetech launched the Macugen® in 2004, a RNA aptamer against the vascular endothelial growth factor (VEGF) for the treatment of wet age-related macular degeneration (AMD)[27]. Barriers to the commercialization of aptamers are essentially two: i) some *in vitro* generated aptamers do not elicit a comparable *in vivo* effect, and ii) the selection process is time-consuming and not still efficient[21]. In spite of these issues, a recent market report projected the global aptamer market to $5.4 billion by 2019.

3.1 Aptamers generation

Aptamer selection requires two steps: upstream screening and downstream screening. The upstream screening step identifies full-length aptamers through the systematic evolution of ligands by exponential enrichment (SELEX), whereas the

downstream screening step aims to isolate the shortest oligonucleotide sequence required for target binding[20].

3.1.1 Upstream screening

In vitro selection or SELEX is the techniques used to isolate aptamers, which was first described by Ellington, Szostak, Tuerk and Gold in 1990[17,28].

The SELEX process consists of three steps, which are cyclically repeated to screen sequences with highest affinity for the target[16]. The preparation of an initial pool of oligonucleotides (library) is followed by the selection of the best aptamer candidate and by its amplification. The process is summarized in **Figure 2**.

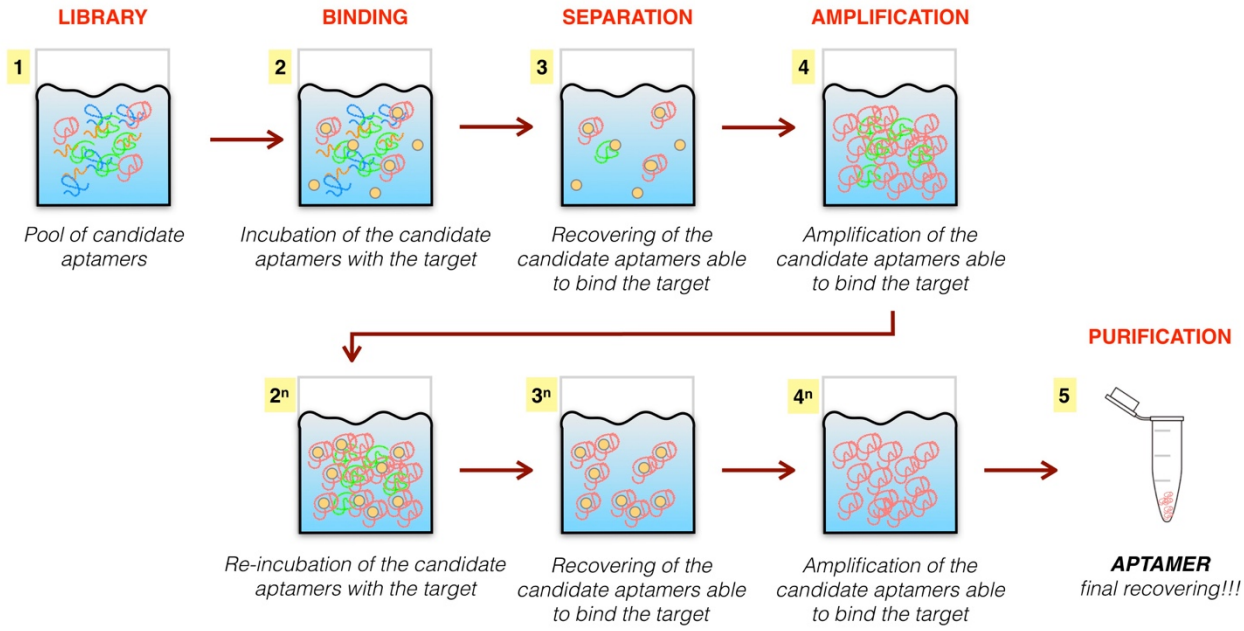

Figure 2. *Aptamer selection process.* Diagram representing the process for aptamer selection.

Library generation – The whole process for nucleic acid aptamer generation starts with the production of a synthetic library, which contains a pool of ~10^{12}-10^{15} different oligonucleotides (ssDNA or RNA sequences), which are able to bind any target molecule. Each single sequence represents a possible aptamer candidate and is made of a central random region of ~25-30 bp, flanked by two defined primers at the 3' and at the 5' ends, necessary for subsequent amplification[20,21]. Aptamer generating libraries can be divided into five types, on the base of the

collected sequences. The most common libraries are the standard, which collect random sequences of 20-60 bp. Structurally-constrained libraries contain sequences with stable regions in order to induce aptamer folding in a certain secondary structure. Libraries based on a known sequence are constituted by oligonucleotides with specific and known sequences inserted in their random region. Finally, libraries based on genomic sequences (genomic SELEX) are created by digesting genomic DNA in order to find protein capable of binding them[29].

Binding and separation – After its generation, the library is incubated with aptamer target. A part of the oligonucleotides in the pool is able to recognize the target and these sequences are considered possible aptamers (partitioning), while unbound sequences are filtered out from the solution and discarded (elution)[20]. Different methods have been developed to discriminate aptamers from specific sequences. First approaches, developed by Gold and co-workers, were based on a nitrocellulose membrane where the target was immobilized[28]. However, alternative strategies based on biochemistry techniques have been developed to replace this approach, e.g. chromatographic affinity or magnetic columns are often used[29-34]. In addition, capillary electrophoresis has been proposed as a selection technique because of speed and high resolution, as it has been demonstrated by Bowser and co-workers to select aptamer against the neuropeptide Y and human immunoglobulin E (IgE) in only 4 cycles[35,36]. Moreover, in recent years, aptamers have been selected against whole cells through the cell-SELEX method, a complex technique, which allowed Gold and colleagues to select U251 glioblastoma cells, and subsequently Kobatakes and colleagues to the identification of SBC3 lung cancer cells[37,38].

Amplification – After the selection of aptamers from a specific oligonucleotide pool, aptamers are amplified by polymerase chain reaction and the products of the amplification are used as new sub-library for the following selection rounds[21].

3.1.2 Downstream screening

After the first step of the SELEX, candidate aptamers are normally ~80 bp long. However, aptamer binding region is only 10-15 bp long and the redundant nucleotides could be deleted through a process called "aptamer truncation", Many strategies have been developed in order to minimize aptamer length without affecting their binding region, and most of them are based on computational biology[26,39]. For example, Giangrande and colleagues truncated an RNA aptamer against the prostate-specific membrane antigen (PSMA) preserving its binding activity and its functionality through the use of a structure simulation and a target docking algorithms, while Green et al. (1996) were able to select the shortest binding sequence of a DNA aptamer anti-platelet derived growth factor (PDGF) through partial fragmentation[40]. Furthermore, other techniques have also been developed eluding the use of computational biology, e.g. Duan and co-workers selected the binding region of the anti-CD133 aptamer to recognize cancer stem cells through the hybridization with complementary oligonucleotides probes of non-essential regions, as well as Wang and co-workers detected the anti-human protein tyrosine kinase 7 (hPTK7)[41,42]. Such selection methods involving aptamer truncation are effective. However, their complexity, the length and their costs still remain a concern[20].

3.2 Biomedical applications of aptamers

The similarities between aptamers and antibodies lead to their application in various field, including research tools, bioassays, food safety, and environment monitoring, as demonstrated by a plethora of reviews recently published on this topic[43-47]. However, a field of major interest for aptamers application is biomedicine, where aptamers can be used as sensors for biomarker discovery, molecular imaging probes, drug delivery systems and drugs, especially in cancer nanomedicine and therapy[16,20].

3.2.1 Aptamers as potential drugs

Although the most studied aptamers are against thrombin, VEGF and PDGF, aptamer applications range from cancer to infectious pathogens.

Therapeutic aptamers in eye disease – The first therapeutic aptamer approved by the FDA was the Pegaptanib, which today is commercially available as Macugen® (Pfizer and Eyetech)[22,23]. The Pegaptanib is a 27 ribonucleotide pegylated RNA aptamer antagonist of the VEGF isoform 165[48]. Since its approval in 2004, the Macugen® has always been used for the treatment of AMD, a degenerative ocular disease that causes vision loss in older adults due to retinal damage. However, the efficacy of this aptamer was then discovered to be important also for the treatment of diabetic macular edema (DME) and of the proliferative diabetic retinopathy (PDR) with promising results in clinical trials[49]. At the present moment, the spectrum of use of Pegaptanib is being broadened to other pathologies such as ischemic diabetic macular edema (MIDME), uveitis, choroid neovascularization secondary to pathologic myopia, and iris neovascularization[50].

The limits of anti-VEGF agents to treat AMD are their inability to promote the regression of new blood vessels, which are the cause for vision loss. To bypass this limitation the E10030 aptamer (Fovista™) was developed by Ophthotech Corp in 2012: the E10030 is a 29 pegylated RNA aptamer able to bind PDGF, which regulated pericytes maturation. The combined administration of E10030 with Pegaptanib showed successful neovascular regression in preclinical models[51].

Therapeutic aptamers for hemostasis – Thrombin is a wide-studied target for anticoagulation, and its *in vivo* inhibition is a major solution to prevent and treat blood clotting abnormalities[20,52]. Anti-thrombin aptamer (TBA), a 15 bp oligonucleotide, was first selected in 1992 by Toole et al. and it was the most studied aptamer for clinical applications in 2012[18,53]. After the evaluation of TBA efficiency *in vivo*, the Nu172 aptamer (ARCA Bipharma) was developed as a potential thrombin inhibitor candidate. Nu172 is a 26 bp aptamer able to prevent fibrinogen cleavage of α-thrombin by interacting with the exosite I. Nu172 is currently in phase II clinical trials to be certified for anticoagulation in invasive medical procedures, coronary artery bypass graft and percutaneous interventions[54].

Therapeutic aptamers for cancer – The goal of new therapeutic approaches in oncology is often to block the neoplastic progression through the inhibition of specific cell-pathways, which lead to cell abnormal proliferation. Several clinical trials have proposed the use of aptamers to specifically bind tumor cells and to stop cancer development. The specific cell membrane receptors that can be blocked in tumors are numerous, but only few have been investigated with aptamers. A pivotal role is played by nucleolin, a protein which is often over-expressed on the surface of cancer cells and that is firstly involved in cell survival, growth and proliferation, as well as in nuclear transport and transcription[55]. In particular, nucleolin seems to manage the internalization of the tumor-homing F3 peptide and its inhibition affects several signaling pathways responsible for abnormal cell proliferation during cancer progression, such as Nf-kB and Bcl-2 pathways[56,57].

AS1411 (Antisoma, PLC) is a 26 bp long aptamer rich in guanosine and screened against nucleolin[24,55]. When AS1411 interacts with surface nucleolin, the complex is internalized and prevents its binding with Bcl-2, thus inducing cell apoptosis. AS1411 has shown good growth-inhibitory properties *in vitro* (**Tab.1**) and the ability to be accumulated in tumor tissue[24,58].

Table 1. ***Dose administered and time exposure of different cell lines to AS1411 aptamer, in order to observe growth inhibition[24,58].***

Cell Line	Description	Dose of AS1411 administered	Time of exposure to AS1411
A549	Human epithelial lung carcinoma	1μmol/l	6 days
DU145	Human epithelial prostate carcinoma	2μmol/l 15μmol/l	6 days 5 days
MDA-MB-231	Human breast adenocarcinoma	15μmol/l	5 days
MCF-7	Human breast adenocarcinoma	15μmol/l	5 days
HeLa	Human cervix adenocarcinoma	15μmol/l	5 days
Primary cells from leukemia	Human leukemia	10μmol/l	7 days
Primary cells from lymphoma	Human lymphoma	10μmol/l	7 days

Therapeutic aptamers in microbiology – When aptamers were first described by Ellington and Gold in 1990, their ability to bind viral proteins was clear, and, consequently, their use to treat viral and bacterial diseases has always been investigated[59,60].

Ebola epidemic of 2014 and other emerging viruses have prompted several research groups to use specific aptamers in the treatment of these diseases by blocking sites essential for virus infectious progression[61-64]. For example, it has been shown that specific aptamers against influenza major targets are able to inhibit or block virus fusion, penetration and replication[65-69]. Aptamers are also thought to be useful to kill multidrug-resistant (MDR) bacteria *in vivo*, possibly by blocking resistance enzymes such as the New Delhi metallo-ß-lactamase (NMD-1) or by inducing the classic pathway of the complement in lieu of antibodies[70,71].

3.2.2 Aptamers as sensors for biomarker discovery

Biomarkers are molecules that change their expression level when physiological conditions are altered, and can thus be used to indicate the progression state of a disease or the risk to develop it. Biomarkers are therefore a tool with high potentiality for disease screening and early diagnosis. However, a very limited number of biomarkers have been thus far discovered and the use of antibodies to identify disease targets is often unfeasible, because their targets are frequently cell epitopes and it is impossible to design and select an antibody against an unknown receptor. As such, aptamer research is moving to fill this gap. To this purpose, target cells are amplified, collected and lysed. The lysate is then incubated with aptamers, while target proteins undergo to a comparative proteomic analysis[20].

In recent years, many research groups have worked to find aptamers that specifically bind biomarkers. In instance the hPTK7 has been discovered as a potent marker for T-cell acute lymphoblastic leukemia, tenascin-C as a biomarker for glioblastoma cells, the immunoglobulin μ (Igμ) heavy chain for Burkitt's lymphoma, whereas the stress-induced phosphoprotein I for ovarian cancer[37,72-74].

3.2.3 Aptamers as molecular imaging probes for diagnostic

Aptamers have also been proposed as detecting agents in diagnostics, both as molecular beacons or as sensors for bio-imaging[16,20].

In 2001, Hamaguchi et al. developed an aptamer beacon for thrombin. An anti-thrombin aptamer was modified with a complementary sequence at its 3' and 5' end to form a stem-loop structure. Furthermore, the 5'-end was labeled with a fluorescent moiety, whereas the 3' with a quencher. In the absence of thrombin the complementary 3' and 5' ends lie in close proximity, resulting in fluorescent quenching, whereas in the presence of thrombin, aptamer acquires its 3D specific conformation, distancing the fluorophore and the quencher, thus allowing the expression of the fluorescence signal in a dose-dependent manner[75].

In the subsequent years, the idea of labelling aptamers with fluorophores was exploit to develop new probes for computerized tomography (CT) and for magnetic resonance imaging (MRI). Moreover, this idea seemed to be appeal to combine aptamers with nanomaterials for CT and MRI analysis, to improve *in vivo* imaging and to promote the accurate targeting as the diffusion of this materials through blood stream[16]. For example, C6 cancer cells were detected by using a Cy3-labeled aptamer against nucleolin transmembrane protein, while aptamers against the prostate specific membrane antigen (PSMA) were conjugated with quantum dots to detect prostate cancer cells[76,77]. Again, in 2013, Kim et al. successfully immobilized a VEGF receptor 2 aptamer on a magnetic nanocrystal surface to detect the angiogenic vasculature in glioblastoma by MRI with high sensitivity and efficiency[78].

3.2.4 Aptamers as drug delivery systems

The ability of aptamers selected through the cell-SELEX to recognize cell antigens have been further exploited to deliver a variety of molecules, mainly drugs, into cells[16]. To this purpose, aptamers can be used alone or in combination with other delivery systems, such as polymers or liposomes, in order to enhance their specificity[20].

Building on their previous work on quantum dots-aptamer complex for PSMA, Min et al. were able to load this construct with doxorubicin, an anticancer drug, and to effectively introduce it inside prostate cancer cells[79]. On the other hand, Levy's

group relied on an aptamer against PSMA to introduce a siRNA in prostate cancer cells, which inhibited gene expression within 30 minutes from the exposure[80].

Alternatively, to enhance polymers specificity as drug delivery system, they can be functionalized with aptamers. This strategy has shown to be promising for clinical applications. Farokhzad et al. encapsulated rhodamine-labeled dextran within a nanoparticle of poly (lactic acid)-block-polyethylene glycol copolymer with a terminal carboxylic acid functional group (PLA-PEG-COOH) conjugated to an aptamer against the PSMA antigen of prostate cancer cells. This system was able to enter PSMA over-expressing cells in less than 2 hours[81]. The same group further generated a nanoparticle with poly (D,L-lactic-co-glycolic acid)-block-poly (ethylene glycol) (PLGA-b-PEG) copolymer conjugated with the A10 aptamer against PSMA to deliver docetaxel inside cancer cells. This system was tested *in vivo* and it was able to induce the complete regression of the tumor in five out seven mice treated[82]. Following these promising results, several others similar constructs based on the conjugation of polymers and aptamers were efficiently tested[83,84].

Liposomes were also engineered with aptamers to deliver cisplatin and taxol inside breast cancer cells. The AS1411 aptamer-liposome bioconjugate system efficiently transported cisplatin inside tumorigenic cells, and effectively killed the target cancer cells, but not the healthy ones[85]. Moreover, if compared to control liposomes, liposomes conjugate with AS1411 aptamer and containing taxol, increased the cellular uptake of the construct[86].

4. Aptamer to enhance material biocompatibility

As widely discussed in the first chapter of this thesis, one of the hot topic in TE is the amelioration of material surface bioactivity to obtain highly-dynamic scaffolds capable of interacting with autologous cells and to positively modulate protein adsorption at the interface[6]. Numerous groups have aimed to reach this goal by immobilizing the minimum integrin recognition sequence for cells on scaffolds, i.e. arginine-glycine-aspartic acid RGD motif, by chemically or physically modifying scaffold surface or by coating scaffold with other biocompatible materials (see **Chapter 3**). However, issues connected to these methods, including the lost in protein mobility, the necessity to use heterologous proteins or the evidence that the effectiveness of the entire ECM proteins in triggering cell adhesion is better than that of small binding domains, require the search of new ones[87-90]. As such, in this section we want to focus on the possibility of using aptamers to improve the bioactivity of scaffold for TE.

The pioneer of this topic was Hoffmann in 2007, who suggested that the fast adhesion of circulating endothelial precursor cells (EPCs) on aptamer-coated vascular grafts was useful to promote endothelium healing and to prevent hyperplasia[91]. Aptamers screened through Cell-SELEX against EPCs were immobilized on vascular prosthesis, which were subsequently incubated with the whole anti-coagulated porcine blood. After the immune-staining for CD31 and CD 144[20], they observed that EPCs were captured on aptamer-enriched implants, but not on the controls.

On this way, five years later, Chen et al. proposed a similar approach by immobilizing aptamers selected against a cell surface receptor on PEG hydrogels to improve the ability of cells to adhere and to colonize the material[92]. Cell proliferation was surprisingly proportional to the concentration of aptamers used for the functionalization.

Considering all these premises, our group have recently proposed the possibility to improve the bioactivity of materials by using ssDNA aptamers selected against fibronectin[93]. Fibronectin is one of the major physiologically occurring proteins in damaged tissues and is mainly involved in cell adhesion and in regeneration process[87]. By using anti-fibronectin aptamers we aimed to ameliorate its adsorption

on scaffolds and as a consequence to exploit it as a docking point for cell adhesion. We believe aptamer coating of biomaterials could be a useful and viable approach to improve the performance of scaffold materials for clinical purposes, because different medical devices could be envisaging able to capture bioactive mediators from patients' blood and concentrate them where they are needed, on the biomaterial itself. At the same time, this technology could represent a revolutionary improvement of traditional biomaterials that can be enriched with exogenous molecules but are not still able to selectively capture a desired molecule.

Here we present two examples of biomaterials we aimed to dope with anti-fibronectin aptamers: a hyaluronic-based hydrogel and a machined titanium surface.

4.1 Improved scaffold biocompatibility through anti-fibronectin aptamer functionalization

In our first work, we attempt to enrich a polyethyleneglycole diacrylate/thiolated hyaluronic acid hydrogel (PEGDA/tHA) with anti-fibronectin aptamers[93]. This material was chosen both because its chemistry allows easy functionalization thanks to freely available acrylate groups through a nucleophilic addition (Michael's addition) and because it naturally presents scant docking points for cell attachment.

Human osteoblasts were cultured on the samples in the presence or in the absence of aptamers for 10 days and assayed for their capacity to proliferate, adhere and migrate inside the hydrogel.

Our results showed that aptamers quite rapidly enriched hydrogels with fibronectin after culturing medium addition. In turn, fibronectin retention consistently promoted the number of adherent cells (**Fig.3a**), which appeared to have a more spread cytoplasm and more numerous mature focal adhesion complexes, already after 2 days of culture (**Fig.3b**).

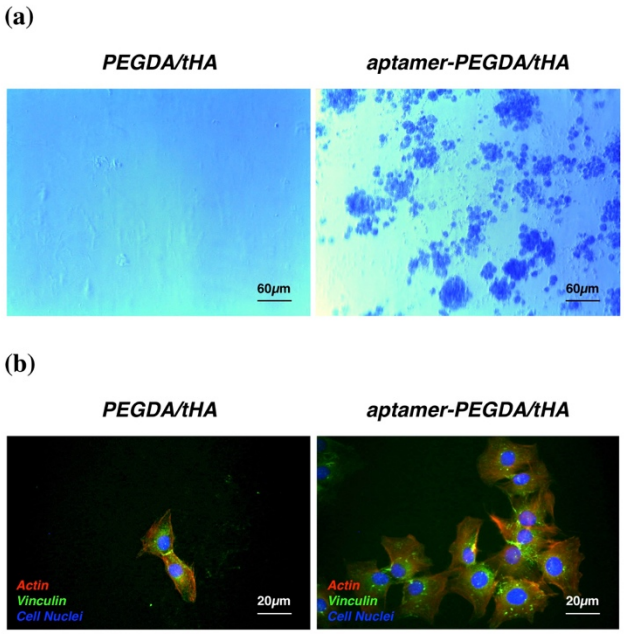

Figure 3. ***Anti-fibronectin aptamers promote cell adhesion and proliferation of human osteoblasts on PEGDA/tHA hydrogel.*** (**a**) Images reporting human osteoblasts staining with Toluidine blue on PEGDA/tHA with or without aptamers 10 days after seeding. (**b**) Typical immunofluorescence images reporting human osteoblasts labeled for cytoskeleton (red), focal adhesions (green) and cell nuclei (blue) 2 days after seeding.

Furthermore, when cells were seeded on the top of specimens, they migrated deeper in the hydrogel when aptamers were present, appearing disposed on multiple focus planes (**Fig.4**).

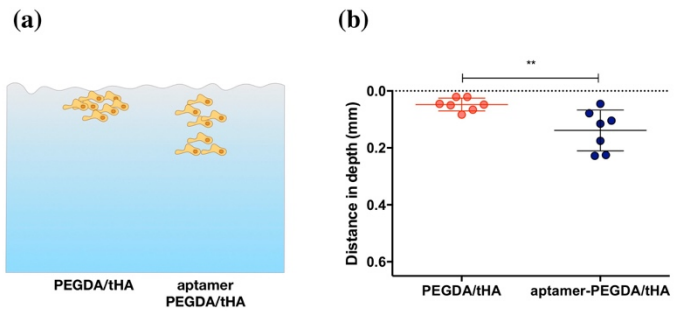

Figure 4. ***Anti-fibronectin aptamers promote cell migration into PEGDA/tHA hydrogel.*** (**a**) 2D graphic reconstruction of the human osteoblasts migration inside the hydrogel. (**b**) Z-plot of cell observed on multiple focus planes. ** p=0.0077.

Noteworthy, our results were appreciated only in the presence of serum in the culturing medium, confirming that aptamers required the presence of fibronectin to accomplish their function. As such, we concluded that anti-fibronectin aptamers promoted scaffold enrichment for this protein, thus ameliorating cell adhesion and scaffold colonization.

4.2 Titanium biomimetic coating through immobilized anti-fibronectin aptamers

Titanium has long been known to be the gold standard material for dental and orthopedic rehabilitation. Upon its insertion, through a microscopic sequence of biological and mechanical events, titanium establishes a structural connection with the bone, which is termed osseointegration, and which in turn depends on different factors, including titanium surface features and surrounding tissues quality[94]. Furthermore, it has been supposed that the size of this intimate relationship between the bone and titanium could be strongly affected by initial serum proteins adsorption and that fibronectin plays a crucial role in this step[95-97]. As such, we decide to regulate the adsorption pattern on titanium by enriching its surface with aptamers.

After having bound anti-fibronectin aptamers on machined titanium surfaces modified via a sol-gel method, we proceeded by culturing human osteoblasts on titanium specimens and to assess their adhesion with scanning electron microscopy (SEM) after 1 day of culture.

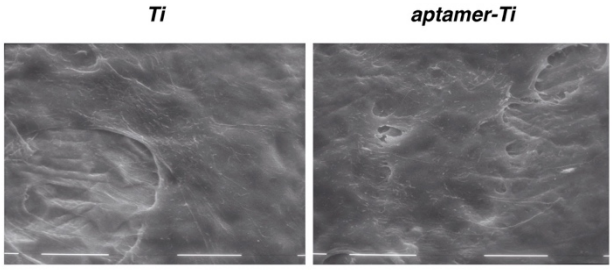

Figure 5. ***Anti-fibronectin aptamers influence cell morphology.*** Typical SEM images showing human osteoblasts on machined titanium surface with or without aptamers after 1 day of culture (Scale bar 10µm).

SEM analysis revealed that cells attached and spread on machined titanium surfaces both in the presence or in the absence of aptamers. However, cells on bare titanium showed an extremely flat shape, with scarcely defined cellular limits, while cells on aptamer-doped titanium were smaller, less elongated and with intercellular edges clearly more visible and more numerous filopodia, appearing that cells attached more willingly to the underlying titanium (**Fig.5**).

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Chapter 5

Anti-fibronectin aptamers improve the colonization of chitosan films modified with D-(+) Raffinose by murine osteoblastic cells

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Abstract

The aim of the present study was to investigate how the enrichment of chitosan films with anti-fibronectin aptamers could enhance scaffold colonization by osteoblasts, by improving their adhesion and accelerating their proliferation.

Chitosan discs were enriched with excess of anti-fibronectin aptamer. Aptamer adsorption on chitosan was monitored by measuring aptamer concentration in the supernatant by spectrophotometry, as well as its release, while functionalization was confirmed by labelling aptamers with a DNA intercalating dye. Chitosan samples were then characterized morphologically with atomic force microscopy and physically with contact angle measurement. Chitosan enrichment with fibronectin was then investigated by immunofluorescence and Bradford assay. Two-% chitosan discs were then enriched with increasing doses of aptamers and used as culturing substrates for MC3T3-E1 cells. Cell growth was monitored by optical microscopy, while cell viability was assessed by chemiluminescence. Cell morphology was investigated by immunofluorescence and by scanning electron microscopy.

Chitosan films efficiently bound and retained aptamers. Aptamers did not affect the amount of adsorbed fibronectin, but affected osteoblasts behavior. Cell growth was proportional to the amount of aptamer used for the functionalization, as well as aptamers influenced cell morphology and their adhesion to the substrate.

Our results demonstrate that the enrichment of chitosan films with aptamers could selectively improve osteoblasts behavior. Moreover, our results support further investigations on this type of functionalization as a suitable modification to ameliorate the biocompatibility of biomaterials for hard tissue engineering.

1. Introduction

Tissue engineering (TE) aims to restore damaged tissues through the development of scaffolds that act as biological substitutes to be quickly colonized by autologous cells[1]. Ideal scaffolds should mimic the structure of natural extracellular matrix (ECM), both mechanically and biologically[2,3]. ECM acts as a structural support for cells and, furthermore, it is a natural reservoir of molecules and growth factors involved in tissue regeneration. As a consequence, a scaffold that successfully mimics ECM should provide appropriate biological stimuli for cellular colonization and proliferation, as well as for scaffold integration in the host tissue without risk of rejection[4]. To improve cell adhesion, small peptide fragments, containing integrin-binding domain (i.e. RGD arginine-glycine-aspartate peptide), are often immobilized on scaffold surfaces[5,6]. These small bioactive molecules promote cell attachment[7,8]. However, to date, most artificial scaffolds still fail to act as effective and specific reservoirs of ECM components. To this purpose, we propose the possibility to use aptamers as docking points, to specifically enrich biomaterials with specific circulating and autologous molecules involved in the regeneration process and naturally occurring in damaged tissues[9].

Aptamers are short oligonucleotides (<100 bp), whose three-dimensional structure is able to bind molecular targets, ranging from ions to entire proteins, with high specificity[10]. In recent years, the literature on aptamers has been growing up, but still few works are focused on their use for enriching biomaterial surfaces[11,12]. With the regard of this, our previous work was a pioneering study on the use of aptamers as innovative molecules to improve scaffold biocompatibility[9].

In the present work, we chose to immobilized aptamers on chitosan substrate, as the rationale reported in **Figure 1**.

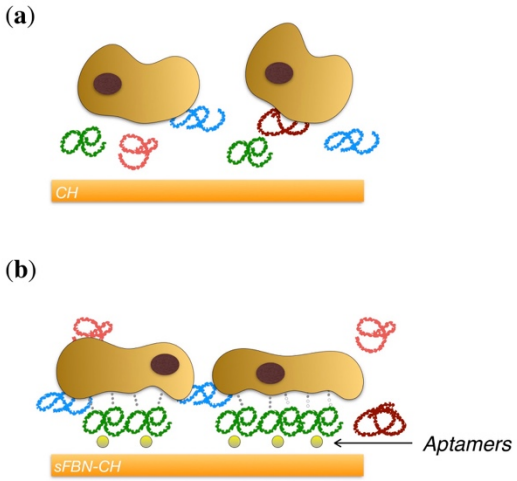

Figure 1. ***Rationale to enrich biomaterials with aptamers.*** (**a**) The non-functionalized scaffold (CH) randomly adsorbs molecules from the extracellular environment, thus impairing cell growth on the substrate. (**b**) Vice versa aptamers are used to enrich scaffold with the desired protein (sFBN-CH). This adsorption is specific and enhances the biological activity of the scaffold, which leads to cell colonization.

Chitosan is derived from the alkaline N-deacetylation of chitin, a natural polysaccharide principally obtained from several marine species, which include crabs and shrimp shells[13]. Among the natural occurring polymers, chitosan has been thoroughly investigated, as we reported in **Chapter 1** of this thesis, because it is biodegradable, biocompatible, it has low toxicity and it can be designed in a large variety of different shapes[14-16]. In particular, chitosan hydrogels present mechanical characteristics similar to that of native natural ECM, and become an optimal candidate for the development of scaffolds for TE applications[17]. Considering our final application, we chose chitosan for two main reasons. Firstly, chitosan has been demonstrated to be an optimal candidate for the fabrication of membrane for guided tissue regeneration (GBR) of periodontal tissue, and secondly chitosan has effective antibacterial activity against several oral pathogens, e.g. *Porphyronomas gingivalis, Prevotella intermedia and Actinobacillus actinomycetemcomintans*[18-22].
In the present study, we enriched chitosan discs with aptamers screened against human and bovine fibronectin (FBN). Features of anti-FBN aptamers are reported in **Figure 2**. We chose to enrich chitosan scaffolds selectively for this protein, because FBN is a natural occurring proteins in wounded tissues and it plays a pivotal role in

cell adhesion, proliferation and migration during regeneration (see **Chapter 3**)[23]. We had aptamers modified at their 3'-end with a thiol group, as recent studies have demonstrated the ability of chitosan to bind sulfur-containing compounds[24].

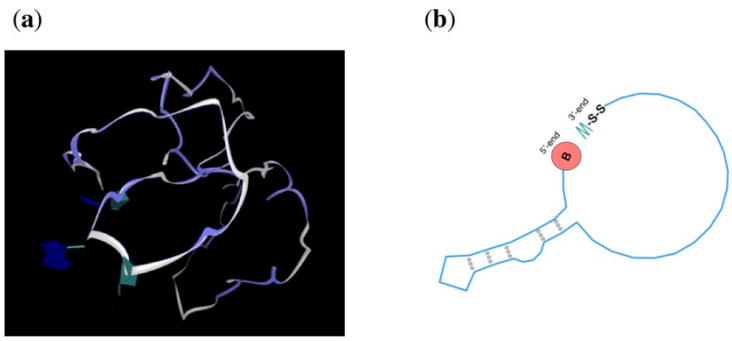

Figure 2. ***Anti-fibronectin aptamer structure.*** (**a**) 3D rendering and (**b**) 2D reconstruction of anti-FBN aptamer (Courtesy of Dr. Rafal Drabek – Base Pair Biotechnologies).

We would highlight that the use of anti-FBN aptamers to enrich chitosan and novel biomaterials, offers some advantages in comparison to their direct coating with FBN or with other ECM molecules, included the possibility of avoiding the loss of protein mobility at the material interface[25].

Considering what all above, the aim of the present study is to assess the ability of anti-FBN aptamers to ameliorate osteoblasts proliferation and colonization of chitosan films.

2. Materials and Methods

Sample preparation

Chitosan – Chitosan films were prepared according to Bettini et al. as previously described[17]. A 1 or 2% chitosan solution were prepared by dissolving purified chitosan powder 90% de-acetylated (A.C.E.F., Piacenza, Italy) in a 1% acetic acid aqueous solution. Then, D-(+) raffinose (Sigma-Aldrich, Saint Louis, MO, USA) was added at a final concentration of 290mM as viscosity modifying agent. One millimeter of each solution was thus spread out onto a microscope slide (12*25mm) to obtain a film with uniform thickness of 0.25mm and dried at 45°C in a ventilated oven for 1h. Films were then transferred in a 5% potassium hydroxide (NaOH, Sigma-Aldrich) gelation solution for 24h.
Chitosan discs were cut off from 1% and 2% films and adapted for 96-multi well plates, rinsed twice in double distilled water and cleaned under UV light for 10min.

Aptamers – ssDNA aptamers screened against human and bovine FBN (Base Pair Biotechnologies, Pearland, TX, USA) and modified with a short carbon chain containing a disulphide bond at the 3'-end and with a biotin at the 5'-end were used in this study. Anti-FBN aptamers were 40 nucleotides long and 12597.5g/mol heavy. Prior to use, the disulphide bond at the 3'-end was reduced with a 2mM, pH 7.8 solution of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Sigma-Aldrich) for 2h, to free thiol-groups able to bind chitosan as reported by Elviri et al. without the requirement of any other cross linker molecule[24]. The excess of TCEP was subsequently removed through a chromatographic column (mini Quick Spin Oligo Columns, Roche Life Science, Branford, CT, USA). Before chitosan functionalization, aptamers were quantitated by spectrophotometry (Nanophotometer, Implen GmbH, München, Germany) and thus diluted in Phosphate Buffer Saline with calcium and magnesium (PBS, Sigma-Aldrich) at the desired working concentration.

sFBN-CH and CH – Chitosan discs were incubated and functionalized with 100µl of the desired aptamer solution for 2h to obtain selective fibronectin chitosan (sFBN-CH). Bare chitosan was used as control and incubated for 2h in PBS (CH).

The process of aptamer adsorption on chitosan, as its potential release were monitored by spectrophotometry (Nanophotometer, Implen GmbH) measuring the absorbance at 260nm. To this purpose, 1% chitosan discs were incubated with 100µl of PBS in which was dissolved an excess of aptamer. Aptamer concentration in the supernatants was then measured after 30min, 1, 1.5, 2 and 2.5h. Aptamer solutions were then discarded and sFBN-CH rinsed twice in fresh PBS, thus incubated with 100µl of fresh PBS. Aptamer concentration was measured after 16, 24, 40 and 48h to evaluate any possible release of aptamer from the functionalized substrate.
Moreover, the presence of aptamers on sFBN-CH was confirmed through the use of a cyanine blue light-fluorescence DNA intercalating dye (SYBR-Safe DNA gel stain, Thermo Fisher Scientific, Waltham, MA, USA). Two-% chitosan discs were treated respectively with 5, 10 and 20µg of anti-fibronectin aptamers. After 2h aptamer solutions were removed, specimens rinsed twice in PBS and incubated with 100µl of a 1:10000 solution of the dye, that labelled the aptamer revealing its presence under UV light.

Sample characterization

Atomic force microscopy – To evidence any modification induced by aptamer grafting on chitosan discs, samples were characterized by atomic force microscopy (AFM) in tapping mode using a Vecco Dimension 3100 microscope (Parksystems, South Korea).

Contact angle measurement – To measure sample wettability, contact angle analysis was performed at room temperature (RT) with a goniometer (AB Lorentzen & Wettre, Kista, Sweden). Specifically, a 4µl drop of double distilled water was positioned on the surface of dry samples and images were recorded within 10s of deposition with a digital camera (G9, Canon, Tokyo, Japan). Digital pictures were then analyzed with the ImageJ software (ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA) for angle determination.

Protein adsorption studies

Immunofluorescence – To highlight FBN presence on chitosan films, CH or sFBN-CH were incubated with a 10% Fetal Bovine Serum solution (FBS, Thermo Fisher Scientific) for 30 min. Subsequently, FBN on specimens was labelled with a rabbit polyclonal anti-fibronectin antibody (F3648, Sigma-Aldrich) and revealed with a FITC-anti-rabbit IgG antibody (ab97048, abcam, Cambridge, UK). Samples were examined using a fluorescence microscope (Axio Imager 2, Zeiss, Jena, Germany). Areas covered with FBN were then quantitated with the ImageJ.

Bradford assay – To quantify the FBN adsorbed, a Bradford assay was developed. To this purpose, CH and sFBN-CH were incubated for 2h in 100µl of 10% FBS solution. After 2h, the amount of FBN in the supernatant was quantitated with Bradford (BIO-RAD Protein Assay, BIO-RAD, Hercules, CA, USA) following the manufacturer's recommendations and the amount of protein bound to the surface was determined by difference. Briefly, 10µl of supernatant were collected and mixed with 200µl of a Bradford Working solution. Specimens were then incubated 2min at 37°C and their absorbance finally read at 620nm with a Multiskan FC plate reader (Thermo Fisher Scientific).

Cell assays

Cell cultures – Cell assays were performed with murine osteoblastic MC3T3-E1 cells obtained from ATCC (LGC Standards srl, Milan, Italy). Cells were cultured in complete complete alpha-MEM (α-MEM, Thermo Fisher Scientific) supplemented with 10% FBS, 1% Penicillin and Streptomycin (PenStrep, Thermo Fisher Scientific) and 1% L-Glutamine (Thermo Fisher Scientific). Cells were plated at a density of 5000 cells/disc and assays were all performed in 96-well plates or in 8-well chamber slides.

Inverted microscopy and Trypan blue staining – To evaluate the influence of aptamers on cell growth, MC3T3-E1 cells were cultured up to 7 days on sFBN-CH functionalized respectively with 5, 10 or 20µg of aptamer. Cells were observed at the inverted microscope (DMIL, Leica, Wetzlar, Germany) and with a stereomicroscope (SMZ25, Nikon, Tokyo, Japan). Photographs were taken at day 3, 4 and 7. To evaluate cell viability, cells were stained with Trypan blue (Trypan blue

stain 0.4%, Thermo Fisher Scientific) at day 7. sFBN-CH and CH areas covered with cells were quantitated after the staining using the ImageJ software.

Cell proliferation assay – To observe cell viability, cells were assayed through chemiluminescence assay (CellTiterGLO, Promega, Madison, WI, USA) 1, 2 and 4 days after seeding following the manufacturer's recommendations. Briefly, medium was removed from wells, cells rinsed in PBS and incubated for 2 min on an orbital shaker with 200µl of 50:50 solution of culturing medium and Lysis buffer. Samples luminescence was then stabilized for 10min in dark conditions and measured with the luminometer (GLOMAX 20/20, Promega).

Immunofluorescence – Cytofluorescence was developed to observe cell morphology and actin fibers organization. After 2 days of culture, cells were fixed in 4% paraformaldehyde solution (PFA, Sigma-Aldrich) for 10 min at RT. After 2 rinses in PBS, cells were permeabilized with 0.1% v/v TritonX-100 (Sigma-Aldrich) for 5min at RT and washed twice in PBS. One-% Bovine Serum Albumin solution (BSA, Sigma-Aldrich) was applied for 30min at RT and subsequently substituted with a 1:200 TRITC-conjugated phalloidin (FAK100, Merck Millipore, Darmstadt, Germany) solution in PBS for 1h at RT for actin staining. After three washes with PBS, nuclear counterstaining was performed by sample incubation with DAPI (FAK100, Merck Millipore). Sample images were taken with a stereomicroscope equipped for fluorescence (SMZ25, Nikon) and analyzed through the documentation D3 software (Nikon).

Scanning electron microscopy – Scanning electron microscopy (SEM) morphological analysis was performed after 2 days of culture too. Cells were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) for 30min at RT, thus washed in Na-Cacodylate buffer for 5min at RT and dehydrated in ethanol (Sigma-Aldrich) at increasing concentrations at RT, 10min each alcohol. Specimens were then sputter-coated with a gold-palladium layer (Plano, Germany) using a SCD 040 coating device (Balzer Union, Wallruf, Germany) and studied using a dual beam Zeiss Auriga

Compact system equipped with a GEMINI Field-Effect SEM column. SEM analysis was performed at 5keV.

Statistical analysis

Data were analyzed using Prism6 (GraphPad, La Jolla, CA, USA). All the values are reported as the mean ± standard deviation of three repeated experiments. Differences among the groups were evaluated with either t-Test, one-way ANOVA or two-way ANOVA statistical tests and with the Tukey or Bonferroni post-test for multiple comparisons. Differences were considered significant when $p<0.05$.

3. Results

Chitosan binds and retains aptamers

A 1% chitosan disc was incubated with 71.95µg of aptamer. The amount of aptamer in the supernatant quickly dropped, reaching a minimum of 4.96µg after 1h (**Fig.3a**), indicating that the aptamer was promptly adsorbed by chitosan. Aptamer concentration in the supernatant was then stable. No ssDNA traces were detected in the supernatant by spectrophotometry, suggesting no release of aptamer from the disc (**Fig.3b**).

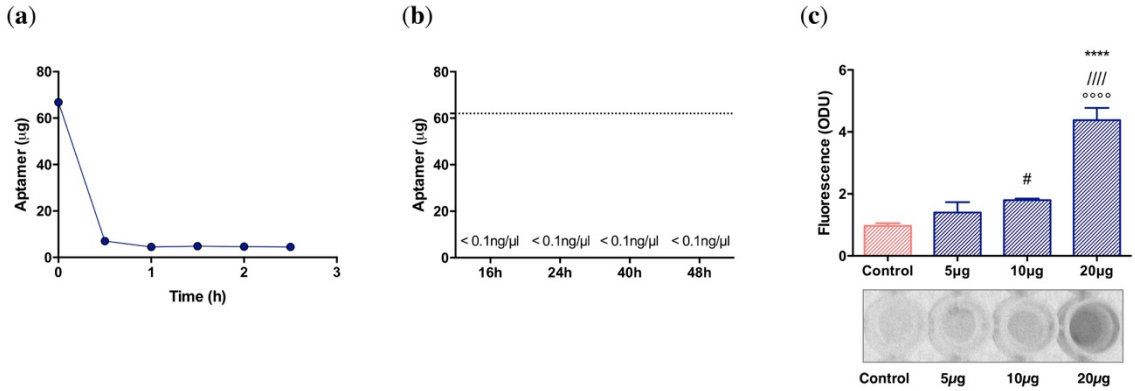

Figure 3. ***Evidence of chitosan functionalization.*** (**a**) Evolution of aptamer in the supernatant. Aptamer was quickly adsorbed by chitosan within 30 min. (**b**) Histograms representing aptamer release in the supernatant. No aptamer release was detected. (**c**) Evidence of aptamer presence on chitosan and ImageJ quantitation. Gel fluorescence depends on the amount of aptamer used for the functionalization. (sFBN-CH (10µg) vs. CH #=p<0.05; sFBN-CH (20µg) vs. CH °=p<0.05; sFBN-CH (20µg) vs. sFBN-CH (5µg) /=p<0.05; sFBN-CH (20µg) vs. sFBN-CH (10µg) *=p<0.05).

Two-% chitosan discs were then incubated with increasing doses of aptamers and the presence of aptamer was detected through an UV-fluorescence DNA intercalating dye. Fluorescence increased with increasing aptamer concentration, suggesting that chitosan bound aptamers in a dose-dependent manner (**Fig.3c**).

Aptamers moderately modify chitosan surface properties

AFM microscopy revealed that the presence of anti-FBN aptamers moderately influenced chitosan surface morphology, with sFBN-CH that showed a moderately

enhanced roughness (**Fig.4a**). Moreover, aptamer grafting had effects on samples wettability: 2% sFBN-CH was lightly more hydrophilic than bare CH (p=0.0272; **Fig.4b**).

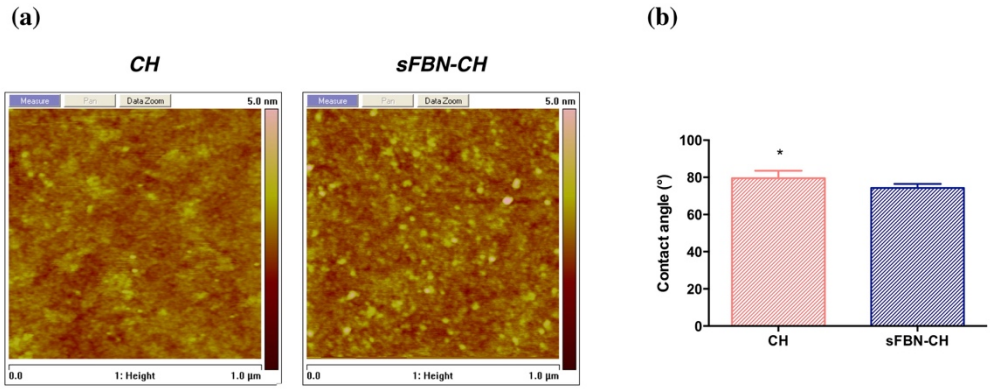

Figure 4. ***Atomic force microscopy and contact angle measurement.*** (**a**) Typical AFM images showing CH and sFBN-CH. (**b**) Histograms representing the contact angle on CH and sFBN-CH. *=p<0.05.

Aptamers did not increase the amount of adsorbed proteins and of FBN

sFBN-CH allowed protein adsorption, even though the amount of adsorbed serum proteins was not significantly different from CH specimens (p=0.0869; **Fig.5c**). Furthermore, results reported in **Figure 5a** show a comparable adsorption of FBN in the presence or in the absence of anti-FBN aptamers. Fluorescence was quantitated through the ImageJ and results are reported in **Figure 5b**.

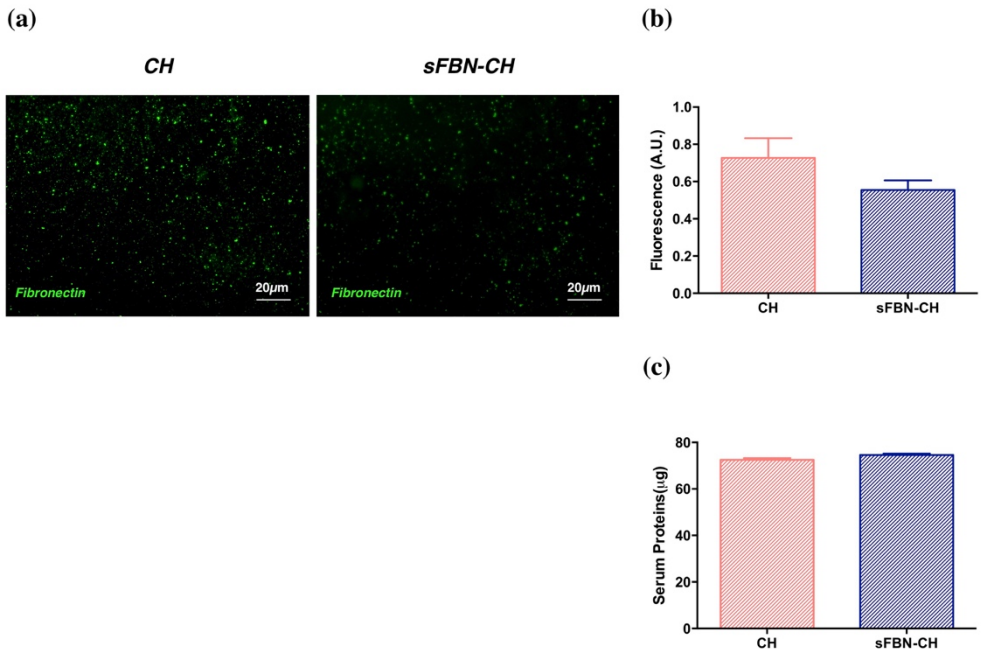

Figure 5. *Protein adsorption to CH and sFBN-CH.* (**a**) Images reporting FBN immune-labeling on CH and on sFBN-CH. (**b**) ImageJ fluorescence quantitation. (**c**) Histograms reporting the amount of serum proteins deposited on CH and on sFBN-CH. *=p<0.05.

Aptamers enhanced cell proliferation

Osteoblast proliferation on sFBN-CH depended on the amount of immobilized aptamers. **Figure 6** show that osteoblasts rapidly proliferated on sFBN-CH functionalized with increasing doses of aptamers in a dose-dependent manner, as reported by the quantitation of chitosan covered area measured with the ImageJ after Trypan blue staining at day 7 (**Fig.7**). After 7 days of culture, osteoblasts on sFBN-CH functionalized with the highest amount of aptamer reached confluence in several areas of the sample. Furthermore, Trypan blue staining showed that cells were viable in all conditions, and highlighted the differences in proliferation rate on the different samples.

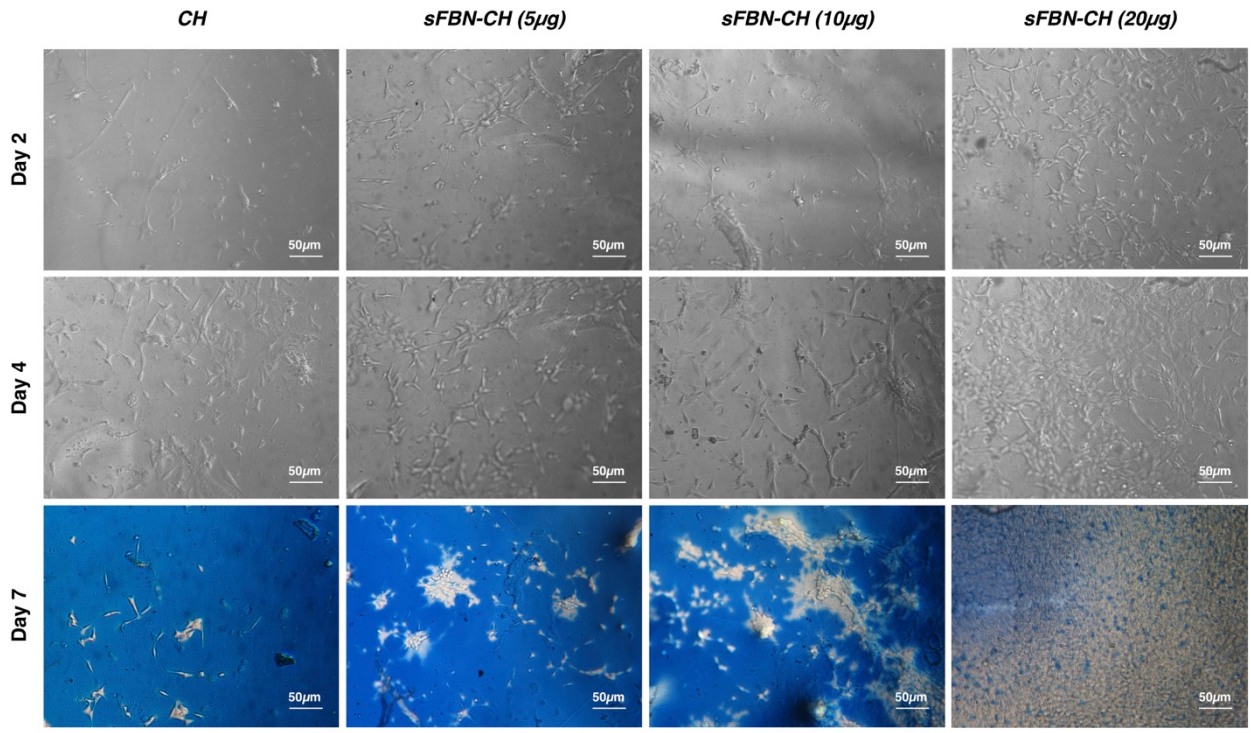

Figure 6. *Cell proliferation on sFBN-CH enriched with increasing aptamer doses.* The rate of cell growth was apparently proportional to the amount of anti-FBN aptamer used to functionalize chitosan scaffolds.

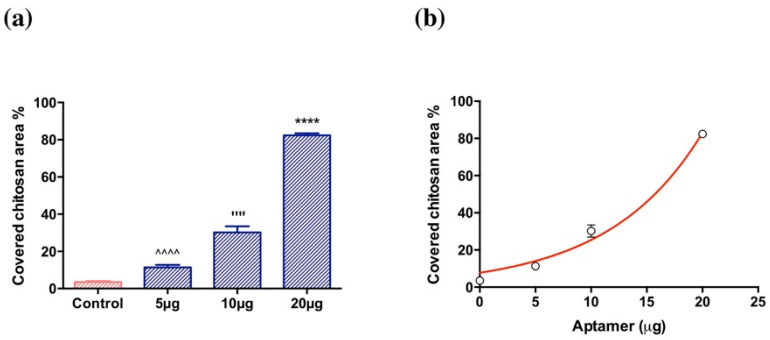

Figure 7. ***Cell proliferation on sFBN-CH enriched with increasing aptamer doses.*** (**a**) Histograms reporting the percent amount of chitosan area covered with cells on chitosan enriched with different amount of aptamers. (**b**) Exponential regression trend of the percent amount of chitosan area covered with cells as a function of aptamer dose used for chitosan functionalization. The rate of cell growth was exponentially proportional to the amount of aptamer used to functionalize scaffolds (R^2=0.9846). (sFBN-CH (5µg) vs. CH ^=p<0.05; sFBN-CH (10µg) vs. CH '=p<0.05; sFBN-CH (20µg) vs. sFBN-CH (10µg) *=p<0.05).

At day 7, the amount of cell-covered areas on sFBN-CH enriched with the highest amount of aptamer and on CH were evaluated through the ImageJ. Strikingly, the 66.90% of sFBN-CH was covered by cells, if compared to only the 5.81% on CH (p<0.0001; **Fig.8**).

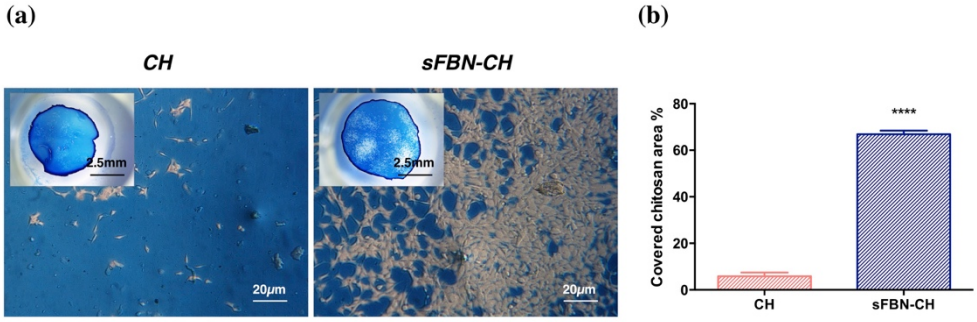

Figure 8. ***Anti-fibronectin aptamers support cell growth.*** (**a**) Images, taken with an inverted microscope and with a stereomicroscope (low magnification), showing murine osteoblastic MC3T3-E1 cells on CH and sFBN-CH. (**b**) Histograms reporting the percent amount of chitosan area covered with cells on CH and sFBN-CH. *=p<0.05.

Chemiluminescence assay (**Fig.9**) confirmed that on sFBN-CH cell viability and proliferation were enhanced, showing significant differences after just 1 day of

culture (p=0.0121) and with remarkably differences after 2 and 4 days of culture (day 2 p<0.0001; day 4 p<0.0001).

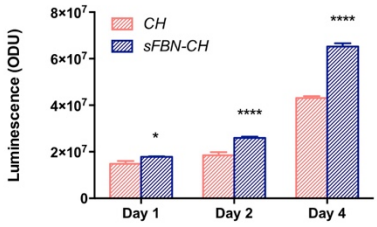

Figure 9. ***Anti-fibronectin aptamers enhance cell proliferation.*** Histograms reporting MC3T3-E1 cells viability on CH and on sFBN-CH. Cells on CH proliferated slower than cells on sFBN-CH. *=p<0.05.

Aptamers affect cell adhesion and morphology

Fluorescence and SEM images are reported in **Figure 10a**.

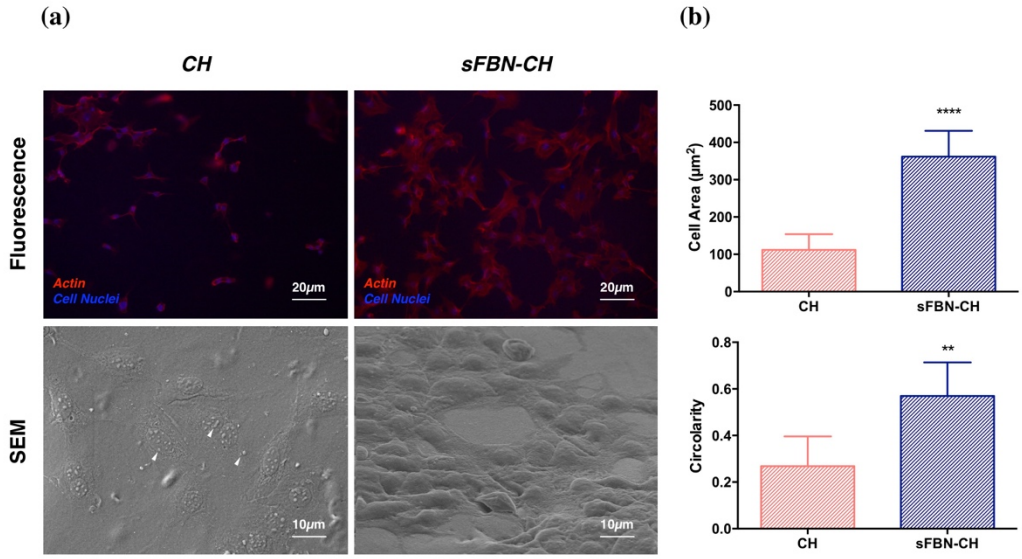

Figure 10. ***Anti-fibronectin aptamers influence cell morphology.*** (**a**) Typical fluorescence and SEM images showing murine osteoblastic MC3T3-E1 cells on CH and on sFBN-CH after 2 days of culture. (**b**) Histograms representing the mean of cell area and of cell circularity measured with the Documentation D3 software. Cells appeared to adhered better on sFBN-CH, showing more flattened shape and less elongated filopodia. *=p<0.05.

For fluorescence cells were stained for cytoskeleton, by labelling actin and for cell nuclei. On sFBN-CH osteoblasts appeared quadrangular and with a spread, but contained shape, typical of osteoblasts. On the other hand, on CH sample they

appeared less spread and thus less attached to the substrate, showing longer filopodia typically used by cells to find more docking points to the surface. Moreover, cells on CH appeared small and with the cytoplasm tense around cell nuclei.

SEM analysis confirmed this consideration. Cells seeded on CH were adhered, but with longer filopodia and thin filaments, and, consistently with this, they appeared less attached to the substrate. On the contrary, on sFBN-CH, cells appeared healthy, well spread and with longer filopodia which allowed their attachment to the substrate. SEM analysis revealed also that cell on CH presents numerous apoptotic vesicles on their surface (white arrows) confirming that these cells were less healthy that on sFBN-CH.

To assess the differences in cell shape, an image analysis was performed and cell area and circularity were measured on both the samples. Both these parameters were significantly different between the specimens (**Fig.10b**). In particular, cell area was confirmed more spread on sFBN-CH ($p<0.0001$), while cell circularity, which biologically is inversely correlated to cell elongation was more evident for sFBN-CH too ($p=0.0050$).

4. Discussion

Current regenerative approaches aim to develop scaffolds that faithfully mimic the ECM both biologically and mechanically[2,3]. Biologically, replicating the extracellular environment requires the enrichment of the scaffold with signals and stimuli that support and accelerate cell colonization and proliferation. For this reason, in our study we propose to immobilize aptamer against FBN, a protein that contains several cell binding domains on chitosan substrate[23].

Chitosan is a naturally occurring polysaccharide widely studied for TE applications, because it is highly biocompatible both *in vitro* and *in vivo*[26-28]. Moreover, in recent years, the interest in chitosan for bone and cartilage TE has rapidly grown and several studies have been published on the use of chitosan films and sponges as substrates for bone cells, including the MC3T3-E1 cell line that we used in the present study.

Bettini et al. proposed a modified protocol for chitosan production through the use of D-(+) raffinose, which, acting as a spacer, helps to increase the water content of chitosan, thus improving its hydrophilicity and biocompatibility[17,23,26-28]. However, in spite of this enhanced compatibility, some cell models such as MC3T3-E1 do not proliferate well on these modified films, as our present and previous data show (see **Chapter 2**)[29]. This can hardly be attributed to defective protein adsorption on chitosan discs, as chitosan can actually adsorb a great amount of proteins from the supernatant. To this purpose, in this study, we proposed to modify FBN selective adsorption by adding aptamers with a thiol group on their 3'-end (**Fig.1**). The rationale for this was to exploit the ability of chitosan to bind sulfur-containing compounds as we previously described[24]. Furthermore, chitosan could be thus seen as an interesting and versatile platform for rapid modification with aptamers, as well as being a useful tool for customized scaffolds to meet individual clinical needs. Indeed, rapidly and stably enriched chitosan could enable the clinician to use a single, universal implantable matrix and decide to functionalize it with different aptamers against different targets according to the specific needs of the surgery to perform.

Consistently with this, we first demonstrated the ability of chitosan films prepared in the presence of D-(+) raffinose to firmly and rapidly bind aptamers. Aptamers were

adsorbed on the surface within 30 min (**Fig.3a**) and no release was detectable with the spectrophotometer after the functionalization (**Fig.3b**), which is suggestive of little dispersion of aptamers from the scaffold, a positive feature for possible *in vivo* implantation. Noteworthy, aptamer-grafting slightly modified chitosan surface properties (**Fig.4**). AFM revealed a little enhance roughness of samples due to aptamer presence, which can be negligible because nanometric and uninfluenced for cell response. Moreover, aptamer grafting enhanced surface wettability in statistically significant way, but which can be eluded, since the presence of a scramble aptamer did not influence subsequent osteoblasts behavior (data not shown). At immunofluorescence, anti-FBN aptamers did not appear to modify the amount of proteins adsorbed from serum by the scaffold (**Fig.5**). We thus proceeded to culture MC3T3-E1 cells on sFBN-CH enriched with different doses of aptamers. This cell line was chosen because it presents with normal features of normal osteoblasts, including contact inhibition and was considered a representative model of osteoblastic behavior. Somewhat strikingly, osteoblastic proliferation was significantly increased when scaffolds were enriched with anti-FBN aptamers (**Fig.6** and **Fig.7**) in comparison to CH, and a substantial difference in the amount of chitosan surface covered by cells was observed in the presence or in the absence of protein-specific aptamers by day 7 (**Fig.8**). Our data were further confirmed by the chemiluminescence viability assay (**Fig.9**), which showed an increasing in on sFBN-CH already at early time points. A possible plausible explanation to our results is that aptamers may provide a pool of ready-to-bind proteins that cells can use to adhere on the substrate and elicit a pro-proliferative effect. Lack of difference in cellular effects between CH and scrambled aptamer coated chitosan (data not shown) is indicative that the effects are not due to the presence of aptamers but rather to bound proteins. This is supported by immunofluorescence and SEM observations. Images (**Fig.10**) showed that osteoblasts on sFBN-CH displayed a morphology highly indicative of a better adhesion. Vice versa, cells on CH samples appeared contracted and rich in apoptotic bodies, which are indicative of impaired viability, confirming chemiluminescence assay results (**Fig.9**).

5. Conclusions

Taken together, our results highlight the ability of aptamers to affect and improve osteoblast behavior on chitosan substrate, even though sFBN-CH did not appear to bind more FBN than CH. As such, other mechanisms involved are probably underlying cell response we observed in this study. A viable hypothesis may be that aptamers promote a suitable conformational adsorption of FBN, thus allowing cells to recognize integrin binding domains (i.e. PHSRN, IKVAV and RGD) required for their ability to adhere to biomaterials. Future studies will now necessary to investigate the mechanism of FBN adsorption on chitosan, in order to well define the role of aptamers.

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Chapter 6

Surface modification of chitosan with anti-fibronectin aptamers: a mass spectrometry probing evidence on fibronectin behavior

In press in Rapid Communications in Mass Spectrometry

Abstract

Protein dynamics are fundamental for scaffold surface bioactivity, even though their direct adsorption on biomaterials often induces their altered functionality. Here, mass spectrometry was successfully applied as current analytical method to study the interactions between a fibronectin fragment and a chitosan substrate enriched or no with aptamers selected against fibronectin. Aptamers have been shown to be an effective method to enhance the bioactivity of biomaterials by acting as receptors for biological stimuli, thus conferring docking points suitable for cell adhesion and scaffold colonization.

First, the dynamics of fibronectin in aqueous solution with or without anti-fibronectin specific DNA aptamer, were described. Thus, the same study was carried out on chitosan-based films, pre-adsorbed with fibronectin in the presence or in the absence of aptamer.

Hydrogen/deuterium exchange mass spectrometry approach allowed both to identify the fibronectin/aptamer interaction regions and to establish any differences in fibronectin flexibility and thus on its biological functionality.

1. Introduction

Biomaterials are widely used to realize scaffolding devices capable of triggering tissue regeneration, including functional restoring[1]. During regeneration, the extracellular matrix (ECM), with its complex and dynamic microenvironment, plays a pivotal role in regulating the mechanism of cell adhesion, growth and proliferation[2-4]. Thus, the direct coating of scaffolds with entire ECM protein or with cell-binding domains contained in ECM molecules (i.e. Arginine-Glycine-Aspartic Acid, RGD), are widely studied options as promising support for a broad range of biomedical applications. Furthermore, it has been demonstrated their effectiveness in significantly affecting cell behavior[5,6].

However, it has been reported that direct functionalization of a surface with entire proteins could results in random non-specific adsorption, with consequent loss of protein mobility and functionality, which in turn led to the activation of undesired reactions[5,6]. Therefore, the biocompatibility of biomaterials is strictly related to the control of proteins adsorption on surfaces and understanding dynamic interaction mechanism may represent an important step for the design of supports with improved efficacy in tissue regeneration.

Among biomaterials of common use, chitosan is a natural polymer derived from the alkaline N-deacetylation of chitin, which is widely used in pharmaceutical and biomedical applications thanks to its useful characteristics of biodegradability, biocompatibility, low toxicity and malleability[7]. Since chitosan molecular structure is very close to that of glycosaminoglycan that compose the ECM, it is an ideal candidate as supportive material for the realization of scaffolds intended for tissue engineering (TE). Additionally, chitosan capacity of enhancing wound healing, together with its intrinsic antibacterial capacity against some common oral pathogens, make of chitosan an optimal candidate for oral tissues regeneration[8,9].

In order to improve the scarce ability of chitosan to act as a valid substrate for cell growth, Bettini et al. described the possibility to prepare chitosan films in the presence of D-(+) raffinose, thus positively contributing to the growth of human lung-derived WI-38 and of human umbilical vein endothelial cells (HUVEC) on this biomaterial[10]. However, the same model was not efficient in supporting the growth of murine MC3T3-E1 osteoblastic cells (**Chapter 2** and **5**)[11,12]. Consequently, we

developed further modifications of this material by exploiting the affinity of chitosan for the binding of sulfur containing components[13]. At the beginning, we positively modulated the adhesion of MC3TE-E1 cells by enriching with thiol-modified gelatin (**Chapter 2**)[11]. Then, we stylishly improved the bioactivity of chitosan by mean of 3'-end thiol modified aptamers selected against fibronectin (FBN) (**Chapter 5**)[12].

In our previous work, we observed that even though aptamers did not determined a significant quantitative increase in the selective adsorption of FBN, their presence evoked an improved adhesion and growth of MC3T3-E1 cells. Thus, we hypothesized that this effect was probably due to the contribution of aptamer in preserving the natural conformation of FBN, without modifying its conformation and maintaining a favorable exposure of the amino acidic adhesion sequences, i.e. RGD motif, for cells.

The aim of this work is to assess the type of interactions established between FBN and chitosan (CH) or between FBN and aptamer-doped chitosan (selective fibronectin chitosan – sFBN-CH) though the use of mass spectrometry (MS). In particular, this work is focused on the application of the hydrogen deuterium exchange mass spectrometry (HDX-MS), which being a useful approach to study protein-ligand interactions and protein dynamics, allows to study FBN-aptamer interaction and the dynamics of its adsorption on CH and on sFBN-CH.

2. Materials and Methods

Anti-FBN aptamer/FBN interaction

Mass spectrometry – To evaluate the molecular weight and the charge distribution spectrum of the anti-FBN aptamer, of the FBN fragment (fFBN) and of the complex anti-FBN aptamer/fFBN, electron spray ionization mass spectrometry (ESI-MS) was used.

To this purpose, aliquots of an anti-FBN aptamer (apt, Base Pair Biotechnologies, Pearland, TX, USA) 83.5µM, of a human plasma 30kDa N-terminal fFBN 16.7µM (Sigma-Aldrich, Saint Louis, MO, USA) and of an anti-FBN aptamer and fFBN (apt/fFBN 83.5µM/1.67µM) aqueous solution were diluted ten times with ultrapure water and analyzed with an Orbitrap instrument equipped with a pneumatically assisted ESI interface (Thermo Scientific, San Jose, CA, USA). The desolvation gas was nitrogen 99.99% pure, that was delivered at 820kPa, while the interface MS parameters were the follows: capillary voltage (40V), electrospray voltage (3.5kV) and capillary temperature (150°C). Direct infusion mode, operating with a syringe pump at a constant flow rate of 1µl/min was applied. MS data were acquired in positive (fFBN and apt/fFBN) or negative (apt) ion mode over the m/z 500-2000 range.

Hydrogen deuterium exchange mass spectrometry – To identify the stoichiometry of the interaction between the anti-FBN aptamer and the fFBN a hydrogen deuterium exchange mass spectrometry (HDX-MS) approach was used.

To this purpose, aliquots of fFBN 16.7µM and of apt/fFBN 83.5µM/16.7µM were diluted ten times in ultrapure water or in deuterated water (Sigma-Aldrich) and analyzed with HDX-MS after 2, 10, 30, 60 and 120 min.

High performance liquid chromatography mass spectrometry – To assess the amino acid sequences of fFBN involved in the bond with the anti-FBN aptamer, high performance liquid chromatography mass spectrometry (HPLC-MS) was used.

To this purpose, aliquots of pure fFBN 16.7µM alone or in the presence of anti-FBN aptamer (83.5µM) were diluted ten times in ultrapure or deuterated water and

digested with a 5µM pepsin (Sigma-Aldrich) solution for 4h at 37°C. For specimens in deuterated water, pepsin digestion was performed 1h after the dilution to allow hydrogen/deuterium (H/D) exchanges.
Once peptides were obtained, their separation was carried out on a Kinetex C18 column (75*2.1mm, 2.6µm – Phenomenex, Torrance, CA, USA) under gradient conditions of aqueous formic acid 0.1% v/v (Sigma-Aldrich) and acetonitrile at a flow rate of 2ml/min.
After the analysis, obtained data were processed by the Proteome Discover™ software (Thermo Scientific).

sFBN-CH/FBN interaction

sFBN-CH and CH – Chitosan specimens were prepared as previously described (**Chapter 2** and **5**)[11,12]. A 2% chitosan solution was prepared by dissolving purified chitosan powder 90% de-acetylated (A.C.E.F., Piacenza, Italy) in a 1% acetic acid solution. D-(+) raffinose (Sigma-Aldrich) was then added at the final concentration of 290mM as viscosity modifying agent. Thus, one milliliter solution was spread out onto a microscope slide (12*25mm) to obtain a uniform film 0.25mm thick and dried at 45°C in a ventilated oven for 1h. Films were then transferred in a 5% potassium hydroxide (NaOH, Sigma-Aldrich) gelation solution for 24h.
Anti-FBN aptamers were modified with a short carbon chain containing a disulphide bond at their 3'-end and with a biotin at the 5'-end. Prior to use, the disulphide bond at the 3'-end was reduced with a 2mM, pH 7.8 solution of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Sigma-Aldrich) for 2h, to free thiol-groups able to bind chitosan as reported by Elviri et al[13]. The excess of TCEP was removed with a chromatographic column (mini Quick Spin Oligo Columns, Roche Life Science, Branford, CT, USA).
Ninety-six well plate fitting chitosan discs were thus incubated with 50µl of aptamer (0.4µg/µl) for 2h to obtain selective binding chitosan (sFBN-CH). Bare chitosan was used as control (CH).

sFBN-CH and CH enrichment with FBN – To understand the role aptamer in the amelioration of FBN adsorption on chitosan, sFBN-CH and CH specimens were

further enriched with fFBN. Briefly, samples were incubated with 50µl of fFBN solution for 1h, thus washed three times with 100µl of Phosphate Buffer Saline (PBS, Sigma-Aldrich) to remove softly bound fFBN.

High performance liquid chromatography mass spectrometry – To assess if chitosan influenced the biological activity of FBN by masking its bioactive peptide when directly adsorbed, HPLC-MS was used.
To this purpose, pure fFBN either adsorbed on CH or on sFBN-CH was digested with a 2.5µg/µl solution of trypsin for 4h at 37°C and pH 7.4. Digestion was then stopped with 1µl of formic acid and obtained peptides were analyzed with the HPLC-MS as described above.

Statistical analysis

Data were analyzed using Prism6 (GraphPad, La Jolla, CA, USA) and reported as the mean ± SD of three repeated experiments.

3. Results and Discussion

Anti-fibronectin aptamer bind the fibronectin fragment with a 1:1 stoichiometry

MS is a leading analytical technique to collect information on biomolecules. With the regard of proteins, they can be accurately identified and characterized for covalent structures, dynamic and non-covalent interactions, using low amounts.

In this work, an aqueous solution of a fFBN (1.67µM) was initially analyzed by direct infusion in ESI-MS system to accurately establish its molecular weight (MW). The positive ion full-scan spectrum of fFBN presented a charge state distribution centered on +21 at m/z 1376.39 (**Fig.1a**), with a calculated average MW of 28884±0.1 Da. Similarly, an aqueous solution of the anti-FBN aptamer was analyzed by acquiring negative ions at low resolution. The charge state distribution was centered at the following values: 901.57 m/z, 972.15 m/z and 1048.84 m/z, which correspond to -14, -13 and -12 charge states, respectively (**Fig.1b**). The calculated average MW of the anti-FBN aptamer was 12631±0.1 Da.

As a further step, the binary solution of fFBN (1.67µM) and anti-FBN aptamer (8.35µM) was analyze by positive ion mode. In this case, the charge state distribution was centered on 1321.98 m/z, and the average MW was assessed at 41478±0.1 Da (**Fig.1c**). This value corresponds to the sum of the anti-FBN aptamer and of the fFBN MWs, indicating that one anti-FBN aptamer interact exactly with one fFBN.

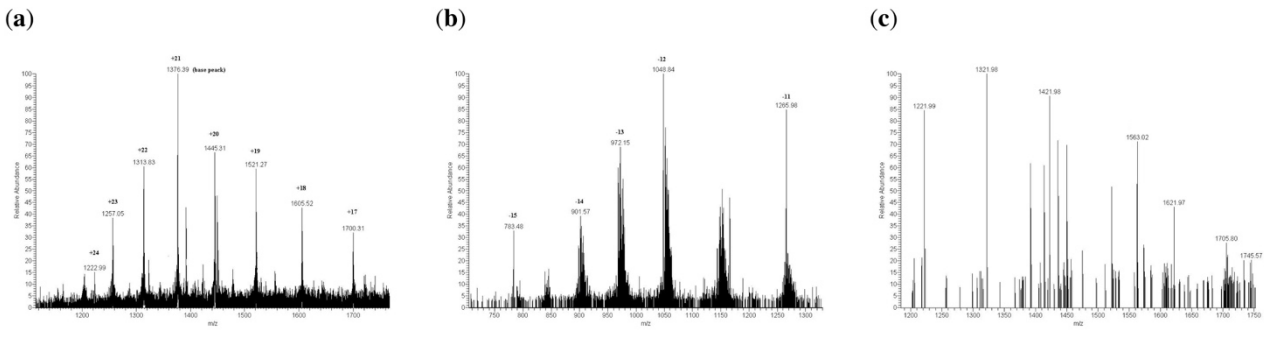

Figure 1. ***Full-scan ESI-MS spectra.*** (**a**) Fibronectin fragment (fFBN). (**b**) Anti-fibronectin aptamer (apt). (**c**) Fibronectin fragment and anti-fibronectin aptamer (fFBN/apt).

Additionally, the HDX-MS approach was used to prove the interaction between fFBN and anti-FBN aptamer. In the presence of anti-FBN aptamers, HDX-MS analysis showed that fFBN exchanged fewer hydrogen atoms, indicating that some of the

hydrogens initially available for the exchange were engaged in apt/fFBN interaction. Kinetic studies demonstrated that pure fFBN or fFBN in the presence of anti-FBN aptamer exhibited two different trend both in terms of exchanged hydrogens and relative rate constants (**Fig.2**). In the case of pure fFBN, after an initial very fast exchange involving approximately 48 hydrogen atoms in 0.5 min (k~4.1±0.8 min^{-1}), a second group of hydrogens exchanged with a reduced rate (k~0.3±0.1 min^{-1}) up to ~71 hydrogens in 60 min. In the presence of the anti-FBN aptamer, an initial fast exchange involved approximately 27 hydrogen atoms in 0.5 min (k~3.3±0.8 min^{-1}) and a second group of hydrogens exchanged with a reduced rate (k~0.4±0.1 min^{-1}) up to ~49 hydrogen atoms in 60 min.

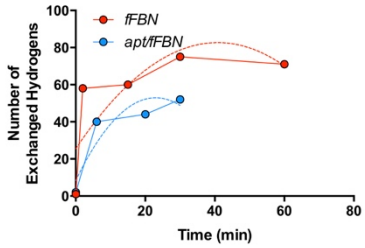

Figure 2. ***Hydrogen/deuterium exchange.*** Kinetics of the H/D exchange of fFBN in the presence or in the absence of anti-FBN aptamer.

Four residues generated by the digestion of the fibronectin fragment are supposed to be involved in the interaction with the anti-fibronectin aptamer

Peptides obtained through the digestion with pepsin of the fFBN in the presence or in the absence of the anti-FBN aptamer were analyzed by HDX-MS. Thus, by comparing the number of exchanged hydrogens (**Tab.1**), possible interaction regions involved in the fFBN/apt bond were identified at the level of the following 4 residues: YRVGDTYERPKDMSI, YVVGETWEKPYQGWMM, SYRIGDTWSKKDNRGNL and YKIGDTWRRPHETGGYM. The QAQQMVQPQSPVAVSQ and the QTTSSGSGSPFTDVR residues were not considered as involved in the interaction with aptamer, since both in the presence or in the absence of anti-FBN aptamer they exchanged with deuterated water the same number of hydrogens (**Fig.3**).

Table 1. ***Table reporting the characteristics of pepsin-generated fibronectin fragment residues in the presence or in the absence of the anti-fibronectin aptamer.***

Peptide	Molecular Weight H_2O [Da]	Molecular Weight D_2O [Da]	Disposable hydrogens for H/D Exchange	Molecular Weight after the interaction with aptamer D_2O [Da]	Exchanged Hydrogens
QAQQMVQPQSPVAVSQ	1726.9	1745.0	18	1745.0	18
YRVGDTYERPKDMSI	1830.0	1851.1	21	1843.1	13
YVVGETWEKPYQWMM	2004.2	2019.3	15	2018.4	14
SYRIGDTWSKKDNRGNL	2010.2	2037.3	27	2026.3	16
YKIGDTWRRPHETGGYM	2067.3	2089.4	22	2082.4	15
QTTSSGSGSPFTDVR	1526.6	1544.7	18	1544.7	0

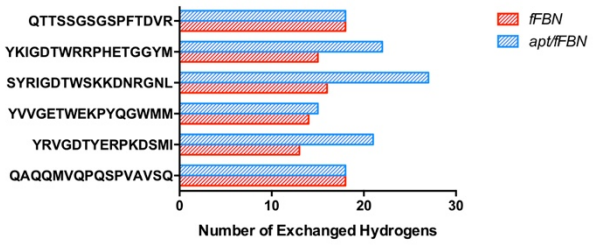

Figure 3. ***Hydrogen/deuterium exchange.*** Histograms reporting the number of hydrogens exchange by pepsin-generated fibronectin fragment residues in the presence or in the absence of the anti-fibronectin aptamer.

Anti-fibronectin aptamer allow higher conformational mobility of fibronectin adsorbed on chitosan thus enhancing its biological activity

fFBN aqueous solution and fFBN adsorbed on CH or on sFBN-CH were digested by trypsin and the obtained residues were analyzed with the HPLC-MS.

Table 2. ***Table reporting trypsin-generated fragment of fFBN adsorbed on CH or on sFBN-CH.***

fFBN peptides from pure fFBN	fFBN peptides from fFBN adsorbed on CH	fFBN peptides from fFBN adsorbed on sFBN-CH
HYQINQQWER	HYQINQQWER	HYQINQQWER
QINQQWER	QINQQWER	QINQQWER
QQWER		
YTGHTYR		
VGDTYERPK	VGDTYERPK	VGDTYERPK
IGDTWR	IGDTWR	IGDTWR
VVGETWEKPYOGW		
IGDTWSKK		
IGDTWSK	IGDTWSK	IGDTWSK
HTSVQTTSSGSGPFTDVR		
HTSVQTTSSGSGPFTDVR	HTSVQTTSSGSGPFTDVR	HTSVQTTSSGSGPFTDVR
HTSVQTTSSGSGPFTDV		
HTSVQTTSSGSGPFT		HTSVQTTSSGSGPFT
HTSVQTTSSGSGPF		
SSGSGPFTDVR		SSGSGPFTDVR
TTSSGSGPFTDVR		

In **Table 2**, the residues obtained from the three specimens are reported. The conformational mobility presented by pure fFBN allowed to obtain a higher number of residues than when it was adsorbed on chitosan both in the presence or in the absence of aptamers (**Fig.4**). As such, it has been supposed that more digestion sites were accessible by trypsin. Consistently with this, it is interesting to note that the number of peptides generated from fFBN adsorbed on CH or on sFBN-CH were comparable, with little more residues generated in the presence of aptamers. Noteworthy, when fFBN is adsorbed on sFBN-CH it loses part of its conformational mobility, but it maintains greater exposure of specific sites to the solvent and to the enzymatic activity of trypsin, than when directly adsorbed on CH. Taken together, these data confirm that aptamer act as a spacer and allows to preserve the pristine conformation of FBN, once adsorbed on chitosan.

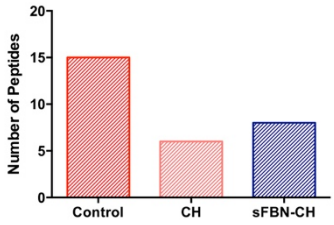

Figure 4. ***Fibronectin fragment trypsin-generated peptides.*** Histograms reporting the number of fFBN residues obtained by trypsin digestion of pure fFBN and of fFBN adsorbed on CH or on sFBN-CH.

4. Conclusions

We previously reported that aptamers did not affect the amount of adsorbed FBN on chitosan (**Chapter 5**)[12]. Consequently, we supposed that aptamers rather influenced the conformation of adsorbed FBN, thus enhancing its biological activity. This hypothesis reflected an enhanced osteoblast adhesion and growth on chitosan.

In this work, we demonstrated through the use of the different MS approaches that YRVGDTYERPKDMSI, YVVGETWEKPYQGWMM, SYRIGDTWSKKDNRGNL and YKIGDTWRRPHETGGYM residues are presumably involved in the interaction with aptamer. Furthermore, MS findings indicated an improved functionality of fFBN when adsorbed on sFBN-CH, supporting our hypothesis of an enhanced conformational adsorption of FBN and possibly explaining why cells preferentially adhered to sFBN-CH than on CH.

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Chapter 7

Functional fibronectin adsorption on aptamer-doped chitosan modulates cell morphology by integrin-mediated pathway

Submitted to Biomedical Materials

Abstract

A decisive step in cell-biomaterial interaction is represented by the adsorption of proteins at the interface and the fine control of this phenomenon may be useful to trigger proper cell response. To this purpose, we can selectively control protein adsorption on biomaterials by means of aptamers. Aptamers selected against fibronectin dramatically enhanced chitosan ability to promote cell proliferation and adhesion, even though the underlying biological mechanism remained unknown. We supposed that aptamers contributed to ameliorate the adsorption of fibronectin in an advantageous geometrical conformation for cells, thus regulating their morphology by the activation of the integrin-mediated pathway. We investigated this possibility by culturing epithelial cells on chitosan enriched with increasing doses of aptamers in the presence or absence of cytoskeleton pharmacological inhibitors. Our results showed that aptamers controlled cell morphology in a dose dependent manner. Simultaneously, when the inhibition of actin polymerization was induced, the control of cell morphology was attenuated, while no differences were detected when cells contractility was challenged. Altogether, our data provide evidence that aptamers contribute to control fibronectin adsorption on biomaterials by preserving its conformation and thus function. Furthermore, our work provides a new insight into a new way to accurately tailor material surface bioactivity.

1. Introduction

Biological tissues are complex systems and their structural and molecular organizations rely on their functions. As such, scaffold design in tissue engineering (TE) to restore lost tissues should be accurately tuned to trigger a dialogue with the surrounding biological milieu, most importantly cells, thus promoting regeneration[1]. A scaffold biomaterial, therefore, should properly support cell growth, proliferation, adhesion, differentiation and new extracellular matrix (ECM) deposition typical of the lost tissue[2].

Chitosan is a polysaccharide derived from the partial de-acetylation of chitin, the main component of crustacean and arthropods exoskeleton. Chitin de-acetylation, which occurs through enzymatic or chemical hydrolysis under severe alkaline conditions, confers to chitosan the capacity to be easily protonated and a unique structural versatility, thus making it an optimal candidate material for TE scaffold fabrication[3,4]. Furthermore, it has been widely described that chitosan possesses high affinity for proteins, which are of the utmost importance in addressing cells responses at the interface[4]. However, our previous efforts highlighted low cell adhesion and proliferation capacity on chitosan (**Chapter 2** and **5**)[5,6]. To justify the mismatch between high protein adsorption and poor cell response, we previously hypothesized (**Chapter 5**) that the cationic properties of chitosan induced a non-specific serum protein adsorption, characterized by a loss function. A shift to retained protein functionality at the interface of chitosan is therefore desirable, and we designed a chitosan modification capable of selectively binding fibronectin (FBN) – sFBN-CH (selective fibronectin chitosan)[6].

FBN was chosen as a target, because abundant in wounded tissues and for its pivotal role in cell adhesion thank to the presence within its structure of cell binding domains, including the minimal recognition sequence for cell integrins (i.e. Arginine-Glycine-Aspartic Acid, RGD)[7]. Integrins are a large family of homologous transmembrane receptors, constituted of two non covalently associated glycoprotein subunits, alpha (α) and beta (ß). After ECM molecules recognition (e.g. FBN), integrins are activated, α and ß parts cluster and the cytoplasmic tail of the ß subunit binds intracellular proteins that form mature focal adhesions, which interact with actin bundles to control cytoskeleton organization and thus cell shape[8].

sFBN-CH was obtained dressing chitosan by means of anti-FBN aptamers, which are oligonucleotides with binding capacity, able to recognize, bind and retain target molecules, including proteins, with high specificity[9]. We showed for the first time the capacity of anti-FBN aptamers biomaterial modification to dramatically improve murine osteoblasts (MC3T3-E1) colonization (**Chapter 5**)[6].

After having demonstrated an amelioration of FBN biological activity once adsorbed on sFBN-CH (**Chapter 6**), the aim of the present work is to assess the molecular mechanisms behind the amelioration of cell behavior at the interface of sFBN-CH. The functional FBN adsorption, should allow cell shape and spreading by actin modulation. As such, we studied the shape of cells on chitosan modified with increasing amount of anti-FBN aptamers and under cytoskeleton modulation.

2. Materials and Methods

Sample preparation

Chitosan – Chitosan discs were prepared as previously described (**Chapter 2**, **5** and **6**)[6,10]. Briefly, a 2% chitosan solution was prepared by dissolving purified 90% de-acetylated chitosan powder (A.C.E.F., Piacenza, Italy) in a 1% acetic acid solution (Sigma-Aldrich, Saint Louis, MO, USA). D-(+) raffinose (Sigma-Aldrich) was then added at a final concentration of 290mM as viscosity modifying agent. One milliliter solution was thus spread out onto a glass support to obtain a uniform film 0.25mm thick and dried at 45°C for 1h in a ventilated oven. Film was thus transferred in a 5% potassium hydroxide (Sigma-Aldrich) gelation solution for 24h and subsequently cut to discs fitting for 48-wells culturing plate.
Chitosan discs were rinsed twice in Phosphate Buffer Saline (PBS, Thermo Fisher Scientific, Waltham, MA, USA) and cleaned under UV light for 10 minutes.

Aptamers – Single-stranded DNA-based aptamers screened against human and bovine FBN were used in this study (Base Pair Biotechnologies, Pearland, TX, USA). Aptamers were modified with a carbon chain containing a disulfide bond on their 3'-end and with a biotin on their 5'-end.
Prior to chitosan functionalization, thiol group at the 3'-end was exposed by reducing the disulfide bond with aptamer reducing buffer (Base Pair Biotechnologies) for 10 min. Excess of reducing agent was then removed with a chromatographic column (mini Quick Spin Oligo Columns, Roche Life Science, Branford, CT, USA) and aptamers finally diluted at their working concentration in aptamer folding buffer (Base Pair Biotechnologies).

sFBN-CH and CH – The surface of chitosan discs was modified with anti-FBN aptamers by exploiting their capacity to spontaneously adsorb sulfur-containing compounds[11]. Each chitosan disc was incubated with 100µl of anti-FBN solution for 1h (sFBN-CH), while unmodified chitosan (CH) incubated 1h with 100µl of aptamer folding solution was used as control.

Four different anti-FBN aptamer amounts were used for the test groups: 5, 10, 20 and 40µg.

Protein adsorption studies

Bradford assay – Bradford assay was used to study the time-course of blood plasma proteins and of pure FBN adsorption to CH and sFBN-CH.

To this purpose, discs were soaked for 1h in 200µl of PBS supplemented with 2% human serum (Pooled Normal Human Serum, Innovative Research, Peary Court, FL, USA) either of pure FBN (F0895, Sigma-Aldrich) at a concentration comparable with that present in a 2% human serum solution (0.02mg/ml). Protein amount in supernatants was quantitated after 5, 15, 30, 45 and 60 min through Bradford assay (BIO-RAD Protein Assay, BIO-RAD, Hercules, CA, USA) according to the manufacturer's recommendations. Ten-µl aliquots were collected at each time point, mixed with 200µl of Bradford Working Solution and incubated at 37°C for 2 min prior to absorbance assessment at 620nm with a micro plate photometer (Multiskan™ FC, Thermo Fisher Scientific). The amount of proteins deposited on the discs was finally calculated by subtracting the residual concentration in supernatant from the initial one.

Western Blot – Western Blot (WB) was developed at the end of the time-course analysis to assess the selectivity of sFBN-CH for FBN.

Samples were briefly rinsed twice in PBS to remove softly bound proteins, incubated with 80µl of Sample Buffer 1X (Tris-HCl 62.5mM pH 6.8, SDS 1.5%w/v, DTT 100mM) and then freeze, thawed, sonicated for 15 min and boiled at 95°C for 10 min to completely recover adsorbed proteins. Equal volumes of recovered proteins were loaded on a 12% polyacrylamide gel (Acrylamide/Bis-Acrylamide 30%, Sigma-Aldrich), electrophoresed 1h at 180V and subsequently blotted on a PVDF membrane (Immobilon-P, Darmstadt, Germany) 1h at 100V. Non-specific sites were firstly blocked 1h at room temperature (RT) in Tris-buffered saline (TBS, Tris-HCl 50mM pH 7.5 and NaCl 150mM) containing 10% of blocking reagent (Roche S.p.A., Segrate, Italy). Then, the membrane was prior incubated overnight with an anti-FBN (F3648, Sigma-Aldrich) antibody diluted 1:800 in 0.1% Tween 20 in TBS supplemented

with 5% bovine serum albumin (BSA, Sigma-Aldrich) and then with a HRP-conjugated secondary antibody (CellSignalling Technology, Danvers, MA, USA) diluted 1:10000. Eventually, immune reactivity was visualized with enhanced Chemiluminescence (Immobilon Western Chemiluminescent HRP, Sigma-Aldrich).

Cell assays

Cell cultures – *In vitro* assays were performed with human cervical (HeLa) cell line (Sigma-Aldrich).

Cells were cultured up to 4 days in 48-well plates at a density of 5000 cells/disc and maintained in complete high glucose Dulbecco modified Eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific) and 1% Penicillin and Streptomycin (PenStrep, Thermo Fisher Scientific).

For the experiments with cytoskeleton inhibitors, blebbistatin and cytochalasin-D were added to the culturing medium at a final concentration of 1µM and 0.1µM, respectively. Cytoskeleton inhibitors concentration were assessed as reported in ***Appendix A.***

Inverted microscopy – To assess the effects of aptamers on cell shape, morphology and adhesion, cells were cultured up to 4 days on CH and sFBN-CH specimens at increasing aptamer concentrations and in the presence or in the absence of cytoskeleton inhibitors.

Cells were daily observed with an inverted microscope (Eclipse TS100, Nikon). Photographs were acquired with a digital camera (Digital Sight DS-Fi2, Nikon) and image analysis was performed with the D3 software (Nikon). In particular, the amount of spread and non-spread cells on chitosan surface was measured after the region of interest identification (ROI). Cells were considered as spread when filopodia were clearly visible and their circularity was lower than 0.8.

Statistical analysis

Data were analyzed using Prism6 (GraphPad, La Jolla, CA, USA) and reported as the mean ± standard debiation of three repeated experiments performed in

multiple replicates. Differences between groups were evaluated with two-way ANOVA statistical test combined to Sidak multiple comparisons post-hoc test, and considered significant when $p<0.05$. Trends were fitted with linear regression approximation with a 95% interval confidence.

3. Results

Anti-FBN aptamers interface modification induces firm FBN adsorption

Blood plasma proteins showed very fast deposition on both CH and sFBN-CH (**Fig.1a**). As a tendency, slight more proteins seemed to be adsorbed on CH (39.2±0.1µg) vs. sFBN-CH (34.5±1.4µg), even though no significant differences were revealed after the statistical analysis (p=0.2034). The time-courses resulted comparable and estimated to hyperbolic trends (CH R^2=0.9789; sFBN-CH R^2=0.9866). Consistently with this, when CH or sFBN-CH specimens were incubated 1h with a solution of pure FBN at serum concentrations, no differences were revealed among the groups (CH 6.6±0.1µg; sFBN-CH 6.0±0.1µg; p=0.2352; CH R^2=0.9547; sFBN-CH R^2=0.9755).

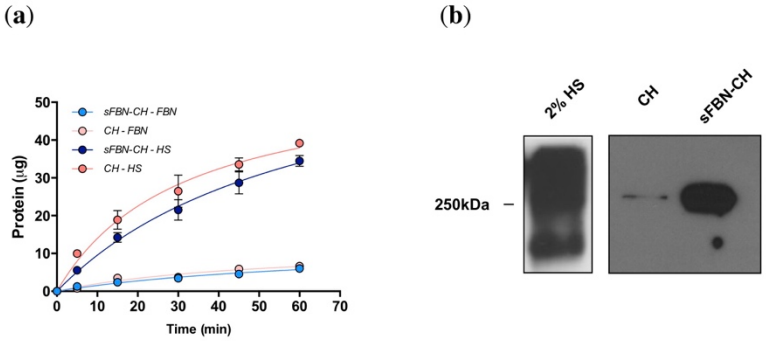

Figure 1. ***Protein adsorption over time and aptamer-doped chitosan selectivity for fibronectin.*** (**a**) Time-course of blood plasma protein and of pure FBN adsorption to CH and to sFBN-CH. (**b**) Western blot analysis of FBN stably adsorbed on CH and on sFBN-CH.

Furthermore, to investigate whether aptamers enhanced the adsorption of FBN, a WB was performed. **Figure 1b** shows that chitosan selectivity for FBN was 34.7-fold promoted by aptamers (O.D. CH=2.8% vs. O.D. sFBN-CH=97.2%).

Anti-FBN aptamers interface modification promotes epithelial cells adhesion in a dose-dependent manner

To investigate if aptamers improve the adhesion of cells to chitosan, the amount of flattened cells was monitored over the time up to day 4 and quantitated by image analysis.

The presence of aptamer dramatically increased the entity of cell spreading starting from day 3 (**Fig.2a**). After 1 day of culture, no spread cells were found both on the CH and on the sFBN-CH sample, as well as no significant differences were detectable ($p>0.9999$). However, 6.93-fold more at day 3 and 3.56-fold more cells at day 4 were spread on sFBN-CH, with statistically different significances (day 3: $p=0.0002$; day 4: $p<0.0001$).

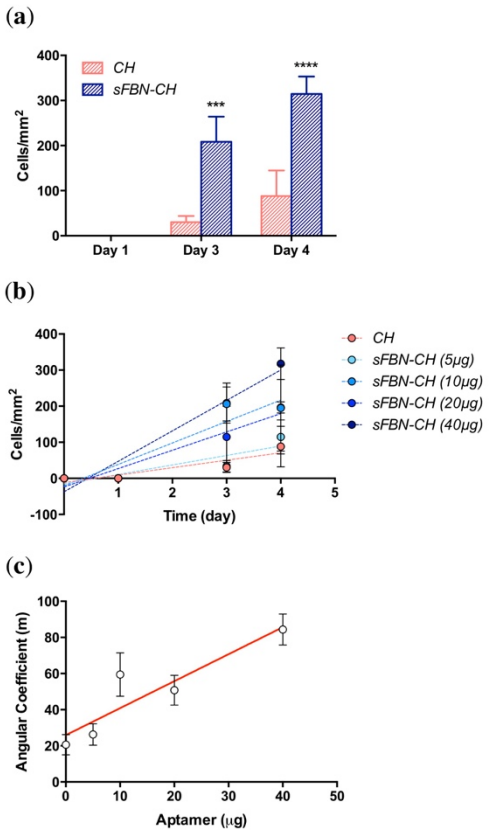

Figure 2. ***HeLa cells spreading on chitosan.*** (**a**) Histograms showing the amount of spread cells CH and sFBN-CH after 1, 3 and 4 days of culture. (**b**) Trend of cell spread rate on chitosan discs implemented with increasing aptamer doses. (**c**) Trend of angular coefficients derived from cell spread rates on chitosan discs implemented with increasing aptamer doses. *=$p<0.05$.

Additionally, when different doses of aptamers were used, the amount of well-spread cells increased proportionally with the amount of total aptamer used, following linear regression trends (**Fig.2b** – CH $R^2=0.5723$; sFBN-CH(5µg) $R^2=0.6621$; sFBN-CH(10µg) $R^2=0.7529$; sFBN-CH(20µg) $R^2=0.7916$; sFBN-CH(40µg) $R^2=0.9068$). Up

to 3 days, the differences with the control were significant only when the highest dose of aptamers was used (CH vs. sFBN-CH(40µg) p<0.0001), while at day 4 the differences against control were significant (CH vs. sFBN(10µg) p=0.0270; CH vs. sFBN-CH(20µg) p=0.0090; CH vs. sFBN-CH(40µg) p<0.0001) except for the minimum dose of aptamer used (CH vs. sFBN-CH(5µg) p>0.9999). In addition to this, at day 4 differences were detected also between sFBN-CH with the maximum dose of aptamer and all the other groups (sFBN-CH(5µg) vs. sFBN-CH(40µg) p<0.0001; sFBN-CH(10µg) vs. sFBN-CH(40µg) p=0.0077; sFBN-CH(20µg) vs. sFBN-CH(40µ) p=0.0280).
The plot of the angular coefficients of the regression lines fitting cells spreading data with different amounts of aptamers revealed a linear regression trend with an estimated R^2 value of 0.8322 (**Fig.2c**), showing linear increase of cell spreading for increasing aptamer concentrations.

Integrin-mediated pathway is at the basis of epithelial cells adhesion at the anti-FBN aptamers modified interface

No differences in morphology of cells exposed to cytochalasin-D or blebbistatin and in untreated cells were detected on bare chitosan (**Fig.3a-b**). However, when inhibiting actin polymerization with cytochalasin-D on sFBN-CH, we observed a significant 1.29-fold or 1.37-fold reduction in the number of spread cells, when compared to untreated cells (p=0.0043) and to cells cultured with blebbistatin (p=0.0007), respectively. These two conditions did not show difference in between each other (p=0.5496) (**Fig.3a-b**).
Furthermore, when the angular coefficients of linear regression trends derived from the use of different aptamer doses were compared (**Fig.3c**), a dip of the fitting curve was detected for cytochalasin-D treated group with significant differences to control and blebbistatin group when 20 or 40µg of aptamers were applied (sFBN-CH(20µg) control vs. cytochalasin-D p=0.0025, blebbistatin vs. cytochalasin-D p=0.0052; sFBN-CH(40µg) control vs. cytochalasin-D p=0.0279, blebbistatin vs. cytochalasin-D p=0.0002), On the other hand, a striking overlapping of control and blebbistatin group fitting lines was observed.

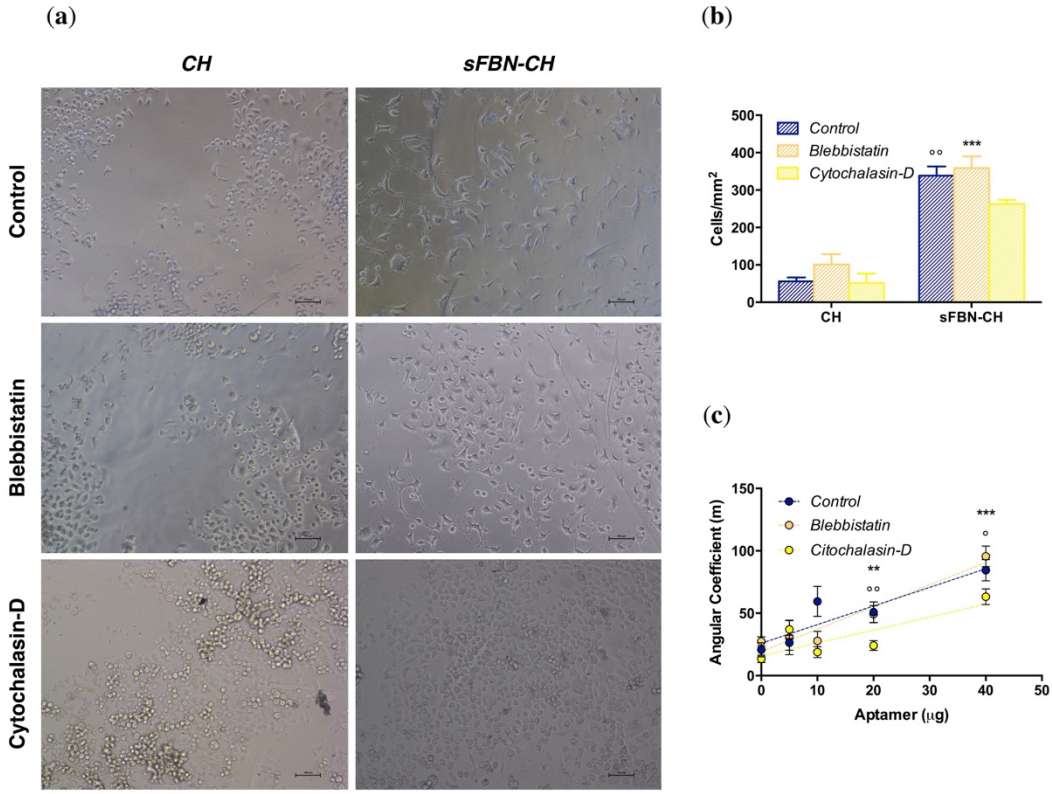

Figure 3. ***HeLa cells spreading modulation on sFBN-CH by blebbistatin and cytochalasin-D.*** (**a**) Representative images acquired with an inverted microscope of cells on CH and sFBN-CH after 4 days of culture in complete medium or in complete medium supplemented with blebbistatin 1µM or cytochalasin-D 0.1µM. (**b**) Histograms showing the mount of spread cells on CH and on sFBN-CH after 4 days of culture in complete medium or in complete medium supplemented with blebbistatin 1µM or cytochalasin-D 0.1µM. (**c**) Trend of angular coefficients derived from cell spread rates on chitosan discs implemented with increasing aptamer doses and cultured in complete medium supplemented with blebbistatin 1µM or cytochalasin-D 0.1µM. °=p<0.05 control vs. cytochalasin-D. *=p<0.05 blebbistatin vs. cytochalasin-D.

4. Discussion

Proteins adsorption on biomaterials occurs shortly after scaffold insertion and anticipates cells attachment. Therefore, the control of this phenomenon at the interface is extremely important when seeking to pilot cells fate[12]. Chitosan has been widely recognized as a suitable biomaterial for scaffold production, even if it possesses a scarce ability to support the attachment of mesenchymal, epithelial and osteoblast-like cells (**Chapter 2** and **5**). Hence, the presence in the literature of different methods to enhance cell response on chitosan through FBN enrichment[6,13-17].

To this purpose, we have previously proposed a novel method to ameliorate the adsorption of autologous FBN on chitosan through the use of selective binding molecules: aptamers (**Chapter 5** and **6**). We obtained a sFBN-CH that did not influence the amount of FBN adsorbed at the interface, but which induced an impressive amelioration of osteoblasts proliferation, adhesion and morphology[6]. We confirmed this finding in the present experiment (**Fig.1a**). However, after two rinses in PBS, WB analysis revealed a higher signal of FBN adsorbed on sFBN-CH, showing a much stronger and stable adsorption pattern. Interestingly, these data comply with the results we obtained in a previous work testing a selective FBN hyaluronic acid (sFBN-HA – **Chapter 4**)[18]. The use of anti-FBN appears therefore a valid general system to obtain materials capable to stably adsorb FBN.

Additionally, our experiments showed striking effects of sFBN-CH on human epithelial cells adhesion. The chitosan modified with the highest dose of anti-FBN aptamers (40µg) dramatically increased the number of well-spread cells with a pancake-like shape at day 3 and 4 (**Fig.2a**). Furthermore, when increasing the amount of anti-FBN aptamers (0, 5, 10, 20 and 40µg), the rate of cell spreading was progressively enhanced, suggesting an arising presence of functional FBN-related binding domains for cells at the interface (**Fig.2b-c**). Consistently with this, when cells were cultured in the presence of actin polymerization inhibitor cytochalasin-D, the rate of cell spreading decreased (**Fig.3**). Interestingly, the effects of cytochalasin-D were more substantial when sFBN-CH with higher doses of anti-FBN aptamers were used. Additionally, when myosin II contractility was inhibited, no significant differences in cell shape were observed. Since myosin II contractility does not directly depend on

integrin activation, which is in turn regulated by proper exposition of FBN RGD binding-motif, these data suggest that sFBN-CH induces a FBN adsorption pattern that is fully functional to eventually influence actin bundles reorganization and cell shape.

The hypothesis of a FBN functional adsorption is supported by the literature. For example, observing the preferential adhesion of valve endothelial cells (VEC) on tissue culture plates (TCPs) covered with increasing doses of FBN, Cuy et al. tried to ameliorate chitosan bioactivity by a FBN coating[17]. Surprisingly, VEC morphology on FBN-coated chitosan was not different from the control and their growth was lower than the growth on TCPs. Noteworthy, no differences were detected between the amount of FBN adsorbed on TCPs and chitosan, suggesting a loss in bioactivity of FBN when adsorbed on chitosan with the respect of TCPs. Consistently with this, Custodio et al. observed that when FBN was covalently immobilized on chitosan via carbodiimide chemistry, the adhesion of SaOS-2 cells was promoted if compared to FBN spontaneous adsorption, confirming the hypothesis of a probable loss in bioactivity when FBN is simply adsorbed on chitosan[16].

5. Conclusions

To conclude, we demonstrated that sFBN-CH was able to dramatically increase the number of adhering cells. A similar modified hyaluronic acid-based hydrogel (sFBN-HA) showed comparable effects, but, interestingly, different mechanisms appear to be involved (**Chapter 4**). While sFBN-HA quantitatively increased the adsorption of FBN, enhancing cell adhesion, sFBN-CH showed a similar amount of adsorbed FBN when compared to the control CH, but with great differences in the strength and functionality of the adsorption, as discussed above. Additional studies, aimed at directly investigating the structural conformation of FBN on CH and on sFBN-CH, clarified the role of function-wise conformation differences of adsorbed FBN (**Chapter 6**).

Appendix A - Supplement

Aim

Preliminary experiments were undertaken to define the concentration of cytoskeleton inhibitors (cytochalasin-D and blebbistatin) that did not elicit signs of cytotoxicity and which allowed the spreading of the cells with no evident changes of morphology.

Materials and Methods

Cell cultures – Experiments were performed with human cervical (HeLa) cell line (Sigma-Aldrich).

Cells were cultured for 4 days in 96-well plates at a density of 2000 cells/disc and maintained in complete high glucose Dulbecco modified Eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific) and 1% Penicillin and Streptomycin (PenStrep, Thermo Fisher Scientific). Simultaneously, cells were treated with increasing amounts of blebbistatin (0.1, 0.5, 1 and 1.5μM: 203390, Sigma-Aldrich) and of cytochalasin-D (0.1, 0.25, 0.5 and 0.75μM: C8273, Sigma-Aldrich).

Chemiluminescence assay – Cell viability was assessed with chemiluminescence (CellTiterGLO, Promega, Milano, Italy). Briefly, samples were incubated with 100μl of a 1:1 solution of culturing medium and lysis buffer for 1 min on an orbital shaker, thus stabilized at RT in dark conditions for 10 min. Eventually, samples were briefly spinned and luminescence quantitated with a luminometer (GloMax® 20/20, Promega).

Crystal violet staining – Cell morphology was assessed by crystal violet (CV) staining, with an inverted microscope (Eclipse TS100, Nikon). To this purpose cells were fixed 20 min at RT in paraformaldehyde 4% (PFA, Sigma-Aldrich), thus stained with a 5mg/ml solution of CV (491502, Carlo Erba, Cornaredo, Italy) prepared in 20% methanol (Sigma-Aldrich) for 10 min and rinsed twice in distilled water. When samples were completely dried, specimens were observed and microphotographs acquired with a digital camera (Digital Sight DS-Fi2, Nikon).

Results

Blebbistatin did not show negative effects on cell viability up to 1.5μM (**Fig.1Sa**). However, cell morphology was affected when doses higher than 1μM were applied (**Fig.2Sb**). On the counterpart, cytochalasin-D showed a negative effect on cell viability, which was significantly reduced at concentrations higher than 0.1μM (**Fig.1Sb** – Day 4: Control vs. 0.25μM p=0,0002; Control vs. 0.5μM p=0.0003; Control vs. 0.75μM p<0.0001). Consistently, after CV staining cell morphology was severely affected by cytochalasin-D. With increasing amount of cytochalasin-D used, cells appeared more elongated, they lost contacts and adhesion among them when organized in cluster and finally they fused forming structures like syncytiums (**Fig.2Sc**). Blebbistatin 1μM and cytochalasin-D 0.1μM were finally chosen as cytoskeleton inhibitor concentrations to use for further experiments.

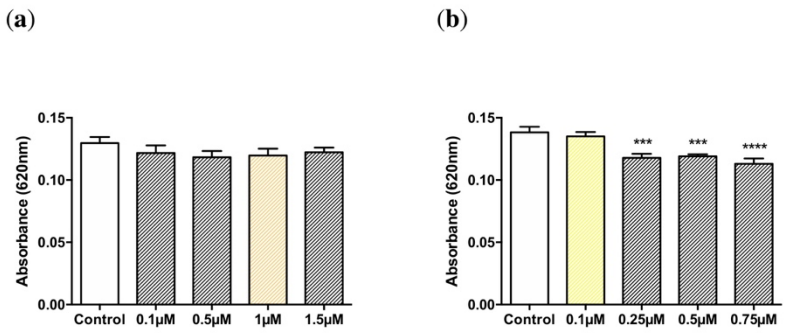

Figure 1S. *Cell viability.* (**a**) Histograms showing HeLa cells viability up to 4 days treating with blebbistatin at increasing concentrations. (**b**) Histograms showing HeLa cells viability up to 4 days treating with cytochalasin-D at increasing concentrations. *=p<0.05.

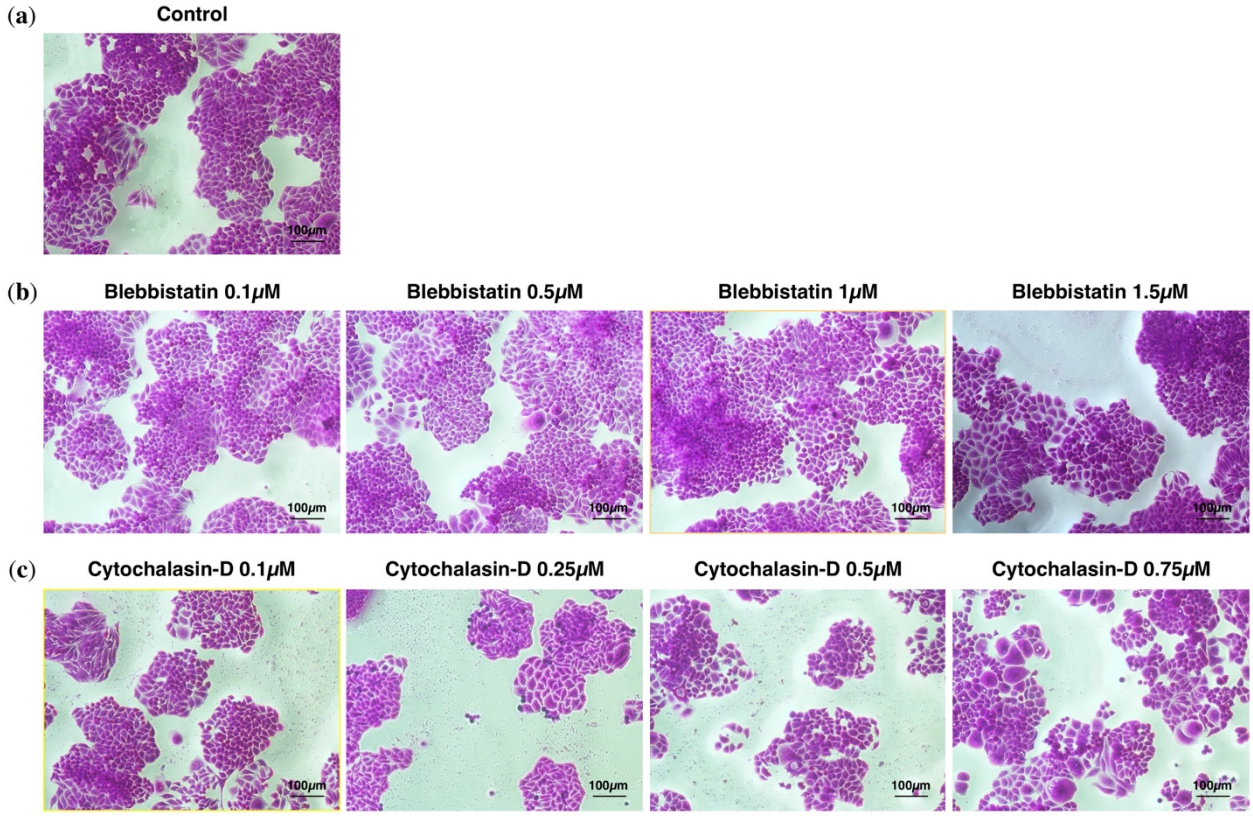

Figure 2S. ***Cell morphology.*** (**a**) Representative images acquired with an inverted microscope of HeLa cells stained with CV after 4 days of culture in complete medium. (**b**) Representative images acquired with an inverted microscope of HeLa cells stained with CV after 4 days of culture in compete medium supplemented with blebbistatin at increasing concentrations. (**c**) Representative images acquired with an inverted microscope of HeLa cells stained with CV after 4 days of culture in compete medium supplemented with cytochalasin-D at increasing concentrations.

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Conclusions

Protein adsorption on biomaterials plays a pivotal role in the subsequent host reactions, implant integration and consequent tissue regeneration. Therefore, its control is a major goal of advanced biomaterial surfaces.

In this work, an innovative approach to control the selective adsorption of proteins at the host-biomaterial interface has been presented. In particular, a new selective-fibronectin binding chitosan (sFBN-CH) as a versatile platform for tissue engineering has been pioneered by decorating chitosan films with aptamers, small binding molecules with high affinity for their target. The rationale beyond the use of this new class of molecules was to confer specificity to the substrate, by retaining autologous cues in a suitable conformational way.

On an *in vitro* level, the major biological aspects related to an advantageous aptamer-mediated adsorption of FBN at the interface, including cell adhesion, spreading, migration and proliferation, have been investigated, except for cell differentiation. The results we obtained are promising and highlight that this approach has a lot of potential to be explored, especially if aptamers against different target would be employed. However, some issues still need to be addressed and they will be a central topic of future research. Of relevance, to clarify the potential of these devices on a clinical level, preclinical *in vivo* test are necessary.

All considered, we strongly believe that aptamer coating of biomaterials will be a useful and viable approach for both research and clinical purposes, in different branch of medicine, both for the discussed properties of chitosan and for the possibility of tailoring the therapy by the means of specific aptamers. Moreover, on a clinical level, different medical devices could be envisaged able to capture specific bioactive mediators from patients' blood and to concentrate them where they are needed, in the amount they are needed, on the material itself. On the other hand, the same technology could be used to confer 3D cell culture system the ability to store proteins, i.e. FBN, taking it from the culturing medium and simultaneously to capture that directly produced and deposed by cells. As such, this is for sure an improvement of traditional biomaterials that can be enriched with

exogenous molecules, but that are not able to selectively capture and retain the desired ones.

We thus appeared to be at the eve of a new promising way for a customized therapy and for a tune tailoring of scaffolding materials in regenerative medicine.

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