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Gene Activated Biomaterials

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Preface

This PhD thesis is part of a general research project focused on the realization of bioactive biomaterials and led by Prof. P.A. Netti. It is organized into an introduction, two chapters describing the state of the art, three chapters describing the performed experiments and discussing the obtained results, and a final chapter with the main conclusions of the research. The two chapters on the state of the art (1 and 2), are focused on bioactive biomaterials and gene activated biomaterials, respectively. Each of the following three chapters (3 trough 5) is relative to one scientific article about the experiments carried out to realize 3D gene activated biomaterials able to recruit external cells, and transfect them once internalized. The article which chapter 3 refers is "Cell Recruitment and Transfection in Gene Activated Collagen Matrices" by Silvia Orsi, Antonia De Capua, Daniela Guarnieri, Daniela Marasco, and Paolo A Netti, published in Biomaterials 2010 (Epub 2009) 31(3) 570-576. Chapter 4 includes the article "Design of novel 3-D gene activated PEG scaffolds with ordered pore structure" by Silvia Orsi, Daniela Guarnieri and Paolo A. Netti, submitted for publication to Journal for Material Science Materials in Medicine, and presently under revision. In chapter 5 is reported the article "Gene activated PEG matrices designed to direct cell migration" by Silvia Orsi, Daniela Guarnieri, Antonia De Capua and Paolo A. Netti, to be submitted for publication.

The work carried out within the PhD program has benefited from the skilled collaboration of biologists and chemists of the CRIB (Centro di Ricerca Interdipartimentale sui Biomateriali dell'Università di Napoli Federico II), as well as from the thorough and bright supervision of Professor P.A. Netti.

Chapter 1

Introduction

1.1 Introduction

Intelligent biomaterials able to induce controlled mechanism of action and reaction within the physiological environment are becoming more and more potentially powerful tools in drug and gene delivery, diagnostics, and tissue engineering [1]. Different approaches have been proposed to generate intelligent biomaterials. Among these approaches, those based on biological solutions have recently attracted the interest of an increasingly number of researchers [2]. These researchers have developed biomaterials that can provide a range of biological signals, or a combination of them, in an homogeneous or spatial-temporal orchestrated manner, in order to control and stimulate the biological microenvironment (bioactive biomaterials) [3].

Incorporation of DNA in biomaterials, as biological signal, [4] is one of the most promising methods among those proposed to bioactivate biomaterials. As genes naturally carry on specific instructions for cells on how to work, it derives that modification of their genetic DNA is a powerful approach for affecting their fate. Transfer of genes encoding for proteins involved in specific cellular processes, suitably isolated and modified (gene transfer) is a promising technology with many therapeutic and research applications such as gene therapy, tissue engineering, and functional genomics [5]. The success of the *in vivo* application of gene transfer is limited by both extracellular and intracellular barriers. Extracellular barriers include stability, transport and cellular association, while the intracellular barriers are related to internalization, endosomal escape, cytoplasmic transport and stability, and nuclear localization [6]. With the final aim of overcoming the barriers, many attempts have been performed in order to develop both viral and non viral vectors for gene transfer. A step forward relatively to traditional delivery systems can be achieved by integrating gene vectors

into biomaterials. Gene transfer from biomaterial can protect gene vectors against extracellular barriers by both protecting them from attack by immune response, and limiting degradation by serum nuclease or protease [7]. Moreover, gene transfer from biomaterials has the potential to maintain effective vector concentration for prolonged times, which extends the opportunity for cellular internalization, and increases the likelihood of gene transfer. In addition it enhances localized gene expression [8], improving the application of gene transfer to many biomedical applications. Gene transfer from most biomaterial systems likely occurs through a combination of vector interactions, with the vector and material designed to regulate these interactions. Gene transfer from biomaterials with physically entrapped or chemical immobilized gene vectors, can result in significantly different transfection profiles, suggesting unique opportunities for each of them in various biomedical applications [9]. Biomaterials with dispersed vectors have the capacity to deliver large quantities of vector with transgene expression correlating to the dose of DNA delivered [4, 10]. In such a case, release occurs trough biomaterial degradation or vector diffusion, or a combination of them. On the contrary, immobilization of vectors to biomaterial prevents the aggregation of vectors and places the vector directly into cellular microenvironment, mimicking the natural process of virus binding to extracellular matrix proteins [11, 12].

While in traditional gene delivery the gene vectors locate the target cells, in gene transfer from biomaterials, the cells locate the vectors following their migration into the biomaterial. The ability to direct cell migration towards vectors within or upon a biomaterial, could be useful for improving cell transfection, because migration may help cells to find vectors. To control and guide cell migration, current approaches imply the formation of time/space controlled gradients of attracting signals [13-15]. In this context, researchers are moving towards the identification of techniques to prepare

biomaterial with assigned gradients of biomacromolecular signals able to guide cell migration into biomaterials [13-19]. The importance of signal gradients in dictating the characteristics of cell migration, in terms of speed and directionality of cell motion, has been recently highlighted and the possibility to control cell fate has been stressed [20-23].

Aim of the research object of this PhD thesis is designing and manufacturing biomaterials able to both guide cell migration and mediate gene transfer. To do so the potency of gene activated biomaterials has been investigated. Furthermore, this strategy has been implemented in order to achieve a control of gene expression, as well as a specific cell recruitment, mostly through the design and construction of different 3D gene activated matrix systems.

The first part of the research has been devoted to realization of a 3D DNA bioactivated collagen matrix by Poly (ethylenimine) (PEI)/DNA (encoding for green fluorescent protein GFP) complex immobilisation in the matrix through biotin/avidin bond. Moreover, a serum based chemotactic gradient within the matrix has been realised in order to directionally attract NIH3T3 cells. In this system, cells are recruited and forced to migrate through the matrix, where they find the bound PEI/DNA complexes and are transfected. 3D cell migration and cell transfection have been monitored through time-lapse videomicroscopy and fluorescence microscopy. Cell transfection has also been quantified through FACS analysis. The obtained results show that the engineered matrix is able to recruit external cells and transfect them once internalised.

In the second part of the research, a DNA bioactivated high porous poly(ethylene glycol) (PEG) matrix by Polyethylenimine (PEI)/DNA (encoding for GFP) complexes adsorption has been realized. As the design of the microarchitectural features of a matrix also contributes to promote and influence cell fate, the inner structure of gene

activated PEG hydrogels has been appropriately designed by gelatine microparticles templating. Furthermore, the microarchitectural properties of the matrix has been analysed by scanning electron microscopy, and 3D cell migration and transfection has been monitored through time-lapse videomicroscopy and confocal laser scanning microscopy.

Based upon the results of the performed experiments, the last part of the research has been devoted to the realization of 3D DNA bioactivated PEG porous matrix by PEG-PEI/DNA (encoding for GFP) complex immobilization. Moreover, in order to spatially guide the cell movement a gradient of the adhesive RGD peptides has been realized inside the matrix. The efficiency of this system is under evaluation in respect to its cell recruitment (effect of RGD gradient on cell migration) and cell transfection (expression of GFP) capability in relation to DNA immobilization into the matrix.

The results of the research carried out have provided the feasibility of preparing gene activated matrices using different both materials and approaches. In particular they have highlighted the suitability of (i) using natural-based (Collagen) as well as synthetic (PEG) materials, (ii) introducing DNA into the matrix by adsorption and (covalent or non) immobilization of the gene vectors, and (iii) influencing cell migration by microarchitectural matrix features or biomacromolecular signals gradients.

On the whole the obtained results show that the realized DNA bioactivated matrices can be used to recruit external cells and transfect them once internalized, and therefore they are useful tools in many biomedical applications.

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Chapter 2

Bioactive Biomaterials

2.1 Introduction

Achievement of any advance in medical treatment of a large variety of pathophysiological conditions, requires the development of new therapeutic agents, as well as their combination with biomaterials that can serve as sensors and carriers [1-4]. Design of intelligent biomaterials able to sense and respond, is a promising path to be followed for development of better diagnostic and therapeutic medical systems. Furthermore, biomaterial-based tissue regeneration scaffolds, biosensors, and drug delivery devices, provide new opportunities to mimic the natural intelligence and response of biological systems [5-8].

Different approaches have been proposed to generate intelligent biomaterials able to induce controlled mechanisms of action and reaction within the physiological environment, and finally to stimulate specific cellular responses at molecular level, and recognize external biological stimuli [8]. To reach this goal an increasing number of researches are carried out with the aim of finding physics-derived solutions such as development of methods for fabricating polymeric particles with variable controlled parameters (i.e. shape [9-11], mechanical properties [12, 13], surface topology and compartmentalization [14]). Conversely biological-derived solutions (bioactive biomaterials) have also been proposed. These solutions are based on development of materials that can provide, in homogeneous or spatial-temporal orchestrated manner a range or a combination of biological signals able to control and stimulate the biological microenvironment around them [15]. Biomaterials are designed as a drug, and the set up of a drug starts from the biological recognition of the process to control. Therefore, the

design of a biomaterial has to be based on the individualization of the function that it has to induce, relatively to the specific application.

Biological activation of materials can take several forms such as incorporation of adhesion factors, incorporation of growth factors, incorporation of enzymatic recognition sites and incorporation of DNA, here briefly summarized and described in more details in the following section. Incorporation of biomimetic adhesion sites is used to promote cell adhesion and migration on or within bioactive materials. The optimal density and spatial distribution of ligands upon or within bioactive materials are important factors to consider when designing biomimetic materials. The selection of the types of cells adhering to a material and their spatial distribution can also be controlled through the selection of the adhesion sites that are incorporated into a bioactive material. Incorporation of growth factors is used because these factors, biologically active proteins, act in highly specific manner and can enhance cell survival, promote cell proliferation, or control cellular phenotype. Their release can occur through a number of mechanisms, including diffusion-based or cell-triggered release, or degradation of the material. An important feature for functional biomaterials, on which is based another approach is that active material enzymatic remodelling can occur in presence of cells. Material remodelling can be designed to occur in response to specific enzymes produced by the cells. Incorporation of DNA into biomaterials can enhance plasmid DNA uptake and subsequent protein expression of genes encoded within the plasmid. It provides the potential for long-term delivery of bioactive signals. The use of plasmid DNA to generate therapeutic proteins within a host tissue, provides several advantages over traditional protein-based approaches. An ideal DNA therapeutic approach would limit the number of doses necessary to achieve long term effects. From a commercialization viewpoint, plasmid DNA manufacturing is simpler and more cost-

effective than recombinant protein production [16]. While DNA-based therapeutics offer promising new modes for treating disease, efficient systems to facilitate gene transfer and sustain gene expression are critical to their success.

2.2 Selection of methods used for the bioactivation of biomaterials

2.2.1 Bioactivity by incorporation of adhesion factors

Biomaterials can be endowed with biological activity through incorporation of adhesion-promoting oligopeptides, if needed by the specific application. Cell recognition of traditional biomaterials (i.e. polytetrafluoroethylene, silicon rubber, or polyethylene) occurs indirectly in vivo. Proteins from body fluids adsorb nonspecifically onto the surfaces of these materials, and some of them (i.e. fibronecting, vitronectin, and fibrinogen) promote adhesion of cells via specific cell-surface adhesion receptors. Direct control of cell adhesion on biomaterials can be enhanced by both preventing nonspecific adhesion of proteins on the material surface and incorporating cell-type-specific adhesion-promoting peptides [17].

Incorporation of adhesion-promoting peptides into biomaterial surfaces has been extensively investigated. These peptides are short primary sequences taken from the receptor-binding domains of adhesion proteins such as laminin and fibronectin. The most commonly studied adhesion peptide is the tri-peptide sequence, RGD. Since the first description of the use of RGD-containing peptides to promote cell attachment [18], RGD has been used extensively to biologically activated surfaces and materials. Cell-type selectivity is a common goal in therapeutic targeting and it may also represent an important goal in tissue engineering as well as in diagnosis.

2.2.2 Bioactivity by incorporation of growth factors

Incorporation of growth factors, powerful regulators of biological functions, is an additional approach for biologically activate biomaterials. For this reason they are being explored as key components of biomaterials and biomaterial systems. Their biological activity depends upon their identity, and how their are presented to the cells in space and time. Some growth factors are more effective when provided to cells through a controlled release process, whereas others are more effective when presented as bolus [19-21]. This difference in behaviour may be related to how the cells traffic and recycle their receptors for the growth factors. Trafficking and recycling may be modulated by altering either the growth factors, or their interactions with a biomaterial that is releasing or incorporating them [22-23].

Controlled release systems have been developed for growth factors, such as those based on traditional biomaterials for angiogenic growth factors delivery in vascular repair [24-26] or for neuronal survival and differentiation factors delivery in neurodegenerative diseases [27]. Many of such growth factors bind heparin, as well as heparin sulphate proteoglycans in the extracellular matrix. To exploit this binding affinity, heparin was conjugated with several biomaterials used for different applications. This immobilised heparin served as an affinity site to bind and slowly release the growth factors in the target site [28].

2.2.3 Bioactivity by incorporation of enzymatic recognition sites

Incorporation of growth factors or DNA into a biomaterial addresses the topic of transmitting biological information from a biomaterial to the neighbouring cells. On the other end it is possible to insert signals into a biomaterial able to receive the information

produced by cells. One such form of information is the enzymatic activity associated with the cell surface during cell migration. Cell migration through natural biomaterials involved in the generation, remodelling and regeneration of tissues, depends mainly upon (i) the sensitivity of the materials to proteases produced by the cells, (ii) the amount of enzyme produced by the cells, and (iii) the amount of materials to be remodelled by the cells as they migrate through them [29].

Variable approaches have been developed to engineer biomaterials that can be remodelled by cells through cell-associated enzymatic activity [30]. Cells naturally remodel the extracellular matrix in development, adaptation and healing, and materials subject to the remodelling activities of cell may enable exploitation of these biological activities in tissue engineering. A method for the chemical incorporation of bioactive signals has been developed for fibrin, a natural biomaterial matrix that can be remodelled proteolytically [29]. Exogenous peptides bear in one domain a substrate for the transglutaminase involved in coagulation, factor XIIIa, and are thus covalently conjugated to the fibrin network as it forms, incorporating the bioactive peptide within the gel. Another domain of the peptide bears a bioactive peptide, for example, with cell adhesion or growth factor binding activity [29]. Trough such a route, it is possible to incorporate the biological activity of a host of non-fibrin proteins (e.g. laminin) as synthetic components added into the platform of the biologically-derived fibrin gel.

Completely synthetic biomaterials, proteolytically degradable and comprising other bioactive components, have been designed. Gels have been formed based on poly(ethylene glycol) chains comprising central oligopeptides, that are substrates for collagenase or plasmin, both involved in cell migration [30]. These water-soluble hybrid chains may than be coupled at their termini to form three-dimensional, completely synthetic elastic gels, degradable by cell-associated enzymatic activity. Additional

biological activity can be conferred upon proteolytically remodable gels by copolymerisation of suitably reactive oligopeptides, such as terminally reactive poly(ethylene glycol) grafted with the adhesion peptide RGD. These approaches permit to construct totally synthetic materials, but with many characteristics of the natural extracellular matrix. Moreover enzymatic recognition introduced on linkers for the binding of other biomacromulecular signals can be used to modulate the release on cell demand (only in presence of cells).

2.2.4 Bioactivity by incorporation of DNA

Bioactivation by DNA incorporation is only briefly summarized in this chapter, as it will the main topics of the Chapter 2 of this thesis.

Immobilization of DNA is another method for incorporating biological signals into biomimetic materials. Typically, plasmid DNA is presented on or within a biomaterial to enhance the efficiency of its uptake, and limit the expression of the protein encoded for by the plasmid to the target tissue of interest [31-33]. DNA incorporation provides an additional degree of control in manipulating the cellular response during tissue repair and regeneration. DNA delivery can be used to express proteins that typically act primarily as an extracellular component in a signalling cascade, such as growth factors. It can also be used for delivering proteins that act intracellularly, such as transcription factors. Release of DNA incorporated into the biomaterial and its uptake by cells can be regulated through modifications of the material.

2.3 Bioactive signal gradients

Spatial patterning of biological signals is a field of growing interest. Several of these bioactive factors are well characterized for different biomedical applications and are known to induce concentration-dependent cell type–specific responses. Furthermore, they usually work in a synchronized manner with other similar factors under physiological conditions. While these factors are traditionally homogeneously delivered, both temporal and spatial control over their delivery is an important requirement for biomedical applications.

Continuous gradients of chemical signals are a form of spatially patterned signals successfully developed and employed in variable field, such as probing directed axonal regeneration [34-41], nerve regeneration [42], controlled cellular migration, and localization and/or alignment involving fibroblasts, endothelial cells, Chinese hamster ovary cells, vascular smooth muscle cells, leukocytes, and neutrophils [43-56]. The chemical signal gradients, in their soluble or immobilized forms induce specific cellular responses, which may include controlled cellular migration (chemotaxis or haptotaxis, respectively), usually in the direction of increasing concentration/surface density of the chemical signal. A positive effect on directed axonal growth has been demonstrated under the influence of various chemical signal gradients, including gradients of IKVAV-containing peptide [57], laminin [34-36], nerve growth factor (NGF) [37-39], combined laminin and NGF [42], and combined NGF and neurotrophin-3 [38, 40].In such conditions neurite extensions were found to be superior in the presence of signal gradients compared to corresponding homogeneously delivered signals. Wound healing is another area of investigation. Controlled movement of fibroblasts takes place under the influence of chemotactic factors secreted by macrophages and platelets [58], and

represents a key area to explore the effect of various chemical signal gradients on the migratory behaviour of fibroblasts, leukocytes, and neutrophils. In addition, the ability of chemical signal gradients (such as an RGD-containing peptide density gradient) to influence the alignment of the fibroblasts, can also be exploited in the tissue engineering of ligaments and tendons [47]. Moreover, gradient substrates can also be used as a screening tool in optimizing the dosage of growth factors that lead to a higher cell proliferation rate or improved juxtracrine signalling [51].

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Chapter 3

Gene transfer challenge in bioactive biomaterials design *Gene Activated Biomaterials*

3.1 Introduction

Altering or manipulating genes or gene expression has tremendous therapeutic potential for the treatment of a variety of disorders. The use of plasmid DNA to generate therapeutic proteins within a host tissue provides several advantages over traditional protein based approaches. Gene transfer approach can be employed to increase the production of specific proteins or block the expression of unwanted proteins. Additionally, gene transfer has the potential to provide protein expression for long periods of time at effective concentrations, and target any cellular process by altering expression of a specific protein. An ideal DNA therapeutics would limit the number of doses necessary to achieve long term effects and, from a commercialization viewpoint, plasmid DNA manufacturing is simpler and more cost-effective than recombinant protein production [1]. While DNA-based therapeutics offer promising new modes for treating disease, critical to the success of these programs are efficient systems to both facilitate gene transfer and sustain gene expression.

Effective in vivo gene delivery requires that the DNA has to be delivered to the desired cell population, efficiently internalized by the cell, and transported to the appropriate cellular compartment. Although the path is known, many barriers exist that limit the efficiency of delivery. The inability to cross biological membranes, such as plasma and nuclear membrane, results from both size and charge density of naked DNA. Vectors for gene delivery are being developed to overcome the barriers of the stability, size and charge density, and consist of modified virus and lipid or polymeric-cationic polymers that package the DNA.

In vivo production and secretion of therapeutic proteins by gene vectors delivery are also limited, because of the presence of extracellular barriers, by the administration

approach. Classical gene vectors administration approach is based on injection [2]. Targeting a cell population or anatomical location by injection or systemic delivery, is a complex goal to be achieved.

A new approach to gene delivery entails the incorporation of DNA or gene vectors in biomaterial systems. Biomaterial devices systems have been proven for various small-molecule drug and protein delivery and have recently been applied to the delivery of nucleic acids. Direct delivery of the vector from a biomaterial can localize transgene expression primarily to the implant site. In addition to localized delivery, the biomaterial can protect the vector against extracellular barriers that reduce their therapeutic efficacy by both protecting them from attack by immune responses, and limiting degradation by serum nucleases or proteases [3].

Biomaterial-mediated gene transfer is a promising technology that has many therapeutic and research applications such as gene therapy, tissue engineering, and functional genomics. In addition to the established challenge of biomaterials-mediated gene transfer in gene therapy, there are evolving challenges associated with the relationships between cellular microenvironment and gene transfer, and between transgene expression and subsequent tissue formation, in tissue engineering and regenerative approaches [4-10]. As well as the are challenges associated with integrated miniaturized lab in high-throughput studies on gene function or activity [11-13]. Therefore research at interface of biomaterials, gene therapy, and tissue engineering has identified several design parameters for vector and biomaterial that must be satisfied in relation to the specific application.

In this chapter the current knowledge on challenges of gene transfer from biomaterials will be summarized. A brief introduction of vector design and biomaterials design,

provides the foundation to examine the advantages and limitations with different delivery modalities and mechanisms.

3.2 Vectors design

Nucleic acids (e.g., DNA, RNA, siRNA) can be delivered alone, or packaged using viral or non-viral vectors to increase expression of therapeutic gene or knockdown expression of a specific gene (i.e. RNAi). For delivery, vector must evade the immune system and be transported to the cell microenvironment for internalization, typically into endosome, from which the vector must escape prior to being degraded as the endosome transitions into a lysosome. To induce expression of an encoded gene, the nucleic acids must dissociate from any packaging component and traffic to the nucleus for expression. For delivery of siRNA or similar strategies, the nucleic acid only needs to be present within the cytoplasm for activity.

Viral vectors are composed of either DNA or RNA surrounded by a capsid, which provides greater efficiency than non-viral vectors, yet provokes an immune response that can lead to clearance of vector or infected cells [14, 15]. A variety of viruses have been utilized as gene delivery vectors, with adenovirus and retrovirus among the most common, and adeno-associated virus (AAV) among the most promising vectors for future therapies. Naked plasmid and non-viral vectors initiate inflammatory responses that are milder than viral vectors, yet lack their intrinsic efficiency. Plasmid alone is able to transfect cells *in vivo*, but generally has a low efficiency *in vitro*. Alternatively, the nucleic acids (e.g., plasmid, siRNA) are complexed with cationic polymers or lipids, with the design of these transfection reagents dependent upon the nucleic acid properties, such as size [16, 17]. Complexation with cationic polymers or lipids protects

against degradation, creates a less negative particle relative to naked plasmid, and facilitates internalization and intracellular trafficking [18, 19] Cationic polymers contain high density of primary amines, which are protonable at neutral pH. This high density of positive charges allows the cationic polymers to form stable complexes. In addition to proving positive charges for DNA complexation, the primary amines also serve as functional groups with which to chemical modify the polymers with ligand and peptides. The most used cationc polymers for gene delivery are Poly-L-lysine (PLL) and Poly(ethylenimine) (PEI). Furthermore mixing cationic lipid results in the collapse of the DNA to form a condensed structure (lipoplex) in which nucleic acid are buried within the lipid. The most used cationic lipids for gene delivery are DOTMA, DOTAP and DOPE.

In selecting among available vectors for delivery to promote the specific needed process, multiple aspects of vector must be considered, such as the immune response to the vector, the target cell population for gene delivery, the required duration of expression, and the stability of the vector. The first aspect concerns the immune response elicited from the vector limits transgene expression [20, 21], yet local inflammatory response can potentially influence the therapeutic process. The extent of the immune response can determine whether the vector could be delivered more than once. The second aspect relates to the different vectors that have differential activity based on cell division. Some viruses can infect both dividing and non-dividing cells (e.g., neurons), whereas other are effective only in dividing cells. Many non-viral vectors are restricted to dividing cells. A third consideration aspect concerns the required duration of expression, which is based on the progenitor cells and the requirements of the therapy. Some viruses integrate their DNA into the chromosome and thus provide permanent expression, whereas many applications require expression

only during specific periods. In addition, the vector must retain its bioactivity throughout the conditions used for biomaterials fabrication. Some viral vectors inactivate rapidly at room temperature and may not be appropriate for incorporation into biomaterials. Non-viral vectors generally have good stability, though plasmid degradation and aggregation of DNA complexes are significant concerns. Finally, the level and duration of gene expression may need to be modulated to avoid side effects resulting from excessive protein activity at the target site, or inappropriate activity at a distant site. Expression can be modulated using inductible promoters that are either tissue specific or activated by small molecules [22].

3.3 Biomaterials design

Gene transfer from biomaterials enables localized expression, as the biomaterial can enhance gene transfer relative to traditional delivery system (e.g., injection) [2]. Targeting a cell population or anatomical location by injection or systemic delivery is complex, but direct delivery of the vector from a biomaterial can localize transgene expression primarily to the implant site. In addition to localized delivery, the biomaterial can protect the vector against extracellular barriers that reduce their therapeutic efficacy, by protecting them from attack by immune responses and limiting degradation by serum nucleases or protreases [23]. Biomaterials have been able to increase the half-life of viral vectors and reduce the immune response that normally targets the virus [24]. Additionally, biomaterial based gene transfer has the potential to maintain the effective levels of the vector for prolonged times, which extends the opportunity for cellular internalization and increases the likelihood of gene transfer.

Sustained release formulations can compensate for vectors lost due to clearance or degradation. Alternatively, interactions between biomaterial and vector can retain vectors locally and prevent clearance. Gene transfer from most biomaterial systems likely occurs trough combination of vector interactions with the matrix, and subsequent release, or cellular uptake directly from biomaterial, with both vector and material designed to regulate such interactions. Gene activated biomaterials can be categorized according to two basic mechanisms by which the DNA is incorporated into biomaterial: vectors dispersion (release) and vectors immobilization.

3.4 Release versus Immobilization

The efficacy of gene activated biomaterials depends upon the interaction mechanism between gene vector and biomaterial. The material and or the vector are being engineered to provide specific interactions that mediate their release or retention in the biomaterial, which can affect their function within the biological system.

Gene transfer from biomaterials with physically or chemically entrapped or immobilized gene vectors, can result in substantially different transfection profiles, suggesting unique opportunities for each of them in various biomedical applications.

Biomaterials with dispersed vectors have the capacity to deliver large amount of vector (mg quantities) with transgene expression correlating to the dose of DNA delivered, and release occurring over a period of weeks or months [25, 26]. In such a case, release from biomaterials occurs by a combination of polymer degradation and vector diffusion.

A critical aspect associated with the encapsulation of gene therapy vectors, is that the biomaterial fabrication method must be compatible with the vector integrity.

Contrary to release approaches, immobilization prevents aggregation of DNA complexes and places the vector directly into the cellular microenvironment, mimicking the natural process of virus binding to extracellular matrix proteins [27, 28]. This approach has been used to efficiently transfect cells with significantly less vector than more conventional methods [29]. One explanation for such an increased efficiency is that by maintaining elevated concentration of DNA directly in the cell microenvironment, significantly less DNA is required to drive transgene expression at levels comparable to release approaches. Likely owing to the low amount of DNA used, this expression is more transient, occurring for relatively short times (days to weeks). In fact, short-lived expression may be advantageous in applications such as initiating a cascade of events, or when prolonged expression may lead to undesirable effects.

Molecular interactions between vector and polymer dictate whether the vector will be bound or released.

Viral and non-viral vectors, which contain negatively charged DNA or RNA, potentially complexed with proteins, cationic polymers, or cationic lipids, interact with polymeric biomaterials. Such an interaction occurs through non-specific mechanisms, including hydrophobic, electrostatic, and van der Waals interactions, that have been well characterized for adsorption and release of proteins from polymeric systems [30]. Non-specific binding depends upon the molecular composition of the vector (e.g. lipid, polymer, protein), and the relative quantity of each vector component (e.g. ratio of amines on the polymer to phosphates in DNA (N/P)). Alternatively, specific interactions can be introduced trough complementary functional groups on the vector and polymer, such as antigen-antibody or biotin-avidin, to control vector binding to the substrate. For examples Poly (L-lysine) (PLL) and Polyethylenimine (PEI) can be modified with biotin residues for subsequent complexation with DNA and binding to a neutroavidin

substrate [31, 32]. Complexes can be formed with mixture of biotinylated and non-biotinylated cationic polymer. Release studies demonstrated that only 25% of immobilized DNA complexes were released over an 8-day period, with approximately 15% released within the first 24 h. For complexes formed with PLL, the number of biotin groups and their distribution among the cationic polymers were critical determinants of both binding and transfection. The number of biotin groups in the complex can be manipulated through the fraction of biotinylated PLL used for complex formation and the number of biotin residues per PLL. Increasing the number of biotin groups per complex can led to increased binding [33]. However, in vitro transfection was maximal when complexes contained biotin residues attached to a small fraction of the cationic polymers [32]. At this condition, less than 100 ng of immobilized DNA can mediated transfection, which was increased 100-fold relative to bolus delivery of similar complexes [33]. Additionally, transfection can be observed only in the location to which complexes were bound, suggesting the possibility of spatially regulating DNA delivery. For complexes formed with PEI, substantial transfection can be observed *in vitro* but was independent of the number of biotin groups present on the complex, suggesting that complex immobilization occurred through nonspecific interactions [32]. Nonspecific binding of DNA complexes to substrates has been employed with other systems to mediate delivery. PLGA and collagen membranes can be coated with phosphatidyl glycerol (1–5%) to support binding of complexes formed with polyamidoamine dendrimers [34]. Vectors can be slowly released from this matrices, to yield transfection in vitro comparable to bolus transfection controls. In vivo studies demonstrated a six- to eightfold enhancement in transfection relative to plasmid DNA delivery. Adsorption of PEI/DNA complexes to silica nanoparticles [35, 36] can result in transgene expression in vitro comparable to that observed by bolus delivery and with

reduced toxicity. Plasmid DNA can also be incorporated into inorganic calcium phosphate coprecipitates that are adsorbed onto PLGA matrices, which can be mostly released by 48 h [37].

The effective affinity of the vector for the biomaterial is determined by the strength of these molecular interactions, which may also be influenced by environmental conditions (e.g. ionic strength, pH), binding-induced conformational changes, or vector unpacking.

3.5 Cellular responses and transgene expression

3.5.1 Inflammation and vector activity

Biomaterial implantation induces a foreign body response, which together with the host response to the vector, may influence gene transcription. The foreign body response following biomaterial implantation can be described according to multiple stages: acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction, and fibrous capsule development [38, 39]. Neutrophils are the first cells to arrive, followed by macrophages. Infiltrating macrophages could potentially have beneficial functions such as secretion of angiogenic and other growth factors that promote cell proliferation, vascularization, and wound healing [40]. This inflammatory response to the material can be accompanied by an inflammatory response to the vector that can result in cell lysis or phagocytosis. Cell infected or transformed with viral vectors stimulate apoptosis by cell-autonomous mechanism or extracellular signals derived from other cells. Infection can also induced the expression of molecular markers that flag the affected cells for killing by for example natural killer cells. Unmethylated CpG motifs on plasmid can trigger antigen-specific immune defences [41, 42]. In

summary, the inflammatory response induced by the biomaterial and gene vectors has the potential to degrade, clear or inactivate the vector, inhibit promoter activity, attenuate gene transcription, eliminate transfected cells, or prevent repeated dosing.

The design of vector and biomaterial may be able to reduce the inflammatory response and thus enhance gene expression. For example, macrophage invasion is dependent on the type of cell adhesion molecules [40], and hydrogels may produce a more mild foreign body response compared with porous sponges. The material may be able to modulate some of the inflammation stemming from the vector, as the material could prevent some antibody responses. Alternatively, the vector design can modulate or reduce the inflammatory response and thus enhance transgene expression, such as by removing CpG motifs from plasmids [43] or incorporating the pharmacological agent dexamethasone into a non-viral vector transfection reagent [44].

3.5.2 Concentration and duration

Non-viral vectors delivered from the biomaterial can induce localized transgene expression with a duration that may be significantly longer than the duration of vector release [2, 45]; however, the precise relationship between delivery and duration of expression must be developed. Low dosages of DNA released from a biomaterial had shorter durations of expression relative to larger dosages, with modest differences in the expression level. Modifications to either the vector or biomaterial properties will likely affect the duration of expression through either controlling the duration of delivery or altering the gene silencing mechanisms.

3.5.3 Spatially patterned gene delivery

The ability to release multiple factors from a biomaterials , with spatial control, may be particularly important for many applications in particular in tissue engineering and functional geneomics. The spatially patterned delivery of DNA encoding for tissue inductive factors in the case of tissue engineering, may be able to spatially direct cellular processes in order to recreate complex tissue architectures. For example, an injury at the bone/cartilage interface requires that both bone and cartilage be restored. Transplanted or endogenous progenitor cells have been shown capable of forming either bone [26, 46] and cartilage [47] when presented with the appropriate factors. Spatial patterning of DNA on biomaterials is very useful in constructing integrated microarrays for functional geneomics.

Several methods have been developed *in vitro* to spatially control gene delivery and obtain patterns of gene expression. The spatially controlled deposition of gene therapy vectors can be achieved by several methods, such as spotting, printing, microfluidics, or pinning (i.e., the aqueous vector solution wets the hydrophilic but not hydrophobic regions) [48-50]. The deposition procedure must retain vector activity, while the vector-material interactions must maintain the vector locally. The potential for spatially controlled gene delivery was demonstrated but, the extension of these techniques to 3D systems remains a significant challenge.

3.6 Challenges and opportunities of gene transfer from biomaterials

Gene activated biomaterials with nonviral and viral vectors generally promotes gene transfer to cells within or adjacent to the implant. Depending on the choice of gene product, the protein produced by transfected cells may function either locally or

systematically. Localized protein production is being used to stimulate an immune response, deliver a suicide gene, or promote wound healing. Alternatively, protein secreted by locally transfected cells can be distributed systemically, which has application to disorder such as hemophilia. The versatility of gene activated biomaterials to alter protein concentrations locally or systematically may impact numerous applications *in vivo* and *in vitro*.

3.6.1 Gene therapy

The aim of gene therapy is to treat diseases involving deficient or mutated proteins by delivering genes that encode intact proteins to target cells and making them express there. This is thus expected to be a new way to treat refractory diseases such as congenital diseases, cancer, and AIDS. Within this strategy, gene vectors are widely used to express the target gene transiently without expecting its insertion into the chromosome for the treatment of those diseases. However, the vector, when given by the usual method, is inactivated and degraded immediately after its contact with cells. Therefore, the vector method is not suitable for the treatment of a disease requiring gene expression over several weeks or more, during which the copy number of the administered gene decreases through dilution by cell division and intracellular degradation occurs. To overcome this shortcoming, repeated administration of the virus vector is required. This, however, imposes a heavy burden on the patients because the delivery of amounts of genetic material in excess of its physiological concentration causes serious side effects [51, 52]. In order for gene therapy to be applicable in clinical medicine, it is imperative that a suitable method for stable controlled release of the required amount of the vector delivered over the desired period of time has to be developed. Biomaterial based gene delivery systems may enhanced delivery

(biomaterial + gene vectors) of the vector and extent the duration of transgene expression to achieve sufficient protein quantities, that act locally or systemically. For these reasons several studies have been focused on the use of biomaterials based gene delivery systems. For example, intranasally delivered nanospheres loaded with plasmid encoding acute respiratory syncytial virus RSV antigens can reduce viral titers and viral antigen load after RSV infection in mice [53]. Additionally, IL-2, IL-12 and TNF-α expression induced by a virus-releasing gelatine sponge can inhibit tumor growth in heterotopic nodules of tumor-bearing mice [54].

3.6.2 Tissue Engineering

Tissue engineering aims at repairing and restoring damaged tissue function employing three fundamental "tools" namely cells, scaffolds and biological signals such as growth factors or DNA [55-61], which however are not always simultaneously used. On the other hand, summoning recent experimental and clinical evidences, it follows that the success of any tissue engineering approach mainly relies on the dedicate and dynamic interplay among these three components, and that functional tissue integrating and regeneration depends upon their sapient integration [59]. Therefore, biomaterial scaffolds have to provide biological signals able to guide and direct cell function through a combination of matricellular cues exposition and growth factors and/or DNA delivery. In particular the integration of DNA into the scaffold provides the potential for long-term bioavailability of bioactive signals as cells themselves produce the proteins needed for the regenerative process. Scaffold realised according to this approach, were developed few decades ago and called gene activated matrices (GAMs) [8-10, 62]. They have been later implemented and used in different tissue engineering applications. In particular a clinical study, using collagen-embedded adenovirus encoding PDGF, has

begun to evaluate the safety and maximum tolerated dose for treatment of diabetic ulcers [63]. Collagen-based delivery of nonviral or viral DNA has been employed in models of bone [8, 26, 64], and nerve regeneration [65]; wound healing [62, 67-69]; muscle repair [70]; and cardiovascular disease [71]. Alternatively, viruses have been tethered to endovascular microcoils [72], stents [73], and heart valve replacement cusps [10] to localize delivery to the arterial wall and avoid spread to distal sites [73]. Porous PLG scaffolds releasing plasmid DNA were able to transfect cells within and around the scaffold, with sufficient expression of PDGF to promote tissue formation [10]. While these studies have illustrated the potential for extending the production of growth factors locally, adapting the delivery strategies to control transgene expression spatially (micrometers to millimeters) or temporally (days to months) may re-create the environmental complexity present during tissue formation [74-75]. The ability to regulate expression of one or more factors in time and space may be critical to the engineering of complex tissue architectures, such as those found in vascular networks and the nervous system. These systems would also increase our understanding of the biology behind tissue formation, which would serve to identify how gene delivery can best augment the regenerative process.

3.6.3 Functional Genomics

Transfected cell arrays represent a high-throughput approach to correlate gene expression with functional cell responses, based on gene delivery from a surface [76]. While traditional nucleic acid analytical methods are limited to “one gene at a time”, DNA microarray technology enables parallel processing of several gene species concurrently. In principle, this system can be employed for numerous studies, such as screening large collections of cDNAs [76] or targets for therapeutic intervention [77].

Transfected cell arrays were formed using a substrate-mediated approach in which plasmids or adenoviruses were mixed with collagen and spotted onto glass slides or into wells [76-78]. Plated cells were transfected and could be analyzed for cellular responses using a variety of imaging or biochemical techniques. Further development of the substrate-mediated approach requires the development of a cost-effective delivery system, that efficiently transfects a wide variety of primary cells and cell lines, while allowing for spatially controlled DNA within the different domains [76, 79].

At the end of 2004, the US Food and Drug Administration (FDA) cleared the AmpliChip ™ Cytochrome P450 Genotyping test (Roche Molecular Systems) for use with the Affymetrix GeneChip Microarray Instrumentation System (Affymetrix). By analyzing expression of a key specific gene (cytochrome P4502D6) within the cytochrome P450 family that plays an important role in metabolism of commonly prescribed drugs, this assay helps predict patient metabolic responses to certain drugs. This information assists physicians in prescribing proper drug dosing in patients at risk for drug toxicity. In 2007, the DNA microarray-based breast cancer prognosis test, MammaPrint® (Agendia, The Netherlands), was cleared by the FDA. MammaPrint®, produced by Agilent's inkjet printing array platform, is part of an *in vitro* diagnostic laboratory service that profiles the expression activity of 70 breast-tumor-associated genes in a surgically-removed suspect breast tumor biopsy, yielding correlative information about the likelihood of tumor recurrence [80]. The assay is only approved as an adjunct to traditional tumor oncology and histological profiling. Its clinical utility is in assisting selection of chemotherapy options to minimize recurrence. Despite some success and rapid development of numerous DNA microarray technologies, numerous challenges remain in understanding the biological and clinical significance, the assay

signal, screened data and various practical issues pertaining to reproducibility, quality control and correlations among different microarray methods and platforms [81-83].

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Chapter 4

Cell Recruitment and Transfection in Gene Activated Collagen Matrices

This chapter is extracted from the article "Cell Recruitment and Transfection in Gene Activated Collagen Matrices" by Silvia Orsi, Antonia De Capua, Daniela Guarnieri, Daniela Marasco, and Paolo A Netti. Biomaterials 2010 (Epub 2009) 31(3) 570-576

4.1 Introduction

One of the main objectives of tissue engineering and regenerative medicine is to produce new functional tissues by realising suitable micro-environments able to promote cellular processes involved in tissue formation. Cells in native tissues exist within a three-dimensional, viscoelastic milieu rich in biological information, and steadily sense and respond to all physiological stimuli [1-3]. In tissue engineering the local micro-environment is simulated by constructing scaffolds with appropriate physico-chemical and biological characteristics [4-6]. The constructed scaffolds, which basically work as 3D mechanical platforms for cell attachment and growth, in practice have to induce an action and reaction controlled mechanism stimulating cellular response at molecular level. The major challenge in designing such scaffolds consists in including a specific combination of signals that are pivotal in specifically promoting cell response and controlling tissue morphogenetic processes. These signals can be soluble macromolecules (e.g. growth factors, chemokines, cytokines), or insoluble factors (e.g. ECM proteins, glycosaminoglycans, and proteoglycans [7-8]) adsorbed or covalent bound to scaffold matrix, and able to induce and guide tissue formation. However, the main drawback of this approach still remains the difficulty to achieve long–term effect of functional molecules because of their short biological half life at physiological conditions. An alternative and more sophisticated approach to elicit specific biological responses, relies on the use of transfected cells able to synthesize and secrete the desired protein *in situ*. Therefore cells genetically induced to secrete proteins may act as point-source delivery systems, allowing a prolonged and more specific effect [9-11]. Cells can be transfected *in vitro* and then transplanted in the damaged tissue or, in alternative, the plasmid DNA, encoding for the inductive factor, can be delivered instead of the protein [12]. However, the direct injection

of the plasmid may lead to a scarce transfection efficiency because of plasmid dispersion to non target tissue. To overcome these limits, DNA incorporation into the scaffold is a versatile alternative approach, as it connects localized transgene expression, promoting expression of tissue inductive factors directly within the local environment, with the requirements for tissue formation, [13]. Such an approach offers several advantages as it circumvents some of the obstacles associated with protein degradation and plasmid deliver. This approach provides the potential for long-term bio-availability of bio-active signals from the tissue itself. Scaffolds realised according with this approach, developed few decades ago and called Gene Activated Matrices (GAMs) [14-18], have been later implemented and successfully used in the field of bone, cartilage and skin tissue engineering [19-21]. In addition, recently these scaffolds have been employed in order to improve the methodology of incorporation and release of nucleic acid within the matrix through formation of DNA complexes with cationic polymers [22-23], or by encapsulating plasmid in nanoparticle release systems [24].

Conversely, directing cell migration towards plasmid complexes within the matrix could be a useful tool for improving cell transfection, because movement through the scaffold may facilitate cells to find matrix embedded plasmid complexes. To control and guide cell migration, current approaches imply the formation of time/space controlled gradients of signals [25-27]. In this context, researchers are moving towards the identification of techniques to prepare scaffolds presenting controlled gradients of biomacromolecular signals able to guide cell migration into the scaffold [25-31]. Recent findings have demonstrated the importance of signal gradients in affecting cell migration, in terms of speed and directionality of cell motion, highlighting the possibility to control cell fate [32-35].

In this article we investigated the potency of collagen GAMs able to recruit and transfect specific cell populations, starting from the idea that once recruited, cells migrate through the matrix, where they find pDNA complexes bound to the matrix, and are transfected. The matrix was functionalised by immobilising biotinilated poly(ethylene imine) (PEI)/DNA complexes into the matrix through the modification of collagen molecules with avidin, and cells were attracted within, and guided through the matrix, by imposing an appropriate gradient of Fetal Bovine Serum (FBS). The efficiency of the GAM system was evaluated in terms of capability of cell recruitment (effect of FBS gradient on cell migration), and cell transfection (expression of green fluorescent protein (GFP)) in relation to DNA immobilization in the extracellular microenvironment.

4.2 Materials and Methods

4.2.1 Materials

PureCol (3mg/ml, 97% type I collagen in solution) was purchased from Inamed (Fremont, CA, USA). The crosslinker succinimidyl-[(N-maleimidopropionamido)-dodecaethyleneglycol] ester (NHS-PEO_{12}-Maleimide), the N-succinimidyl-S-acetylthiopropionate (SATP) and the Avidin (ImmunoPure Avidin) for the collagen molecules modification, were all purchased from PIERCE (Rockford, IL, USA). Bovine Serum Albumin (BSA), Lactalbumin and Ovalbumin, three fluorescein conjugates model serum proteins, were purchased from Molecular Probes (Invitrogen; Eugene, Oregon, USA). Biotinylated linear PEI (JetPEI-Biotin), fluorescein-conjugated linear PEI (JetPEI-fluoF), and tetramethylrhodamine-conjugated linear PEI (JetPEI-fluoR) were purchased

from Polyplus-transfection SA (7mM ammine content, Illkirch, France). pshuttle plasmid DNA encoding for green fluorescent protein (GFP) was purified from bacteria culture using Qiagen (Santa Clara, CA).

4.2.2 Collagen Modification – Conjugation to avidin

To obtain a DNA-activated collagen matrix exploiting biotin/avidin bond we developed a conjugation procedure between avidin and collagen. The procedure consisted of three steps: Functionalisation of Avidin, Collagen derivation with hetero-functionalised spacer and conjugation of Avidin with Collagen, that are described below.

4.2.2.1 Thiol-Functionalisation of Avidin

Avidin protein was dissolved in 0.10 M Phosphate Buffered Saline (PBS) and 0.15 M sodium chloride, pH = 7.2-7.5 at a concentration of 0.037 mM. A stock solution of SATP was prepared in dimethyl sulphoxide (DMSO) at a concentration of 55 mM. Avidin and SATP were reacted in 1: 50 molar proportion for 1 hour at room temperature. The excess of SATP was removed from the derivatised mixture by zeba spin desalting column (Pierce Biochemical, Rockford, IL, USA). The thioester function of SATP moiety was hydrolyzed, just before conjugation to collagen, with NH_2OH 0.4M in PBS for 2 h at room temperature, releasing free thiol groups on avidin (Fig. 4.1a).

Functionalisation of avidin protein purity and integrity was assesed by electrospray ionization mass spectrometry (ESI-MS).

4.2.2.2 Collagen derivation with hetero-functionalised spacer

In order to obtained suitable functionalized-collagen we employed a PEG hetero-cross-linker (Fig.4.1b).

N-hydroxysuccinimide group of the NHS-PEO_{12}-Maleimide was reacted with primary amine groups of collagen type I monomers in 15: 1 molar proportion (Fig. 4.1b). This reaction was carried out, under constant stirring, in PBS (pH 6.5) for 24 h at 4°C to prevent the spontaneous fibrillogenesis of collagen that can occur at neutral pH and higher temperature [36]. The excess of NHS-PEO_{12}-Maleimide was removed from the derivatized mixture by dialysis in PBS (pH 6.0) 0.1 M. The availability of the maleimide group on collagen was indirectly confirmed by the conjugation of the derivatised collagen protein with a FITC-cysteine containing model peptide (FITC-β-Ala-Glu-Cys-Gly) in a molar ratio equal to 0.036. The efficiency of this reaction was detected by RP-HPLC (Waters2795) equipped with a Photodiode Array detector (Waters 2996), utilising a narrow bore 50x2 mm C18 Biobasic column, 300 Å, 3 µm (ThermoElectron), and applying a gradient of CH_3CN, 0.1% TFA (Solvent B) from 5 to 70% , respect to solvent A (H_2O, 0.1% TFA) over a period of 20 min.

4.2.2.3 Conjugation of thiol-avidin protein with maleimide-collagen

The maleimide group of the collagen-PEO_{12}-Maleimide derivative was reacted with the sulfhydryl group introduced by SATP on avidin (Fig. 4.1c). This reaction was carried out in PBS (pH 6.0) for 24 h at 4°C under constant stirring in a large defect of avidin, employing a molar ratio of 1:10.

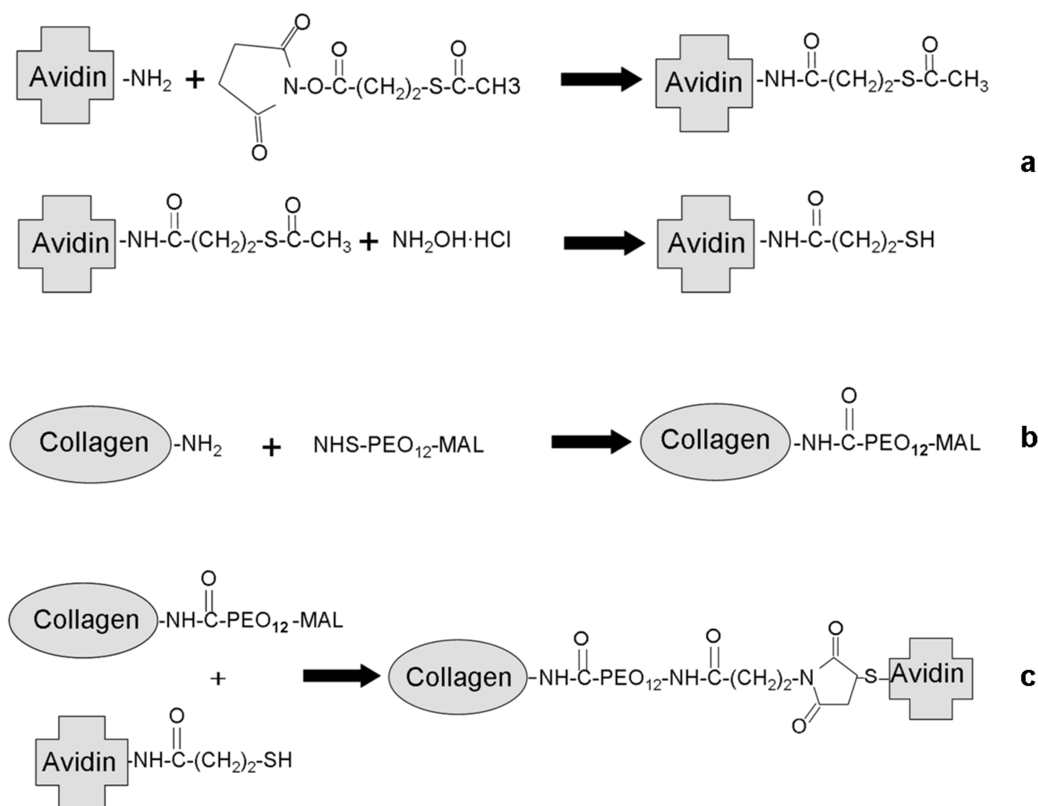


Figure 4.1. a) Reactions chemistry for the two step thio-functionalisation of avidin. See text for details; b) Reaction chemistry for collagen derivation with hetero-functionalised spacer; c) Reaction chemistry for the conjugation of thiol-avidin protein with maleimide-collagen.

4.2.3 PEIpDNA Complexes formation and characterisation

DNA (3 µg) diluted in 100 µl of Dulbecco's Modified Eagle Medium (DMEM, Gibco) was complexed with PEI (6 µl = 4 µl of PEI-Biotin + 2 µl of PEI-fluo) diluted in 100 µl of DMEM, at a nitrogen/phosphate ratio (N/P) of 5. The solution containing the cationic polymer was added to the DNA solution. All the mixture was incubated for 20 min at room temperature.

The ability of PEI (PEI-Biotin and PEI-fluo) to condense DNA was verified by gel electrophoresis.

The particle size of transfection complexes was measured by dynamic light scattering (DLS) using an ALV CGS3000 correletor. For particle sizing, complexes were diluted in the appropriate buffer (DMEM) to give a final DNA concentration of 15 µg/ml.

4.2.4 Realization of gene activated collagen matrix

Gene activated collagen matrix was realised by immobilising PEIpDNA complexes into the matrix. PEIpDNA complexes were mixed with avidinated collagen molecules to induce formation of a specific bond between biotin molecules on PEIpDNA complexes and avidin molecules. The mixture was cast and incubated at 37°C and 5% CO_2 for 30 min for gelification. Another formulation approach was performed as control by simply mixing the PEIpDNA complexes with the unmodified collagen molecules, and then casting and incubating the resulting solution at 37°C and 5% CO_2 for 30 min.

4.2.5 3D Cell Migration Assay/Cell Tracking

A 3D chemotactic assay suitable for directly monitoring, through a predesigned time lapse procedure, 3D cell migration in our gene activated collagen matrices, was designed. In such an assay, FBS as chemotactic agent was included. The assay was constructed with the design requirements of imposing a FBS gradient and maintaining both cell viability and good optical quality over the entire time-length of the experiment. The system was realised by using a common cell culture Petri dish, which was filled with a silicon mould. The central part of the silicon mould was excavated to generate a container for the gel (Fig. 4.2). The experiments were performed by confining the cells into one portion of the collagen matrix, and inducing the serum to diffuse within the matrix only in one direction,

by putting the acellularised portion of the gel into contact with a chamber, realised with a second silicon mould, containing the FBS.

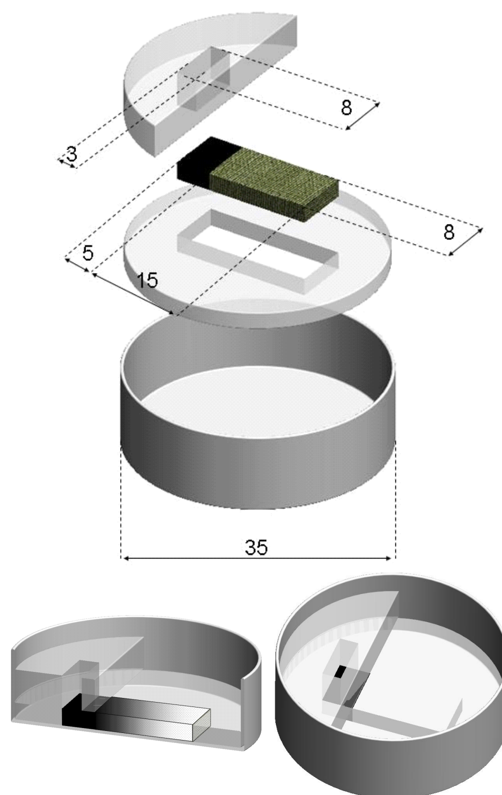


Figure 4.2. Chemotactic assay (linear dimensions in mm).

In order to detect the time when the FBS reaches the portion of the matrix containing the cells, which permits determination of when and where to begin the cell migration experiment, timescales of FBS concentration profile in our collagen matrix were defined. FBS gradient dynamics was obtained through experimental determination of the diffusion coefficient (D) of three fluorescent serum model proteins (BSA, Ovalbumin and Lactalbumin) by a home-made Fluorescent Recovery After Photobleaching (FRAP) apparatus [37].

3D migration of NIH3T3 cells within the collagen matrix was monitored by time-lapse experiments performed using an inverted phase contrast microscope (IX50, Olympus)

equipped with an incubation chamber (37 ºC, humidified, 5% CO_2 atmosphere), a x-y-z computer-controlled stage (PROSCAN; Prior, USA) and a charge coupled device (CCD) coolsnap camera (RS Photometrix, USA). Camera and computerized stage were synchronized by a specific code to follow several cells during the same experiment. Images, captured every 10 minutes over a 24 h time-interval, were analyzed by using the image analysis software Metamorph 5.0. Cells trajectories were reconstructed by tracing the centroid of each of them through time, using an automated image analysis algorithm. X, Y and Z values of individual cell centroid were stored in a text file.

Speed and directionality of cell motion were evaluated. Speed was measured according to the persistent random walk model described by Stokes [38]. The persistence of cell movement was assessed by calculating the directionality index, which is the ratio between displacement vector and trajectory length of a cell.

Directionality of cell motion relatively to the gradient direction was determined by evaluating the distribution of angles between cell displacement vector and gradient axis. These angles were measured by analysing text files with a dedicated MatLab (The Mathworks) routine.

4.2.6 Complexes release

To verify and evaluate the immobilization of the PEIpDNA complexes in the collagen, complexes loading efficiency and release studies were performed on both formulation matrices (with and without avidin). Samples were immersed in H_2O and incubated at 37°C. After scheduled time intervals (0, 4, 24, 48 and 72 h), water was removed, and its PEIpDNA concentration was detected via a standard curve, by measuring the fluorescence at 535 nm in a multi-well plate spectrofluorimeter (Perkin-Elmer, Wallac 1421). The values measured at 0 h were used to determine the complexes loading efficiency, that was

expressed as the percent difference between the total complexes amount and the 0 h measured amount. For each experimental time point, experiments were repeated in triplicate on different samples, and the percentage of PEIpDNA complexes released was calculated with respect to the encapsulated amount.

4.2.7 Cell culture and transfection

Transfection experiments were carried out on NIH3T3 mouse embryo fibroblasts cultured in both sample (with avidin) and control (without avidin) collagen matrices.

NIH3T3 cells were grown in DMEM containing 10% (*v/v*) FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin (HyClone, UK) and, in a humidified atmosphere at 37°C and 5% CO_2. 1.4 * 10^5 cells per ml were re-suspended in PEIpDNA collagen solution before collagen gelification. After the formation of the bio-activated collagen matrices, cell culture medium was added above cellular constructs and these were cultured at 37°C and 5% CO_2.

To evaluate and compare transfection efficiency in both formulation samples, qualitative and quantitative analyses were carried out. Samples were firstly investigated by confocal laser scanning microscope (CLSM) (LSM510, Zeiss) at 24, 48 and 72 h in order to detect the distribution of GFP expressing cells. The percentage of transfected cells at 48h was then measured by using Flow-activated Cell Sorting (FACS) analysis (CellSorter FACSCanto II). Samples preparation for FACS analysis included variable steps. 48h after transfection, collagen gel matrices were digested by [2.5 mg/ml] Collagenase A solution (Roche Diagnostics Corporation) for 40 min at 37 °C. The action of this enzyme was blocked by adding FBS and cells were centrifuged twice for 5 min at 1000 rpm to remove digested collagen. Cell pellet was resuspended in cold PBS and analysed by FACS.

4.3 Results

4.3.1 Collagen modification-Conjugation to avidin

To obtain a DNA-activated collagen matrix, exploiting biotin-avidin bond, avidin was conjugated to collagen by introducing a thiol group on avidin and using a hetero-bifunctional crosslinker.

Since cysteine residues on avidin protein are engaged in disulphuric bonds, we create a chemical moiety bearing free thiol groups by using SATP. Firstly the N-Succinimidyl group of SATP was bound to free amino primary group of avidin and then hydrolization of the thioesther group induced the free thiol functions on the protein.

The achievement of SATP derivatised-avidin protein was confirmed by ESI-MS analysis in which deconvoluted mass-spectrum indicated the addition of 133 amu deriving from peptide bond formation between free amine group and SATP.

The availability of the maleimide group on collagen was assessed by the conjugation of the derivatised collagen protein with a FITC-cysteine containing model peptide (FITC-β-Ala-Glu-Cys-Gly). RP-HPLC analyses at 443 nm, that is the emission wavelength of Fluorescein isothiocyanate, assessed the conjugation even at very low concentration of peptide (Fig. 4.3).

Results showed tripeptide fluoresceinate peak to higher retention times increasing reaction time from 0 to 3h. These results are an evidence of the conjugation of free thiol-containing peptide to the collagen.

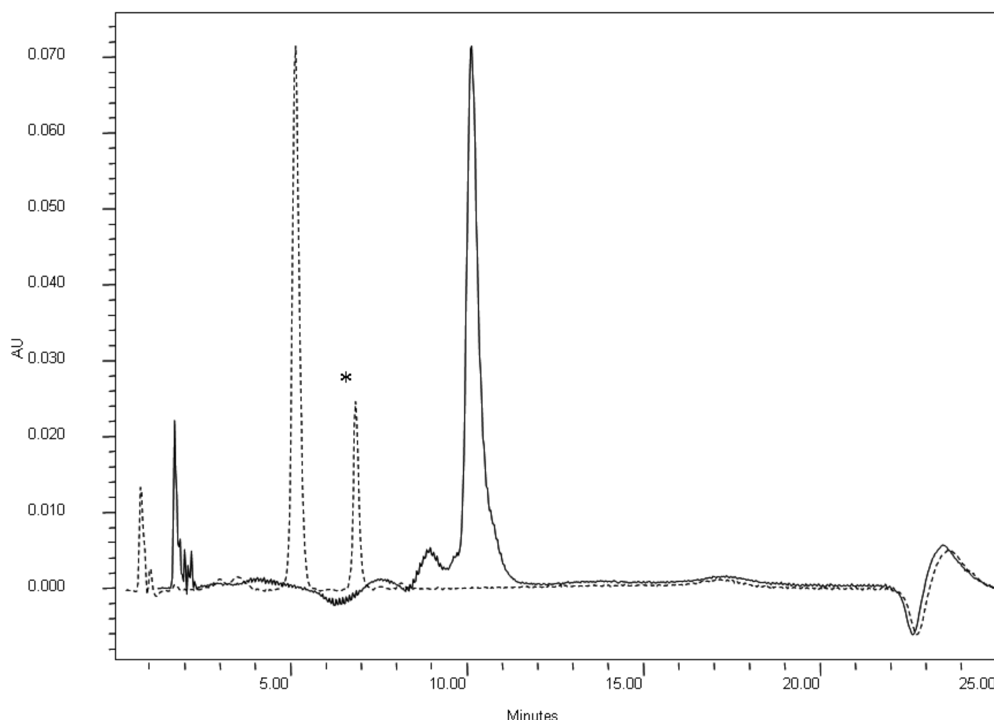


Figure 4.3. HPLC analysis at 443 nm of the conjugation reaction between the FITC-Cys-containing model peptide and the maleimide-derivatized collagene at t = 0 h (dashed line) and t = 3 h (solid line). The peak indicated by (*) is an impurity.

4.3.2 PEIpDNA complexes characterization

The ability of PEI (PEI-biotin and PEI-fluo, in 2: 1 volume ratio) to complex with DNA at N/P=5 in DMEM was established through the complete elimination of electrophoretic mobility of the DNA during gel electrophoresis indicating that the DNA was fully condensed (data not shown).

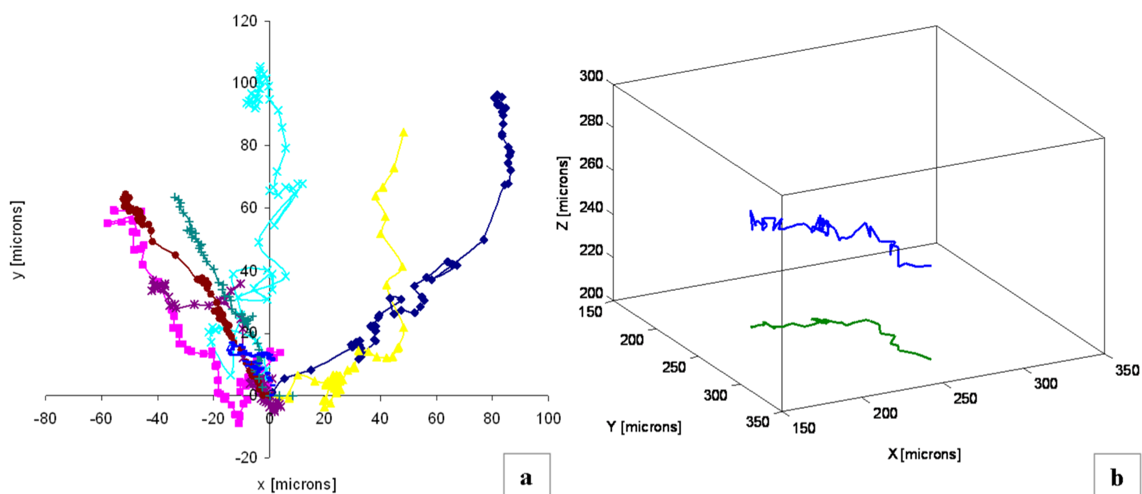
The diameter of PEIpDNA complexes (N/P = 5 in DMEM) evaluated with DLS was equal to 309.12 ± 33.42 nm.

4.3.3 3D cell migration

Measured D values of BSA (66 kDa), Ovalbumin (45 kDa) and Lactoalbumin (39 kDa) in 2.4 mg/ml collagen gel are $7.91 \pm 0.11 \times 10^{-7}$, $1.01 \pm 0.01 \times 10^{-6}$, $1.26 \pm 0.01 \times 10^{-6}$ cm^2/s,

respectively. As BSA is the slowest protein, we have used its D value to determine the characteristic time needed for all FBS molecules to rich the cellularised matrix portion. The time (τ) for significant diffusion along a distance (L) of 3 mm is, from the formula $\tau \sim L^2/D$, around 30 h. This result allows us to define how to induce a cell sensible FBS gradient in our matrices and when to start the time-lapse experiment.

Examples of cell tracks obtained from an assay prepared with our gene activated collagen matrices containing FBS gradient, are shown in Figures 4.4a,b. The NIH3T3 cells migrated through the collagen matrix preferentially up the gradient describing well defined and elongated trajectories.



Figures 4.4. a and b Examples of 3D cell tracks obtained from images analysis of time-lapse experiments carried out by using an assay prepared with gene activated collagen matrices containing FBS gradient, reported in 2D (a) and in 3D (with xy projection) (b). Y axis coincides with the gradient direction. In Fig. 4 a the starting point of all cell paths has been located in correspondence of the origin of the axis.

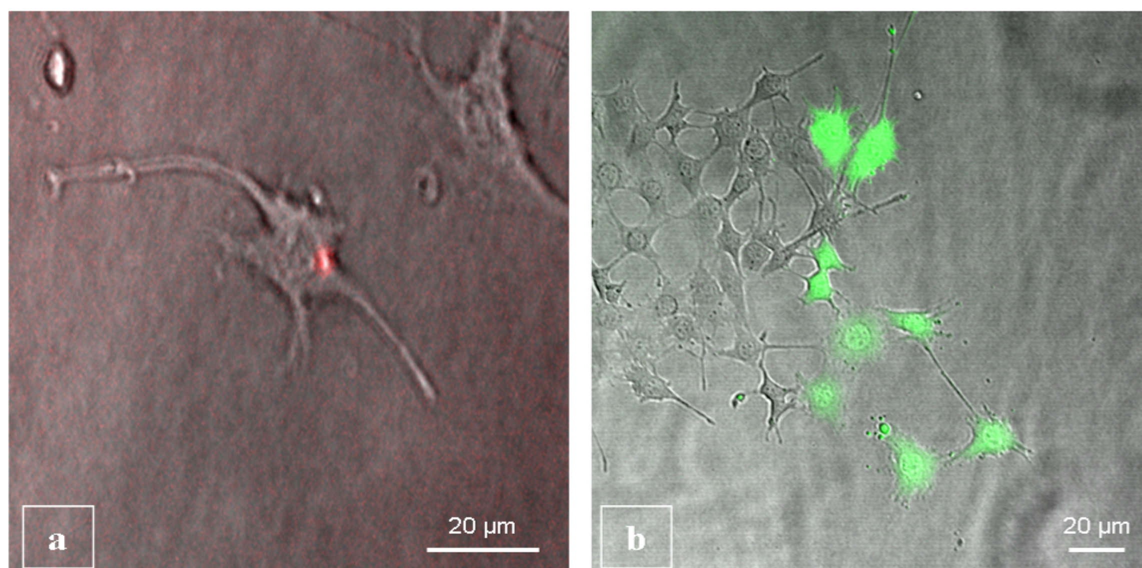
Migratory characteristics were assessed using three parameters: cells speed, directionality index and average angle of cells displacement with respect to the gradient direction. The evaluated values for these parameters are 0.19 ± 0.02 µm/min, 0.89 ± 0.03 and 25.3 ± 6.3°, respectively.

4.3.4 Complexes loading efficiency

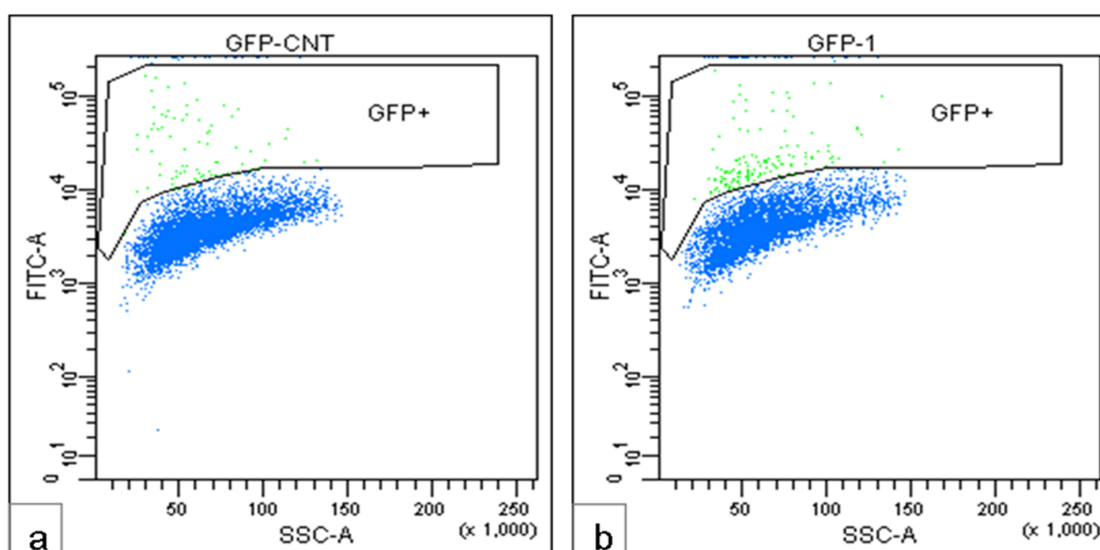
Complexes loading efficiency tests revealed that the percentage of PEIpDNA complexes encapsulated in samples with avidin was higher than in samples without avidin. More specific, complexes loading efficiency was about 70% in samples realised with avidinated collagen, while about 40% in samples obtained using unmodified collagen. PEIpDNA complexes release tests in water showed that during the experimental time period (4 up to 72 h), no complexes were released from samples of both formulations.

4.3.5 Transfection analysis

In order to follow the fate of PEIpDNA complexes, we use both biotinilated and rhodaminated PEI. CLSM analyses showed the presence of PEIpDNA complexes inside the cells after 24 h of culture in avidinated collagen matrices (Fig. 5a). Additionally, after 24, 48 and 72 h of culture NIH3T3 cells into the matrix were found to express the transgene (GFP) (Fig. 4.5b). To quantify number of GFP expressing cells, we performed FACS analysis (Figures 4.6a, b). Results showed that the percentage of GFP expressing cells increased from 1.3 ± 0.5 in control samples without avidin up to 2.8 ± 0.4 in samples with avidin.



Figures 4.5 a and b. CLSM picture of PEIpDNA complexes inside the cells after 24 h of culture in bound collagen matrices(a), and CLSM picture of NIH3T3 inside the matrix after 24 h of culture expressing GFP (b). Both pictures were realised by merging transmission and fluorescence images of the same areas.



Figures 4.6 a, b. FACS analysis results for avidin free (a) and with avidin (b) samples that show the percent of transfected (GFP+) cells.

4.4 Discussion

Assessment of the fundamental relationships among gene delivery, transgene expression, and tissue formation remains a significant challenge in the design of tissue engineering scaffolds. Gene delivery can stimulate local protein production capable of activating processes that may play important roles in tissue development and physiology [39]. Engineering of mature and functional tissues may depend on the ability to direct cells into spatially complex arrangements on length scales ranging from micrometers to centimetres and guide dynamic organization, maturation, and remodelling of cells [40].

Combination of the ability to recruit and guide the migration of cells with the capacity to influence cell fate by gene transfer, has the potential to enhance tissue engineering challenge. To this aim we developed a 3D DNA bioactivated collagen matrix by PEI/DNA complexes immobilization in a collagen matrix through biotin/avidin bond. In particular we developed a conjugation procedure between avidin and collagen in order to immobilise biotinilated PEI/DNA complexes within the collagen matrix. Moreover, we realised a serum based chemotactic gradient within the scaffold in order to directionally attract NIH3T3 cells.

Our results show that spatial constraints of FBS components diffusion through our collagen modified gel using our assay and consequent maintenance of a concentration gradient over 72 h, enables significant chemotactic migration of cells. In our system chemotactic cell migration occurs over a significant distance. This requires that a concentration gradient of FBS exists over many hours. In particular our results show that FBS gradient in our systems influenced also cell migration path. Cell trajectories result from randomly oriented steps in the presence of a uniform FBS concentration (data not shown), while from cell steps preferentially along the gradient direction under a FBS gradient. To accurately

analyse cell migration in the case of FBS gradient we estimated from single cell trajectory the value of several migration parameters: cell speed (0.19 μm/min), directionality index (0.89) and average angle of cell displacement with respect to the gradient direction (25°).

It has been shown that a typical speed for cells in collagen and fibrin gels is less than one cell diameter per hour [31]. The value of the NIH3T3 speed detected in our collagen matrix (0.19 μm/min→11,4 μm/h) is an indicator of the good performance of the chemotactic assay. As well as the values of both directionality index and average angle of cell displacement show that our assay allows a considerable directional guidance for cell migration: cells move in the direction of the gradient, and their trajectory is fairly linear and form an average angle with the gradient direction of 25°.

Cell density is a very important factor in setting the experimental conditions because of the poor specificity of the FBS as NIH3T3 chemotactic agent. Furthermore low cell density also avoids any significant gel compaction during the observation period. Restricting measurements to regions of the gel deeper than 1mm has mitigated any surface induced alignment effect. Lack of any contact guidance due to these possibilities was supported by the random and slower cell migration results obtained from uniform FBS concentrations, data not shown [41].

Although the chemotactic responses represented here cannot be directly compared to those found in the literature due to unmatched experimental conditions, this simple approach can be use to develop studies that combine migratory response with gene transfer.

Once tested the ability of our collagen matrix to guide cell migration, we have focused our attention upon the evaluation of the effectiveness of the immobilisation of DNA complexes into the matrix and, than, to the transfection efficiency of this matrix. Complexes loading efficiency study indicates that the percentage of PEIpDNA complexes encapsulated in the matrices was about 70% and 40% in samples with avidin and without avidin, respectively.

Moreover release study of complexes in water points out that no complexes were released from both kind of samples for all throughout the entire experiment. Therefore within the cellular microenvironment there is a complexes bioavailability in the samples with avidin higher than within avidin free samples. These different values may result from chemical modification induced on the collagen molecules of avidinated samples. Segura et al. [42] have suggested that binding of biotinylated complexes to avidinated substrate results from a combination of specific and non-specific interactions, with the former depending upon bounding of biotin residues attached to the cationic polymers, to avidin molecules on the substrate. According in our avidin free samples all the complexes had to be free or non-specifically bound to the unmodified collagen. In light of this consideration, and taking into account that our samples with and without avidin restrain 70% and 40% of the complexes, respectively, it derives that in our samples with avidin at least 30% of complexes is specifically bound.

Interestingly, substrate immobilization of PEI/DNA complexes inhibits their aggregation, which can occur rapidly both in vivo and in vitro and reduces the activity of DNA complexes [43].

Internalization of DNA complexes from the substrate can occur either by release of complexes from the substrate, or by direct internalization of the immobilized complexes [42]. Complexes release may be mediated by dissociation of biotin- avidin interaction, or by enzymatic degradation of collagen or avidin. The collagen molecules and the avidin proteins may be digested by cell-releasing protease. This consideration find a support in the increasing trend of complex release in cell conditioned medium (data not shown). The second hypothesis involves internalization of the DNA directly from the substrate, likely through a process of vector unpacking. This process implies DNA dissociation from the cationic polymer and has to occur intracellularly in order to facilitate DNA expression

[44]. However, a partial unpacking process may occur extracellularly, in which DNA complexes dissociate from the collagen-bound cationic polymer to enable DNA cellular internalization.

The results obtained through our transfection efficiency tests show that complexes immobilization in the matrix provides an higher efficiency of transfection, relative to their simple dispersion. Based upon the results of our loading efficiency and release experiments, the enhanced transfection efficiency is here related to the higher bioavailability in the cellular microenvironment of PEIpDNA complexes in the samples with avidin than in the free avidin ones. The specific binding of the complexes in the samples with avidin has generated two-fold increase in transfection efficiency, according to complexes concentration within the matrices during the experiments.

Manipulating complex properties for substrate-mediated delivery can be employed to regulate the transfection profile (number of transfected cells, transgene expression) [22].

Biomaterial scaffolds that support cell adhesion, guide cell migration and are also capable of efficient gene delivery, can provide a fundamental tool for localized transfection, which can stimulate and direct cellular processes that lead to tissue formation. Several strategies employ biomaterials to provide a sustained release of DNA or DNA complexes [13]; however, DNA complexes can also be immobilized to concentrate the DNA at the biomaterials surface and prevent distribution to non-target tissue. Through our study we have set up a strategy to immobilize DNA into collagen hydrogels for transfect recruited cells, in order to increase the transfection efficiency and prevent the distribution of DNA to non-target tissue.

The ability to guide cell migration and spatially control gene transfer in the scaffold can be employed to create spatial gradients in the expression of various tissue inductive factors (e.g. growth factors, matrix molecules), that are characteristic of many developing tissues.

This spatial control of cell attachment and transfection can be a powerful tool with broad applicability to tissue engineering.

4.5 Conclusions

This study demonstrates that a forced migration through the matrix may be achieved by imposing a FBS concentration gradient, and that transfection efficiency may be enhanced by immobilising DNA complexes in the cellular microenvironment. This combined approach offers the possibility to realise matrices that not only provide a mechanical support to neo-tissue growth, but also guide this process by both directing cellular motion and transferring DNA.

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Chapter 5

Design of novel 3-D gene activated PEG scaffolds with ordered pore structure

This chapter is extracted from the article “Design of novel 3-D gene activated PEG scaffolds with ordered pore structure” by Silvia Orsi, Daniela Guarnieri and Paolo A. Netti, submitted for publication to Journal for Material Science Materials in Medicine, and presently under revision.

5.1 Introduction

Tissue engineering aims to promote the healing of diseased or injured tissues trough the use of a scaffold that supports cellular infiltration, contains bioactive signals, and is able to guide invading cells through tissue formation [1, 2]. The success of any tissue engineering approach mainly relies on the delicate and dynamic interplay among extracellular matrix (ECM) proteins, cytokines, growth factors, cell-cell contacts and mechanical stimuli that, when sapiently integrated and orchestrated within the scaffold, results in tissue or organ formation [3]. Therefore, biomaterial scaffolds have to provide mechanical and structural, as well as biological signals able to guide and direct cell functions. To provide mechanical and structural signals, they need to have a structure of interconnecting pores, and to combine tissue-like elasticity with enhanced pathways for mass transport and cell migration [4]. In detail the scaffold-assisted regeneration of specific tissues has been shown to be strongly dependent on scaffold's surface/volume ratio, as well as on pore size and interconnectivity. Indeed, these microarchitectural features significantly influence cell morphology, binding and phenotypic expression, as well as extent and nature of nutrient diffusion and tissue ingrowth [5-8]. It has also been suggested that the pore dimension may directly affect some biological events and, consequently different tissues require optimal pore size for their regeneration [5-7]. Therefore, scaffolds with significantly different micron-scale porosities are needed for regeneration of highly structured biological tissues. Furthermore, soluble macromolecules (e.g. growth factors, chemokines, cytokines) or insoluble factors (e.g. ECM proteins, glycosaminoglycans, and proteoglycans) have to be absorbed or covalently bound to scaffolds to allow them to provide biological signals. Although peptides and growth factors are generally used as the bioactive signals in tissue engineering, the employment of DNA is an alternative or complementary approach to

introduce bioactive signals into scaffolds [9-14]. This approach provides the potential for long-term bio-availability of bio-active signals as cells themselves produce the proteins needed for the regenerative process. Scaffolds realised following this approach, were developed few decades ago and called Gene Activated Matrices (GAMs) [15-18]. They have been later implemented and successfully used in the field of bone, cartilage and skin tissue engineering [19-21]. More recently these scaffolds have been employed to implement the methodology of incorporation and release of nucleic acid within the matrix through formation of DNA complexes with cationic polymers [22] or by encapsulating plasmid in nanoparticle release systems [23].

In this article we present and discuss the results of a study aimed at preparing DNA bioactive Poly(ethylene glycol) (PEG) porous hydrogel scaffold for tissue engineering. PEG hydrogels with finely controlled porous architectures were prepared via gelatine particles templating and then functionalised by poly(ethylene imine) (PEI)/DNA complexes adsorbtion. Furthermore, by controlling the gelatine microparticles spatial size distribution, the particle templating technique [24] was implemented in order to create and tailor porosity and pore size gradients within the porous architecture of the hydrogels. The ability of templated bioactive hydrogels to support cell attachment and migration through the interconnected structure, was evaluated, using fluorescently marked cells, by confocal laser microscopy and time-lapse videomicroscopy, respectively. Moreover the efficiency of these novel gene activated hydrogels was detected in terms of cell transfection (expression of green fluorescent protein (GFP)) in relation to DNA availability in the extracellular microenvironment.

5.2 Materials and Methods

5.2.1 Gelatine microparticles preparation

Gelatine type B (Sigma-Aldrich, Mw = 176 KDa) with an isoelectric point (IEP) of 5.0, was used for microparticle fabrication. In particular, 5 g of gelatine were dissolved in 45ml ddH_2O by mixing and heating (60°C). This aqueous gelatine solution was added dropwise to 250 ml of oil (Cotton Seed Oil Sigma-Aldrich) while stirring at 500 rpm. The temperature of the emulsion was then lowered to around 15°C with constant stirring. After 30 min, 100 ml of chilled acetone (4°C) was added to the emulsion. After 1 h, the resulting microparticles were collected by filtration, washed with acetone to remove residual oil, and mechanically sieved for size separation.

5.2.2 Hydrogels preparation

To generate porous hydrogels, we introduced 50% (v/v) of home-made uncrosslinked gelatine microparticles of specific diameter size into steel gaskets adhered to a glass slide. Then we poured a PBS solution containing 20-40% (w/v) of PEG diacrylate (PEGDA) (Sigma-Aldrich, Mw = 700 Da) and 3% of a UV light-sensitive radical (Irgacure 2959 Ciba, Switzerland) around the microparticles. This mixture was exposed to long-wavelength ultraviolet (UV) light (365 nm, 10 mW/cm^2) for 5 min in order to polymerize the diacrylate. After polymerization, the gelatine beads were leached away from the hydrogels using water at 37°C over 24 h. To obtain hydrogels with different pore size, templating particles of variable diameter size range (53-75; 75-150; 150-210; 210-300; 300-500 μm) were used. Moreover, two types of pore size distribution structure were realised: one with a stepwise porosity size gradient, characterised by two areas, each with a specific pore size, and another with a continuous gradient. In the first case, a partition was

inserted in the middle of the gasket and then each of the two areas were filled with 75-150 and 300-500 μm diameter microparticles. In the second case, the gasket was filled with microparticles sized 75-150, 150-212, and 212-300 μm from bottom upwards.

5.2.3 Microstructural Analysis

Hydrogel morphologies were investigated by Scanning Electron Microscopy (SEM) and image (imageJ®) analyses. Samples were serially dehydrated (50, 75, 85, 95% ethanol at 30 min each; 100% overnight), cross-sectioned, gold-sputtered, and analysed by SEM (S440, LEICA) at an accelerating voltage of 20 kV, and variable magnifications. The porosity was analysed in terms of pore size, shape and spatial distribution. In particular the mean pore diameter and the normalized pore size distribution were estimated by 2D image analysis procedures, tracing not less than 100 pores for each sample and correcting the software value, calculated with the hypothesis of spherical shape, with the factor $4/\pi$, according to the ASTM D3576.

5.2.4 Protein residual quantification

To verify if the radical-based polymerization mechanism leads to non specific covalent gelatine incorporation, a BCA protein assay (Micro BCA™ Pierce, Rockford, Illinois) on the final hydrogels was performed according to manufacture procedure.

5.2.5 Cell seeding and culture

NIH3T3 cells were cultured at 37°C and 5% CO_2 in DMEM (Invitrogen, Gaithersburg, MD) supplemented with 10% (v/v) FBS, 100U/ml penicillin and 0.1mg/ml streptomycin (HyClone, UK), in a humidified atmosphere at 37°C and 5% CO_2. Scaffolds for cell-culture experiments (d = 15mm and h = 2mm) were pre-treated by incubation in DMEM

supplemented with 10% (v/v) FBS for 24 h. Before seeding, NIH3T3 cells were stained with green Cell Tracker (Molecular Probes) according to manufacturer's procedures, in order to improve cell detection within scaffolds, and then trypsinized, harvested and centrifuged. 10^5 cells, resuspended in 200 µl of medium, were statically seeded onto samples representative of scaffolds with different pore dimension. After seeding, the scaffolds were incubated for 2 h in a humified atmosphere (37°C, 5%CO_2), and subsequently, 1.5 ml of cell-culture medium was added to each sample. The samples were analysed by confocal laser scanning microscope (CLSM) (LSM510, Zeiss) after 24, 48 and 72 h of culture to investigate cell adhesion and penetration. Images were acquired by using a 20x objective, HeNe laser (λ = 543 nm) and z-stack function. Qualitative analyses of cell movement into the scaffold were also performed by time-lapse videomicroscopy using a fluorescence microscope and 10x objective, in order to evaluate cell behaviour in 3D porous structure. Images were acquired every 10 min over 6 h. Long term viability of cells in hydrogels from 24h to 21days was analyzed as a function of pore dimension by Alamar Blue assay (Invitrogen). 10^5 cells were statically seeded onto 3 different pore dimensions samples (templating gelatine microparticles diameter of 53-75, 75-150, 150-300 µm). Experiments were repeated in triplicate foe each pore dimension.

5.2.6 Complexes formation

Plasmid DNA encoding for green fluorescent protein (GFP), purified from bacteria culture using Qiagen extraction kit (Santa Clara, CA), was complexed with PEI (Linear PEI 7mM ammine content, Polyplus-transfection, Illkirch, France) at a nitrogen/phosphate ratio (N/P) of 5. Both plasmid DNA and PEI were diluted with NaCl (150 mM) and than mixed by adding PEI solution to DNA solution.

5.2.7 3D cell transfection

Scaffolds preparation and cell seeding for cell-transfection experiments were performed using the previously described procedures, but incubating the scaffolds with 200 µl of complexes (N/P = 5) solution for 24 h in order to induce complexes adsorption, and using unstained cells (without cell-tracker). Before cells seeding, complexes solution was completely removed and then the scaffolds were washed to remove non-adsorbed complexes. Retained complexes were quantified as a function of the pore size via a standard curve, by measuring the fluorescence at 535nm of the washed water in a multi-well plate spectrofluorimeter. For this analysis, DNA was complexed with fluorescein-conjugated linear PEI, JetPEI-fluoF (Polylus-transfection, Illkirch, France). The detected values were used to determine the complexes retaining efficiency, that was expressed as the percent difference between the amount of total and non absorbed complexes. Experiments were repeated in triplicate for each pore dimension.

The efficiency of gene transfer by the DNA activated matrix was detected through fluorescence microscopy. Samples were investigated by CLSM at 24, 48, 72 and 96 h of culture in order to detect the distribution of GFP expressing cells.

5.3 Results and Discussion

Assessment of the fundamental relationships among gene delivery, structural scaffold features, and tissue formation, remains a challenge in designing tissue engineering scaffolds. Gene delivery can stimulate local protein production able to activate processes that may play important roles in tissue development and physiology [25]. Furthermore 3D

structural properties of a scaffold can influence cellular organization and distribution; therefore the functionality of the engineered tissue [26-29].

Combination of the ability to tailor and control scaffolds structure with the capacity of influencing cell fate by gene transfer, has the potential to enhance tissue engineering challenge. To this aim we developed 3D DNA bioactivated PEG hydrogels with well defined pore structure. A preliminary evaluation of the morphological features of hydrogels was assessed by SEM analysis of both samples surfaces and cross sections. All samples are characterised by an extremely interconnected internal porous structure and well–defined porous external surfaces (Fig. 5.1).

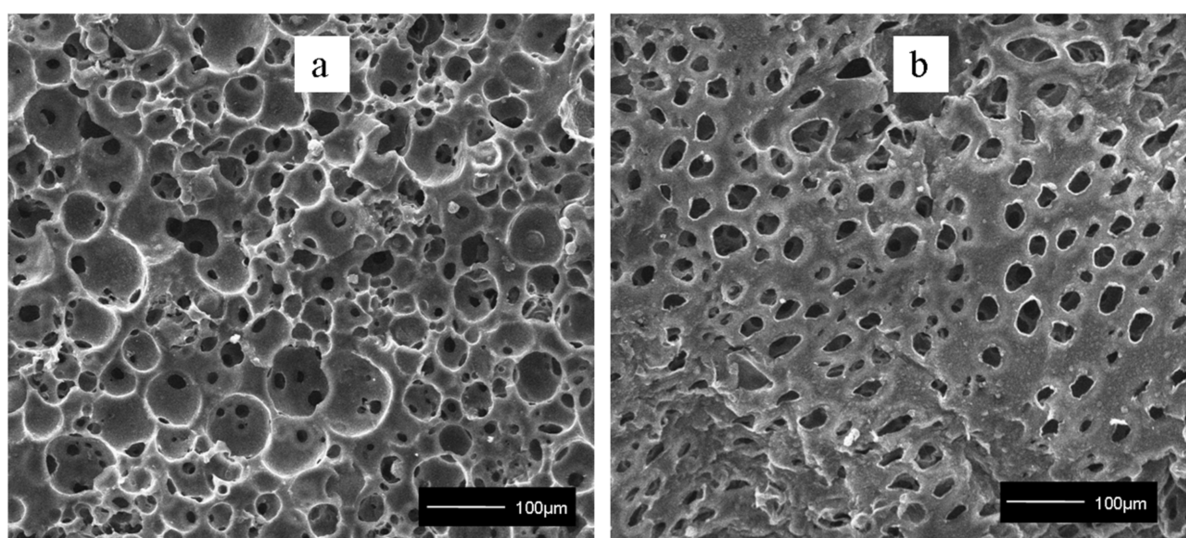

Figure 5.1. SEM micrographs of a) cross section and b) surface of PEG hydrogel obtained using templating particles in the size range 75-150 µm.

Image analysis investigation has shown a porosity of about 80%. The results of such investigation are affected by the intrinsic error of the used procedure that entails loss of small fundamental details resulting from the use of 2D images to represent solid objects. However, this procedure has proved a valid tool for the description of additional morphological parameters such as pore size and distribution. Pore size coherently varies

with particles diameter, given that scaffolds uniformly shrank by ~ 40%, upon dehydratation (Tab. 5.1).

Templating particles diamiter [μm]	Dehydrated pore mean diameter [μm]
53-75	40± 9
75-150	81 ± 17
150-210	112 ± 16
210-300	171 ± 20
300-500	277 ± 26

Table 5.1. Templeted PEG hydrogel pore size.

Moreover SEM analyses of samples obtained with different particles dimensions have shown hydrogels with pore dimension gradients (Figures 5.2 and 5.3). This is an evidence that the proposed procedure permits to create and tailor porosity and pore size gradients into the matrix. In particular, as expected from the preparation procedure, hydrogels with both two pore dimensions (Fig. 5.2) and a continuous pore dimension gradient (Fig. 5.3) have been obtained. Pore size for the former hydrogels has a bimodal distribution with two modes clustering around 80 and 280 μm (Fig. 5.4). The ability to generate 3D porous matrices with well-controlled anisotropic architectures is highly desirable in designing tissue engineering scaffolds.

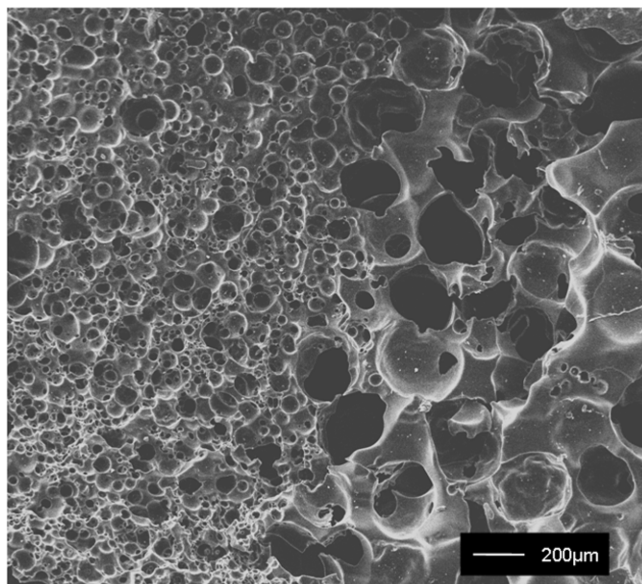

Figure 5.2. SEM micrograph of stepwise pore size gradient hydrogel prepared with 75-150 μm and 300-500 μm templating particles.

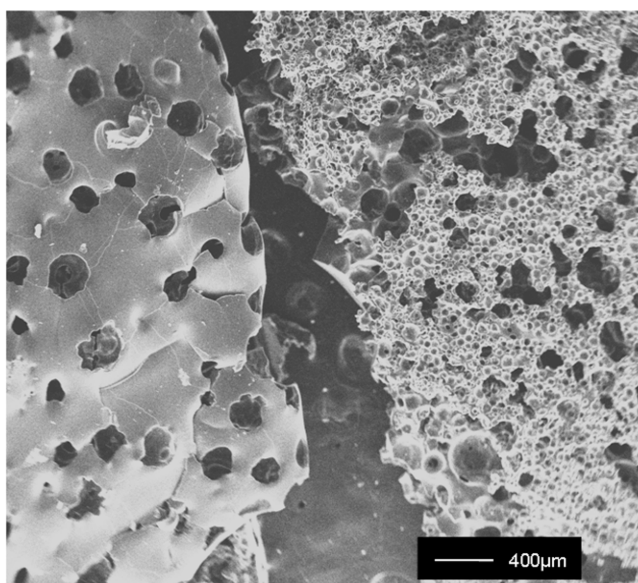

Figure 5.3. SEM micrograph of front and bottom surfaces of a continuous pore size gradient hydrogel prepared using microparticles sized 75-150, 150-212, 212-300 μm from bottom upwards.

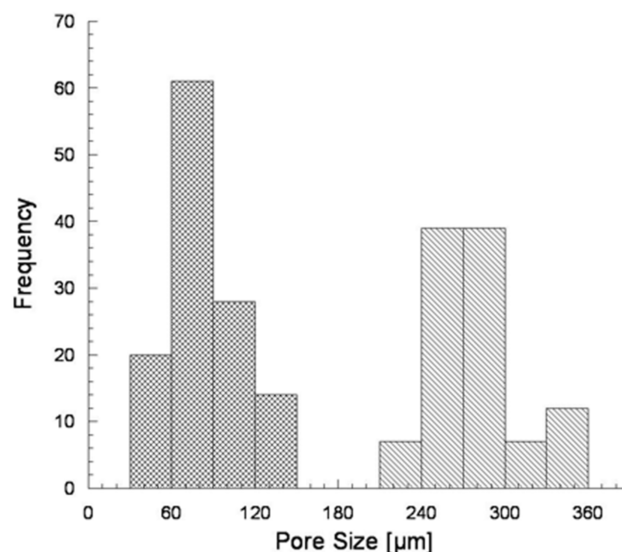

Figure 5.4. Pore-size frequency distribution of stepwise gradient PEG hydrogel.

Porous scaffolds characterized by pore size gradients offer the great advantage of reproducing the spatial organization of cells and extracellular matrix of highly complex 3D tissues, such as bone and cartilage [30-33].

Quantification of gelatine non specific covalent incorporation in the porous hydrogels has shown that independently from the templating microparticles diameter, 690±170 µg of gelatine are retained in each hydrogels, this quantity rapresents less then 0.5% of the whole gelatine used to prepare the hydrogels.

The results of CLSM analyses have shown a strong effect of pore dimension on both cell morphology and infiltration. In particular the number of cells able to deeply penetrate the matrices after 72 h of culture increase with pore dimension (Fig. 5.5). Moreover at the same time point of culture cells appear better spread as the pore dimension increase, indicating a better interaction with the matrix.

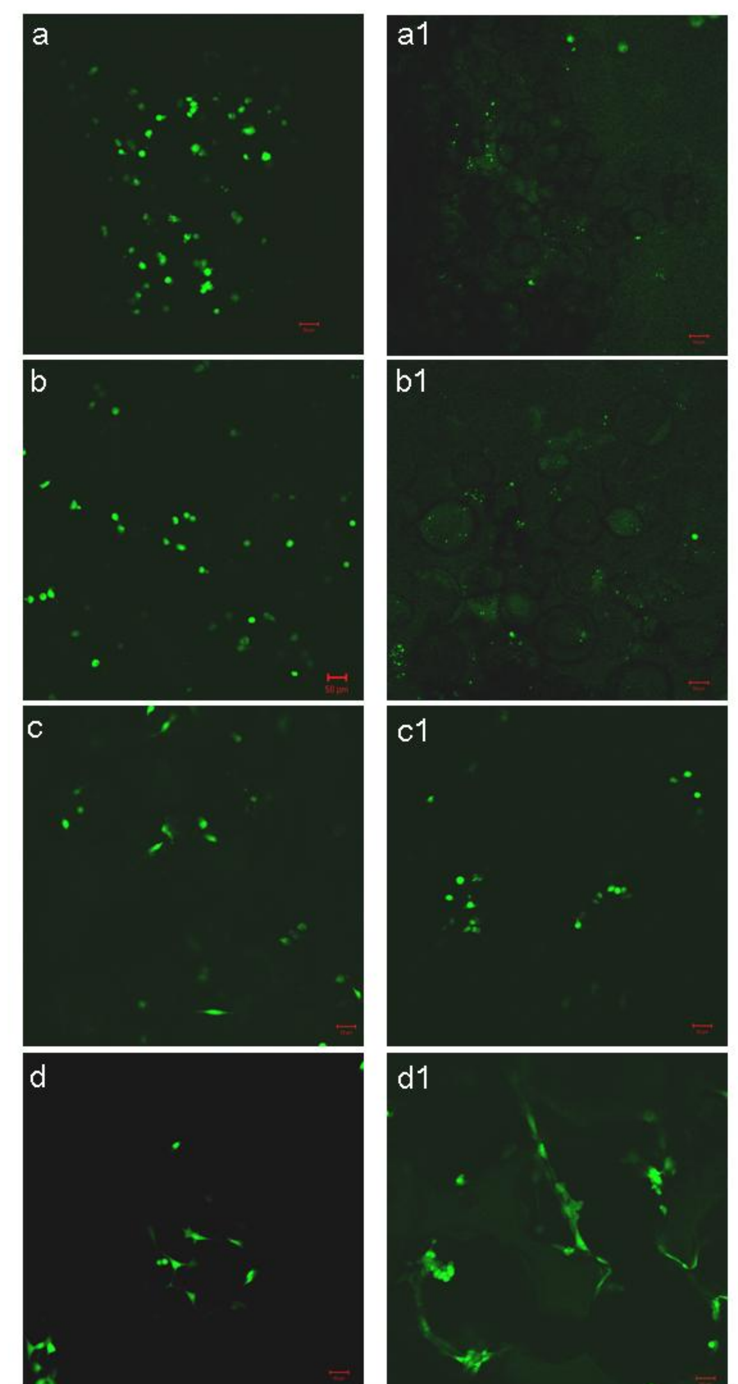

Figure 5.5. CLSM images of cells within 3D matrices. Images a through d refer to the cell seeding surface, while a1 through d1 to the opposite surface. From top to bottom the image couples are related to matrices obtained using as templating agent gelatine microparticle with diameter of 53-75, 75-150, 150-300 and 300-500 µm, respectively.

This last result has been corroborated from z-stack analysis (Fig. 5.6), yz projections have shown that cells are located at variable focus planes along the z-axis and preferentially distributed within matrix pores (Fig. 5.6b).

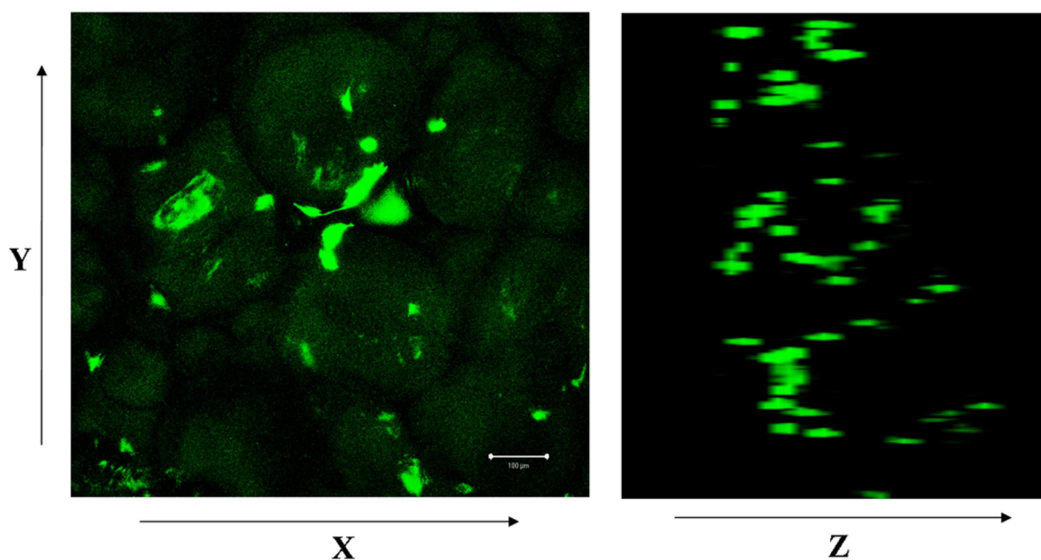

Figure 5.6. CLSM z sectioning images of cells within 3D porous matrices obtained using gelatine microparticles of 150-300 µm in diameter: a) xy and b) yz projection of 30 overlapped consecutive z-slices

Additionally, NIH3T3 cells moved inside the 3D scaffold following its micro-architecture (Fig. 5.7), likely because of combination of serum protein adsorption to PEG and 3D porous structure properties.

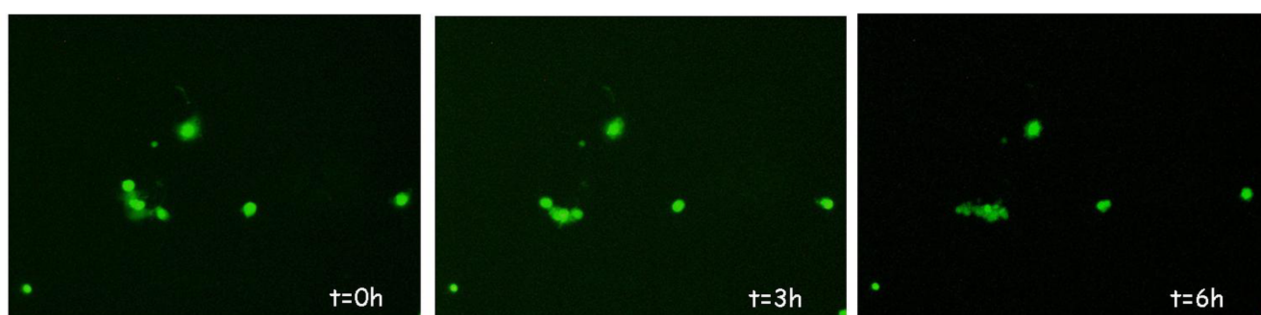

Figure 5.7. Time-lapse video microscopy clips spanning 6h of 3D cell migration into a PEG porous scaffold obtained using gelatine microparticles of 150-300 µm in diameter. Magnification 10x

The Alamar Blue assay has provided that after 22 days of static culture, cells are still vital in all samples. However the rate and trend of cell proliferation are different in the scaffolds with different pore dimension (Fig. 5.8). In particular results shown that in the samples with smallest and largest pore size, the proliferation trends reach a peak after 16 days of culture, while in the sample with intermediate pore size cell proliferation increases throughout the entire experiment. Based upon the results of CLSM analysis, these trends can be explained as dependent on the ability of cells to colonize the scaffolds. In the sample with the smaller pore dimension, cells colonize prevalently the surface that after 16 days of culture is completely cellularized. In the samples with the largest pore dimension, cells completely colonize the scaffold and after 16 days are confluently. On the contrary in the sample with intermediate pore dimension, cells slowly and continuously penetrate the scaffold.

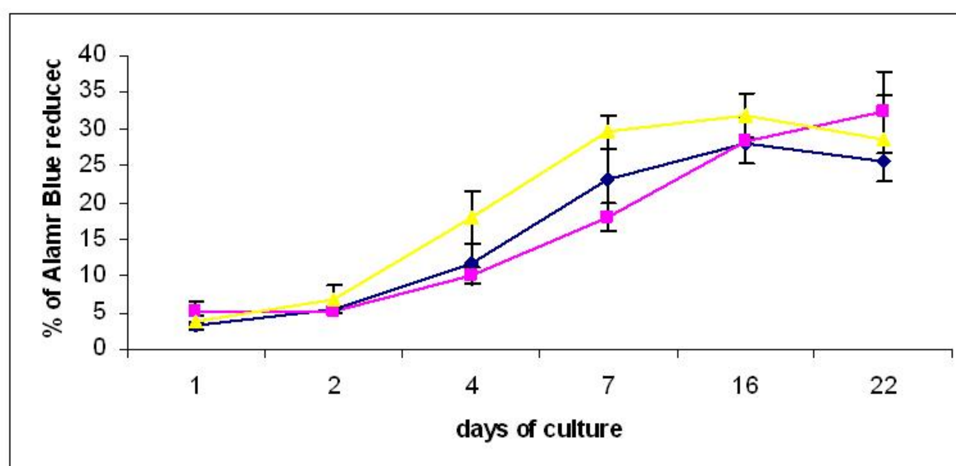

Figure 5.8. % of Alamar Blue reduced during 22 days of culture in samples relized with gelatine templating microparticles with diameter of 53-75 (yellow), 75-150 (blue), and 150-300 (pink) µm.

In order to follow the fate of PEIpDNA complexes adsorbed into the PEG scaffolds, both fluoresceinated and rhodaminated PEI were used. Complexes retention efficiency tests have revealed that the percentage of PEIpDNA complexes adsorbed is 79,1±1, 78,9±3, 75,5±1 and 73,8±2 in hydrogels obtained by using gelatine microparticles of diameters in

the 53-75, 75-150, 150-300 and 300-500 µm ranges, respectively. As expected, pore size and number of complexes entrapped in the scaffold, are inversely correlated. CLSM analyses have shown the presence of PEIpDNA complexes inside the cells seeded into the PEG scaffold after 24 h of culture (data not shown). After 48 h of culture, NIH3T3 cells into the matrix were found to express the transgene (GFP) (Fig.5.9). Additionally, during the course of the experiment (up to 96 h of culture) the number of GFP transfected NIH3T3 cells increased (data not shown).

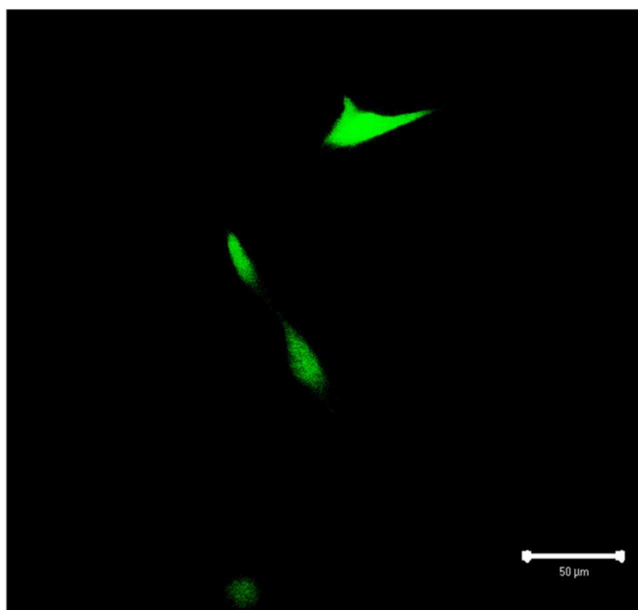

Figure 5.9. GFP expressing cells after 96h of culture into DNA bioactive 3D PEG porous scaffold obtained using gelatine microparticles of 150-300 µm in diameter. xy projection of 30 overlapped consecutive z-slices

5.4 Conclusions

The major result of the research carried out is the production of a novel method for preparing gene activated scaffolds with ordered and highly interconnected macroporosity.

Using this method, the attractive features of hydrogels (i.e. biochemical versatility, tissue-mimetic mechanical properties, and hydrophilicity), may be combined with the benefits resulting from their induced both ability of influencing cell fate (affecting their processes by DNA incorporation), and interconnected macroporous structure (including improved nutrient transport, and space for cell migration). Furthermore, the elaborated method is a step forward in the production of gene activated matrices with ad hoc microarchitectural features and under very mild physical-chemical conditions.

5.5 References

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Chapter 6

Gene activated PEG matrices designed to direct cell migration

This Chapter is a draft manuscript of the article “Gene activated PEG matrices designed to direct cell migration” by Silvia Orsi, Daniele Guarnieri, Antonia De Capua and Paolo A. Netti, in preparation to be submitted for publication to Acta Biomateralia**.**

6.1 Introduction

Non-viral gene transfer is a promising technology that can be applied in many therapeutic and research fields such as gene therapy, tissue engineering, and functional genomics. The success of this technology is limited by barriers such as extracellular stability and transport, cellular association and internalization, endosomal escape, cytoplasmic transport and stability, and nuclear localization [1]. Matrix-mediate gene transfer can protect gene vectors against extracellular barriers that reduce their efficacy by both defending them from attack by immune response and limiting degradation by serum nuclease or protease [2]. Moreover, matrix-based transfer has the potential to maintain the effective levels of the vector for prolonged times, extending the opportunity for cellular internalization and increasing the likelihood of gene transfer. In addition gene transfer from matrices enables localized expression, as the matrix can enhance gene transfer relative to traditional delivery system (e.g. injection) [3], improving the use of gene transfer in many biomedical applications. In traditional gene delivery, the gene vectors locate the target cells, while in matrix-mediate gene transfer the cells locate the vector following their migration into the matrix. Directing cell migration towards plasmid complexes within a matrix, could be a useful tool for improving cell transfection, because movement through the matrix may facilitate cells to find complexes. To control and guide cell migration, current approaches imply the formation of time/space controlled gradients of signals [4-6]. In this context, researchers are seeking for the identification of techniques to prepare matrices with controlled gradients of biomacromolecular signals able to guide cell migration into the matrices [4-10]. Recent studies have demonstrated the importance of signal gradients in affecting cell migration, in terms of speed and directionality of cell motion, highlighting the possibility to control cell fate [11-14].

The potency of PEG gene activated matrix able to recruit and transfect specific cell populations has been investigated, starting from the idea that once recruited, cells migrate through the matrix, where they find pDNA complexes bound to the matrix, and get out transfected. The matrix has been functionalised by immobilising poly(ethylene imine) (PEI)/DNA complexes through the modification of PEI molecules with acrylated PEG. In order to attract the cells within, and guide them through the matrix, an appropriate gradient of the adhesive RGD peptides has been realized. The efficiency of this system is under evaluation respect to its of cell recruitment (effect of RGD gradient on cell migration), and cell transfection (expression of green fluorescent protein (GFP)) capability in relation to DNA immobilization in the matrices.

6.2 Materials and Methods

6.2.1 Gelatine microparticles preparation

Gelatine type B (Sigma-Aldrich, Mw = 176 KDa) with an isoelectric point (IEP) of 5.0 was used for microparticle fabrication. In particular, 5 g of gelatine were dissolved in 45ml ddH_2O by mixing and heating (60°C). This aqueous gelatine solution was added dropwise to 250 ml of oil (Cotton Seed Oil Sigma-Aldrich) while stirring at 500 rpm. The temperature of the emulsion was then lowered to around 15°C with constant stirring. After 30 min, 100 ml of chilled acetone (4°C) was added to the emulsion, and after 1 h, the resulting microparticles were collected by filtration, washed with acetone to remove residual oil, and mechanically sieved for size separation.

6.2.2 Synthesis of acryloyl-PEG-RGD

The peptide sequence Gly-Arg-Gly-Asp-Ser (RGD) was synthesized by the solid-phase method using standard 9-fluorenylmethoxycarboxyl (Fmoc) chemistry protocols on Rink-amide MBHA resin (scale of 2.0 mmol), using standard Fmoc protection for amino acids sidechains,

on a scale of 2.0 mmol. The peptide was conjugated to acryloyl-PEG-N-hydroxysuccinimide (acryloyl-PEG-NHS, 3400 Da, >95% pure; Nektar Therapeutics, San Carlos, CA). Conjugation was carried out in solution by mixing the NHS-activated PEG with the peptide in Phosphate Buffered Saline (pH 7.0, 50 mM) at 1:1 molar ratio, over night. The conjugated acryloyl-PEG-peptide was dialyzed and lyophilized before use. Conjugation of RGD to acryloyl-PEG was confirmed by MALDI-TOF analysis, performed with a Voyager PerSeptive BioSystem.

6.2.3 Hydrogels preparation

To generate porous hydrogels, we introduced 50% (v/v) of home-made uncrosslinked gelatine microparticles (d = 150-300µm) into steel gaskets adhered to a glass slide. Then we poured a PBS solution containing 40% (w/v) of PEG diacrylate (PEGDA) (Sigma-Aldrich, Mw = 700 Da), 1mM of PEG-RGD and 3% of a UV light-sensitive radical (Irgacure 2959 Ciba, Switzerland) around the microparticles. This mixture was exposed to long-wavelength ultraviolet (UV) light (365 nm, 10 mW/cm^2) for 5 min in order to polymerize the diacrylate. After polymerization, the gelatine beads were leached away from the hydrogels using water at 37°C over 24 h.

In order to generate an RGD gradient within the matrix, a partition was inserted in the central part of the gasket. After introduction of microparticles two PBS solutions,

containing the same percentage of PEGDA (40% (w/v)), and Irgacure (3%), but different amount of PEG-RGD (0,5mM and 2,5mM), were poured each in one of the two areas.

6.2.4 Synthesis of PEG-PEI conjugate

A solution of PEI HCl (25000 Da, Sigma-Aldrich) 0.7 μmol dissolved in 1 mL of 20 mM HEPES, at pH 7.1, was reacted with 50 equiv of acryloyl-PEG-NHS dissolved in 0.7 mL of DMSO. After 1 h incubation, PEI conjugates were isolated by a cation-exchange column and then dialyzed. The characteristics of the obtained product were investigated by NMR spectroscopy.

6.2.5 Complexes formation

Plasmid DNA encoding for green fluorescent protein (GFP), purified from bacteria culture using Qiagen extraction kit (Santa Clara, CA), was complexed with PEI (Linear PEI 7mM ammine content, Polyplus-transfection, Illkirch, France) and PEI-PEG at a final nitrogen/phosphate ratio (N/P) of 5. Both plasmid DNA and PEI (PEI and PEI-PEG) were diluted with NaCl (150 mM) and than mixed by adding PEI solution to DNA solution. The bioactivity of PEI-PEG/DNA complexes was verified performing 2D transfection analysis on NIH3T3 cells cultured in Petri culture dishes at 37°C and 5% CO_2 in DMEM (Invitrogen, Gaithersburg, MD) supplemented with 10% (v/v) FBS, 100U/ml penicillin and 0.1mg/ml streptomycin (HyClone, UK), in a humidified atmosphere at 37°C and 5% CO_2. Samples were investigated by CLSM at 24, 48 and 72h of culture, in order to detect the distribution of GFP expressing cells.

6.2.6 Realization of gene activated PEG matrix

Gene activated PEG matrix was realised by immobilising PEIpDNA complexes into the matrix. PEIpDNA complexes were mixed with PEGDA, PEG-RGD molecules and UV light-sensitive radical and then poured around the gelatine microparticles. The mixture was then exposed to the UV light for polymerization. After polymerization, the gelatine beads were leached from the hydrogels using water at 37°C over 24 h.

6.2.7 Microstructural Analysis

Hydrogel morphologies were investigated by Scanning Electron Microscopy (SEM) and image (imageJ®) analyses. Samples were serially dehydrated (50, 75, 85, 95% ethanol at 30 min each; 100% overnight), cross-sectioned, gold-sputtered, and analysed by SEM (S440, LEICA) at an accelerating voltage of 20 kV, and variable magnifications. The porosity was analysed in terms of pore size, shape and spatial distribution. In particular the mean pore diameter was estimated by 2D image analysis procedures, tracing not less than 100 pores for each image and correcting the software value, calculated with the hypothesis of spherical shape, with the factor $4/\pi$, according to the ASTM D3576.

6.2.8 Cell seeding and culture

NIH3T3 cells were cultured at 37°C and 5% CO_2 in DMEM (Invitrogen, Gaithersburg, MD) supplemented with 10% (v/v) FBS, 100U/ml penicillin and 0.1mg/ml streptomycin (HyClone, UK), in a humidified atmosphere at 37°C and 5% CO_2. Before seeding NIH3T3 cells were stained with green Cell Tracker (Molecular Probes) according to manufacturer procedures, in order to improve cell detection within matrices. Then cells were trypsinized, harvested and centrifuged. 10^5 cells, resuspended in 200 μl of medium, were statically seeded onto matrices. After seeding, the matrices were incubated for 2 h in a humified

atmosphere (37°C, 5%CO_2). Subsequently, 1.5 ml of cell-culture medium was added to each sample. After 24 and 48 h of culture, samples were analysed by confocal laser scanning microscopy (CLSM) (LSM510, Zeiss) to investigate cell adhesion and penetration as well as RGD gradient effect on cell migration within the matrices. Images were acquired by using a 20x objective, HeNe laser (λ = 543 nm) and z-stack function.

6.2.9 3D cell migration

Qualitative evaluation of cell migration within the matrices characterized by RGD gradient was performed through CLSM analyses. To carry out these analyses cells were accurately seeded only in the area containing 0.5mM RGD. Occurrence of cells in the higher RGD concentration area (2.5mM) was investigated at specific time points.

6.2.10 3D cell transfection

In order to verify the bioactivity of the PEI-PEG/DNA complexes bound to the PEG matrices, a 2D transfection analysis was performed on NIH3T3 cells cultured on PEG gene activated non porous matrices (obtained without using gelatine templating microparticles). To verify the bioactivity as well as the bioavailability of PEI-PEG/DNA complexes immobilised into the PEG porous matrices, a 3D cell transfection analysis was performed on NIH3T3 cells cultured in PEG porous gene activated matrices. In both cases samples were investigated by CLSM at 24, 48, 72 and 96 h of culture, in order to detect the distribution of GFP expressing cells.

6.3 Results and Discussion

This report investigates the efficacy of gene transfer from bioactive biomaterials able to recruit and guide cells and influence their fate by DNA incorporation. To pursue the research aim PEG matrices with modulated structural features have been produced. In this matrices have been introduced a spatial presentation of adhesive RGD peptides, as well as gene vectors through their immobilization them to the matrix. This process has been based on the idea that, once recruited, cells migrate through the matrix and get out transfected.

A preliminary evaluation of the structural features of hydrogels was assessed by SEM analyses of samples cross sections. Samples are characterised by an extremely interconnected internal porous structure (Fig. 6.1). Image analysis investigation has shown a porosity of about 80%. This result is affected by the intrinsic error of the applied procedure that entails loss of small fundamental details resulting from the use of 2D images to represent solid objects. This procedure has also been used to determine pore size, obtaining a mean value of 140 ± 20 μm. This result is coherent with templating particles dimension, given that matrices uniformly shrank by ~ 40% upon dehydratation.

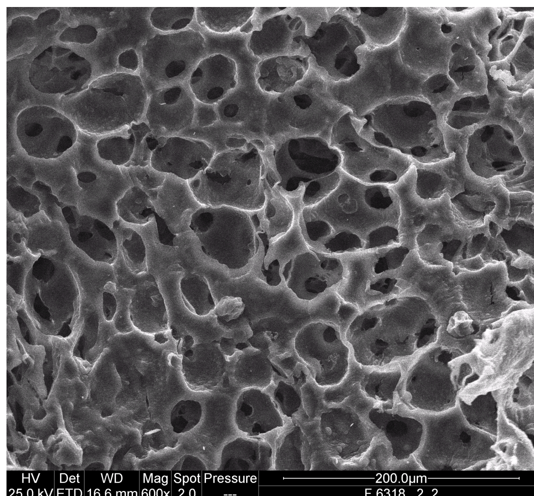

Figure 6.1. SEM micrograph of PEG hydrogel cross section obtained using templating particles in the size range 150-300 µm.

Moreover z-stack projections have indicated that a considerable number of NIH3T3 fibroblasts was able to colonize our 3D porous RGD functionalized PEG matrices. In particular cells are well spread suggesting a good interaction with the matrix (Fig. 6.2).

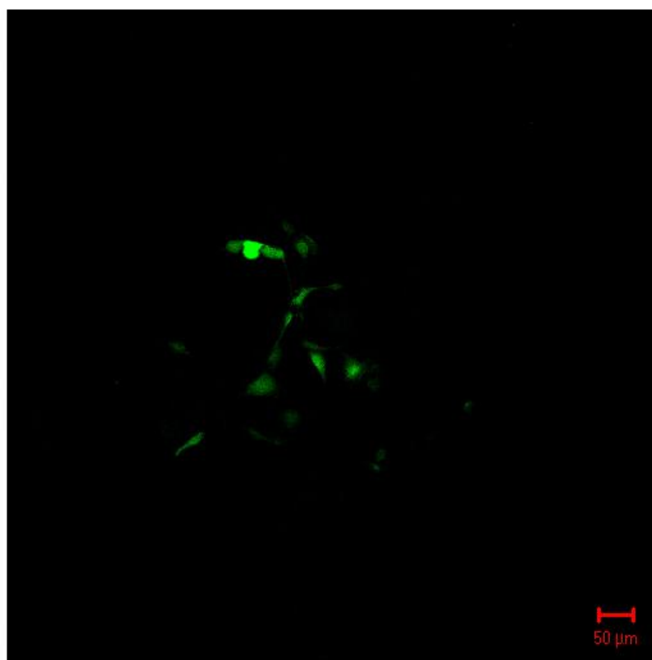

Figure 6.2. CLSM image of cells within 3D porous RGD functionalized PEG matrix after 24 h of culture.

The preliminary results of CLSM analyses carried out on samples with RGD gradient, have shown that cells are able to move inside the matrices following the RGD gradient. In particular NIH3T3 cells have been found in the higher RGD concentration area (2.5 mM) of the samples after 96h of culture.

Before investigating the ability of complexes bound to the matrix to transfect cells, the bioactivity of PEI-PEG/DNA complexes was testified by the occurrence of GFP expressing cells in 2D cell transfection tests performed in cell culture Petri dishes (Fig. 6.3). This result is here taken as an evidence that occurrence of PEGAC molecules on the complexes does not exclude the bioactivity of the complexes.

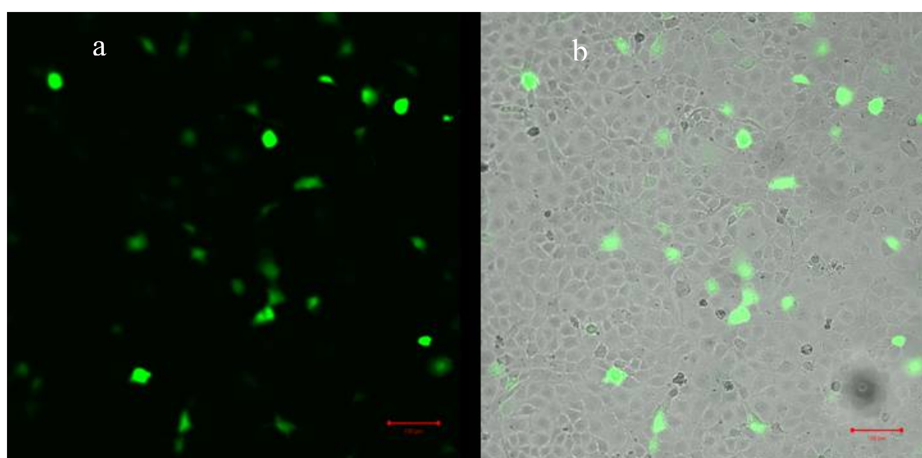

Figure 6.3. a: CLSM pictures of NIH3T3 cells after 48 h of culture in Petri dish expressing GFP. b: image realized by merging transmission and fluorescence images of the same area.

The use of PEG conjugated polycations instead of only polycations, to condense DNA offers some advantages, including improvement in water solubility, resistance against DNAse, and reduced cytotoxicity, and allows the conjugation of biomacromulecular signals for specific targeting [15, 16].

The bioactivity of PEI-PEG/DNA complexes bound to the PEG through the acrylate end of the PEG molecules on the complexes and the acrylate ends of the PEGDA used for the matrix, was first testified by the presence of GFP expressing cells after 48h of culture in

2D transfection experiments. These experiments were performed on NIH3T3 cells, cultured on gene activated non porous matrices (Fig. 6.4). This result points out that the defined immobilization procedure we have does not invalidate the bioactivity of the complexes.

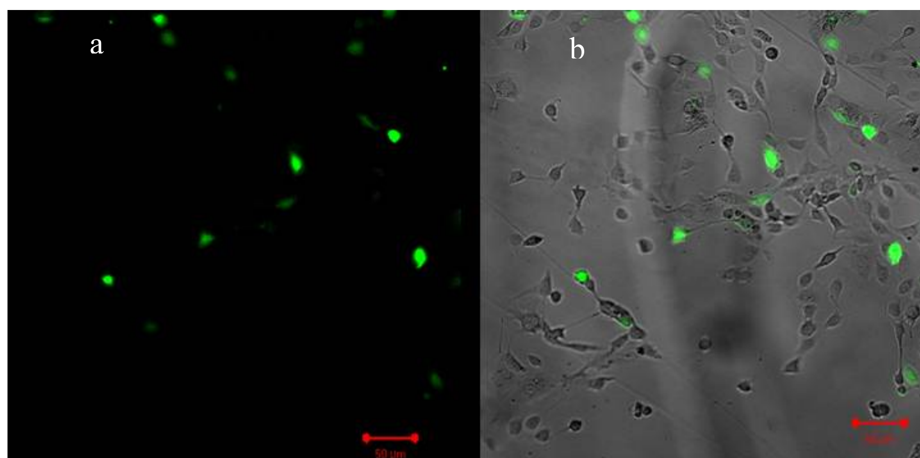

Figure 6.4. a: CLSM images of NIH3T3 cells after 48 h of culture on PEG with PEIPEGpDNA complexes bound matrix expressing GFP. b: image realized by merging transmission and fluorescence images of the same area.

The bioactivity and bioavailability of PEI-PEG/DNA complexes bound to the PEG in the porous matrices were then checked monitoring GFP expressing cells in 3D transfection experiments carried out on NIH3T3 cells cultured inside the matrices after 72h of culture (Fig. 6.5).

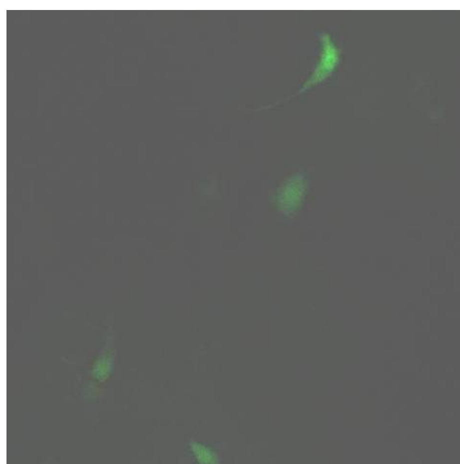

Figure 6.5. CLSM pictures of NIH3T3 cells after 72h of culture in PEG gene activated porous matrices (PEIPEGpDNA complexes bound matrix) expressing GFP

6.4 Conclusions

The preliminary results of the performed experiments show that we are setting up an innovative method for preparing gene activated matrices with a spatial predefined presentation of adhesive peptides and immobilised DNA complexes. This approach has the potential to be a step forward in the production of gene activated matrices under very mild physical-chemical conditions. However, the very preliminary character of the obtained results imposes caution in their interpretation.

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Chapter 7

Conclusions

Conclusions

The PhD research project was aimed at designing and realizing biomaterials bioactivated with DNA, suitable for biomedical applications (i.e. gene therapy, tissue engineering and functional genomics). The potency of these materials, named gene activated biomaterials, was investigated. In particular, the possibilities for their implementation was verified in order to achieve both a controlled gene expression, through DNA immobilization instead of its simple dispersion, and a specific cell recruitment. Such an implementation was finalised at obtaining systems in which the external cells are recruited and forced to migrate into the material, where they find the bounded DNA and are transfected. To reach the aim of the research project, different solutions have been proposed and variable experiments have been performed. In particular, one research line of the entire project has been devoted at designing and realizing 3D gene activated collagen matrices. The results of the activities carried out in the framework of this research line have demonstrated that a forced migration through the matrix may be achieved by imposing a FBS concentration gradient, and that transfection efficiency may be enhanced by immobilising DNA complexes in the cellular microenvironment. A second research line of the project has been devoted at producing 3D gene activated PEG matrices with ordered, highly interconnected macroporosity using a templating microparticles process and with adsorbed DNA. The result obtained within this research line have highlighted that these systems can combine the benefits of influencing cell fate, by both DNA adsorbed to the matrix and interconnected porous structure, with the attractive properties of hydrogels (i.e. biochemical versatility, tissue-mimetic mechanical properties, and hydrophylicity). The third research line has been devoted to the realization of 3D DNA bioactivated PEG porous

matrices with immobilised DNA, and an appropriate RGD gradient. Despite their preliminary character, the obtained results stress that the realized systems have the potential to attract cells and guide their migration within the matrix, as well as to locally transfect them.

On the whole, the results of the activities carried out in the framework of the PhD research project show that engineered gene activated (both biologically derived or synthetic biomaterials based) matrices are able to recruit external cells and transfect them once internalized, therefore they can help in many biomedical applications.