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Biodegradable polymeric systems for controlled release of growth factors in regenerative medicine

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ABSTRACT

With advances in biotechnology, bioengineering, and chemistry, a wide variety of new, more potent and specific active molecules are being created. Because of common problems such as low solubility, high potency, and/or poor stability of many of these new molecules, the means of drug delivery can impact efficacy and potential for commercialization as much as the nature of the molecule itself. the controlled release systems (DDS's) are one of the most promising technologies to solve these problems because offer several potential advantages over traditional methods of administration. First, drug release rates can be tailored to the needs of a specific application; for example, providing a constant rate of delivery or pulsatile release. Second, DDS provide protection of drugs, especially proteins, that are otherwise rapidly destroyed by the body. Finally, DDS can increase patient comfort and compliance by replacing frequent (e.g., daily) doses with infrequent (once per month or less) injection. the last twenty years the DDS in addition to being used for the release of drugs are also used in the field of tissue engineering for controlled release of specific molecules, such as growth factors or cytokines.

In the first chapter of this thesis the drug delivery systems have been investigate for the control of the concentration of signal molecules within tridimensional scaffold for the induction of angiogenesis in bone regeneration processes. To achieve a good bone tissue regeneration is essential to have a three dimensional structure, called scaffold, with specific physical and mechanical properties, such as high porosity, high interconnected pore network, high biocompatibility but especially capable of releasing in a controlled way bioactive molecules such as proangiogenic factors (VEGF). For this reason we have developed a bottom up approach founded on the assembly of building blocks by solvent induced microparticle sintering to realize multifunctional polymer scaffolds with predefined pore dimension

and fully percolative pathway, able to include interspersing DDS for the release of angiogenic factor or similar molecules. This approach offers the possibility to realize scaffolds that not only meet all the requirements in terms of controlled microstructure, chemical stability and mechanical response necessary to support neo-tissue growth, but also provide a guidance of cell and tissue processes by presenting bioactive agents in a predefined chrono-programmed manner.

In the second chapter of this thesis has been developed intraocular drug delivery systems for the controlled release of growth factors for the treatment of different ocular diseases. The traditional treatment of these pathologies consists of daily administration of drugs but, the main limit of these conventional treatments is bound to the fact that blood levels of the drug falling into the therapeutic range are obtained right after administration. In other words, there is a risk that the drug action lasts too little to have a significant effect on the patient.

To overcome this issues and to optimize the clinical performance we have developed PLGA micro e nanoparticles system for the controlled release of specific factor: Nerve Growth Factor (NGF) to humor vitreous. This factor has been shown to have a protective effect on the cells of the visual system, opening interesting therapeutic prospects for degenerative diseases currently no effective treatments, such as the age-related macular degeneration, diabetic retinopathy, retinitis pigmentosa and glaucoma.

INTRODUCTION – GENERAL PART

1. Time Controlled Delivery System

One of the main lines of research in the field of pharmaceutical technology concerns the development of formulations that can release drugs in the body in amounts and at controlled rates. There are already numerous pharmaceutical forms from which the release can be delayed, or by which the speed and duration of release of the active molecules can be programmed. That formulations are defined controlled release systems (DDS's) and allow the adjusting the blood level of the drug, in order to avoid phenomena of under or over dosing and minimize unwanted side effects.

The use of this DDS's can provide several advantages over traditional methods of administration. First, drug release rates can be tailored to the needs of a specific application; for example, providing a constant rate of delivery or pulsatile release. Second, DDS's provide protection of drugs, especially proteins, that are otherwise rapidly destroyed by the body. Finally, DDS's can increase patient comfort and compliance by replacing frequent (e.g., daily) doses with infrequent (once per month or less) injection.

However, there are potential disadvantages that should not be overlooked. Disadvantages of using such delivery systems include possible toxicity of the materials used, dose dumping, requirement of surgical procedures to implant or remove the system, and higher manufacturing costs. DDS main advantages and disadvantages are summarized in Table 1.

A large number of classes of drugs that include chemotherapeutic drugs[1], immunosuppressant's[2], anti-inflammatory agents[3,4], antibiotics[5,6], opioid antagonists[7], steroids[8,9], hormones[10] can benefit from temporal or distribution controlled release. Recently, the need to develop new controlled release strategies has been intensified by advances in the design of peptide drugs and the use of growth factors in therapy.

DDS advantages	DDS disadvantages
Extension of the duration of action and bioavailability of the drug	Possibility of toxicity of the materials
Minimization of drug degradation and loss	Harmful degradation products
Prevention of drug's adverse side-effects	Necessity of surgical intervention either on systems application or removal
Reduction of dosing frequency	Patients discomfort with DDS device usage
Minimization of drug concentration fluctuations in plasma level	High cost of final product
Improved drug utilization	
Improved patient compliance	

Table 1 : DDS advantages and disadvantages

DDS can be produced by using natural or synthetic polymers, which can be biodegradable or non-biodegradable (see Fig. 1). These polymeric systems can be used in the release of drugs, proteins and cells.

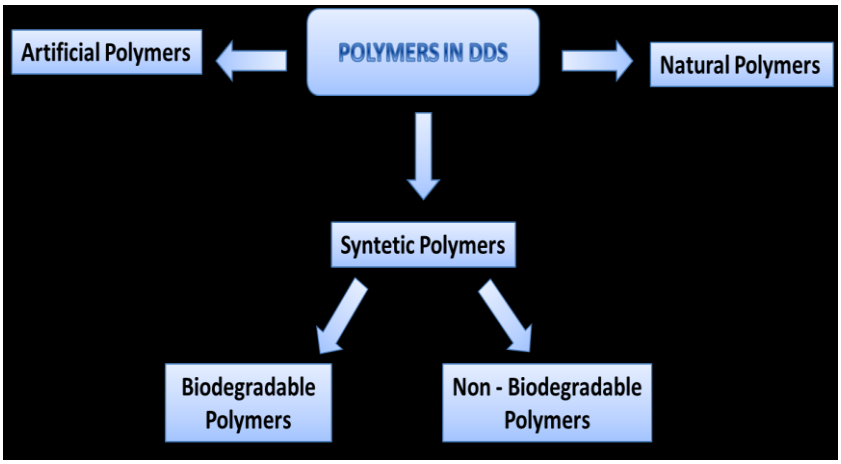

Figure 1 : Type of Polymer used for DDS production

The polymers used in DDS should present a set of properties that make them suitable materials to interact with the human body, but the biodegradability is one of the most important features. Biodegradable polymers are particularly attractive for application in DDS since, once introduced into the human body, they do not require removal or additional manipulation. Their degradation products are normal metabolites of the body or products that can be metabolized and easily cleared from the body[11,12].

Moreover, synthetic polymers offer a wide variety of compositions with adjustable properties. These materials open the possibility of developing new DDS with specific properties (chemical, interfacial, mechanical and biological) for a given application, simply by changing the building blocks or the preparation technique.

1.1. PLGA Drug Delivery System

Poly(D,L-lactic-co-glycolide), PLGA and its various derivatives have been the center focus for developing nano/microparticles encapsulating therapeutic drugs in DDS applications[1,4,5,7] due to their inherent advantages over the conventional devices that include extended release rates up to days, weeks or months, in addition to their biocompatibility/biodegradability and ease of administration via injection.

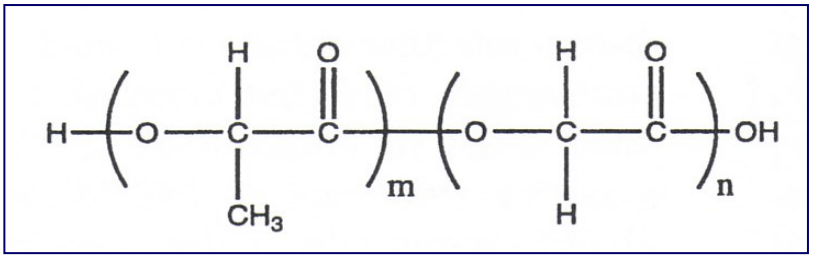

Figure 2: PLGA Structure

Macromolecular drugs such as proteins, peptides, genes, vaccines, antigens, human growth factors, etc., are successfully incorporated into PLGA or

PLGA based nano/microparticles. Despite the formulations already available in the market, yet research in this area has advanced greatly due to the advantages of PLGA polymers over other systems. Also, the American Food and Drug Administration (FDA) have approved a very large number of drug delivery products based on this biomaterial. Table 2 lists injectable PLGA depots marketed in the US [13]. Controlled drug release can be easily achieved by adjusting the polymer parameters such as molecular weight, monomer ratio, drug loading, excipient loading, glass transition temperature, and several other formulation variables. These advantages have also led to various medical and pharmaceutical applications including sutures, dental repairs, fracture fixation, ligament reconstruction, vascular grafts, and controlled drug delivery carriers.

Drug	*Trade name*	*Company*	*Polymer*	*Route*	*Application*
buserelin acetate	Profact®Depot, Suprefact®Depot	Hoechst Marion Roussel	PLGA	s/c implant	Prostate cancer
goserelin acetate	Zoladex□Depot	Astra Zeneca	PLGA	s/c implant	Prostate cancer, endometrioses
leuprorelin acetate	Lupron®Depot, Enantone®Depot, Enantone®Gyn Depot Trenantone®	Takeda-Abbott	PLGA PLA	3-month depot suspension, 1-month suspension 3-month suspension	Prostate cancer, endometrioses
octreotide acetate	Sandostatin LAR®Depot	Novartis Pharma	PLGA	s/c suspension	GH suppression, anti cancer
triptorelin	Decapeptyl® Depot	Debiopharma	PLGA	s/c depot injection	LHRH agonist, prostate cancer
recombinant human growth hormone	Nutropin®Depot, [discontinued commercialisation since 06/2004]	Genentech-Alkermes	PLGA	monthly s/c injection	Growth hormone deficiency

Table 2 : Examples of peptide/protein controlled, release systems based on PLGA.

1.2. PLGA Micro and Nanoparticles

The importance of the copolymers of PLA and PLGA is greatly increased in consequence of the possibility to prepare them from particle systems for the delivery of drugs. The advantages of use of particle systems include, in fact, the possibility of administering the medication directly to the site of action where the system forms a deposit from which the active ingredient can be released and act for a prolonged period of time.

Within particles, two different categories can be distinguished: microparticles and nanoparticles. The distinction between both classes is often confusing and even polemic. Lately, the most accepted classification reports that particles up to 100 nm are considered nanoparticles while the ones from 1 μm up to 1,000 μm are classified as microparticles.

Particulate systems as DDS can be prepared using different types of materials like polymers, lipids or peptides. Particles prepared from either natural or synthetic polymers have been extensively investigated to be applied as DDS. Their synthesis can be achieved from different techniques[14]. Among them, are emulsion polymerization, solvent evaporation, ionic gelation, self-assembly, nanoprecipitation and supercritical fluid technology[15].

The release of the active principle to be based microspheres of biodegradable polymers is governed by the diffusion of the active principle through the polymer matrix and the biodegradation of the polymer matrix itself.

The mechanism of the release of active molecules from PLGA microspheres is mainly governed by autocatalytic bulk degradation process-based. It was also shown that this mechanism is affected by many physical and chemical factors, such as the initial pH, ionic strength and temperature of the external environment, the ratio of the copolymers, the molecular weight, the crystallinity and the size of the species.

In the combined diffusive-erosive mechanism an important role is played in the environment of the activity of the water release. The particle, in contact with the aqueous environment, hydrates and water that gets inside solubilizes the drug, which begins to spread through the macroporous structure of the microspheres of PLGA (feature sizes: 10^{-10} - 10^{-9} nm), whose existence and characteristics depend on the techniques of preparation of the microspheres (i.e., multiple emulsion technique).

The release of macromolecules can be prevented by the microporosity of the system, since the size of the micropores can be comparable to those of the molecule to release. Following the degradation of the polymer micropores increase in size until it is allowed the transport of the macromolecule[16].

1.3. Protein and Peptide in PLGA particles : Instability and Inactivation problems

Many proteins and peptide are fragile molecules that undergo different pathways of instability and they have limited half-life in vitro and in vivo and need to be carefully handled. The integrity of protein structures is essential for their proper function in physiological or pathological conditions. Unexpected unfolding or degradation of proteins may lead to inactive, sometimes even toxic products. Protein instability, generally, can be divided into two chemical and physical processes. Chemical instability involves the formation and destruction of covalent bonds, which usually occurs in the primary structure and disulfide bonds. Chemical process includes hydrolysis (proteolysis), deamination, racemization, oxidation, disulfide formation and β-elimination. The degradation products result from chemical instability must be carefully characterized for safe use of the proteins. Physical stability refers to proteins' ability to retain their secondary, tertiary and quaternary structure, which can be lost by reversible or irreversible denaturation through a loss of tertiary structure, aggregation and adsorption.

A major issue with PLGA delivery systems is protein stability during preparation, storage and release. There are several factors associated with this polymer that may cause destabilization of proteins. The potential inactivation mechanisms involved and stabilization approaches during protein encapsulation and release have been reviewed[17–20]. During microsphere preparation, proteins are exposed to conditions that are known to cause denaturing and aggregation, namely high shear, elevated temperature, exposure to the air/liquid interface, organic solvents and the oil in water (O/W) interface. Higher energy emulsification methods such as by sonication, homogenization and vortex are detrimental to proteins[21,22]. The addition of an aqueous protein solution to an organic solvent can lead to denaturing of proteins. Proteins, which are surface active, can diffuse to the

O/W interface and aggregate non-covalently or covalently upon exposure of the hydrophobic core and subsequent disulfide scrambling. A solid state protein is desirable when an organic solvent exposure is necessary and when the surface-association is the dominating mechanism for protein instability. The conformation of lyophilized protein is "trapped" when it is added to organic solvent, because the lack of lubricating water[23]. In addition, decreased mobility of proteins upon dehydration stabilizes them against shear and elevated temperature encountered during emulsification.

During release from polymer microparticles, proteins are exposed to many stresses that can compromise the physical and chemical stability of proteins. These include protein rehydration, exposure to soluble oligomers, low pH, interactions between protein and polymer, loss of stabilizing excipients, and physiological temperature.

Interactions between protein and polymer, such as adsorption can also affect the stability of proteins. Adsorption occurs by a hydrophobic interaction between the polymer and the hydrophobic interior of proteins and can often lead to the formation of insoluble aggregates or irreversible conformational changes[24]. Even when adsorption is reversible, it may accelerate other deleterious reactions by exposing previously buried residues or increasing side chain mobility. Adsorption can be minimized by the addition of other proteins or surfactants that compete with the protein for hydrophobic interactions with the polymer[25].

1.3.1. Stabilization strategies of Growth Factor in PLGA Delivery System

Several stabilizers that have been proven to effectively improve protein stability in the polymer have been summarized in table 3. However, individual protein needs to be carefully studied for better selection of

stabilization strategies, but in general we can intervene on two fronts: during the phase of preparation and/or during the release phase.

Stage	Stress factor	Stabilization approach	Stabilization mechanism
Preparation	Water/organic solvent interfaces	Add sugars, polyols, PEG	Increase of Gibbs free energy of unfolding, shielding from interfaces by preferential hydration
		Increase protein loading	Reduction of interface/protein ratio
		Add other proteins	Competition for interfaces
		Avoid emulsification, use non-aqueous process	Absence of water/organic solvent interfaces
		Pre-encapsulate protein in hydrophilic core	Shielding from interfaces
Preparation	Protein-PLGA contacts	Add other proteins	Competition for PLGA
		Pre-encapsulate protein in hydrophilic core	Shielding from PLGA
		Hydrophobic ion pairing	Shielding from PLGA
Preparation	Shear	Add surfactants	Competition for interfaces
		Reduce homogenization time	Minimized exposure to shear
		Avoid sonication, use other homogenization method	Absence of cavitation stress
Preparation	Drying	Add lyoprotectants	Increase of Gibbs free energy of unfolding, water substitution
		Avoid lyophilization, use other drying method	Absence of freezing step
Storage	Moisture	Reduce residual solvent level	Minimized mobility and water-induced degradation
	Dehydration	Add lyoprotectants	Increase of Gibbs free energy of unfolding, water substitution
Release	Acidification	Add basic compounds	Buffering
	Protein-PLGA contacts	Add other proteins	Competition for PLGA
		Add surfactants	Shielding from PLGA
		Add sugars	Increase of Gibbs free energy of unfolding, shielding from PLGA by preferential hydration

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Table 3 : Approaches to protect microencapsulated protein against stress factor during precipitation, storage and release

During the preparation, the addiction of excipients, such as BSA [26], PEG400 [27] and other, to the inner aqueous phase that compete with the water/organic solvent interface can prevent emulsification-induced denaturation and aggregation. This approach may be particularly useful when PLGA microparticles are loaded with low amounts of therapeutically potent proteins. On the other hand, increasing the protein concentration during emulsification was shown to result in higher absolute amounts of aggregated protein at the interface, indicating that multi-layer interfacial adsorption can occur [28].

Common surfactants have not been very successful as stabilizer in the emulsification step because they seem to have an insufficient competition with the protein for the water/organic solvent interface, or promotion of

organic solvent/ protein contacts through hydrophobic contacts with both components.

Another approach to protect proteins against degradation during emulsification has been pre-entrapment in a hydrophilic core, which is subsequently encapsulated in PLGA microparticles [29]. Although this approach generally improved release characteristics, in none of these studies data on the protein structure were reported.

During the release phase specific stabilization approaches to minimize protein degradation associated with the direct environment of (degrading) PLGA are often needed. To inhibit acid-induced protein degradation inside the microparticles during release (if occurring at all *in vivo*), co-incorporation of poorly water-soluble basic inorganic salts, such as magnesium hydroxide [30], calcium carbonate and sodium bicarbonate [31].

Finally the addition of poloxamers, such as Pluronic F68, induces a decrease of the interactions of the macromolecule with the free carboxyl residues of PLGA and at the same time, it reduces the effects of the decrease in pH in the microenvironment of the particle, as evidenced by an increased release rate of protein [32].

2. Regenerative Medicine and the importance of Growth Factors release

As a consequence of disease or trauma, the tissues and organs in our bodies may be unable to perform their anatomical and metabolic functions. Until recently the use of implants, and in severe cases transplants, is the only way to deal with these pathological conditions [33].

In recent years, a new branch of regenerative medicine called tissue engineering is under development. This term, officially coined in 1988 by the *National Science Foundation*, indicates that this multidisciplinary field produces biological substitutes containing living and functional cells for regeneration, maintaining or improving performance of the tissue [34]. Tissue engineering represents the meeting point of different disciplines such as medicine, biology, engineering, and chemistry with the common purpose for obtaining or replacing organs or parts of organs in the human body. This will make possible to have a viable alternative to transplantation, as this can have many fundamental problems such as the chronic shortage of donors, the phenomena of organ rejection and the continued need to take immunosuppressive drugs [33].

Tissue engineering (TE) aims at the repairing and restoring damaged tissue function employing three fundamental "tools", namely cells, scaffolds and growth factors (GFs) which, however, are not always simultaneously used [35] [36]. On the other hand, summoning recent experimental and clinical evidences indicate that the success of any TE approach mainly relies on the delicate and dynamic interplay among these three components and that functional tissue integration and regeneration depend upon their sapient integration [37]. Future generation of scaffolds will have to provide not only the adequate mechanical and structural support but also have to actively guide and control cell attachment, migration, proliferation and differentiation. This may be achieved if the functions of scaffold are extended to supply biological signals

able to guide and direct cell function through a combination of matricellular cue exposition and GF sequestration and delivery [37]. Therefore an ideal scaffold should possess a three-dimensional and well defined microstructure with an interconnected pore network, mechanical properties similar to those of natural tissues, be biocompatible and bio-resorbable at a controllable degradation and resorption rate as well as provide the control over the sequestration and delivery of specific bioactive factors to enhance and guide the regeneration process.

To overcome the problem of the in vivo instability of growth factors that are used to induce tissue regeneration, it is vital to use technology to develop the administration form. Indeed, when a solution of the growth factor is injected into the site requiring regeneration, the biological effect cannot be always predicted. This is because the growth factor is rapidly diffused away from the injection site. To enhance the in vivo efficacy of the growth factor, the drug delivery system (DDS) is promising. It is possible that when used in combination with an appropriate DDS technology, the growth factor enhances the in vivo proliferation and differentiation of key cells that promotes tissue regeneration. For example, the controlled release of a growth factor at the site of action over an extended time period is performed by incorporating the factor into an appropriate carrier. It is also possible that the growth factor is protected against proteolysis, as it is incorporated in the release carrier for prolonged retention of the activity in vivo. DDS technology can be also useful for half-life prolongation, absorption improvement and targeting, applicable in tissue engineering using protein and genes.

2.1. ECM – Extra Cellular Matrix

The extracellular matrix represents the secreted product of resident cells within each tissue and organ and is composed of a mixture of structural and

functional proteins arranged in a unique, tissue specific three-dimensional ultrastructure. These molecules, mainly proteins, provide the mechanical strength required for proper function of each tissue and serve as a conduit for information exchange (i.e. signaling) between adjacent cells and between cells and the ECM itself. The ECM is in a state of dynamic reciprocity [38] and will change in response to environmental cues such as hypoxia and mechanical loading.

In addition to the direct regulation of cell function by ECM molecules, cell function is also dependent on the signaling of various growth factors and cytokines. Growth factors and cytokines are general terms for soluble molecules, in contrast to the typical solid-state for ECM molecules, which regulate functions such as proliferation, migration, differentiation and apoptosis.

These molecules are secreted by cells, to act in an autocrine nature on the producing cells, or in a paracrine fashion on neighboring cells. Due to the central importance of these molecules in cell biology, they and their receptors and intracellular signal transduction pathways are major targets for manipulation in tissue engineering and regeneration strategies. Growth factors and cytokines interact with the ECM in a variety of ways, which allows one to potentially intervene in cell-growth factor interactions by modulating the ECM. The ECM can serve as a reservoir for many factors, due to their binding of the factors and protection from degradation.

The ECM may also be involved in growth factor presentation to cells, and in regulating expression of the factors by cells. Specific examples of these interactions include the binding of growth factors such as FGF, vascular endothelial growth factor (VEGF), heparin-binding epidermal growth factor (HB-EGF), and HGF to heparan sulfate or heparin present in the ECM to form tight complexes.

2.2. Growth factor : Type and Mechanism of action

Growth factors are soluble-secreted signalling polypeptides capable of instructing specific cellular responses in a biological environment. The specific cellular response trigged by growth factor signalling can result in a very wide range of cell actions, including cell survival, and control over migration, differentiation or proliferation of a specific subset of cells.

The signal transmission mechanism initiates with the growth factor that is secreted by the producer cell which then goes to instructs cell behavior through binding to specific transmembrane receptors on the target cells (figure 3). The machinery that transduces the growth factor-binding signal to the cell nucleus involves a complex array of events involving cytoskeleton protein phosphorylation, ion fluxes, changes in metabolism, gene expression, protein synthesis and ultimately an integrated biological response [39].

Growth factors differ from other oligo/polypeptide molecules, such as insulin and hormones, in the mode of delivery and response elicited. Typically, growth factors do not act in an endocrine fashion; they exhibit short-range diffusion through the extracellular matrix and act locally owing to their short half-lives and slow diffusion. The ability of a growth factor to deliver a particular message to a distinct subpopulation of cells is not exclusively determined by the identity of the growth factor and its ability to diffuse through the extracellular matrices (ECMs); it is also determined by the target cell number, type of receptors and the intracellular signal transduction subsequent to factor binding. The same growth factor can convey

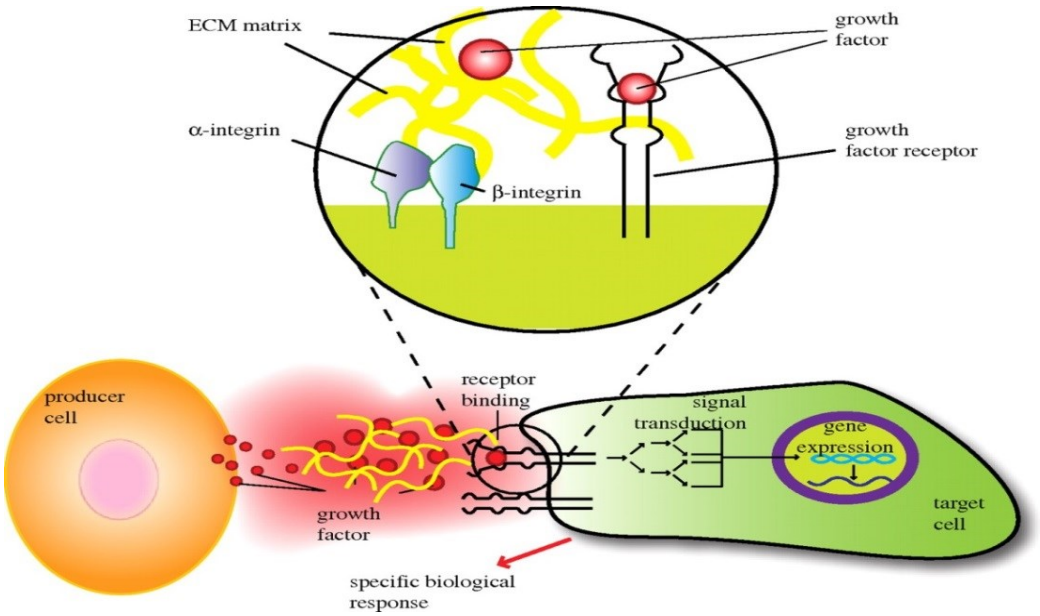

Figure 3 : Cross talk between cells mediated by growth factors and ECM. The producer cell secretes soluble growth factors that bind to target cell receptors

different instructions depending on the receptor type to which it binds, and on the cell type to which it binds. Moreover, the same receptor can translate different messages depending on the intracellular transduction pathways, which can differ from one cell type to another. The ultimate response of a target cell to a particular soluble growth factor can also be governed by external factors, including the ability of the factors to bind to ECM, ECM degradation and growth factor concentration and cell target location [40].

2.3. ECM – mimicking Scaffold

A natural ECM or its derivatives may actually not be the ideal scaffold for tissue engineering applications because tissue engineering should be an accelerated regeneration process compared to the natural development program. Mature tissue matrix often does not possess the highly interconnected macro or micropore structures to allow for quick and uniform cell population throughout, which is essential for a tissue engineering/repair process. Therefore certain artificially designed scaffold features (such as

porosity, pore size, interconnected pores, etc.) are necessary for optimal tissue engineering applications (accelerated tissue regeneration). In addition, there are always concerns over the possible immune rejection and pathogen transmission when a natural ECM is used [41].

Because of the impossibility of using natural ECM although the central role that it plays in morphogenesis tissue, it is essential, during the design of a three-dimensional scaffold, re-create all of its features.

In particular, to mimic the functional and microstructural characteristics of the ECM, a scaffold must present: high degree of porosity, high surface to volume ratio, high degree of pore interconnection, appropriate pore size [42,43] and finally release in a controlled manner various active molecules, such as cytokines and growth factors [44,45].

2.4. Strategies for presentation of growth factors

There are several strategies which we can control the growth factors release from the scaffold but in any case some points should be considered :

- the loading capacity corresponds to the amount of growth factor that can be mixed or grafted into the scaffold;
- the distribution relates to the way the growth factor is dispersed, which will influence the release kinetics;
- the binding affinity defines how tightly the growth factor binds the system; this must be sufficiently low to allow release, but high enough to prevent uncontrolled release;
- the release kinetics: its control allows the appropriate dose of growth factor to reach the cells over a given period of time;
- the long-term stability: the system should enable growth factors to maintain their structure and activity over a prolonged period of time;

- the economical viability: such biomaterials must further be easy to manufacture and to handle, and be cost-competitive.

Two distinct strategies for biomaterial presentation of growth factors in tissue engineering have been pursued:

- Chemical immobilization of the growth factor into or onto the matrix
- Physical encapsulation of growth factors in the delivery system.

Chemical methods have been investigated, but often result in the denaturation and activity lowering of protein because the functional groups of protein are chemically modified by immobilization reaction. Therefore, physical methods are preferable for growth factor integrity. In fact, such a physical immobilization is often observed in growth factors existing in the body [46]. Some growth factors are naturally stored in the body; they are stored ionically with the acidic polysaccharides of ECM (such as heparin sulfate and heparin) because most of them have a positively charged site on the molecular surface. It is recognized that, when needed, the complexed growth factor is water solubilized by the enzymes secreted from the surrounding cells released from the ECM complex during ECM degradation. This complexation also protects growth factors from their denaturation and general enzymatic degradation in vivo.

CHAPTER 1

INDUCTION OF ANGIOGENESIS PROCESS IN 3D - SCAFFOLD FOR BONE REGENERATION

1. Introduction

Currently, the repair of critical bone defects resulting from trauma, infections, tumors or congenital malformations is one of the most pressing problems in orthopedic field. Actually, autogenous bone grafting is still the most widely used practice for bone repair because leads a good osteoconduction and osteoinduction, in addition to the complete absence of immunogenic reactions after surgery[47]. However, autogenous bone grafts are associated with the morbidity of donors[48], additional surgical procedures for harvest, and limitations in the quantity and available bone size.

Bone tissue engineering can become a good and promising alternative to conventional methods leading to overcoming the problems related to donor site morbidity and size limitations. However, while reconstruction of small to moderate sized bone defects using engineered bone tissues is technically feasible, and some of the currently developed concepts may represent alternatives to autologous bone grafts for certain clinical conditions, the reconstruction of large volume defects remains challenging. Increasing evidences have demonstrated that the key factor for the poor repair with tissue-engineered bone is poor vascularization[49]. Bone is a highly vascularized tissue that relies on the supply of essential nutrients and oxygen from blood vessels for maintaining skeletal integrity. The development of a microvasculature and microcirculation is critical for the homeostasis and regeneration of living bone, without which, the tissue would simply degenerate and die[50].

To achieve a good bone tissue regeneration is essential to have a three dimensional structure, called scaffold, with specific physical and mechanical properties, such as high porosity, high interconnected pore network, high biocompatibility but especially capable of releasing in a controlled way bioactive molecules such as proangiogenic factors (VEGF) .

The bioactivation of the scaffold can be obtained or through simple diffusion of bioactive molecules across the polymer matrix, or through electrostatic or covalent immobilization of the molecule on the surface, but both techniques, however, have inherent limitations[35]. In the case in which the GF is dispersed directly inside the matrix, the subsequent release of the macromolecule will follow a kinetics that depends on the nature of the polymer that constitutes the scaffold and that will be difficult to control and predict. On the other hand, the immobilization strategies should take into account the structure and the site of action of GF, in order to immobilize the macromolecule and without compromising its activity[35]. Moreover, in both strategies, the activity of the macromolecule can be seriously compromised during the process of manufacture of the scaffold, in addition to not be properly protected from enzymatic degradation when in contact with the biological microenvironment in vivo.

In any case, the use of DDS is strategic for the control of the concentration of signal molecules within tridimensional scaffolds[51]. Among the DDS, the microparticles based on PLGA appear to be excellent candidates for the delivery of GF in scaffolds for tissue engineering applications[52]. The microparticles made of PLGA can, in fact, protect the encapsulated molecule and release it at specific time intervals and for long periods of time during the development of the tissue. Furthermore, the possible micropositioning oriented in space of the particles inside the scaffold can make possible the control of tissue growth both in the extension and in the organization.

1.1. Vascularization in early bone development

Bone formation and development occurs through two distinct processes: intramembraneous and endochondral ossification and the vascularization is the prerequisite for both the processes[53]. In intramembranous bone formation,

mesenchymal stem cells (MSCs) can be transported through capillaries and differentiate directly into mature osteoblasts.

These osteoblasts then deposit bone matrix and lead to bone formation. On the other hand, during the endochondral ossification, the chondrocytes secret angiogenic growth factors promoting the invasion of blood vessels, which then bring along a number of highly specialized cells and replace the cartilage mold with bone and bone marrow[54].

Vasculature also plays a key role in bone formation by the production of growth factors that control the recruitment, proliferation, differentiation and function of various cells including osteoblasts and osteoclasts[55]. During the bone remodeling, the osteoblasts play an important role in the balance of

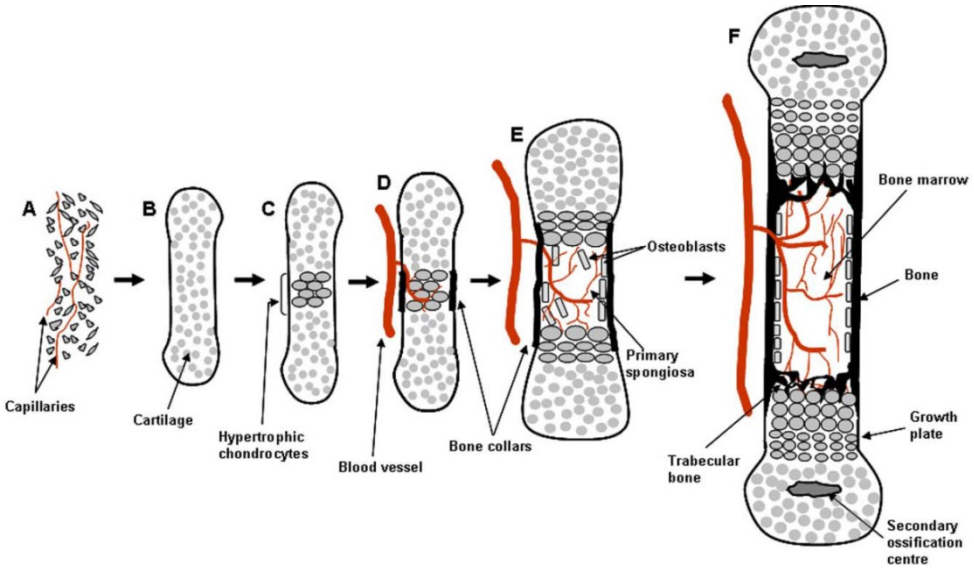

Figure 4 : A schematic diagram of endochondral bone formation. (A) Mesenchymal cells condense and (B) differentiate into chondrocytes forming an avascular cartilage model of the future bone. (C) At the centre of condensation the chondrocytes cease proliferating and become hypertrophic. (D) Perichondral cells adjacent to the hypertrophic chondrocytes differentiate into osteoblasts forming a bone collar. The hypertrophic cartilage regulates the formation of mineralised matrix; the release of angiogenic factors to attract blood vessels and undergoes apoptosis. (E) The coordination of osteoblasts and vascular invasion form the primary spongiosa. The chondrocytes continue to proliferate with concomitant vascularisation resulting in a coordinated process that lengthens the bone. Osteoblasts of the bone collar will eventually form cortical bone; while osteoblasts precursors located in the primary spongiosa will eventually form trabecular bone. (F) At the ends of the bone, secondary ossification centres develop through cycles of chondrocyte hypertrophy, vascular invasion and osteoblast activity. Columns of proliferating chondrocytes form in the growth plate beneath the secondary ossification centre. Finally, expansion of stromal cells and hematopoietic marrow starts to take place in the marrow space[56,57]

resorption and bone deposition by secreting osteoprotegerin that is an inhibitor of osteoclast activity. However, the mature osteocytes lose the capability to produce this molecule[58]. Blood vessels transport osteoprogenitor cells for the deposition of new bones[59]. The invading vasculature, thus, serves as both a reservoir and a conduit for the recruitment of essential cells involved in bone remodeling, and provides critical signals necessary for bone morphogenesis[60].

It follows that the balance between cartilage formation and vascular invasion is of fundamental importance during bone development and any change in this balance leads to growth plate thickening and a reduction in bone formation[61].

1.2. The role of VEGF in bone Regeneration

Among all the growth factors that come into play during the processes of development, remodeling and repair of bone tissue, VEGF plays a crucial role in the phase of vascularization.

From a structural point of view, the VEGF is a 46-kDa glycoprotein that comprises six related proteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor. The most studied member of the VEGF family is VEGF-A. this family exist in different isoforms derived from the same gene by alternative splicing of messenger RNA and defined on the basis of the length of the amino acid chain, such as VEGF121, VEGF165, VEGF189, VEGF209 in humans[62].

VEGF plays several effects during the bone regeneration both directly and indirectly on osteoblast differentiation improving the bone formation. The vegf, in fact, acts directly on osteoblasts by promoting the osteoblast migration, proliferation and differentiation, and also playing a mediating role of different osteoinductive factors, such as TGF, IGF-I e FGF-2, which

upregulate VEGF expression in osteoblasts[63–65]. VEGF also acts on osteoblasts also indirectly by stimulating the endothelial cells on the one hand and on the other to produce anabolic factors that improve the bone formation.

Treatment with an anti-VEGF chimeric protein was found to inhibit blood vessel formation during endochondral ossification and led to compromised bone formation[66]. VEGF may be considered the major factor coupling between osteogenesis and angiogenesis[66].

2. 3D – Scaffold for bone regeneration

In order to regenerate the natural tissue you have to make three-dimensional structures that induce tissue regrowth with specific stimuli. Recent studies show that the isolated cells are hardly capable of organize themselves spontaneously to form complex tissues in the absence of three-dimensional structures able to guide and stimulate their activity[67]. In most cases, the cultured cells examined tend to multiply and proliferate only in two dimensions The three-dimensional tissue regeneration requires, therefore, a support (scaffold) that emulates the extracellular matrix for the organization of cells in complex structures. The role of the scaffold is to induce tissue regeneration by providing a "temporary guide" for cell growth, under appropriate culture conditions, to facilitate the process of differentiation[68].
These structures must show, under ideal conditions, good qualities of mechanical stability, preserving the specific characteristics of biological compatibility. Consequently, during the design phase must consider two distinct sets of parameters: the biological parameters, related to the interaction with the cells which it comes in contact, and engineering parameters, related to the mechanical and microstructural features of the system. In particular, the main parameters that must be considered are:

- **Biocompatibility** One of the primary requirements of any bone scaffolds is biocompatibility linked to the need to improve and / or restore a specific biological function, without interfering or interact in a harmful way with the physiological activities of the organism. An ideal bone scaffold must be osteoconductive where the scaffold allows the bone cells to adhere, proliferate, and form extracellular matrix on its surface and pores. The scaffold should also be able to induce new bone formation through bio-molecular signaling and recruiting progenitor cells, a property known as osteoinduction. Furthermore, an ideal scaffold needs to

form blood vessels in or around the implant within few weeks of implantation to actively support nutrient, oxygen, and waste transport[69].

- **Mechanical properties** The mechanical properties of an ideal bone scaffold should match host bone properties and proper load transfer is important as well. Mechanical properties of bone vary widely from cancellous to cortical bone. Young's modulus of cortical bone is between 15 and 20 GPa and that of cancellous bone is between 0.1 and 2 GPa. Compressive strength varies between 100 and 200 Mpa for cortical bone, and between 2 and 20 MPa for cancellous bone. The large variation in mechanical property and geometry makes it difficult to design an 'ideal bone scaffold'[69].

- **Pore size** A must have property for scaffolds is interconnected porosity where the pore size should be at least 100 mm in diameter for successful diffusion of essential nutrients and oxygen for cell survivability. However, pore sizes in the range of 200–350 mm are found to be optimum for bone tissue in-growth. Furthermore, recent studies have indicated that multi-scale porous scaffolds involving both micro and macro porosities can perform better than only macro porous scaffolds. Unfortunately, porosity reduces mechanical properties such as compressive strength, and increases the complexity for reproducible scaffold manufacturing.

- **Bioresorbability** Bioresorbability is another crucial factor for scaffolds in bone tissue regeneration. An ideal scaffold should not only have similar mechanical properties that of the host tissue, but also be able to degrade with time in vivo, preferably at a controlled resorption rate and eventually creating space for the new bone tissue to grow. The degradation behavior of the scaffolds should vary based on applications such as 9 months or

more for scaffolds in spinal fusion or 3–6 months for scaffolds in cranio-maxillofacial applications. Naturally, design and manufacturing of multi-scale porous scaffolds having ideal composition including targeted biomolecules, mechanical properties and related bioresorbability are some of the key challenges today towards their successful implementation in bone tissue engineering.

2.1. Bottom-up approach for tridimensional scaffold realization

Based on the engineering and biological parameters listed above, one of the main objectives to be achieved during the design of scaffolds is to guarantee accurate control over their macroscopic properties (e.g., geometry, mechanical strength, density, porosity) and microstructural (e.g., size, interconnection of the pores) and on their bioactivation (surface functionalization, release of growth factors).

For this purpose there are several techniques that depend on the type of polymer used and the application intended. In recent years, many studies in the literature have focused on the development of porous scaffolds by sintering of ceramic or polymeric microparticles, in order to overcome several limitations that are found in conventional techniques, mainly related to the mechanical properties, pore interconnection, as well as the use of toxic solvents. For this reason, in this project we propose a bottom up approach founded on the assembly of building blocks by solvent induced microparticle sintering to realize multifunctional polymer scaffolds with predefined pore dimension and fully percolative pathway, able to include interspersing microdepot for the release of bioactive molecules. This approach offers the possibility to realize scaffolds that not only meet all the requirements in terms of controlled microstructure, chemical stability and mechanical response necessary to support neo-tissue growth, but also provide a guidance of cell

and tissue processes by presenting bioactive agents in a predefined chrono-programmed manner.

This technique of sintering polymeric microspheres is based on partial dissolution of the microsphere surface, based on Flory–Huggins solution theory, to form porous three dimensional structures for tissue engineering applications.

Flory–Huggins solution theory deals with the interplay between monomer:monomer and monomer: solvent interaction in a polymer:solvent mixture. By a specific solvent/non-solvent mixture is possible to stabilize a balance between polymer dissolution and precipitation. During this intermediate state the microsphere surfaces were susceptible to bonding with adjacent microspheres prior to precipitation of the intertwined surface chains and sintering of the microspheres.

The use of specific solvent/non-solvent mixture under mild temperature during the sintering polymeric microspheres overcome some limitations when treating with thermolabile bioactive molecules, such as growth factors, which tend to denature with temperature.

The concept of sintering of microparticles originates from the process of densification of powders, at the base of the methods of production of ceramic materials, and is based on the fact that the system is able to redistribute their mass as a result of the formation of gradients of matter towards areas with high concentration of crystalline defects, triggered by appropriate conditions of temperature, leading to a densification of the system.

The simplest case of a single phase sintering takes place when an aggregate of particles is heated. Must be analyzed two simple conditions: an initial state represented by two particles in contact and a final situation in which the pores are closed in a continuous matrix. The shrinkage, densification and elimination of the pores occurs at a rate determined by the initial size of the

particle, from the value of surface energy and viscosity of the material. To obtain porous materials the sintering must be stopped at the first stage, by choosing appropriate parameters.

When the spheres begin to coalesce, the adhesion from spheres has a radius of curvature smaller than the curvature of the particle surface, leading to a negative pressure which causes a viscous flow of the material along the inter-particle region (Fig. 5)[70].

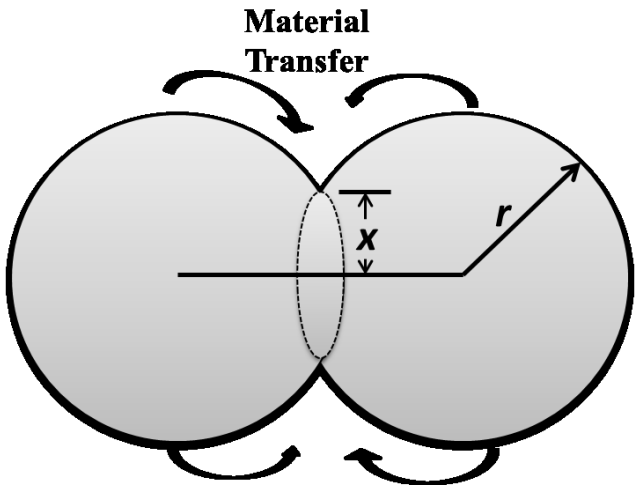

Figure 5 : schematization of a junction point formed between two beads in contact

There are a number of different pathways for the transport of material towards the area of the junction point during the initial stage. Some of these lead to densification, which consists in the process of narrowing that determines the approach of the centers of the microparticles. The other transport mechanisms lead to coarsening, which consists in the growth of the junction between the particles, resulting in a reduction of the specific surface area without any shrinkage. Two possible routes for the transport of matter take place by evaporation/condensation or by surface diffusion. These mechanisms of coarsening determine the displacement of material from the surface of the particles to the junction points, leading to a reduction of surface energy[70].

The transport mechanism that determines the speed of growth of the junction points greater will be the dominant mechanism and cause coarsening or densification. The particle size is very important in determining the speed, in that, in the equation of sintering there is a dependence from the inverse particle radius *r*. At constant temperature and composition of the equation of the speed for the densification and coarsening is:

$$\left(\frac{x}{r}\right)^n = \frac{k}{r^p}t$$

where, k is a constant that contains the surface tension and the diffusion coefficient, *n* varies between 2 and 6 and between 2 and 4[70].

3. Materials and Methods

3.1. Materials

The acid poly (D, L-lactic-co-glycolic acid) (PLGA) 50:50 (Resomer ® RG 504H, i.v. 0:32 to 0:44 dl/g) was purchased from Evonik® (Germany). The poloxamer188 (Pluronic ® F68, Mw 8400, HLB 29), the polycaptolactone, the polyvinyl alcohol (Mowiol 40-88) bovine serum albumin (BSA), heparin sodium salt (Hp), the substrate liquid ABTS (2,2 '-azino-bis (3 - etilbenzotiazolin-6-sulfuric) acid) and phosphate buffer (0.01M Na_2HPO_4, 0.0027 M KCl and 0.137 M NaCl, pH 7.4) were purchased from Sigma Aldrich (USA). The VEGF growth factor with the respective ELISA kit for the quantitative determination were provided by Peprotech (New Jersey, USA). All solvents used are of analytical grade and HPLC grade and were supplied by Sigma Aldrich (USA). The water used was filtered through 0.22 μM filter (Millipore, USA).

3.2. Microparticles Preparation

3.2.1. Polycaprolactone Microparticles

The PCL microparticles were prepared by the technique of oil in water (O/W) single emulsion with solvent evaporation. Briefly, a polymer solution, obtained by dissolving the polycaprolactone in dichloromethane (20 ml, 10% w/v), is added dropwise into 400ml of a aqueous PVA solution (Mowiol ® 40-88) with 0.1% of Tween 21. The presence of such emulsifying agents in the aqueous phase is essential for reducing the phenomena of coalescence and aggregation of the microspheres during the evaporation of the solvent. The two solutions are mixed for three hours at a suitable speed, using an electronic stirrer (RZR 2102, Heidolph, Germany), so as to allow the complete evaporation of volatile organic solvent and in order to obtain the

dispersed microparticles. After, this microparticles are filtered and washed three times with distilled water to remove the emulsifying agents, and finally allowed to dry under a chemical hood.

3.2.2. PLGA microparticles for controlled release of VEGF

The microspheres of PLGA containing VEGF were prepared by the technique of multiple emulsion/solvent evaporation (W/O/W) with growth factor theoretical loading of 0.01% (0.01 mg to 100 mg of VEGF microparticles). VEGF has been encapsulated in combination with stabilizing agents: the bovine serum albumin (BSA) and heparin sodium salt (Hp). A sterile solution in PBS, pH 7.4, containing VEGF, Hp and BSA (ratio VEGF / Hp / BSA 1:1:70 w / w / w) (250 µl) , was emulsified by homogenization at 8000 rpm for 1 minutes (Ultraturrax T25 Basic, probe 8G, IKA, Germany), in 10 ml of a solution of PLGA and poloxamer 188 (5:1 ratio) in dichloromethane (10% w/v) .

The emulsion thus obtained was added to the external phase consisting of 40 ml of an aqueous PVA solution (0.5% w/v) (Mowiol® 40-88), and emulsified by an electronic stirrer (RZR 2102, Heidolph, Germany), at 400 rpm for 3 hours, at room temperature, in order to facilitate the evaporation of the organic solvent and the precipitation of the polymer in the form of microparticles. Thereafter, the microparticles were isolated and washed with 30 ml of distilled water 3 times, by centrifugation at 5000 rpm for 10 minutes at 4°C, and subsequently subjected to a lyophilization cycle of 24 hours (0,001 atm , -60°C) (Freeze Dryer Alpha 1-4 LD plus, Martin Christ, DE).

3.3. Dimensional and Morphological analysis of microparticles

a) Scanning electron microscopy observation

The morphology and the internal structure of the microspheres was visualized using scanning electron microscopy (SEM) (Zeiss, FEG Ultraplus, Germany). A small quantity of plain microspheres was sprinkled on the SEM stubs and coated using a SEM coating system (Cressingthon, 208 HR, UK) with 10nm of platinum-palladium under an argon atmosphere. Samples were then observed for their surface morphology by SEM and photographs were taken at 20000 Kv and different magnification. One stub was made for each batch and many pictures were taken of each stub.

b) Particle size analysis

The average diameter and the particle size distribution was determined using a laser granulometer (Mastersizer 2000, Malvern Instrument, UK) from previously lyophilized microspheres suspended in an aqueous solution of PVA 0.5% (w/v). For each formulation the average diameter of the particles was expressed in microns ± standard deviation (SD) of the measurement carried out on three different batches of microspheres.

3.3.1. VEGF Encapsulation efficiency and Quantitative analysis

VEGF encapsulated and released from the microspheres was quantified by specific immunoenzymatic assay ELISA (*Enzyme-linked immunosorbent assay*), according to the procedure recommended by the manufacturer (ELISA Kit®, R&D Systems, USA). The standard solution of VEGF or the sample to be analyzed to appropriately diluted unknown concentration (200 uL) and 50 µ l of dilution buffer provided by the manufacturer (*RD1W*) were added to each well of the multiwell plate coated with a specific monoclonal

antibody of VEGF. The loaded plate was incubated for 3 hours at room temperature and then washed 3 times with a washing solution (*Wash Buffer*). Following the wash, we proceed with the addition of VEGF specific polyclonal antibody conjugated to the enzyme peroxidase. The plate is maintained in incubation at room temperature for 2 hours and then, after three washes, to each well to be analyzed is adds the substrate solution composed of hydrogen peroxide and chromium-tetramethylbenzidine (ratio 1:1). After 20 minutes of incubation at room temperature and subsequent washing, the colorimetric reaction is stopped with 50 µl of a solution of sulfuric acid 2 N. The optical absorbance was measured by spectrophotometer to the wavelength λ = 450nm.

- **Encapsulation efficiency of microparticles**

The amount of VEGF encapsulated within the microspheres was determined by degradation of the polymer matrix by basic hydrolysis, in a solution of sodium hydroxide. One mg of microspheres was suspended in 1 ml of 0.1 N NaOH and maintained at room temperature for about 24 hours. The obtained solutions were centrifuged at 4500 rpm for 15 minutes at 4°C, and the supernatant analyzed for the content of VEGF by ELISA, as previously described. The results were expressed as percentage encapsulation real or actual loading (mg VEGF encapsulate for mg of microspheres) and encapsulation efficiency (mg encapsulated real VEGF / VEGF theoretical X 100 mg) ± standard deviation (SD) of the measurement carried out on three different batches.

- **In vitro Release kinetics**

In vitro release studies of VEGF were made by suspending 5 mg of lyophilized microparticles in 1 ml of PBS pH 7.4, previously filtered through filter pores of 0.22 µm in diameter (Millex®, Millipore, USA). The samples

were incubated in a incubator at 37 ° C and 30 RPM, to mimic the conditions of release of the microspheres dispersed in a scaffold.

At regular intervals of time, the samples were centrifuged at 300 rpm for 5 minutes at 4 ° C and the release media removed and replaced with the same volume of fresh PBS pH 7.4. The samples taken were analyzed by ELISA, as described previously, to determine the amount of growth factor released. The results were expressed as ng of VEGF released per mg of microparticles ± standard deviation (SD) of the measurement carried out on three different batches.

3.3.2. VEGF released bioactivity

The HUVEC cells were previously prepared and characterized in Human Endothelial-SFM Basal Growth Medium in the presence of HI-FBS, EGF (100 μg/ml), Hp (100 μg/ml), sodium penicillin and sulfate streptomicin (respectively 10.000 U/mL penicillin e 10.000 μg/mL of streptomicin).

For the assay of VEGF proangiogenic activity on Matrigel has been used a plate with 24 wells. For each well have been added 230 μl of Matrigel (13 mg/ml) (R&D Systems), the plate was been incubate for 30 minutes at 37°C in order to obtain the formation of a gel. Subsequently, the HUVEC cells were stratified on this gel at a concentration of $8x10^4$ cells\ml of serum-free medium, in presence of VEGF microspheres (mg of microspheres corresponding to 20 ng/ml di VEGF released) or in presence of free growth factor (20 ng/ml).

The cells were incubate at 37°C to promote adherence to the gel layer. After 6 hours of incubation, is possible observe, at inverse microscopy, the formation of capillary network. Finally the cells were fixed with PBS containing glutaraldehyde at 0,2% (v/v) paraformaldehyde at 1% (w/v), photographed and analyzed by a specific software (*Scion Image*).

3.4. 3D – Scaffold

3.4.1. Scaffold Preparation: Microparticles Solvent Sintering

Once optimized the process of the microparticles fabrication was proceeded to their compaction using a random packing of such particles, in order to obtain a three-dimensional porous structure. Approximately 75 mg of PCL microparticles, previously sieved to obtain a suitable size range, are placed in a teflon mold with a diameter of 10 mm and height of 2 mm. Subsequently a solution of solvent/non solvent (30/70 v/v) are cast on the particles spreading by capillarity within the interstices formed between the microparticles. This solution was left to evaporate from the molded microspheres for 30 minutes under a chemical hood before and being placed under vacuum for an additional 24 hours in order to allow the complete evaporation of the solvent/non-solvent sintering solution (Fig.6).

For the achievement of the bioactive scaffolds, 5mg of PCL microparticles are replaced with the same mg of microspheres containing VEGF.

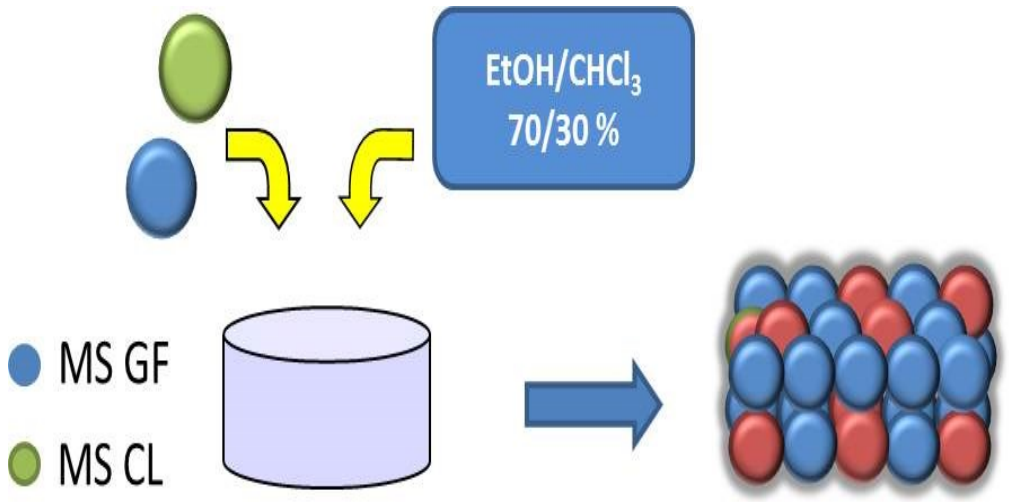

Figure 6 : schematization of the process of scaffolds realization

The different types of scaffolds are summarized in Table 4:

Type	Dimensional range (µm)
scaffold Protein-free	200-300
scaffold Protein-free	300-355
scaffold Protein-free	355-425
scaffold Protein-free	200-425
Bioactive scaffold	200-300
Bioactive scaffold	200-425

Table 4 : different types of scaffolds realized by solvent sintering

3.4.2. Surface Treatment

The surface hydrophobicity is well known as one of the key factors to govern cell response. More studies were showed that more hydrophilic is the surface of material films much more is the cell adhesion on the surface, for example, osteoblast adhesion was reported decrease when the contact angle of surface increased from 0° to 106°[71,72]. Moreover the preferential adsorption of some serum proteins (like fibronectin and vitronectin in culture medium and/or from cell secretion) onto the hydrophilic surfaces containing hydroxyl and carboxylic acid groups may be the reason for the better cell adhesion, spreading, and proliferation[73,74]. For this reason, we have modified the surface of the scaffold by two different treatments :

a) hydrolysis of the surface witch Ethanol\0.5N NaOH mix solution (15min, 200 RPM) to increase the percentages of hydroxyl (–OH) and carboxylic acid (–COOH) groups on the surface area.

b) O_2 plasma treatment. The plasma treatment was carried out on a

Gmbh Plasma Deposition System (Model Femto Diener, 13.53 Mhz, DE) under O_2 at 20%. The chamber was evacuated to less than 10 Pa before filling with oxygen. After the pressure of the chamber had stabilized to a proper value, glow discharge plasma was created by controlling the electrical power at a radio frequency of 13.56 MHz for a predetermined time (3min).

Static contact angle measurements were used to study the effect of different treatments on the surface of bioactive scaffolds, fabricated by solvent sintering. The surface wetting characteristics were examined with a drop-shape analysis system. Deionized water droplets (~ 4 μL) were delivered to the sample surface by a syringe at room temperature and the droplet configuration was captured with a camera. From the measured angle between the droplet baseline and the tangent at the water/air boundary, a contact angle was calculated as the average of the measured left and right contact angles. For statistical analysis, six contact angle measurements were obtained from three different surface regions of two identical samples.

3.4.3. Morphological characterization

a. Scanning electron microscopy observation

The SEM analysis of the sintered scaffolds was carried out to study the morphology and qualitatively evaluate the extension of the junctions between the microparticles and the porosity in terms of distribution, pore size and degree of interconnection. Briefly a section of scaffold was sprinkled on the SEM stubs and coated using a SEM coating system (Cressingthon, 208 HR, UK) with 15nm of platinum-palladium under an argon atmosphere. One stub was made for each type of scaffold and seven pictures were taken of each stub at different magnification.

b. Atomic Force Microscopy observation

The atomic force microscope is a member of the scanning probe microscopy techniques that utilize a probing tip that scans the surface of a sample. The measured interaction between the sample and the probe (e.g. current in scanning tunneling microscopy or force in AFM) renders a three-dimensional image of the sample surface[75].

This microscope was used to study the surface morphology of treated and untreated scaffold. The samples were analyzed with a Nanowizard II microscope (JPK, Santa Barbara, CA). AFM images were recorded in contact method at room temperature in air using silicon cantilevers. Height phase images were recorded with the maximum available number of pixels (512×512). Three-dimensional images were acquired using Nanowizard image processing software. The roughness were carried out using the cross section analysis..

3.4.4. Micro – CT characterization

A precise and quantitative analysis of porosity of the sintered scaffolds, in terms of distribution, pore size and degree of interconnection has been obtained by microcomputed tomography (micro-CT), a radiographic method for the production of cross sectional images of bodies.

The information originates from the X-ray attenuation measurement that undergo crossing the sample. The result of this process is the formation of two-dimensional image (projection). Now if the sample or the detection system are rotated, you will get many projections how many are the angles of rotation. The composition of these projections (using sophisticated algorithms) provides an image of a slice of the sample. If you rotate one of the components of the system, you add a translation motion of the system X-ray source-sample-detector you can get the individual slices at different

heights; overlaying this slices it is possible to obtain a reconstruction of the entire volume of the sample and then the full view of its internal structures[76].

Three samples for each type of sintered scaffolds was subjected to topographical analysis and through the images of the section collected, implemented through dedicated software (CTAn) it was possible to calculate several characteristic parameters (total porosity, average pore size and pore distribution) based on the size of the individual pixels that defines the image resolution.

3.4.5. Mechanical characterization

To evaluate the mechanical properties of the sintered scaffolds was performed a test of static compression through the use of a dynamometer (Istron, Model 5942) in order to determine the strength, the elastic modulus and deformation for each type of sample. The compression tests were performed, in reference to ASTM 695/2°, on samples of cylindrical geometry with a diameter of 10 mm and a height of 2 mm. It is used a preload of 0.5N and e load cell of 1N, setting a speed of the moving crosshead of 1 mm/min.

3.4.6. PCL/PLGA microparticles distribution study

To evaluate the distribution of the PLGA microspheres, containing the growth factor, inside the scaffold, we are performed confocal microscopy analysis. Specifically, were made PLGA microspheres with the same procedure as above, but substituting the poloxamer 188 with one labeled with Rhodamine B. Subsequently this microspheres have been used for the preparation of several scaffolds (n = 10) and observed at confocal microscopy (λ = 485). The images obtained were analyzed and the

distribution of the marked microspheres in the volume of the scaffolds was calculated.

3.4.7. Cells adhesion test on scaffold surface treated

To evaluate the influence of the two treatments on the process of cell adhesion were carried out specific tests. The different types of scaffolds (untreated, plasma and EtOH/NaOH treated) are placed in 24multiwell plate and plated with human umbilical endothelial vein cells (HUVECs) suspended in medium in order to obtain a surface concentration of $5 \cdot 10^4$ cells/cm^2 . It is explicitly noted that is not added in the culture medium serum, which contains several adhesion proteins and growth factors that may adversely affect the adhesion test. In this way, then, you are sure that any cell adhesion is due solely to the contribution of the type of treatment.

The different scaffolds are, at this point, placed in an incubator at 37 °C with a controlled atmosphere at 5% CO_2. After 6h of incubation is carried out cell counts adhere to different scaffolds, through the Bürker chamber. In reality, this detection is the result of a cross-check between the number of cells that are located on the bottom of the container and in suspension, that, by the difference of the number of total cells plated, giving those who actually have adhered to the scaffolds.

To observe cell morphologies, a part of the scaffolds seeded with cells, to 6h from the seeding, were fixed in 2.5% glutaraldehyde (SIC, Rome, Italy) in 0.1 M sodium cacodylate (SIC), pH 7.3, washed three times for 10 min in the same buffer, post-fixed in 1% osmium tetroxide (SIC) in 0.1 M sodium cacodylate, pH 7.3, on ice for 1 hour, washed three times for 10 min in the same buffer and dehydrated in ascending series of ethanol on ice. At the end of dehydration, samples were put in critical point drying (CPD Leica,) placed on aluminum stub with carbon tape, coated with 20 nm of gold metal

by sputter coater (208 HR sputter coater cressington) and observed at a Zeiss ultraplus scanning electron microscopy.

To observe cell proliferation, a part of the scaffolds seeded with cells, to 6h from the seeding, were fixed in 4% paraformaldehyde and subsequently labeled with phalloidin tetramethylrhodamine B isothiocyanate and Sytox green(Invitrogen) in accordance with the procedures recommended by the manufacturers. The samples were observed under a confocal microscope(Leica, model TCS SP5 II).

3.4.8. Bioactive scaffold "In Vitro" Release kinetics

In vitro release studies of VEGF from sintered scaffolds were made by suspending one scaffold in 1 ml of PBS pH 7.4, previously filtered through filter pores of 0.22 μm in diameter (Millex®, Millipore, USA). The samples were incubated in an incubator at 37 ° C and 30 RPM, to mimic the in vivo conditions. At regular intervals of time, the release media removed and replaced with the same volume of fresh PBS pH 7.4. The samples taken were analyzed by ELISA, as described previously, to determine the amount of growth factor released. The results were expressed as ng of VEGF released per scaffold ± standard deviation (SD) of the measurement carried out on three different batches.

3.4.9. Cells – Bioactive Scaffold Interaction analysis

The human endothelial cells (HUVECs) were used for the experiments of cell-material interaction. Approximately two hundred thousand cells were suspended in 200μl of culture medium containing 20% fetal bovine serum (FBS), a vascular endothelial growth factor (lg / mL), 1% pen/strep (10,000 U / mL penicillin and 10,000 lg / mL streptomycin). The HUVECs were

seeded in a static way on bioactive scaffolds with PLGA microspheres containing VEGF. As a control, we used the same PCL scaffolds containing empty PLGA microspheres.

After about two hours, time necessary to allow the cells to adhere to the material, was added to the above scaffold a complete medium containing 5% fetal bovine serum (in this time in order to assess the proliferative effect due to the only VEGF released from the microspheres). The samples kept in incubator for cells in static conditions after 1 and 7 days of culture were blocked in formalin for 20 min at room temperature. She was later performed a staining of actin microfilaments with phalloidin tetramethylrhodamine B isothiocyanate and of cell nuclei with Sytox green (Invitrogen) in accordance with the procedures recommended by the manufacturers. The samples were observed under a confocal microscope(Leica, TCS SP5 II).

4. Results and Discussion

4.1. Microparticles characterization

The morphology of all types of microspheres produced was evaluated by SEM. Are shown the micrographs relating to PCL and PLGA microspheres, in particular, for each type are shown two micrographs at different magnification, in order to emphasize the size distribution and surface morphology of the individual microspheres.

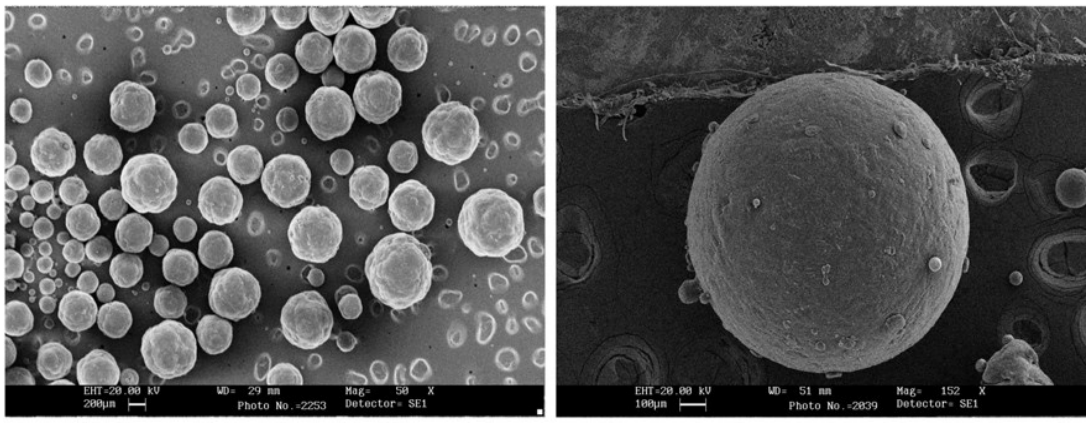

Figure 7 : micrographs of Polycaprolactone microparticles at different magnification

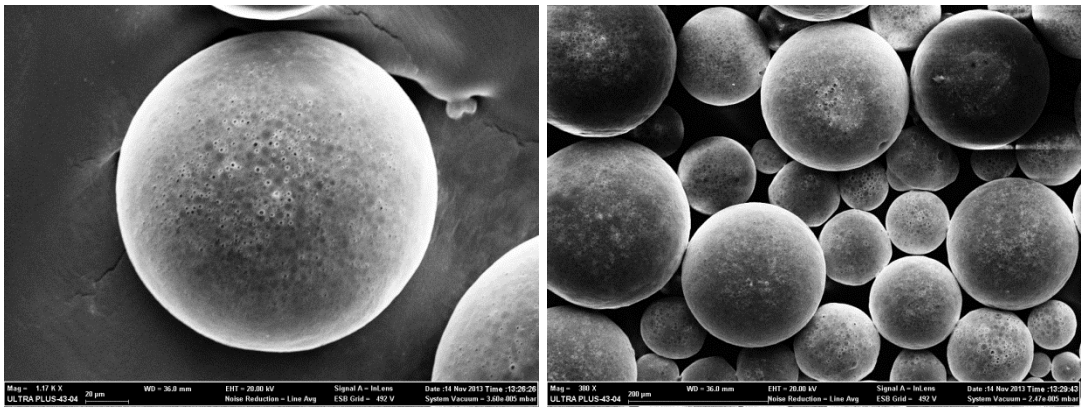

Figure 8 : micrographs of PLGA micropheres at different magnification

From the micrographs shown (Fig. 7) it is found that the Polycaprolactone microparticles are spherical and non-porous. These properties are suitable for

the production of scaffolds for sintering, because microspheres of regular shape facilitate the formation of porous structures interconnected, while the surface porosity is not a necessary condition. Moreover, it is observed, a rather broad size distribution that does not allow an accurate control of the properties of the scaffold, because the presence of microparticles with wide dimensional range may involve an occlusion of the pores.

From the micrographs shown (Fig. 8) it is found that the PLGA microspheres are spherical and porous. These characteristics are suitable to obtain a controlled and prolonged release of the dispersed growth factor. In fact the presence of a dense pore networks, which goes from the inside to the surface of the microspheres, as shown by the sections analyzed (Fig. 9), allows to control the release of the factor in the desired mode.

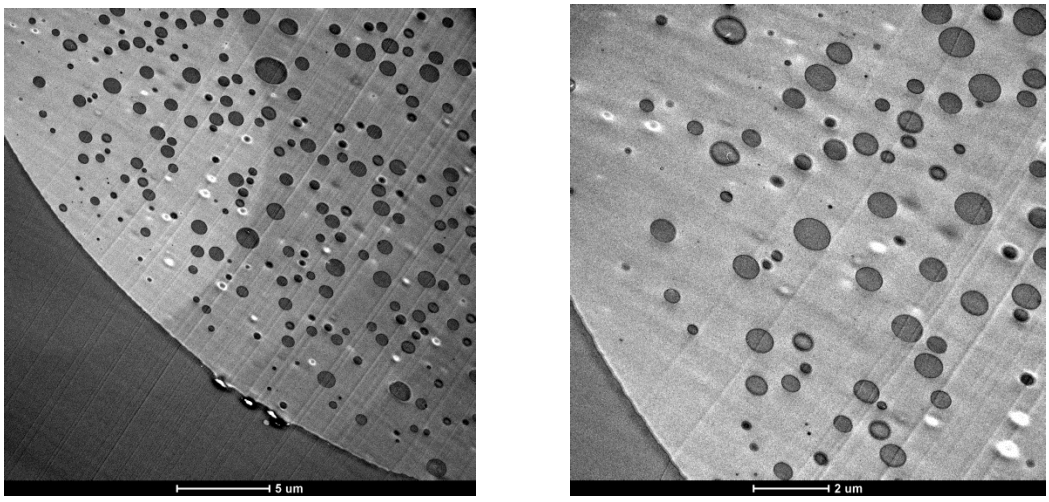

Figure 9 : micrographs of sections of PLGA micropheres at different magnification

4.1.1. VEGF encapsulation efficiency and “In Vitro” release kinetics

The microparticles based on PLGA containing VEGF were prepared by the technique of multiple emulsion/solvent evaporation (W/O/A), widely used for the microencapsulation of proteins and growth factors.

The specific formulation conditions adopted have allowed to obtain microparticles able to encapsulate VEGF with an efficiency of encapsulation of about 55% (55.14% ± 2.16 ng VEGF for mg of microspheres).

The release profile of VEGF by microparticles is shown in Figure X and is expressed as ng of VEGF released in active form for 1 mg of microspheres as a function of time.

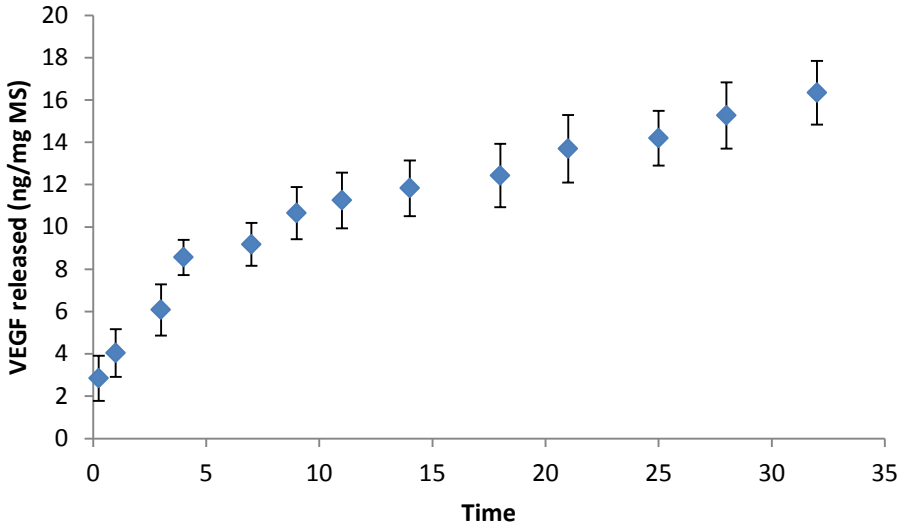

Figure 10 : Release profile of VEGF by PLGA microparticles (ng\mg of MS)

As can be seen from the graph, the microparticles show an initial release of VEGF, in the first day of incubation, rather slow (<5 ng/mg), followed by a controlled release of VEGF sustained for over a month. In particular, the particles are able to provide constant release of VEGF of about 0.5 ng/mg daily for all period evaluated.

These results suggest that the microparticles manufactured have excellent potential, when placed in three-dimensional substrates, promoting the activation of angiogenic processes, through careful control of the concentrations of GFs inside the scaffold.

4.1.2. VEGF released bioactivity

One of the basic requirements of a controlled release system of growth factor is to safeguard the maintenance of bioactivity during the encapsulation process. In this case the bioactivity of VEGF released from microparticles was evaluated by the Matrigel assay, as previously described. With this assay it was possible to quantify the proangiogenic response in function of the length and extent of the capillary network formed after 1 day of incubation in cell culture medium without proangiogenic agents (figure 11-A), containing soluble VEGF (Figure 11-B) or microspheres loaded with VEGF (Figure 11-C).

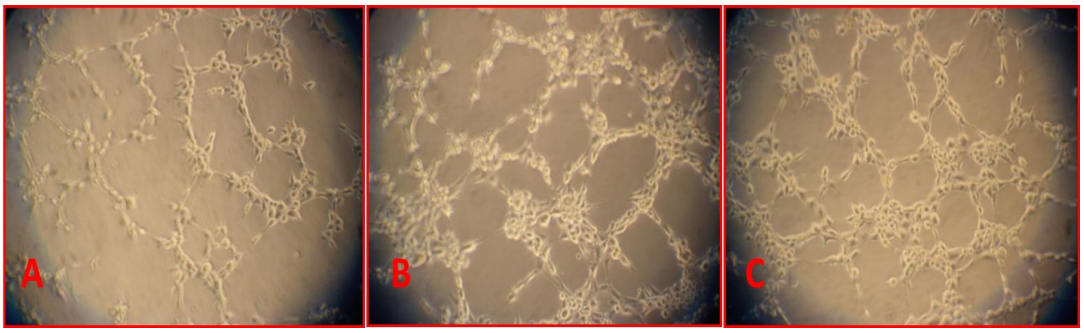

Figure 11 : In vitro assay on Matrigel. The images (A, B, C) represent the vascular network formed after 6 hours of incubation: A) in the absence of VEGF, B) with free VEGF (20ng/ml), C) with microspheres containing VEGF

.

Finally, the graph in Figure 12, shows that the number of capillary structures formed in the presence of microparticles containing VEGF is equal to that obtained in the presence of the same amount of free VEGF.

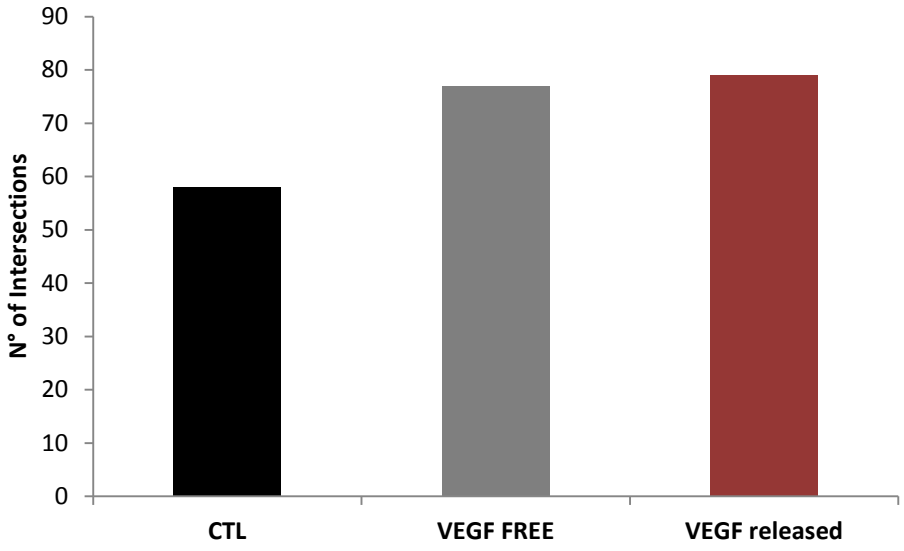

Figure 12 :Number of intersections formed after 6 hours of incubation in the absence of VEGF, with free VEGF (20ng/ml) and with microspheres containing VEGF

4.2. Scaffold characterization

In order to modulate the driving force that regulates the compaction of adjacent microspheres, we selected a suitable solvent-non-solvent pairs such that the solvent was more volatile than the non-solvent and the two were miscible with each other. In the specific case, the selected solvent was chloroform and the selected non-solvent was ethanol. The choice of chloroform (solvent) was dictated by its high ability to dissolve the PCL, the PLGA and its fast kinetics of evaporation; at the same time, the use of ethanol (non-solvent) has permitted a reduction of the power of chloroform dissolution.

The effect of solvent concentration (mg MS/µl of solvent) was studied and the results are shown in Figure 13, which depicts the effect of the concentration of the sintering solution on the degree of sintering between the microspheres.

The scaffold sintered at concentrations less than 1% (W/V) did not survive demolding and were discarded from the analysis. The scaffold sintered with the 1% (W/V) solvent composition remained intact after demolding suggesting that the microspheres were indeed sintered even though it is difficult to see the region where the microspheres are joined together. As the concentration increases from 1% (W/V) the bonding region between the microspheres becomes more evident. At a concentration of 2% (W/V) the sintering causes occlusion of the open porosity visible at lower concentrations.

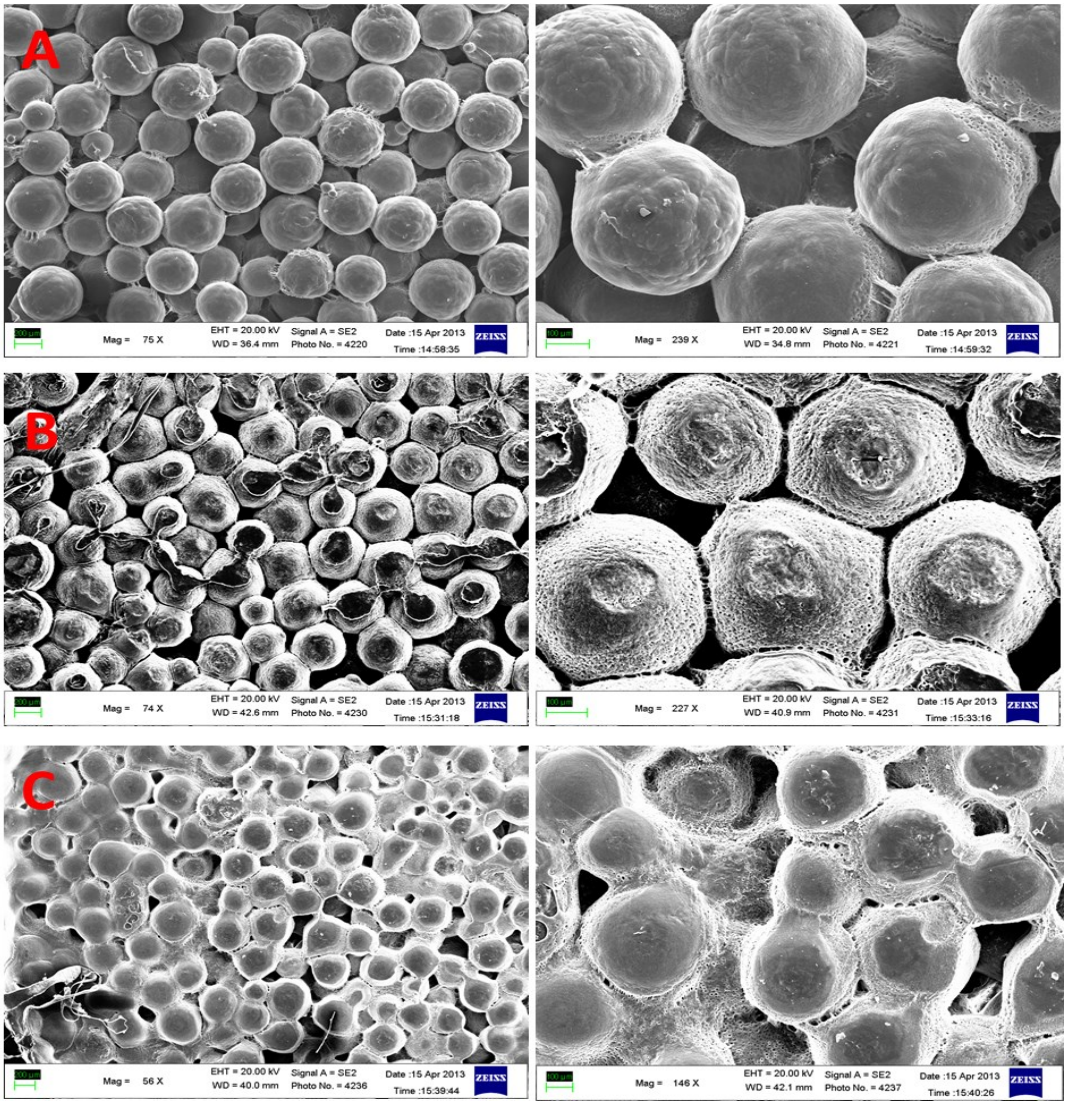

Figure 13 : Micrographs at different magnification of scaffold sintered at different concentrations of solvent / non-solvent (ul / mg MS): a) 1%, B) 1.5% and C) 2%.

The SEM analysis showed, in all samples, that the microparticles are distributed randomly, with points junction connection arranged uniformly throughout the volume (Fig. 14, 15 and 16).

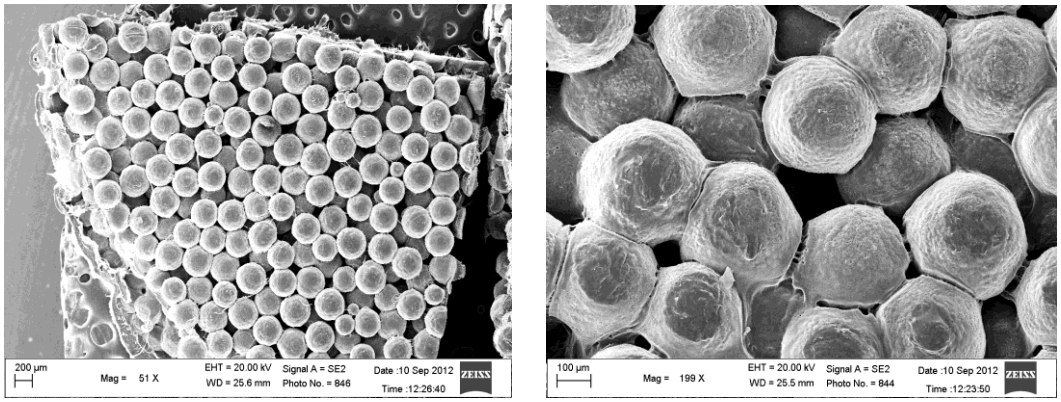

Figure 14 : Micrographs at two different magnifications of scaffolds made with microspheres in the range 300 to 355 µm.

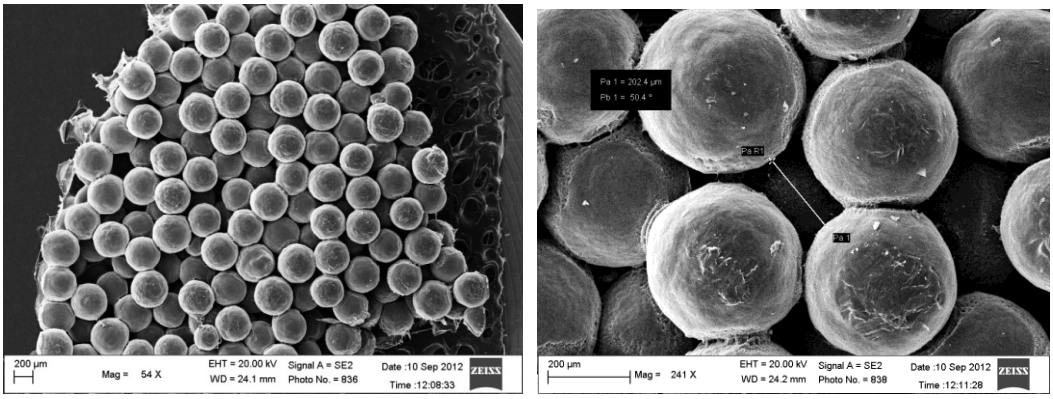

Figure 15 : Micrographs at two different magnifications of scaffolds made with microspheres in the range 355 to 425 µm

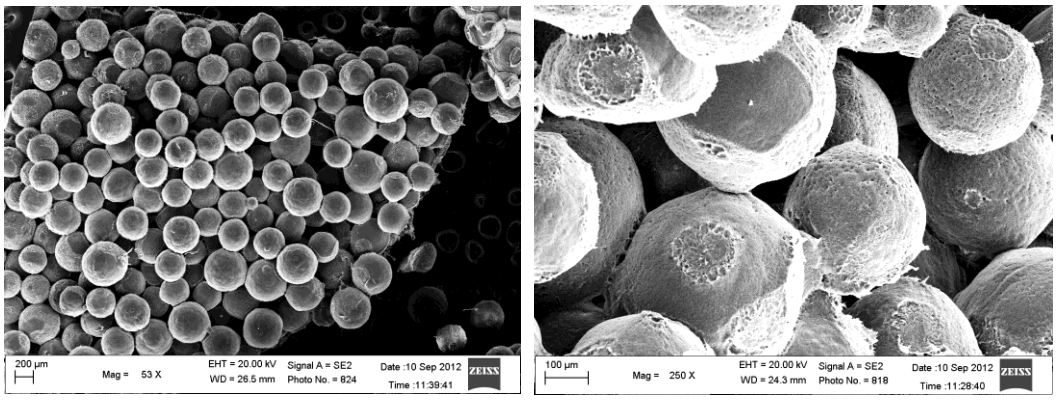

Figure 16 : Micrographs at two different magnifications of scaffolds made with microspheres in the range 200 to 425 µm

It 'also evident from these micrographs, the formation of points junction, characterized by such dimensions as to ensure a high bond strength between the microspheres. These junctions, although are well developed and elongated, are confined in the neighborhood of the junction points, resulting in a fully interconnected structure.

Bioactive scaffolds have been obtained through the compaction of PCL microparticles, obtained by single emulsion and PLGA\VEGF microparticles, obtained by the double emulsion, using the same parameters used for the realization of scaffold protein-free.

The images obtained by confocal microscopy show that the microparticles containing the growth factor are uniformly distributed in the total volume of the scaffold and predicted a homogeneous release of the factor into the surrounding environment (Fig. 17).

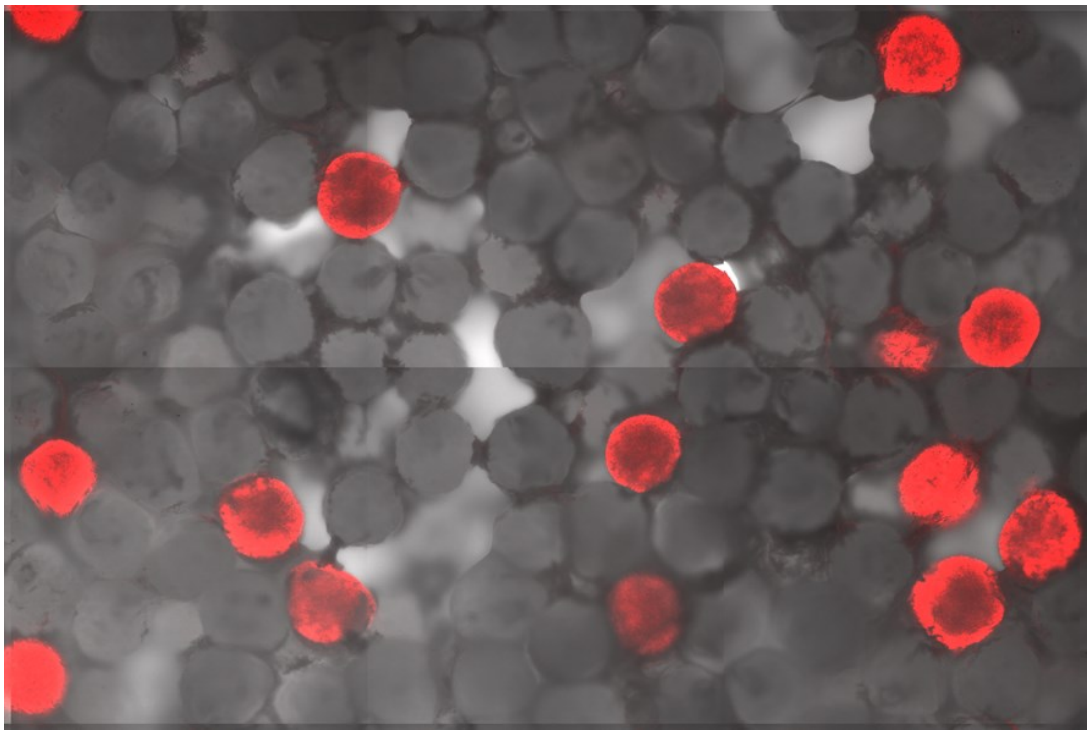

Figure 17 : Sections of scaffold manufactured with pcl microparticles and plga/Rhod microparticles. The image show that the plga microparticles are uniformly distributed in the total volume of the scaffold

4.2.1. Porosimetric analysis by Micro-CT

A qualitative and quantitative evaluation of the porosity was performed by analysis techniques of 3D images with the help of a computerized microtomograph. Through the images of the sections of the sample was possible to calculate some characteristic parameters of the structural properties of the scaffold (volume fraction, average pore size and pore distribution), based on the size of the individual pixels that defines the resolution of the image. The table 5 shows the values obtained from the analysis of images made using dedicated software (CTan):

Scaffold Types	Microsphere Diameter (µm)	Total Porosity (%)
Protein-free Scaffold	200-425	29.5 ± 2.1
Protein-free Scaffold	355-425	36.46 ± 1.7
Protein-free Scaffold	300-355	34.65 ± 2.2
Protein-free Scaffold	200-300	33.30 ± 0.7
Bioactive Scaffolds	200-425	30.23 ± 1.3
Bioactive Scaffolds	200-300	34.76 ± 0.6

Table 5 : Relationship between the size of the microparticles used and the porosity of the scaffolds produced as assessed by micro-CT

The results show a total porosity decreasing with decreasing size of the microparticles used for the realization of the scaffold. Scaffold made with only the particles with broader range show a porosity of less than all the others and this may be attributable to the greater packing to which the microspheres are meeting and which result in a reduction of the total porosity of the scaffolds.

Passing from Scaffold protein-free to those bioactive, made with the same size range of microspheres, the total porosity does not vary, showing that the microspheres of PLGA not interfere with the packaging process.

it was also possible to reconstruct the three-dimensional images of the cross sections of the sample using an appropriate software (CTVol) able to reproduce the original structure. Is shown below the relative structure of the scaffold protein-free obtained by sintering of microparticles with size range 200 to 300 μm:

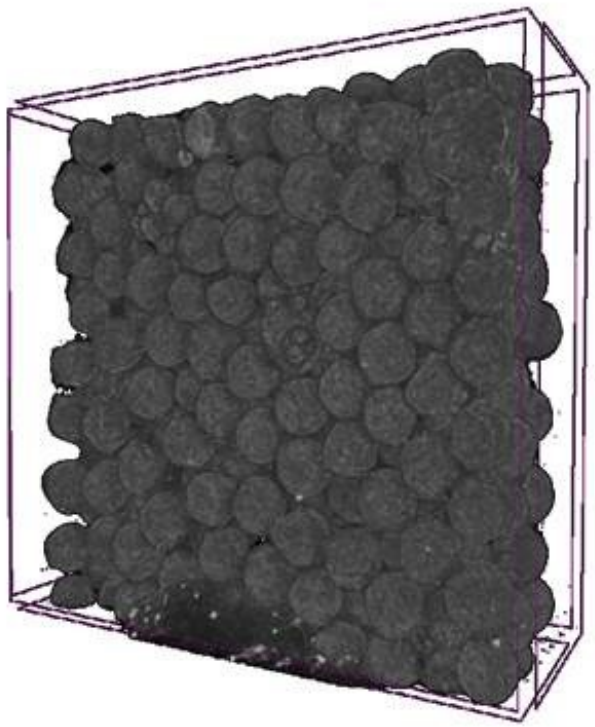

Figure 18 : 3D representation of the scaffold protein-free obtained by solvent sintering of microparticles with a size range 200 to 300 μm.

4.2.2. Surface treatment

The surface property of a material especially the degree of hydrophilicity or hydrophobicity plays a very important role in cell adhesion during the initial period of cell seeding. This determines the successful formation of tissue constructs leading to the subsequent cell proliferation, differentiation and new tissue in-growth.

Because scaffolds are composed of PCL and PLGA, two highly hydrophobic polymers, to facilitate the process of cell adhesion and proliferation, were subjected to two types of treatments: chemical and plasma treatments.
To study the effect of the different treatments, the water contact angles on the surface of scaffold were measured by a sessile drop technique, previous described (fig. 19).

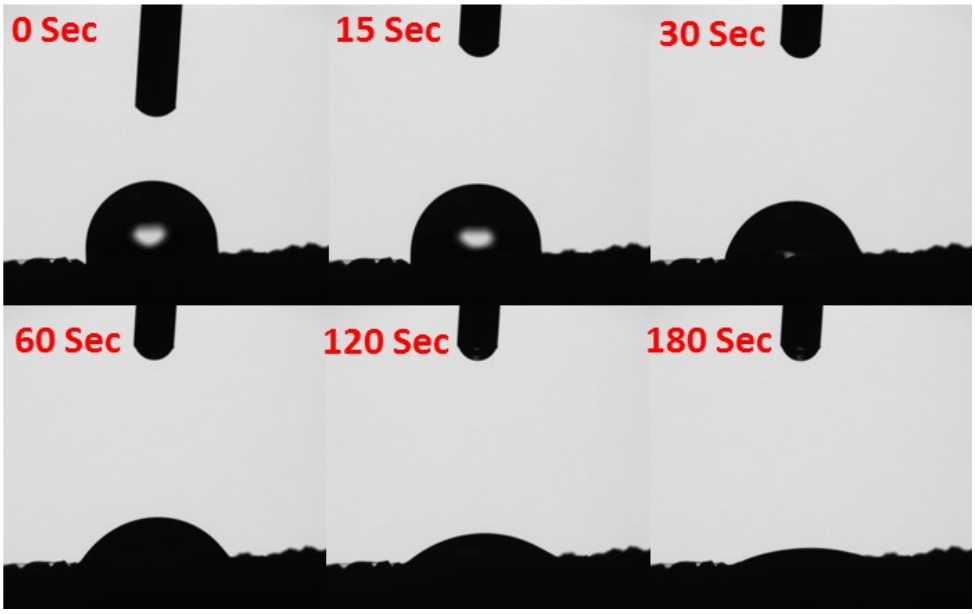

Figure 19 : Water contact angle images at different time point on scaffold protein free untreated

The results of this analysis are listed as a function of time in Table 6.

Time Point (Sec)	untreated (± S.D.)	EtOH\NaOH treated (± S.D.)	Plasma treated (± S.D.)
0	93.787 ± 3.601	34.683 ± 8.407	4.806 ± 9.621
15	71.824 ± 3.812	0	0
180	21.442 ± 3.342	0	0

Table 6 : Measured static contact angle data at different time point on untreated, EtOH/NaOH treated and plsma treated PCL scaffold surface.

These results clearly show that chemical methods as well as plasma treatments are able to enhance the attachment and growth of cells. Plasma surface modification is effective in enhancing cell–material interactions as a Ethanol\sodium hydroxide treatments.

the greater hydrophilicity of the scaffold plasma treated compared with the scaffold alkaline hydrolysis treated, can be explained by the greater penetration of the plasma that resulted in a deeper modification of the surface with the introduction of a greater number of polar components, such as hydroxyl (—C—OH), carbonyl (—C═O), carboxyl [—(C═O)—OH] oxygen groups, on the scaffold surface and the cleaving of the C—C and C—H bond in the polymers backbone. These polar functional groups not only increase the wettability and solid surface energy of the polymer surface but also improve the early cell attachment and protein adsorption.

This hypothesis was also confirmed by the study of the treated surfaces through analysis of atomic force and scanning electron microscopy. The micrographs and the AFM images shown in figures 20, 21 and 22, show that while the treatment of alkaline hydrolysis involves no substantial change in the surface compared to the control, the plasma treatment causes a structural change of the surface, with the formation of numerous growths

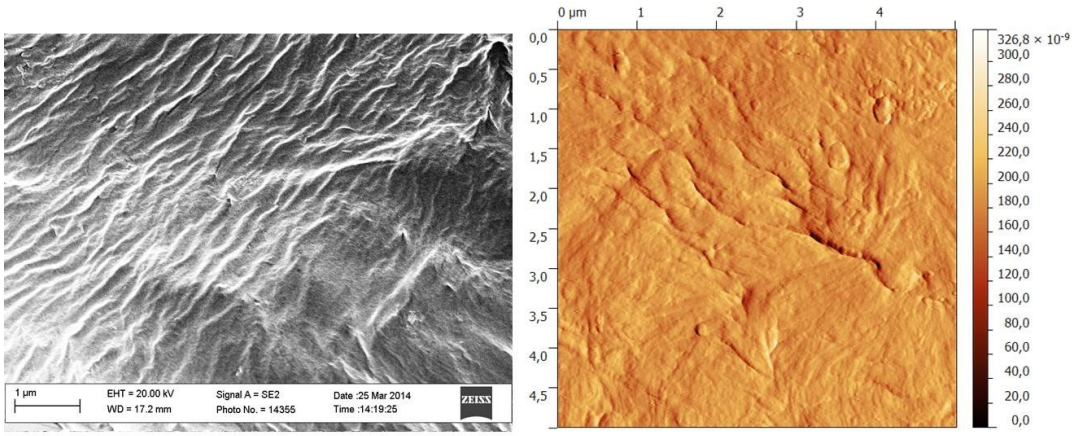

Figure 20 : Micrograph and AFM image of the surface of the scaffold protein free untreated

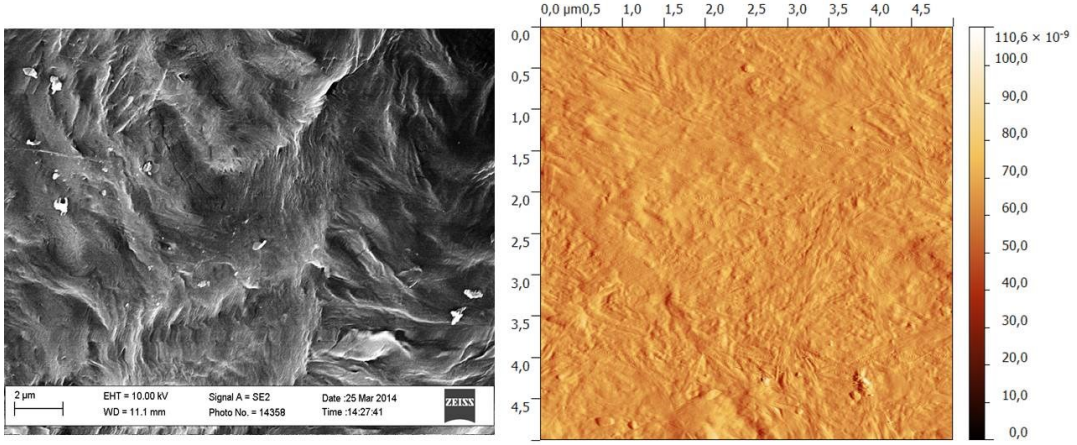

Figure 21 : Micrograph and AFM image of the surface of the scaffold protein free EtOH\NaOH treated

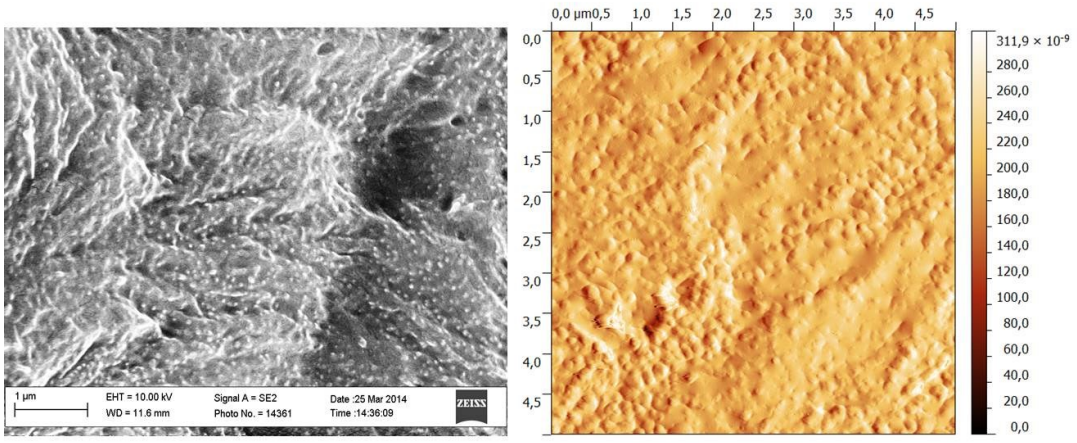

Figure 22 : Micrograph and AFM image of the surface of the scaffold protein free Plasma treated

In order to observe the effect of surface treatment on the adhesion, proliferation, and differentiation behavior of cells, the HUVECs cells were cultured on the scaffold with different treatments.

Regarding the morphology of the cells, at 6 hours after seeding, does not seem to vary a lot from one treatment to another (Figure 23). In particular in all three Scaffold all cells have a spherical shape with the presence of lamellipodia and filopodia.

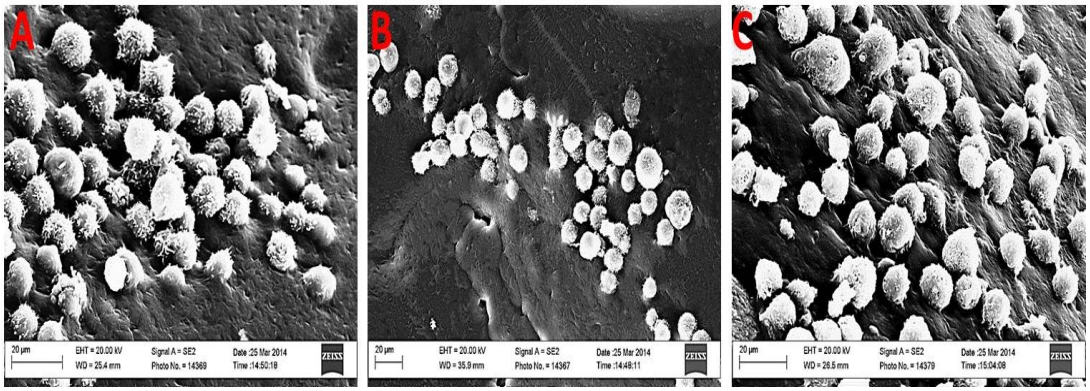

Figure 23 : Micrographs of cells on scaffolds: (A) untreated, (B) EtOH / NaOH treated and (C) Plasma treated. In all three micrographs the cells morphology is equal, indicating that the different treatments do not affect the morphology.

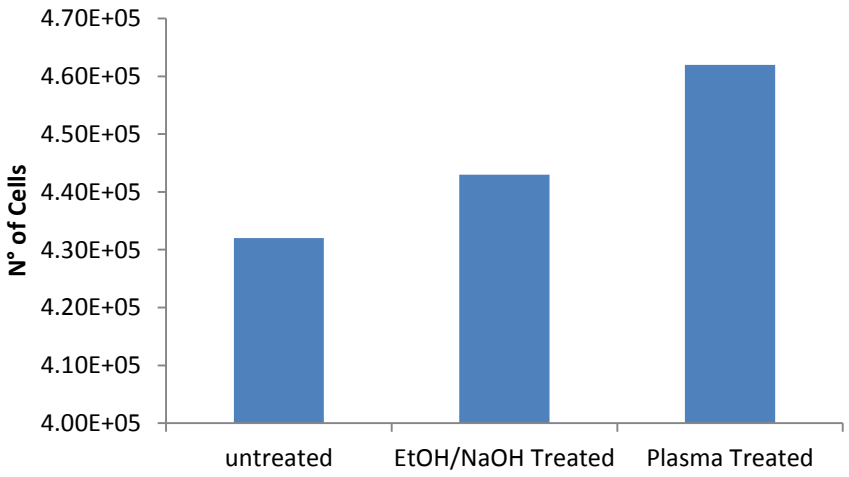

Table 7 : type of treatment performed on the surface of the scaffold in function of cell adhesion

Conversely, to change is the number of cells (6 hours after seeding), that adhere on the surface of the scaffold. Reporting the type of treatment performed on the surface of the scaffold as a function of cell adhesion (Tab 7) may be observed that, in both the scaffolds treated, cell adhesion is greater than the non-treated, in particular is maximum for plasma. This, as already pointed out above, may be due to the greater number of polar components and the increased roughness of the plasma surface treatment.

4.2.3. Mechanical properties

The mechanical strength of the scaffolds sintered, as well as the influence of the size of the microparticles used on such properties, was evaluated by mechanical compression test . The results obtained are reported in the following table:

Tipologia Scaffold	**Diametro Microparticelle (µm)**	**Modulo Elastico (Mpa)**
Scaffold Protein-free	200 - 425	4.26 ± 0.31
Scaffold Protein-free	355- 425	2.36 ± 0.54
Scaffold Protein-free	300 - 355	2.41 ± 1.7
Scaffold Protein-free	200 - 300	2.73 ± 3.2
Scaffold Protein-free EtOH/NaOH treated	200 - 300	2.05 ± 2.74
Scaffold Protein-free plasma treated	200 -300	4.61 ± 2.31

Table 8 : Relationship between the size of the microparticles used and the mechanical properties of the scaffolds made.

The samples tested show an elastic modulus of between 2 MPa and 4.5 MPa approximately. From the mechanical characterization performed on the untreated samples were not observed significant changes in the elastic modulus to vary the range of microspheres used, except in the sample with the range 200-425 which. This result can be attributed to the increase in the number of junction points and, therefore, of structural constraints in the structure of the scaffold.

In the samples that were subjected to a surface treatment we can see that while in the scaffolds treated with EtOH/NaOH the elastic modulus does not vary compared to untreated, the scaffolds treated with plasma show a doubles value of the elastic modulus. This phenomenon can be attributed to the fact that the plasma treatment penetrates deeper into the polymer backbone, making it more rigid.

4.2.4. "In Vitro" Release kinetics from bioactive scaffold

The release profile of VEGF by bioactive scaffold is shown in Figure 24 and is expressed as ng of VEGF/scaffold released in active form in function of time. We observe an initial release of VEGF, in the first day of incubation, rather slow (<6 ng/scaffold), followed by a controlled release of VEGF sustained for over a month. In particular, the scaffold are able to provide a constant release of VEGF of about 2.5 ng/scaffold daily for all period evaluated.

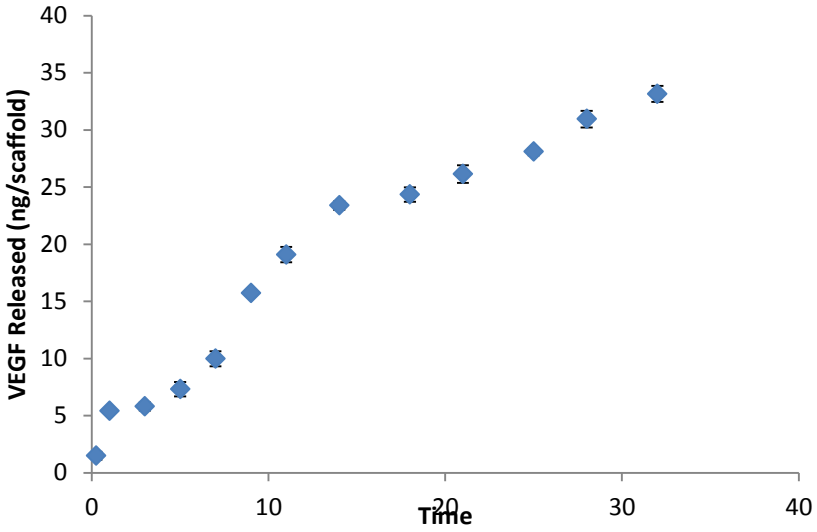

Figure 24 : Release profile of VEGF by bioactive scaffold (ng\scaffold)

Graph 25 compares the kinetics of release of VEGF from individual bead and scaffolds, expressed both as ng VEGF/mg of microspheres. This comparison shows that the trend of the release kinetics is practically the same, but much slower for the beads inserted into the scaffold. This phenomenon can be ascribed to the process of sintering that through the formation of the junction points between microspheres, reduces the surface area available to the free factor. In addition, the tortuosity of the path to which the growth factor meets once released by the microsphere further slows its kinetics.

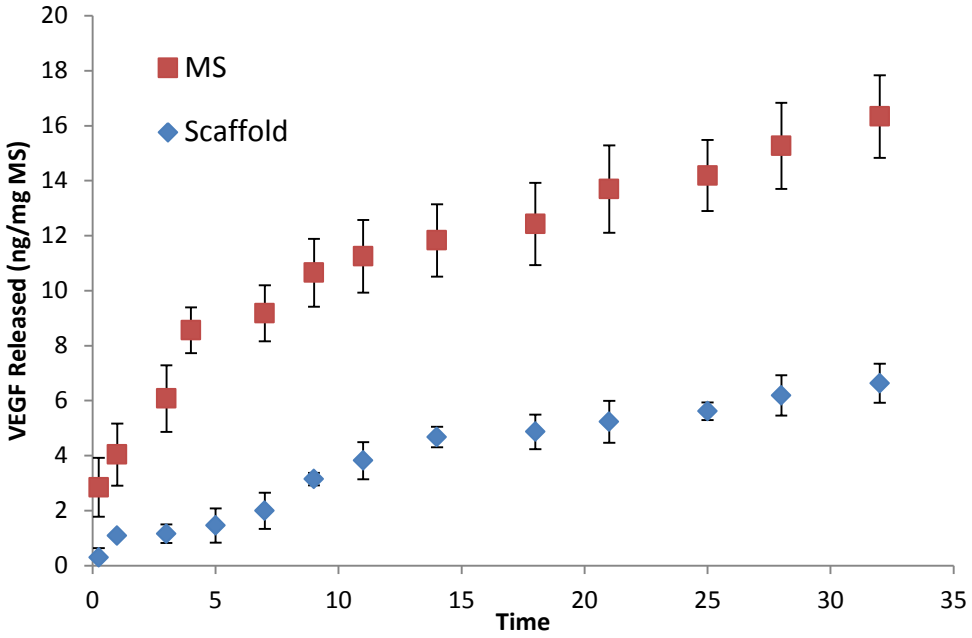

Figure 25 : Comparison between the release kinetics of VEGF from scaffolds and single microparticles (ng VEGF / mg MS)

These results suggest that the bioactive scaffolds made from microparticles containing VEGF, previously described, have enormous potential in inducing the formation of new blood vessels and consequently to promote the formation of a highly vascularized tissue.

4.2.5. Cell adhesion and proliferation studies

Tests were carried out on cell invasion in the scaffold. On the surface of the scaffolds were seeded endothelial cells, as previously described. After one and seven days cellularizzati samples containing microspheres with and without VEGF were fixed with formalin. Subsequently, immunofluorescence was performed using phalloidin to stain the actin cytoskeleton in red and Sytox green to color the nuclei in green. The images were made with two-photon confocal microscope. As can be seen in Fig 26, one day after the sowing we do not have a significant difference between free protein scaffolds

and bioactive scaffolds. In both cases, at the time a day, we can only evaluate the adhesive effect of the cells to scaffolds mediated by medium containing fetal serum.

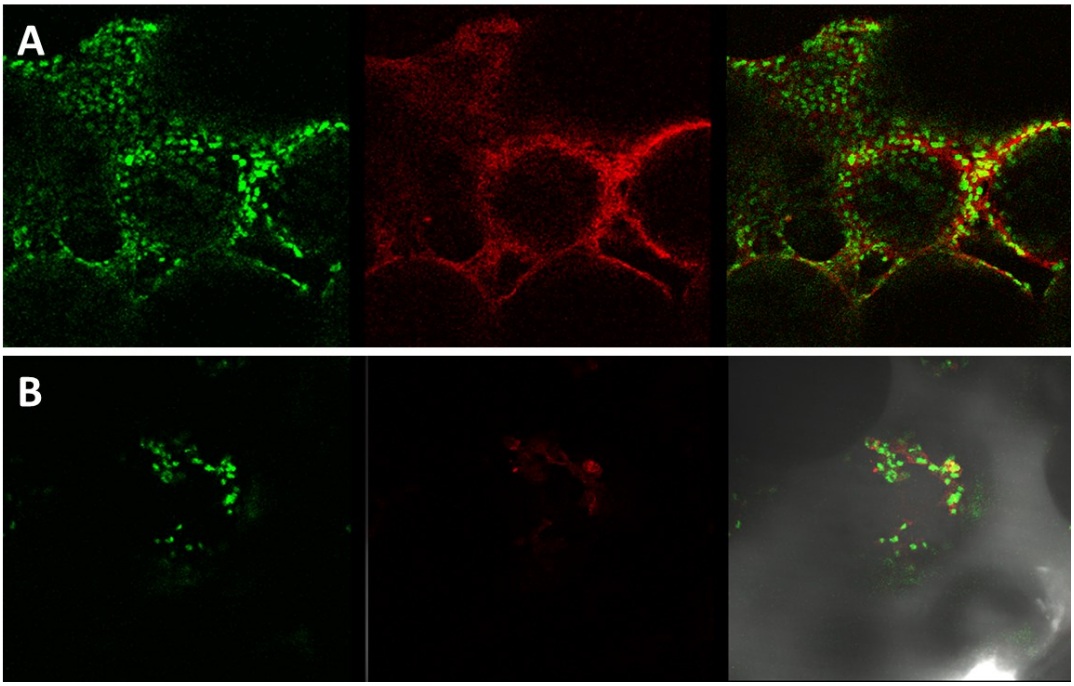

Figure 26 : Confocal microscopy images by staining with phalloidin (red) and Sytox green (green) of the scaffold protein free (A) and bioactive scaffold (B) a one day.

After 7 days, however, the bioactive scaffolds have a homogeneous cell distribution and especially an increase of cells inside the scaffold compared to the time a day. it is noteworthy to point out that the scaffold protein-free has a lower number of cells when compared to the same time a day scaffold (Fig. 27).

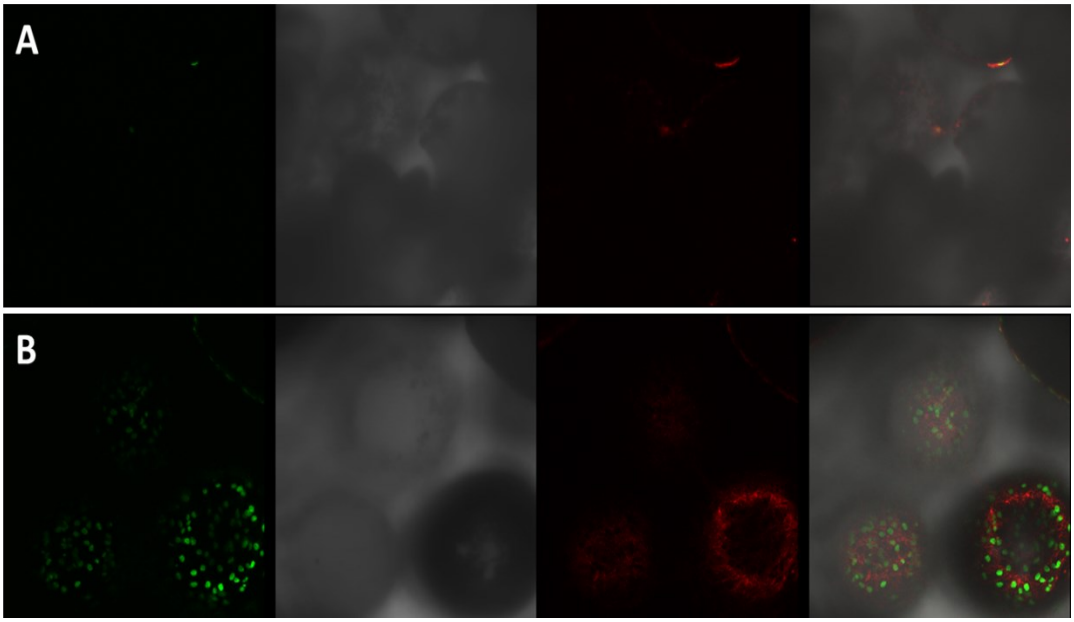

Figure 27 : Confocal microscopy images by staining with phalloidin (red) and Sytox green (green) of the scaffold protein free (A) and bioactive scaffold (B) a 7 days

5. Conclusions

In this research work has been proposed and optimized an innovative approach for the realization of three-dimensional bioactive scaffold for the regeneration of bone tissue. This approach consists in the sintering of biodegradable Polycaprolactone microparticles and PLGA microspheres containing VEGF (bottom-up approach).

The first stage of this work has been focuses on the study and optimization of the process of preparation of polycaprolactone microparticles. Through the use of the process of single emulsion has been possible to achieve spherical, non-porous and dimensions suitable microparticles, for their use in the process of sintering.

Subsequently it is passed to the realization of PLGA microspheres able to release in a controlled manner the VEGF (Vascular endothelial growth factor) through the optimization of the process parameters of the double emulsion/solvent evaporation with which the microparticles were obtained. Such microspheres have been shown able to issue constant concentrations of GF in the time by means daily doses of about 0.5 ng / ml, in order to obtain a efficient proangiogenic stimulus.

This research work then focused on the realization of three-dimensional scaffolds through chemical compaction (through the use of a suitable mixture of solvents) of the microspheres obtained and optimized in the first phase. To identify the optimal conditions of preparation, the scaffolds obtained by solvent sintering were fully characterized in terms of morphology (SEM, confocal microscopy, porosity, computed microtomography) and mechanical properties (compression test).

To further improve the characteristics of such Scaffold, in terms of increased adhesion and cell proliferation, were made two types of surface treatments. From the comparison of these two treatments can be seen that both lead to an increase in the number of adherent cells, but in the case of treatment with

plasma was also able to improve the mechanical properties (from 2.73 to 4.61 MPa).

The last phase of this thesis concerned the study of the kinetics of growth factor release from scaffolds. Studies *in vitro* have clearly demonstrated that the scaffolds are made to have a huge potential in inducing the formation of new blood vessels and the formation of highly vascularized tissue.

The use of a bottom-up approach has allowed the creation of structures with microstructural properties can provide adequate support to the regeneration of bone tissue and to guide this regeneration processes through the controlled release in space and time of VEGF.

CHAPTER 2

CONTROLLED RELEASE SYSTEMS OF NERVE GROWTH FACTOR FOR THE TREATMENT OF OCULAR DISEASE

1. Introduction

Diseases of the eye vary from minor conjunctivitis to irreversible visual impairment caused mainly by diseases of the posterior eye. The most prevalent eye diseases which cause visual impairment include age-related macular degeneration, diabetic retinopathy, glaucoma, dry eye and retinitis pigmentosa.

The increase in the number of patients affected by these diseases has determined, in recent years, a renewed in scientific and institutional interest in relation to these diseases. The causes of this increase are manifold. In first place, there is a progressive increase in life expectancy, which has led to the exponential growth of ocular diseases associated with aging. In addition the great scientific and technological progress of ophthalmology, in recent decades, has led to a reduction in patients destined to blindness. However, the number of patients with partial residual vision, insufficient to ensure complete autonomy has also increased.

Currently, many pharmaceutical companies have focused their attention on the design of new pharmaceuticals systems for the treatment of these eye diseases. From cornea to choroid, new drugs have been formulated to address a great variety of ocular diseases.

Yet without good drug delivery systems, these drugs are less effective than they could be, possibly even ineffective or able to cause serious side effects. This problem is caused in the eye due to the great differences in tissue types that need to be targeted for therapy in different disease situations and the significant uptake barriers posed by ocular structures and biochemical inactivation systems. These barriers include simple but effective sclera/corneal tissue constraints on drug entrance into the eye and the selective permeability of the blood–ocular barriers.

Another important aspect of drug delivery is “targeting.” To maximize efficacy and safety, drugs need to be directed as best as possible to a specific tissue or cell type once ocular penetration has been achieved. It is usually difficult to have the drug self-target, but a drug delivery system can sometimes be designed to achieve this goal.

The first barrier of eye is the tear film. Less than 5% of most drugs delivered in this way enter into the eye and the remaining 95% is swept away through tear drainage. Certainly, drug modification and special compounding can significantly enhance penetration, but for drugs that are expensive, such delivery may not be justified especially, since this route generally does not allow for penetration back to the posterior segment. On the other hand, several techniques such as mixed micellar formulations are showing promise that drug delivery to the posterior segment can, in fact, be both practicable and practical.

Beyond this, there are both hydrophilic and hydrophobic barriers to drug delivery through the cornea. The stroma is a good water barrier, while epithelial and endothelial cells function as effective lipid barriers with only partial penetration of biphasic/nonpolar drugs.

For posterior segment disease, the most common route of delivery presently used is intravitreal injection. Advantage of this type of direct treatment is that a high level of drug is delivered to the retina. Negatives include the possibilities of infection, retinal detachment, the development of cataract or glaucoma, a relatively short time of action, and the lack of patient enthusiasm for such delivery. Nonetheless, frequent administration of drugs via this route can lead to retinal detachment, retinal haemorrhage, endophthalmitis and increased intraocular pressure. To minimize some of these complications, novel drug-delivery systems have been developed in the form of

biodegradable or non-biodegradable micro and nanoparticles, which can be placed long term in the vitreous.

A particle systems offers several inherent advantages over other systems, such as naked injection of drug. For example, the composition, size, and surface properties of the particle can be controlled to give maximal drug content, penetration, and uptake as well as afford a level of protection against drug toxicity. Because of their size, one of the most interesting and useful properties of micro and nanoparticles is their potential ability to cross the blood–ocular barriers and their nature also allows for protection of the drug during transport, improving drug lifetime within the eye. To date, most of the materials used have been non degradable but advances are rapidly being made in the formulation of biodegradable substrata that could further enhance delivery as well as not leave behind a residual footprint.

This project will be focused on the study of design, formulation, set up, characterization and production of micro and nanoparticles' systems for the controlled release of Nerve Growth Factor (NGF), that appears to play a crucial role in the treatment of various eye diseases.

1.1. Ocular Anatomy

The eye is an isolated sensory organ consisting of diverse tissue and cell types. The basic structure (Fig. 1) is essentially a globe consisting of three concentric layers. The outermost layer is called the fibrous tunic and is divided into the cornea and the sclera. Inside this external covering is the vascular tunic or uvea, consisting of the iris and the adjacent ciliary body in the anterior segment and the choroid in the posterior segment of the eye. These structures are responsible for ocular nutrition through the systemic blood supply. Finally, the innermost layer is the nervous tunic, mainly consisting of the neural retina. Within these concentric layers are the fluid or gel-containing reservoirs: the anterior chamber and the smaller posterior chamber, containing the aqueous humor and the larger vitreous cavity filled with vitreous gel. The anterior segment is mainly composed of the cornea, lens, iris, ciliary body and aqueous humor, while the posterior segment comprises the photoreceptive retina, posterior sclera, choroid and vitreous humor. The lens divides the two segments, and is common to both[77].

1.1.1. The Cornea

The cornea forms the strongest refractive entity of the eye, contributing two-thirds of the refractive power of this organ[78]. It is completely avascular and is composed of three main layers. These layers, beginning at the anterior of the cornea are the epithelium, the stroma, the endothelium. The stroma makes up 90% of cornea's structure and is composed of collagen fibers, proteoglycans and approximately 78% water. Without interweaving, the collagen fibers are laid down at right angles to each other. This arrangement allows for the transmittance of 99% of incident visible radiation. The corneal surface is bathed in a thin tear film that helps to protect the eye from irritants and noxious substances and also acts as an effective drug barrier, quickly diluting and sweeping away drops applied to the eye. However, the cornea is the

principal route into the eye for topically applied drugs even though it serves as a major barrier to drug absorption, affording both a physical barrier and a metabolic barrier to substances that might enter the eye. The cellular structure of the cornea along with the tear film pose both hydrophilic and lipophilic barriers to substance penetration. Penetration is difficult due to zonula occludens, or tight junctions between the epithelial cells. The hydrophobic nature of the corneal epithelium is probably the greatest impediment to intraocular drug bioavailability. Interestingly, the cornea is rich in nerve fibers but is avascular. Thus, in spite of the substantial uptake barriers that keep out unwanted substances, the lack of vascularization necessitates the selective uptake of nutrients (including oxygen) through the tear film or the aqueous humor. On the positive side for getting drugs into the eye, the cornea expresses specific transport systems that can facilitate drug entrance into (or out of) the tissue. Physically, the cornea is striate in nature, consisting of five discrete layers, two of which are laminar cell layers. This essentially makes the cornea a lipid–water–lipid sandwich. On the surface are epithelial cells attached to a layer of basement membrane called Bowman's membrane, a laminate structure mainly composed of collagen fibers. This pairing is one of the first lines of defense of the eye, forming a barrier that is difficult to penetrate. Underneath the epithelium is a thick layer of matrix material called the stroma. It is mainly acellular with only a few cells, such as keratocytes, and is about 500 microns thick, accounting for about 90% of the corneal thickness. It is composed of mostly water (~80%), regularly arrayed collagen fibers (~15%), and mucopolysaccharides (~5%). Finally, on the interior surface is a layer of endothelial cells attached to Descemet's membrane. It is a latticework of collagen fibers coming together in a nodal pattern. It acts as a barrier to the entrance of potentially toxic organisms into the eye but allows for passage of water and nutrients. The endothelium is composed of simple, mostly hexagonally shaped cells, only a single cell layer in thickness. However, it performs the important task of fluid regulation for the stroma,

maintaining a state of deturgescence, compacting the collagen fibers and maintaining tissue transparency. Physiologically, it has no true elastic fibers and thickens with age (birth: 3 microns; adult: 12 microns; aged: up to 30 microns). There is a barrier in the endothelium to penetration by aqueous humor, that is, a tight seal, composed of macula occludens junctions. Endothelial cells are rich in mitochondria to produce the energy needed to pump sodium and water out of the cornea to maintain proper thickness and thus transparency.

1.1.2. The Lens

The lens is the transparent biconvex epithelial tissue that is responsible for accommodation and refraction of light. This structure is avascular and is made up of a dense inner cortex and an elastic outer capsule. The bulk of the lens is made up of elongated lens fiber cells which are precisely aligned to maintain transparency and a high refractive index.

1.1.3. The Iris

The iris is a colored muscular tissue attaching peripherally to the ciliary body. It controls the amount of light entering the pupil. The iris also divides the anterior segment into the anterior and posterior chambers. The stroma of the iris contains the dilator and sphincter muscles. The dilator muscle relaxes the iris to increase the amount of light that enters the pupil, while the sphincter muscle constricts the iris to achieve the opposite effect.

1.1.4. The Ciliary Body

The ciliary body is located between the choroid and iris. It is divided into two sections, the anterior pars plicata and the posterior pars plana. The pars plicata comprises the ciliary muscles and ciliary processes. The ciliary muscles are attached to the zonules of the lens by the ciliary processes. These muscles pull on the zonules to change the shape of the lens in order to adjust

the accommodation. In addition to connecting the ciliary muscles to the zonules, the ciliary processes also produce the aqueous humor that flows into the anterior chamber through the pupil.

1.1.5. The aqueous humor

The aqueous humor is a flowing fluid that fills the anterior chamber, the space between the cornea and iris. This fluid is produced by the ciliary processes and is ejected through the trabecular meshwork into the canal of Schlemm which leads into episcleral veins. The ciliary processes of the human eye produce aqueous humor at a rate of 2 to 2.5 μL/min. The production of the aqueous humor results in the intraocular pressure (IOP) of the eye. The composition of the aqueous humor is similar to that of blood plasma. A major function of the aqueous is to provide nutrients for the cornea and lens.

1.1.6. The Retina

The retina is the light sensory tissue which lines the posterior two-thirds of the eye. It contains the photochemicals and neurologic connections which convert the light entering the eye to neural impulses that are relayed to the brain via the optic nerve. The retina is supplied with blood by a layer of large choroidal vessels and capillaries. Immediately inside this vascular layer is the retina pigment epithelium (RPE) layer and the inner neurosensory layer (rods, cones and ganglion cells). The RPE is a single layer of tightly joined cells which forms a barrier that prevents the diffusion of some substances between the choroid and the neurosensory layer. The photoreceptor cells of the retina are the rods and cones. The rods, the majority of which are in the periphery of the retina, are responsible for peripheral and low luminance vision. The cones, clustered mainly in the macula, provide color vision and central detailed vision. Inner to the rods and cones is the bipolar layer which serves as neural transmitters to the ganglion cell layer. The ganglion cell

layer is the last neural connector which converges to form the optic nerve that ultimately transmits the neural impulses.

1.1.7. The Choroid

The choroid is the dense network of blood vessels that covers the retina. These blood vessels are of two types. The first category is the choriocapillaris, which are small capillaries that form the innermost layer of the choroid. The second group of blood vessels is composed of larger arteries and veins, which are posterior to the choriocapillaris. The blood flow from the choroid provides nutrients to the RPE.

1.1.8. The Sclera

The sclera is the white colored part of the eye. This tissue is composed mainly of collagen fibers. disorganized manner. This haphazard arrangement results in the opacity of the sclera. The main function of this tissue is to provide a protective layer for the internal components of the eye.

1.1.9. The Vitreous Humor

The vitreous humor is a transparent, gel-like structure that accounts for about four fifths of eye's volume. Towards the anterior segment of the eye, it is bordered by the lens, zonules and ciliary body. In the posterior segment, the vitreous humor is enclosed by and adjoins the retina. It serves as a mechanical buffer for the surrounding tissues, and assists in the maintenance of intraocular pressure. Transparency of the vitreous body provides an uninhibited path for light to be transmitted to the retina.

1.2. Ocular Diseases

Many sight threatening diseases occur within the posterior segment causing vision impairment and even irreversible blindness [6]. Some of these diseases include age-related macular degeneration (AMD), retinitis pigmentosa, endophthalmitis, and diabetic retinopathy.

1.2.1 Age-related Macular Degeneration

Age-related macular degeneration, or AMD for short, affects the macula in people over the age of 50. The macula is the part of the retina that lies in the back of the eye and is responsible for fine detailed central vision. This disease causes progressive deposition of abnormal material called “drusen” within the macula. Over time, it may progress into two main clinical forms, namely the “dry” and “wet” forms. Either can lead to sight loss. The “dry” form occurs when there is progressive loss of cells in the macula area. In this case, there is usually a gradual deterioration of vision and it may take several years before vision is significantly affected. The “wet” form occurs when there is abnormal growth of blood vessels within the macula area. These blood vessels usually leak fluid, or may bleed and develop a scar. In this case there is usually rapid progression of visual loss (90% chance of severe visual loss in the affected eye within 2 years). Some individuals with “dry” disease may go on to develop “wet” disease.

1.2.2 Retinitis Pigmentosa

The Retinitis Pigmentosa (RP) is a retinal degenerative disease that affects the photoreceptors (rods and cones) the cells which, when hit by light, transform your image into an electrical pulse that travels along the optical paths to reach the brain where are interpreted. This is a hereditary disease that is transmitted genetically by the Legge of Mendel. It is caused by a genetic mutation that causes a defect in loading of photoreceptors that degenerate,

causing progressive loss of vision. The risk of having sick children depends on the type of inheritance.

1.2.3 Endophthalmitis

Endophthalmitis is an inflammatory condition of the intraocular cavities (ie, the aqueous and/or vitreous humor) usually caused by infection. Noninfectious (sterile) endophthalmitis may result from various causes such as retained native lens material after an operation or from toxic agents. The 2 types of endophthalmitis are endogenous (ie, metastatic) and exogenous. Endogenous endophthalmitis results from the hematogenous spread of organisms from a distant source of infection (eg, endocarditis). Exogenous endophthalmitis results from direct inoculation of an organism from the outside as a complication of ocular surgery, foreign bodies, and/or blunt or penetrating ocular trauma.

1.2.4 Diabetic Retinopathy

Diabetic retinopathy is retinopathy (damage to the retina) caused by complications of diabetes, which can eventually lead to blindness. It is an ocular manifestation of diabetes, a systemic disease, which affects up to 80 percent of all patients who have had diabetes for 10 years or more.

1.3 Nerve growth Factor

Nerve growth factor (NGF) is the first discovered member of the neurotrophin family[79]. NGF is essential for the development and phenotypic maintenance of neurons in the peripheral nervous system (PNS) and for the functional integrity of cholinergic neurons in the central nervous system (CNS)[80].

The aminoacid and messenger RNA sequences of this neurotrophin have been classified and indicate that NGF is a highly conserved molecule that shares considerable homology within different species[81]. The mature, active form of NGF descend from proteolitic cleavage of a precursor form (ProNGF), that have important roles during development and in adult life, having both pro-apoptotic and neurotrophic properties[82].

NGF exerts its biological action by challenging the specific receptor tropomyosin kinase receptor A (TrkA), which is a typical tyrosine kinase receptor[83]. The major cytosolic/endosomal pathways activated by the TrkA are Ras-mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), phosphatidylinositol 3 kinase (PI3K) -Akt, and Phospholipase C (PLC) -γ [84]. NGF also binds to and activate the low affinity, non-selective p75 pan-neurotrophin receptor (p75NTR). This receptor is a transmembrane glycoprotein that regulates signaling through TrkA[85]; binding of NGF to p75NTR activates additional signaling pathways that, in the absence of co-expressed TrkA, may signal a cell to die via apoptosis[86]. Signaling pathways by p75NTR are the Jun kinase signaling cascade, NF-κB and ceramide generation[87].

The discovery of NGF dated the ‘50s of the last century and was awarded with the Nobel prize in 1986. In 1953, Rita Levi-Montalcini, working in the Victor Hamburger laboratory at Washington University (Saint Louis, MA, USA), grafted a piece of mouse sarcoma tissue in to chick embryos whose

wing buds had been extirpated. She discovered that the tumor tissue produced a soluble factor that promoted the growth of nearby sensory and sympathetic ganglia. Collaborating with the biochemist Stanley Cohen, they isolated the substance responsible and named it NGF. For over 35 years, NGF has been considered as a very powerful and selective growth factor for sympathetic and sensory neurons and for cells derived from the neuronal crest[88].

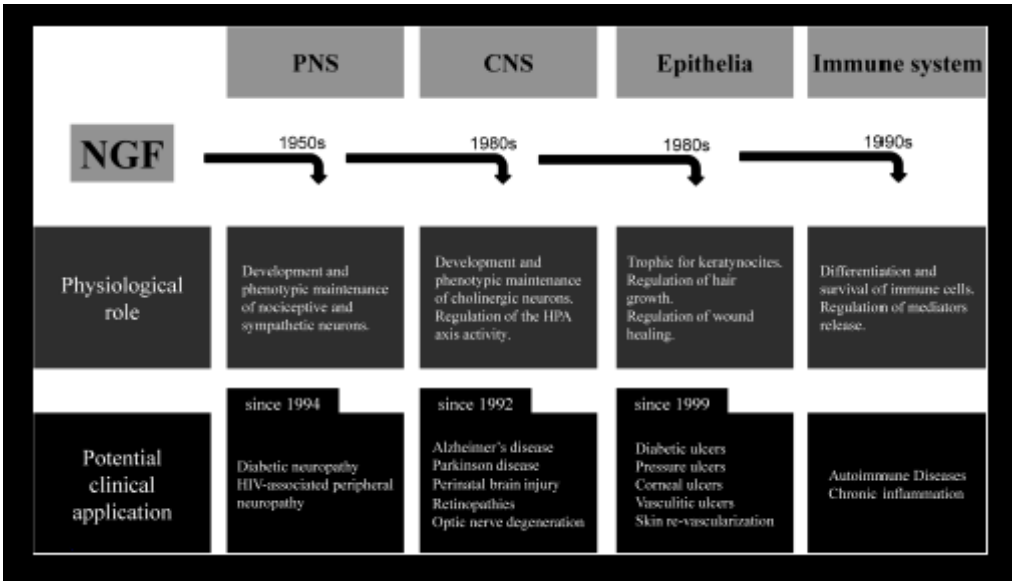

Figure 28 : The huge amount of research data produced since its discovery in the 1950s

In these neurons, NGF dynamically controls neurotransmitters and neuropeptides synthesis. In sympathetic neurons the production of norepinephrine is regulated by NGF through selective induction of tyrosine hydroxylase (TH)[89]. In the dorsal root ganglion (DRG) the expression of neuropeptides such as Substance P (SP) and Calcitonin Gene-Related Peptide (CGRP) by primary sensory neurons is under NGF control and in vivo deprivation of NGF, as a result of nerve transection or anti-NGF treatment, causes a marked decrease in SP and CGRP synthesis[90].

NGF supply from the innervation field influences the neuronal plasticity that allows the adult nervous system to modify its structure and functions in

response to stimuli. Indeed, the constitutive synthesis of NGF in adult tissues correlates with PNS neurons phenotypic features, such as innervation density, cell body size, axonal terminal sprouting, dendrites arborization, induction or inhibition of neuropeptides and neurotransmitters or transmitter-producing enzymes[91].

In the central nervous system (CNS), the greatest amount of NGF is produced in the cortex, the hippocampus and in the pituitary gland; although significant quantities of this neurotrophin are also produced in other areas, including the basal ganglia, thalamus, spinal cord and in the retina[92]. The NGF plays a pivotal role in the survival and function of cholinergic neurons of the basal forebrain complex (BFC)[93], such functions include attention, arousal, motivation, memory and consciousness. Since BFC neurons are highly affected in Alzheimer's disease (AD), NGF has been indicated as a potential protective and/or curative factor for neurodegenerative disorders associated with these neurons[94]. In the CNS, NGF also regulates phenotypic features in noradrenergic nuclei of hypothalamus and brainstem, participating in the central regulation of autonomic response and in the modulation of stress axis activity.

Cells of the immune-hematopoietic system also produce and utilize NGF. Since the early description of the effects of NGF on mast cells, the role played by the neurotrophin in the regulation of immune functions and immune cells behavior has been greatly characterized. NGF receptors are expressed in immune organs and on immune cell populations allowing NGF to modulate cell differentiation and regulate the immune response[95]. NGF affects the survival and/or differentiation and/or phenotypic features of hematopoietic stem cells, granulocytes, lymphocytes and monocytes. NGF concentrations in the tissues change during inflammation and inflammatory mediators induce NGF synthesis in a variety of cell types. Enhanced production of NGF has been reported in inflamed tissues of patients with

inflammatory and autoimmune diseases[96] but the reasons why NGF concentration is enhanced and how this can affect inflammatory responses are far from being fully understood.

1.3.1 Nerve growth factor in the eye

1.3.1.1 Anterior Segment

The first demonstration of the presence of NGF in the anterior segment of the eye dates back to 1980-1983 when Ebendal demonstrated for the first time the presence of NGF in the iris of the adult rat (5-10 pg / iris NGF). By studying the interactions between the sympathetic nerve endings and sensory target organs, Ebendal demonstrated that surgical denervation of sensory and / or sympathetic resulted in an increase in the amount of NGF in the iris, the greater the sensory denervation with respect to symphathetic. These observations suggested that the sensory nerves would influence the levels of NGF in the territories of innervation through uptake / removal of the factor or feedack exerting a negative impact on the production or use of NGF.

Ebendal also noted a sharp increase in NGF in the iris of the rat isolated and placed in culture. On the basis of this observation, Barth observed an increase in the iris in culture of NGF to 200 times compared to normal values in vivo and release of a factor in the medium. Also showed that the increase of NGF in culture derived from a synthesis and release by the iris, in fact, the production of NGF was blocked by inhibitors of the synthesis and mRNA transcription. One could therefore hypothesize several mechanisms responsible for the observed after NGF removal or denervation of the iris: an increase in the synthesis of NGF and / or a reduction in its degradation or removal from the cut nerve endings.

In 1986 Heuman demonstrated in vitro increased levels of mRNA for NGF in the iris of the rat formally proving that it was the same iris to increase the production of NGF in culture. Contrary to the data on the in vivo studies, the

results of Shelton of iris in culture confirmed a significant increase in the levels of NGF messenger in the iris tissue and the author concluded that the significant increase in the expression of NGF gene was primarily responsible for the sharp increase in NGF observed in the iris in culture.

Based on the evidence that NGF was a key molecule in the interaction between certain types of neurons and their target tissues, in 1985. The injection of NGF into the anterior chamber of the eye leading to an increase of production of substance P by the iris, mimicking the effects of sympathectomy, and also an increase in the activity of tyrosine hydroxylase with a slight increase in Acht.

In order to investigate whether also the neurotransmitters could influence the production of NGF by the target tissues of the nerves Hellweg studied the effects biogenic amine neurotransmitter and neuropeptide synthesis of NGF on the concentration and comparing the reaction to the trauma caused by dissection iris. Among the many substances tested none was able to induce strong initial increase in the synthesis of NGF observed in culture. In addition Hellweg proved that cutting the iris in culture in small pieces the production of NGF increased two hundred and fifty times the control, showed that this increase was the iris trauma primarily responsible for the observed transient increase of NGF. Subsequently we began to assess the presence and the function of NGF also level of the ocular surface. The first evidence of the presence of NGF at the level the corneal got Ebendal studied the levels of NGF mRNA in the chicken embryos[97]. She was later demonstrated the presence of NGF in the corneal epithelium and endothelium and in keratocytes in the human cornea and in the adult rat , these same cells were able to directly synthesize NGF. These observations suggested a role of NGF in tropism and in maintaining the integrity of the corneal epithelium in basal conditions[98].

Employing a model experimental lesion of the corneal epithelium has been demonstrated that tissue reacts with a corneal insult mechanical corneal increased levels of NGF, due to the direct synthesis by the same cornea. These evidences show that NGF represents a corneal layer one molecule of "communication" between the sensory innervation and epithelial cells, influencing each other's function and tropism in a manner similar to that observed in the iris tissue.

The corneal ulcers are caused by the loss of corneal sensory innervation and are accompanied by a reduction in the sensitivity to repeated disepitelizzazioni with ulcer formation seriously affecting visual function. For this condition does not exist at effective drug therapy. The topical administration of NGF resulted in a re-epithelialization of the ulcer with a recovery of corneal sensitivity of all patients treated without local or systemic side effects important, during or after the treatment. Recently, in addition to the role of NGF in the pathophysiology of the cornea and the iris, it has been demonstrated a potential role of this neurotrophin on other tissues of the anterior segment of the eye (crystalline lens and ciliary body) in addition to the presence NGF in the aqueous humor.

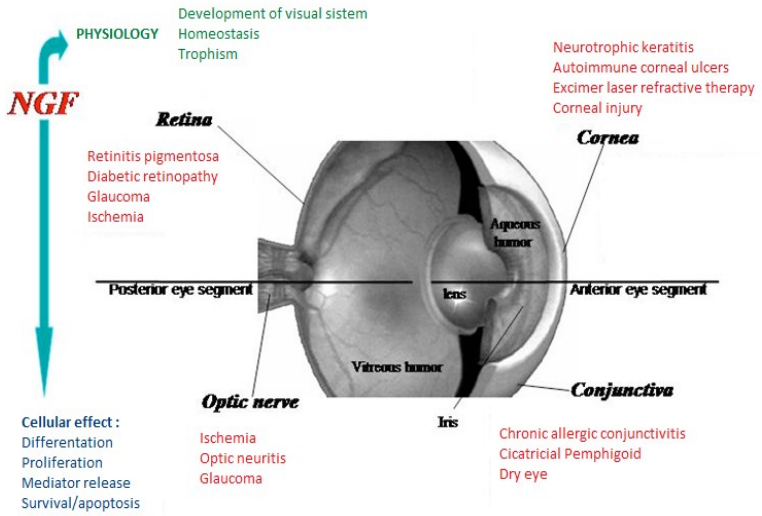

Figure 29 : NGF and Visual system

1.3.1.2 Posterior Segment

Some growth factors, and between these NGF, act on cells of the retina both during development and in the adult state. The synthesis of NGF and the presence of specific receptors was found in the retina of the chick embryo[99] and rats developing, where NGF appears to play a crucial role in the development and differentiation of the retina. Following the production of NGF has been demonstrated by the ganglion cells, Muller cells in the retina and pigment epithelium of adult rats[100] , and has been highlighted the presence of NGF receptors also on cells of the retina of adult rats : pigmented epithelium and Muller cells, ganglion cells and Muller cells[101]. Also has been demonstrated the presence of anterograde and retrograde transport of NGF receptor by the ganglion cells in the rat.

The addition of NGF to the culture medium of human RPE cells induce an increase in intracellular calcium levels and changes in the shape and intra-cytoplasmic distribution of actin, indirect indices of stimulation to proliferation and cell migration [102]. Moreover, in a study of a mutant strain of mice, the administration of NGF that was able to delay the degeneration of photoreceptors.

Several studies have evaluated the action of NGF on the growth and differentiation of retina in vitro. Numerous studies in animal models have focused on the role of NGF in protecting ganglion cells by a mechanical or ischemic damage. All work showed an efficacy of neuropeptide inhibiting the degeneration of these cells, in particular the study on rats showed that treatment with NGF allows survival of ganglion cells and nerve fibers of the optic nerve for about 7 weeks, has shown that in an experimental model of ocular hypertension in the rabbit the administration of NGF leads to a reduction of the loss of ganglion cells, on the other hand, the administration of anti-NGF, which inhibit the action of endogenous NGF, increase the

retinal damage induced hypertension. The results of this study suggest a protective role of NGF on retinal ganglion cells subjected to hypertensive damage.

1.3.1.3 Retinal and Optical Nerve

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1.3.1.4 Nerve Growth Factor in Ocular Disease

In recent years, numerous studies year demonstrated that the retinal cells are sensitive to the action of NGF. In 1979, Turner and Delaney first demonstrated that NGF is able to reduce or avoid the damage of the retinal cells after resection of the optic nerve. Recently, other studies have shown that the NGF administration induces functional recovery of the retinal ganglional cells in an animal model of acute retinal ischemia[103]. In this study, visual acuity and contrast sensitivity were comparable normal animal and also a significant number of cells were protected from ischemic injury after administration of NGF. The mechanism basis of this effect is not clear although it has been speculated that the NGF reduces l' entry of calcium ions in retinal neurons after protecting ischemia in this way from being damaged. Another explanation is that the possible NGF can interfere with the mechanisms that regulate apoptosis. In support of this hypothesis has been demonstrated that NGF reduces the ganglional cell apoptosis after resection of the optic nerve in newborn rats. It is also possible that the protective role of NGF on the cells of visual system is a part of a cascade of events that involve the interaction between different cell types such as neurons, glia, retinal pigment epithelium, opening interesting therapeutic prospects for degenerative diseases currently no effective treatments, such as the degeneration age-related macular, diabetic retinopathy, retinitis pigmentosa and glaucoma.

1.4 Drug Delivery Systems into the Vitreous

The administration of drugs and proteins in the district of the back of eye can be carried out through four possible routes: topical, systemic, intraocular and periocular. In the case of topical administration, the difficulty of the drug to penetrate through the cornea, aqueous humor and the lens, almost always involves non-therapeutic levels of the drug to the retina. The blood ocular barriers, consisting of the blood aqueous and blood retinal barriers, are the main obstacle for the effective treatment of most intraocular diseases by drug delivery using the systemic route. This route requires the administration of large doses of drugs to achieve therapeutic levels in the vitreous-retina, and can be associated with significant systemic side effects. The periocular drug delivery sites, are also hindered by the blood ocular barrier, but require smaller amount of drugs than the systemic route, and sometimes can achieve therapeutic drug levels in the vitreous-retina with lower systemic side effects. Nevertheless, poor penetration of many drugs into the eye not only limits the number of medications available for the treatment of ocular diseases, but also limits the extent to which those available drugs can be used without incurring undesirable systemic side effects. Therefore, due to the poor accessibility of some drugs to the vitreous by indirect routes, the direct intravitreous delivery of drugs is required for the effective treatment of some posterior segment diseases.

When you want to administer active principles or proteins to the retina, the most simple and direct way is given by intravitreal injection. This type of administration has the advantage that the active principle remains localized and the exposure to the systemic circulation is very limited, because impeded by several barriers present. The second advantage is given by the relative safety of this method and by the increasingly minimally invasive surgical procedures in which the patient must undergo.

Moreover, since the eye is a confined organ and the vitreous humors is a gelatinous character, we could say that the vitreous can be considered as a "reservoir" of drugs, particularly those with higher molecular weight, because the ILM can be considered a barrier only to molecules greater than 40 kDa. As amply shown, the elimination of substances from the vitreous can occur through two main roads: posterior (active route) and anterior (by aqueous humors). Importantly, large molecules such as antibodies are removed through the posterior path and appear to pass the ILM.

The speed with which a drug is eliminated from the vitreous is dependent on the one hand by its diffusivity and the other from its retinal permeability. Obviously, the structural state of the vitreous affect the elimination, as well as the integrity of the ocular barrier will affect its retinal permeability. From this it follows that the half-life of free large molecules inside the vitreous can be up to 4-5 days.

Most attractive is the fact that the human vitreous represents about 80% of the eye volume (around 4 mL), offering a wide liquid empty space for the delivery of implants or devices with limited interference with vision.

The limits to the drug delivery in the vitreous humor depending on the physicochemical properties of the drug and on the liquefaction state of the vitreous, the drug might not diffuse homogenously and high, even toxic concentrations could be reached focally. Once in the vitreous, active compounds may not diffuse passively into the retina and some retinal cells may not be targeted efficiently through intravitreous injections.

Being anionic, polycationic formulations may form aggregates and induce an inflammatory reaction. Other high-molecular-weight positively charged molecules may be unstable in the vitreous, and protection against degradation may be required. Retinal inflammation and glial cells activation may result from any intervention into the vitreous or with polymers or their bioproducts,

which interfere with the drug activity per se. Finally, rarely, tractions induced by implants or injection procedures themselves may create retinal traction, tears, and retinal detachment.

To minimize some of these complications, novel drug-delivery systems have been developed in the form of biodegradable or non-biodegradable micro- and nanoparticles, which can be placed long term in the vitreous.

2. Materials and Methods

2.1. Materials

The acid poly (D, L-lactic-co-glycolic acid) (PLGA) 50:50 (Resomer ® RG 504H, i.v. 0:32 to 0:44 dl/g) was purchased from Evonik® (Germany). The poloxamer188 (Pluronic ® F68, Mw 8400, HLB 29), the polycaptolactone, the polyvinyl alcohol (Mowiol 40-88) bovine serum albumin (BSA), heparin sodium salt (Hp), Vitamin E TPGS, Lisozyme and phosphate buffer (0.01M Na_2HPO_4, 0.0027 M KCl and 0.137 M NaCl, pH 7.4) were purchased from Sigma Aldrich (USA). The NGF growth factor was donated by Dompé (Aquila, Italy). The ELISA kit for the quantitative determination were provided by Biotech (California, USA). All solvents used are of analytical grade and HPLC grade and were supplied by Sigma Aldrich (USA). The water used was filtered through 0.22 μM filter (Millipore, USA).

2.2. Methods

2.2.1. PLGA microparticles for controlled release of NGF

The PLGA microparticles containing NGF were prepared by a modified multiple emulsion/solvent evaporation technique (W/O/W) with different theoretical loading and stabilizing agents. This multiple emulsion consists of a three-phase system in which the oil droplets, containing an internal aqueous phase, are dispersed in an external aqueous phase. The preparation method consists of two stages of emulsion that allow to encapsulate a drug efficiently.

Briefly, a sterile buffer solution (50 mM NaH_2PO_4 .H_2O, 100 mM NaCl, pH 7.2) containing Nerve Growth Factor (conc. 0.98mg/ml) stabilizing agent and polyetilenglycol 400 in the ratio 1:40:1 (v/w/v) was emulsified by

homogenization at 17.500 rpm for 1 minutes (Ultraturrax T25 Basic, probe 8G, IKA, Germany), in 2.5 ml of a solution of PLGA in Ethyl Acetate (20% w/v). The emulsion thus obtained was added to the external phase consisting of 5 ml of an aqueous PVA solution (2% w/v) (Mowiol® 40-88) doped with 300 µl of Ethyl Acetate, and emulsified by homogenization at 8.500 rpm for 40 minutes. At the end, this double emulsion was added to trehalose water solution (5% w/v) and subjected to agitation by electronic stirrer (RZR 2102, Heidolph, Germany), at 500 rpm for 3 hours, at room temperature, in order to facilitate the evaporation of the organic solvent and the precipitation of the polymer in the form of microparticles. Thereafter, the microparticles were isolated and washed with 30 ml of distilled water 4 times, by centrifugation at 6000 rpm for 15 minutes at 4^0C and subsequently subjected to a lyophilization cycle of 24 hours (0,001 atm , -60°C) (Freeze Dryer Alpha 1-4 LD plus, Martin Christ, DE).

Different formulations are made varying the water phase of first emulsion, using various types of stabilizing agent to protect the nerve growth factor. The principal stabilizing agent used were: Bovine Serum Albumin, Lysozyme, Heparin and Vitamin E.

Type	**Stabilizing Agent**	**Theoretical Loading % (mg NGF\100mg MS)**
Micro A	HSA, PEG400	0.0098, 0.0147, 0.0196, 0.049
Micro B	Heparin, HSA and PEG400	0.0098
Micro C	Vitamin TPGS	0.0098

Figure 30 : Type and properties of microparticles containing NGF

2.2.2. Dimensional and Morphological analysis of microparticles

a) Scanning electron microscopy observation

The morphology and the internal structure of the microspheres was visualized using scanning electron microscopy (SEM) (Zeiss, FEG Ultraplus, Germany). A small quantity of plain microspheres was sprinkled on the SEM stubs and coated using a SEM coating system (Cressingthon, 208 HR, UK) with 10nm of platinum-palladium under an argon atmosphere. Samples were then observed for their surface morphology by SEM and photographs were taken at 5 Kv and different magnification. One stub was made for each batch and many pictures were taken of each stub.

b) Confocal microscopy observation

The distribution of HSA-Rhod and NGF-Flu within the microparticles was assessed by analysis by confocal microscopy (STED) (Leica TCS SP5 II), with Zeiss lens 20X / 3 NA (Carl Zeiss, DE). Different pictures and z/ stacks were taken for all type of microparticles formulated.

c) Particle size analysis

The average diameter and the particle size distribution was determined using a laser granulometer (Mastersizer 2000, Malvern Instrument, UK) from previously lyophilized microspheres suspended in an aqueous solution of PVA 0.5% (w/v). For each formulation the average diameter of the particles was expressed in microns ± standard deviation (SD) of the measurement carried out on three different batches of microspheres.

2.2.3. Encapsulation efficiency

The amount of total protein and NGF encapsulated within the microparticles was determined by degradation of the polymer matrix by extraction method.

Ten mg of microspheres was suspended in 1 ml of Ethyl Acetate\Buffer solution (50 mM NaH_2PO_4 .H_2O, 100 mM NaCl, pH 7.2) and vortexed at room temperature for about 30 sec. The solutions were centrifuged at 4500 rpm for 15 minutes at 4°C, and the supernatant analyzed for the content of proteins.

For the analysis of the total amount of proteins present in the different types of realized microparticles was used a spectrofluorometric assay (FluoroProfile Kit, Sigma Aldrich) while for the analysis of NGF was used an specific immunofluorescence assay ELISA (Enzyme-linked immunosorbent assay).

For the total amount of protein was used the FluoroProfile quantification kits as per the manufacturer's instructions using Human serum albumin (HSA), Nerve growth factor (NGF) to generate standard curves.

NGF encapsulated in the microspheres was quantified by specific immunoenzymatic assay ELISA (*Enzyme-linked immunosorbent assay*), according to the procedure recommended by the manufacturer (RayBio Human Beta-NGF ELISA Kit). The standard solution of NGF or the sample to be analyzed to appropriately diluted unknown concentration (100 µL) were added to each well of the multiwell plate coated with a specific monoclonal antibody of NGF. The loaded plate was incubated for 2.5 hours at room temperature and then washed 4 times with a washing solution (*Wash Solution*). Following the wash, we proceed with the addition of 100 µl of biotinylated antibody to each well. The plate is maintained in incubation at room temperature for 1 hours and then, after three washes, to each well to be analyzed is adds the TMB One-Step Substrate Reagent. After another 30 minutes of incubation at room temperature in the dark with gentle shaking, to each well is adds50 µl of Stop Solution. The optical absorbance was measured by spectrophotometer (PelkinElmer) to the wavelength λ = 450nm.

2.2.4. "In vitro" release Kinetics

In vitro release studies of NGF were made by suspending 20 mg of lyophilized microparticles containing NGF fluorescein-labeled (theoretical loading 0.0098%) in 1 ml of PBS pH 7.4, previously filtered through filter pores of 0.22 μm in diameter (Millex®, Millipore, USA). The samples were incubated in a incubator at 37°C and 30 RPM. for mimic the conditions of release. At regular intervals of time, the samples were centrifuged at 3000 rpm for 5 minutes at 4 °C and the release media removed and replaced with the same volume of fresh PBS pH 7.4. The samples were analyzed by spectrofluorimeter (λ_{max}=492nm) to determine the amount of nerve growth factor released.

2.3. PLGA nanoparticles for controlled release of NGF

The PLGA nanoparticles containing NGF were prepared by the technique of multiple emulsion/solvent evaporation (W/O/W) with a theoretical loading 0.0065% (0.065 mg to 100 mg of NGF microparticles). Briefly, a sterile buffer solution containing Nerve Growth Factor (conc. 0.98mg/ml) and Vitamin E in the ratio 1:1 (v/v) was emulsified by homogenization at 17.500 rpm for 1 minutes (Ultraturrax T25 Basic, probe 8G, IKA, Germany), in 5 ml of a solution of PLGA and Poloxamer 188 (ratio 2:1) in Ethyl Acetate (1.5% w/v). The emulsion thus obtained was added to the external phase consisting of 5 ml of an aqueous PVA solution (2% w/v) (Mowiol® 40-88) doped with 300 μl of Ethyl Acetate, and emulsified by homogenization at 21.500 rpm for 1 minutes. At the end, this double emulsion was added to trehalose water solution (5% w/v) and subjected to agitation by electronic stirrer (RZR 2102, Heidolph, Germany), at 500 rpm for 2.5 hours, at room temperature, in order to facilitate the evaporation of the organic solvent and the precipitation of the

polymer in the form of nanoparticles. Thereafter, the microparticles were isolated and washed with 30 ml of distilled water 2 times, by centrifugation at 12000 rpm for 10 minutes at 4^0C and subsequently subjected to a lyophilization cycle of 24 hours (0,001 atm , -60°C) (Freeze Dryer Alpha 1-4 LD plus, Martin Christ, DE).

2.3.1. Dimensional and Morphological analysis of nanoparticles

a) Osservazione al microscopio a trasmissione elettronica (TEM)

The morphology of the nanoparticles was analyzed with the aid of a Transmission Electron Microscope (TEM) (Tecnai FEI, Eindhoven, Netherlands). Briefly, 10 μl of a suspension of nanoparticles (1 mg/ml) were laid on a 300 mesh copper grid with membrane Lacey Carbon, specific support for analysis by TEM. The nanoparticles were subsequently treated with phosphotungstic acid (2% w/v), the support was dried at room temperature and analyzed.

b) Analysis of the size and ζ potential

The dimensions and the ζ potential of the nanoparticles obtained with the two different protocols were determined by a Zetasizer® Nano (Malvern Instruments, UK). In particular, the dimensions have been assessed using the technique of Photon Correlation Spectroscopy (PCS), analyzing Doppler Anemometry (LDA), appropriate dilution of the sample with a salt solution KCl 1N.

2.3.2. Encapsulation efficacy

The encapsulation efficiency of NGF in the nanoparticles was determined by extraction of the protein in ethyl acetate/Buffer solution, as described previously for the microparticles. Briefly, 10 mg of lyophilized nanoparticles

was solubilized in 1 ml of ethyl acetate, and NGF was extracted with 1 ml of Buffer solution (50 mM NaH_2PO_4 .H_2O, 100 mM NaCl, pH 7.2). Following quantitative determination by spectrofluorometric assay (FluoroProfile Kit, Sigma Aldrich).

2.3.3. "In vitro" release kinetics

The profiles of in vitro release of NGF from PLGA and Poloxamer based nanoparticles was determined by incubating 5 ml of a suspension nanoparticles (2 mg/ml), at 37 ° C in static conditions. At intervals of time regular, 500 µl of this suspension were collected, filtered through filters with porosity of 0.22 µM and analyzed by FluoroProfile Kit, as previously described. The total volume of the release media was restored with fresh PBS pH 7.4 at each time interval.

2.3.4. Freeze-drying Studies of nanoparticles

There is strong evidence that nanoparticles in aqueous media form colloidal suspensions that have a limited stability over time. In order to increase the stability and to simplify the conservation of these particle systems have been conducted studies of lyophilization of nanoparticulate suspensions, containing NGF and white, in the presence of cryoprotectants of sugar nature, in particular trehalose and sucrose.

The nanoparticles were suspended in an aqueous solution containing trehalose or sucrose at 5% (w/v). The studies were conducted using suspensions of nanoparticles at the concentration of 1mg/ml. The suspension obtained was frozen at -80 ° C and subjected to a cycle of lyophilization of about 48 hours. The lyophilized powder was resuspended in water filtered and appropriately diluted in order to be analyzed by Zetasizer® Nano (Malvern Instruments, UK), in order to assess the size and the polydispersity index.

3. Results and Discussions

3.1. Properties of microparticles containing Nerve Growth Factor

The most common techniques used for the preparation of microparticles provide the formation of emulsions between aqueous phases containing the bioactive agents and an organic phase containing the polymer. A good process should lead to microparticles formulation with a high encapsulation efficiency, desired size, and with a sustained release kinetics. Furthermore, the encapsulated bioactive agent must maintain its stability. This is particularly important when the active agent is a growth factor. In fact, if the growth factor is denatured during the formulation process, it will be therapeutically inactive, and might cause unpredictable side effects, such as immunogenicity or toxicity[104].

The most used organic solvent in double emulsion technique is methylene chloride (MC), however, evidence of stability of NGF in different solvents has detected that the MC leads to a loss of activity of about 20-30%. To solve this problem it was used ethyl acetate (EA) as organic solvent for the process of double emulsion. Compared to the more hydrophobic MC, EA usually plays a less deteriorative effect on bioactivity of the entrapped proteins. So far, only protein C has been found more sensitive to EA than to MC. However, most researchers in this field still choose MC as the organic solvent because of its desirable physical properties, such as ability to dissolve large amounts of biodegradable polymers, low solubility in water (2.0%, w/v) and low boiling point convenient to be removed by evaporation (39.8 °C). In contrast, the relatively high solubility in water (8.7%, w/v) and the high boiling point (76.7 °C) of EA limited its application in W/O/W double emulsion technique. The relatively high solubility of EA in water usually gives rise to a fast diffusion of EA from oil droplets into the outer aqueous phase during the re-emulsification and solidification, which easily leads to

polymer precipitation rather than the formation of microparticles. On the other hand, the higher boiling point indicates a longer removal time to solidify microparticles by evaporation, which means a longer contact time between proteins and ethyl acetate, resulting in more loss in protein bioactivity[105].

To further improve the stability of the NGF different stabilizing agents were added to the aqueous phase containing the growth factor: Polyetilenglycole 400 (PEG400), Human serum Albumin (HSA), Heparin (hp) and Vitamin E TPGS.

The PEG400 and HSA were identified as a stabilizing agents because with their amphiphilic properties, preserves the growth factor from the aggregation to the water/oil during the process of emulsification (sacrificial lamb approach). Instead, the heparin interacting with the specific binding domain present on growth factors (Hp binding domain), stabilizes the active three-dimensional structure of the macromolecule and promotes its interaction with specific receptors located on cell membranes. Finally, the Vitamin E TPGS is a water-soluble source of vitamin E, that presents a structure consisting of a lipophilic and a hydrophilic portion, therefore results to have remarkable amphiphilic properties. Moreover, its lipophilic alkyl tail (polyethylene glycol) and hydrophilic polar head portion (tocopherol succinate) are bulky and have large surface areas. The hydrofile-lipophile balance (HLB) of TPGS is about 13 and it melts at 37–41 °C and is heat stable under temperature 200°C. Such characteristic makes it a good emulsifier able to minimize the interactions between the aqueous phase containing the growth factor and the oil phase containing the polymers. In addition, numerous studies have demonstrated the remarkable antioxidant ability of this vitamin and its beneficial effects on Age-related Macular Degeneration (AMD).

The properties of the formulations of microparticles produced are shown in Table 9.

Type	Excipients	Microparticles average diameter (µm ± S.D.)	Total Protein Encapsulation Efficency (% ± D.S.)	NGF Encapsulation Efficiency (% ± D.S.)
Micro A	HSA, PEG400	3.5 ± 1	76 ± 3.4	30.2 ± 6.4
Micro B	Heparin, HSA and PEG400	5.2 ± 1	64.1 ± 1.7	27.8 ± 1.4
Micro C	Vitamin TPGS	8.0 ± 1	----------	29.3 ± 2.8
Micro D	---------	13.6 ± 1	----------	2 ± 2.9

Table 9 : Properties of microparticles containing NGF

From the results shown in Table 9, it is possible to observe that the addition of stabilizing agents greatly increase the efficiency of encapsulation (about 30%) compared to control (about 2%), confirming the hypothesis previously expressed.

The particles prepared by encapsulating BSA and NGF labeled with fluorescent probes, respectively BSA rhodamine-labeled (BSA-Rhod) and NGF fluorescein-labeled (Fluo-NGF), were analyzed by confocal microscopy (Leica, TCS SP5 II), in order to observe the distribution of molecules within the microparticles. The analysis shows that the NGF is uniformly distributed throughout the volume of the microparticle (green signal) and that the HSA (red signal) is distributed mainly on the surface of the interior cavities of the microparticle (Fig. 31).

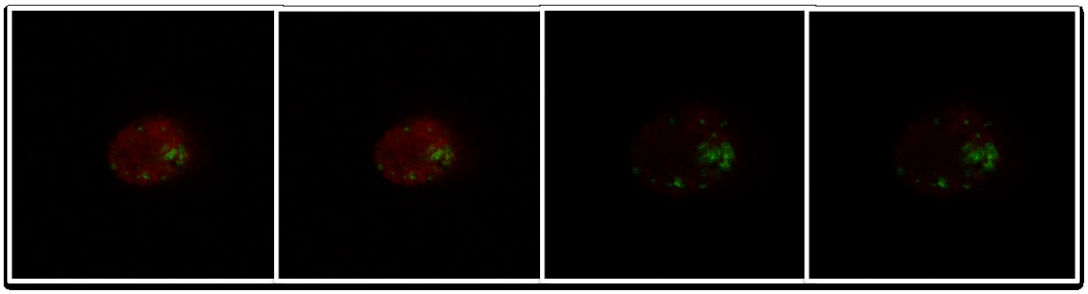

Figure 31 : Confocal image of microparticles containing BSA-Rhod and NGF-Fluo

Finally, As shown in the micrographs (Fig. 32) the formulation conditions of the microparticles have been optimized in order to obtain particles with a diameter smaller to 10 μm, such that they can diffuse into the vitreous humor without affecting the vision .

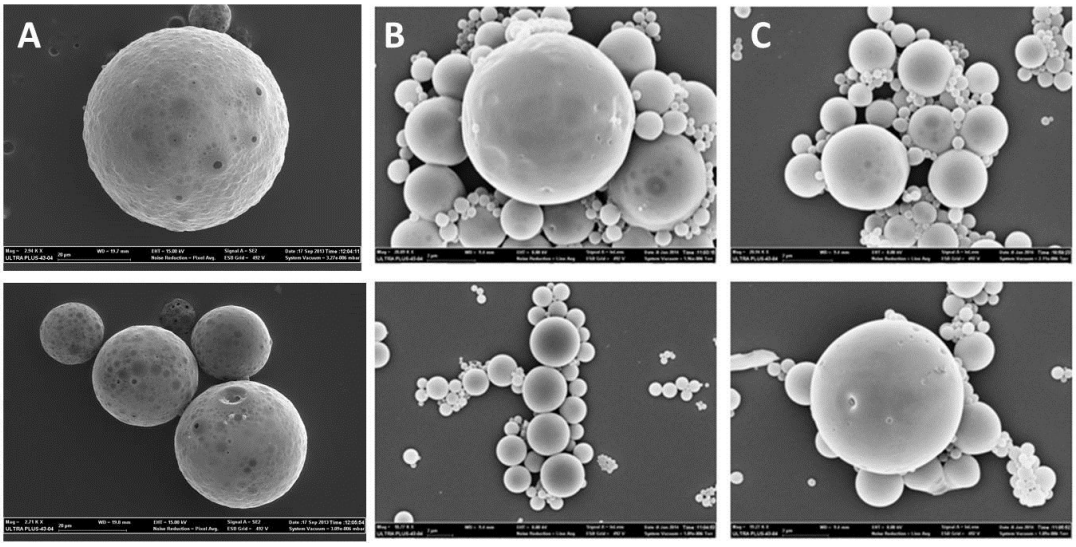

Figure 32 : Micrographs of different type of microparticles : (A)MS(with Vitamin E TPGS, (B) MS with HSA and PEG400, (C) MS with HSA,Hp and PEG400

3.1.1. Nerve Growth Factor release kinetics from microparticles

The kinetics of in vitro release of NGF from the microparticles was evaluated in PBS pH 7.4, at 37 ° C, under static conditions. Taking into account the different properties of the microparticles studied, has been conducted the

study on the kinetics of release of NGF only for the formulation Micro A, characterized by the best encapsulation efficiency.

The release profile of NGF from the microparticles is shown in figure X, expressed as µg of NGF released in active form for 1 mg of microparticles as a function of time. As can be seen from the figure, the microparticles show an fast initial release of NGF, in the first day of incubation, followed by a sustained controlled release of 37 ng\mg NGF throughout the period under consideration.

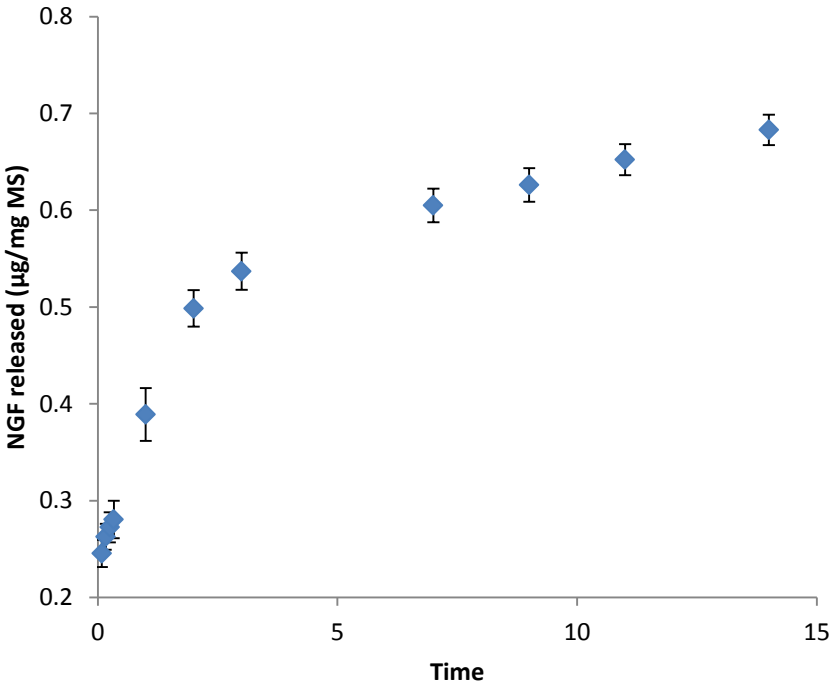

Figure 33 : Release profile of NGF from PLGA-based micrparticles in PBS pH 7.4 a 37°C. The results were expressed as µg NGF released per 1 mg of microparticles, mean ± SD of three replicates

3.2. Properties of nanoparticles containing Nerve Growth Factor

Nanoparticle-based blend of PLGA/Poloxamer, containing NGF were prepared using the modified technique of emulsion/solvent diffusion. This technique, although it is the most widely used for the fabrication of micro- and nanoparticles, may include some risk for the activity of growth factors such as NGF. In fact, the formation of an emulsion between the aqueous phase containing the active principle and the organic phase containing the mixture of polymers, can promote the localization of the GF to the water/oil interface, minimizing the mobility of the molecule and favoring the aggregation phenomena. On the other hand, the biodegradation of the PLGA involves the formation of water-soluble derivatives of acidic nature, responsible for the fall of the pH value within the nanoparticles. Furthermore, the anionic nature of the PLGA used in the preparation of such nanosystems can promote the formation of undesired interactions polymer/protein. These phenomena not only affect the characteristics of the control of the release of active principle, but may alter the native structure of the macromolecule leading to a loss of the activity of GFs.

In order to minimize these risks, it was decided to add an excipient to the polymer phase: the poloxamer 188. Several studies have shown that this excipient having a stabilizing activity when encapsulated within nanoparticle systems. Further studies have shown that the poloxamer 188 is able to minimize the iterations between the carboxylic residues of PLGA and the growth factor encapsulated and is also able to reduce the effects of the decrease of the pH of the microenvironment due to the degradation of PLGA.

Finally, studies conducted on PLGA\Poloxamer 188 nanoparticles containing nucleic acids have shown that the presence of poloxamer on their surface results in a reduction of the negative charges of the surface which results in a

lessening of the phenomena of particle aggregation and phagocytosis by macrophages.

Beside the use of poloxamer 188, which further strategy stabilizer, the NGF has been encapsulated together with vitamin E TPGS, a water-soluble source of vitamin E previously described.

the analysis of the morphology obtained by transmission electron microscopy (TEM) showed that the nanoparticles prepared are spherical, characterized by a regular morphology and a smooth surface, in agreement with particles usually obtained from such preparation technique (Fig. 34). This aspect is of particular importance considering the prospect of use of these nanoparticles as controlled release systems of NGF to eye level.

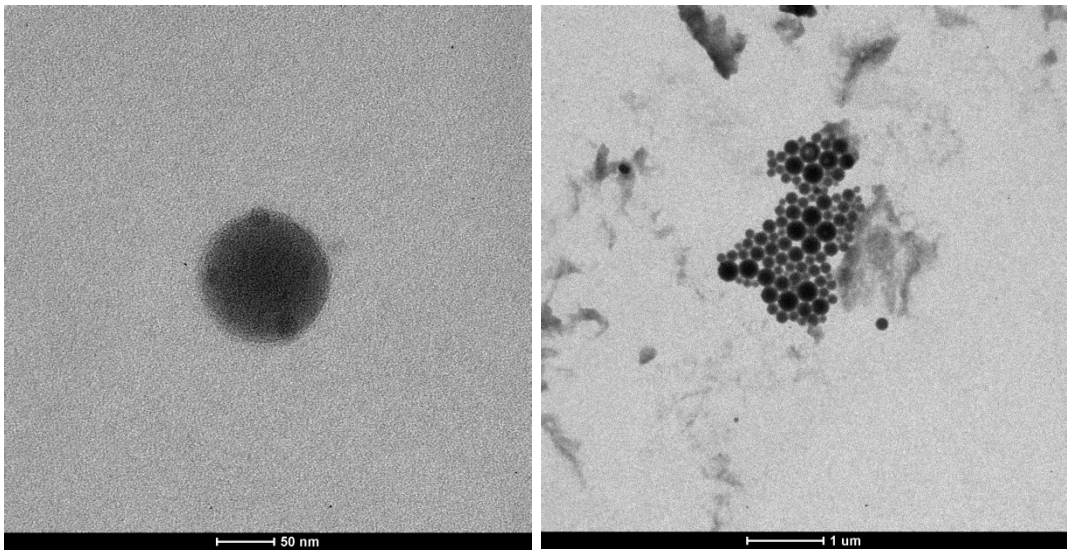

Figure 34 : Microgrphs at different magnification of nanoparticle containing NGF

From the results shown in Table X it is possible to observe that while varying the ratio between PLGA and poloxamer 188 in the oil phase, the particles do not exhibit significant differences in the values of average diameter, polydispersity index and production yield, resulting in all cases in agreement with the properties of nanoparticles based on PLGA/Poloxamer prepared by the same technique formulation. Such systems, in fact, generally show average diameters less than 300 nm, and polydispersity indices of about 0.1.

Type	PLGA/ Pol 188 Ratio	Encapsulation Efficancy (% ± D.S.)	production yield (% ± D.S.)	ζ Potential (mV ± D.S.)	SIZE (nm ± D.S.)	PDI
Nano A	5:1	33.4 ± 2	79 ± 2	-30.3 ± 1.6	239.8 ± 1	0.026 - 0.032
Nano B	3:1	31.1 ± 1	74 ± 4	-30.1 ± 1.8	233.4 ± 1	0.066 - 0.071
Nano C	2:1	30.3 ± 2	78 ± 2	-31.2 ± 1.3	226.4 ± 1	0.055 - 0.060

Table 10 : Properties of nanoparticles containing NGF

The efficiency of encapsulation remains more or less unchanged at around a value of about 31%. These values are in agreement with those obtained from the microparticles, confirming the hypothesis that the use of ethyl acetate in the formulation decreases the efficiency of the process of the double emulsion/solvent evaporation. We note that the decrease of the ratio between PLGA and Poloxamer also decreases the efficiency of encapsulation. This may be attributable to the fact that decreasing the amount of poloxamer increases the share of the growth factor in contact with the oil phase.

3.2.1. Nerve Growth Factor release kinetics from nanoparticles

The release profiles of NGF, respectively from the formulations Nano A, Nano B and C, were obtained by suspending the nanoparticles in PBS pH 7.4 and incubating the samples at 37 ° C in static conditions.

All formulations show an initial release of the GF (burst) of approximately 33% of the total amount encapsulated in the nanoparticles. Burst so high are typical of formulations based on PLGA by nanometric dimensions. The first phase of the release is dominated, in fact, by means of dissolution in the release of the protein located on the surface of the particle and/or macropores internal to it. Being nanometric systems, the surface exposed to the release media is particularly large, therefore the diffusion of the GF is favored, thus

achieving relatively higher proportions in the first hours of incubation. At the initial burst, however, follows a sustained release for the whole period examined (Fig. X).

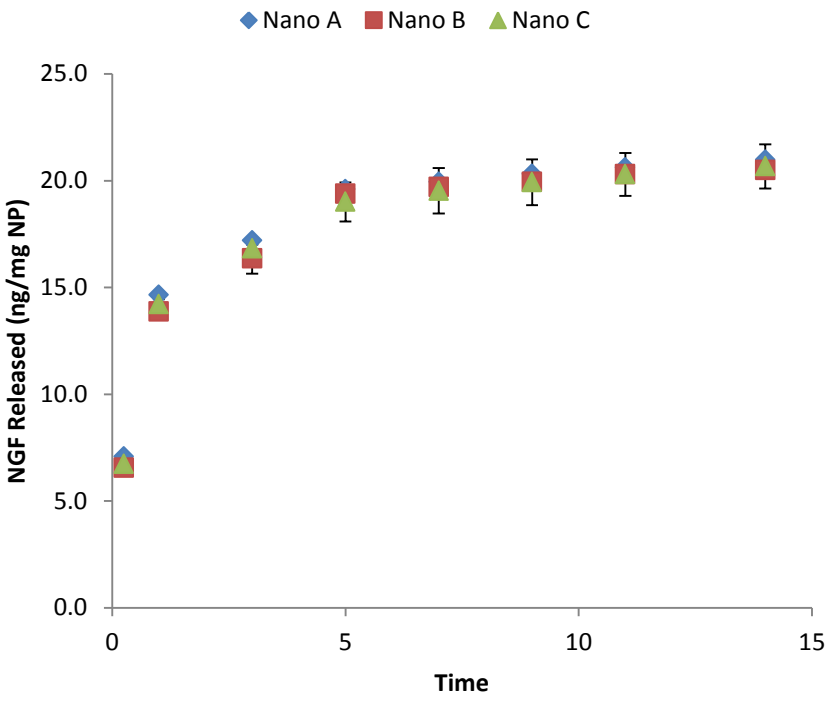

Figure 35 : Release profile of NGF from all type of PLGA-based nanoparticles in PBS pH 7.4 a 37°C. The results were expressed as µg NGF released per 1 mg of microparticles, mean ± SD of three replicates

3.2.2. Freeze-drying Studies of nanoparticles

In order to prolong the stability of the nanoparticles in suspension, lyophilization studies have been conducted in the presence of trehalose and sucrose at 5% w/v. The use of a cryoprotectant is essential during the process of freezing and lyophilization to avoid phenomena of nanoparticles aggregation.

The lyophilization studies were conducted on a suspension of 1mg/ml of nanoparticles in all three formulations. The lyophilized powders obtained were suspended in water and analyzed by Zetasizer® Nano to assess the size

and polydispersity index (PI) after the lyophilization process. The results obtained are depicted in the graph X and Y.

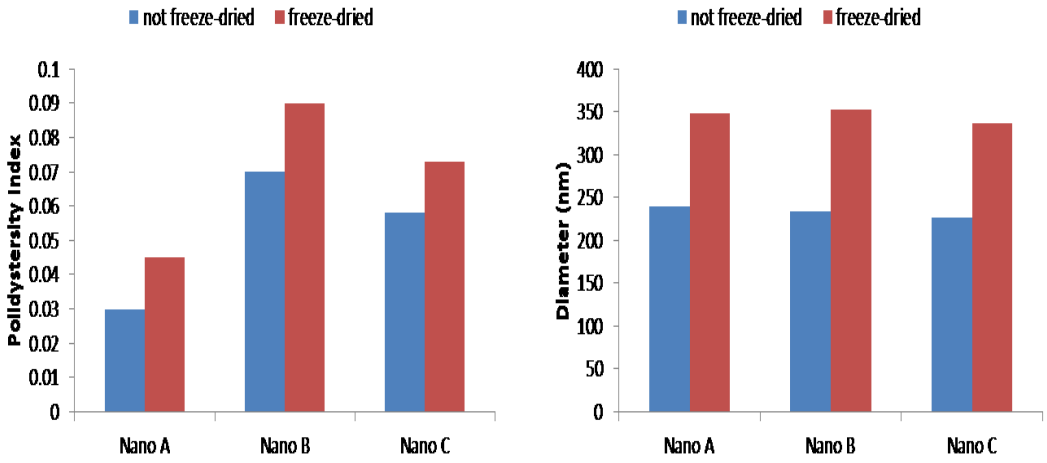

Figure 36 : Studies of freeze/drying of nanoparticles containing NGF in the presence of Sucrose 5% w/v.

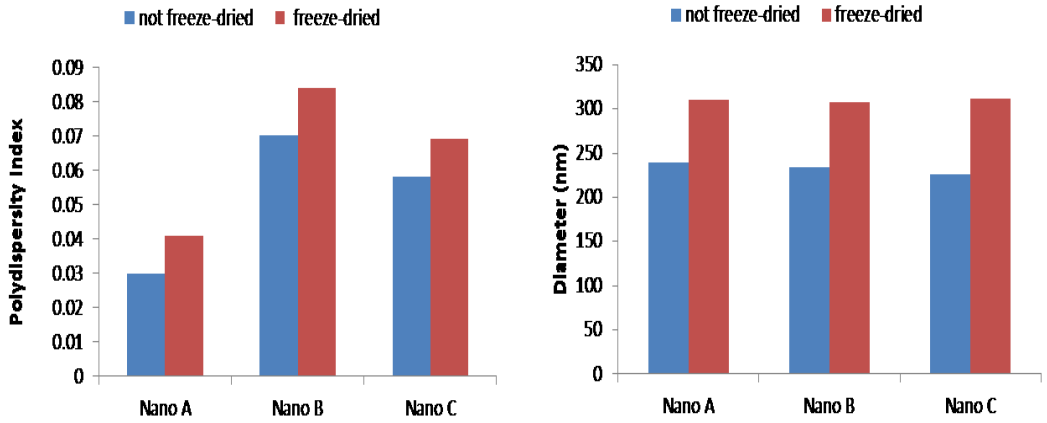

Figure 37 : Studies of freeze-drying of nanoparticles containing NGF in the presence of trehalose 5% w/v

From the graphs we can see that the size and PDI of lyophilized formulations have a slight increase compared to the control formulations. These increases, however, are not indicative of a phenomenon of aggregation, suggesting that the original features of the lyophilized formulations remain almost unchanged, in all cases analyzed.

From the graphs it is also noted that there isn't significant difference between freeze-dried samples in the presence of trehalose and sucrose, demonstrating that both sugars are excellent cryoprotectant agents. Nevertheless, the trehalose remains the cryoprotectant elected for subsequent studies of lyophilization, not only for the excellent results obtained, but also because appears to be one of the more inert and most used excipients.

4. Conclusions

An intraocular drug delivery systems for the controlled release of growth factors for the treatment of different ocular diseases is proposed in this work. The traditional treatment of these pathologies consists of daily administration of drugs, but, the main limit of these conventional treatments is bound to the fact that blood levels of the drug falling into the therapeutic range are obtained right after administration. In other words, there is a risk that the drug action lasts too little to have a significant effect on the patient.

To overcome these issues and to optimize the clinical performance we have developed PLGA micro e nanoparticles system for the controlled release of a specific factor: Nerve Growth Factor (NGF) to the humor vitreous. This factor has been shown to have a protective effect on the cells of the visual system, opening interesting therapeutic prospects for degenerative diseases currently no effective treatments, such as the age-related macular degeneration, diabetic retinopathy, retinitis pigmentosa and glaucoma.

We demonstrated that the encapsulation efficiency could be increased by adding water-soluble excipients to the inner aqueous phase prior to emulsification. In particular, have been used and evaluated different excipients such as the HSA for microparticulate systems and Vitamin E TPGS for nanoparticle systems.

We showed that encapsulation of NGF in PLGA particles provides a sustained release formulation, maintaining concentrations of the macromolecule at pico/nanomolar levels. The micro-and nanoparticles developed, keep a control of the concentration of growth factor constant in time by daily doses of approximately 37 ng/mg for microparticles and 0.3ng\mg for nanoparticles

Taking into account that doses of factor excessive or uncontrolled may cause side effects, an important result of this study is that particle systems for the

controlled release of NGF are capable of providing a slow and sustained release of the active, allowing to obtain a cellular response to NGF better than simply dispersed and at higher concentrations.

These results demonstrate that NGF-containing in micro e nanoparticles are an effective means for delivering this molecule into the eye and their use may be a promising strategy in the treatment of different ocular disease

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