Gene Delivery Using Dendrimer/pDNA Complexes Immobilized in Electrospun Fibers Using the Layer-by-layer Technique

MASTER DISSERTATION

Kirthiga Ramalingam
MASTER IN NANOCHEMISTRY AND NANOMATERIALS

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Thesis submitted to the University of Madeira for obtaining the degree of

Master in Nanochemistry and Nanomaterials

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and co-supervision of Dr. Shili Xiao

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September 2014

Kirthiga Ramalingam

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ABSTRACT

Tissue engineering is an important branch of regenerative medicine that uses cells, materials (scaffolds), and suitable biochemical and physicochemical factors to improve or replace specific biological functions. In particular, the control of cell behavior (namely, of cell adhesion, proliferation and differentiation) is a key aspect for the design of successful therapeutical approaches. In this study, poly(lactic-co-glycolic acid) (PLGA) fiber mats were prepared using the electrospinning technology (the fiber diameters were in the micrometer range). Furthermore, the electrospun fiber mats thus formed were functionalized using the layer-by- layer (LbL) technique with chitosan and alginate (natural and biodegradable polyelectrolytes having opposite charges) as a mean for the immobilization of pDNA/dendrimer complexes. The polyelectrolyte multilayer deposition was confirmed by fluorescence spectroscopy using fluorescent-labeled polyelectrolytes. The electrospun fiber mats coated with chitosan and alginate were successfully loaded with complexes of pDNA and poly(amidoamine) (PAMAM) dendrimers (generation 5) and were able of releasing them in a controlled manner along time. In addition, these mats supported the adhesion and proliferation of NIH 3T3 cells and of human mesenchymal stem cells (hMSCs) in their surface. Transfection experiments using a pDNA encoding for luciferase showed the ability of the electrospun fiber mats to efficiently serve as gene delivery systems. When a pDNA encoding for bone morphogenetic protein-2 (BMP-2) was used, the osteoblastic differentiation of hMSCs cultured on the surface of the mats was promoted. Taken together, the results revealed that merging the electrospinning technique with the LbL technique, can be a suitable methodology for the creation of biological active matrices for bone tissue engineering.

KEYWORDS: Electrospinning; layer-by-layer; gene delivery; human mesenchymal stem cells; osteoblastic differentiation

RESUMO

A engenharia de tecidos constitui um ramo importante da medicina regenerativa que usa células, materiais (matrizes) e fatores bioquímicos e físico-químicos adequados para melhorar ou substituir funções biológicas. Em particular, o controlo do comportamento celular (nomeadamente da adesão, proliferação e diferenciação celulares) é um aspeto muito importante a considerar no design de abordagens terapêuticas. Neste estudo, foram preparadas matrizes de poli(D,L-ácido láctico-co-glicólico) (do inglês, "PLGA") usando a técnica de electrofiação (os diâmetros das fibras situaram-se na escala micro). Adicionalmente, as matrizes foram funcionalizadas usando a técnica de camada-sob-camada (em inglês, "layer-by-layer", LbL) usando quitosano e alginato (polielectrólitos naturais e biodegradáveis com cargas opostas) como uma forma de imobilizar complexos de DNA plasmídico (pDNA) e dendrímeros. A deposição das multicamadas de polielectrólitos foi confirmada por espectroscopia de fluorescência usando polielectrólitos marcados com sondas fluorescentes. As matrizes revestidas com quitosano e alginato conseguiram ser carregadas com complexos de pDNA e dendrímeros de poli(amidoamina) (PAMAM) (geração 5) e de os libertar de forma controlada ao longo do tempo. Adicionalmente, estas matrizes mostraram ser capazes de suportar a adesão e proliferação de células NIH 3T3 e de células humanas estaminais mesenquimatosas à sua superfície. As experiências de transfecção com pDNA que codificava a enzima luciferase mostraram a capacidade das matrizes para funcionar como sistemas de entrega de genes. Quando foi usado um pDNA que codificava a proteína morfogenética do osso-2 (do inglês, "BMP-2"), a diferenciação osteoblástica das células estaminais mesenquimatosas foi promovida. No seu conjunto, os resultados revelaram que a técnica de electrofiação, juntamente com a de LbL, constituem metodologias adequadas para a criação de matrizes biologicamente ativas para a engenharia de tecido ósseo.

KEYWORDS: Electrofiação; camada sob camada; entrega de genes; células estaminais mesenquimais humanas; diferenciação osteoblástica

CONTENTS

LIST OF ACRONYMS

ALG – Alginate

ALP – Alkaline phosphatase

BL – Bilayer

BMP-2 – Bone morphogenic protein-2

bFGF – basic fibroblast growth factor

CHI – Chitosan

DMF – Dimethylformamide

DMEM – Dulbecco's modified Eagle's medium

2D,3D – Two dimensional, Three dimensional

EDC – 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

EDTA – Ethylenediaminetetraacetate

FITC – Fluorescein isothiocyanate

GFP – Green fluorescent protein

MW – Molecular weight

α-MEM – α-Minimum essential medium

hMSCs – Human mesenchymal stem cells

LbL – Layer-by-layer

Luc – Luciferase

PBS – Phosphate buffered saline

pDNA – Plasmid deoxyribonucleic acid

PEI – Poly(ethylene imine)

PEG – polyethylene glycol

PLGA – Poly (lactic-co-glycolic acid)

R-B – Rhodamine B

NHS – N-Hydroxysuccinimide

THF – Tetrahydrofuran

VEGF – Vascular endothelial growth factor

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CHAPTER 1

INTRODUCTION

1.1. The electrospinning technique

Tissue engineering, one of the key branches of regenerative medicine, is an interdisciplinary field that joins the area of materials engineering with that of life sciences towards the development of functional substitutes for injured or damaged tissues (Figure 1).[1] In its classic approach, tissue engineering combines cells, materials (scaffolds), and suitable biochemical and physico-chemical factors to obtain a biological tissue made *in vitro* that can be later implanted in the human body.[2] Among these, the scaffolds, where cells attach to generate the new tissue, play an important role. Scaffolds consisting of 2D or 3D networks can be prepared. Scaffolds are not only responsible for the structural support of the tissue under construction, but can also be used to provide biochemical and physico-chemical signals that are perceived by cells, and thus influence their proliferation and differentiation.[1-3] Scaffolds having a high surface area to volume ratio are usually good candidates for tissue engineering as they present a large area for tissue growth. Scaffolds should thus present adequate mechanical properties, and also the right porosity, pore size and pore interconnectivity to be used as a functional biomaterials in tissue engineering.[4]

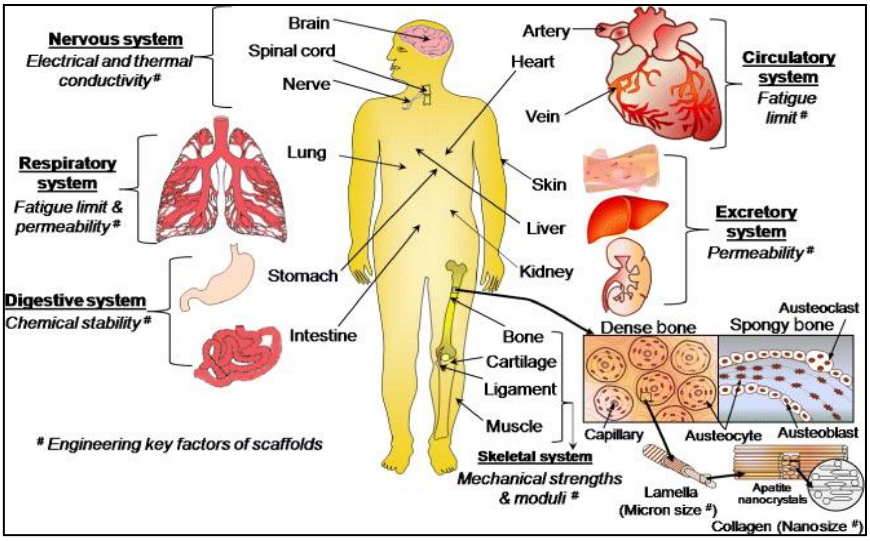


Figure1. Schematic representation of applications of tissue engineering.[5]

Recently, nano/micro fibrous scaffolds have drawn much attention due to their excellent physical and chemical properties, such as high aspect ratio, large specific surface area and high porosity.[6,7] These scaffolds may be used to mimic the extracellular matrix microenvironment and be useful in tissue engineering/regeneration applications.[8] In order to produce nano/micro fibrous mats/scaffolds, various procedures have been assayed, like mechanical drawing, template-based synthesis, phase separation, self-assembly and electrospinning.[9] Among these methods, electrospinning has been widely investigated by researchers as a cost-effective technology that can produce continuous non-woven fibrous mats with fiber diameters in the nano or microscale, a feature that distinguishes it from the classical non-woven fiber fabrication techniques.[10] Electrospun fibrous scaffolds possessing excellent properties (high surface-to-volume ratio, tunable porosity), provides the possibility to be prepared in different sizes and shapes.[11,12] In addition, the fibrous scaffolds can be made of different polymers/materials and its composition can be controlled to achieve the desired properties.

Electrospinning was first proposed by Anton Formhals in 1934.[13] However, only in the 90's it started to have the deserved attention.[14] The basic electrospinning system consists of four parts, namely the syringe pump, a source of high voltage, a spinneret, and a grounded collector.[15] Figure 2 shows a schematic representation of an electrospinning machine. The syringe pump is used to pressurize the polymer solution in the syringe. Throughout the process of fiber formation, a high voltage (tens of kV) is applied to the end of the capillary containing the liquid solution (usually a polymer solution). Once the electric field intensity increases, the hemispherical surface of the liquid at the end of the capillary extends, presenting a conical shape known as the "Taylor cone".[16] By increasing the electric field further, the critical value at which the repulsive electrostatic force overcomes the surface tension is reached, and a jet of liquid is ejected from the tip of the Taylor cone. Then, the strand of polymer solution goes through a process of instability and lengthening, during which solvent evaporates.[17] The fibers are then deposited on the collector, resulting in a non-woven fibrous layer.[18] Fibers can be deposited onto the collector in a random or in a ordered manner according to the desired applications.[19]

Electrospinning is, thus, a relatively simple method of producing nano/micro fiber mats where several parameters can be adjusted to tune the properties of the electrospun fibers. These parameters are generally divided into three groups.[20] First, one should consider the parameters related with the solution. Solution variables include the viscosity, the concentration, the molecular weight of the polymer, the surface tension, the dipole moment, the dielectric constant, and the conductivity (some of these properties are interdependent). Second, there are variables associated with the electrospinning process itself. These factors include the applied voltage, the distance of the electrode from the collector, the flow rate, the collector composition and

geometry, and the design of the needle tip. Finally, environmental variables should be considered, like the temperature, and the relative humidity.

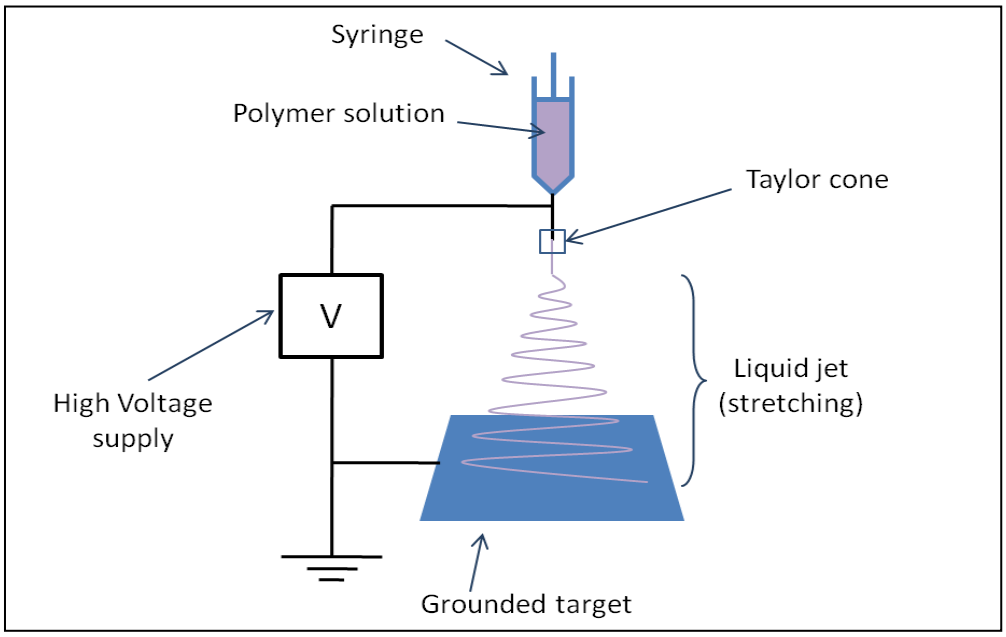


Figure 2. Schematic representation of electrospinning.[21]

Through electrospinning, various organic and inorganic/polymer hybrid composite nano/micro fibers have been produced for biological applications.[22,23] An important advantage of the electrospinning technique is that it can be used to prepare bioactive scaffolds for tissue engineering applications through the immobilization of growth factors and/or differentiation factors (or the genes that codify them) in the fibers by chemical or physical methods.[24] In this process, the bioactive molecules (proteins or genes) can be complexed (or chemically linked) to nanocarriers (non-viral vectors) to facilitate their uptake by cells.[25] For example, PEI/pDNA polyplexes (plasmids encoding VEGF and bFGF were used together with PEI as non-viral vector) emulsified in a mixture of PELA (obtained by bulk ring opening polymerization of lactide/PEG) and PEG (polyethylene glycol) solutions were electrospun to produce pDNA

containing nanofibers. The nanofibrous scaffolds thus prepared were able to promote cell attachment and viability, cell transfection and protein expression (extracellular secretion of collagen type IV and laminin). *In vivo* studies showed that, in comparison with pDNA polyplex infiltrated fibrous mats, these bioactive nanofibers diminished the inflammation reaction and enhanced the generation of microvessels and formation of mature vessels.[26] In another study, the bone morphogenetic protein-2 (BMP-2) plasmid was immobilized in fiber scaffolds in three different ways: after fiber preparation, by dripping with naked pDNA (A) or pDNA/chitosan polyplexes (B); by mixing the pDNA/chitosan complexes with the solution containing the fiber components before electrospinning (C). After, they analyzed each situation in terms of potential for bone regeneration *in vivo* (in nude mice). The experiments revealed that, after 4 weeks of treatment, the scaffolds of the group A had a better performance than the scaffolds of the group B. The scaffolds of group C released the pDNA more slower than the rate of bone healing and, so, it was not possible to take conclusions from those experiments. Overall, the results showed the potential of the electrospun nanofibers to deliver bioactive compounds. [27]

Indeed, the scaffolds that can release signaling molecules continuously after implantation in the body are expected to enhance tissue regeneration in the defect area and conduct to a more efficient therapy.

1.2. The layer-by-layer self-assembly technique (LbL)

The layer-by-layer (LbL) self-assembly technique is a simple, powerful and highly versatile technique that has a huge scientific and technologic interest since it can be used for the

preparation of functional materials with tailored properties. The LbL self-assembly technique was first introduced in the year 1992 by the scientist Gero Decher.[28] In a typical LbL assembly process, polyelectrolytes (polyanions and polycations) with opposite charges are alternately deposited over a surface based on their electrostatic interaction. The process leads to the formation of multilayered composite films with desired thickness. Figure 3 illustrates the LbL assembly process on fibrous scaffolds.

The major advantage of the LbL technique is that the thickness, morphology, and composition of the resultant films can be controlled by varying the number of polymer adsorption cycles and by changing the composition of the layer components.[29] By using the LbL assembly approach, a broad range of materials including polymers, nanoparticles, and biomolecules are able to be assembled onto substrates of varying geometry (e.g., planar surfaces, colloids, and fibers, etc.) to form functional nanostructured materials.[30] In particular, the combination of the LbL assembly technique with electrospun fibers has drawn much attention for producing multifunctional biomaterials to be used in tissue engineering. For example, Luo *et al.* fabricated novel carbon nanotube-containing nanofibrous polysaccharide scaffolding materials via the combination of electrospinning and LbL self-assembly techniques for tissue engineering applications.[31] In that study, electrospun cellulose acetate nanofibers were alternatively assembled with positively charged chitosan and negatively charged multiwalled carbon nanotubes or sodium alginate via the LbL technique. It showed that the incorporation of multiwalled carbon nanotubes in the multilayered cellulose acetate fibrous scaffolds tended to endow the fibers with improved mechanical properties and promote fibroblast attachment, spreading, and proliferation when compared with the native fibrous mats. The LbL technique is, indeed, suitable for biomedical applications since all the process is conducted in water and

because of the possibility to use it for the immobilization of bioactive molecules (growth and differentiation factors or the genes that codify them).[32,33] The technique may even allow the controlled delivery of bioactive molecules along the time.

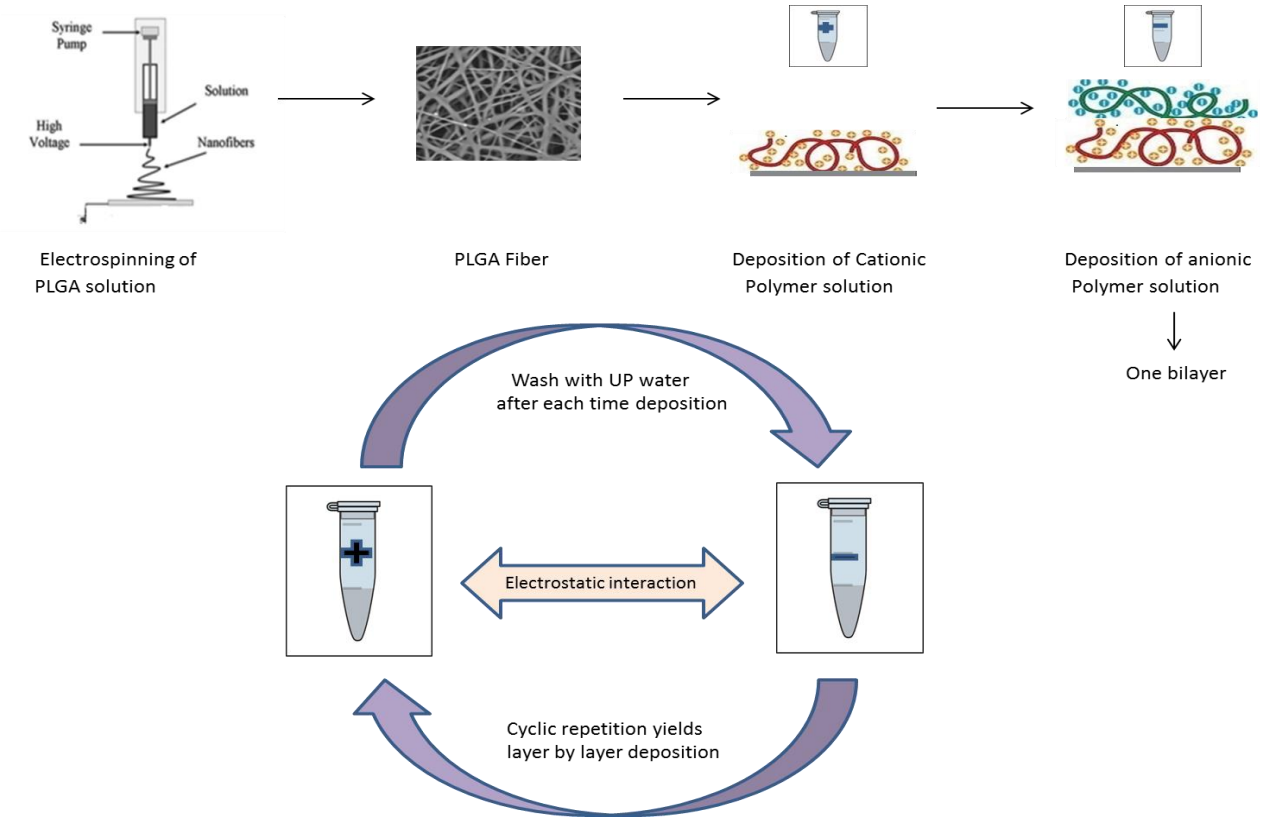


Figure 3. Illustration of the LbL technique applied to fiber mats. Adapted from ref.[34]

1.2.1. Polyelectrolytes used in the LbL technique

A polymer is the chemical association of small structural units called monomers. Hence, it is considered to be a large molecule. Polymers can be naturally derived (e.g. collagen, chitosan,

cellulose, etc.), as well as synthetically produced (e.g. polystyrene, polyethyleneglycol, polyacrylate, etc.).[35] Polyelectrolytes are polymers made of monomers that contain groups that can be ionized when the polymer is dispersed in an aqueous solution. These polymers can be further sub-divided into those where the charge density depends on pH (weak polyelectrolytes) and those where the charge density is independent of pH (strong polyelectrolytes).

In the present study, the polycation chitosan and the polyanion sodium alginate were used as polyelectrolytes for the LbL technique. This pair of polyelectrolytes is extensively used in biomedical applications.[36-38]

Alginate

Alginate (or alginic acid) is a naturally available anionic polysaccharide polymer which was first discovered by Stanford in 1881. Usually, for commercial purposes, alginate is obtained from brown seaweed.[39] Alginate has a linear structure and is composed of two types of uronic acids, namely guluronic (G) and mannuronic (M) acids (Figure 4). Indeed, it is a linear polymer made of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid, being a block copolymer of the two monomers.[40] The number ratio between M and G strongly determines the properties of alginate such as its swelling behavior, gel strength, etc..[41] The alginic acid as such is insoluble in water, so it is treated with salts, like sodium, potassium or ammonium, to become soluble in water.

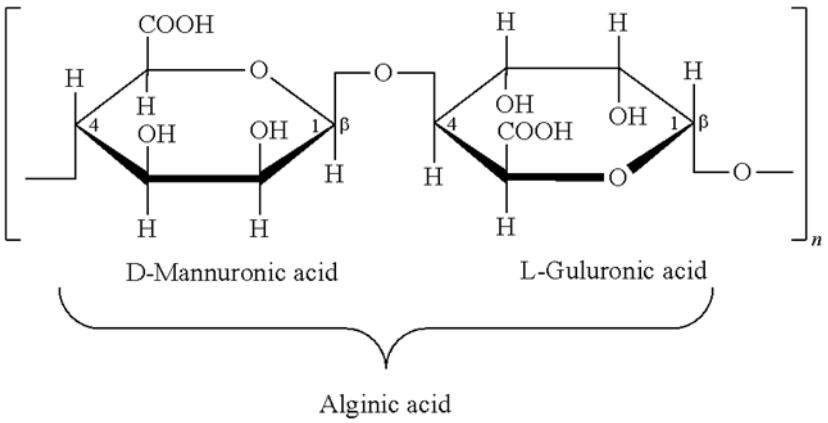


Figure 4. Chemical structure of alginic acid.

Due to its easy ionization in water, biocompatibility and biodegradability, alginate has found wide applications in the biomedical field, food and beverage industry, cosmetics, animal food, textile industry, etc..[42]

Chitosan

Chitosan is a well known cationic polysaccharide polymer that is produced by deacetylation of chitin which was discovered in 1811 by Henri Braconnot who worked on mushrooms. Chitin is naturally available from the exoskeleton of marine organisms such as crab, shrimps, etc.. It is also available from insects and plants.[43] Chitosan is a linear polymer composed of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine (Figure 5). The degree of deacetylation of chitosan is significant for its properties.[44,45] Chitosan has a high density of amino groups that confer it a positive charge at neutral pH.[46] Its biodegradability makes it widely used in the biomedical field, like in drug delivery, gene delivery, tissue engineering, etc..[47,48]

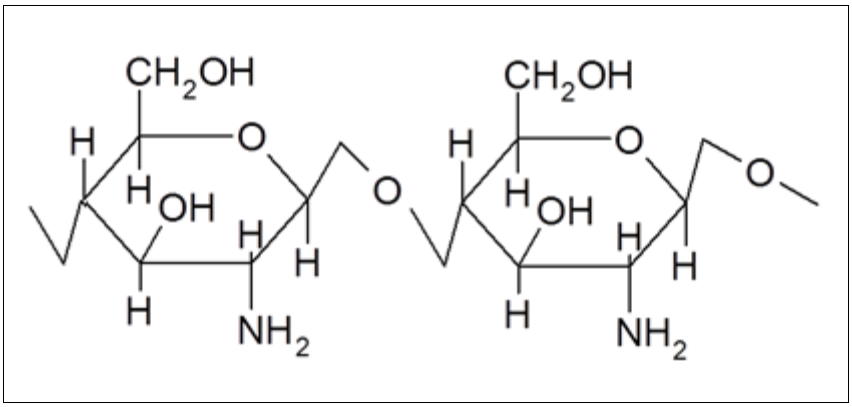


Figure 5. Chemical structure of chitosan.

1.3. Poly (lactic-co-glycolic acid) (PLGA)

Poly(lactic-co-glycolic acid) (PLGA) is a polymer largely applied in the biomedical field due to its biodegradability properties, that is, the ester linkages established between monomers can be easily hydrolyzed in the aqueous physiological environment (Figure 6).[49] The PLGA monomer molar ratio is an important parameter that is responsible for the polymer mechanical strength, degree of crystallinity, swelling behavior and capacity to be hydrolyzed.[50] Importantly, for the monomer molar ratio 50:50 (moles of lactic acid:moles of glycolic acid), the PLGA is reported to be hydrolyzed faster than for other monomer molar ratios.[51] It is reported that PLGA can undergo complete biodegradation in aqueous media.[52]

Nowadays, PLGA fibers are produced using the electrospinning technique to form tissue scaffolds.[53-55] However, the properties of the scaffolds are also strongly dependent on their preparation conditions. For instance, the mechanical strength of a scaffold and its porosity features are key factors in tissue engineering. If the mechanical properties/porosity features of a scaffold are not good enough, then the scaffold will not successfully meet its purposes.[56] The PLGA polymer is reported as being biocompatible, biodegradable and as adequate to form

porous scaffolds with the right level of porosity and pore interconnectivity, thus being very promising in tissue engineering approaches.[57,19]

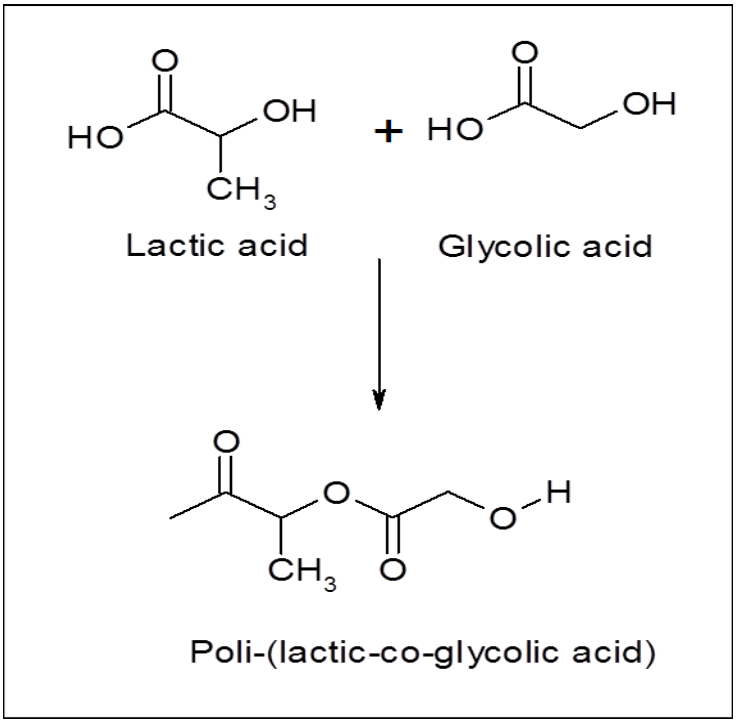


Figure 6. Structure of PLGA.

Due to its excellent properties, PLGA is approved by the European Medicine Agency as well as by the US Food and Drug Administration. It is also used in other biomedical applications aiming at tissue regeneration such as drug and gene delivery.

1.4. Poly(amidoamine) (PAMAM) dendrimers

Dendrimers are molecules with a special geometry as they grow radially from a central core, generation by generation, forming star-like structures (Figure 7). They have three distinct parts: the core, the central shell and the outer groups.[58] As the generation of the dendrimer grows, the

dendrimer starts to adopt a spherical shape.[59] There are two well established possible ways for the synthesis of dendrimers, namely convergent and divergent methods.[60] In the divergent method, the synthesis starts from the core of the dendrimer and proceeds outwards. In the convergent method, the dendrimer branches are first synthesized and then step-by-step assembled from the periphery towards the core of the final dendrimer. One important characteristic of dendrimers is that they have cavities in their interior (that, for instance, can be used to accommodate drugs) and a high number of terminal (surface) groups that can be functionalized (for carrying drugs or for linkage to specific chemical compounds that allow cell targeting).[61] Furthermore, in comparison to the classical polymers, the dendrimers have a well-defined shape and molecular weight (low polidispersity). For these reasons, dendrimers have been studied for a wide range of biological and medical applications (drug delivery, gene delivery, bioimaging, etc.).[62,63]

The poly(amidoamine) (PAMAM) dendrimers are the most studied dendrimers for biomedical applications.[64] Native PAMAM dendrimers have primary amines at their surface and tertiary and secondary (amides) amines in their interior. At the physiological pH, these dendrimers are positively charged and can interact with anionic molecules. In gene delivery studies, a plasmid DNA (pDNA) that codifies a certain bioactive protein may be complexed with PAMAM dendrimers and thus form dendriplexes.[65] Being negatively charged, the pDNA alone has difficulties in penetrating the membrane of cells that also possesses a negative charge due to the glycoproteins at their surface. In dendriplex formation, the charge of the pDNA molecule is neutralized and the molecule is compacted, thus favoring DNA uptake by cells.[66] It is reported that the efficiency of transfection mediated by dendrimers is generation and cell type dependent.

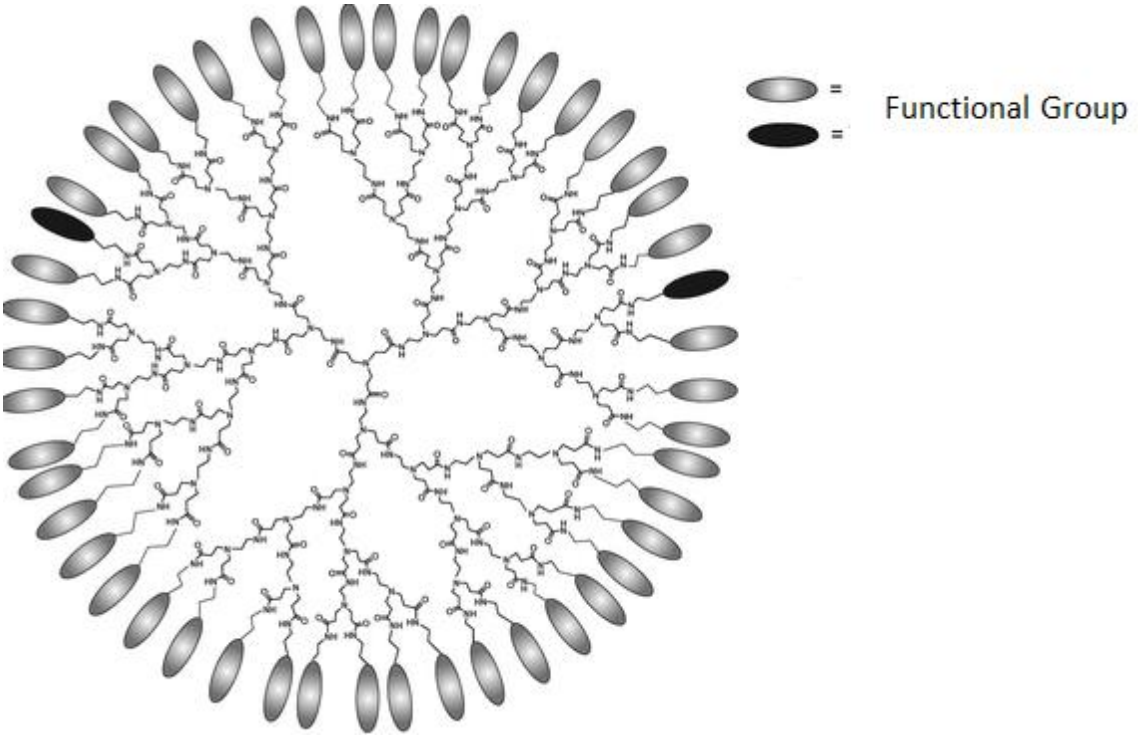


Figure 7. Schematic diagram of a generation 3 poly(amidoamine) (PAMAM) dendrimer. Note the presence of the functional end groups at the perifery. Adapted from ref. [67].

Dendrimers can even be functionalized at the surface to diminish their cytotoxicity (cationic dendrimers can be particularly toxic) or to specifically interact with cell surface receptors and facilitate the cell uptake process.[68] Indeed, numerous studies showed the efficacy of dendrimers as drug delivery vectors.[69]

1.5. Human mesenchymal stem cells (hMSCs)

Stem cells are cells that have the ability to regenerate themselves and to differentiate into different types of cells.[70] Stem cells exist in the embryo and also in the adult organism where

they participate in the regeneration of tissues and organs (hence, they can be termed as "cells giving life to particular organs").[71,72]

Because they have a wide ability for differentiation, stem cells have a particular interest in tissue engineering. For this purpose, autologous, allogeneic or xenogenic cells can be used. However, autologous cells are, in principle, more appropriated to be implanted in the human body as they don´t give rise to immunological reactions.[73,74] As such, stem cells can be removed from a patient, be propagated *in vitro* and then be used in tissue engineering constructs.

Mesenchymal stem cells (MSCs) are multipotent stem cells that can be obtained from the adult (without raising ethical concerns) and that, upon certain stimuli, can differentiate into several cell types, like adipocytes, chondroblasts, and osteoblasts.[75] These cells have also the advantage of proliferating very well *in vitro* and of being immunosuppressive (as such, cells from a different individual can be used in a patient).[76] Human mesenchymal stem cells (hMSCs) are thus very promising for tissue engineering, in particular for bone tissue engineering.

In order to differentiate into the desired cell type, the mesenchymal stem cells must be submitted to specific stimuli that can be of biochemical nature. Dexamethasone, for instance, is often use *in vitro* to induce the differentiation of MSCs into bone forming cells (osteoblasts).[77] Bone morphogenetic protein-2 (BMP-2) is also known for its ability to direct MSCs differentiation towards the osteoblastic lineage both *in vitro* and *in vivo*.[78] When bioactive proteins are used to direct MSCs differentiation, one should consider two possibilities: to use the protein itself or to use the gene codifying the protein.[79] The use of genes instead of proteins presents an important advantage. Indeed, as proteins easily denaturate and lose their activity, a high quantity must be used which can cause side effects in the body, whereas when genes are

used, the cells will produce the proteins at a physiological concentration. Indeed, a study by Santos *et al.* showed that it is possible to deliver the BMP-2 gene into MSCs using PAMAM dendrimers as gene delivery vectors to differentiate the cells into osteoblasts. This study further showed that a low transfection level may be sufficient to accelerate cell differentiation as cells will after communicate among them through paracrine mechanisms. Hence, it is possible to take the patient's own stem cells, genetically modify them *in vitro* and then implant them in the body attached to a scaffold. Alternatively, a bioactive scaffold can be used, from where genes codifying growth/differentiation factors will be release and transfect the stem cells existent in the body which will be recruited for the implant site. This second approach has the disadvantage that other types of cells can be transfected.

1.6. Objectives of the thesis

This master project aimed to study the possibility of using poly(lactic-co-glycolic acid) (PLGA) electrospun fiber mats as vehicles for gene delivery into human mesenchymal stem cells (hMSCs) having in view applications such as bone tissue engineering. Complexes of PAMAM dendrimers and plasmid DNA (codifying the bone morphogenetic protein-2, BMP-2) were immobilized in the electrospun fiber mats using polymers with opposite charges (chitosan and alginate) and the layer-by-layer (LbL) technique. hMSCs were then cultured in the gene-loaded electrospun fiber mats and their proliferation and differentiation towards the osteogenic lineage was evaluated.

In more detail, the project aimed:

a) The preparation of PLGA fiber mats using the electrospinning technique and their characterization by Scanning Electron Microscopy (SEM) and Contact Angle measurement;

b) The evaluation of the possibility of using chitosan and alginate as polymers for the coating of the PLGA fiber mats using the LbL technique; this was done using Fluorescence Spectroscopy;

c) The study of the ability of PAMAM dendrimers (generation 5) to compact pDNA through the PicoGreen® assay (pDNA/dendrimer complex formation);

d) The determination of the capacity of the coated PLGA fiber mats to load pDNA/dendrimer complexes; for this, the PicoGreen® assay was also used;

e) The study of the cumulative release of pDNA along time from the coated PLGA fiber mats; for this, the electrostatic interaction between the pDNA and the PAMAM dendrimers was disrupted and the amount of pDNA was obtained using the PicoGreen® assay;

f) The evaluation of the cell adhesion and cell proliferation on non coated and coated PLGA fiber mats; this was done using NIH 3T3 cells and the resazurin reduction assay;

g) The evaluation of the transfection efficiency using a pDNA codifying the luciferase enzyme and NIH 3T3 cells cultured on coated PLGA fiber mats;

h) The study of the proliferation and differentiation of hMSCs cultured over the coated PLGA fiber mats loaded with pDNA codifying for the BMP-2 protein.

CHAPTER 2

EXPERIMENTAL PART

2.1. Materials, reagents and cell culture media

Poly (D,L-lactide-co-glycolide) (PLGA) (M_W = 71,000, 1.25 g/cm^3, IV = 0.59 dl/g was bought from Jinan Daigang Company (China). Sodium alginate (ALG) (M_W = 50-120kDa), chitosan (CHI, from crab shells, 85% deacetylated), tetrahydrofuran (THF), dimethylformamide (DMF), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and N-Hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich®. Poly(amidoamine) (PAMAM) dendrimers, generation 5, with amino termini and an ethylenediamine core (G5) were acquired to Dendritech Inc. (USA). The dialysis membranes were from Spectrum® Laboratories and the filters used for sterilization (0.22μm) were from VWR®. The Quant-iT™ PicoGreen® dsDNA Kit was from Invitrogen®. The experiments also used: phosphate buffer saline solution (PBS; Gibco®, without calcium or magnesium), Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich®), α-Minimum Essential Medium (α-MEM; Gibco®), trypsin-EDTA solution (0.05% w/v, Gibco®), ascorbic acid (Sigma-Aldrich®), beta-glycerophosphate (Sigma-Aldrich®), dexamethasone (Sigma-Aldrich®), Reporter Lysis Buffer (RLB, Promega®), luciferase assay system (Promega®), bicinchoninic acid assay (BCA, Sigma-Aldrich®), sodium chloride (Merck®), sodium carbonate (Merck®), sodium bicarbonate (Merck®), magnesium chloride (Sigma-Aldrich®), p-nitrophenyl phosphate (Sigma-Aldrich®), acetic acid (Riedel-de Haen®), rhodamine B (Gibco®), and fluorescein isothiocyanate (FITC, Sigma-Aldrich®).

2.2. Preparation of PLGA mats by electrospinning

A PLGA (27.5% w/v) polymer solution was prepared by dissolving the PLGA solid in tetrahydrofuran (THF)/dimethylformamide (DMF) (3/1) solvent mixture.[80] The solution was left overnight for homogenization protected from light.

The homogenized PLGA polymer solution was fed into the syringe of the electrospinning equipment (FM1108, Beijing Future Material Sci-tech Co. Ltd, Ad. ZhongGuancun, Beijing P.R.China, Figure 8) and a high voltage (20 kV) was applied to the polymer solution. During this process, a charged jet was ejected from the needle and then undergone extensive stretching and thinning along with a rapid solvent evaporation. The distance between the needle tip to the collector plate was 15cm. While the jet traveled towards the grounded collector, the fibers were formed and collected on the aluminium foil (Figure 9). The fibrous mats were dried in the desiccator for a complete evaporation of the solvent (usually 2 days) and detached from the foil before use. The experimental conditions used in the electrospinning process are summarized in Table 1.

Figure 8. Electrospinning equipment.

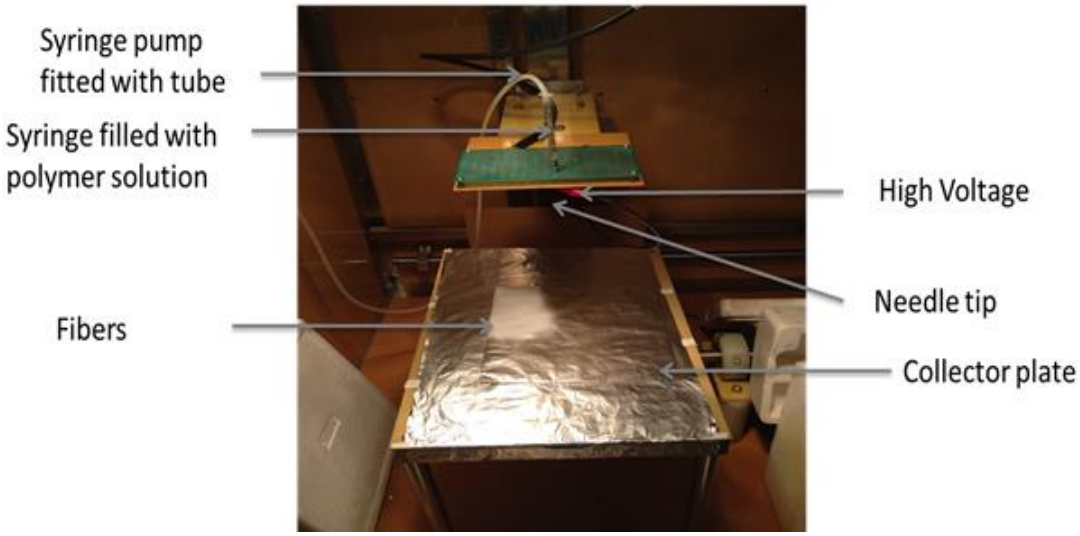


Figure 9. Electrospinning equipment (magnification of the internal constituents).

Table 1. Parameters used in the preparation of the electrospun PLGA fibers.

CONDITIONS USED	
Concentration of PLGA	27.5%
Collecting distance	15 cm
Syringe	1 ml
Nozzle size	8 mm
Voltage	+20 kV / -2.50 kV
Flow rate	0.004 mm/s
Relative Humidity (%)	38 to 40

2.3. Characterization of the electrospun PLGA fibers

2.3.1. SEM analysis of the electrospun mats

The morphology of the PLGA fibers was analyzed by scanning electron microscopy (SEM, JEOL, JSM-5600LV).[81] Before SEM observations, the samples were sputtered with gold (a film with a thickness of 10 nm was formed in their surfaces). The fiber diameters were measured using the Image J 1.40G software.

2.3.2. Porosity of the electrospun mats

The porosity of the electrospinning fibrous mats was calculated according to the following equations:[81]

$$\rho(g/cm^3) = m(g) \div d(cm) \times s(cm^2)$$

$$\text{Porosity (\%)} = (1 - \rho/\rho_o) \times 100\%$$

where ‘ρ’ is the apparent density of the electrospun scaffold, ‘m’ is the mass of the fiber, ‘d’ is the thickness of the scaffold, ‘s’ is the area of the electrospun scaffolds, and ρ_o is the density of the bulk polymer PLGA, which is 1.25 g/cm.[3,82] The electrospun fibrous mats were cut into 20 x 20 mm (8 samples) for these experiments. Each sample was weighed individually and it’s thickness was measured using the Vernier calipers (Trade Tools Ltd., China). To enhance the accuracy of the fiber thickness, all the 8 samples of the fiber mats were placed together in the same position and then the thickness was measured from the side corners, middle. Together with the area of the sample fibrous mats, these parameters were applied in the above mentioned equation to determine the apparent density. Then, the obtained apparent density is applied in the second equation to determine the porosity.[83]

2.3.3. Water contact angle (WCA) measurements

The electrospun PLGA fibers were cut into 20x20 mm and placed on the sample platform of a Drop shape analyzer (KRUSS, DSA100).[84] One sessile droplet of distilled water with 4μL volume was dropped onto randomly selected areas for each sample at ambient temperature and humidity. The static values of the water contact angle were measured when the droplet was stable. At least four measurements on each film were averaged for data analysis.[85] The Water contact angle was measured with DSA 100 Instrumental software.

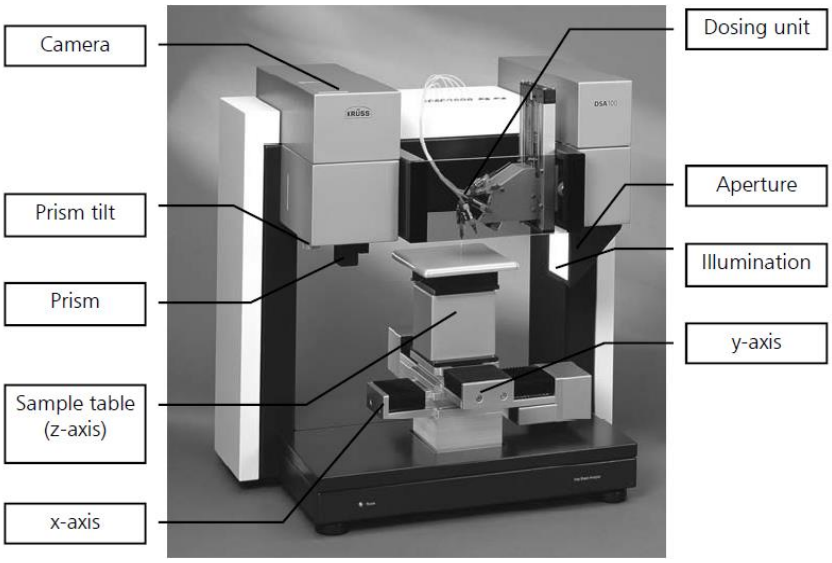


Figure10. Drop shape analyser – Adopted from KRUSS, DSA100 software program.

2.4. Preparation of chitosan and alginate solutions

2.4.1 Preparation of pristine sodium alginate and chitosan solutions

For the experiments, an alginate solution (1 mg/ml) was prepared in 0.15M NaCl. The pH of the alginate solution was adjusted to be 7.2 with 0.1M NaOH. A chitosan solution (1mg/ml) was also prepared. The chitosan powder was dissolved in 0.1M acetic acid and 0.15M NaCl. This solution was gently stirred overnight to become homogeneous. The pH of the chitosan solution was 3.4. This was not adjusted to physiological pH.

2.4.2. Preparation of rhodamine B labeled chitosan solution

The conjugation of chitosan with rhodamine B was summarized in figure 11.[86] Chitosan was dissolved in 20 ml of 0.1M acetic acid to prepare a 1% w/v chitosan solution and, in the next day, an equal volume of methanol was added. A rhodamine B solution was also prepared by dissolving 8 mg of the product in 4 ml of methanol. Before mixing with chitosan, rhodamine B was activated with a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) mixture for 3 hours (the concentration of EDC and NHS was two folds the concentration of rhodamine B). Then, the activated Rhodamine B was added dropwise to the chitosan solution under vigorous stirring at room temperature, in the dark, for 3 days. After that, the resulting solution was adjusted with NaOH (0.5M) to pH=10 in order to precipitate the conjugated compound. Then the compound was transferred to 15 ml tubes and centrifuged for 10 minutes at 15000 rpm. The supernatant was removed and the pellet was washed using ultrapure water. This step was repeated until no fluorescence supernatant was detected (7 centrifugations were done). Once the centrifugation was completed, the precipitate was dissolved in 10 ml of 0.1M acetic acid again and dialysed against 0.1M acetic acid solution for 3 days, followed by lyophilization to get the rhodamine B-conjugated chitosan compound. For the stock solution, the compound was dissolved in the mixture solution of 0.1M acetic acid and 0.15M sodium chloride. The final solution concentration was brought to 1mg/ml before use.

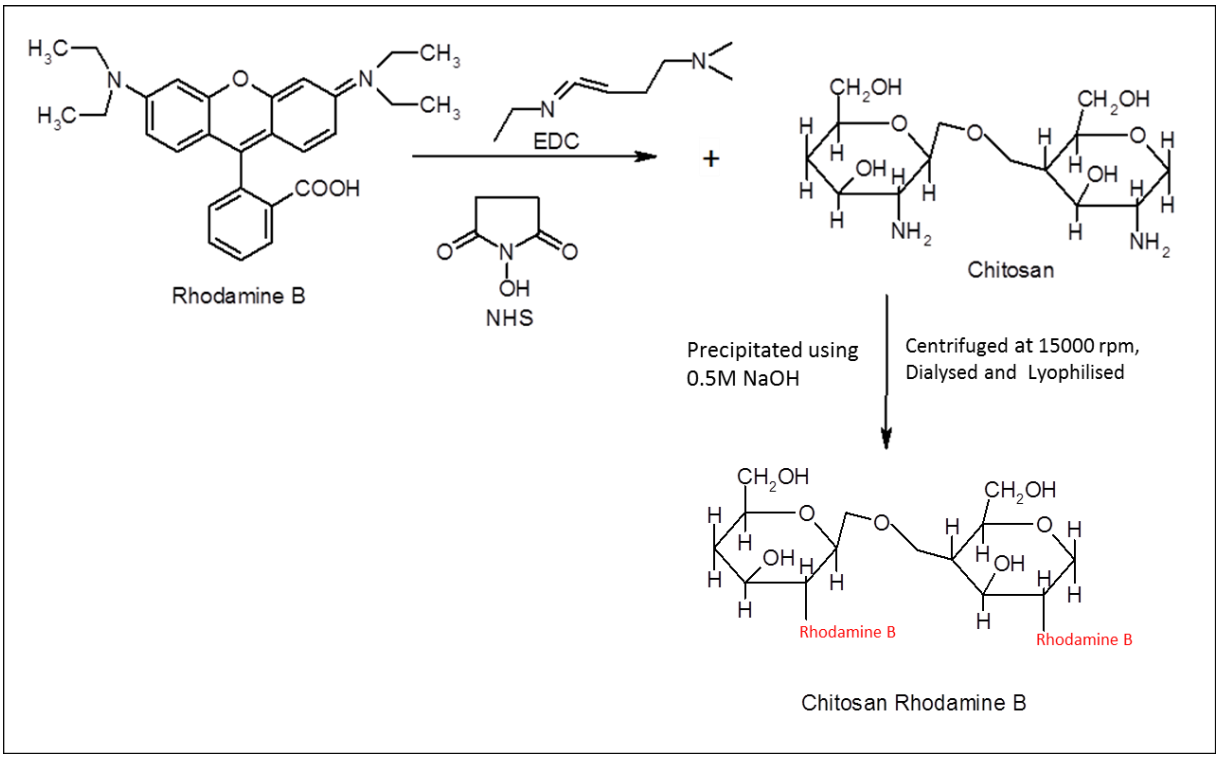


Figure 11. Conjugation of chitosan with rhodamine B.

2.4.3. Preparation of fluorescein isothiocyanate (FITC) labeled sodium alginate solution

The conjugation of alginate with FITC was summarized in figure 12.[87] An aqueous solution 1% w/v of sodium alginate was mixed with EDC/NHS (3:1) for the activation of the carbonyl groups on alginate in acetic acid buffer (pH 4.9) for 30 min, followed by addition of hexamethylenediamine (60 mg) and reaction for another 4 h. The mixture was precipitated in 2-propanol to remove the unreacted diamine. The alginate-amine derivative was then reacted with FITC (2 mg) in pH 9.0 sodium bicarbonate solution for 4 h and then dialyzed against distilled water for 2 days. The resulting alginate-FITC solution was lyophilized.

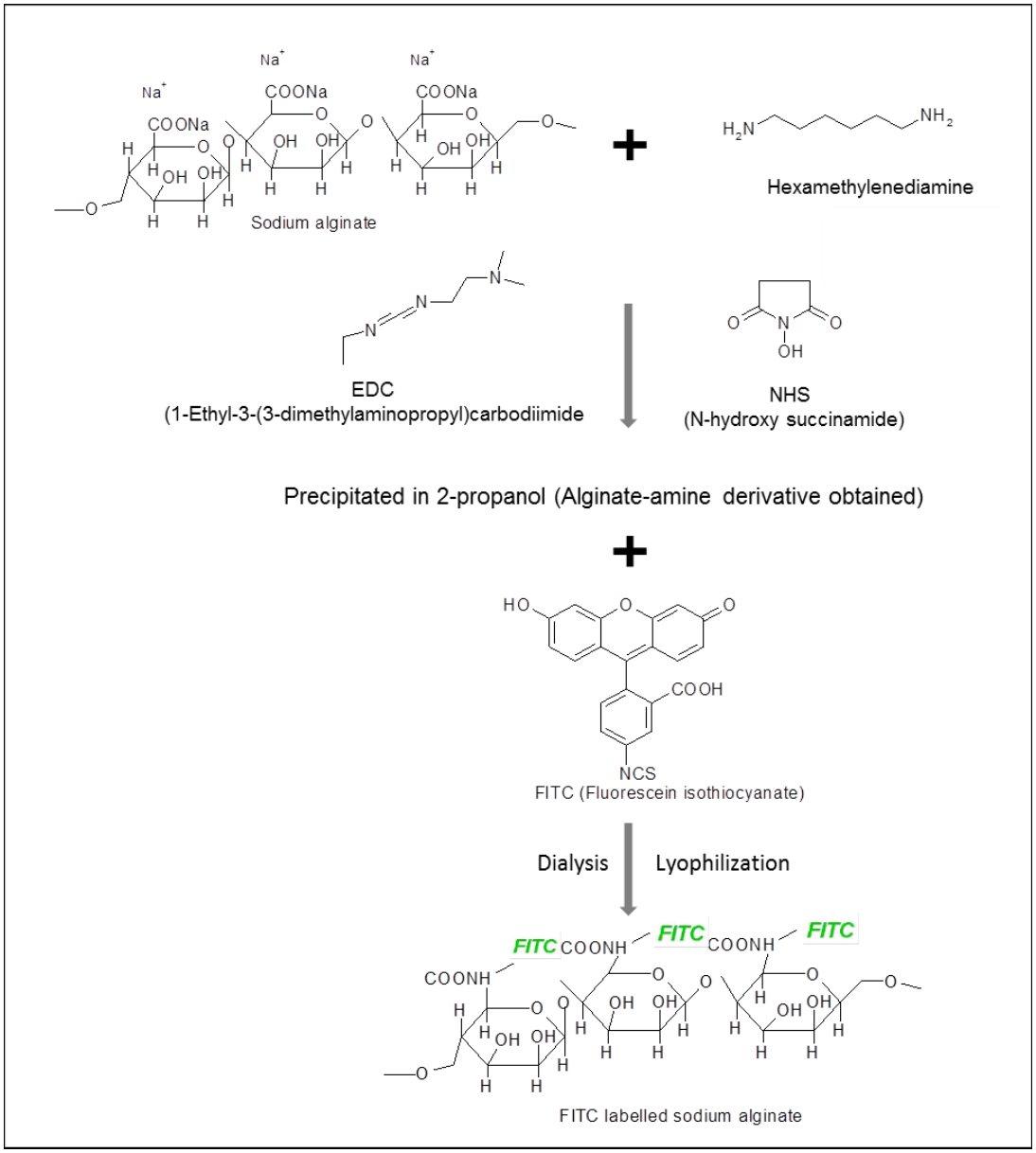


Figure12. Labeling of sodium alginate with FITC.

For the stock solution, the alginate-FITC compound was dissolved in 0.15M sodium chloride. The final solution concentration was brought to 1mg/ml before use.

2.5. Surface functionalization of the PLGA fibers using the LbL technique

The PLGA fiber mat produced using the electrospinning technique and deposited onto a aluminium foil was removed after drying and was cut into 1.5 x 1.5 cm^2 for use. Before functionalization, the PLGA fiber mats were used as covers of glass coverslips and placed inside the wells of 24-well cell culture plates.[88] The functionalization of the PLGA fibers with polyelectrolytes using the layer-by-layer approach was similar to what is described in the literature.[89-91] In a typical procedure, the PLGA fibrous mats were first immersed into chitosan(1mg/ml) solution for 10 min, followed by rinsing with water three times (each rinsing step took 2 minutes). Then, the substrates were immersed in the negatively charged alginate(1mg/ml) solution for 10 min, followed by similar rinsing steps in water. The immersion/rinsing cycles were repeated until the desired number of bilayers was achieved.[92] In this work, a bilayer is obtained after one deposition cycle (so, it consists in one layer of chitosan and one layer of alginate, by this order). Two bilayer-coated PLGA fibers were used in the experiments done in this thesis.

The polyelectrolyte deposition process over the PLGA fiber mats was followed by fluorescence spectroscopy by using rhodamine B-labeled chitosan (excitation wavelength 540nm and emission wavelength 625nm) and FITC-labeled alginate (excitation wavelength 495nm and emission wavelength 519nm). To avoid possible crossing interferences in the absorption and emission processes, the study was first made with rhodamine B-labeled chitosan and pristine alginate. After, a similar study was done with pristine chitosan and FITC-labeled alginate. The fluorescence measurements were made using a microplate reader (Victor^3 1420, Perkin Elmer).

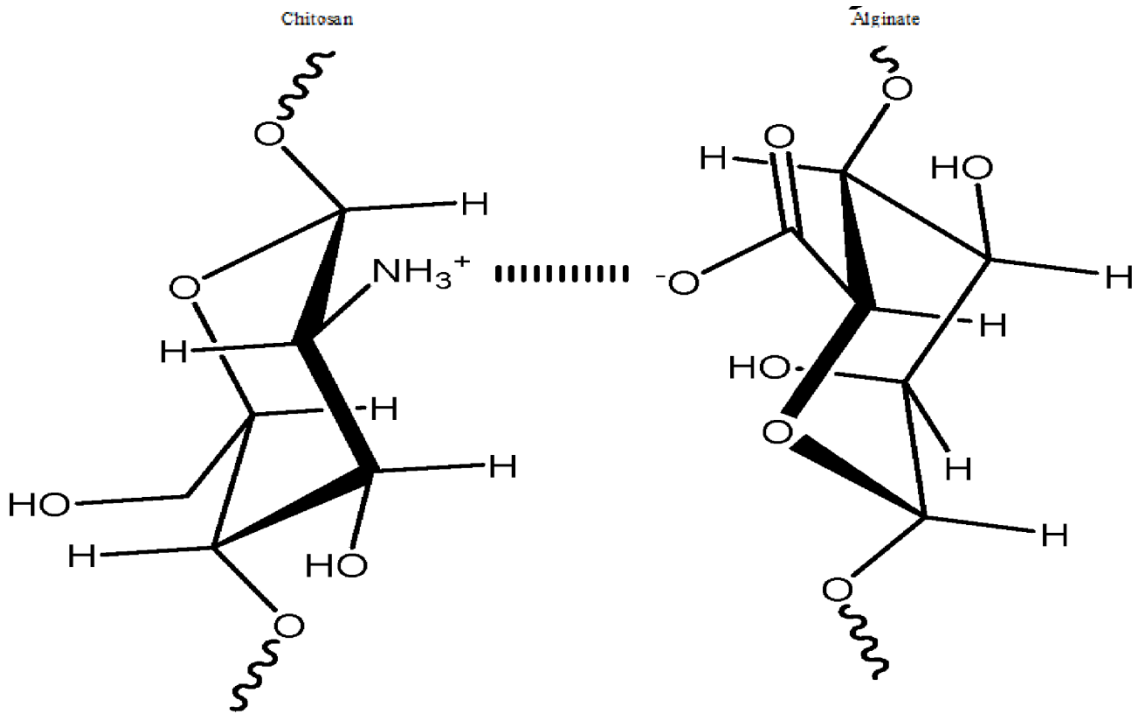


Figure 13. Interaction between positive charged chitosan and negative charged alginate.[93]

2.6. Plasmid propagation and isolation

Two kinds of pDNAs were used in the present work: (a) a pDNA encoding luciferase (Luc) and enhanced green fluorescent protein (EGFP) (pEGFPLuc, 6.4 kb); (b) a pDNA encoding the bone morphogenetic protein-2 (BMP-2) (pcDNA3.1/Hist©/hBMP2, 8.5 kb, kindly donated by Prof. Yasuhiko Tabata, University of Kyoto, Japan). Both plasmids were amplified in *Escherichia coli* host strain, DH5α, grown overnight in Luria-Broth Base medium containing ampicillin. Afterwards, the plasmids were isolated and purified using a Qiagen Plasmid Maxi Kit (Qiagen). The isolated plasmids were dissolved in a small amount of ultra-pure water and the concentration of plasmid DNA in solution was determined from the UV absorbance at 260 nm.[94] For

evaluation of plasmid purity, the absorbance ratio at 260nm and 280nm was measured and was always found to be in the range of 1.7 and 1.9.

2.7. Dendrimer/pDNA complex assembly

Dendrimer/pDNA complexes were prepared at several N:P ratios (where N= number of primary amines in the conjugate; P= number of phosphate groups in the pDNA backbone) by mixing adequate volumes of a solution of poly(amidoamine) (PAMAM) dendrimers (G5, generation 5, with amino termini and an ethylenediamine core) (1mg/ml) and a pDNA solution (0.1µg/µl).[95] PBS solution (pH= 7.4) was always used to prepare these solutions. Complex solutions were vortexed and allowed to incubate for 20 minutes at room temperature prior to experiments. The N:P ratios selected to study pDNA compactation were 0, 0.5, 1, 2, 4, 6 and 8, respectively. The N:P ratio used when pDNA was immobilized in the electrospun fiber mat was 5. At this ratio, the size of the complexes was measured at 633 nm on a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments) using a detection angle of 173°. Zeta potential measurements were also performed using a detection angle of 17° and were calculated using the Smoluchowsky model for aqueous suspensions. The data presented are means of three independent measurements.

2.8. PicoGreen® intercalation assay

The ability of the dendrimers to compact pDNA was studied using the PicoGreen® assay.[96] PicoGreen® is a fluorescent dye that emits light when bonded to double strand DNA. When the

DNA is completely free in solution, the intensity of the fluorescence attains a maximum level. When the DNA is compacted due to complexation with the dendrimer, PicoGreen® cannot bind DNA and the fluorescence intensity decreases.

PicoGreen® was diluted 200x in Tris-EDTA buffer (10mM Tris, 1mM EDTA, pH = 7.5) according to the manufacturer's instructions. Then, 100 µl of this solution was added to 100µl of each dendrimer/pDNA complex solution. The resultant mixture was incubated for 5 minutes at room temperature in the dark. The resultant mixture was then transferred to a 96-well plate and PicoGreen® fluorescence was measured using a microplate reader (Victor^3 1420, Perkin Elmer) at excitation wavelength 485nm and emission wavelength 535nm. A blank was prepared with 100µl of PBS and 100µl of the diluted PicoGreen® solution. Three independent experiments were performed and the % of relative fluorescence (%F) was determined using the following equation:

$$\%F = \frac{F_{sample} - F_{blank}}{F_{DNAonly} - F_{blank}} \times 100$$

The fluorescence from free DNA was considered to be 100% (N:P ratio 0).

2.9. Immobilization of the pDNA/dendrimer complexes on the PLGA fiber mats

Fiber mats without and with polyelectrolyte coverage (2BL, 2 bilayers of CHI/ALG polymers) were used in the experiments. The desired amount of dendrimer/pDNA complex (N/P=5, pDNA = 1µg/well or 2µg/well) was dispersed in 400µl PBS solution and added to each well of a 24-well plate containing the fiber mats, followed by 30 min incubation at room temperature. The amount of dendrimer/pDNA complex loaded on the PLGA fiber mats was indirectly determined

by measuring the quantity remaining in solution. For that, 100µl of the gathered supernatant was added of an equal volume of sodium alginate solution (6mg/ml) and shaked gently for 1 h (37°C, 217 rpm/min) to disrupt the electrostatic interactions between the dendrimer and the pDNA. Then the amount of pDNA in solution was quantified using the PicoGreen® assay. For the quantification, a calibration curve was constructed using a series of pDNA standard solutions with increasing concentrations, in the presence of sodium alginate (6mg/ml).

2.10. pDNA/dendrimer complex release from the PLGA fiber mats

After loading the fiber mats with the complexes and quickly rinsing with ultrapure water to remove the non-bonded complexes, 400µl PBS solution was added to each well and the release of the complexes was studied over a period of 24 h.[97] The experiments were conducted at 37°C, under shaking. An initial load of 2ug/well pDNA was used in each well. Samples were taken at different time intervals and analyzed using the PicoGreen® assay after disruption of the electrostatic forces existent between the dendrimers and the pDNA, and using the methodology described in the previous section.

2.11. Biological assays

2.11.1. Cells and cell culture

The main equipment used in the biological assays was a CO_2 incubator (Nuaire, AutoFlow IR Direct Heat), a laminar flow chamber (Nuaire, Class II A/B3), an inverted optical microscope (Olympus, CK40), an inverted fluorescence microscope (NIKON, TE2000), and a microplate

reader (PerkinElmer, Victor3™). All cell culture dishes were from NUNC. All cell cultures were incubated at 37°C, in a humidified atmosphere of 95% air and 5% carbon dioxide. NIH 3T3 cells (a cell line derived from primary mouse embryonic fibroblasts) were cultured in DMEM (Sigma-Aldrich®) and hMSCs were cultured in α-MEM (Gibco®) supplemented with 10% fetal bovine serum (FBS; Gibco®) and 1% of an antibiotic-antimycotic solution (Gibco®, with 10,000 units penicillin/ml, 10 mg streptomycin/ml and 25µg amphotericin B/ml). The primary cultures of hMSCs were established from small pieces of human trabecular bone rich in bone marrow which were obtained from patients during surgery interventions after trauma. Only bone that would have been discarded was used, with the approval of the Dr. Nélio Mendonça Hospital (Funchal main hospital) ethics committee. The trabecular bone was kept in sterilized tubes, refrigerated, in α-MEM supplemented with 10% FBS and 1% antibiotic–antimycotic solution, for 3 to 4 h. Then, primary cultures were prepared by gently shaking the tubes with the bone marrow pieces to obtain a cell suspension, which was spread by cell culture dishes. The culture medium was removed and changed by new one after 1 day to remove the non-adherent hematopoietic cells. The medium was always changed twice a week until confluence was achieved. The cultures were then passaged and frozen in liquid nitrogen using the standard procedure.

Cell passages were always done using trypsin (0.05% w/v) and following the standard protocol. The media and solutions used in the cell culture assays were prepared as follows.

Dulbecco's Modified Eagle's Medium (DMEM)

A stock solution of DMEM was prepared by adding the commercial DMEM powder and $NaHCO_3$ to distilled water following the instructions present in the DMEM bottle label. The pH of this solution was first adjusted to 7.4 using 1M NaOH or 1M HCl solution and the medium was then sterilized by filtration (0.22µm filter). Complete DMEM (250ml) medium was prepared by mixing 25 ml (10% v/v) of FBS, 2.5 ml of antibiotic solution (1% v/v) and 222.5ml of DMEM (stock). The fresh complete DMEM solution was kept refrigerated at 4^0C before use.

α-Minimum Essential Medium (α-MEM)

A stock solution of α-MEM was prepared by adding the commercial α-MEM powder and $NaHCO_3$ to distilled water following the instructions present in the α-MEM bottle label. The pH of DMEM solution was first adjusted to 7.4 using 1M NaOH or 1M HCl solution and then sterilized by filtration (0.22µm filter). Complete α-MEM (200ml) medium was prepared by mixing 20ml of FBS (10% v/v), 2ml of the antibiotic-antimycotic solution (1% v/v), and 178ml α-MEM stock solution. The fresh complete α-MEM solution thus prepared was kept refrigerated at 4^0C before use. For osteogenesis induction, the complete α-MEM medium was further supplemented with ascorbic acid (250µM), beta-Glycerophosphate (5mM), and dexamethasone (10^{-9} M).

Phosphate buffer saline (PBS)

The phosphate buffer saline (PBS) solution was prepared by dissolving 9.55g of PBS powder in 1L of distilled water. The pH of PBS solution was first adjusted to 7.4 using 1 M NaOH or 1 M HCl solution and then sterilized by filtration (0.22μm filter).

2.11.2. Cell attachment and proliferation on PLGA fiber mats

Cell attachment and proliferation on the PLGA electrospun mats was evaluated using NIH 3T3 cells directly cultured on the electrospun PLGA fibrous mats.[98] The mats were cut, mounted on circular glass cover slips (diameter of 14 mm) and then fixed in 24-well plates. For sterilization, the mats were initially left under UV radiation overnight, followed by soaking with 75% ethanol solution for 1.5 h and, ultimately, washed three times with PBS solution. The electrospun mats were then soaked in culture medium overnight prior to cell seeding. For the cell attachment and proliferation experiments, NIH 3T3 cells were detached from the culture dish using trypsin-EDTA solution (0.05% w/v) and seeded on the top of the mats at a concentration of 2×10^4 cells/well. Both fiber mats with and without polyelectrolyte coverage (2BL, 2 bilayers of CHI/ALG polymers) were used in the experiments. Control experiments were conducted with cells cultured directly on the plastic surface of the cell culture dishes and on the glass surface of the coverslips.

Cell adhesion was evaluated at 1 h, 3 h, and 6 h of cell culture. Cell proliferation was studied from 1 day until 7 days in culture. Cell adhesion/proliferation was studied based on the resazurin reduction cell metabolic assay. The resazurin reagent was prepared by mixing 0.02g of resazurin with 200ml of PBS solution (resazurin stock solution). This solution was sterilized with

a 0.22µm membrane inside the laminar flow hood chamber. The prepared resazurin solution was distributed into small tubes and stored at -20^{0}C. At the appropriate time point, the medium in each cell culture well was replaced by fresh medium (1ml) containing 10% v/v of resazurin stock solution and the cells were further incubated for 3 h. Subsequently, 100µl of the resultant medium was transferred to 96-well plates (white, FluoroNunc® 96 well plates) and the resorufin fluorescence (λ_{ex}= 530nm, λ_{em}= 590nm) was measured in the microplate reader. All experiments were conducted in triplicate. Blank experiments were done using wells containing PLGA electrospun mats but without seeded cells.

2.11.3. *In vitro* gene transfection study using a pDNA encoding luciferase (Luc)

The preparation, sterilization and pDNA/dendrimer complex (prepared with the pEGFPLuc plasmid) loading of the PLGA fibrous mats were performed according to the procedures already mentioned. In these studies, NIH 3T3 cells (2×10^4 cells/well) were seeded in the wells of 24-well plates containing pDNA/dendrimer-loaded fibrous mats, with and without polyelectrolyte coverage. Control experiments were performed using fibrous mats samples without pDNA/dendrimer-loading, with and without polyelectrolyte coverage (2 bilayers). Another control experimented consisted in cells directly cultured on the glass surface of the cover slips. In all cases, cells were allowed to grow for 48 h and then cell viability was measured (using the resazurin reduction method previously described) and further harvested for luciferase activity and total protein content analysis. During cell harvesting, cells were first washed once with PBS solution, and then 100µl of lysis buffer (1x, Reporter Lysis Buffer, Promega) was added to each

well. Homogenized cell lysates were obtained after three cycles of freezing (-20°C, 20 min)/thawing (room temperature, 90 rpm shaking). Cell lysates were transferred to an eppendorf for luciferase and protein analyses. Luciferase activity in cell lysates were measured using the Promega's luciferase assay reagent (the supplier's instructions were followed).[99] For each sample, the microplate reader was set for 3 s delay with signal integration for 10 s. The amount of protein in cell lysates was determined using the bicinchoninic acid assay (BCA assay) using bovine serum albumin as a standard.[99]

2.11.4. *In vitro* gene transfection study using a pDNA encoding the bone morphogenetic protein-2 (BMP-2)

These experiments were very similar to those described in the previous subsection but used hMSCs (passage 3) and a pDNA encoding the bone morphogenetic protein-2 (BMP-2) (pcDNA3.1/Hist©/hBMP2). Also, cell culture was performed along 21 days. For these experiments, α-MEM containing ascorbic acid (250 µM) and beta-glycerophosphate (5mM) was used. Osteogenesis positive control experiments were also conducted using dexamethasone in the cell culture medium (10^{-9} M).[100] For all cases, the medium was replaced every 3 days and cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Cell viability was analyzed after 14 days of cell culture using the resazurin reduction assay. Alkaline phosphatase (ALP) activity was measured after 14 and 21 days of culture, as well as the total protein content (BCA assay). The hMSCs were first rinsed 3 times with PBS. Then, 200 µl of Reporter Lysis Buffer was added to each well and homogenized cell lysates were

obtained after three cycles of freezing (-20°C, 20 min)/thawing (room temperature, 90 rpm shaking). Cell lysates were stored at -20°C for further analysis. Alkaline phosphatase (ALP) activity was determined using a colorimetric assay involving the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol (yellow). Briefly, 20 µl of the cell lysates were mixed with 200 µl of ALP substrate and incubated for 1 h at 37°C in the dark. Then, 10 µl of 0.02M NaOH was added to each well to stop the hydrolysis reaction. The absorbance was read at 405 nm and the p-nitrophenol content was calculated from a standard calibration curve. The results are presented using arbitrary units but express the number of moles of p-nitrophenol produced by unit of time and by µg of protein.

CHAPTER 3.

RESULTS AND DISCUSSION

3.1. Preparation and characterization of the electrospun PLGA fibers

Fiber mats made of poly (L-lactic-co-glycolic acid) (PLGA) were successfully prepared using the electrospinning technique and experimental conditions reported in the literature. Indeed, the polymer solution properties and the electrospinning processing parameters are of vital importance for the surface and mechanical properties of the electrospun fibers, as well as for their uniformity.[101] Figures 14A and 14B show the morphology of the obtained electrospun fiber mats after visualization by scanning electron microscopy (SEM). We can observe that the mats mainly consist in fibers with diameters in the micrometer range.

The porosity of the fiber mats was also experimentally determined and, as expected, its value was very high (97.47±0.05%). Thus, this indicates that the mats should be appropriate for tissue engineering applications since cells can growth inside the pores. Furthermore, by detailed analysis of the SEM images, one can conclude that the pores are interconnected, which is a very important requirement in tissue engineering.

The PLGA fiber mats were also characterized by contact angle measurement to evaluate its wettability properties which can strongly determine the interactions of a surface with biomolecules and cells. The contact angle was found to be $115^0 \pm 5^0$, which indicates that the PLGA fiber mat surface is relatively hydrophobic. This is in accordance with the literature data.[102]

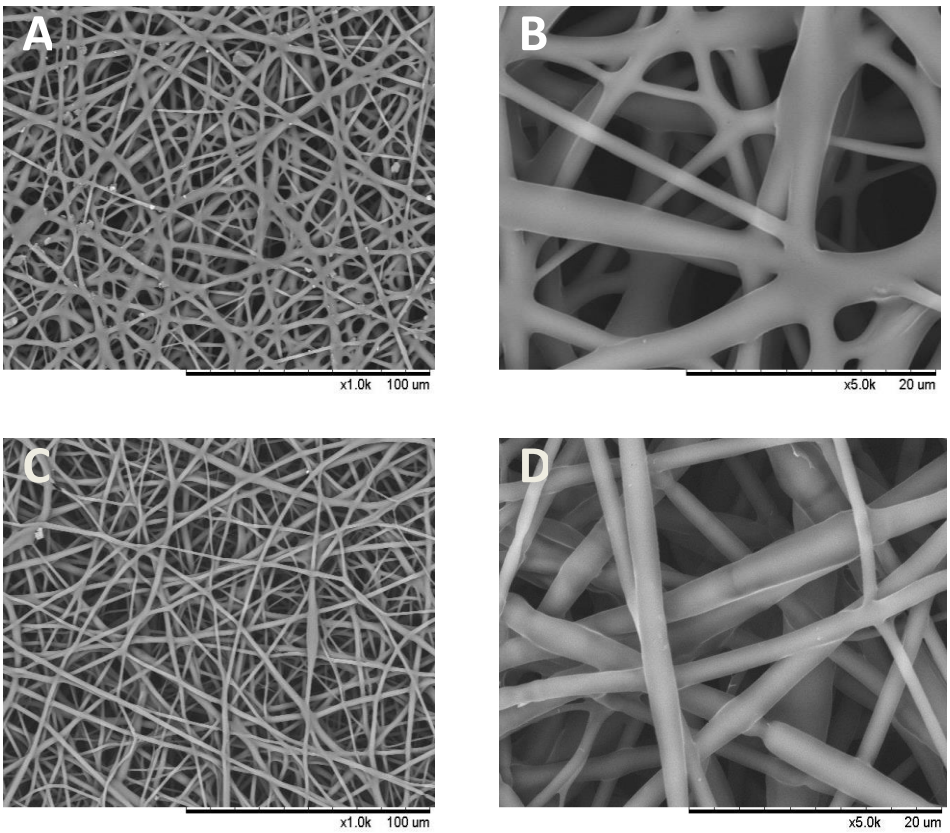
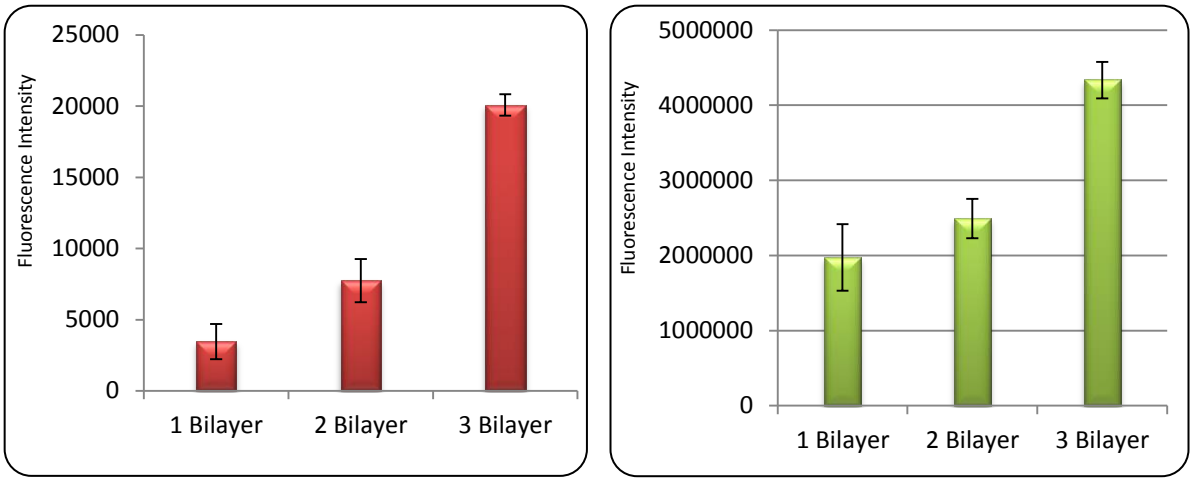


Figure 14. SEM images of electrospun PLGA fiber mats before (A and B) and after the deposition of a bilayer of 2 polyelectrolytes (C and D). B and D are the magnified images of A and C, respectively.

3.2. Modification of the electrospun PLGA fiber mats with polyelectrolytes using the LbL technique

As a mean of immobilizing the pDNA/dendrimer complexes (that are positively charged),[103] the surface of the electrospun fiber mats was modified using the LbL technique and chitosan (cationic) and alginate (anionic) as oppositely charged polyelectrolytes. Each bilayer consisted in the deposition of a first layer of chitosan and a second layer of alginate. After coating the surface

of the PLGA fiber mats with two polyelectrolyte bilayers, no significant change was observed in their morphology (Figure 14C and D). However, the contact angle decreased in some extent (Table 2) in relation to non-coated fiber mats as polyelectrolyte deposition enhanced the wettability of the surface due to the charged nature of the polyelectrolytes. On the other hand, there was no significant change in the contact angle with the increase of the number of polyelectrolyte bilayers deposited onto the mats.



A B

Figure 15. (a) Fluorescence intensity of PLGA fiber mats coated with rhodamine B-labeled chitosan/alginate as a function of the number of bilayers; (b) Fluorescence intensity of PLGA fiber mats coated with chitosan/FITC-labeled alginate as a function of the number of bilayers.

The success of the LbL process was confirmed by performing independent experiments with rhodamine B-labeled chitosan plus pristine alginate (Figure 15A), and pristine chitosan with

FITC-labeled alginate (Figure 15B). It is obvious that the fluorescence intensity increased with the number of polyelectrolyte bilayers (a linear increase was even observed when rhodamine B-labeled chitosan was used), which demonstrates the successful coating of the PLGA fiber mats using the LbL technique.

Table 2. Water contact angle of non-coated and coated PLGA electrospun fiber mats.

Note: Random areas of the fiber mats were analyzed and the average value is indicated.

Number of bilayers	Water contact angle
0	$115^0 \pm 5^0$
1	$106^0 \pm 11^0$
2	$101^0 \pm 10^0$
3	$106^0 \pm 6^0$

3.3. pDNA/dendrimer complex immobilization and release from the electrospun PLGA fiber mats

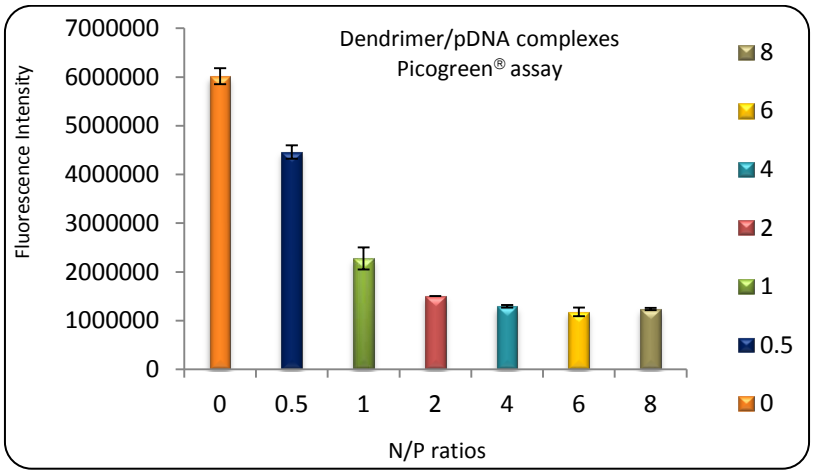
PAMAM dendrimers with amino termini (G5, generation 5) have been shown to efficiently bind pDNA through electrostatic interactions since they are positively charged at the physiological pH.[104] These molecules have the ability to neutralize the charge of the pDNA and also of compacting it. In fact, these dendrimers have a high density of surface active amines that make

them good gene delivery carriers and, also, confer them buffering capabilities that help them to escape from the acidic endosomal compartments (through the known "proton-sponge effect").[105]

In the present work, the capacity of G5 PAMAM dendrimers to condense pDNA was confirmed (Figure 16A) by preparing the complexes at different N:P ratios and evaluating the amount of "free pDNA" using the PicoGreen® assay (this is a fluorescent dye that intercalates in the double helix of DNA). The results show that a maximum of pDNA compactation is achieved for N:P ratios higher than 4. As such, a N:P ratio of 5 was chosen for all the following experiments. At this N:P ratio, the hydrodynamic diameter and surface zeta potential of the complexes were experimentally determined in PBS solution, being 422 ± 20 nm and +6.5 ± 0.9 mV, respectively. Indeed, it is reported that complexes of G5 PAMAM dendrimers and the same type of plasmid (pEGFPLuc, 6.4 kb), at the N:P ratio of 5, present a size around 80 nm and a zeta potential value higher than +25 mV.[106] The differences between the present results and those found in the literature may be due to the different buffer used in the preparation of the complexes. What is important is to highlight the fact that, by being positive, these nanoparticles can be deposited on the top of the polyelectrolyte coated electrospun fiber mats that have alginate as the last deposited polymer.

The complex loading efficiency of the electrospun fiber mats was studied for uncoated and coated samples and for two different initial pDNA concentrations in the wells (1 µg/well and 2 µg/well). The loading process consisted in the incubation of the fiber mats with a solution containing the pDNA/dendrimer complexes for 30 min (this time period was optimized for maximum loading), followed by washing to remove the complexes in excess. The amount of pDNA present in the withdrawn solution was determined using the PicoGreen® assay (after disrupting the electrostatic forces within the complex with alginate) and the quantity of loaded

pDNA was calculated by difference from the initial amount of pDNA (in a complexed form) added to the wells.



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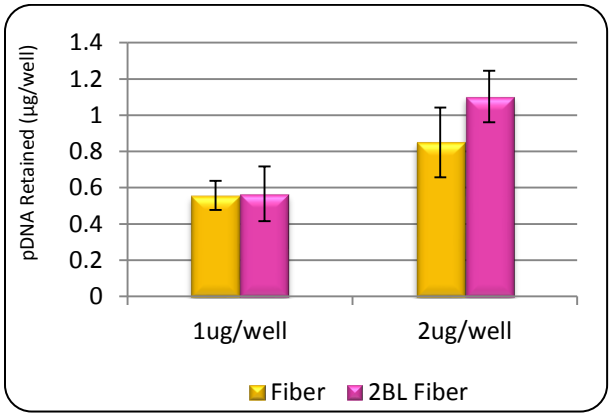

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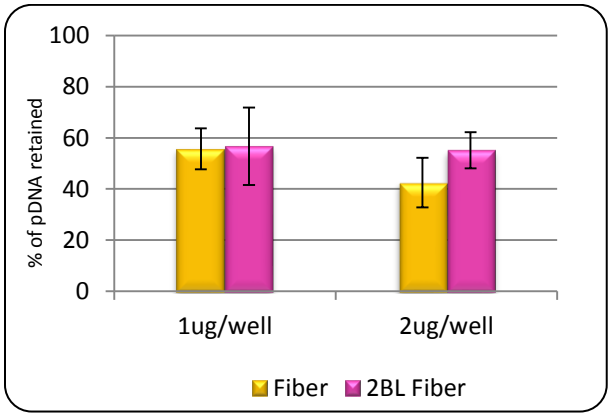


C

Figure16. Study of pDNA/dendrimer compactation at different N:P ratios (A), and amount of pDNA retained in the fiber mats (pDNA loading) as a function of the quantity of pDNA added to the wells (B,C); in C, the amount of pDNA retained is expressed as a percentage of the quantity of pDNA added to the wells.

Figures 16B and 16C summarize these results, together with Table 3. From Figure 16B and Table 3, one can conclude that a higher quantity of pDNA/dendrimer complex was retained in the electrospun fiber mats when 2 µg of pDNA was added to each well. This probably means that there were still spaces available for adsorption at the surface of the mats when the 2 µg/well pDNA concentration was used. Also, when the 2 µg/well pDNA concentration was used, the amount of pDNA retained was higher for the coated electrospun fiber mats than for non-coated ones. This might be due to the stronger electrostatic adsorption between the negatively charged alginate at the surface and the positively charged dendrimer/pDNA complexes (6.5 ± 0.89 mV). Interestingly, the percentage of pDNA retained in the fibers in relation to the quantity initially added to the wells, was always around 50% (Figure 16C and Table 3) which may indicate that an equilibrium is established between the adsorbed complexes and the ones that are kept in solution. When the concentration of the pDNA solution increases from 1 µg/well to 2 µg/well, more complexes will adsorb to the mat surface but, still, the same proportion of complexes will remain in solution. Similar results were obtained by other authors that used polyethylenimine (PEI)/DNA complexes immobilized on tissue engineering scaffolds made of poly(lactide-co-glycolide).[107]

Table 3. pDNA loading on PLGA fiber mats.

	Amount of loaded pDNA per well		% of the amount of pDNA added to the well	
Amount of pDNA added to the well	1µg	2µg	1µg	2µg
Non-coated PLGA mats	0.56 µg	0.85 µg	56 ± 8%	42 ± 10%
Coated (2BL) Fiber PLGA mats	0.57 µg	1.1 µg	57 ± 15%	55 ± 7%

Based on these results, a pDNA concentration of 2µg/well was always used to load the electrospun fiber mats in the following experiments.

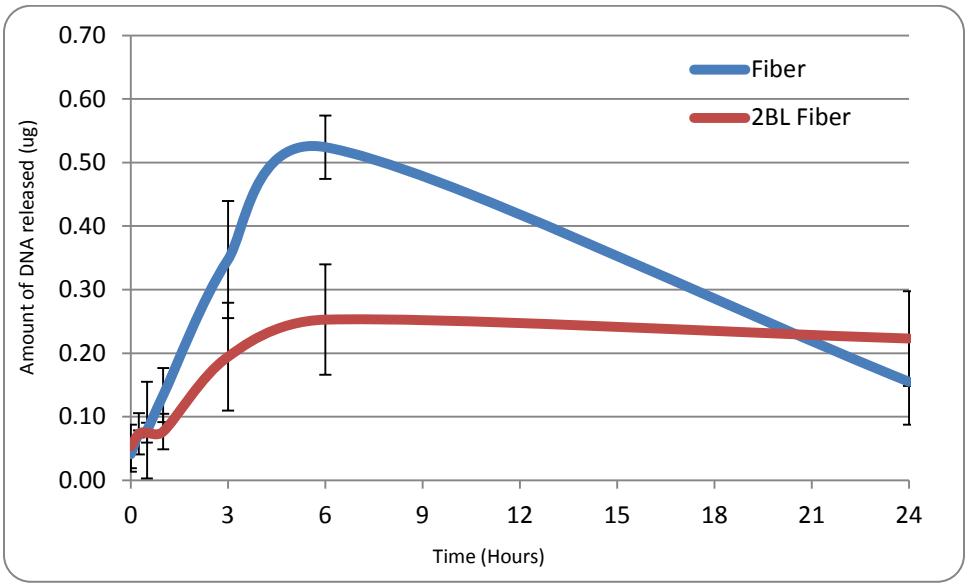


Figure17. Cumulative release of pDNA from non-coated and coated electrospun fiber mats in PBS solution, at 37ºC.

Figure 17 shows the cumulative release of pDNA from the PLGA fiber mats with and without surface modification. These results were obtained by adding PBS solution to the complex loaded electrospun fiber mats and by following the amount of complex released using the PicoGreen® assay (after disrupting the electrostatic forces within the complex with alginate). It was clear that pDNA release for the coated PLGA fiber mats occurred in a much more sustained manner when compared to the non-coated samples, especially in the time window of 30 min to 6 h. This might be ascribed to the strong electrostatic interaction between the pDNA/dendrimer complexes and the negatively charged PLGA surface. After 24h, the amount of pDNA detected in solution decreased significantly for the non-coated samples whereas remained

constant for the coated ones. The results at this time point were surprising since higher values of pDNA were expected for both cases. Probably, these results can be explained as follows. After 6h, the amount of complexes released from the non-coated samples almost reached the total amount initially loaded. So, these complexes will be a long time in solution, being more susceptible to electrostatic disruption and pDNA degradation (degraded pDNA will not be detected using the PicoGreen® assay). On the other hand, the coated samples released the complexes at a slower rate and, as a consequence, the pDNA was protected from degradation for a longer period of time. Taken together, these *in vitro* results point out that the coated electrospun fiber mats will be more adequate as vehicles for the delivery of pDNA/dendrimer complexes due to their sustained release behavior and, thus, capability to protect the pDNA.

3.4. Cell attachment and proliferation on PLGA fiber mats

The ability of the non-coated and coated electrospun fiber mats to support the attachment and proliferation of cells was evaluated using NIH 3T3 cells (a fibroblast cell line) and the resazurin reduction assay. The resazurin reduction assay is based on the capacity of metabolic active cells (living cells) to transform the compound resazurin into resorufin which is fluorescent. The fluorescence intensity is, then, directly proportional to the cell metabolic activity and, thus, to the number of viable cells. It allows, thus, an indirect measure of cell attachment and proliferation over the fiber substrates.

Figures 18A and 18B show the results of cell attachment as a function of time (until 6h of cell culture) and of the type of surface (cells were seeded on the top of the plastic surface of cell culture wells and coverslips, as well as on the top of non-coated and coated electrospun fiber

mats). Whereas in Figure 18A the cell attachment is represented by the fluorescence intensity of resorufin, in Figure 18B the cell attachment is plotted as a percentage of the metabolic activity shown by the cells cultured in the plastic surface of the cell culture wells. Apparently, cells attached better to the plastic surface of cell culture wells or glass surface of the coverslips (used as controls) then on the top of the fiber mats. However, these two types of surfaces are difficult to compare using dye-based assays since the fiber mats are susceptible to dye absorption.[108] That is, the resorufin produced during the assay used for cell attachment quantification may be absorbed by PLGA fibers and, so, its fluorescence signal will be reduced. However, one can compare the non-coated and coated electrospun fiber mats which, in the present case, were shown to be able of supporting cell attachment without significant differences between them. The fluorescence intensity grew along the first 6 hours of cell culture revealing a progressive attachment process. Figures 19A, 19B also illustrate these findings.

Similar results were obtained for cell proliferation. The quantitative data presented in Figures 18C and 18D show that cells were able to growth in number in the four substrates studied, at least until seven days in culture. However, at days 3 and 7, the number of cells present on the top of coated electrospun fiber mats was a little bit smaller than the number of cells present on the top of non-coated surfaces. Although this difference is very small, it was significant and is probably related with the electrostatic repulsion between the negative charge of the last alginate layer that is covering the PLGA fibers and the glycolipids and glycoproteins existent in the cell membranes which also have a negative charge.

The observation of the cultures by microscopy techniques also showed a good coverage of the surfaces by NIH 3T3 cells after 3 days of culture (Figures 19C, 19D), corroborating the quantitative data obtained through the resazurin reduction assay.

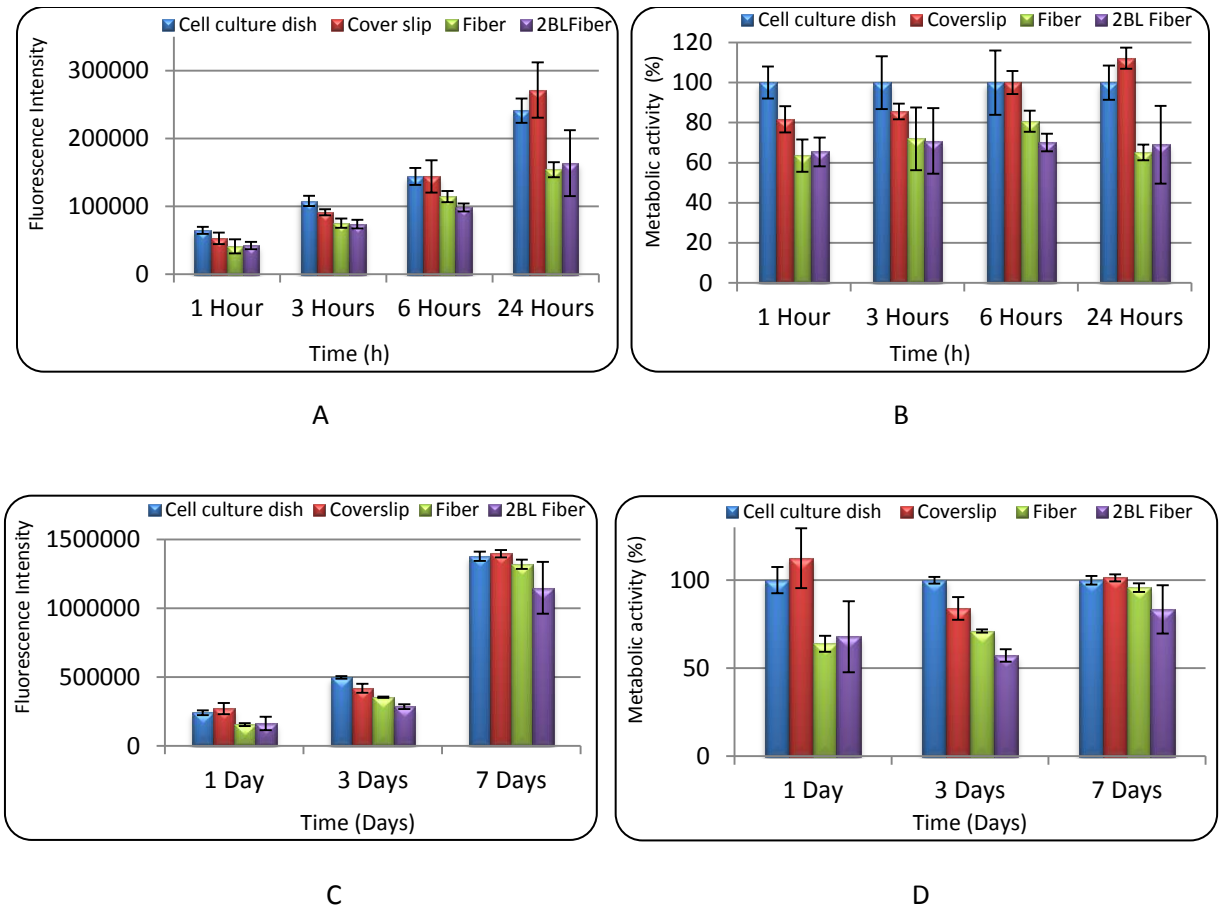


Figure 18. NIH 3T3 cells attachment (A and B) and proliferation (C and D) on the plastic surface of the culture dishes, on the glass surface of the coverslips, and on non-coated and 2 bilayer-coated electrospun fiber mats. The values represent means from 4 replicates ± S.E.M..

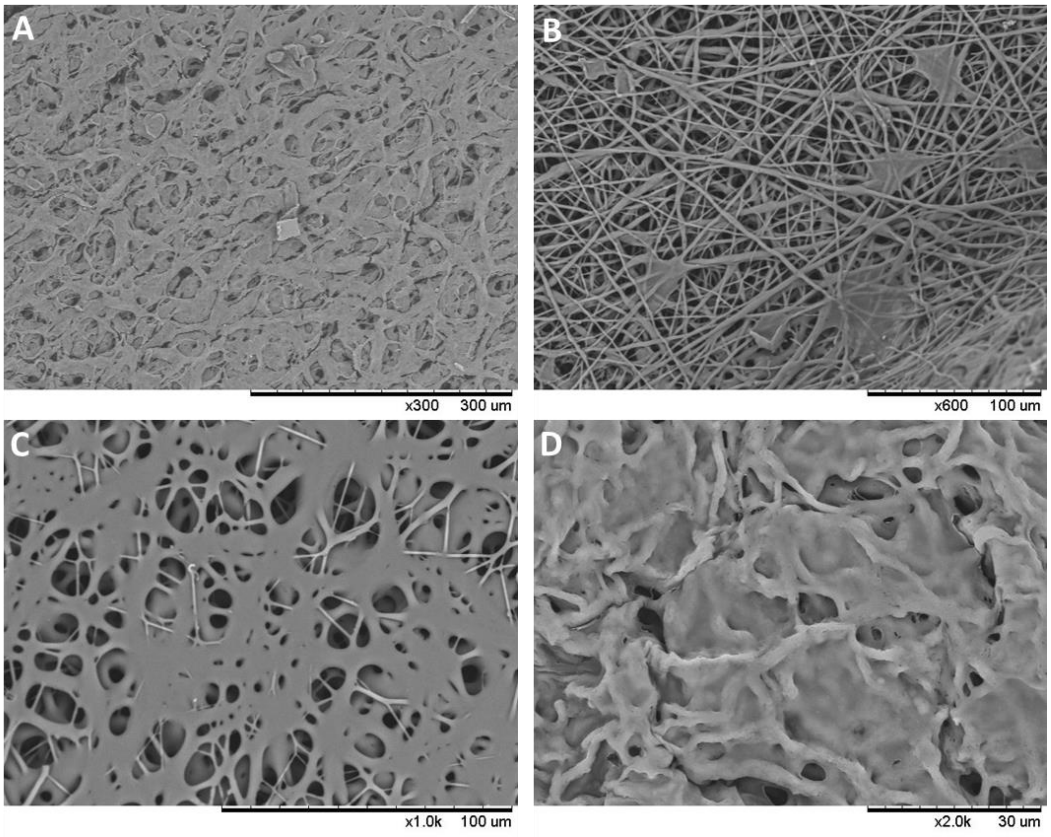


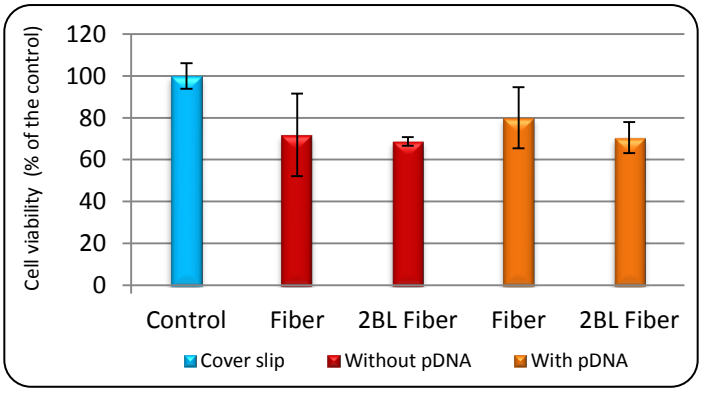
Figure 19. Scanning electron microscopy images of NIH 3T3 cells on the non-coated electrospun fiber mats (A,C) and 2-bilayer-coated electrospun fiber mats (B,D). Images were taken after 6h (A, B) and 3 days (C,D).

3.5. *In vitro* gene transfection studies

Two types of gene transfection studies were performed with polyelectrolyte coated (2 bilayers) and non-coated electrospun fiber mats.[109] The first one used a pDNA encoding for the luciferase enzyme and NIH 3T3 cells (Figure 20). The second one used a pDNA encoding for bone morphogenetic protein-2 (BMP-2) and human mesenchymal stem cells (hMSCs) (Figure 21).

Transfection experiments using the luciferase reporter gene

The transfection efficiency achieved by the pDNA coated electrospun fiber mats was assessed after 48h of cell culture. After this period, NIH 3T3 cells viability was not significantly different in the 4 different types of surface studied, that is, in pDNA/dendrimer complex coated and non-coated, and polyelectrolyte coated and non-coated electrospun fiber mats (Figure 20A).



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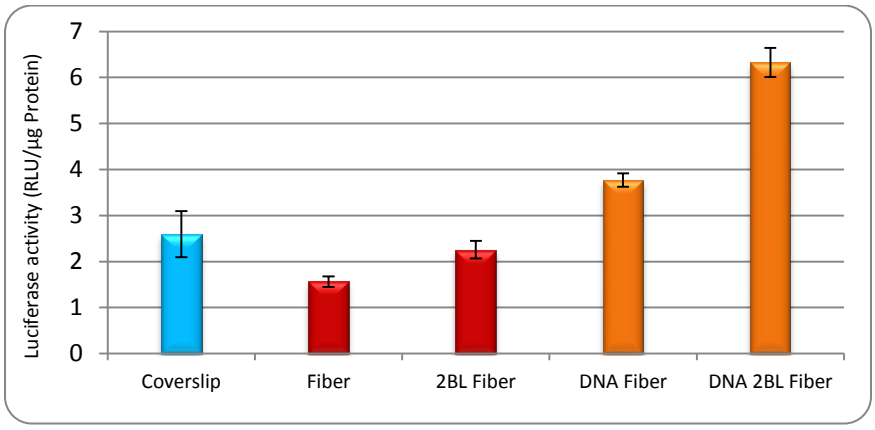


B

Figure 20. NIH 3T3 cells viability (A) and luciferase activity (B) after 48h of culture and for the pDNA/dendrimer complex non-loaded and loaded electrospun fiber mats. The values represent means from 4 replicates ± S.E.M..

In respect to luciferase enzyme activity, as expected, the levels were significantly higher for the electrospun fiber mats coated with pDNA/dendrimer complexes than for the pDNA/dendrimer complex non-coated surfaces (used as controls). Furthermore, the level of luciferase activity was enhanced for the surfaces coated with polyelectrolytes as a mean of immobilizing the pDNA/dendrimer complexes (Figure 20B). These findings are in agreement with the previous results that revealed a slightly higher pDNA loading in this type of surfaces and also, a sustained release of the loaded pDNA which may be beneficial for its stability and cellular internalization. Indeed, the transfection efficiency achieved by polyelectrolyte coated electrospun fiber mats was about 1.5 times higher than that observed when the polyelectrolyte layers were not present.

Transfection experiments using the BMP-2 gene

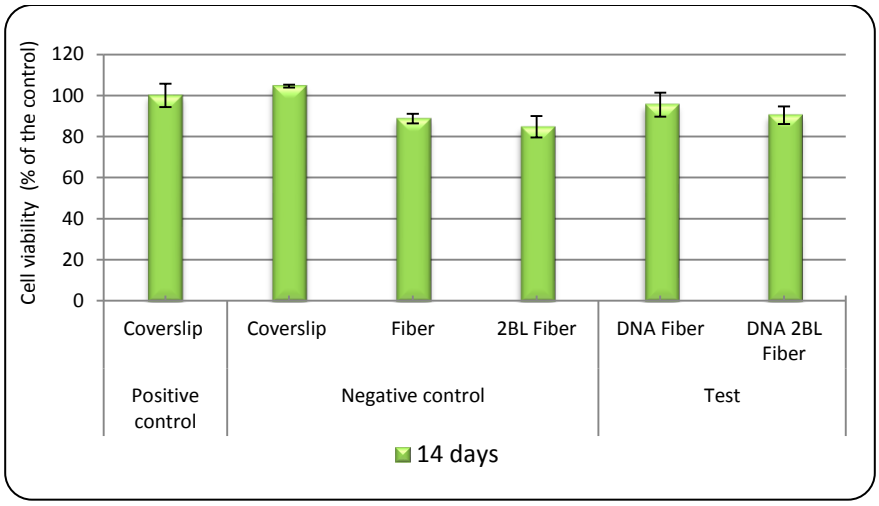
Even if the levels of luciferase activity previously obtained were not impressive, it was hypothesized that they could be sufficient for a clinical relevant application (e.g., tissue engineering) such as the differentiation of hMSCs towards the osteoblastic lineage. The same methodology for cell transfection was generally followed although the time of the experiments has been extended having in consideration the lower growth rate of this type of cells and the characteristic behavior of the osteogenesis process occurring *in vitro*. As such, the cells were cultured along 21 days in the surface of the electrospun fiber mats loaded with pDNA/dendrimer complexes. For comparison, cells were also cultured in the top of the plastic surface of culture dishes or over the electrospun fiber samples in the presence of dexamethasone (positive control of osteogenesis) which is known to be an osteogenesis inductive factor.[110] Experiments were further done without dexamethasone and without pDNA using hMSCs cultured in the top of the

plastic surface of the cell culture dishes or over electrospun fiber mats (these were considered negative controls of the osteogenesis process).

hMSCS viability was analyzed at day 14 of cell culture (Figure 21A) using the resazurin reduction assay. These results showed that the presence of dexamethasone did not affect cell viability and, again, that cell viability was apparently higher when cells were cultured in the plastic surface of the culture dishes than when electrospun fiber mats were used. The justification for this finding is the same as presented before and has to do with the probable absorption of resorufin by the fibers.[108] However, the different experiments that used electrospun fibers can be compared among them. In this case, one can conclude that cell viability was not significantly different in all situations under study.

Alkaline phosphatase (ALP) activity was measured at days 14 and 21 after cell seeding. This parameter is well established as an early marker of the osteogenesis process *in vitro* and is known to present high values between 2 to 3 weeks of cell culture, before the occurrence of mineralization.[111] In the present work, cells were lysed and ALP activity was determined in their extracts. The results were normalized for the total protein content to get an indication of the ALP activity per cell, that is, of the extent of cellular differentiation. Figure 21B reveals that hMSCs differentiation towards the osteoblastic lineage was generally enhanced in the experiments done in the presence of dexamethasone (positive control) when compared with those performed in its absence and in the absence of pDNA/dendrimer complexes (negative control, without differentiation factors). When pDNA/dendrimer complexes were immobilized in the electrospun fiber mats without polyelectrolyte coating, the values of ALP activity were higher than those of the positive control (with dexamethasone). However, when pDNA/dendrimer complexes were immobilized in the electrospun fiber mats with polyelectrolyte coating, the extent of ALP

activity stayed in the middle of the correspondent positive and the negative control. These results can be explained by the fact that dexamethasone is a biochemical factor that is immediately available in the cell culture medium whereas that does not happens with BMP-2 in the case where pDNA/dendrimer complexes are used. Indeed, BMP-2 will only be produced after complex internalization, release of the pDNA from the dendrimer, entry of pDNA in the cell nucleus, transcription of the gene, and translation of the mRNA into the active protein. It is, thus, a slower process but that is expected to have longer time biological effects (due to the sustained release of complexes by the fibers) and less side effects (in comparison to the use of massive quantities of differentiation factors or other drugs that can be used for the same purpose). Because complex release is faster in the electrospun fiber mats without polyelectrolyte coating, an enhanced ALP activity in respect to the positive control is observed for those samples. Interestingly, when analyzing the results obtained for the different positive controls, it seems that the simple presence of the polyelectrolytes in the electrospun fiber mats have a positive effect on differentiation. So, even if complex release from polyelectrolyte coated electrospun fiber mats was slower, the positive effect on differentiation caused by the presence of the polyelectrolyte layers conducted to non-significant differences of ALP activity (at both days 14 and 21) between polyelectrolyte non-coated and coated substrates having immobilized complexes. *In vivo*, however, one should expect prolonged biological effects when polyelectrolyte coated electrospun fiber mats are used due to their sustained release properties.



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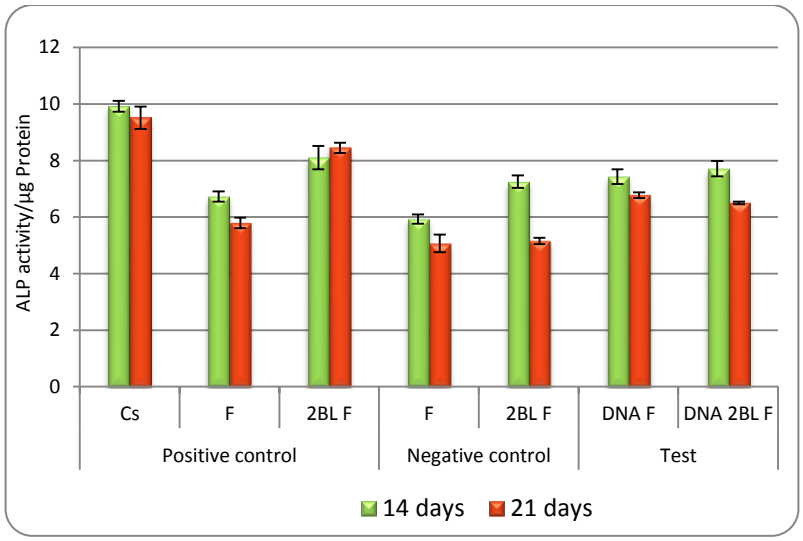

B

Figure 21. hMSCs viability at day 14 (A) and alkaline phosphatase (ALP) activity at days 14 and 21 (B) for pDNA/dendrimer complex non-loaded and loaded electrospun fiber mats, with and without polyelectrolyte coating. The values represent means from 4 replicates ± S.E.M..

CONCLUSIONS

As a general conclusion, the merging of the electrospinning and the LbL techniques constitutes a promising approach for the development of biologically active surfaces for biomedical applications, namely for tissue engineering.

In more detail:

a) PLGA fiber mats were prepared using the electrospinning technique; their characterization by Scanning Electron Microscopy (SEM) revealed that the diameter of the fibers was in the micrometer range, and the water contact angle measurements showed that their surface was relatively hydrophobic.

b) The electrospun fiber mats were successfully coated with several layers of chitosan and alginate using the LbL technique which decreased in a small extent the hydrophobic character of the surface and simultaneously allowed the immobilization of pDNA/dendrimer complexes.

c) pDNA/dendrimer complex release from the polyelectrolyte coated electrospun fiber mats was controlled along the time, protecting the pDNA from degradation.

d) The polyelectrolyte coated electrospun fiber mats supported the growth and attachment of cells; in addition, the presence of pDNA/dendrimer complexes in the surface of the fibers had no deleterious effect over cell viability.

e) The polyelectrolyte coated electrospun fiber mats were able of serving as vehicles for the effective delivery of the BMP-2 gene, which resulted in the enhanced differentiation of hMSCs towards the osteoblastic lineage.

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