Research Doctorate School in Biological and Molecular Sciences

Ph.D. in BIOMATERIALS – *XXV Cycle (2010-2012)*

"Polymeric Nanoparticles for the Controlled Administration of Bioactive Protein Agents"

Antonella Lisella

Supervisor: Dr. Federica Chiellini

Tutor: Dr. Cesare Errico

Laboratory of Bioactive Polymeric Materials for Biomedical and Environmental Applications (BIOlab) – Department of Chemistry and Industrial Chemistry (University of Pisa)

To my Family

Table of Contents

1. <u>Chapter I</u>: Introduction

1.1. Drug Delivery Technology

Nanotechnology can be defined as "the design, characterization, production and application of materials, devices and systems by controlling shape and size of the nanoscale" [1]. There is no single field of nanotechnology. The term broadly refers to fields such as biology, physics, chemistry, and any others involved in the controlled manipulation, measurement and production of materials and devices at the nanoscale order, characterized by new proprieties and functions [2]. Particular interest in nanotechnology is in the healthcare area, where nanomedicine has emerged as a new research field, which could have deep effect in the current treatment of various diseases. Nanomedicine is defined as the application of nanotechnology to healthcare [3]. Within nanomedicine, drug delivery systems (DDS) have assumed an important role in the past three decades, and significant advances have been made in this multidisciplinary field due to the necessity to develop new therapeutic strategies. Indeed, the aim of DDS are hence the maximization of the therapeutic efficacy, minimization of the adverse effects of the drugs of interest and the delivery of drugs in controlled manner [4]. The conventional pharmaceutic preparations, and the common drugs administration routes (e.g. oral, parenteral, subcutaneous, intravenous, intramuscular, topical, etc.) suffer from some problems that can be summarized in three main points: (a) typically drugs are administered at determined intervals, in order to maintain the therapeutic effects. The time between each administration causes a drop of drug concentration in the body that results toxic and affects the therapeutic benefits (Figure 1), (b) many therapeutically compounds are destroyed by the regular defense mechanisms of the body before they arrive at target sites, (c) the eukaryotic cells are characterized by bilayer lipid membrane, that represents for the drugs a barrier to overcome in order to get their effects.
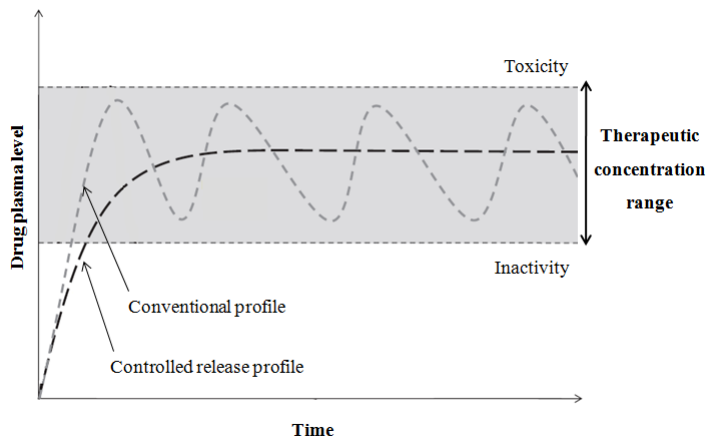

Figure 1: Drug plasma level concentrations obtained with conventional administration and controlled drug release [5].

In order to solve these problems, novel DDS are designed to encapsulate therapeutic agents, with the purpose of achieving the follow advantages:

- improved delivery of poorly water-soluble drugs;
- targeted delivery of drugs in a specific manner;
- delivery of large macromolecule drugs to intracellular sites of action;
- co-delivery of two or more drugs or therapeutic modality for combination therapy;
- potentially decrease the amount of drug to administer, thus reducing the side effects;
- allow the administration of non conventional drugs;

The first device used as DDS was described in the 1960s as lipid vesicles, that later became known as liposomes [6]. Afterwards, a variety of other organic and inorganic biomaterials for DDS were developed. In the 1980s the first examples of cell specific targeting of liposomes were described, and after more complex DDS were found able to responding to specific conditions, such as changes in pH or temperatures, to trigger drug release [7]. To date there are more than twenty nanotechnology therapeutic products that have been approved by the Food and Drug Administration (FDA) for clinical use, and more are in clinical trial [8]. Among DDS, the formulation of nanosized drug delivery devices have become an important area of research, because they have the ability to deliver a wide variety of drugs such as proteins, peptides, DNA and RNA, to several areas of the body for sustained periods of time.

The characteristics of DDS depend on the nature of the materials that constitute the delivery system. Organic macromolecules have highly tunable physical-chemical characteristics and in some cases, polymeric materials can be further processed or functionalized [9]. For this reason, at present, the main class of DDS is represented by polymeric based systems; however the promising applications of nanomedicine are not limited to drug delivery, but include also *in vitro* diagnostics, *in vivo* imaging and tissue engineering. Some of these fields are becoming realities or are actually being used today, while others are generating promise in their early phases of development and are expected to experience vigorous growth in the future. As recognition of the importance of these fields, it is expected that the global market of nanoscale applications in the medical field could grow to $70-160 billion by 2015 [10].

1.1.1. Polymeric Biomaterials

A biomaterial can be defined as "any matter, surface, or construct that interacts with biological systems" [11]. The study of these materials is called biomaterials science and include science fields like medicine, biology, chemistry and tissue engineering. The important prerequisite to qualify a material as a biomaterial is that it should be biocompatible. Biocompatibility is the capability of materials to coexist with living tissues or organisms without producing toxicity, injury, or immunological response [12]. Consequently, a wide range of materials encompassing all the classical materials such as metals, ceramics, glasses, and polymers have been investigated as biomaterials. Among

these, polymers are the most versatile class of biomaterials that have been extensively investigated for medical and related applications [13]. The use of polymeric materials as biomaterials has evolved over the past decades and their use in medicine dates back almost to the birth of the of polymer science. The combination between advanced organic chemistry and polymer synthesis has led to the development of tools for the design of polymer architecture and the grafting of bioactive molecules. In this way, polymers offer the advantage, compared with other types of biomaterials, such as metals and ceramics, to be engineered and processed in different manners in order to allow unique chemical structures with specific functions for desired applications [14]. The employ of polymeric biomaterials has found a multitude of uses, especially as drug delivery systems and tissue engineering scaffolds. Drug delivery systems, as described in section 1.1, are a tool to administrate pharmaceutical agents in order to improve their therapeutic effect. The role of polymers in drug delivery covers multiple aspects, from the enhancement of the physical-chemical stability of the drug to the regulation of drug release and targeting. In fact, the use of polymers as drug delivery carriers allows the controlled release of the drug, creates easier dosage regimes that consequently improve the absorption, distribution and the elimination of the drug. Many active agents such as proteins, antibody, vaccine and gene based drugs, may not be delivered using common administrations routes and polymers are currently used as physical carriers for these drugs [15].

Tissue engineering has emerged as an interdisciplinary field that applies the principles of engineering and life sciences toward the development of tissue substitutes or maintaining tissues and organs functions [16]. Indeed, tissue engineering purposes involve replacement or repair of damaged or failed tissues with viable ones by creation of an environment, which promotes the native capacity of cells to integrate, differentiate and proliferate. Even in this field, polymers offer a number of advantages, over other materials in scaffold preparations, such as the obtainment for the desiderate degradation kinetics and the possibility to fabricate different shapes with desired pore morphologic features. Furthermore, polymers can be designed with chemical functional groups that can induce tissue growth and their regeneration [17].

Biodegradable polymers, used in both drug delivery and tissue engineering, can be generally classified as natural and synthetic, based on their origin. Natural polymers seem to be the obvious choice for biomedical applications due to their excellent biocompatibility, as structurally they closely mimic native cellular environments, have unique mechanical properties, and are biodegradable by an enzymatic or hydrolytic mechanism. However, natural polymers suffer from a number of disadvantages, such as risk of viral infection, antigenicity, sourcing and processing that could generate variation in properties from batch to batch [18]. Synthetic polymers, on the other hand, offer ample advantages due to their synthetic flexibility and the possibility to develop polymers having a wide spectrum of properties with excellent reproducibility. Furthermore, the controlled degradation rate of these polymers is easier to reach due to the possibility to vary their structure. The fields of application of synthetic and natural

polymeric biomaterials is not only limited to drug delivery and tissue engineering, but is indeed very wide, including surgical field, implant materials and in a large numbers of medical devices, such as in dental, orthopedic, dermatological, ophthalmic and cardiovascular applications [19]. Moreover, a large interest come from the concept of "smart" polymers originated from the ability of certain synthetic polymers to mimic the response of physiological polymers such as DNA and proteins. In fact, the "smart" polymers have the capability to change according to their surrounding environment because can be sensitive to a large number of factors such as temperature, humidity, pH, intensity of light and change in electrical or magnetic field [20]. Biomedical polymers classification and their main applications are summarized in Table 1[21] [22] [23].

TABLE 1: Synthetic and natural polymers classification and applications in DDS.	
Synthetic polymers	
Polymer	**Main applications**
Poly (glycolic acid); Poly (lactic acid) and their copolymers	Used in sutures, drug delivery systems and tissue engineering. Copolymerized to regulate degradation time.
Polyhydroxybutyrate	Biodegradable, used as matrix for drug.
Poly (caprolactone) and copolymers	Delivery systems, cell microencapsulation.
Poly(alkylene succinate)	Properties can be tuned by chemical modification, copolymerization and blending.
Polyanhydrides	Biodegradable, useful in tissue engineering and for the release of bioactive molecules.
Poly(ortho esters)	Surface–eroding polymers. Application in sustained drug delivery, ophthalmology.
Polycyanoacrylates	Biodegradable, depending on the length of the alkyl chain. Used as surgical adhesives, glues, and in drug delivery.
Polyacrylonitrile	Dialysis membranes.
Polyphosphazenes	Can be tailored with versatile side–chain functionality. Applications in drug delivery.
Polyamides	Used for sutures and hemofiltration membranes. Inhibitors of DNA transcription.
Polyurethanes	Used for prostheses, vascular grafts, catheters tissue adhesives, artificial cardiac structures, coatings.

Polypropylene	Membrane plasmapheresis, sutures, external medical devices.
Polyethylene	Used for orthopaedic implants and catheters.
Poly(methyl methacrylate)	This and its copolymers are used as dental implants, in bone replacement and as intraocular lenses.
Poly(hydroxyethyl methacrylate)	Used for contact lenses, ocular prostheses, skin coatings, catheters.
Polytetrafluoroethylene (Teflon®)	Used for vascular grafts, clips and sutures facial prosthesis, coatings.
Polydimethylsiloxanes	Used for implants in plastic surgery, orthopedics, blood bags, pacemakers, drug delivery devices, membrane oxygenators.
Poly(N-vinyl pyrrolidone)	Hydrogels for controlled release of drugs from metal stents, or in blood substitutes.
Polysulfone	Heart valves, penile prostheses.
Polyvinyl chloride	Used for tubing, blood bags.
Polystyrene	Used as substrates for cell cultures.
Poly(ethylene oxide)	Different polymer derivatives and copolymers used in a variety of biomedical applications.
Poly (ethylene glycol)	Several applications as spacer, in bioconjugations, coatings, excipient in many pharmaceutical products.
Polylysine	Applied in gene delivery, formation of conjugate vectors.
Polyethylenimine	Applied in gene delivery, formation of conjugate vectors.
Polyamidoimine	Vectors of nucleotide transfer and gene delivery.
Natural polymers	
Polymer	**Main applications**
Proteins and protein-base polymers	
Collagen	Sutures, drug release.
Gelatin	Used in the preparation of gels and in drug release.
Albumin	Drug stabilizer and drug release.
Polysaccharides and vegetal derived polymers	

Carboxymethylcellulose	Drug release, dialysis membranes, cell immobilization (ionotropic gelation and formation of polyelectrolyte complexes).
Starch	Drug delivery.
Cellulose sulphate	In polyelectrolytic complexes for imunoisolation.
Agarose	Used in clinical analysis and as matrix.
Alginate	Biocompatible. Applied in gel preparation and to immobilize cell matrix and enzymes. For the release of bioactive molecules, injectablem icrocapsules for neurodegenerative diseases and hormones deficiency.
Polysaccharides and human/animal derivatives	
Hyaluronic acid	Moisturizing agent, wound dressing, artificial tears in ophthalmology, various orthopaedic applications.
Heparin and glycosaminoglycans	Thrombolytic anticoagulant properties. Used in surgery for ionotropic gelation and in capsules preparation.
Microbial Polysaccharides	
Dextran and derivatives	Excellent reological properties, plasma expander. Used in drug delivery.
Chitosan and derivatives	Produced generally by partial deacetylation of chitin. Polycationic polymer. Biocompatible, not toxic. Used in gel and film preparation. Applied in drug delivery.

1.1.2.Polymer Drug Delivery Systems

Polymeric systems are the most versatile class of materials and have an enormous impact on pharmacological therapy. In recent years both natural and synthetic polymers have been investigated as carriers for controlling drug release. The first generation DDS were based on non degradable polymers matrix, although the disadvantage of this systems was that the release kinetics was incontrollable and totally dependent of the diffusive behavior of the drug through the polymer matrix [24]. The second generation of DDS, based on natural or synthetic biodegradable polymers, allowed the controlled release of the encapsulated drug and the possibility to address the active agents to the target site. The choice of the polymer is a key point to achieve a controlled release, because the properties of the DDS depend on the physical, chemical and biological proprieties of their constituents. The polymer matrix can be engineered in order to target a selected tissue and to obtain the precisely modulated release kinetic [25]. Nowadays, a third generation of DDS is under investigations. These systems can be design by multi

component materials specifically engineered to avoid biological barriers and stimulate cellular responses such as direct cell proliferation and differentiation [26].

Based on the nature of the carriers, controlled drug delivery devices can be classified into two main categories: diffusion controlled release systems and degradable delivery systems. In diffusion controlled release systems, the active agent release within an aqueous solution is inhibited by the insoluble polymer matrix. The drug is released either by passing through the pores or between polymer chains, and these are the processes that control the release rate. Diffusion controlled systems are of two types: reservoir (membrane systems) and matrix systems (Figure 2).

- A reservoir is generally spherical, cylindrical or dislike in shape and consists of a drug core in powdered or liquid form surrounded by a polymeric film. This layer of non-biodegradable polymeric material represents the only barrier through which the drug slowly diffuses. The proprieties of the drug and the polymer govern the diffusion rate of the drug release. The reservoir can be a microporous or macroporous polymer film. Its composition changes from one component to a mixture of polymers, or to a heterogeneous matrix in which hydrophilic polymer particles are dispersed in a hydrophobic polymer matrix [27].

- In the matrix type of diffusion control system, the drug is uniformly distributed throughout the polymer matrix and is released from the matrix at a uniform rate as drug particles dislodge from the polymer network. Drugs can be physically embedded in polymers at large enough concentrations to create a series of interconnecting pores through which the drug can slowly diffuse. In these systems, the matrix may consist of hydrophobic or viscous hydrophilic polymers in which the solid drug is dispersed. These release systems are cheap and readily available, since they are prepared simply by mixing the polymer matrix and the drug. The release rate is based on the diffusion of the drug molecules to the device surface where they are delivered. This process takes place as long as the higher concentration of the drug in the system core affords a constant flow of drug molecules through the matrix. In this dissolution-diffusion process, the interface between the drug reservoir and the release moiety progressively moves towards the core of the device [28].
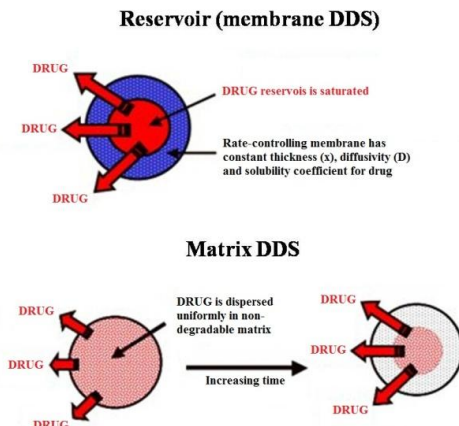
Reservoir (membrane DDS)

Matrix DDS

Figure 2: A schematic representation of the reservoir and matrix DDS. In membrane DDS, the drug is contained in a core, which is surrounded by a polymer membrane, and it is released by diffusion through this rate-controlling membrane. Reservoir devices have a coating that controls the release rate (modified from [24]).

In degradable delivery systems the drug molecules, which are initially dispersed in the polymer, are released as the polymer starts eroding or degrading. Similar to the reservoir system, the degradable reservoir system has a drug-loaded core surrounded by a polymer coating that degrades or erodes. These systems combine the advantage of long-term constant rate drug release with bioerodability or biodegradability. Polymer surface and bulk degradation are two typical modes of degradations. In a surface-degrading polymer, degradation is confined to the outer surface of the device. In a bulk-degrading polymer, however, degradation occurs homogeneously throughout the material. Water is an important factor during hydrolysis and thus water intrusion into the device is of significant importance for the study of degradation kinetics as well as release kinetics [29]. The use of these systems avoids the problems related to the physiological excretion or mechanical removal of the non degradable drug delivery devices after their function is completed. In general, an almost constant release profile over long periods can be achieved by fine modulation of geometric, chemical, degradation, and porosity characteristics of the device. In this respect, degradable drug delivery systems can be formulated in different shapes, i.e. cylinders, sticks, microcapsules, microspheres, fibers, films, and nanoparticles [30].

1.1.3. Polymeric Nanoparticles

Polymeric nanoparticles (NPs) have been extensively studied as drug carriers in the pharmaceutical field. NPs are frequently defined as solid, colloidal particles in the range 10-1000nm with the capability to carry drugs to the target site or release drugs in a controlled way in the body [31]. The first generation of particulate DDS was primarily based on liposomes and polymer-drug conjugates. Liposomes, discovered in the 1960s, can be defined as a spherical vesicle with a lipid bilayer membrane structure, which can

encapsulate both hydrophilic and hydrophobic drugs [32]. Polymer-drug conjugates, where the drug or bioactive compound is covalently linked to the macromolecular backbone, first appeared in 1970s and also have been extensively investigated [33]. Both liposomes and polymer-drug conjugates have provided the foundations for the field of advanced drug delivery based on nanotechnology. However, these systems are characterized by several disadvantages, such as poor encapsulation efficiency and bioavailability of drugs, difficulty to address the systems to target site and high production cost. Nowadays, the development of NPs based on polymeric materials, offers the tools for overcoming these barriers. Indeed, polymeric NPs systems offer the capability to achieve controlled release of the drugs, improve the stability of active agents (the polymeric shell protects the drugs against degradation factors such as pH, enzymes, light) and their subcellular size allows relatively higher intracellular uptake than other particulate systems. Other advantages of NPs as active substance carriers include high drug encapsulation efficiency, low polymer content compared to other DDS and the possibility to synthesized NPs from materials that are either biocompatible or biodegradable [34]. The term NPs is a collective term given for any type of polymeric NPs. According to the structural organization, NPs can be classified as nanocapsules and nanospheres. Polymeric nanospheres may be defined as a matrix particles whose entire mass is solid and molecules may be adsorbed at the sphere surface or encapsulated within the particle. Polymeric nanocapsules are vesicular systems, acting as a kind of reservoir, in which the entrapped drugs are confined to a cavity consisting of a liquid core (either oil or water) surrounded by a solid polymer shell [35]. A schematic representation of nanosphere and nanocapsules is shown in Figure 3.
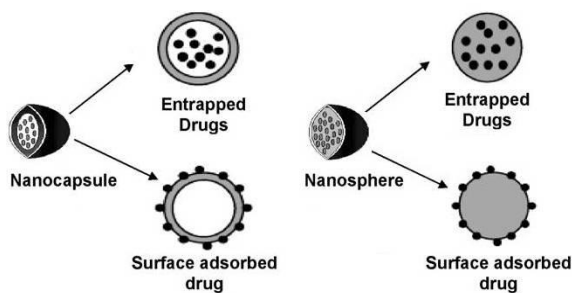
Figure 3: Structure of nanocapsules and nanospheres [36].

The design of polymeric NPs depends on the therapeutic application, target site (organs, tissues, cellular or subcellular organelles) and the route of administration. Although injection is the most common way of delivering NPs, these nanocarriers can be also delivered through the skin, oral, nasal, pulmonary, vaginal, rectal, ocular and buccal routes [37]. In every routs of administration, NPs have to be able to allow their delivery beyond the different biological barriers, such as skin, mucus, blood, extracellular matrix, and cellular or subcellular barriers. With respect to these factors, the proper selection of the polymers drastically affect the NPs intracellular fate, safety, biocompatibility, release kinetics and the capability to overcome biological barriers [38]. Biocompatibility and

biodegradability of NPs are essential for the safety of patients and should be tested both *in vitro* and *in vivo*. These restrictions are firm with systemic administration of NPs, whereas they are less significant for NPs designed for topical applications. The use of biodegradable materials avoids the problems related to the physiological excretion or mechanical removal of the delivery device after drug depletion. The presence of a biodegradable matrix can provide a further control of the release rate, by joining the typical diffusive mechanism with tunable polymer degradation [39].

The most commonly used biodegradable polymers are poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly (ε-caprolactone) (PCL) and the copolymer poly (lactic-*co*-glycolic acid) (PLGA) of PLA and PGA. Other than these polymers, natural hydrophilic polymers, such as chitosan, sodium alginate and gelatin have also been used to prepare drug loaded NPs. However, the aliphatic polyesters based on lactic and glycolic acids are unquestionably the most widely employed polymers and are approved by the FDA for human use [40]. Their high biodegradability and biocompatibility are well assessed. In fact, it is known that lactide/glycolide polymer chains are cleaved by hydrolysis to form natural metabolites (lactic and glycolic acids), which are eliminated from the body through the Krebs cycle [41].

Polymeric NPs are being developed to improve the diagnosis and treatment of a wide range of diseases, ranging from cancer, viral infections, cardiovascular diseases to pulmonary and urinary tract infections. A growing number of applications are still under investigation in order to integrate polymer science with molecular cell biology, both for developing new approaches to human therapies and as basic tools in biological and medical research [42].

1.1.3.1.   Nanoparticles Preparation Technique

The properties of NPs have to be optimized according to the particular application. In order to achieve the properties of interest, the method of preparation plays a key role. Thus, it is highly advantageous to have several preparation techniques to obtain NPs with the desired properties. The choice of preparation method is made on the basis of a number of factors such as the type of polymeric system, area of application, size requirement, etc. For instance, a polymeric system that is developed for an application in the biomedical or environmental fields should be completely free from additives or reactants such as surfactants or traces of organic solvents. In addition, the preparation process should guarantee for the chemical stability and biological activity of the incorporated drug. There are several methods for the preparation of NPs:

- **Emulsion/Solvent Evaporation**: this is the most common method used for the preparation of NPs. The preparation of nanocarriers follows the general protocol of dissolving the polymer in a water immiscible, volatile organic solvent which is then emulsified with an aqueous phase to stabilize the system. The organic solvent is then evaporated inducing the formation of polymer particles from the organic phase droplets. This technique has been successful

for encapsulating hydrophobic drugs. However, results for incorporation of hydrophilic bioactive agents are yet poor. A modification of this procedure has led to protocols that favor the encapsulation of hydrophilic compounds, the single oil-in-water emulsion (o/w) and the double (water-in-oil)-in-water, (w/o/w) emulsion techniques [43]. In the single emulsion method, the drug and the polymer are dissolved in an organic solvent and the resulting solution is added into an aqueous solution to make an o/w emulsion with the aid of amphiphilic macromolecules such as surfactant. The solvent in the emulsion is removed by evaporation, resulting in formation of compact NPs. In the double emulsion technique, a primary emulsion is prepared by dispersing the aqueous phase into an organic solvent containing a dissolved polymer. This is then reemulsified, under vigorous stirring, in an outer aqueous phase containing a stabilizer, thus forming a w/o/w emulsion. The emulsifications are carried out using either high speed homogenizers or sonicators. The w/o/w emulsion system has been used widely for the development of protein delivery systems. Both in single and double emulsions, several parameters can influence the properties of the particles produced, such as nature of polymer, polymer molecular weight and stirring speed. The main drawback of these methods is the removal of excipients post production. Any residual organic solvents will have toxicological implications. Another limitation is that surfactant must be present for preparation of nanoparticles in order to stabilize the system [44].

- **Salting Out:** is an emulsion-based approach, with the advantages that avoids surfactants and chlorinated solvents. In brief, a saturated salt solution containing a stabilizing agent such as polivinilalcool (PVA) is added under stirring to an acetone solution of the polymer. An o/w emulsion forms as the salt prevents the water and acetone mixing. Sufficient water is then added to allow the acetone to diffuse into the external aqueous phase and induce NPs formation. From the perspective of drug encapsulation, this method is most appropriate for water insoluble drugs, although the loading of water soluble drugs can be possible by altering the pH of the aqueous phase. However, a limitation of this method is represented by the presences of salts, because their presence affects the stability of many bioactive agents, such as proteins and peptides [45].

- **Phase separation**: this method involves the phase separation of a polymer solution by adding an organic non-solvent. Drugs are first dissolved in a polymer solution. An organic non-solvent is added to this solution (e.g. silicon oil, vegetable oil, light liquid paraffin) under continuous stirring, by which the polymer solvent is gradually extracted and soft coacervate of droplets containing the drug are generated. The rate of adding non-solvent affects the extraction rate of the solvent, the size of the particles and encapsulation efficiency of the drug. The coacervate phase is then hardened by exposing it into an excess amount of another non-solvent such as hexane, heptane, and diethyl ether. The final characteristics of the colloidal products are affected by the molecular weight of the polymer, viscosity of the non-solvent, and polymer

concentration. The main disadvantage of this method is a high possibility of forming large aggregates. Extremely sticky coacervate droplets frequently adhere to each other before complete phase separation. This technique is promising for the preparation of protein-loaded microcapsules [46].

- **Nanoprecipitation:** the basic principle of this technique is based on the interfacial deposition of a polymer, dissolved in a semi-polar solvent (i.e., ethanol, acetone, hexane, methylene chloride or dioxane), into an aqueous, non-solvent solution, under moderate stirring. Acetone is the most frequently employed polymer solvent in this method. Sometimes, binary solvent blends, acetone with small amount of water, blends of acetone with ethanol and methanol are used. In this method, the NPs are obtained as a colloidal suspension that forms when the semi-polar solvent phase is added slowly to the aqueous phase. The advantage of this method is that it does not require sonication or very high temperatures conditions that might damage the active agents. However, this technique is mostly suitable for compounds having a hydrophobic nature, but displays very limited solubility in water. In order to overcome this barrier, modifications of this technique are under investigation to improve the versatility of the nanoprecipitation, particularly with respect to the encapsulation of hydrophilic drugs [47].

- *Co*-**precipitation method:** the *co*-precipitation method is an original and straightforward procedure developed by the research group where this PhD thesis has been carried out. It is particularly advantageous for the loading of protein drugs into a polymer matrix. In this case, the polymer is dissolved in a water/miscible organic solvent and added dropwise to an aqueous solution containing the selected protein and appropriate stabilizers. During the *co*-precipitation process, the polymeric material gives rise to micro-phase separation because of its low water solubility and the concurrent interaction with protein molecules leads to NPs formation. Indeed, this methodology does not entail the use of chlorinated solvents and it does not require vigorous shear mixing, preventing appreciable denaturation of the entrapped protein molecule that typically occurs with other techniques [48].

- **Dialysis Method:** the dialysis method is a simple and effective preparation method for small and narrow size distributed NPs. In this method, polymer is dissolved in an organic solvent and placed inside a dialysis tube with proper molecular weight cut-off. Dialysis is performed against a non-solvent miscible with the former miscible solvent. The displacement of the solvent inside the membrane is followed by the progressive aggregation of polymer due to a loss of solubility leading to the formation of an homogeneous suspensions of NPs. The solvent used in the preparation of the polymer solution affects the morphology and particle size distribution of the NPs [35].

- **Self Assembling:** in this method NPs can be obtained by interaction between charged polymers and oppositely charged molecules. Such association depends on many factors including coulombic interactions, hydrophobicity of the polymer-molecule pair, and the conformational features of the polymer.

Typically, self assembled complexes are formed by polyions with opposite charges. The solution behaviour of these complexes strongly depends on their composition. Electroneutral complexes that contain equivalent amounts of polyion units and monomers are water-insoluble. Nonstoichiometric complexes containing an excess of one of the components are generally soluble in water [45].

1.1.3.2. Targeting nanoparticles

The most prominent advantage of nanosystems as drug carrier over conventional drug delivery systems is the option to improve selective delivery of drugs to the site of action, mechanism called drug targeting. In the very general sense, drug targeting leads to the accumulation of therapeutic agent at the target organ or tissue in selective and quantitative manner, independently of the sites and routes of its administration. Ideally, under such conditions, the local concentration of the drug at pathological site should be high, while its concentration in other site should be below certain minimal levels to prevent side effects.

Drug targeting can be classified into active, passive and physical targeting [49].

Active targeting can be achieved by modification of the carriers' surface with targeting moieties. In detail, antibodies or other specific adhesion molecules (e.g. polypeptides, oligosaccharides, viral proteins, and molecules of endogenous origin) are bound to the surface of NPs and antigens or other specific adhesion molecule counterparts on the surface of cells can be recognized. The drug should specifically be delivered to the site of action followed by potential intracellular uptake (Figure 4-A) [50].

Passive targeting can be achieved without further integration of a specific targeting moiety on the NPs surface. This kind of targeting is generally based on the relation between the size and shape of the nanosystems and tissue characteristics, such as permeability [51]. Moreover, passive targeting occurs through different administration routes, for instance after intravenous application. The passive targeting through intravenous administration route of NPs may be achieved by extravasation in the diseased tissue or organ. NPs are recognized by the Reticulo Endothelial System (RES), mainly liver and spleen, and taken up by macrophages in the bloodstream which leads to fast removal from the systemic circulation. The characteristics of NPs, like size and surface charge, play a key role to reach efficient uptake into macrophage cells. The macrophage is a specialized host defense cell whose contribution to pathogenesis is well known. Therefore, particle uptake into those immune related cells could allow the selective accumulation of the NPs in inflammatory diseases [52] (Figure 4-B).
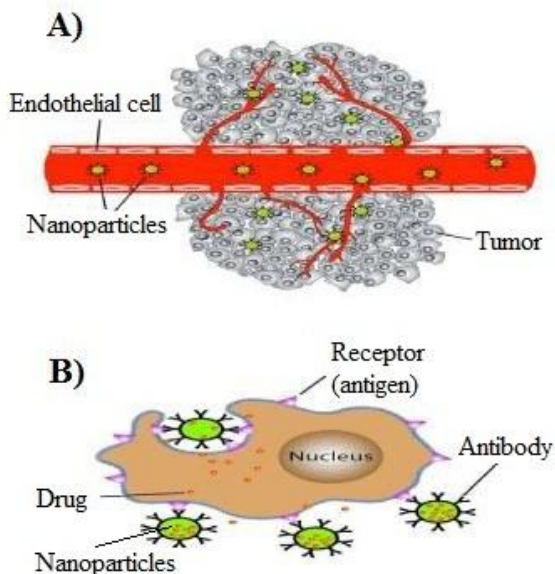
Figure 4: Examples of passive and active NPs targeting. (A) Passive tissue targeting by nanoparticles in blood vessels. Particle directed in the target tissue as a result of leaky vessels and ineffective lymphatic drainage; (B) Active cellular targeting of nanoparticles with conjugated antibodies [53].

Physical targeting can be achieved by endogenous or exogenous physical factors that can mediate targeted drug delivery. The targeting effect is based on the fact that pathological area differs from healthy area for physical parameters (e.g. temperature and pH). For instance, inflamed or tumor areas usually show acidosis and hyperthermia and the use of stimuli-responsive NPs, that disintegrate under specific pH/temperature conditions, allow for the releasing of the drug in the target area [54]. The physical targeting through exogenous factor can be achieved by the use of external physical forces, for example a magnetic field. For this purpose an innovative strategy consists in the administration of NPs with magnetic proprieties, which are able to reach the target site by applying an external magnetic field (Figure 5). Iron oxides are the most widely used sources of magnetic materials. Iron oxides have several crystalline polymorphs known as α-Fe$_2$O$_3$ (hematite), β-Fe$_2$O$_3$, γ-Fe$_2$O$_3$ (maghemite), ϵ-Fe$_2$O$_3$, Fe$_3$O$_4$ (magnetite) and some others (amorphous and high pressure forms). Nevertheless, only maghemite and magnetite found the greatest interest for bio-applications [55]. Magnetic NPs are now widely studied in the field of gene and anti-cancer therapies [56]. These magnetic systems are also employed in medicine as an important tool in the field of diagnostic. For precise diagnosis of disease, many imaging modalities such as fluorescence optical imaging, magnetic resonance imaging (MRI), positron-emission tomography (PET), and computed tomography (CT) have been developed and improved with the use of magnetic NPs [57].
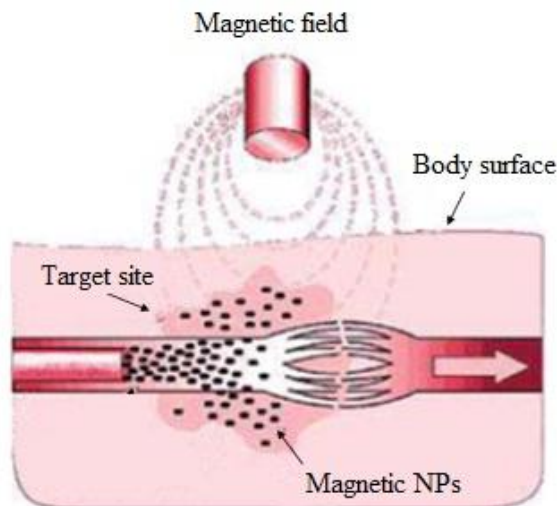
Figure 5: Schematic representation of physical drug targeting using magnetic NPs. NPs are delivered to the desired site by externally applied magnetic field. The magnetic field aids in particle localization and retention at targeted site by extravasation of the particles into the surrounding tissue (modified from [58]).

1.2. Therapeutic Agents

Therapeutic agents currently on the market can be placed into four categories: small molecules (which are chemically synthesized compounds); natural products (which are small, naturally occurring molecules that are generally isolated from plants, fungi); biotherapeutics (which are macromolecules that occur naturally or are engineered based on a biological template, such as proteins and peptides); and nucleic acids based therapeutics (which are DNA or RNA, and antisense molecule designed to treat infections) [59]. In the common administrations, these agents are often destroyed during intestinal transit or inadequately absorbed and therefore become ineffective. Moreover, the uncontrolled level of these agents could cause concentration spikes, thereby harming the body. Nanotechnology is offering solutions to these fundamental problems in the drug administration, in particular with the use of NPs as DDS (see section 1.1).

1.2.1. Conventional, Chemically Synthesized Drugs

Therapeutic agents, are prepared or synthesized by performing various chemical reactions using a starting material and changing its molecular structure by reactions with other chemicals [60]. The starting material for organic synthesis can be a simple compound obtained from oil and natural gas or more complex chemicals isolated in large amounts from plant and animal sources. Historically, most molecules aimed to treat or alleviate symptoms of pathological conditions, were isolated from natural products. However, in recent years, the necessity of developing commercially and large-scale production of therapeutic agents requires their chemical synthesis. Thus, the goal of

chemical synthesis is to supplement the supply of a drug that is commonly isolated in small amounts from natural sources and make these particular products commercially available. In the last 30 years, chemical synthesized drugs have become an integral and valuable part of modern medicine. Indeed, synthesized drugs have played an important role in the treatment of many important human diseases, such as cancer, diabetes, ischemic diseases and bacterial and viral infections. Moreover, these drugs have a good track record with patient safety and in some instances are more efficacious and safer than conventional medicines due to the ability to target specific molecules within the human body [61].

Many anti-cancer drugs derive from chemical synthesis and have been loaded into nanoparticulate systems. The advantages of using polymer based nanosystems for the administration of anti-cancer drugs are the possibility to tune their pharmacokinetics according to the specific tumor treatment, gain cost-effective utilization of the therapeutically active species and minimize inherent toxicity to other non-targeted tissues [62]. The co-formulation of chemotherapeutics and adjuvant agents into polymeric NPs is a promising approach to achieve anticancer drug retention in tumor sites and maximize the effectiveness of tumor treatment [63].

Doxorubicin, an anthracycline antibiotic, is generally administered in the form of hydrochloride salt intravenously. However, doxorubicin is characterized by acute side-effects, which include nausea, vomiting, and heart arrhythmias, neutropenia (a decrease in white blood cells). In order to reduce its adverse effects doxorubicin was loaded into poly (ε-caprolactone) / polyethylene glycol poly (etherester-urethane) (PEGCL) based NPs. In this work, it has been demonstrated that further to maintain doxorubicin chemical stability, its therapeutic activity was increased [64].

Cisplatin is an effective drug for treating a wide variety of cancers, but many tumors develop resistance to this drug, limiting its benefits for the patients [65]. Cisplatin, encapsulated in NPs with lysosomal pH-sensitive cores, have shown to be able to reduce the intestinal/mesentery tumors in treated mice [66].

1.2.2. Administration of Peptides and Proteins

The development of modern pharmaceuticals requires not only identification of new therapeutic mechanisms, but also safe and efficient ways for drug administration. For example, the importance of biotechnological drugs, especially protein drugs, has increased during the last few years, and is expected to increase further as a result of massive current efforts on human genomics and proteomics. If the history of use of the protein drugs is observed, first peptide drugs used were porcine or bovine insulin for diabetes, starting from 1930 [67]. With the advancement of molecular biology since 1970s several therapeutic proteins have been developed and produced, such as Growth Factors (GFs), hormones, erythropoietin, colony stimulating factors, interferon, viral or bacterial proteins (as vaccines) and monoclonal antibodies [68]. Proteins and other types of biological macromolecules are, however, easily degradable both chemically and

enzymatically, and may also lose their biological activity through conformational changes and aggregation. In order to maintain the biological activity of such biotechnological drugs they must frequently be administered together with drug carriers. However, for several years, large molecules such as proteins were not considered feasible candidates for controlled release systems because they were considered too large to slowly diffuse through most polymeric materials. Nowadays, a number of studies have demonstrated that NPs can also enhance the oral bioavailability of encapsulated therapeutic peptides and proteins, such as insulin and GFs. Daily subcutaneous injections of insulin are tedious treatment for patients with insulin-dependent diabetes. Considering the number of patients affected by this disease, several efforts have been done to make insulin orally administrable [69]. NPs delivery systems may provide a solution for oral administration of insulin. In fact, it has been demonstrated that with the use of NPs is possible to allow the protection of insulin against the harmful gastric environment (chemical degradation), and prevent enzymatic degradation [70].

GFs represent another important class of therapeutic proteins. GFs have strong tissue inductive properties, but their short half-life combined with high clearance rates have motivated the development of delivery systems capable of maintaining elevated concentrations to achieve therapeutic efficacy. A summary of NPs and other DDS loaded with GFs are reported in table 2 [71]. The market for synthetic therapeutic peptides rose from €5.3 billion in 2003 to €8 billion in 2005. It has been estimated that it will reach €11.5 billion in 2013. This excludes peptides, proteins and antibodies extracted from natural sources or produced by recombinant DNA technology, cell-free expression systems, transgenic animals and plants and enzyme technology [72].

TABLE 2: Summary of i*n vitro* investigations of NPs loaded with GFs		
Growth Factor	**NPs system**	**Observations**
bFGF (FGF-2)	PCL-PEG-PCL	After an initial burst of ~60%, bFGF was released for 8 days with 70% of total bFGF release
	Tetronic®-PCL-heparin micelle	bFGF was released for over 2 months due to specific interaction with heparin
	Chitosan/dextran sulfate/PLL	Nanocapsules were made by layer-by-layer method. bFGF activity was retained in the capsules
	Chitosan/ tripolyphosphate	~70% of bFGF was released at 24 h. bFGF integrity was not affected by the encapsulation procedure

BMP-2	PEI-coated BSA	BMP-2 release was controlled by PEI coating concentrations. PEI coating reduced the initial burst release compared to the uncoated NPs. BMP-2 activity was retained
	PLL-coated BSA	BMP-2 activity was retained
	Calcium phosphate NPs in PLGA microparticles	A sustained release of BMP-2 for over 7 weeks in a reasonably linear profile
	Dextran	Released BMP-2 stimulated proliferation and differentiation of rabbit bone marrow stem cells
KGF-2 (FGF-10)	Dextran sulfate/polycations	≥80% of the encapsulated KGF-2 was released over 11 days for all formulations. The released protein enhanced the proliferation of EC, compared to free protein solution.
EGF	Polystyrene/Poly (methacrylic acid)	EGF was released as a pseudo-zero order pattern after initial burst effect of 50%
	G5 PAMAM dendrimers	The PAMAM-EGF conjugate stimulated cell growth to a greater degree than free EGF
	PEG-coated DPPC/CHO liposomes	Enhanced EGF resistance to proteases, higher permeability of EGF across Caco-2 cells
	DPPC/LPC liposomes	Increased osteoclast recruitment and enhanced teeth movement
IGF-I	PLGA	Preparation methods affected the IGF-I release
BMP-7	Core/shell NPs composed of liposome (CHO and DDAB) /sodium alginate/chitosan	Burst release was reduced by layer-bylayer coating of liposome, and up to >85% of BMP-7 was released over 4 weeks. The preosteoblast differentiation was enhanced.
TGF-β3	Heparin/PLL	Promote neocartilage formation after 4-week cultivation with MSCs
VEGF	Polycations/dextran sulfate	75% of VEGF was released in a 10-day period for all NP formulations
	PLGA/Pluronic F-127/heparin	A linear, sustained release of VEGF without burst release was achieved for 37 days with 85% of the loaded VEGF being released
	Lecithin/F-127	Sustained released of VEGF was affected by the lecithin amount in the system

1.2.3. Oligonucleotides and Plasmids

The better understanding of the biological pathways involved in the development of several pathologies is leading to new therapeutic approaches aiming at eradicating these

diseases at the source. The DNA molecule has been one of the most important sources not only for the understanding of the fundamental basis of human life but also for the development of a novel group of therapeutics modeled on its endogenous structure. Although most of the DNA-based drugs are in early stages of clinical trials [73], this class of compounds has emerged in recent years to yield extremely promising candidates for a wide range of diseases, including cancer, AIDS, neurological disorders such as Parkinson's disease and Alzheimer's disease, and cardiovascular disorders [74]. Elucidation of the human genome has also provided a major impetus in identifying human genes implicated in diseases, which may lead to the development of DNA-based drugs for gene replacement or potential targets for gene ablation. In addition, using genomic data, potent DNA-based drugs may be developed for personalized medicine [75].

DNA-based therapeutics include plasmids containing transgenes for gene therapy, oligonucleotides, ribozymes, DNAzymes, aptamers and small RNA, termed "short interfering RNAs" (siRNAs) [76]. RNA interference (RNAi) is a biological mechanism whereby the presence of double-stranded RNA (dsRNA) interferes with the expression of a particular gene that shares a homologous sequence with the dsRNA [77]. The RNAi machinery, first discovered in plants, was later demonstrated in the roundworm *Caenorhabditis elegans* by delivery of dsRNA using a microinjection technique. The introduction of dsRNA molecules could produce the interfering activity and result in the highly specific inhibition of complementary gene expression in *C. elegans* [78]. Recent studies have provided insights into the molecular mechanisms of RNAi, in which dsRNA induces the silencing of homologous mRNA. In the cytoplasm of mammalian cells, an enzyme known as Dicer initiates RNA silencing by breakdown of long dsRNA to generate small interfering RNA (siRNA) of about 21–23 nucleotides in length. The resulting siRNAs are incorporated into an RNA-induced silencing complex (RISC) and unwound into a single-stranded RNA (ssRNA), which is followed by the degradation of sense strand ssRNA [79]. The concept of "gene silencing" is not unique to siRNA, but researchers have been silencing genes using single-stranded antisense oligodeoxyribonucleotides (ODNs) for decades. In the classical antisense strategy, the antisense strand of a DNA fragment corresponding to a target gene is delivered inside a cell where it binds complementarily with the sense strand of a target mRNA, producing a partially double-stranded ODN/mRNA complex degraded by endogenous cellular nucleases and preventing the recognition of the mRNA by ribosomes [80]. siRNA and plasmids DNA(pDNAs) are both double-stranded nucleic acids, have anionic phosphodiester backbones, same negative charge to nucleotide ratio, and can interact electrostatically with cationic agents. pDNAs are often several kilo base pairs long, possess a molecular topography which allows them to be condensed into small, nanometric particles when complexed with a cationic agent. pDNA, siRNA, and ODNs based therapies require the intracellular delivery of the active agent to the target cells. Concerning pDNA, it also requires the integration (in some cases) into the host cell genome and the subsequent expression of the resulting transgene. siRNA and ODNs have only to travel to the cytosol to reach the target mRNA [81]. The administration of

naked pDNA, siRNA and ODN is mainly limited by their short half-life *in vivo*, rendering them typical candidates for drug delivery technology applications [82].

In the past, the use of genetically altered natural viruses as gene carriers had been investigated, but they showed safety and immunogenicity limitations, and high costs. Non-viral vectors, mainly based on cationic lipids and polymers, are the most investigated strategies.

This cationic material can electrostatically interact with negatively charged DNA and generate self-assembled nanocarriers with several advantages such as scale manufacture, low immunogenic response, and the capacity to carry large inserts [83]. The most frequently studied cationic polymers include poly(ethylenimine) (pEI), poly(2-dimethylaminoethyl methacrylate) (pDMAEMA), and poly-L-lysine (pLL). Generally, their transfection efficiency is lower than that of viral vectors, and to date, their systemic administration has resulted in a toxic response incompatible with clinical applications [84]. They are non-degradable, with consequent risk of accumulation in the body, most of them show cytotoxicity depending on their chemical structure and molecular weight, likely due to adverse interactions with cellular membranes [85]. The use of biodegradable carriers would limit the accumulation of macromolecular material and promote the release of the active agent into the cytosol, following the degradation of the system itself. Sufficiently long lifetime of the carrier is required to reach the target site after systemic administration, as well as degradation of the system, preferably upon a certain intracellular trigger (pH, redox potential, presence of enzymes), to release the DNA efficiently into the cell [86].

1.3. Biomedical and Pharmaceutical Applications

1.3.1. Angiogenesis

Angiogenesis is the physiological and vital mechanism required for new blood vessels formation [87]. This complex process involves activation, migration and proliferation of endothelial cells from pre-existing vessels, beginning during embryogenesis and continuing throughout life in health and disease body, because is essential for tissue regeneration [88]. However, angiogenesis is also an essential step in pathologic processes, in which its spatial and temporal regulation is compromised. The excessive or inadequate angiogenesis leads to pathology conditions such as tumors and chronic inflammatory disorders [89].

The study of angiogenesis is having a profound impact on the biological and medical world. The recorded history of vasculogenesis and vascular remodeling dates back to at least two centuries ago. In 1787, the British doctor John Hunter was the first to observe the growth of blood vessels. He suggested that the new vascularization is regulated by metabolic compounds both in health and disease [90]. During the 19th century, a lot of works were focused on micro-anatomical description of vascular beds and microcirculatory structure, in different organs and species. Also the concepts of vascular

regulations, in response to metabolic stimulation, started to be a new field of interest. The next major phase of attention to angiogenesis came in the late 20th century, in particular with the work of doctor Judah Folkman. In his work, he introduced the term angiogenesis and hypothesized that tumor growth is dependent upon angiogenesis. Finally, more than 30 years after his publication, angiogenesis is a focal point in cancer research and treatment of many diseases such as peripheral and myocardial ischemia, chronic wounds and healing of skin ulcers [91]. Nowadays, angiogenesis process is widely studied and understanding the mechanism of its regulation is a new challenge for the development of new therapies. Angiogenesis is a highly regulate process and is controlled by the net balance between many molecules that have positive and negative regulatory activity including GFs. GFs play a key role in maintaining angiogenesis balance, because when they are produced in excess in comparison to the angiogenetic inhibitors, the balance is tipped in favor of blood vessel growth [92]. When inhibitors are present in excess, angiogenesis is stopped. The normal, healthy body maintains a perfect balance of angiogenesis modulators and when this balance is compromised the body undergoes to pathological conditions [93].

Angiogenesis may be divided into four stages: (I) activation of the endothelial cells that leads to a localized degradation of the basal membrane of the parent vessel and of the extra-cellular surrounding matrix; (II) oriented migration of endothelial cells in the extracellular matrix; (III) proliferation of endothelial cells; (IV) differentiation of these cells with organization into tubular structures with a new basal lamina. Through these stages the new capillaries form a new vascular network (Figure 6) [94].
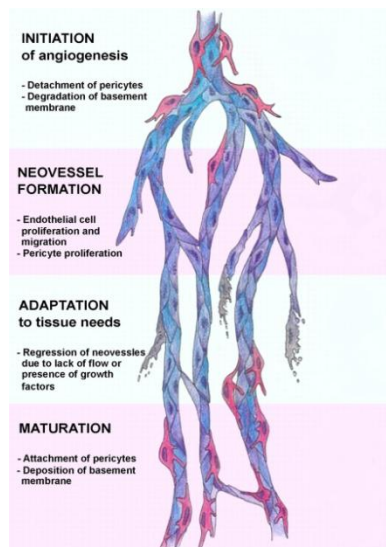

Figure 6: Schematic representation of angiogenesis process (modify from [95]).

1.3.2.Mechanisms of Angiogenesis Regulation

Blood vessels are part of the circulatory system that transports blood throughout the body. There are several types of blood vessels including arteries, veins and capillaries [96]. These blood vessels have a common internal structure. The outer layers are connective tissue and contain the nerves that control the muscles. The next inner layers are elastic fiber and connective tissue containing vascular smooth muscle. These muscles control the diameter of the blood vessel and allows the blood vessel to expand and contract, controlling the amount of blood flow and blood pressure [97]. The inner most layer consists of endothelial cells surrounded by a basement membrane [98]. The endothelial cells are among the most quiescent and genetically stable cells of the body and they are in direct contact with the blood [99]. During angiogenesis, endothelial cells can proliferate rapidly, being one of the main events that are required for the formation of new capillary blood vessel. Vascular endothelial cell proliferation is associated with degradation of basement membrane, which leads to sprouting of pre-existing micro vessels [100]. These vessels invade the extra cellular matrix, form tubes and finally the tips of the tubes connect to create loops that are capable of conducting the blood flow [101]. The molecular mechanisms responsible for angiogenesis are extraordinarily complex: multiple genes must coordinately express their products in appropriate amounts and in an appropriate time-dependent manner. Table 3 lists some GFs and cytokines that have been recognized to be involved in the process of angiogenesis [102]; [103];[104]. Some major GFs and their receptors will be introduced in detail in the following paragraph.

TABLE 3: Growth factors and cytokines involved in angiogenesis process	
Angiogenic Growth Factors and Cytokines	**Abbreviation**
Acidic fibroblast growth factor	aFGF
Angiopoietin	Ang
Basic fibroblast growth factor	bFGF
Heparin-binding epidermal growth factor	HB-EGF
Insulinlike growth factor	IGF
Placental growth factor	PLGF
Platelet-derived growth factor	PDGF

Vascular endothelial growth factor	VEGF
Hepatocyte growth factor	HGF
Transforming growth factor-beta	TGF-β
Granulocyte macrophage colony-stimulating factor	GM-CSF
Monocyte chemoattractant protein-1	MCP-1
Interleukin 8	IL-8
Interleukin 20	IL-20
Leptin	LP
Platelet-derived endothelial cell growth factor	PD-ECGF
Pleiotrophin	PTN
Progranulin	PGRN
Tumor necrosis factor-alpha	TNF-α
Follistatin	FST

1.3.2.1. Growth Factors

Growth factor is a term used to describe a broad range of structurally different molecular families and individual proteins best known for their ability to enhance cell proliferation and growth. They have an array of putative functions during the development including regulating tissue morphogenesis, angiogenesis and cell differentiation. They also play an important role in the maintenance of tissue homeostasis and wound healing in the adult. Their activities are mediated via binding to transmembrane receptors that often contain cytoplasmic tyrosine kinase domains [105]. Some of the most important GFs that work in the angiogenic process, such as PDGF, VEGF and FGF, have been identified in platelets.

PDGF was discovered more than two decades ago and is the major mitogens for many cell types of mesenchymal origin (e.g. fibroblasts and smooth muscle cells). Nowadays, five different isoforms, generated by alternative splicing are known PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD [106]. All PDGF are small proteins with molecular weight in the range of 28-30 kDa, and all isoforms have a common structure characterized by the typical growth factor domain involved in the dimerization of the two subunits, and in receptor binding and activation [107]. PDGF receptors (PDGFRs)

consist of - α and -β subtype, both of which are belonged to tyrosine kinase receptors (RTKs). Interactions between PDGF and PDGFRs are complex, for example, PDGFR-α is activated by PDGF-AA, AB, BB and CC, and PDGFR-β is activated by PDGF-BB and DD. On the other hand, the heterodimer PDGFR-αβ can be activated by PDGF-AB, BB and with low affinity with PDGF-CC and DD. In general, PDGF-A and C are mainly expressed by neuronal, epithelial and muscle cells. PDGF-B is more related to megakaryocytes, endothelial cells and neurons. Binding of PDGFs to PDGFRs leads to receptor dimerization and establishes the docking sites for various signaling molecules which are responsible to create a signaling network that initiates multiple cellular responses. These cascade activations of different signal molecules lead to cell growth, cell migration and antiapoptosis [108]. PDGFR- α and β mediates different biological events. PDGFR-α signaling is mainly important in gastrulation and development of lung, intestine, central nervous system and skeleton. Correspondingly, the β receptor initiates signaling primarily involved in the blood vessel system and hematopoiesis, but leads to similar mitogenic and migration effects [109]. All isoforms of PDGF stimulate angiogenesis, in particular PDGF-BB. The mechanisms of angiogenesis regulation by PDGF-BB are complex and involve a multi steps signaling. After the binding to its receptor on vascular endothelial cells, PDGF-BB activates the intracellular signal transduction pathways for the endothelial cells proliferation and migration. These processes lead to vascular tube formation and the recruitment of smooth muscle cells that initiate the stabilization of new blood vessels [110]. PDGF-BB also increases the expression of several angiogenic factors that include increased VEGF expression in fibroblasts and endothelial cells [111].

In 1983 the VEGF was first identified as a protein able to induce angiogenesis and currently it is one of the most studied growth factor [112]. The VEGF family includes six glycoproteins derived from an alternative splicing of the single gene consisting of eight exons. These isoforms differ for their amino acid length and, most importantly, for their ability to bind cellular heparan sulfates. This binding is critical to the VEGF biology because the loss of heparin binding results in a substantial loss of mitogenic activity of VEGF [113]. All VEGF ligands mediate their angiogenic effect via the RTKs, such as VEGFR-1, VEGFR-2 and VEGFR-3. When VEGF binds the extracellular domain of their receptor, a cascade of downstream proteins is activated after the dimerization and autophosphorylation of the intracellular RTKs. The VEGF receptors have an extracellular portion consisting of 7 immunoglobulin-like domains, a single transmembrane spanning region, and an intracellular portion containing a split tyrosine-kinase domain [114]. Within this family, the most relevant isoforms are represented by VEGF-A, VEGF-C and VEGF-D. VEGF-A has been regarded as the major player for angiogenesis and it binds to VEGFR-1 and VEGFR-2, of which VEGFR-2 is the major mediator of the mitogenic and angiogenic effects of VEGF-A [115]. However, the precise role of the VEGFR-1 receptor in angiogenesis remains elusive. VEGF-C and VEGF-D activate VEGFR-3 receptor, essential for lymphatic endothelial cell growth, migration and survival [116]. The importance of VEGF family and their receptors emerge from their lack or deletion during the development stages. Indeed loss of a single

VEGF allele in mouse models leads to the developmental deformities in the forming vasculature and embryonic death between days 11 and 12. Moreover, mice lacking either VEGFR-1 or VEGFR-2 even die earlier, between embryonic days 8.5 and 9.5 [117].

Another important set of proteins that mediates angiogenesis is FGFs family. FGFs are soluble GFs which have both acidic (aFGF) and basic (bFGF) variety. To date, more than twenty distinct FGFs have been discovered, numbered consecutively from 1 to 23 [118]. Indeed, FGF-23 has been identified as the last member of FGFs family and it is involved in the regulation of phosphate metabolism [119]. FGFs have a molecular weight range from 17 to 34 kDa, and most of isoforms, are characterized by an internal core region of similarity with 28 highly conserved amino-acid residues important for the receptor binding [120]. FGFs mediate their signals through four structurally related RTKs on cell surface (FGFR-1, 2, 3 and 4) to induce numerous biological effects such as mitogenic, chemotactic and angiogenic activity in cells of mesodermal and neuroectodermal origin [121]. Nevertheless, one of the best-characterized functions of FGFs is the induction of new blood vessels, and together with the VEGF, FGFs are the most important regulators of these processes. bFGF may contribute in angiogenesis in two primary ways: by modulating endothelial cell activity and by regulating VEGF expression. In brief, in one way, bFGF is involved in the proteolytic digestion of extracellular matrix by invading endothelial cells; a second way is by inducing expression of VEGF in which has been found to be dependent on bFGF dose [122]; [123].

Like PDGF and VEGF, bFGF binds with high affinity with glycosaminoglycans, in particular with heparin (Hp) [124]. Hp is a linear mucopolysaccharide composed of alternating units of sulfated d-glucosamine and d-glucuronic acid and this esterified sulfuric acid component gives to Hp its acidic property and electronegative charge [125]. Furthermore, Hp is a naturally occurring anticoagulant synthesized and secreted primarily by the mast cells in the body. Hp assumes an important biological function due to its capacity to bind different molecules, including extracellular matrix proteins, enzymes, and protease inhibitors.

In recent years, a large interest comes from the capability of the Hp to bind several GFs and cytokines involved in the angiogenesis process, thus affecting their biological activity [126]. The negative charges of Hp, provided by sulfo and carboxyl groups, are involved in the interactions between Hp and proteins. In fact, the proteins able to interact with Hp are characterized by the presence of clusters of positively charged basic amino acids that form ion pairs with spatially defined negatively charged sulfo or carboxyl groups of the Hp, and this particular region is called heparin-binding domain [127]. A number of studies have been undertaken to determine whether there is a consensus sequence of basic amino acids arranged in a specific way in heparin-binding domain. Instead, two consensus sequences of amino acids have been found: XBBXBX and XBBBXXBX where B is a basic residue and X is a hydropathic residue [128]. The interaction between Hp and proteins, such as angiogenesis related GFs, is also important to potentiate their biological activities. Indeed, several investigations have demonstrated

that when GFs interact with Hp, their bioavailability is improved. This is due to Hp that allows the localization of GFs at the cell surface or in the extracellular matrix and promotes the interaction between GFs and their respective receptors. In addition, the interaction of Hp with GFs has been shown to protect them from heat and acid inactivation and from enzymatic degradation [129, 130].

1.3.3. Therapeutic Angiogenesis

The term therapeutic angiogenesis was first introduced to describe the stimulation of neovascularization in order to treat or prevent pathological clinical situations characterized by local hypovascularity [131]. Traditionally, therapeutic angiogenesis has been achieved by surgical methods (i.e. the transposition of autologous tissues with uncompromised vasculature). However, a significant number of patients have still shown clinical situation characterized by severe general ischemic conditions, which make these symptomatic patients not suitable for the traditional surgery treatments. Nowadays, the advances in nanotechnology field may add a new clinical tool to achieve therapeutic angiogenesis by pharmaceutical route, such as the administration of GFs as alternative treatment for these diseases [132].

1.3.3.1.   Peripheral Arterial Disease

Peripheral arterial disease (PAD) is referred to the obstruction of large arteries in the arms or legs, is a pandemic condition that lead to an insufficient tissue perfusion and has the potential to cause loss of limb or even, in severe cases, loss of life [133]. Smoking, high cholesterol, diabetics and high blood pressure are also significant risk factors for PAD. Moreover PAD can result from atherosclerosis, inflammatory processes leading to stenosis, an embolism, or thrombus formation [134]. The incidence of PAD in Europe reaches 1% per annum over the age of 65 years and 30% of deaths worldwide are attributed to PAD. In the United States 1.4 million suffer from this pathology with an estimated 350876 new cases diagnosed each year [135]. However, in settings such as acute limb ischemia, this pandemic disease can be life threatening and can require emergency intervention to minimize mortality [136]. The common treatments for PAD are represented by surgical revascularization and angioplasty, but limb amputations are still performed for the treatment of severe cases [137]. Although surgical and endovascular techniques give good initial results for symptomatic PAD, the progressive nature of atherosclerosis and bypass grafts commonly lead to patients re-presenting with worsening claudication or critical limb ischemia (CLI), such as a more severe form of PAD and principal cause of lower limb amputations [138, 139]. No effective pharmacological measures are available for this situation and in many cases the only option is limb amputation. In order to achieve limb salvage and in view of the poor results with current therapies, much interest has been shown in developing alternative treatment within therapeutic angiogenesis such as gene therapy, cell therapy and the use of drug delivery systems [140]. Gene therapy involves the insertion of genetic material, plasmid or viral vector, into dysfunctional cells or tissues by intramuscular or intra-

arterial injection. The target of gene therapy is aimed to induce locally expression of the GFs naturally involved in the process of vascular growth and repair. The most commonly GFs used for gene therapy are VEGF and FGF [141]. Various isoforms of VEGF have been delivered to patients in clinical studies and the isoform VEGF-A165 was first tested in humans in 1996 following several successful preclinical trials with animal ischaemic hindlimb models [142]. Concerning the use of FGF, the isoforms FGF-1 and FGF-2 have been studied most in gene therapy for PAD. FGF-1 plays a key role in new vessel development and is widely used for gene therapy. In particular clinical trials have demonstrated that in patients treated with injections of FGF-1 the amputation rate has been decreased from 55% to 37% [143]. Gene therapy based on injection of FGF-2, using a nonviral gene transfer approaches, demonstrated to significantly increase the blood flow and angiogenesis in a rat model of hindlimb ischemia [144]. As alternative strategy to conventional treatment for PAD, in recent years, there has been an increased interest also in cell therapy. The use of stem cell as an agent to induce new revascularization emerged in 1997 with the discovery, in adult human peripheral blood, of the circulating endothelial progenitor cells (EPCs) and their ability to successfully promote vascularization in ischemic tissues [145]. Current investigations in regenerative medicine are evaluating the potential of stem/progenitor cells, such as bone marrow derived stem cells, from a variety of non-hematogenous sources, tissue-resident stem cells, and pluripotent stem cells [146]. The therapeutic application of bone marrow mononuclear cells has confirmed that these cells support collateral vessel formation in animal models [147]. The clinical trials for the use of bone marrow mononuclear cells, administrated in patients by intramuscular injection, have demonstrated the improvement of blood perfusion in ischemic limbs and reduction of amputation rates [148]; [149]; [150]. Nowadays, many studies are in progress to improve the integration and survival of the transplanted cells in order to obtain new cell therapies for the treatment of PAD [151].

The increase of interest in the field of nanotechnology, to overcome the problems related with gene and cell therapy, involved the use of NPs for the delivery of pro-angiogenic compounds in the treatment of PAD. The most studied NPs carrier are represented by those used for the delivery of angiogenesis related GFs, even if in recent years also nucleic acid, such as RNAs are under development [152]. To date, NPs loaded with bFGF and VEGF respectively have been developed. The bFGF loaded NPs have shown the capacity to induce capillary growths in a hind-limb ischemia mouse model, treated for 4 weeks of subcutaneous injection of bFGF NPs [153]. Regarding the use of VEGF loaded NPs it has been demonstrated that injection in mice' ischemic limbs induces the growth of new blood vessels [154]. Several other NPs for GFs delivery have been developed, including IGF-1 and TGF-β3 loaded NPs [155].

1.3.3.2.   Chronic Wounds

Chronic wounds occur when a wound does not heal in an orderly set of stages. If within three months, a wound is not heal is often considered chronic. In acute wounds, there is a

precise balance between production and degradation of molecules, such as collagen, necessary for normal healing. In chronic wounds this balance is lost and, the phase of degradation plays the main role, and consequently healing of chronic wound may never occur (Figure 7). In today's society, chronic wounds represent a major health care burden. It has been estimated that 15% of individuals with diabetes mellitus will develop lower extremity ulcers and 14–24% of diabetic patients with foot ulcers will eventually undergo amputation. Approximately 100000 limb amputations are performed in diabetic patients each year in the United States [156]. Understanding causes of chronic wound is not easy, because vary from patient to patient, and are related with particular patient's conditions, such as poor blood circulation, diabetes or can develop in nursing home patients who are confined to bed. These wounds cause in patients an acute pain, physical stress, and in the severe cases may result in a serious infection, gangrene, and may even require amputation [157]. There are three main types of chronic wounds: venous ulcers, diabetic ulcers, and pressure ulcers [158]. Venous ulcers usually occur in the legs, account for the majority of chronic wounds, and mostly affect the elderly. They are caused by improper function of tiny valves in the veins that normally prevent blood from flowing backward. The dysfunction of these valves impedes the normal circulation of blood in the legs, causing tissue damage and impaired wound healing [159]. Diabetic patients are particularly susceptible to developing ulcers. People with advanced diabetes have a diminished perception of pain in the extremities due to nerve damage. Diabetes also impairs the immune system and damages capillaries [160]. The major causes of foot ulcers in diabetic patient can be attributed to neuropathy, mechanical stresses and ischemia [161]. Pressure ulcers typically occur in people who are bedridden or whose mobility is severely limited. Pressure ulcers are caused by a loss of blood circulation that occurs when pressure on the tissue is greater than the pressure in capillaries, thereby blood flow to the tissue is completely or partially obstructed [162].

The conventional treatments for chronic wounds often are based on topical therapies with antibiotics or anti-inflammatory drugs but with limited success. Currently, other therapies are under investigations and involve the use of GFs. The use of GFs could be one of the best strategies for the treatment of these pathological conditions because defects in angiogenesis are always present in chronic wounds, and GFs based therapies have shown to promote angiogenesis and accelerate healing [163]. These novel strategies to treat chronic wounds are based on synthetic medical devices that mimic the natural healing process and also provide options for controlled GFs or cell delivery. To specifically address GFs to the target site, recent approaches have focused on the incorporation of GFs within platelet gels (PG) or within polymer based NPs [164]. PG provides, *in situ*, exogenous additional GFs that can accelerate tissue reparation and regeneration [165]. In a study on a 57-year-old man with type 2 diabetes and a wound of six months duration, the treatment with PG lead to the complete closure of the ulcer by the fourth week [166]. Due to their low stability, the administration of GFs generally requires a vector, and the recent expansion in the field of nanotechnology is offering a wide variety of opportunities in this field. Recently, NPs have attracted much attention due to their unique properties: reduce GFs side-effects, provide an extended availability

of GFs at the local environment, and protect them against degradation by proteolytic enzymes [167].

Gene therapy and stem cell research have become areas of interest for developing new strategies for wound healing, but still remain challenges in the selection of optimal cells targeting, development of sequential therapeutic methods, and identification of factors which may be detrimental to the introduction of genes. Further research is needed on the intrinsic molecular mechanisms that keep stem cells pluripotent or direct them along particular differentiation pathways [168].
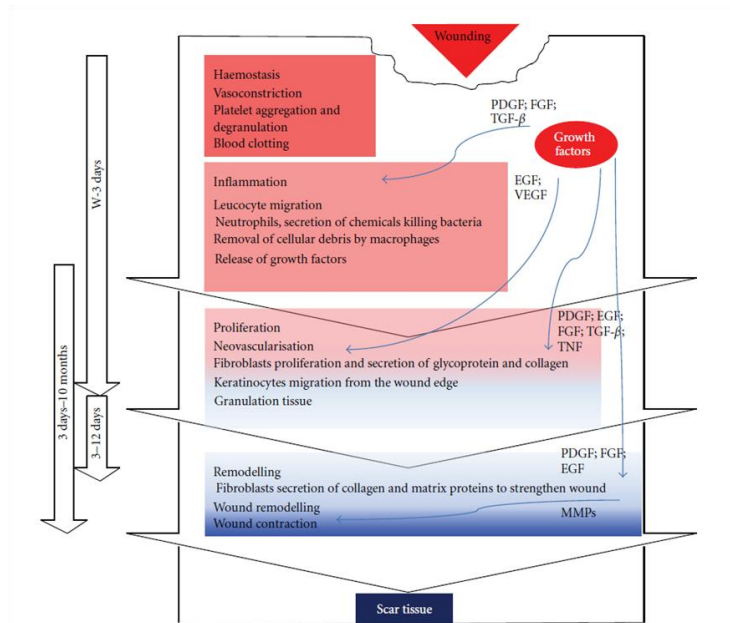

Figure 7: Schematic representation of processes involved in wound healing (modified from [169]).

1.3.3.3. Ischemic Heart Disease

Ischemic heart disease (IHD), also called coronary artery disease (CAD), is a condition that affects the supply of blood to the heart. The coronary arteries are the only existent blood supply to the heart muscle [139]. In a pathological situation, a blockage in the coronary arteries reduces suddenly the supply of blood in a portion of the heart muscle, and consequently leads to the death of that area, resulting in a heart attack. In the pathophysiology of IHD, two processes are involved: supply and demand of myocardial oxygen. Myocardial ischemia occurs when there is imbalance between supply and demand for oxygen. In fact, in some conditions, the impairment of oxygen is secondary to decreased blood flow and is the main responsible for myocardial ischemia [170]. The major risk factors of the incidence of IHD could be smoking, diabetes mellitus, high cholesterol levels, hypertension, stress and genetic predisposition factors [171]. IHD is the most common cause of morbidity and mortality in the Western countries [172]. There have been enormous advances in surgical methods for the treatment of IHD, such as angioplasty, coronary artery bypass graft and revascularization by percutaneous coronary intervention [173]. However, surgical technologies are not suitable for all people suffering from IHD. Many patients are not optimal candidates for surgical treatment because of co-morbid diseases, unsuitable anatomy, or the risks of the procedure [174]. Thus, is an important challenge finding additional or alternative treatments for IHD. Nowadays, the progresses in therapeutic angiogenesis offer the basis for the development of new therapies through the use of pro-angiogenic GFs. Controlled released systems, such as NPs, offer a potent tool for the administration of GFs. The sustained release of GFs may be necessary for the maintenance of stable revascularization and thus increase treatment efficacy with a lower dose of GFs. Indeed, the therapeutic angiogenesis approach is supported by a preclinical evidence showing improvements in blood flow, revascularization and myocardial function after angiogenic GFs delivery in different animal models of myocardial disease [175]. Moreover, the use of NPs, make it a targeted approach for the treatment of IHD. Targeted NPs may be injected intravenously, circulate in the body for long periods, and bind to desired tissues. In the infarcted heart NPs have been used to target macrophages and blood vessels [176].

Other new strategies, such as gene and stem cells therapies, are also currently under investigation as an alternative approach for the treatment of IHD. Nevertheless, even if the clinical trials are promising, gene and stem cells therapies need to be better understood. Several obstacles have contributed to their limited effectiveness, such as problems with viral vectors and immune responses as regards the gene therapy, and isolation, identification and *in vivo* expansion for stem cells therapy [177]; [178].

1.4. Platelet Rich Plasma

1.4.1. Platelets

Platelets are small discoid blood cells (1-3 μm) and are only about 20% of the diameter of red blood cells [179]. The average platelet count ranges from 1.5 to 3.0 x 10^{-5} ml of circulating blood, and the *in vivo* half-life time of platelets is about 7 days. Platelets are synthesized in the bone marrow, the same as the red cells and most of the white blood cells [180]. Platelets are produced from very large bone marrow cells called megakaryocytes by pinching off pieces of cytoplasm and extruded into the circulation. As megakaryocytes develop into giant cells, they undergo a process of fragmentation that results in the release of over 1,000 platelets per megakaryocyte [181]. Platelets are actually not true cells but merely circulating fragments of cells, but they contain many structures that are critical to stop bleeding. Platelets contain proteins similar to muscle proteins, a ring of contractile microtubules (cytoskeleton) around their periphery containing actin and myosin, which allow them to change shape and become active [182]. Platelets activation occur when they are stimulated by a break in the blood vessel wall. The platelets change their shape, become round and extend long filaments (pseudopods), that allow to make contact with the broken vessel wall or with other platelets, and in this way they are able to form a plug to seal the broken blood vessel [183]. Normally, in the resting state, platelets are nonthrombogenic and require a trigger before they become a potent and an active player in hemostasis and wound healing.

Inside the platelet, a number of intracellular structures are present containing glycogen, lysosomes, and two types of granules known as dense granules and α-granules able to secrete other proteins [184]. Dense granules are 3-8 granules per platelet, are small (~150 nm) and appear as dense bodies due to their high calcium and phosphate contents. In addiction dense granules contain adenine nucleotides and serotonin and P-selectin. During platelets activation, the membrane of these granules interacts with the platelet plasma membrane and they merge together. This event allows the subsequent release of granule's content into the extracellular environment. The released constituents contribute to recruit other platelets (aggregation) and also contribute to local vasoconstriction [185]. The importance of the dense granules into the normal hemostasis is shown in two rare human pathological conditions, such as Hermansky-Pudlak syndrome and Chediak-Higashi syndrome, in which the deficiency of these granules is associated with predisposition to bleeding [186]; [187]. Alpha-granules are the largest platelet granules (~200-400 nm), are 50-60 per platelet and are responsible of the granular aspect of the platelets' cytoplasm. In the α-granules are present the majority of platelet factors involved in hemostasis, inflammatory process and wound healing (table 4) [188]. Further, α-granules demonstrate heterogeneity in their constituents and specific sub-populations, probably due to the fundamental role that alpha-granules play in platelets aggregation, thrombosis and regulation of cell proliferation via release of various GFs. During platelets activation the alpha-granules release occurs with an intricate process because platelets contain complex machinery for granule release in which play an

essential role proteins receptor and membrane lipids [189]. The process involving coalescence in the platelet center, formation of a fusion pore that represents the initial site of mixing of two membranes and this fusion pore undergoes to rapidly expand until completely fusion of granule membrane with platelet membrane and consequent release of granule contents [190].

TABLE 4: Alpha-granule contents in human platelets	
Platelets alpha-granules contents	
Adhesion molecules	Platelet Endothelial Cell Adhesion Molecule-1 (PECAM- 1/CD31) Fibrinogen von Willebrand Factor (vWF) Thrombospondin-1 (TSP1) Vitronectin, Fibronectin
Mitogenic factors	Platelet-derived Growth Factor (PDGF) Vascular Endothelial Growth Factor (VEGF) Transforming Growth Factor-β (TGF-β)
Membrane proteins	P- selectin CD40L Glycoprotein IIb/IIIa (GPIIb/IIIa, aIIbβ3 integrin, CD41/CD61)
Coagulation factors	Fibrinogen, Plasminogen, Protein S, Kininogens Factors V, VII, XI, XIII
Protease inhibitors	C1 inhibitor Plasminogen activator inhibitor-1 (PAI-1) Tissue factor pathway inhibitor (TFPI)
Leucocyte recruitment	Chemokines: PF4, RANTES, β- thromboglobulin, ENA-78, SDF - 1α

1.4.2. Preparation of Platelet Rich Plasma

Platelet Rich Plasma (PRP) is blood plasma that has been enriched with platelets. It is prepared from a unit of autologous whole blood by means of extracorporeal blood processing techniques [191]. The blood is collected in the presence of an anticoagulant and centrifuged. An initial centrifugation at low speed separates the blood into three layers as a function of density: the top plasma layer, the middle layer (consisting of white blood cells and platelets), and the bottom layer (consisting of red blood cells). The top and middle layers are then centrifuged at high speed to separate Platelet Poor Plasma (PPP) and PRP (Figure 8). The volume of PRP collected is approximately 10% of the total blood used. Before application, PRP must be activated to release the GFs from the alpha-granules of the platelets [179]. The most common method of platelet activation for clinical use is to add calcium chloride/thrombin and store the PRP for 8 h at room temperature [192]. Other platelet activation methods include freeze/thaw, or addiction of trigger molecules, such as collagen and adenosine diphosphate (ADP). The choice of the

platelet activator would affect the timing and level of GFs release. Furthermore, the higher concentration of platelets in PRP would result in a greater level of GFs release. Other factors may influence the efficacy of PRP are centrifugation protocols, choice of anticoagulant, platelet integrity, and the presence of other cell types [193].
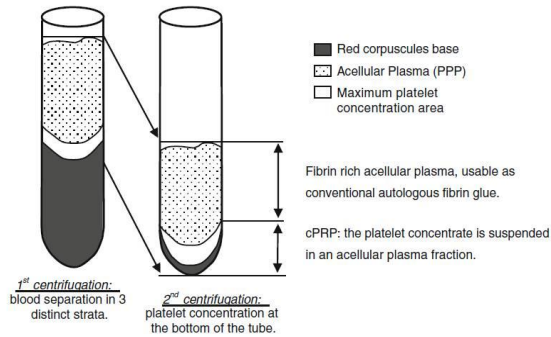

Red corpuscles base
Acellular Plasma (PPP)
Maximum platelet concentration area

Fibrin rich acellular plasma, usable as conventional autologous fibrin glue.

cPRP: the platelet concentrate is suspended in an acellular plasma fraction.

1st centrifugation: blood separation in 3 distinct strata.

2nd centrifugation: platelet concentration at the bottom of the tube.

Figure 8: Technology of PRP preparation [194].

1.4.3. Use of Platelet Rich Plasma in Animal and Clinical Studies

PRP has been clinically used in humans since the 1970s for its healing properties attributed to the presence of autologous GFs and secretory proteins that may enhance the healing process [195]. PRP is made from the patient's own plasma and hence is completely safe, relatively inexpensive, and expected to release the right mixture of GFs for therapeutic use. In humans, PRP has been investigated and used as clinical tool for several types of medical treatments, including nerve injury, tendinitis, osteoarthritis, cardiac muscle injury, bone repair and regeneration, plastic surgery, and oral surgery [196]. PRP has also received attention in the popular media as a result of its use in treating sports injuries in professional athletes [197]. A number of studies have examined the effects of PRP on tissue regeneration and healing. Different studies, in diverse animal models, have demonstrated the capability of the PRP to promote and accelerate healing. A recent study on a rabbit model has indicated that after 4 and 12 weeks, the animals treated with PRP have shown an improved of the cartilage and bone formation [198]. PRP has also been demonstrated to improve the healing of acute traumatic wounds. Studies performed in a rat Achilles tendon rupture model and in equine with tendons rupture also demonstrated the efficacy of PRP to promote the healing. In particular, the study carried out in the rat model has shown an increase of the tendon strength and stiffness by 30% after 1 week [199]; [200]; [201].

Clinical use of PRP has been also investigated in human subjects. However, the data regarding the efficacy of PRP in human are more limited. This is because of the data are not related only to the use of PRP, but to all autologous platelet concentrate, such as platelet gel and fibrin glue. Moreover, human studies are difficult to describe because reported cases may not have controls, have small sample sizes, and do not have a

standardized preparation of PRP. The available studies suggested that the use of PRP in human, promote wound healing and also could lead to decrease of the post-operative infections, pain, and blood loss. There have been publications on the use of PRP for several clinical applications, including periodontal and oral surgery, maxillofacial surgery, heart bypass surgery, and treatment of chronic skin ulcers [202]; [203]; [204]; [205]. As such, wound healing enhancement by PRP exists, however, a small collection of clinical studies have demonstrated a significant enhancement of hard- and soft-tissue healing with the use of PRP. In plastic surgery, the use of PRP has been largely limited to fibrin glues, primarily used to obtain hemostasis and adherence of skin flaps. In fact, a diverse use of fibrin glue for closure of surgery incisions, such as in face and neck lifts and breast reductions and augmentations, resulted in numerous advantages. These included the elimination of the need for drains, a reduction in post-operative pain and swelling, and improved wound healing [206]. In wounds treated with PRP, faster ulcers reepithelialized has been observed in patients treated with PRP, compared with patients with similar wounds, treated with placebo [207]. Moreover, PRP has been useful for the treatment not only of chronic wounds, but also in acute trauma wounds [208]. Treatment with PRP holds great promise. PRP does appear to be effective in the treatment of chronic injuries in wide medical fields.

1.4.4. Platelet Lysate

Platelets Lysate (PL) can be defined as a platelet derivate, obtained by platelets collection from blood samples, in which the presence of GFs and cytokines can promote tissue regeneration and cell proliferation [209]. There is increasing interest in the use of PL as a therapeutic biological product in the field of regenerative medicine as well as for various applications in cell cultures and cell therapy as a replacement of FBS (Fetal Bovine Serum). Currently, the major therapeutic applications of PL are to stimulate bone regeneration in oral, maxillofacial, plastic and orthopaedic surgery, or to accelerate wound healing of soft tissues, in particular in the treatment of ischemic leg ulcers [210]; [211]; [212]. For such clinical applications, PL obtained from an autologous single-donor, is used as a topical product, as such or after activation by exogenous thrombin to induce the release and temporary entrapment of the GFs into a fibrin rich biomaterial [164].

FSB is the most widely used serum for animal cell culture, mainly due to its high concentration of GFs and low concentration of immunoglobulins. However, there are several problems with the use of serum such as availability, cost, unknown composition, variations between batches and complications of the purification of products [213]. In the last decade research has focused on alternatives to serum based media to overcome problems associated with the use of serum. In the 1970s it was demonstrated that platelet components promoted cell growth *in vitro* [214]. Based on this knowledge, PL has been studied as an alternative source for supplementation of cell culture medium. In fact, the use of PL containing multiple GFs has been proven by a number of researches to be

superior to FSB or other serum preparations, in terms of costs and proliferation rate, as regarding mesenchymal stem cells (MSCs) [215]; [216]; [217].

PL can be obtained from PRP. The general procedure is to separate platelet fractions from the plasma by centrifugation steps and concentrate to a density of at least $1x10^{-9}$ platelets/ml. Platelets can be activated with thrombin or lysed by repeated freeze-thaw cycles. Both mechanisms result in the release of GFs and mitogens that are stored in the platelets [218].

1.5. References

1.   Albanese A., Tang PS., Chan WCW. "The effect of nanoparticle size, shape, and surface chemistry on biological systems". Annual Review of Biomedical Engineering 2012;14(1):1-16.

2.   Sanchez F., Sobolev K. "Nanotechnology in concrete-A review". Construction and Building Materials 2010;24(11):2060-2071.

3.   Riehemann K., Schneider SW., Luger TA., Godin B., Ferrari M., Fuchs H. "Nanomedicine-challenge and perspectives". Angewandte Chemie International Edition 2009;48(5):872-897.

4.   Farokhzad OC., Langer R. "Impact of nanotechnology on drug delivery". ACS Nano 2009 2009/01/27;3(1):16-20.

5.   Liechty WB., Kryscio DR., Slaughter BV., Peppas NA. "Polymers for drug delivery systems". Annual Review of Chemical and Biomolecular Engineering 2010;1(1):149-173.

6.   Discher DE., Eisenberg A., "Polymer vesicles". Science 2002;297(5583):967-973.

7.   Timko BP., Whitehead K., Gao W., Kohane DS., Farokhzad O., Anderson D. "Advances in drug delivery". Annual Review of Materials Research 2011;41(1):1-20.

8.   Davis ME., Chen Z., Shin DM. "Nanoparticle therapeutics: an emerging treatment modality for cancer". Nature Reviews Drug Discovery 2008;7(9):771-782.

9.   Ikkala O., Brinke G., "Functional materials based on self-assembly of polymeric supramolecules". Science 2002;295(5564):2407-2409.

10.   Shi J., Votruba AR., Farokhzad OC., Langer R. "Nanotechnology in drug delivery and tissue engineering: from discovery to applications". Nano Letters 2010;10(9):3223-3230.

11.   Williams DF., "On the nature of biomaterials". Biomaterials 2009;30(30):5897-5909.

12.   Kohane DS., Langer R. "Biocompatibility and drug delivery systems". Chemical Science 2010;41(52):441-446.

13.   Nair L., Laurencin C. "Polymers as biomaterials for tissue engineering and controlled drug delivery". Advances in Biochemical Engineering/Biotechnology 2006;102:47-90.

14.   Baldwin AD., Kiick KL. "Polysaccharide-modified synthetic polymeric biomaterials". Peptide Science 2010;94(1):128-140.

15.   Liu S., Maheshwari R., Kiick KL. "Polymer-based therapeutics". Macromolecules 2009 2009/01/13;42(1):3-13.

16.   Kohane DS., Langer R. "Polymeric biomaterials in tissue engineering". Pediatric Research 2008;63(5):487-491.

17.   Jagur-Grodzinski J. "Polymers for tissue engineering, medical devices, and regenerative medicine. Concise general review of recent studies". Polymers for Advanced Technologies 2006;17(6):395-418.

18.   Seeherman H., Wozney JM. "Delivery of bone morphogenetic proteins for orthopedic tissue regeneration". Journal of Interferon & Cytokine Research 2005;16(3):329-345.

19.   Huebsch N., Mooney DJ. "Inspiration and application in the evolution of biomaterials". Nature 2009;462(7272):426-432.

20.   Sun T., Qing G. "Biomimetic smart interface materials for biological applications". Advanced Materials 2011;23(12):H57-H77.

21.   Chiellini F., Piras AM., Errico C., Chiellini E. "Micro/nanostructured polymeric systems for biomedical and pharmaceutical applications". Nanomedicine 2008;3(3):367-393.

22.   Kim S., Kim J-H, Jeon O., Kwon IC., Park K. "Engineered polymers for advanced drug delivery". European Journal of Pharmaceutics and Biopharmaceutics 2009;71(3):420-430.

23.   Tian H., Tang Z., Zhuang X., Chen X., Jing X. "Biodegradable synthetic polymers: Preparation, functionalization and biomedical application". Progress in Polymer Science 2012;37(2):237-280.

24.   Hoffman AS. "The origins and evolution of "controlled" drug delivery systems". Journal of Controlled Release 2008;132(3):153-163.

25.   Raffa V., Vittorio O., Riggio C., Cuschieri A. "Progress in nanotechnology for healthcare". Minimally Invasive Therapy & Allied Technologies 2010;19(3):127-135.

26.   Serda RE., Godin B., Blanco E., Chiappini C., Ferrari M. "Multi-stage delivery nano-particle systems for therapeutic applications". Biochimica et Biophysica Acta 2011;1810(3):317-329.

27.   Kost J., Langer R. "Responsive polymeric delivery systems". Advanced Drug Delivery Reviews 2001;46(0):125-148.

28.   Siepmann J., Siepmann F. "Modeling of diffusion controlled drug delivery". Journal of Controlled Release 2012;161(2):351-362.

29.   Fu Y., Kao WJ. "Drug release kinetics and transport mechanisms of non-degradable and degradable polymeric delivery systems". Expert Opinion on Drug Delivery 2010;7(4):429-444.

30.   Wischke C., Schwendeman SP. "Degradable polymeric carriers for parenteral controlled drug delivery fundamentals and applications of controlled release drug delivery". In: Siepmann J, Siegel RA, Rathbone MJ, editors. Fundamentals and Applications of Controlled Release Drug Delivery. Springer US, 2012. p. 171-228.

31.   Letchford K., Burt H. "A review of the formation and classification of amphiphilic block copolymer nanoparticulate structures: micelles, nanospheres, nanocapsules and polymersomes". European Journal of Pharmaceutics and Biopharmaceutics 2007;65(3):259-269.

32.   Farokhzad OC. "Nanotechnology for drug delivery: the perfect partnership". Expert Opinion on Drug Delivery 2008;5(9):927-929.

33.   Li C., Wallace S. "Polymer-drug conjugates: recent development in clinical oncology". Advanced Drug Delivery Reviews 2008;60(8):886-898.

34.   Petros RA., De Simone JM. "Strategies in the design of nanoparticles for therapeutic applications". Nature Reviews Drug Discovery 2010;9(8):615-627.

35.   Vauthier C., Bouchemal K. "Methods for the preparation and manufacture of polymeric nanoparticles". Pharmaceutical Research 2009;26(5):1025-1058.

36.   Kumari A., Yadav SK., Yadav SC. "Biodegradable polymeric nanoparticles based drug delivery systems". Colloids and Surfaces B: Biointerfaces 2010;75(1):1-18.

37.   Almeida AJ., Souto E. "Solid lipid nanoparticles as a drug delivery system for peptides and proteins". Advanced Drug Delivery Reviews 2007;59(6):478-490.

38.   Parveen S., Misra R., Sahoo SK. "Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging". Nanomedicine: Nanotechnology, Biology and Medicine 2012;8(2):147-166.

39.   Elasabahy M., Wooley KL. "Design of polymeric nanoparticles for biomedical delivery applications". ChemInform 2012;43(27):NO-NO.

40.   Makadia HK., Siegel SJ. "Poly lactic-*co*-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier". Polymers 2011;3(3):1377-1397.

41.   Rasiah IA., Parlane N., Grage K., Palanisamy R., Jahns AC., Atwood JA. "Biopolyester particles: preparation and applications". Encyclopedia of Industrial Biotechnology. In: Flickinger MC. Editor. John Wiley & Sons, Inc., 2009.

42.   Lee BI., Qi L., Copeland T. "Nanoparticles for materials design: Present & future". Journal of Ceramic Processing Research 2005;6(1):31-40.

43.   Sanchez-Dominguez M., Pemartin K., Boutonnet M. "Preparation of inorganic nanoparticles in oil-in-water microemulsions: A soft and versatile approach". Current Opinion in Colloid & Interface Science 2012;17(5):297-305.

44.   Mora-Huertas CE., Fessi H., Elaissari A. "Polymer-based nanocapsules for drug delivery". International Journal of Pharmaceutics 2010;385(1–2):113-142.

45.   Rao JP., Geckeler KE. "Polymer nanoparticles: preparation techniques and size-control parameters". Progress in Polymer Science 2011;36(7):887-913.

46.   Khosla G. "Nanoparticles – an overview of preparation and characterization". Novel Science International Journal of Pharmaceutical Sciences 2012; 1(8):0-0.

47.   Bilati U., Allémann E., Doelker E. "Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles". European Journal of Pharmaceutical Sciences 2005;24(1):67-75.

48.   Chiellini F., Bartoli C., Dinucci D., Piras AM., Anderson R., Croucher T. "Bioeliminable polymeric nanoparticles for proteic drug delivery". International Journal of Pharmaceutics 2007 Oct 1;343(1-2):90-97.

49.   Piras AM., Errico C., Dash M., Ferri M., Chiellini E., Chiellini F. "Polymeric nanoparticles for targeted release of conventional and macromolecular drugs (preparation and characterization aspects)". In: V. RR, editor. Nanoparticles: Synthesis, Characterization and Applications: American Scientific Publishers, 2011. p. 229-335.

50.   Ulbrich W., Lamprecht A. "Targeted drug-delivery approaches by nanoparticulate carriers in the therapy of inflammatory diseases". Journal of The Royal Society Interface 2010;7(Suppl 1):S55-S66.

51.   Singh R., Lillard Jr JW. "Nanoparticle-based targeted drug delivery". Experimental and Molecular Pathology 2009;86(3):215-223.

52.   Panyam J., Labhasetwar V. "Biodegradable nanoparticles for drug and gene delivery to cells and tissue". Advanced Drug Delivery Reviews 2012;64, Supplement(0):61-71.

53.   Barakat NS. "Magnetically modulated nanosystems: a unique drug-delivery platform". Nanomedicine : Nanotechnology, Biology, and Medicine 2009;4(7):799-812.

54.   Morachis JM., Mahmoud EA., Almutairi A. "Physical and chemical strategies for therapeutic delivery by using polymeric nanoparticles". Pharmacological Reviews 2012;64(3):505-519.

55.   Chomoucka J., Drbohlavova J., Huska D, Adam V., Kizek R., Hubalek J. "Magnetic nanoparticles and targeted drug delivering". Pharmacological Research 2010;62(2):144-149.

56.   Gao J., Gu H., Xu B. "Multifunctional magnetic nanoparticles: design, synthesis, and biomedical applications". Accounts of Chemical Research 2009;42(8):1097-1107.

57.   Koo H., Huh MS., Sun I-C., Yuk SH., Choi K., Kim K. "*In vivo* targeted delivery of nanoparticles for theranosis". Accounts of Chemical Research 2010;44(10):1018-1028.

58.   Saiyed Z., Telang S., Ramchand C. "Application of magnetic techniques in the field of drug discovery and biomedicine". BioMagnetic Research and Technology 2003;1(1):2.

59.   Gad SC. "Development of therapeutic agents handbook". Wiley, New Jersey 2011.

60.   Carlson EE. "Natural products as chemical probes". ACS Chemical Biology 2010;5(7):639-653.

61.   Rägo L. "Biosimilar medicines and safety: new challenges for pharmacovigilance". The International Journal of Risk and Safety in Medicine 2009;21(1):5-11.

62.   Torchilin V. "Tumor delivery of macromolecular drugs based on the EPR effect". Advanced Drug Delivery Reviews 2011;63(3):131-135.

63.   Creixell M., Peppas NA. "Co-delivery of siRNA and therapeutic agents using nanocarriers to overcome cancer resistance". Nano Today 2012;7(4):367-379.

64.   Dessy A., Piras AM., Alderighi M., Sandreschi S., Chiellini F. "Doxorubicin loaded polyurethanes nanoparticles". Nano Biomedicine and Engineering 2012;4(2):82-88.

65.   Pogribny IP., Filkowski JN., Tryndyak VP., Golubov A., Shpyleva SI., Kovalchuk O. "Alterations of microRNAs and their targets are associated with acquired resistance of MCF-7 breast cancer cells to cisplatin". International Journal of Cancer 2010;127(8):1785-1794.

66.   Xu P., Van Kirk EA., Murdoch WJ., Zhan Y., Isaak DD., Radosz M. "Anticancer efficacies of cisplatin-releasing pH-responsive nanoparticles". Biomacromolecules 2006 2012/12/18;7(3):829-835.

67.   Moser EG., Morris AA., Garg SK. "Emerging diabetes therapies and technologies". Diabetes Research and Clinical Practice 2012;97(1):16-26.

68.   Vlieghe P., Lisowski V., Martinez J., Khrestchatisky M. "Synthetic therapeutic peptides: science and market". Drug Discovery Today 2010;15(1-2):40-56.

69.   Chen M-C., Sonaje K., Chen K-J., Sung H-W. "A review of the prospects for polymeric nanoparticle platforms in oral insulin delivery". Biomaterials 2011;32(36):9826-9838.

70.   Ramachandran R., Paul W., Sharma CP. "Synthesis and characterization of PEGylated calcium phosphate nanoparticles for oral insulin delivery". Journal of Biomedical Materials Research Part B: Applied Biomaterials 2009;88B(1):41-48.

71.   Zhang S., Uludag H. "Nanoparticulate systems for growth factor delivery". Pharmaceutical Research 2009;26(7):1561-1580.

72.   Pichereau C., Allary C. "Therapeutic peptides under the spotlight". European Biopharmaceutical Review 2005;(WINTER):88-93.

73.   Xu L., Anchordoquy T. "Drug delivery trends in clinical trials and translational medicine: Challenges and opportunities in the delivery of nucleic acid-based therapeutics". Journal of Pharmaceutical Sciences 2010;100(1):38-52.

74.   Patil SD., Rhodes DG., Burgess DJ. "DNA-based therapeutics and DNA delivery systems: A comprehensive review". The AAPS Journal 2005;7(1):E71-E77.

75.   Chan IS., Ginsburg GS. "Personalized medicine: progress and promise". Annual Review of Genomics and Human Genetics 2011;12(1):217-244.

76.   Burnett JC., Rossi JJ. "RNA-Based therapeutics: current progress and future prospects". Chemistry & Biology 2012;19(1):60-71.

77.   Carthew RW., Sontheimer EJ. "Origins and mechanisms of miRNAs and siRNAs". Cell 2009;136(4):642-655.

78.   Hannon G. "RNA interference". Nature 2002;418(6894):244-251.

79.   Oh YK., Park TG. "siRNA delivery systems for cancer treatment". Advanced Drug Delivery Reviews 2009;61(10):850-862.

80.   Kurreck J. "Antisense technologies". European Journal of Biochemistry 2003;270(8):1628-1644.

81.   Zamore PD., Haley B. "Ribo-gnome: the big world of small RNAs". Science 2005;309(5740):1519-1524.

82.   Cho YN., Brumbach JS., Ramalingam M., Haidar ZS. "RNAi therapeutics: current status of nanoncologic siRNA delivery systems". Journal of Bionanoscience 2011;5(1):1-17.

83.   Guo X., Huang L. "Recent advances in nonviral vectors for gene delivery". Accounts of Chemical Research 2011 2012/12/06;45(7):971-979.

84.   Vader P., Aa L., Engbersen JJ., Storm G., Schiffelers R. "Physicochemical and biological evaluation of siRNA polyplexes based on PEGylated Poly(amido amine)s". Pharmaceutical Research 2012;29(2):352-361.

85.   Luten J., van Nostrum CF., De Smedt SC., Hennink WE. "Biodegradable polymers as non-viral carriers for plasmid DNA delivery". Journal of Controlled Release 2008;126(2):97-110.

86.   Hillaireau H., Couvreur P. "Nanocarriers' entry into the cell: relevance to drug delivery". Cellular and Molecular Life Sciences 2009;66(17):2873-2896.

87.   Risau W. "Mechanisms of angiogenesis". Nature 1997;386(6626):671-674.

88.   Karamysheva A. "Mechanisms of angiogenesis". Biochemistry (Moscow) 2008;73(7):751-762.

89.   Chung AS., Ferrara N. "Developmental and pathological angiogenesis". Annual Review of Cell and Developmental Biology 2011;27(1):563-584.

90.   Skalak TC. "Angiogenesis and microvascular remodeling: a brief history and future roadmap". Microcirculation 2005;12(1):47-58.

91.   Cimpean AM., Ribatti D., Raica M. "A brief history of angiogenesis assays". The International Journal of Developmental Biology 2011;55(4-5):377-382.

92.   Sneider EB., Nowicki PT., Messina LM. "Regenerative medicine in the treatment of peripheral arterial disease". Journal of Cellular Biochemistry 2009;108(4):753-761.

93.   Li WW., Talcott KE., Zhai AW., Kruger EA., Li VW. "The role of therapeutic angiogenesis in tissue repair and regeneration". Advances in Skin & Wound Care 2005;18(9):491-500.

94.   Otrock ZK., Mahfouz RAR., Makarem JA., Shamseddine AI. "Understanding the biology of angiogenesis: review of the most important molecular mechanisms". Blood Cells, Molecules, and Diseases 2007;39(2):212-220.

95.	Markkanen JE., Rissanen TT., Kivelä A., Ylä-Herttuala S. "Growth factor-induced therapeutic angiogenesis and arteriogenesis in the heart–gene therapy". Cardiovascular Research 2005;65(3):656-664.

96.	Conway EM., Collen D., Carmeliet P. "Molecular mechanisms of blood vessel growth". Cardiovascular Research 2001;49(3):507-521.

97.	Zamir M. "Shear forces and blood vessel radii in the cardiovascular system". The Journal of General Physiology 1977 April 1, 1977;69(4):449-461.

98.	Tortora GJ., Nielsen MT. "Principles of human anatomy". Hoboken, NJ: J. Wiley, 2009.

99.	Budhiraja R., Tuder RM., Hassoun PM. "Endothelial dysfunction in pulmonary hypertension". Circulation 2004 January 20, 2004;109(2):159-165.

100.	Adams RH. "Molecular control of arterial–venous blood vessel identity". Journal of Anatomy 2003;202(1):105-112.

101.	Shinkaruk S., Bayle M., Lain G., Deleris G. "Vascular endothelial cell growth factor (VEGF), an emerging target for cancer chemotherapy". Current Medicinal Chemistry - Anti-Cancer Agents 2003;3(2):95-117.

102.	Barrientos S., Stojadinovic O., Golinko MS., Brem H., Tomic-Canic M. "Growth factors and cytokines in wound healing". Wound Repair and Regeneration 2008;16(5):585-601.

103.	Carmeliet P., Jain RK. "Molecular mechanisms and clinical applications of angiogenesis". Nature 2011;473(7347):298-307.

104.	Perlingeiro RCR. "Regulation of vasculogenesis and angiogenesis". Coronary Heart Disease. In: Vlodaver Z, Wilson RF, Garry DJ, editors.: Springer US, 2012. p. 261-270.

105.	Lee K., Silva EA., Mooney DJ. "Growth factor delivery-based tissue engineering: general approaches and a review of recent developments". Journal of The Royal Society Interface 2011 February 6, 2011;8(55):153-170.

106.	Li L., Blumenthal DK., Terry CM., He Y., Carlson ML., Cheung AK. "PDGF-induced proliferation in human arterial and venous smooth muscle cells: Molecular basis for differential effects of PDGF isoforms". Journal of Cellular Biochemistry 2011;112(1):289-298.

107.	Fredriksson L., Li H., Eriksson U. "The PDGF family: four gene products form five dimeric isoforms". Cytokine Growth Factor Rev 2004;15(4):197-204.

108.   Pennock S., Kazlauskas A. "Vascular endothelial growth factor a competitively inhibits platelet-derived growth factor (PDGF)-dependent activation of PDGF receptor and subsequent signaling events and cellular responses". Molecular and Cellular Biology 2012;32(10):1955-1966.

109.   Hoch RV., Soriano P. "Roles of PDGF in animal development". Development 2003;130(20):4769-4784.

110.   Risau W., Drexler H., Mironov V., Smits A., Siegbahn A., Funa K. "Platelet-derived growth factor is angiogenic *in vivo*". Growth Factors 1992;7(4):261-266.

111.   Wang D., Huang H-JS., Kazlauskas A., Cavenee WK. "Induction of vascular endothelial growth factor expression in endothelial cells by platelet-derived growth factor through the activation of phosphatidylinositol 3-Kinase". Cancer Research 1999;59(7):1464-1472.

112.   Giacca M., Zacchigna S. "VEGF gene therapy: therapeutic angiogenesis in the clinic and beyond". Gene Therapy 2012;19(6):622-629.

113.   Reardon DA., Turner S., Peters KB., Desjardins A., Gururangan S., Sampson JH. "A review of VEGF/VEGFR-targeted therapeutics for recurrent glioblastoma". Journal of the National Comprehensive Cancer Network 2011;9(4):414-427.

114.   Ferrara N., Gerber HP., LeCouter J. "The biology of VEGF and its receptors". Nature medicine 2003;9(6):669-676.

115.   Donnem T., Al-Saad S., Al-Shibli K., Delghandi MP., Persson M., Nilsen MN. "Inverse prognostic impact of angiogenic marker expression in tumor cells versus stromal cells in non–small cell lung cancer". Clinical Cancer Research 2007;13(22):6649-6657.

116.   Olsson A-K., Dimberg A., Kreuger J., Claesson-Welsh L. "VEGF receptor signalling ? In control of vascular function". Nature Reviews Molecular Cell Biology 2006;7(5):359-371.

117.   Gille H., Kowalski J., Li B., LeCouter J., Moffat B., Zioncheck TF. "Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2)". Journal of Biological Chemistry 2001;276(5):3222-3230.

118.   Itoh N., Ornitz DM. "Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease". Journal of Biochemistry 2011;149(2):121-130.

119.   Fukumoto S. "Physiological regulation and disorders of phosphate metabolism - pivotal role of fibroblast growth factor 23". Internal Medicine 2008;47(5):337-343.

120.   Ornitz DM., Itoh N. "Fibroblast growth factors". Genome Biology 2001;2:1-12.

121.   Turner N., Grose R. "Fibroblast growth factor signalling: from development to cancer". Nature Reviews Cancer 2010;10(2):116-129.

122.   Presta M., Dell'Era P., Mitola S., Moroni E., Ronca R., Rusnati M. "Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis". Growth Factor Reviews 2005;16(2):159-178.

123.   Jih Y-J., Lien W-H., Tsai W-C., Yang G-W., Li C., Wu L-W. "Distinct regulation of genes by bFGF and VEGF-A in endothelial cells". Angiogenesis 2001;4(4):313-321.

124.   Powers CJ., McLeskey SW., Wellstein A. "Fibroblast growth factors, their receptors and signaling". Endocrine-Related Cancer 2000;7(3):165-197.

125.   Sasisekharan R., Venkataraman G. "Heparin and heparan sulfate: biosynthesis, structure and function". Current Opinion in Chemical Biology 2000;4(6):626-631.

126.   Ori A., Free P., Courty J., Wilkinson MC., Fernig DG. "Identification of heparin-binding sites in proteins by selective labeling". Molecular & Cellular Proteomics 2009;8(10):2256-2265.

127.   Capila I., Linhardt RJ. "Heparin–protein interactions". Angewandte Chemie International Edition 2002;41(3):390-412.

128.   Rullo A., Nitz M. "Importance of the spatial display of charged residues in heparin–peptide interactions". Biopolymers 2010;93(3):290-298.

129.   Rolny C., Spillmann D., Lindahl U., Claesson-Welsh L. "Heparin amplifies platelet-derived growth factor (PDGF)- BB-induced PDGF α-receptor but not PDGF β-receptor tyrosine phosphorylation in heparan sulfate-deficient cells". Journal of Biological Chemistry 2002;277(22):19315-19321.

130.   Schultz GS., Wysocki A. "Interactions between extracellular matrix and growth factors in wound healing". Wound Repair and Regeneration 2009;17(2):153-162.

131.   Hockel M., Schlenger K., Doctrow S., Kissel T., Vaupel P. "Therapeutic angiogenesis". Archives of surgery (Chicago, Ill : 1960) 1993;128(4):423-429.

132.   Chu H., Wang Y. "Therapeutic angiogenesis: controlled delivery of angiogenic factors". Therapeutic Delivery 2012;3(6):693-714.

133.   Hodnett PA., Ward EV., Davarpanah AH., Scanlon TG., Collins JD., Glielmi CB. "Peripheral arterial disease in a symptomatic diabetic population: prospective comparison of rapid unenhanced MR angiography (MRA) with contrast-enhanced MRA". American Journal of Roentgenology 2011;197(6):1466-1473.

134.   Ridker Pm SMJRN. "Novel risk factors for systemic atherosclerosis: A comparison of c-reactive protein, fibrinogen, homocysteine, lipoprotein(a), and standard cholesterol screening as predictors of peripheral arterial disease". The Journal of American Medical Association 2001;285(19):2481-2485.

135.   Girolami B., Bernardi E., Prins MH., Ten Cate JW., Prandoni P., Simioni P. "Antiplatelet therapy and other interventions after revascularisation procedures in patients with peripheral arterial disease: a meta-analysis". European Journal of Vascular and Endovascular Surgery 2000;19(4):370-380.

136.   Lane JS., Vittinghoff E., Lane KT., Hiramoto JS., Messina LM. "Risk factors for premature peripheral vascular disease: Results for the National Health and Nutritional Survey, 1999-2002". Journal of Vascular Surgery 2006;44(2):319-325.

137.   Burns P., Gough S., Bradbury AW. "Management of peripheral arterial disease in primary care". British Medical Journal 2003;326(7389):584-588.

138.   Diehm C., Schuster A., Allenberg JR., Darius H., Haberl R., Lange S. "High prevalence of peripheral arterial disease and co-morbidity in 6880 primary care patients: cross-sectional study". Atherosclerosis 2004;172(1):95-105.

139.   Choi D., Hwang K-C., Lee K-Y., Kim Y-H. "Ischemic heart diseases: current treatments and future". Journal of Controlled Release 2009;140(3):194-202.

140.   Ouma GO., Jonas RA., Usman MHU., Mohler ER. "Targets and delivery methods for therapeutic angiogenesis in peripheral artery disease". Vascular Medicine 2012;17(3):174-192.

141.   Khan TA., Sellke FW., Laham RJ. "Gene therapy progress and prospects: therapeutic angiogenesis for limb and myocardial ischemia". Gene Therapy 2003;10(4):285-291.

142.   Cao Y., Hong A., Schulten H., Post MJ. "Update on therapeutic neovascularization". Cardiovascular Research 2005;65(3):639-648.

143.   Nikol S., Baumgartner I., Van Belle E., Diehm C., Visona A., Capogrossi MC. "Therapeutic angiogenesis with intramuscular NV1FGF improves amputation-free survival in patients with critical limb ischemia". Molecular Therapy 2008;16(5):972-978.

144.   Ferraro B., Cruz YL., Baldwin M., Coppola D., Heller R. "Increased perfusion and angiogenesis in a hindlimb ischemia model with plasmid FGF-2 delivered by noninvasive electroporation". Gene Therapy 2010;17(6):763-769.

145.   Asahara T., Murohara T., Sullivan A., Silver M., Van Der Zee R., Li T. "Isolation of putative progenitor endothelial cells for angiogenesis". Science 1997;275(5302):964-966.

146.   Blum A., Balkan W., Hare JM. "Advances in cell-based therapy for peripheral vascular disease". Atherosclerosis 2012;223(2):269-277.

147.   Matoba S., Matsubara H. "Therapeutic angiogenesis for peripheral artery diseases by autologous bone marrow cell transplantation". Current Pharmaceutical Design 2009;15(24):2769-2777.

148.   Tateishi-Yuyama E., Matsubara H., Murohara T., Ikeda U., Shintani S., Masaki H. "Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial". The Lancet 2002;360(9331):427-435.

149.   Amann B., Luedemann C., Ratei R., Schmidt-Lucke JA. "Autologous bone marrow cell transplantation increases leg perfusion and reduces amputations in patients with advanced critical limb ischemia due to peripheral artery disease". Cell Transplantation 2009;18(3):371-380.

150.   Murphy MP., Lawson JH., Rapp BM., Dalsing MC., Klein J., Wilson MG. "Autologous bone marrow mononuclear cell therapy is safe and promotes amputation-free survival in patients with critical limb ischemia.. Journal of Vascular Surgery 2011;53(6):1565-1574.e1561.

151.   Dimmeler S., Losordo D. "Stem Cells Review Series". Circulation Research 2011;109(8):907-909.

152.   Pecot CV., Calin GA., Coleman RL., Lopez-Berestein G., Sood AK. "RNA interference in the clinic: challenges and future directions". Nature Reviews Cancer 2011;11(1):59-67.

153.   Jeon O., Kang S-W., Lim H-W., Hyung Chung J., Kim B-S. "Long-term and zero-order release of basic fibroblast growth factor from heparin-conjugated poly(l-lactide-*co*-glycolide) nanospheres and fibrin gel". Biomaterials 2006;27(8):1598-1607.

154.   Kim J., Cao L., Shvartsman D., Silva EA., Mooney DJ. "Targeted delivery of nanoparticles to ischemic muscle for imaging and therapeutic angiogenesis". Nano Letters 2011;11(2):694-700.

155.   Roy RS., Roy B., Sengupta S. "Emerging technologies for enabling proangiogenic therapy". Nanotechnology 2011;22(49):494004.

156.   Fonder MA., Lazarus GS., Cowan DA., Aronson-Cook B., Kohli AR., Mamelak AJ. "Treating the chronic wound: a practical approach to the care of nonhealing wounds and wound care dressings". Journal of the American Academy of Dermatology 2008;58(2):185-206.

157.   Nwomeh BC., Yager DR., Cohen IK. "Physiology of the chronic wound". Clinics in plastic surgery 1998;25(3):341-356.

158.   Mustoe T. "Understanding chronic wounds: a unifying hypothesis on their pathogenesis and implications for therapy". The American Journal of Surgery 2004;187(5, Supplement 1):S65-S70.

159.   Baker SR., Stacey MC., Jopp-McKay AG., Hoskin SE., Thompson PJ. "Epidemiology of chronic venous ulcers". British Journal of Surgery 1991;78(7):864-867.

160.   James GA., Swogger E., Wolcott R., Pulcini Ed., Secor P., Sestrich J. "Biofilms in chronic wounds". Wound Repair and Regeneration 2008;16(1):37-44.

161.   Hinchliffe RJ., Valk GD., Apelqvist J., Armstrong DG., Bakker K., Game FL. "A systematic review of the effectiveness of interventions to enhance the healing of chronic ulcers of the foot in diabetes". Diabetes/Metabolism Research and Reviews 2008;24(S1):S119-S144.

162.   White-Chu EF., Flock P., Struck B., Aronson L. "Pressure ulcers in long-term care". Clinics in Geriatric Medicine 2011;27(2):241-258.

163.   Harding KG., Morris HL., Patel GK. "Healing chronic wounds". British Medical Journal 2002;324(7330):160-163.

164.   Burnouf T., Su CY., Radosevich M., Goubran H., El-Ekiaby M. "Blood-derived biomaterials: fibrin sealant, platelet gel and platelet fibrin glue". ISBT Science Series 2009;4(1):136-142.

165.   Crovetti G., Martinelli G., Issi M., Barone M., Guizzardi M., Campanati B. "Platelet gel for healing cutaneous chronic wounds". Transfusion and Apheresis Science 2004;30(2):145-151.

166.   McAleer JP., Sharma S., Kaplan EM., Persich G. "Use of Autologous Platelet Concentrate in a Nonhealing Lower Extremity Wound". Advances in Skin & Wound Care 2006;19(7):354-363.

167.   Babensee JE., McIntire LV., Mikos AG. "Growth factor delivery for tissue engineering". Pharmaceutical Research 2000;17(5):497-504.

168.   Gauglitz GG., Jeschke MG. "Combined gene and stem cell therapy for cutaneous wound healing". Molecular Pharmaceutics 2011;8(5):1471-1479.

169.   Bjarnsholt T., Kirketerp-Møller K., Jensen PØ., Madsen KG., Phipps R., Krogfelt K. "Why chronic wounds will not heal: a novel hypothesis". Wound Repair and Regeneration 2008;16(1):2-10.

170.   Eibel B., Rodrigues CG., Giusti II., Nesralla IA., Prates PRL., Sant'Anna RT. "Gene therapy for ischemic heart disease: review of clinical trials". Revista Brasileira de Cirurgia Cardiovascular 2011;26(4):635-646.

171.   Pisters R., Lane DA., Marin F., Camm AJ., Lip GYH. "Stroke and thromboembolism in atrial fibrillation-systematic review of stroke risk factors and risk stratification schema". Circulation Journal 2012;76(10):2289-2304.

172.   Ali M., Atula S., Bath PMW., Grotta J., Hacke W., Lyden P. "Stroke outcome in clinical trial patients deriving from different countries". Stroke 2009;40(1):35-40.

173.   Eisen HJ. "Surgical ventricular reconstruction for heart failure". The New England Journal of Medicine 2009;360(17):1781-1784.

174.   Venugopal JR., Prabhakaran MP., Mukherjee S., Ravichandran R., Dan K., Ramakrishna S. "Biomaterial strategies for alleviation of myocardial infarction". Journal of the Royal Society, Interface / the Royal Society 2012;9(66):1-19.

175.   Yi FWH., Jia GL., Guo WY., Lü AL., Wang HC., Zhang RQ. "Effect of nanoparticle with vascular endothelial growth factor gene transferred into ischemic myocardium: experiment with rabbits". Zhonghua Yi Xue Za Zhi 2006;88(8):510-514.

176.   Dvir T., Timko BP., Kohane DS., Langer R. "Nanotechnological strategies for engineering complex tissues". Nature Nanotechnology 2011;6(1):13-22.

177.   Hoover-Plow J., Gong Y. "Challenges for heart disease stem cell therapy". Vascular Health and Risk Management 2012;2012(8):99-113.

178.   Lavu M., Gundewar S., Lefer DJ. "Gene therapy for ischemic heart disease". Journal of Molecular and Cellular Cardiology 2011;50(5):742-750.

179.   Everts PA., Knape JT., Weibrich G., Schonberger JP., Hoffmann J., Overdevest EP. "Platelet-rich plasma and platelet gel: a review". The Journal of Extra-Corporeal Technology 2006;38(2):174-187.

180.   Würtz M., Hvas AM., Kristensen SD., Grove EL. "Platelet aggregation is dependent on platelet count in patients with coronary artery disease". Thrombosis Research 2012;129(1):56-61.

181.   Malara A., Balduini A. "Blood platelet production and morphology". Thrombosis Research 2012;129(3):241-244.

182.   Li Z., Delaney MK., O'Brien KA., Du X. "Signaling during platelet adhesion and activation". Arteriosclerosis, Thrombosis, and Vascular Biology 2010;30(12):2341-2349.

183.   Jennings LK. "Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis". Thrombosis and Haemostasis 2009;102(2):248-257.

184.   Gobbi G., Vitale M. "Platelet-rich plasma preparations for biological therapy: applications and limits". Operative Techniques in Orthopaedics 2012;22(1):10-15.

185.   Jedlitschky G., Greinacher A., Kroemer HK. "Transporters in human platelets: physiologic function and impact for pharmacotherapy". Blood 2012;119(15):3394-3402.

186.   McVey Ward D., Griffiths GM., Stinchcombe JC., Kaplan J. "Analysis of the lysosomal storage disease Chediak–Higashi syndrome". Traffic 2000;1(11):816-822.

187.   Huizing M., Anikster Y., Gahl WA. "Hermansky–Pudlak syndrome and related disorders of organelle formation". Traffic 2000;1(11):823-835.

188.   Sánchez-González DJ., Trejo-Bahena NI. "Platelet-rich plasma peptides: key for regeneration". International Journal of Peptides 2012;2012:1-10.

189.   Battinelli EM., Markens BA., Italiano JE. "Release of angiogenesis regulatory proteins from platelet alpha granules: modulation of physiologic and pathologic angiogenesis". Blood 2011;118(5):1359-1369.

190.   Blair P., Flaumenhaft R. "Platelet α-granules: Basic biology and clinical correlates". Blood Reviews 2009;23(4):177-189.

191.   Landesberg R., Roy M., Glickman RS. "Quantification of growth factor levels using a simplified method of platelet-rich plasma gel preparation". Journal of Oral and Maxillofacial Surgery 2000;58(3):297-300.

192.   Schallmoser K., Strunk D. "Preparation of pooled human platelet lysate (pHPL) as an efficient supplement for animal serum-free human stem cell cultures". Journal of Visualized Experiments : JoVE 2009(32).

193.   Harrison S., Vavken P., Kevy S., Jacobson M., Zurakowski D., Murray MM. "Platelet activation by collagen provides sustained release of anabolic cytokines". American Journal of Sports Medicine 2011;39(4):729-734.

194.   Dohan DM., Choukroun J., Diss A., Dohan SL., Dohan AJJ., Mouhyi J. "Platelet-rich fibrin (PRF): A second-generation platelet concentrate. Part I: Technological concepts and evolution". Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology 2006;101(3):e37-e44.

195.   Cole BJ., Seroyer ST., Filardo G., Bajaj S., Fortier LA. "Platelet-rich plasma: where are we now and where are we going?". Sports Health: A Multidisciplinary Approach 2010;2(3):203-210.

196.   Smith RG., Grassmann CJ., Campbell M.S. "Platelet-rich plasma: properties and clinical applicationes". The Journal of Lancaster General Hospital 2007;2(2):73-78.

197.   Foster TE., Puskas BL., Mandelbaum BR., Gerhardt MB., Rodeo SA. "Platelet-rich plasma: from basic science to clinical applications". The American Journal of Sports Medicine 2009;37(11):2259-2272.

198.   Sun Y., Feng Y., Zhang CQ., Chen SB., Cheng XG. "The regenerative effect of platelet-rich plasma on healing in large osteochondral defects". International Orthopaedics 2010;34(4):589-597.

199.   Schnabel LV., Mohammed HO., Miller BJ., McDermott WG., Jacobson MS., Santangelo KS. "Platelet Rich Plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons". Journal of Orthopaedic Research 2007;25(2):230-240.

200.   Longo UG., Lamberti A., Maffulli N., Denaro V. "Tissue engineered biological augmentation for tendon healing: a systematic review". British Medical Bulletin 2011;98(1):31-59.

201.   Aspenberg P., Virchenko O. "Platelet concentrate injection improves Achilles tendon repair in rats". Acta Orthopaedica Scandinavica 2004;75(1):93-99.

202.   Rabago D., Best TM., Zgierska AE., Zeisig E., Ryan M., Crane D. "A systematic review of four injection therapies for lateral epicondylosis: prolotherapy, polidocanol, whole blood and platelet-rich plasma". British Journal of Sports Medicine 2009;43(7):471-481.

203.   Lacci KM., Dardik A. "Platelet-rich plasma: support for its use in wound healing". The Yale Journal of Biology and Medicine 2010;83(1):1-9.

204.   Andia I., Abate M. "Platelet-rich plasma injections for tendinopathy and osteoarthritis". International Journal of Clinical Rheumatology 2012;7(4):397-412.

205.   Everts PAM., Hoogbergen MM., Weber TA., Devilee RJJ., Van Monfort G., De Hingh IHJT. "Is the use of autologous platelet-rich plasma gels in gynecologic, cardiac, and general, reconstructive surgery beneficial?". Current Pharmaceutical Biotechnology 2012;13(7):1163-1172.

206.   Cervelli V., Gentile P., Scioli MG., Grimaldi M., Casciani CU., Spagnoli LG. "Application of platelet-rich plasma in plastic surgery: clinical and *in vitro* evaluation". Tissue Engineering - Part C: Methods 2009;15(4):625-634.

207.   Kurokawa I., Mizutani H., Kusumoto K., Nishijima S., Tsujita-Kyutoku M., Shikata N. "Cytokeratin, filaggrin, and p63 expression in reepithelialization during human cutaneous wound healing". Wound Repair and Regeneration 2006;14(1):38-45.

208.   Kazakos K., Lyras DN., Verettas D., Tilkeridis K., Tryfonidis M. "The use of autologous PRP gel as an aid in the management of acute trauma wounds". Injury 2009;40(8):801-805.

209. Fekete N., Gadelorge M., Fürst D., Maurer C., Dausend J., Fleury-Cappellesso S. "Platelet lysate from whole blood-derived pooled platelet concentrates and apheresis-derived platelet concentrates for the isolation and expansion of human bone marrow mesenchymal stromal cells: production process, content and identification of active components". Cytotherapy 2012;14(5):540-554.

210. Ranzato E., Mazzucco L., Patrone M., Burlando B. "Platelet lysate promotes in vitro wound scratch closure of human dermal fibroblasts: Different roles of cell calcium, P38, ERK and PI3K/AKT". Journal of Cellular and Molecular Medicine 2009;13(8 B):2030-2038.

211. Del Fante C., Perotti C., Bonferoni MC., Rossi S., Sandri G., Ferrari F. "Platelet lysate mucohadesive formulation to treat oral mucositis in graft versus host disease patients: A new therapeutic approach". AAPS Journal 2011;12(3):893-899.

212. Plöderl K., Strasser C., Hennerbichler S., Peterbauer-Scherb A., Gabriel C. "Development and validation of a production process of platelet lysate for autologous use". Platelets 2011;22(3):204-209.

213. Johansson L., Klinth J., Holmqvist O., Ohlson S. "Platelet lysate: a replacement for fetal bovine serum in animal cell culture?". Cytotechnology 2003;42(2):67-74.

214. Aldén A., Gonzalez L., Persson A., Christensson K., Holmqvist O., Ohlson S. "Porcine platelet lysate as a supplement for animal cell culture". Cytotechnology 2007;55(1):3-8.

215. Doucet C., Ernou I., Zhang Y., Llense J-R., Begot L., Holy X. "Platelet lysates promote mesenchymal stem cell expansion: A safety substitute for animal serum in cell-based therapy applications". Journal of Cellular Physiology 2005;205(2):228-236.

216. Capelli C., Domenghini M., Borleri G., Bellavita P., Poma R., Carobbio A. "Human platelet lysate allows expansion and clinical grade production of mesenchymal stromal cells from small samples of bone marrow aspirates or marrow filter washouts". Bone Marrow Transplantation 2007;40(8):785-791.

217. Rauch C., Feifel E., Amann EM., Spötl HP., Schennach H., Pfaller W. "Alternatives to the use of fetal bovine serum: human platelet lysates as a serum substitute in cell culture media". Alternatives to Animal Experimentation 2011;28(4):305-316.

218. Gad SC. "Pharmaceutical manufacturing handbook: regulations and quality". John Wiley & Sons, New Jersey 2008.

I. AIM OF THE WORK

The research work carried out in the present PhD thesis is part of a long-standing research activity regarding the design and synthesis of polymeric materials for applications in Pharmaceutical and in Regenerative Medicine. This activity is ongoing in a research group comprising chemists, biologists, pharmaceutical technologists and engineers at the Laboratory of Polymeric Materials for Biomedical and Environmental Applications (BIOLab) of the Department of Chemistry and Industrial Chemistry of the University of Pisa.

Controlled drug delivery technology represents one of the most rapidly advancing areas of science in which several fields, such as chemistry, pharmaceutical technology, and medicine are contributing to human health care. For many years, fundamental and applied investigations have focused on the development of pharmaceutical formulates allowing for maximization of the therapeutic efficacy and minimization of the adverse effects of the drugs of interest.

The research project that constitutes the body of the present thesis involves multidisciplinary skills. The focal point consists in the realization of biocompatible polymeric nanoparticles (NPs) designed for the targeted release of proteins, in particular Growth Factors (GFs). The study has been undertaken within the framework of the research project funded by the Tuscany Region: "Biosurgery – Nanostructured materials based on fibrin and platelet factors able to promote angiogenesis". The six months stage at the Laboratory of Microbiology, School of Life Sciences, Tsinghua University (Beijing, China), was part of the project funded by the European Community, entitled "Hyaluronan-based injectable material for tissue engineering" (Hyanji scaffold).

The main goal of the research project is to produce GFs loaded NPs in order to obtain nanocarriers for the administration of these proteins in the treatment of ischemic diseases. Ischemic diseases remain among the most prominent health problems in the western countries. Defects in blood supply compromise tissues and organs functions causing several pathologic conditions, such as ischemic limbs and chronic ulcers. Therapeutic angiogenesis is a promising strategy for the treatment of these diseases. It is based on the utilization of angiogenic related GFs to enhance the natural healing processes in ischemic tissues. Two recombinant GFs, Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor (bFGF) were selected for the preparations of NPs. Moreover, since it is known that platelets are a great natural source of angiogenic related GFs, the possibility to encapsulate Platelet Lysate (PL), as natural source of GFs was investigated.

In these research activities two polymers, with different characteristics (biodegradable and bioerodible) were employed. Poly (lactic-*co*-glycolic acid) [PLGA] and Poly (methacryloylglycylglycine-OH-*co*-hydroxypropylmethacrylamide) [Poly (Gly-*co*-HPMA)] are polymeric matrices selected to carry out this project, in order to obtain nanosystems that allow respectively a fast (e.g. few days) and a slow (e.g. several weeks)

drug release. Moreover, a proteic model drug, Human Serum Albumin (HSA) was encapsulated with the intention of giving a better understanding of the suitability and versatility of the methods for the encapsulation of proteic drugs. In order to improve the maintenance of bioactivity of the encapsulated GFs, Heparin sodium salt (Hp) was used as stabilizing agent in all of the NPs formulations. NPs morphology, size, surface charge and encapsulation efficiency were determined, as well as the evaluation of drug release profiles. In the perspective of a practical application in the pharmaceutical field, the NPs were submitted to *in vitro* biological evaluation in order to investigate the cytocompatibility and bioactivity of the prepared nanosystems.

The second research activity was carried out at Tsinghua University during a six months stage founded by the European Community. The research activities was aimed at the preparation of NPs by using an innovative biosurfactant, the small bacteria protein Phasin P (PhaP). PhaP is small bacteria amphiphilic protein, located on the surface of polyhydroxyalkanoates (PHAs), with the capability to non-specifically bind hydrophobic polymers. Biomolecules with amphiphilic proprieties, as well as PhaP, are classified as biosurfactants. Most of the synthetic surfactants currently used are petroleum based or chemically synthesized while PhaP can be conveniently produced by recombinant microorganisms and offering the possibility to reduce the costs of production and avoid the use of toxic chemical compounds. Two different polymeric materials, PLGA, and Poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx) were used for the NPs formulation. In order to prepare NPs endowed with targeting moieties, magnetite (Fe_3O_4) was loaded in the nanosystems as a physical targeting agent. This technology allows the possibility to drive NPs to a specific tissue in the body by the applications of simple external magnetic field. The prepared NPs were characterized in term of size, Z-potential and morphology.

II. GLOSSARY

Polymers
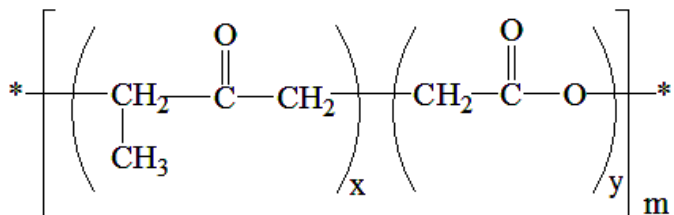
Poly (DL-lactide-co-glycolide)

(PLGA)
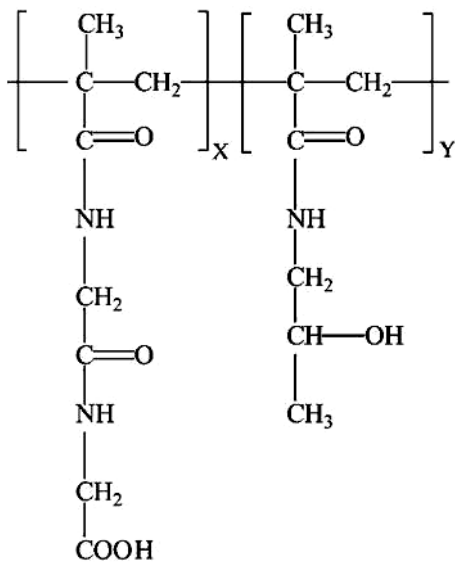
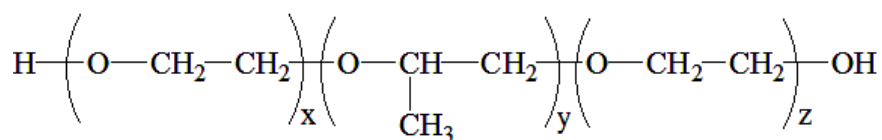*Poly (methacryloylglycylglycine-OHx-co-hydroxypropylmethacrylamide)*
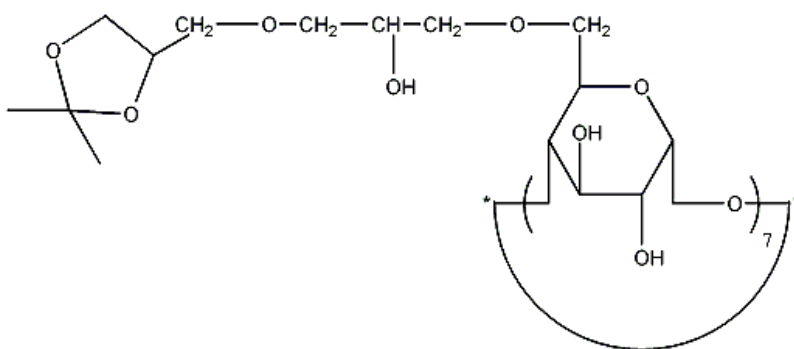
Poly (Gly$_x$-co-HPMA$_y$)
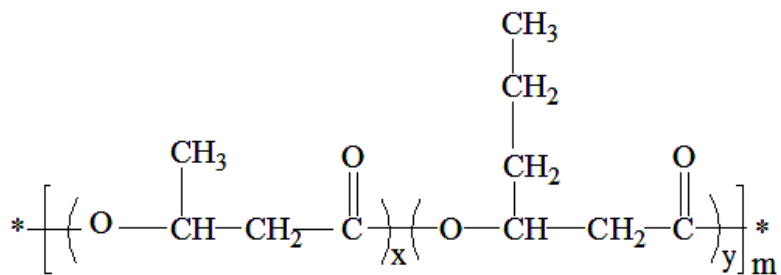
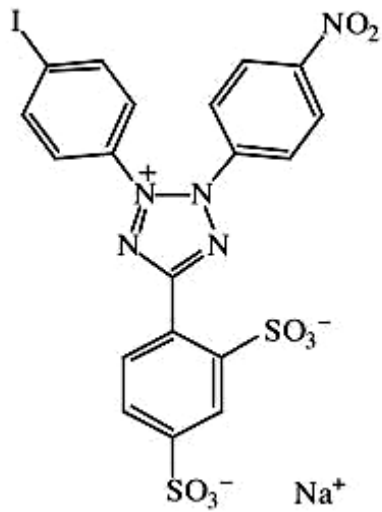*Poly (3-hydroxybutyrate-co-3-hydroxyhexanoate)*

(PHBHHx)

Stabilizers

Pluronic F-127

O–Glycidyl–O–isopropylidenglycerol grafted β–cyclodextrin

(GIG–βCD)

Reagents

*Tetrazolium salt (**WST-1**)*
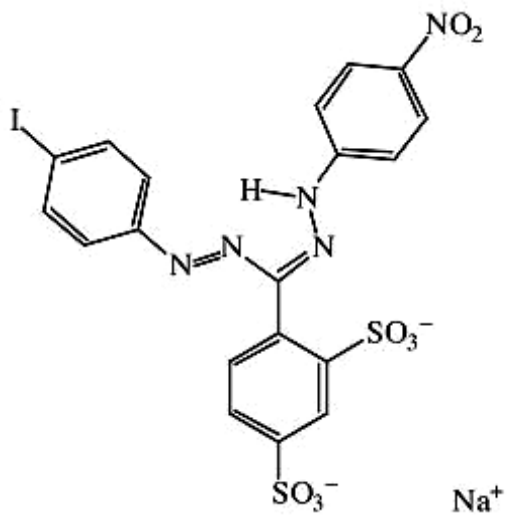
*Formazan (**WST-1**)*

III. LIST OF ABBREVIATIONS

ATCC = American Type Culture Collection

bFGF = Basic Fibroblast Growth Factor

BMP = Bone Morphogenetic Proteins

BSA = Bovine Serum Albumin

CAD = Coronary Artery Disease

CLI = Critical Limb Ischemia

CT = Computed Tomography

DCM = Dichloromethane

DDS = Drug Delivery Systems

DMEM = Dulbecco's Modified Eagles Medium

E.E. = Encapsulation Efficiency

EGF = Epidermal Growth Factor

ELISA = Enzyme-Linked Immuno Sorbent Assay

FBS = Fetal Bovine Serum

FDA = Food and Drug Administration

GFs = Growth Factors

GIG-βCD = *O*–Glycidyl–*O*–isopropylidenglycerol grafted β–cyclodextrin

Hp = Heparin sodium salt

HSA = Human Serum Albumin

HSA-FITC = Human Serum Albumin - Fluorescein Isothiocyanate Conjugate

IGF = Insulin-like Growth Factor

IHD = Ischemic Heart Disease

KGF = Keratinocyte Growth Factor

MRI =Magnetic resonance imaging

MSC = Mesenchymal Stem Cells

N% = Number percent

NPs = Nanoparticles

O/W = Oil in water emulsion

ODNs = Oligodeoxyribonucleotides

PAMAM = Poly (amidoamine)

PBS = Phosphate Buffered Saline

PCL = poly (ε-caprolactone)

PCL-PEG-PCL = poly (ε-caprolactone) – poly (lactic acid) –poly (glycolic acid)

PDGF = Platelet-derived Growth Factor

PDGF-AB = Platelet-derived Growth Factor Isoform AB

PDGFRs = PDGF Receptors

PEI = Polyethylenimine

PET = Positron-emission Tomography

PG = Platelet Gels

PGA = Poly (glycolic acid)

PGA = Poly (glycolic acid)

PhaP = Phasin P

PHAs = Polyhydroxyalkanoates

PHB = Poly-3-hydroxybutyrate

PHBHHx = Poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate)

PHBV = Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate)

PHO = Poly (3-hydroxyoctanoate)

pI = Isoelectric point

PI = Polydispersity Index

PL = Platelet Lysate

PLA = Poly (lactic acid)

PLGA = Poly (Lactic-*co*-Glycolic Acid)

PLL = Poly (L-lysine)

Poly (Gly-*co*-HPMA) = Poly (methacryloylglycylglycine-OHx-*co*-hydroxypropylmethacrylamide)

PPP = Platelet Poor Plasma

PRP = Platelet Rich Plasma

PVD = Peripheral Vascular Disease

RES = Reticulo-endothelial System

RISC = RNA-induced Silencing Complex

RTKs = Tyrosine Kinase Receptors

SD = Standard Deviation

SEM = Scanning Electron Microscopy

TEM = Transmission Electron Microscopy

V% = Volume percent

VEGF = Vascular Endothelial Growth Factor

W/O = Water-in-oil emulsion

W/O/W = Water-in-oil-in-water emulsion

2. <u>Chapter II</u>: PLGA based Nanoparticles Loaded with Growth Factors

2.1. Introduction

Wound healing is a complex and multipart process of restoring cellular structures and tissue after any injury. In human body wound healing process can be divided into 3 distinct phases: the inflammatory phase, the proliferative phase and the remodeling phase [1]. Each phase is strictly regulated by various molecules, and growth factors (GFs) play a key role in this regulation process [2]. In particular, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been identified as the major regulators of vasculogenesis and angiogenesis during development, tissues repair and regeneration [3]. Wound healing occurs naturally in a healthy body, but it is compromised in people with diabetes or cardiovascular diseases. In recent years, the increase of incidence of these diseases, have led to discover the potential use of recombinant proteins as therapeutic agents. However the common administration routes for these new biotechnological drugs have some limitations. Their short half-life results in a low availability of GFs at the site of action that do not satisfy the physiological requirements for tissue repair [4]. A solution to these problems is given by controlled release technologies. Polymeric nanoparticles (NPs) constitute a versatile drug delivery system allowing for the tuning of the release profile of a drug by an appropriate selection of the carrier [5].

Poly (lactic-*co*-glycolic acid) (PLGA) (Figure 1) has attracted immense interest over the last two decades, as a polymer for NPs preparation, due to its favorable properties such as good biocompatibility, biodegradability, low immunogenicity, low toxicity and mechanical strength [6]. Moreover, PLGA is easily formulated into different devices for delivering a variety of drugs such as vaccines, peptides, proteins, and macromolecules. The US Food and Drug Administration (FDA) have approved a very large number of drug delivery products based on this biomaterial [7]. PLGA is a copolymer synthesized from their cyclic dimers, (i.e. dl-lactide and glycolide), by ring-opening polymerization. During polymerization, successive monomeric units (of glycolic or lactic acid) are linked together in PLGA by ester linkages, thus yielding a linear, amorphous aliphatic polyester product [8]. PLGA in the body is hydrolyzed to produce the original monomers, lactic acid and glycolic acid, which are the byproducts of various metabolic pathways under normal physiological conditions. These monomers are easily metabolized via the Krebs cycle and eliminated. Thus, there is very minimal systemic toxicity associated with using PLGA for drug delivery or other biomedical applications [9].
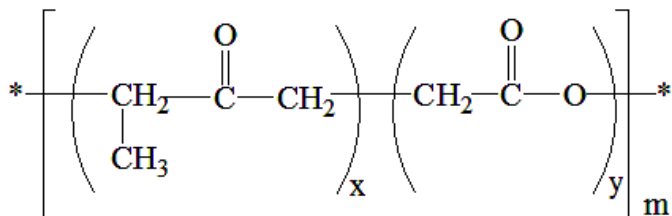
Figure 1: Structure of PLGA

In this study PLGA based NPs loaded with bFGF and VEGF and were prepared by a modified solvent diffusion technique. Heparin (Hp) and human serum albumin (HSA) were employed in NPs formulations as stabilizing agents. Nanoparticles were characterized in term of size, morphology, Z-potential and GFs encapsulation efficiency.

2.2. Material

PLGA [lactide:glycolide (50:50)] was purchased from Purac-Biochem (Amsterdam, NL). Pluronic® F-127 and Hp were obtained from Sigma-Aldrich (Milan, IT). HSA was kindly provided by Kedrion SpA (Castelvecchio Pascoli, Italy). The bFGF, VEGF and related ELISA kits were purchased from R&D Systems (Rovereto, IT). All solvents used were commercially available (Carlo Erba, Milan, Italy).

2.3. Methods

2.3.1. Nanoparticles formulations

Preparation of Unloaded PLGA based NPs

PLGA$_{50:50}$/Pluronic based NPs (in ratios 1:1) were prepared by a modified solvent diffusion technique. Briefly, 1.25% w/v of PLGA and 1.25% w/v of Pluronic F-127 were dissolved in 2 ml of dichloromethane (DCM). This organic solution was emulsified by vortex mixing for 30 seconds with 200 µl of deionized water for the preparation of unloaded NPs. 4 µg of Hp and 200 µg of HSA, added as stabilizing agents, were included in the 200µl of water before the emulsification processes. The resulting water-in-oil emulsion was poured in to 25 ml of ethanol under magnetic stirring, and the resulting suspension was diluted with 25 ml of water. After solvent evaporation, under vacuum at 30˚C, NPs were concentrated in aqueous medium.

Preparation of PLGA based nanoparticles loaded with VEGF and bFGF

PLGA$_{50:50}$/Pluronic based NPs (in ratios 1:1) were prepared by a modified solvent diffusion technique. Briefly, 1.25% w/v of PLGA and 1.25% w/v of Pluronic F-127 were dissolved in 2 ml of DCM. The organic solution was mixed by vortex agitation for 30 s with 200 µl of a water solution containing: 300 ng of bFGF, 300 ng of VEGF, 4 µg of

Hp and 200 μg of HSA as stabilizing agents. The resulting water-in-oil emulsion was poured in to 25 ml of ethanol under magnetic stirring and the resulting suspension was diluted with 25 ml of water. After solvent evaporation under vacuum at 30˚C, NPs were concentrated in aqueous medium.

2.3.2. Nanoparticles characterization

Purification of nanoparticles

The NPs suspensions were purified by using an ALC PK121R refrigerated centrifuge. NPs were centrifuge at 8000g for 1h at 4˚C. The obtained pellet of purified NPs was resuspended in appropriate media depending on their use.

Dimensional analysis

NPs size analyses were carried out by using a Coulter Beckman LS230 Laser Diffraction Particle Size Analyzer, equipped with small volume module plus. Samples were added into the cell until 30–50% obscuration of PIDS detector. Three runs were performed on each sample.

Z-potential analysis

Z-potential analyses were carried out by using Coulter Beckman Nano DelsaTM C at 25˚C after evaluation of pH of NPs suspensions. Z-potential values were calculated as the mean value of 6 replications for each NPs formulation.

Morphological analysis

NPs morphology was evaluated by scanning electron microscopy (SEM) (JEOL LSM5600LV). The samples were prepared from purified and lyophilised NPs. Gold sputtering was performed before SEM analysis.

Freeze-drying

After freezing at –20 °C, nanoparticles were lyophilised in 5 Pascal Lio 5P lyophilisator, equipped with RV8 (Edwards) vacuum pump.

Absorbance measurements on microplates

Microplate absorbance measurements were performed by means of Benchmark Bio–Rad Microplate Reader. All data were processed by using Microplate Manager III (Biorad) and Igor Pro (Wavemetrics).

Determination of GFs encapsulation efficiency

The supernatants of GFs loaded NPs were collected and used for evaluating the amounts of bFGF/VEGF that were not encapsulated into NPs by following the manufacturer instructions of the bFGF and VEGF ELISA kits respectively. The difference between the amount of bFGF/VEGF employed for the NPs preparation and the amount of bFGF/VEGF measured in NPs supernatant represented the bFGF/VEGF loaded into NPs. The encapsulation efficiency was calculated as percentage of bFGF/VEGF loaded in the NPs with respect to total amount of bFGF/VEGF added during the formulation of NPs.

2.4. Results and Discussion

Wound healing is quite a complicated process involving epidermal regeneration, fibroblast proliferation, neovascularization and synthesis [10]. Studies have shown that exogenous application of GFs may decrease the healing period and improve the quality of wound healing [11, 12]. In this study PLGA based NPs loaded with bFGF and VEGF were prepared by a modified solvent diffusion technique in order to obtain a GFs controlled delivery system to enhance wound healing. In order to improve the maintenance of bioactivity of the encapsulated GFs, Hp and HSA were employed in NPs formulations as stabilizing agents. The interaction of Hp with GFs has been shown to potentiate its activity and protect GFs from heat and enzymatic degradations [13], while HSA has a protective effect on GFs, since it reduces their exposure to water/organic solvent interfaces during NPs preparation [14].

Preparation and characterization of PLGA based NPs

Results related to the mean diameter size of unloaded NPs and bFGF/VEGF loaded NPs, their Z-potential values and GFs encapsulation efficiencies are reported in Table 1. NPs showed submicron size and monodispersed diameter distributions (Figure 2). The degree of interaction between biological systems and NPs depends mainly on their size. Indeed, NPs with smaller diameter size present larger contact areas with the target sites. Thus, the size of the developed nanosystems, below 150 nm, is appropriate for their future application, since they can promote a better and faster wound healing.

Table 1	Size ± S.D. (nm)	Z-potential (mV)	Encapsulation efficiency (%)	
			bFGF	VEGF
Unloaded PLGA NPs	116 ± 16	-21.9 ± 0.52	-	-
bFGF/VEGF PLGA NPs	130 ± 18	-4.6 ± 0.06	75 %	97%

Table 1: Size analysis, encapsulation efficiency and Z-potential of PLGA based NPs.
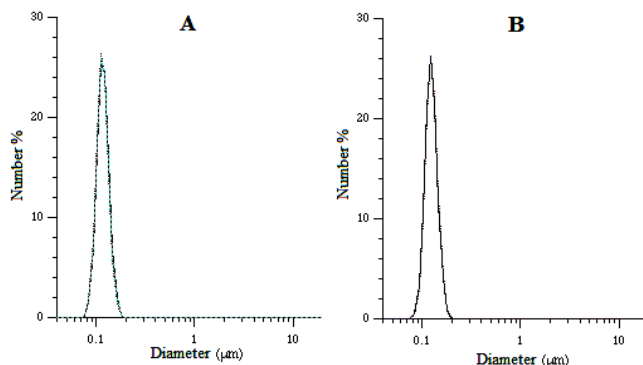
Figure 2: Diameter distribution of PLGA based NPs. Unloaded NPs (A) and bFGF/VEGF loaded NPs (B).

NPs were characterized by negative Z-potential values. The measured Z-potential value of -21.9 ± 0.52 mV of the unloaded NPs, was in the range expected for colloidal systems based on polyesters loaded with Hp, Indeed, both the carboxylic moieties presents at the extremity of the polymer chains and the sulfated groups distributed along the backbone of the glycosaminoglycan chains, conferred negativity to the particles surface. The addition of GFs to the formulation significantly decreased its Z-potential value, from -21.9 to -4.6 mV. The observed different values of Z-potential between unloaded and loaded NPs can be related to the presence of GFs bound to the particles surfaces that masked the anionic nature of Hp. This result confirmed that Hp effectively interacts with the GFs and suggested that the formation of Hp-GFs complexes is one of the factors that promoted the encapsulation of GFs. Z-potential data are commonly used to predict and control dispersion stability of NPs in aqueous dispersion state. High Z-potential values (positive or negative) lead to stable NPs suspensions, because the repulsion between the particles prevent their aggregation [15]. Thus we expect that these systems will be stable during storage.

bFGF and VEGF showed different encapsulation efficiency probably due to the disparity in electrostatic charge of both molecules at NPs formulation pH (~5.5). VEGF is less positively charged than bFGF (pI = 7.3 for VEGF and pI = 9.6 for bFGF), and therefore, Hp could interact more with bFGF and enhance its encapsulation into NPs.

The observed GFs encapsulation efficiency values were high, 75% for VEGF and 95% bFGF, making the NPs preparation methods adopted in this work suitable the preparation of nanosystems loaded with peptide or protein drugs. The obtained high encapsulation efficiencies could be attributed to the utilization of HSA in the formulation that was used as stabilizing agent. Likely, thanks to its amphiphilic proprieties, HSA shielded the GFs during NPs preparation, reducing their exposure to the water/oil interface [16].

The morphology of NPs was assessed by SEM. Figure 3 shows NPs with spherical shape and characterized by a well-dispersed distribution (Figure 3).
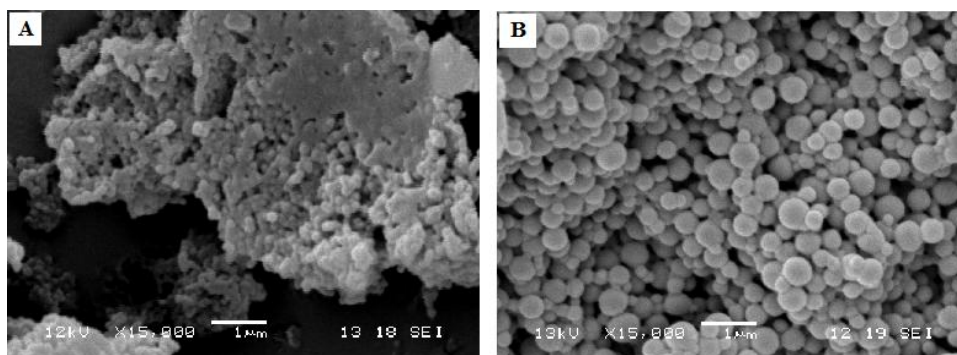

Figure 3: Morphological analyses (SEM micrograph) of PLGA based NPs. Unloaded NPs (A) bFGF/VEGF loaded NPs (B).

2.5. Conclusions

This study was aimed at the preparation and characterization of PLGA based NPs as carriers for the administration of GFs, such as bFGF and VEGF. NPs were successfully prepared by a modified solvent diffusion technique.

Hp and HSA were used as stabilizing agents for the NPs preparation, in order to obtain high GFs encapsulation efficiency and the stabilization of the resulting dispersions. In fact, Hp can stabilize GFs through the formation of Hp-GFs complexes that protect GFs, and help them to preserve their biological activity. HSA was used to avoid the interaction of bFGF/VEGF with the organic solvent during the NPs preparation.

The prepared nanosystems were characterized by monomodal distribution of the diameters, submicron size (below to 150 nm), negative Z-potential values and good spherical morphology. No appreciable differences were observed between the unloaded and GFs loaded NPs. As exception, the Z-potential value of the GFs loaded NPs showed a significant decrease as confirm of the interaction between Hp and GFs.

The high encapsulation efficiency of bFGF and VEGF into NPs makes these nanosystems very promising as GFs delivery devices, and in more broad terms for protein delivery. The successful encapsulation of both bFGF and VEGF might result in a synergic proangiogenic activity, important for the future application of these NPs system in the field of therapeutic angiogenesis, such as for the enhancement of wound healing.

2.6. References

1.   Arwert EN., Hoste E., Watt FM. "Epithelial stem cells, wound healing and cancer". Nature Reviews Cancer 2012;12(3):170-180.

2.   Sneider EB., Nowicki PT., Messina LM. "Regenerative medicine in the treatment of peripheral arterial disease". Journal of Cellular Biochemistry 2009;108(4):753-761.

3.   Bao P., Kodra A., Tomic-Canic M., Golinko MS., Ehrlich HP., Brem H. "The role of vascular endothelial growth factor in wound healing". Journal of Surgical Research 2009;153(2):347-358.

4.   Mahapatro A., Singh D. "Biodegradable nanoparticles are excellent vehicle for site directed in-vivo delivery of drugs and vaccines". Journal of Nanobiotechnology 2011;9(1):1-11.

5.   Mishra B., Patel BB., Tiwari S. "Colloidal nanocarriers: a review on formulation technology, types and applications toward targeted drug delivery". Nanomedicine 2010 Feb;6(1):9-24.

6.   Anderson JM., Shive MS. "Biodegradation and biocompatibility of PLA and PLGA microspheres". Advanced Drug Delivery Reviews 1997;28(1):5-24.

7.   Chien-Hua Niu, Yuan-Yuan Chiu. "FDA perspective on peptide formulation and stability issues". Journal of Pharmaceutical Sciences 1998;87(11):1331-1334.

8.   Kiremitçi-Gümüşderelioğlu M., Deniz G. "Synthesis, characterization and *in vitro* degradation of poly(DL-lactide)/poly(DL-lactide-*co*-glycolide) film". Turkish Journal of Chemistry 1999;23:153-161.

9.   Kitchell JP., Wise DL., Kenneth J., Widder RG. "Poly(lactic/glycolic acid) biodegradable drug-polymer matrix systems". Methods in Enzymology: Academic Press, 1985; 436-448.

10.   Harding KG., Morris HL., Patel GK. "Healing chronic wounds". British Medical Journal 2002;324(7330):160-163.

11.   Zhou W., Zhao M., Zhao Y., Mou Y. "A fibrin gel loaded with chitosan nanoparticles for local delivery of rhEGF: preparation and in vitro release studies". Journal of Materials Science: Materials in Medicine 2011;22(5):1221-1230.

12.   Chu Y., Yu D., Wang P., Xu J., Li D., Ding M. "Nanotechnology promotes the full-thickness diabetic wound healing effect of recombinant human epidermal growth factor in diabetic rats". Wound Repair and Regeneration 2010;18(5):499-505.

13.   Ashikari-Hada S., Habuchi H., Kariya Y., Kimata K. "Heparin regulates vascular endothelial growth factor165-dependent mitogenic activity, tube formation, and its receptor phosphorylation of human endothelial cells". Journal of Biological Chemistry 2005; 280(36):31508-31515.

14.   Van de Weert M., Hennink WE., Jiskoot W. "Protein instability in poly(Lactic-*co*-glycolic acid) microparticles". Pharmaceutical Research 2000;17(10):1159-1167.

15.   Schwegmann H., Feitz AJ., Frimmel FH. "Influence of the zeta potential on the sorption and toxicity of iron oxide nanoparticles on *S. cerevisiae* and *E. coli*". Journal of Colloid and Interface Science 2010;347(1):43-48.

16.   Borselli C., Ungaro F., Oliviero O., D'Angelo I., Quaglia F., La Rotonda M. "Bioactivation of collagen matrices through sustained VEGF release from PLGA microspheres". Journal of Biomedical Materials Research Part A 2009;92A(1):94-102.

3. <u>Chapter III: PLGA based Nanoparticles Loaded with Platelet Lysate</u>

3.1. Introduction

Significant clinical problems occur when blood supply is severally compromised, and tissues or organs oxygen deprivation (ischemia) can cause malfunctions and even the death of the patients. Angiogenesis is an important physiological process required for the growth of new blood vessels, and represents a fundamental route for wounds healing and for restoring blood flow to tissues after injury [1]. The regulation of the angiogenic process is complex and involves a mixture of molecules and cellular events, in which Growth Factors (GFs) play a fundamental role for the activation, proliferation and migration of the endothelial cells and subsequent generation of new blood vessel [2]. Platelet derivatives have been extensively used over the past twenty years to induce a functional angiogenesis *in vitro* [3, 4]. Platelet lysate (PL), which is an haemoderivative rich in GFs deriving from the lysis of platelets, has been proposed in clinical practice for the treatment of several ischemic conditions [5]. PL is obtained from Platelet Rich Plasma (PRP) after its freeze-thawing destruction. One of the advantages of using PL is that provides a mixture of autologous GFs in their biological concentration to assist the body in healing, and promotes vascularization with greater similarly to the natural processes [6]. Platelet-derived growth factor (PDGF) is the major constituent of PL. PDGF is a potent mitogen for cells of mesenchymal origin, including smooth muscle cells and glial cells and it plays a significant role in blood vessel formation [7]. PDGF family includes 5 isoforms: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD, all expressed in platelets except PDGF-DD [8].

Protein drugs, such as GFs, can be easily chemically and enzymatically degraded, and may also lose their biological activity through conformational changes and aggregation. In order to maintain the biological activity of such biotechnological drugs they must be frequently administered. Nowadays, controlled delivery systems represent the best approach to protein drug administration [9]. In particular, protein drugs can be encapsulated within colloidal polymeric carriers, such as nanoparticles (NPs), which can protect them against degradation and ensure their transport and delivery to the specific site of action at a controlled rate [10].

Poly (lactic-*co*-glycolic acid) (PLGA) has attracted immense interest over the last two decades for NPs preparation due to its favorable properties such as good biocompatibility, biodegradability, low immunogenicity, low toxicity and mechanical strength [11, 12]. The US Food and Drug Administration (FDA) approved a very large number of drug delivery products based on PLGA [13].

Fibrin gel, prepared from fibrinogen and thrombin, the key proteins involved in blood clotting, is one of the first biomaterials used to prevent bleeding and promote wound healing [14]. Due to its excellent features, such as elasticity and capability to easy self-

assemble under physiological conditions, fibrin gel is a potent tool for clinical applications [15]. However, one of the disadvantages of using fibrin gel as a drug delivery system is that most of entrapped drug is released in less than several hours [16]. Recently, to overcome this problem NPs were incorporated into fibrin gels to obtain combined controlled drug delivery devices that allow a better control over the drug release profile [17-19].

In this study we report the preparation of PLGA based NPs loaded with Platelet Lysate (PL) as source of angiogenic related GFs by a modified solvent diffusion technique. Different concentrations of PLGA and surfactant were used to determine the best conditions for the preparation of PL loaded NPs. The prepared NPs were incorporated into a fibrin matrix with the intention of producing a controlled system able to delivery PL directly to the wound site as skin dressing, for treatment of chronic wound.

In order to ensure the maintenance of bioactivity of the encapsulated PL, heparin (Hp) was used as stabilizing agent. Several studies demonstrated that GFs present in their architecture an heparin-binding domain [20, 21] and the interaction of Hp with GFs has shown to potentiate its activity and to protect GFs from heat and enzymatic degradation [22, 23].

The prepared NPs were submitted to physical and chemical characterizations. The mean particles size, stability of NPs and Z-potential values were evaluated by dynamic light scattering and electrophoretic light scattering. Morphological analyses were performed by scanning electron microscope (SEM). Colorimetric detection method was used to investigate the total protein loading into NPs and encapsulation efficiency. ELISA assay was used to elucidate the specific growth factor's encapsulation efficiency. The study was carried out using PDGF as reference growth factor in PL. Release kinetic profile was investigated by using a model protein drug, fluorescein isothiocyanate conjugate human serum albumin (HSA-FITC) under physiological conditions.

3.2. Material

PLGA$_{50:50}$ [lactide:glycolide (50:50); inherent viscosity CHCl$_3$ 0.5g/dl: 0.4 dl/g] was obtained from Purac-Biochem (Amsterdam, NL). HSA, PRP and fibrin gel kits were kindly provided by Kedrion SpA (Castelvecchio Pascoli, Italy). Pluronic® F-127, HSA-FITC and Hp were purchased from Sigma-Aldrich (Milan, Italy). The Micro BCA Protein Assay kit was obtained from Pierce (Rockford, IL). Human PDGF-AB ELISA assay kit was purchased from Abnova (Taipei, Taiwan). Phosphate Buffer Saline solution (PBS 10X) was prepared by dissolving 2.0 g of KCl, 2.0 g of KH$_2$PO$_4$•H2O, 80 g of NaCl, and 15.6 g of Na$_2$HPO$_4$•12H$_2$O in 1 liter of distilled water. The pH was adjusted to 7.4 with 10 N NaOH and the resulting solution was sterilized in autoclave (121°C for 20 min) before use and storage. Physiological solution was prepared by dissolving 9.0 g of NaCl in 1 litre of distilled water. The solution was sterilized in autoclave (121°C for 20 min) before use and storage. All solvents used were commercially available (Carlo Erba, Milan, Italy).

3.3. Methods

3.3.1. Preparation of Platelet Lysate

Platelet Lysate (PL) was prepared as reported by following: platelets presents in PRP were isolated by centrifugation at 10000g for 15 minutes, at 4°C, resuspended in aqueous medium and submitted to a freeze-thaw cycle (in order to obtain platelets lyses and subsequent growth factors release) and finally centrifuged at 10000g for 15 minute, 4°C. The GFs water rich supernatant was used for NPs preparation.

3.3.2. Preparation of nanoparticles

Preparation of Unloaded PLGA based nanoparticles

$PLGA_{50:50}$/Pluronic based NPs (in ratios 1:1) were prepared by a modified solvent diffusion technique. Briefly, 2.5% w/v or 1.25% w/v of PLGA and 2.5% w/v or 1.25% w/v of Pluronic F-127 were dissolved in 2 ml of dichloromethane (CH_2Cl_2). This organic solution was emulsified by vortex mixing for 30 seconds with 200 µl of deionized water for the preparation of unloaded NPs. The resulting water-in-oil emulsion was poured in to 25 ml of ethanol under magnetic stirring, and the resulting suspension was diluted with 25 ml of water. After solvent evaporation, under vacuum at 30°C, NPs were concentrated in aqueous medium.

Preparation of PLGA based nanoparticles loaded with Human Serum Albumin

$PLGA_{50:50}$/Pluronic based NPs (in ratios 1:1) were prepared by a modified solvent diffusion technique. Briefly, 2.5% w/v or 1.25% w/v of PLGA and 2.5% w/v or 1.25% w/v of Pluronic F-127 were dissolved in 2 ml of dichloromethane (CH_2Cl_2). This organic solution was emulsified by vortex mixing for 30 seconds with 200 µl of HAS (0.5%) for the preparation of HSA loaded NPs. The resulting water-in-oil emulsion was poured in to 25 ml of ethanol under magnetic stirring, and the resulting suspension was diluted with 25 ml of water. After solvent evaporation, under vacuum at 30°C, NPs were concentrated in aqueous medium.

Preparation of PLGA based nanoparticles loaded with Platelet Lysate

$PLGA_{50:50}$/Pluronic based NPs (in ratios 1:1) were prepared by a modified solvent diffusion technique. Briefly, 2.5% w/v or 1.25% w/v of PLGA and 2.5% w/v or 1.25% w/v of Pluronic F-127 were dissolved in 2 ml of dichloromethane (CH_2Cl_2). This organic solution was emulsified by vortex mixing for 30 seconds with 200 µl of PL for the preparation of PL loaded NPs. The resulting water-in-oil emulsion was poured in to 25 ml of ethanol under magnetic stirring, and the resulting suspension was diluted with 25

ml of water. After solvent evaporation, under vacuum at 30˚C, NPs were concentrated in aqueous medium.

<u>Preparation of Unloaded PLGA based nanoparticles in presence of Heparin</u>

$PLGA_{50:50}$/Pluronic based NPs (in ratios 1:1) were prepared by a modified solvent diffusion technique. Briefly, 1.25% w/v of PLGA and 1.25% w/v of Pluronic F-127 were dissolved in 2 ml of dichloromethane (CH_2Cl_2). This organic solution was emulsified by vortex mixing for 30 seconds with 200 μl of deionized water for the preparation of unloaded NPs. 2 μg of Hp, added as stabilizing agent, was included in the 200μl of water before the emulsification processes. The resulting water-in-oil emulsion was poured in to 25 ml of ethanol under magnetic stirring, and the resulting suspension was diluted with 25 ml of water. After solvent evaporation, under vacuum at 30˚C, NPs were concentrated in aqueous medium.

<u>Preparation of PLGA based nanoparticles loaded with Platelet Lysate in presence of Heparin</u>

$PLGA_{50:50}$/Pluronic based NPs (in ratios 1:1) were prepared by a modified solvent diffusion technique. Briefly, 1.25% w/v of PLGA and 1.25% w/v of Pluronic F-127 were dissolved in 2 ml of dichloromethane (CH_2Cl_2). This organic solution was emulsified by vortex mixing for 30 seconds with 200 μl of PL for the preparation of PL loaded NPs. 2 μg of Hp, added as stabilizing agent, was included in the 200μl of PL solution, before the emulsification processes. The resulting water-in-oil emulsion was poured in to 25 ml of ethanol under magnetic stirring, and the resulting suspension was diluted with 25 ml of water. After solvent evaporation, under vacuum at 30˚C, NPs were concentrated in aqueous medium.

<u>Preparation of PLGA based nanoparticles loaded with Fluorescein Isothiocyanate Conjugate Human Serum Albumin</u>

$PLGA_{50:50}$/Pluronic based NPs (in ratios 1:1) were prepared by a modified solvent diffusion technique. Briefly, 1.25% w/v of PLGA and 1.25% w/v of Pluronic F-127 were dissolved in 2 ml of dichloromethane (CH_2Cl_2). This organic solution was mixed by vortex agitation for 30 seconds with 200 μl of HSA-FITC (1 mg) for the fluorescent marker loaded NPs. The resulting water-in-oil emulsion was poured in to 25 ml of ethanol under magnetic stirring, and the resulting suspension was diluted with 25 ml of water. After solvent evaporation, under vacuum at 30˚C, NPs were concentrated in aqueous medium.

3.3.3. Nanoparticles characterization

Purification of nanoparticles

The NPs suspensions were purified by using an ALC PK121R refrigerated centrifuge. NPs were centrifuge at 8000g for 1h at 4˚C. The obtained pellet of purified NPs was resuspended in appropriate media depending on their use.

Dimensional analysis

NPs size analyses were carried out by using a Coulter Beckman LS230 Laser Diffraction Particle Size Analyzer, equipped with small volume module plus. Samples were added into the cell until 30–50% obscuration of PIDS detector. Three runs were performed on each sample.

Z-potential analysis

Z-potential analyses were carried out by using Coulter Beckman Nano DelsaTM C at 25˚C after evaluation the of the NPs suspensions pH. Z-potential values were calculated as the mean value of 6 replications for each NPs formulation.

Morphological analysis
The NPs morphology was evaluated by scanning electron microscopy (SEM) (JEOL LSM5600LV). The samples were prepared from purified and lyophilised NPs. Gold sputtering was performed before SEM analysis.

Freeze-drying

After freezing at –20 °C, nanoparticles were lyophilised in 5 Pascal Lio 5P lyophilisator, equipped with RV8 (Edwards) vacuum pump.

Absorbance measurements on microplates

Microplate absorbance measurements were performed by means of Benchmark Bio–Rad Microplate Reader. All data were processed by using Microplate Manager III (Biorad) and Igor Pro (Wavemetrics).

Determination of protein encapsulation efficiency and loading

Proteins encapsulation efficiency was calculated from the proteins amount that was detected in the supernatant recollected after centrifugation of the NPs samples. Standard micro BCA protein assay was used to determine the amount of free proteins (HSA) in the NPs supernatant and the amount of proteins contained in the PL employed for the NPs preparation. The difference between the amount of protein employed for the NPs preparation, and the amount of proteins measured in NPs supernatant represented the

proteins loaded into NPs. This value was necessary for the calculation of the encapsulation efficiency and drug loading. The encapsulation efficiency was calculated as percentage of proteins loaded in the NPs with respect to total amount of proteins added during the formulation of NPs (Equation 1). Drug loading was calculated as percentage of proteins loaded in NPs with respect to the dry weight of recovered NPs (Equation 2). A calibrations curve of Bovine Serum Albumin (BSA) was prepared by following the manufacturer instructions. All samples were measured at 565nm. Mean values were obtained from three different batches.

Equation 1:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Mass of drug in NPs}}{\text{Mass of drug used in NPs formulation}} \cdot 100$$

Equation 2:

$$\text{Loading (\%)} = \frac{\text{Mass of drug in NPs}}{\text{Mass of NPs recovered}} \cdot 100$$

In order to determine the percentage of released protein, we evaluated the HSA-FITC loading % of the $2.6 \pm 0.5\%$ in the PLGA based NPs.

Encapsulation efficiency of Platelet Derived Growth Factor Isoform AB

The amount of PDGF-AB in the NPs was determined both in PL and hydrolyzed NPs samples. Briefly, 1mg of the isolated NPs was hydrolyzed in 1ml of NaOH (0.05N). This hydrolytic process was maintained under stirring for 1 hour at room temperature. The obtained samples were opportunely diluted and analyzed by ELISA assay kit. Mean values were obtained from three different batches.

Stability of nanoparticles

Stability of NPs in water was investigated by dynamic light scattering using Coulter Beckman LS230 Laser Diffraction Particle Size Analyzer. Nanoparticles suspensions were stored under static conditions at 4°C for 4 weeks. The average size was analyzed at specified time intervals, within one month, in loaded and unloaded NPs prepared in presence or absence of Hp.

In vitro HSA-FITC release kinetics from PLGA nanosystems

In order to determine the percentage of released protein, we first evaluated the HSA-FITC loading into NPs by an indirect method. The unloaded drug was quantified by measuring the free drug found in the supernatant of the prepared drug-loaded NPs spectrofluorimetrically with excitation and emission wavelengths of 485 and 535 nm,

respectively. Using the amount of unloaded drug, the drug-loaded quantity was determined (total drug added - free drug). HSA-FITC loading was estimated in 2.6 ± 0.5% into the PLGA based NPs.

The HSA-FITC loaded NPs were resuspended in 10ml of Phosphate Buffer Saline (PBS, 0.01M, pH 7.4), and incubated at 37˚C under magnetic stirring. At appropriated time points, samples were centrifuged at 8000g for 15 minutes at room temperature and the supernatant were collected for the determination of HSA-FITC release. NPs pellets were then resuspended with 10 ml of fresh PBS. The released HSA-FITC concentration in the PBS medium was assessed spectrofluorimetrically.

Incorporation of nanoparticles into fibrin matrix

The fibrin matrix kit consisted of thrombin and fibrinogen in separate vials, plus separate solutions of calcium chloride ($CaCl_2$) and potassium dihydrogen phosphate (KDP). The PL loaded NPs incorporated fibrin gel was prepared as follows. Thrombin was reconstituted in a $CaCl_2$ solution and then drawn into a syringe. Fibrinogen, containing Factor XIII, was reconstituted in the KDP solution in order to obtain 30 IU/ml of fibrinogen concentration and then 10 mg of PL loaded NPs were added to this suspension. The PL-NPs-fibrinogen suspension was kept under magnetic stirring until the complete dissolution of NPs. The NPs- fibrinogen suspensions were placed respectively into 24-well plate and when the thrombin solution was added, the gelification phenomenon occurred immediately due to the conversion of fibrinogen to fibrin, and the plate was incubated at 37 ˚C for 1 h to complete polymerization. The fibrin matrices were prepared with two different concentrations of thrombin, 625 UI/ml and 100 UI/ml respectively, in order to study the effect of thrombin concentrations on the fibrin matrix formation.

3.4. Results and Discussion

In this work, we prepared platelet's GFs loaded PLGA based nanoparticles by a modified solvent diffusion technique. This technique is very suitable for the encapsulation of proteins, because avoid the use of high energy procedures that could cause structural damages of the proteins [24]. PL obtained from several purification steps of PRP was used as source of GFs.

NPs formulations studies, comprising the variation of the polymer concentrations for the NPs preparation, were performed in order to define the best formulation conditions. HSA was used as model drug with the intention of obtaining information on the suitability and versatility of the employed method for the encapsulation of proteic drugs.

Characterization of PLGA based NPs

NPs were prepared by using 2.5% w/v and 1.25% w/v of PLGA as matrix. All prepared nanosystems were characterized by a narrow monomodal distribution of diameters

(Figure 1). Table 1 shows the mean diameters size, encapsulation efficiency and PL loading of the PLGA based NPs. No appreciable size differences were evidenced between unloaded, HSA and PL loaded NPs.

The morphological evaluations were carried out by SEM and the obtained results are shown in Figure 2. All the prepared nanosystems showed similar physical characteristics and the presence of HSA and PL did not affect the diameter distributions and size of NPs that resulted below 200 nm. All the prepared NPs were characterized by good spherical shape (Figure 2).
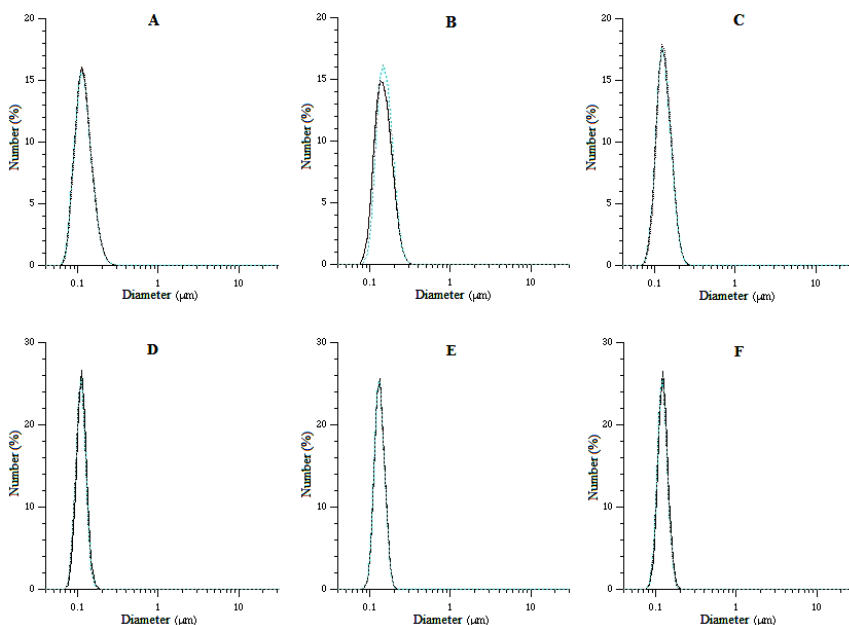
Figure 1: Diameter distribution of PLGA based NPs. Unloaded NPs (A), HSA loaded NPs (B) and PL loaded NPs (C) prepared with 2.5% w/v of PLGA. Unloaded NPs (D), HSA loaded NPs (E) and PL loaded NPs (F) prepared with 1.25 % w/v of PLGA.

Table 1		Size ± S.D. (nm)	Encapsulation efficiency (%)	Loading (%)
2.5% w/v PLGA based NPs	Unloaded NPs	143 ± 26	-	-
	HSA loaded NPs	140 ± 29	53 ± 2%	2.25 ± 0.2%
	PL loaded NPs	167 ± 41	85 ± 4%	1.73 ± 0.3%
1.25% w/v PLGA based NPs	Unloaded NPs	118 ± 16	-	-
	HSA loaded NPs	139 ± 20	79 ± 5%	2.10 ± 0.4%
	PL loaded NPs	130 ± 18	89 ± 6%	2.21 ± 0.3%

Table 1: Dimensional analysis, encapsulation efficiency and loading of PLGA based NPs.
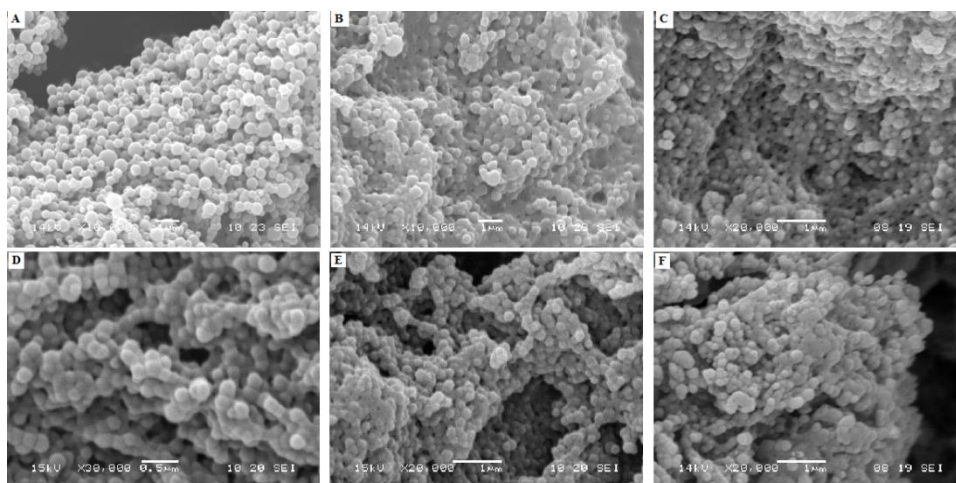
Figure 2: Morphological analyses (SEM micrograph) of PLGA based NPs. Unloaded NPs (A) HSA loaded NPs (B) and PL loaded NP (C) prepared with 2.5% w/v of PLGA. Unloaded NPs (D), HSA loaded NPs (E) and PL loaded NPs (F) prepared with 1.25 % w/v of PLGA.

Based on these results, the NPs formulated with 1.25% w/v of PLGA were chosen for further studies since they were characterized by good proprieties and allowed to reduce the amount of polymeric material employed, thus reducing production costs. Moreover, lower amounts of polymers means reducing the potential toxicity risks of prepared NPs. Hp was used as stabilizing agent for the preparation of the following set of NPs formulations, in order to improve the maintenance of bioactivity of the encapsulated PL. The unloaded and PL loaded PLGA based NPs, prepared in presence of Hp were characterized by a monomodal distribution of diameters (Figure 3) and spherical morphology (Figure 4). No considerable difference with the NPs prepared with the

addition of Hp was evidenced. Table 1 shows the mean diameters size, Z-potential, encapsulation efficiency and PL loading of the PLGA based NPs prepared in presence of Hp.
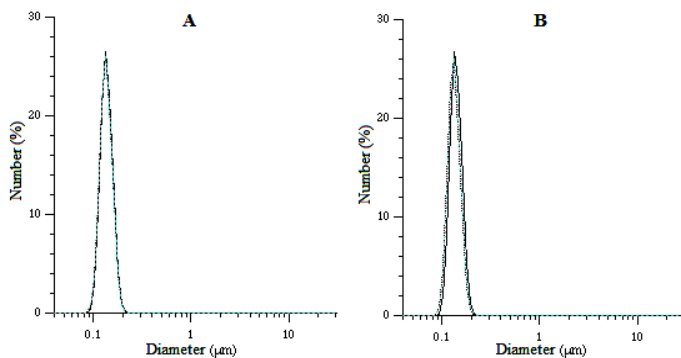
Figure 3: Diameter distribution of PLGA based NPs prepared with 1.25% w/v of polymer and in presence of Heparin. Unloaded NPs (A) and PL loaded NPs (B).
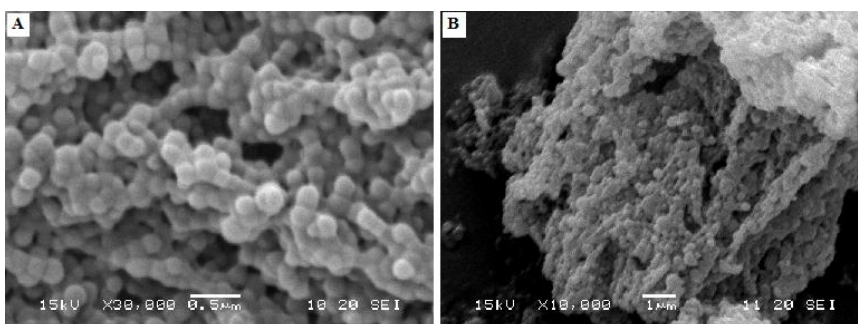
Figure 4: Morphological analyses (SEM micrograph) of PLGA based NPs prepared with 1.25% w/v of polymer and in presence of Heparin. Unloaded NPs (A) and PL loaded NP (B).

Table 2		Size ± S.D. (nm)	Encapsulation efficiency (%)	Loading (%)	Z-potential ± S.D. (mV)
1.25% w/v PLGA based NPs prepared in absence of Heparin	Unloaded NPs		See table 1		-20,6 ± 1.26
	PL loaded NPs				-9.7 ± 0.36
1.25% w/v PLGA based NPs prepared in presence of Heparin	Unloaded NPs	143 ± 20	-	-	-26.6 ± 0.59
	PL loaded NPs	137 ± 19	73 ± 1.8 %	1.9 ± 0.04%	-16.7 ± 0.23

Table 2: Size analysis, encapsulation efficiency, loading and Z-potential of PLGA based NPs prepared in absence and presence of Heparin.

The size of NPs resulted below 200 nm and was not affected by the presence of Hp.

All the prepared NPs were characterized by negative Z-potential values. These negative surface charges can be attributed to the presence of the end carboxyl groups of the polymer on the NPs surfaces. The Z-potential is an indication of the repulsive force that is present on nanoparticle surface and can be used to predict the long-term stability of the NPs. If all the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other minimizing the tendency to aggregate. However, if the particles have low zeta potential values then there is no force that prevent the particles coming together and flocculating [25, 26].

The presence of Hp affected PL loading and encapsulation efficiency. Indeed, the addition of the stabilizing agent decreased the drug loading from $2.2 \pm 0.3\%$ to $1.9 \pm 0.04\%$, and the encapsulation efficiency from $89 \pm 6\%$ to $73 \pm 1.8\%$. We observed that the particles Z-potential was reduced when the NPs were prepared in presence of Hp, suggesting that part of the encapsulated Hp was anchored onto the NPs surface, increasing its anionic nature. Thus, it is likely that the addition of Hp inhibited the loading of PL proteins that are negatively charged at the NPs formulative conditions (pH: 6). The loading of these proteins, such as glycoprotein IIb/IIIa (pI = 5) [27], was hampered by electrostatic repulsions. On the contrary, proteins characterized by a positive charge at this pH, such as GFs (e.g. PDFG, VEFG, bFGF pI= 7-10) [28], were attracted by Hp and their encapsulation was increased. Furthermore, the PDGF family presents an efficient binding site for Hp, and the formation of an Hp-GF complex could have further favored its incorporation into NPs [23]. In order to verify this hypothesis, the PDGF-AB was selected for the following analysis.

Encapsulation efficiency of Platelet Derived Growth Factor Isoform AB

In order to specifically evaluate the effect of Hp on the encapsulation efficiency of the GFs, we selected PDGF-AB as reference GF in PL. This analysis was carried out by PDGF-AB human ELISA kit and the results are reported in Table 3. The detected amount of PDGF-AB in PL was 7000 ng/ml. The encapsulation efficiency of PDGF-AB was effectively increased from 14% to 31% when Hp was added to the formulation.

Table 3	PDGF-AB (ng/ml of NPs suspension)	PDGF-AB EE%
PDGF-AB in PL	7000 ng/ml	-
PL loaded PLGA based NPs	8.8	14 %
PL loaded PLGA based NPs with Heparin	21.3	31 %

Table 3: Encapsulation efficiency of PDGF-AB in the PLGA based NPs prepared in absence and presence of Heparin.

Stability of nanoparticles

A successful drug delivery system requires efficiency and stability during storage, thus stability studies are imperative for new pharmaceutical products. In these studies we analyzed the stability of unloaded and PL loaded NPs, prepared in absence and presence of Hp. NPs were stored in aqueous medium at 4°C by monitoring their size for 4 weeks. Results showed that, after 4 weeks, the size of PL loaded NPs remain stable for all the NPs formulations (Figure 5). The stability of prepared NPs could be attributed to their surface charge, since the repulsion forces between the NPs prevent their aggregation.
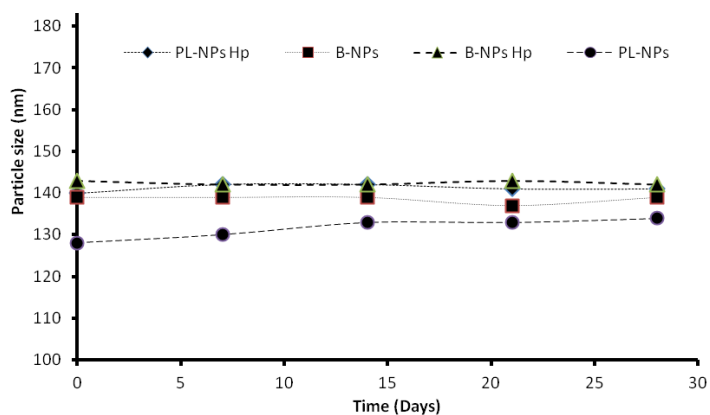
Figure 5: Stability of unloaded and PL loaded PLGA based NPs prepared in absence and presence of Hp in water.

In vitro HSA-FITC release kinetics from PLGA nanosystems

HSA-FITC was selected as a model protein for the *in vitro* release studies. The release profile of the prepared nanosystem was determined in PBS 1X medium at 37°C to reproduce the physiological conditions. The result of the release studies are displayed in Figure 6. The release kinetic was characterized by two phases of release, an initial burst (40%) followed by sustained release up to 90% in 13 days. These results are in accordance with the literatures [29-31]. Release profiles provided by PLGA based NPs are typically characterized by a biphasic curve: (a) in the first phase, an initial drug release is associated to the diffusion of the encapsulated drug localized onto the NPs surface. (b) In the second phase, drug is released progressively [32].
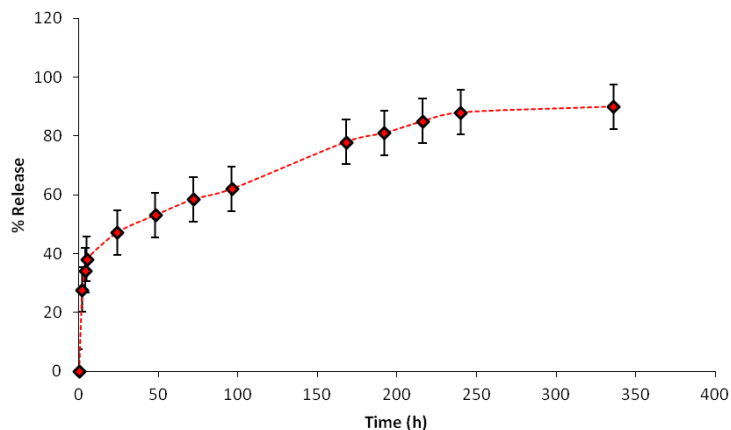
Figure 6: HSA-FITC release profile from PLGA based NPs.

Incorporation of nanoparticles into fibrin matrix

Fibrin gels loaded with the developed PL-NPs were prepared in order to obtain a controlled release device that can be topically applied at the wound site. Gels were obtained by using two different concentrations of thrombin (Figure 7). The gelification phenomenon occurred immediately due to the conversion of fibrinogen into fibrin and PL-NPs were successfully entrapped into the matrices.

The composition of fibrin gel, in terms of fibrinogen and thrombin concentrations, represents an important parameter that determine the release rate from fibrin matrices [15]. As the concentration of thrombin is increased in the composite gel, a dense cross-linked fibrin network is formed, and the release from the fibrin network is slowed [33].

We examined the concentrations effect of thrombin on the PL-NPs-loaded fibrin gel formation in order to obtain fibrin matrices with different release properties. No significant difference in fibrin matrices formation were observed between the two preparations with different thrombin concentrations; the prepared fibrin matrices resulted equally suitable to be used as devices for PL-NPs incorporation, thus offering the possibility to modulate the PL release at a desired rate.
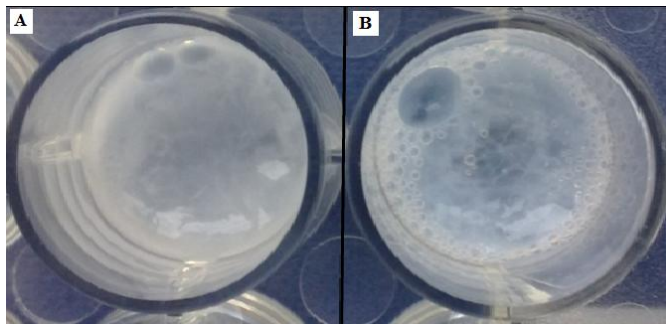
Figure 7: Fibrin matrices with PL loaded PLGA based NPs prepared with 625 UI/ml (A) and 100 UI/ml (B) of thrombin respectively.

3.5. Conclusions

This study was aimed at the preparation and characterization of PLGA based NPs as carriers for the administration of PL. NPs were successfully prepared by a modified solvent diffusion technique. Initially we prepared NPs with two different polymer and surfactant concentrations in order to select the optimal formulation conditions. Based on these results, the formulations with 1.25% w/v of polymer and surfactant were chosen for the further analysis.

Hp was used as stabilizing agent for the NPs preparation, in order to obtain an improvement of PL encapsulation and stabilization of the dispersions. The prepared nanosystems were characterized by monomodal distribution of the diameters, submicron size (below to 200 nm), negative Z-potential values and good spherical shape. The addition of Hp to the formulations did not affect the dimensions and morphologies of the prepared nanosystems. On the contrary, Z-potential values were increased while the encapsulation efficiency and drug loading were decreased. Likely, the high negative charge density of Hp increased the negativity of the particles, and inhibiting, by electrostatic repulsions the encapsulation of the negatively charged PL proteins. As confirm, positively charged proteins such as PDGF-AB, displayed an opposite behavior. Indeed, PDGF-AB was selected to specifically evaluate the effect of Hp on encapsulation efficiency, and we observed an increase of the EE% value which rose from 14% to 30%.

The stability of the prepared NPs was studied over 4 weeks. The nanosystems showed a good stability in water and maintained their size below 200 nm, with no appreciable difference between the loaded and unloaded NPs both in presence and absence of Hp. *In vitro* release studies were carried out by using HSA-FITC NPs drug release was characterized by an initial burst followed by sustained release up to 90% in 13 days. The developed NPs were successfully incorporated into fibrin matrices prepared by using two different concentrations of thrombin.

The developed combined-system seems to be promising for the delivery of PL. It possesses the advantages of both PLGA based NPs and fibrin gel scaffolds, including: protection of PL from proteolysis; sustained and controlled release; biologic adhesiveness and biocompatibility. This is especially true for topical applications systems, for whom the rate of GFs release have a critical importance in the promotion of the wounds healing process.

3.6. References

1.   Ferrara N., Kerbel RS. "Angiogenesis as a therapeutic target". Nature 2005;438(15):967-974.

2.   Risau W. "Mechanisms of angiogenesis". Nature 1997;386(6626):671-674.

3.   Giusti I., Rughetti A., D'Ascenzo S., Millimaggi D., Pavan A., Dell'Orso L. "Identification of an optimal concentration of platelet gel for promoting angiogenesis in human endothelial cells". Transfusion 2009;49(4):771-778.

4.   Roy S., Driggs J., Elgharably H., Biswas S., Findley M., Khanna S. "Platelet-rich fibrin matrix improves wound angiogenesis via inducing endothelial cell proliferation". Wound Repair Regeneration 2011;19(6):753-766.

5.   Cole BJ., Seroyer ST., Filardo G., Bajaj S., Fortier LA. "Platelet-rich plasma: where are we now and where are we going?". Sports Health: A Multidisciplinary Approach 2010;2(3):203-210.

6.   Alsousou J., Thompson M., Hulley P., Noble A., Willett K. "The biology of platelet-rich plasma and its application in trauma and orthopaedic surgery: a review of the literature". Journal of Bone & Joint Surgery, British Volume 1, 2009;91-B(8):987-996.

7.   Colciago A., Celotti F., Casati L., Giancola R., Castano SM., Antonini G. "*In vitro* effects of PDGF isoforms (AA, BB, AB and CC) on migration and proliferation of SaOS-2 osteoblasts and on migration of human osteoblasts". International journal of biomedical science 2009;5(4):380 - 389.

8.   Werner S., Grose R. "Regulation of wound healing by growth factors and cytokines". Physiological Reviews 2003;83(3):835-870.

9.   Balasubramanian V., Onaca O., Enea R., Hughes DW., Palivan CG. "Protein delivery: from conventional drug delivery carriers to polymeric nanoreactors". Expert Opinion on Drug Delivery 2010;7(1):63-78.

10.   Farokhzad OC., Langer R. "Impact of nanotechnology on drug delivery". ACS Nanotation 2009;3(1):16-20.

11.   Anderson JM., Shive MS. "Biodegradation and biocompatibility of PLA and PLGA microspheres". Advanced Drug Delivery Reviews 1997;28(1):5-24.

12.   Govender T., Stolnik S., Garnett MC., Illum L., Davis SS. "PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug". Journal of Controlled Release 1999;57(2):171-185.

13.   Chien-Hua N., Yuan-Yuan C. "FDA perspective on peptide formulation and stability issues". Journal of Pharmaceutical Sciences 1998;87(11):1331-1334.

14.   Janmey PA., Winer JP., Weisel JW. "Fibrin gels and their clinical and bioengineering applications". Journal of Royal Society Interface 2009;6(30):1-10.

15.   Zhou W., Zhao M., Zhao Y., Mou Y. "A fibrin gel loaded with chitosan nanoparticles for local delivery of rhEGF: preparation and in vitro release studies". Journal of Materials Science: Material Medicine 2011; 22(5):1221-1230.

16.   Park K., Shalaby WSW., Park H. "Biodegradable hydrogels for drug delivery". Lancaster, Pennsylvania: Technomic Pulications, 1993.

17.   Hall H. "Modified fibrin hydrogel matrices: both, 3D-scaffolds and local and controlled release systems to stimulate angiogenesis". Current Pharmaceutical Design 2007;13(35):3597-3607.

18.   Chung YI., Kim SK., Lee YK., Park SJ., Cho KO., Yuk SH. "Efficient revascularization by VEGF administration via heparin-functionalized nanoparticle-fibrin complex". Journal of Controlled Release 2010 May 10;143(3):282-289.

19.   Chung Y-I., Tae G., Hong Yuk S. "A facile method to prepare heparin-functionalized nanoparticles for controlled release of growth factors". Biomaterials 2006;27(12):2621-2626.

20.   Munoz EM., Linhardt RJ. "Heparin-binding domains in vascular biology". Arteriosclerosis Thrombosis, and Vascular Biology 2004;24(9):1549-1557.

21.   Cardin AD., Weintraub HJ. "Molecular modeling of protein-glycosaminoglycan interactions". Arteriosclerosis, Thrombosis, and Vascular Biology 1989;9(1):21-32.

22.   Sommer A., Rifkin DB. "Interaction of heparin with human basic fibroblast growth factor: Protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan". Journal of Cellular Physiology 1989;138(1):215-220.

23.   Rolny C., Spillmann D., Lindahl U., Claesson-Welsh L. "Heparin amplifies platelet-derived growth factor (PDGF)- BB-induced PDGF α-receptor but not PDGF β-receptor tyrosine phosphorylation in heparan sulfate-deficient cells". Journal of Biological Chemistry 2002;277(22):19315-19321.

24.   Santander-Ortega M., Csaba N., González L., Bastos-González D., Ortega-Vinuesa J., Alonso MJ. "Protein-loaded PLGA–PEO blend nanoparticles: encapsulation,

release and degradation characteristics". Colloid & Polymer Science 2010;288(2):141-150.

25.   Schwegmann H., Feitz AJ., Frimmel FH. "Influence of the zeta potential on the sorption and toxicity of iron oxide nanoparticles on *S. cerevisiae* and *E. coli*". Journal of Colloid and Interface Science 2010;347(1):43-48.

26.   Rouissi T., Tyagi RD., Brar SK., Prevost D., John RP., Surampalli RY. "Efficient and simple method for determination of suspendibility of bio-inoculant suspensions". Bioresource Technology 2011;102(22):10754-10758.

27.   Charo IF., Fitzgerald LA., Steiner B., Rall SC., Bekeart LS., Phillips DR. "Platelet glycoproteins IIb and IIIa: evidence for a family of immunologically and structurally related glycoproteins in mammalian cells". Proceedings of the National Academy of Sciences 1986;83(21):8351-8355.

28.   Goretzki L., Burg MA., Grako KA., Stallcup WB. "High-affinity binding of basic fibroblast growth factor and platelet-derived growth factor-AA to the core protein of the NG2 proteoglycan". Journal of Biological Chemistry 1999;274(24):16831-16837.

29.   Geng H., Song H., Qi J., Cui D. "Sustained release of VEGF from PLGA nanoparticles embedded thermo-sensitive hydrogel in full-thickness porcine bladder acellular matrix". Nanoscale Research Letters C7 - 312 2011;6(1):1-8.

30.   Prasad S., Cody V., Saucier-Sawyer J., Fadel T., Edelson R., Birchall M. "Optimization of stability, encapsulation, release, and cross-priming of tumor antigen-containing PLGA nanoparticles". Pharmaceutical Research 2012;29(9):2565-2577.

31.   Corrigan OI., Li X. "Quantifying drug release from PLGA nanoparticulates". European Journal of Pharmaceutical Sciences 2009;37(3-4):477-485.

32.   Makadia HK., Siegel SJ. "Poly lactic-*co*-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier". Polymers 2011;3(3):1377-1397.

33.   Jeon O., Kang S-W., Lim H-W., Hyung Chung J., Kim B-S. "Long-term and zero-order release of basic fibroblast growth factor from heparin-conjugated poly(l-lactide-*co*-glycolide) nanospheres and fibrin gel". Biomaterials 2006;27(8):1598-1607.

4. <u>Chapter IV: Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based Nanoparticles Loaded with Platelet Lysate</u>

4.1. Introduction

Therapeutic angiogenesis is a promising therapy based on the stimulation of neovascularization, which can be used in the treatment or prevention of clinical situations characterized by local hypovascularity. The angiogenic process involves growth factors (GFs) activation of endothelial cells that leads to proliferation, migration and stabilization of vessels to form a mature vascular network [1].

Nowadays, platelets derivates are under investigations in the field of therapeutic angiogenesis as one of the most concentrated natural source of GFs [2]. Platelet lysate (PL), which is an haemoderivative rich in GFs deriving from the lysis of platelets, has been proposed in clinical practice for the treatment of several ischemic conditions [3]. PL is obtained from Platelet Rich Plasma (PRP) after its freeze-thawing destruction. One of the advantages of using PL is that it provides a mixture of autologous GFs in their biologically concentration to assist the body in healing, and promotes vascularization with greater similarly to the natural processes [4].

The successful administration of therapeutic proteins, such as GFs, are often impeded by several difficulties, bound to their insufficient stability and shelf life, costly production, immunogenic and allergic potential, as well as poor bioavailability and sensitivity towards proteases. Nowadays, controlled delivery systems represent the best approach to protein drug administration. In particular, protein drugs can be encapsulated within injectable colloidal polymeric carriers, such as nanoparticles (NPs), which can protect them against degradation and ensure their transport and delivery to the specific site of action at a controlled rate [5].

Hydroxypropyl methacrylamide (HPMA) based co-polymers are a class of biomedical polymers obtainable through synthetic pathways. They can be tailored to their final specific application by modulating the choice of the co-monomers and are amenable to bio-functionalization with biological targeting moieties thanks to the presence of reactive functional groups [6]. Water-soluble polymers such as HPMA copolymers are frequently employed as drug carriers because there are able to improve the solubility of hydrophobic compounds, to reduce non-specific toxicity, and to increase the therapeutic index of low molecular weight anticancer drugs [6, 7].

The hydrophilicity of the polymer was balanced by adding different percentages of biodegradable oligopeptide side-chains to the HPMA backbone [8]. These side-chains had also the potential to be used as drug attachment/release sites [9]. However, the HPMA backbone is not biodegradable and consequently the molecular masses of HPMA co-polymers have been limited to 40 kDa or below to ensure renal elimination [10].

These characteristics, along with the fundamental properties of biocompatibility, endow these copolymers with good chances of success as matrices for NPs formulation.

In our laboratories we successfully prepared NPs based on the co-polymers poly (methacryloylglycylglycine-OHx-*co*-hydroxypropylmethacrylamide$_y$) [poly (Gly$_x$-*co*-HPMA$_y$] (Figure 1) loaded with Human Serum Albumin (HSA) as a model protein drug [11].
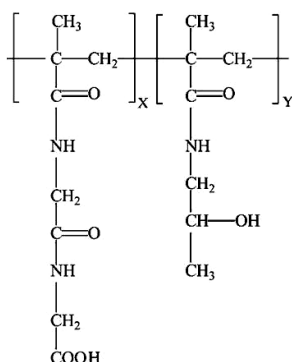

Figure 1: Structure of poly (Gly$_x$-*co*- HPMA$_y$).

Fibrin gel, a network formed when fibrinogen is activated by thrombin, represents another potential drug-delivery system. Due to its excellent features, such as elasticity and capability to easy self-assemble under physiological conditions, fibrin gel is a potent tool for clinical applications [12].

Fibrin gel with loaded with a drug can be easily injected, forming a drug reservoir for local delivery at the desired site. In several studies, fibrin gel was used as matrix to deliver GFs [13, 14]. However, due to GFs instability when administrated in gel formulations [15], systems based on the incorporation of GFs loaded NPs into fibrin gels are under investigation [16].

The objective of the present study was to investigate the suitability the copolymer poly (methacryloylglycylglycine-OH$_{25}$-*co*-hydroxypropylmethacrylamide$_{75}$) [poly (Gly$_{25}$-*co*-HPMA$_{75}$)] for the preparation of NPs loaded with PL as a source of GFs. PL used for NPs preparations was obtained from several purification steps of PRP, the portion of plasma enriched with platelets.

The prepared NPs were designed to be subcutaneously injected into ischemic limbs. According to this future application, we studied the possibility to include the prepared PL-NPs in a fibrin matrix. The prepared NPs should be characterized by a fast release profiles since the drug will be further retain by the fibrin matrix before reaching the site of action. This system can be defined as combined injectable controlled delivery system for the release of PL directly at the ischemic site.

In order to ensure the maintenance of bioactivity of the encapsulated GFs, heparin sodium salt (Hp) was used as stabilizing agent during NPs preparation. Several studies demonstrated that GFs present in their architecture a heparin-binding domain [17, 18] and the interaction of Hp with GFs showed to potentiate its activity and to protect GFs from heat and enzymatic degradation [19, 20].

The prepared NPs were submitted to physical and biological characterizations. The mean particles size and stability of NPs were evaluated by dynamic light scattering. Morphological analyses were performed by Scanning Electron Microscope (SEM). Colorimetric detection method was used to evaluate the protein loading into NPs and the process encapsulation efficiency. Release kinetic profile was carried out by using Fluorescein Isothiocyanate Conjugate Human Serum Albumin (HSA-FITC) as model protein drug. Moreover, a careful *in vitro* biological investigation of the cytotoxicity of the prepared NPs was carried out.

4.2. Material

Poly (Gly$_{25}$-*co*-HPMA$_{75}$) and 1-*O*-glycidyl-2,3-*o*- isopropylidenglycerol-(β-cyclodextrin) (GIG-(βCD) were supplied by Polymer Laboratories Ltd. (UK). HSA, PRP and fibrin gel kit were kindly provided by Kedrion SpA (Castelvecchio Pascoli, Italy). HSA-FITC and Hp were purchased from Sigma-Aldrich (Milan, Italy). The Micro BCA Protein Assay kit was obtained from Pierce (Rockford, IL). Phosphate Buffer Saline solution (PBS 10X) was prepared by dissolving 2.0 g of KCl, 2.0 g of KH$_2$PO$_4$•H$_2$O, 80 g of NaCl, and 15.6 g of Na$_2$HPO$_4$•12H$_2$O in 1 liter of distilled water. The pH was adjusted to 7.4 with 10 N NaOH and the resulting solution was sterilized in autoclave (121°C for 20 min) before storage. PBS 10X was used diluted ten folds with sterile distilled water (PBS 1X) Physiological solution was prepared by dissolving 9.0 g of NaCl in 1 liter of distilled water. The solution was sterilized in autoclave (121°C for 20 min) before use and storage. Dulbecco's Modified Eagle's Medium (DMEM), Bovine Calf Serum (BCS), Penicillin/Streptomycin (P/S) solutions were purchased from Gibco. Complete Dulbecco's Modified Eagle's Medium (Complete DMEM) was prepared by adding penicillin (100 U/mL), streptomycin (100μg/ mL), glutamine (4 mM) and 10% calf serum to DMEM. The Balb/3T3 Clone A31 (ATCC CCL163) mouse embryo fibroblast cell line was purchased from ATCC (American Tissue Culture Collection) and propagated following the instructions provided by the supplier. The cell proliferation reagent WST-1 was purchased from Roche Molecular Biochemicals and stored at -20°C in the dark. All solvents used were commercially available (Carlo Erba, Milan, Italy).

4.3. Methods

4.3.1. Preparation of Platelet Lysate

Platelet Lysate (PL) was prepared as reported by following: platelets presents in PRP were isolated by centrifugation at 10000g for 15 minutes, at 4˚C, resuspended in aqueous

medium and submitted to a freeze-thaw cycle (in order to obtain platelets lyses and subsequent GFs release) and finally centrifuged at 10000g for 15 minute, 4˚C. The GFs water rich supernatant was used for NPs preparation.

4.3.2. Preparation of nanoparticles

Preparation of Unloaded Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based nanoparticles

The preparation of Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs was carried out by co-precipitation technique [11]. In brief, 12.5 mg of polymer was dissolved in 2 ml of a water/ethanol solution (1:4) and was added by a syringe equipped with 22 G needle to a solution of 7 mg of GIG-βCD, 5 mg of HSA in 5 ml of distilled water kept under magnetic stirring at room temperature.

Preparation of Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based nanoparticles loaded with Platelet Lysate

The preparation of Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs was carried out by co-precipitation technique. In brief, 12.5 mg of polymer was dissolved in 2 ml of a water/ethanol solution (1:4) and was added by a syringe equipped with 22 G needle to a solution of 7 mg of GIG-βCD, 5 mg of HSA and 200 μl of PL in 4.8 ml of distilled water kept under magnetic stirring at room temperature.

Preparation of Unloaded Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based nanoparticles in presence of Heparin

The preparation of Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs was carried out by co-precipitation technique. In brief, 12.5 mg of polymer was dissolved in 2 ml of a water/ethanol solution (1:4) and was added by a syringe equipped with 22 G needle to a solution of 7 mg of GIG-βCD, 5 mg of HSA and 4 μg of Hp in 5 ml of distilled water kept under magnetic stirring at room temperature.

Preparation of Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based nanoparticles loaded with Platelet Lysate in presence of Heparin

The preparation of Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs was carried out by co-precipitation technique. In brief, 12.5 mg of polymer was dissolved in 2 ml of a water/ethanol solution (1:4) and was added by a syringe equipped with 22 G needle to a solution of 7 mg of GIG-βCD, 5 mg of HAS, 200 μl of PL and 4 μg of Hp in 4.8 ml of distilled water kept under magnetic stirring at room temperature.

Preparation of Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based nanoparticles loaded with Fluorescein Isothiocyanate Conjugate Human Serum Albumin

The preparation of Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs was carried out by co-precipitation technique. In brief, 12.5 mg of polymer was dissolved in 2 ml of a water/ethanol solution (1:4) and was added by a syringe equipped with 22 G needle to a

solution of 7 mg of GIG-βCD, 4.5 mg of HSA and 0.5 mg of HSA-FITC in 5 ml of distilled water kept under magnetic stirring at room temperature.

4.3.3. Nanoparticles characterization

Purification of nanoparticles

The NPs suspensions were purified by using an ALC PK121R refrigerated centrifuge. NPs were centrifuge at 8000g for 30 minutes at 4˚C. The obtained pellet of purified NPs was resuspended in appropriate media depending on their use.

Dimensional analysis

NPs size analyses were carried out by using a Coulter Beckman LS230 Laser Diffraction Particle Size Analyzer, equipped with small volume module plus. Samples were added into the cell until 30-50% obscuration of PIDS detector. Three runs were performed on each sample.

Morphological analysis

The NPs morphology was evaluated by scanning electron microscopy (SEM) (JEOL LSM5600LV). The samples were prepared from purified and lyophilised NPs. Gold sputtering was performed before SEM analysis.

Freeze-drying

After freezing at –20 °C, nanoparticles were lyophilised in 5 Pascal Lio 5P lyophilisator, equipped with RV8 (Edwards) vacuum pump.

Determination of protein encapsulation efficiency and loading

Proteins encapsulation efficiency was calculated from the proteins amount that was detected in the supernatant recollected after centrifugation of the NPs samples. Standard micro BCA protein assay was used to determine the amount of free proteins (HSA or HSA-FITC) in the NPs supernatant and the amount of proteins contained in the PL employed for the NPs preparation. The difference between the amount of protein employed for the NPs preparation, and the amount of proteins measured in NPs supernatant represented the proteins loaded into NPs. This value was necessary for the calculation of the encapsulation efficiency and drug loading. The encapsulation efficiency was calculated as percentage of proteins loaded in the NPs with respect to total amount of proteins added during the formulation of NPs (Equation 1). Drug loading was calculated as percentage of proteins loaded in NPs with respect to the dry weight of recovered NPs (Equation 2) [21]. A calibrations curve of Bovine Serum Albumin (BSA) was prepared by following the manufacturer instructions. All samples were measured at 565 nm. Mean values were obtained from three different batches.

Equation 1:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Mass of drug in NPs}}{\text{Mass of drug used in NPs formulation}} \cdot 100$$

Equation 2:

$$\text{Loading (\%)} = \frac{\text{Mass of drug in NPs}}{\text{Mass of NPs recovered}} \cdot 100$$

Stability studies of nanoparticles

Stability of unloaded and PL loaded NPs, prepared in absence and presence of Hp, was studied in water medium by dynamic light scattering using Coulter Beckman LS230 Laser Diffraction Particle Size Analyzer. NPs suspensions were stored under static conditions at 4°C. The average size was analyzed at specified time intervals.

In vitro HSA-FITC release kinetics from Poly (Gly25-*co*-HPMA75) nanosystems

The HSA-FITC loaded NPs were purified by centrifugation and the resulting pellet was resuspended in 7ml of PBS 1X. Purified NPs suspension was divided into aliquots of 1ml into individual tube and incubated at 37°C under magnetic stirring. At different time points, samples were centrifuged at 8000g for 15 minutes at room temperature and the obtained supernatants were collected. The released HSA-FITC concentration in the collected supernatants was assessed spectrofluorimetrically with excitation and emission wavelengths of 485 and 535nm, respectively.

Incorporation of nanoparticles into fibrin matrix

The fibrin matrix kit consisted of thrombin and fibrinogen in separate vials, plus separate solutions of calcium chloride ($CaCl_2$) and potassium dihydrogen phosphate (KDP). The PL loaded NPs incorporated fibrin gel was prepared as follows. Thrombin was reconstituted in a $CaCl_2$ solution and then drawn into a syringe. Fibrinogen, containing Factor XIII, was reconstituted in the KDP solution in order to obtain 30 IU/ml of fibrinogen concentration and then 3 mg of PL loaded NPs were added to this suspension. The PL-NPs-fibrinogen suspension was kept under magnetic stirring until the complete dissolution of NPs. The NPs- fibrinogen suspensions were placed respectively into 24-well plate and when the thrombin solution was added, the gelification phenomenon occurred immediately due to the conversion of fibrinogen to fibrin, and the plate was incubated at 37 °C for 1 h to complete polymerization. The fibrin matrices were prepared with two different concentrations of thrombin, 625 UI/ml and 100 UI/ml respectively, in order to study the effect of thrombin concentrations on the fibrin matrix formation.

4.3.4. Biological investigations

Absorbance measurements on microplates

Microplate absorbance measurements were performed by means of Benchmark Bio–Rad Microplate Reader. All data were processed by using Microplate Manager III (Biorad) and Igor Pro (Wavemetrics).

CO_2 incubator

Cell cultures were performed in a Hera Cell (Heraeus Instruments S.p.A.) CO_2 Incubator in 5% CO_2 enriched atmosphere.

Laminar flow cabinet

Experiments that required sterile conditions were performed under a Hera Safe HS12 Bioclass II (Heraeus Instruments S.p.A.) laminar flow cabinet.

Optical Microscopy (OM)

Optical microscopy observations were performed by Nikon Eclipse TE 2000 inverted microscope equipped with epifluorescence lamp and Nikon D Eclipse C1 confocal system.

Determination of IC_{50} of unloaded Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based nanoparticles

For the determination of the IC_{50} (50% inhibitory concentration, that is the material concentration at which 50% of cell death in respect to the control is observed) of the prepared NPs a subconfluent monolayer of 3T3 fibroblast was trypsinized using a 0.25% trypsin, 1mM EDTA solution, centrifuged at $200 \times g$ for 5 min, re-suspended in growth medium and counted. Appropriate dilution was made in order to obtain 3×10^3 cells per 100 μl of medium, the final volume present in each well of a 96 well plate. Cells were incubated at 37 ˚C, 5% CO_2 for 24 h until 60–70% confluence was reached. The medium from each well was then removed and replaced with medium containing a different concentration (1-10 mg/ml) of unloaded NPs. Control cells were incubated with fresh growth medium and wells containing only growth medium were used as blanks. After 24h of incubation with medium containing the NPs sample, cells were analyzed for viability with Cell Proliferation Reagent WST-1.

Determination of IC_{50} of Platelet Lysate loaded Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based nanoparticles

For the determination of the IC_{50} of PL loaded NPs a subconfluent monolayer of 3T3 fibroblast was trypsinized using a 0.25% trypsin, 1mM EDTA solution, centrifuged at $200 \times g$ for 5 min, re-suspended in growth medium and counted. Appropriate dilution was

made in order to obtain 3×10^3 cells per 100 μl of medium, the final volume present in each well of a 96 well plate. Cells were incubated at 37 ˚C, 5% CO$_2$ for 24 h until 60–70% confluence was reached. The medium from each well was then removed and replaced with medium containing a different concentration (1-10 mg/ml) of PL loaded NPs. Control cells were incubated with fresh growth medium and wells containing only growth medium were used as blanks. After 24h of incubation with medium containing the NPs sample, cells were analyzed for viability with Cell Proliferation Reagent WST-1.

4.4. Results and Discussion

The preparation of bioeliminable polymeric NPs for drug delivery were carried out by using the co-precipitation technique. Co-precipitation is an original technique developed in our laboratory. Co-precipitation is based on the dropwise addition of an organic polymer solution to an aqueous protein solution under gentle magnetic stirring; the progressive interaction between the two components gives rise to the formation of a NPs suspension. In comparison to similar methods for NPs preparation, the co-precipitation method does not imply the use of chlorinated solvents or energetic mixing, both of which are known to cause appreciable protein denaturation [11] [22]. This method turned out to be particularly suitable for the preparation of nanocarriers for protein drugs delivery.

<u>Preparation and characterization of Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs</u>

The co-precipitation technique described above yields both unloaded (blank) and PL loaded NPs in presence and absence of Hp. In all formulations, NPs were characterized by narrow monomodal distribution an average diameter size below 200 nm (Table 1). The diameters were not affected by the addition of Hp (Figure 2). The obtained size represents a good characteristic for further applications of these NPs, because this diameter size fall within the limit size of 220 nm approved by the Food and Drug Administration (FDA) for NPs administered by injection [23]. Furthermore, NPs with diameters ranging from 100 to 200 nm are characterized by high cellular uptake since they can easily cross the physiological barriers and have access to different cell districts [24, 25].

SEM analysis of the purified and lyophilized solid pellets showed a homogeneous distribution of spherical NPs (Figure 3). The nanosystems prepared in absence and presence of Hp did not show appreciable differences.

PL loaded NPs prepared in absence of Hp were characterized by encapsulation efficiency (EE %) of 55 ± 6 % and PL loading of 5.6 ± 0.9 %. In presence of Hp, the EE % of PL was 68 ± 2.5% and PL loading was 7.2 ± 3.3 %. The increase of in EE % and loading % can be attributed to the presence of Hp, which gave stability to PL and improved its encapsulation into NPs. It has been demonstrated that Hp in solution can bind GFs by ionic interactions [26] and the formation of these Hp-GFs complexes might have increased the GFs encapsulation into NPs [27].

Based on these results, the NPs formulations prepared in presence of Hp were chosen for the following set of analysis.
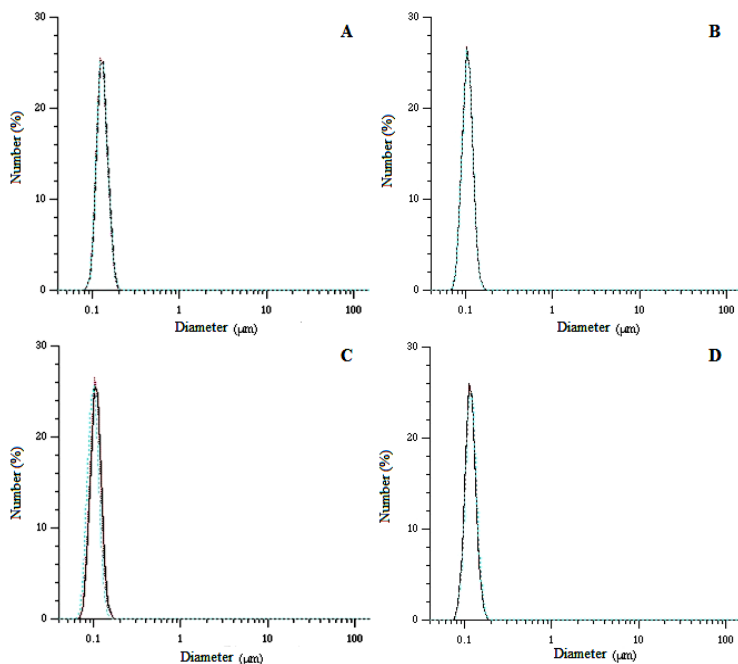

Figure 2: Diameter distribution of Poly (Gly$_{25}$ -*co*-HPMA$_{75}$) based NPs. Unloaded NPs (A) and loaded with PL (B). In C and D are shown NPs prepared with heparin unloaded and loaded with PL respectively.

Table 2	Size ± S.D. (nm)
Unloaded NPs	135 ± 19
PL loaded NPs	111 ± 15
Unloaded NPs with Heparin sodium salt	111 ± 15
PL loaded NPs with Heparin sodium salt	125 ± 18

Table 2: Dimensional analysis of Poly (Gly$_{25}$-*co*- HPMA$_{75}$) based NPs.
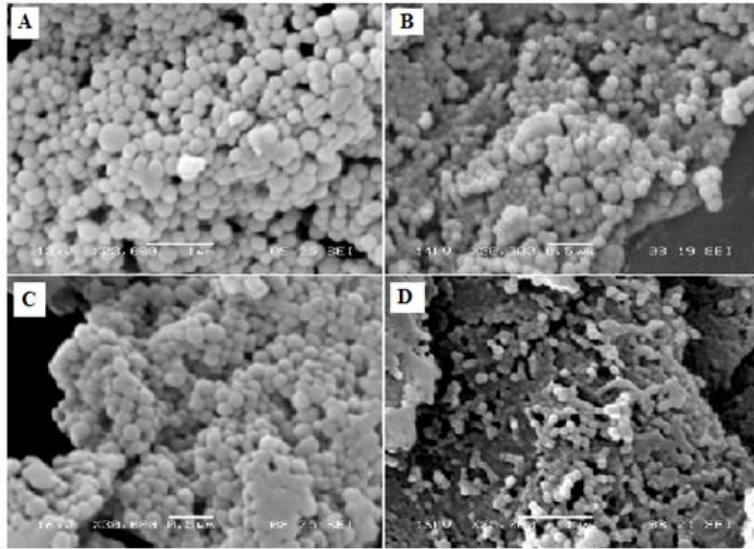
Figure 3: Morphological analyses (SEM micrograph) of Poly (Gly$_{25}$ -*co*-HPMA$_{75}$) based NPs. Unloaded NPs (A) PL loaded NPs (B), unloaded with heparin (C) and PL heparin loaded NPs (D).

NPs Stability Studies

NPs stability is one the most critical issues for their applications to biological systems, as their general tendency is to aggregate upon storage. In these studies we analyzed the stability of unloaded and PL loaded NPs, prepared in absence and presence of Hp. NPs were stored in aqueous medium at 4°C by monitoring their size for 4 weeks. The results shown in Figure 4 indicated that all formulations maintained their size below 200 nm and remain stable in water for 4 weeks.
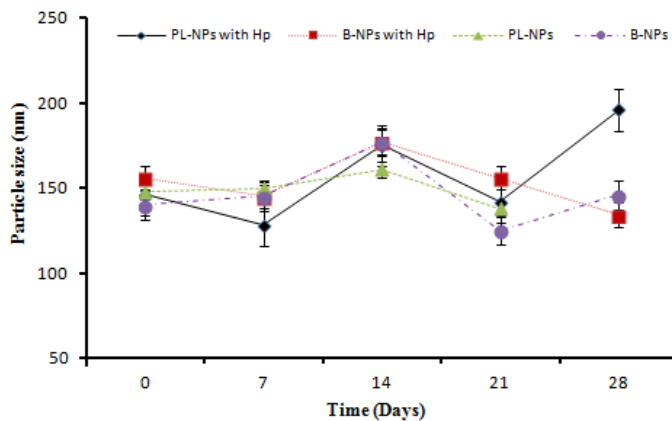
Figure 4: Stability analyses of unloaded and PL loaded Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs prepared in absence and presence of Hp in water medium.

Preparation and characterization of HSA-FITC loaded Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs

The drug release profile of Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs was evaluated by using HSA-FITC as model protein. HSA-FITC loaded NPs were prepared and characterized in terms of size and morphology. The morphological analysis of the purified and lyophilized solid pellets showed that spherical homogeneously distributed HSA-FITC loaded NPs were successfully prepared (Figure 5/A). Dimensional analysis carried out by light scattering showed that NPs dispersions were constituted of monodispersed nanoparticles having an average diameter of 125 ± 79 nm (Figure 5/B) and an HSA-FITC EE% of the 85 ± 2.6%. The physical properties of HSA-FITC loaded NPs were comparable to that of PL loaded NPs, thus making this protein a good model for the study of the *in vitro* release kinetics of proteins from Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs.
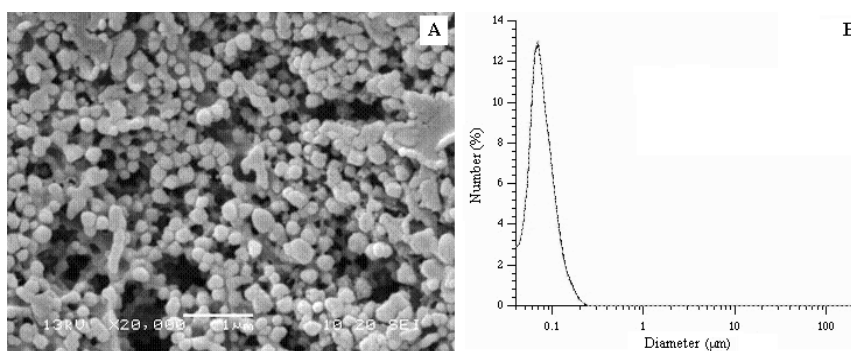
Figure 5: SEM image of HSA-FITC loaded Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs (A). Diameter distribution of HSA-FITC loaded Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs (B).

In vitro HSA-FITC release kinetics from Poly (Gly$_{25}$-*co*-HPMA$_{75}$) nanosystems

The experimental conditions to evaluate the protein release profile were set up in order to reproduce the physiological environment in terms of temperature, pH and salt concentrations. A PBS 1X pH 7.4 was chosen as experimental medium because of its buffer strength, during NP release studies [11].

The *in vitro* protein release profile from NPs is shown in Figure 6. HSA-FITC release from NPs was characterized by an initial burst of 30 % followed by a sustained release of 70 % in 3 days. The burst release might be attributed to the fraction of the protein which is adsorbed or weakly bound to the NPs surface. After this first release phase, the release of HSA-FITC was almost completed in 3 days. This fast release was what we expected for this formulation that should be incorporated into a fibrin matrix and then subcutaneously injected into the ischemic limbs.
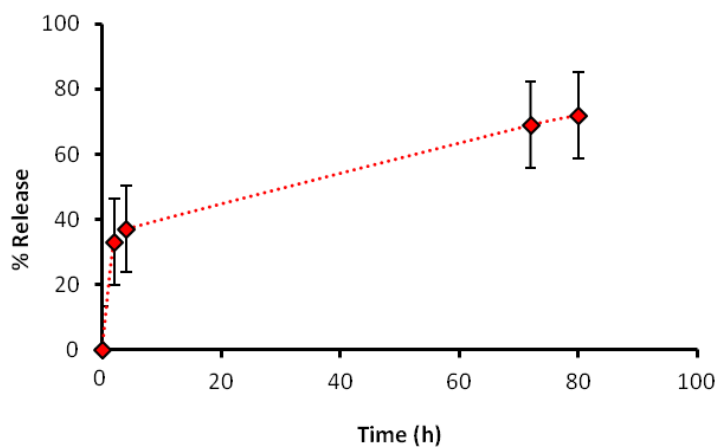
Figure 6: HSA-FITC release profile from Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs.

Incorporation of nanoparticles into fibrin matrix

In order to obtain injectable or implantable systems for the treatment of ischemic diseases, fibrin gels loaded with the developed PL-NPs were prepared. Gels were obtained by using two different concentrations of thrombin (Figure 7). The gelification phenomenon occurred immediately due to the conversion of fibrinogen into fibrin and PL-NPs were successfully entrapped into the matrices.

The composition of fibrin gel, in terms of fibrinogen and thrombin concentrations, represents an important parameter for the release rate of fibrin matrices [28]. In fact, the release can be controlled by adjusting the content of thrombin or fibrinogen. As the concentration of thrombin is increased in the composite gel, a dense cross-linked fibrin network is formed, and the release from the fibrin network is slowed [29].

We examined the effect of thrombin concentrations on the PL-NPs-loaded fibrin gel formation in order to obtain fibrin matrices with different release properties. No significant difference in fibrin matrices formation were observed between the two preparations with different thrombin concentrations; the prepared fibrin matrices resulted equally suitable to be used as devices for PL-NPs incorporation, thus offering the possibility to modulate the PL release at a desired rate.
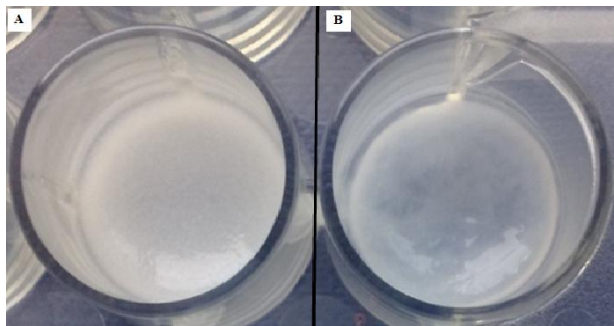
Figure 7: Fibrin matrices with PL loaded poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs prepared with 625 UI/ml (A) and 100 UI/ml (B) of thrombin respectively.

Determination of IC$_{50}$ of Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based nanoparticles

In order to evaluate the biocompatibility of NPs, *in vitro* experiments were carried out using the balb 3T3 mouse embryo fibroblasts clone A31 cell line, following the procedures of ISO 10993 - Part 5: "Test for cytotoxicity - *in vitro* methods". Cytotoxicity, defined as the "*in vitro* evaluation of toxicological risks using cell culture", is a rapid, standardized, sensitive and inexpensive way to assess the *in vitro* biocompatibility of materials to be used in biomedical applications [30]. The prepared Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs were easily re-suspended in DMEM, thus allowing for the investigation of their IC$_{50}$. The NPs were suspended in DMEM at different concentrations ranging between 1 and 10 mg/ml. Cells were incubated for 24h with NPs and then tested for cell viability using WST-1 tetrazolium salt assay. The obtained results, reported in Figure 7, showed that the NPs formulations did not exhibit significant cytotoxic effects. The IC$_{50}$ values were higher than 10 mg/ml, with no considerable difference between the unloaded and PL loaded NPs. In fact, several studies demonstrated that PL is not toxic and it can be used in cell culture as substitute of Fetal Bovine Serum (FBS). PL contains a number of growth promoting substances, including GFs, which makes it an attractive as alternative medium for cell culture [31];[32].
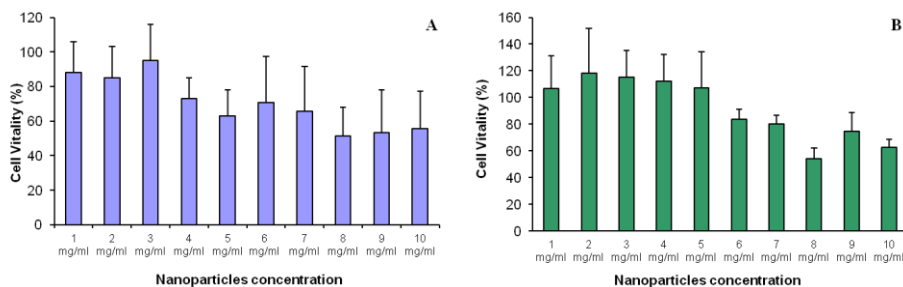
Figure 7: Cell viability measured by WST-1 assay in A31 cell line in presence of increasing concentrations of unloaded Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs (A) and PL loaded NPs (B).

4.5. Conclusions

This study was aimed at the preparation and characterization of Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs as carriers for the administration of PL. NPs were successfully prepared by the co-precipitation technique, a versatile method that appears advantageous for the preparation of nanocarriers loaded with non-conventional drugs such as proteins and peptides. Hp was used as stabilizing agent for the NPs preparations, in order to obtain an improvement of PL encapsulation and stabilization of the dispersions. All the NPs formulations displayed a monomodal distribution of diameters with an average size below to 200 nm and good spherical shape. The addition of Hp to the formulations did not affect the dimensions and morphologies of the prepared nanosystems. PL was successfully encapsulated in the prepared NPs with an EE % of 55 %, which was raised to 68 % by adding Hp. Alike, PL loading % raised from 5.6 % to 7.2 % by adding Hp. These results demonstrated the capability of Hp to improve the PL encapsulation into NPs.

The stability of the prepared NPs was studied within 4 weeks. The nanosystems showed a good stability in water and maintained their size below 200 nm, with no appreciable difference between the loaded and unloaded NPs both in presence and absence of Hp. *In vitro* release studies were carried out by using HSA-FITC NPs drug release was characterized by an initial burst followed and a sustained release of 3 days. The developed NPs were successfully incorporated into fibrin matrices prepared by using two different concentrations of thrombin. This system may be useful to achieve a controlled release of PL at the ischemic site. Careful *in vitro* cytotoxicity test performed on NPs showed their excellent compatibility. They were characterized by an IC$_{50}$ higher than 10 mg/ml and no differences in cell viability were observed between PL loaded and unloaded NPs, which highlighted the excellent cytocompatibility of PL. Taking together the above results, we believe that the PL loaded Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs could be a very promising injectable system for the controlled release of PL for the treatment of ischemic diseases.

4.6. References

1.   Li WW., Talcott KE., Zhai AW., Kruger EA., Li VW. "The role of therapeutic angiogenesis in tissue repair and regeneration". Advances in Skin & Wound Care 2005;18(9):491-500.

2.   Everts PA., Knape JT., Weibrich G., Schonberger JP., Hoffmann J., Overdevest EP. "Platelet-rich plasma and platelet gel: a review". The Journal of Extra-Corporeal Technology 2006;38(2):174-187.

3.   Cole BJ., Seroyer ST., Filardo G., Bajaj S., Fortier LA. "Platelet-rich plasma: where are we now and where are we going?" Sports Health: A Multidisciplinary Approach 2010;2(3):203-210.

4.   Alsousou J., Thompson M., Hulley P., Noble A., Willett K. "The biology of platelet-rich plasma and its application in trauma and orthopaedic surgery: e review of the literature". Journal of Bone & Joint Surgery, British Volume 2009 August 1, 2009;91-B(8):987-996.

5.   Farokhzad OC., Langer R. "Impact of nanotechnology on drug delivery". ACS Nano 2009;3(1):16-20.

6.   Inoue K., Kawaguchi M., Yamanaka R., Higuchi T., Ito R., Saito K. "Evaluation and analysis of exposure levels of di(2-ethylhexyl) phthalate from blood bags". International Journal of Clinical Chemistry and Diagnostic Laboratory Medicine 2005;358(1-2):159-166.

7.   Duncan R. "Development of HPMA copolymer-anticancer conjugates: clinical experience and lessons learnt". Advanced Drug Delivery Reviews 2009;61(13):1131-1148.

8.   Drobník J., Kopeček J., Labský J., Rejmanová P., Exner J., Saudek V. "Enzymatic cleavage of side chains of synthetic water-soluble polymers". Macromolecular Chemistry and Physics 1976;177(10):2833-2848.

9.   Duncan R., Rejmanova P., KopeÄek J., Lloyd JB. "Pinocytic uptake and intracellular degradation of N-(2-hydroxypropyl) methacrylamide copolymers a potential drug delivery system". Biochimica et Biophysica Acta - General Subjects 1981;678(1):143-150.

10.   Duncan R., Vicent MJ., Greco F., Nicholson RI. "Polymer-drug conjugates: towards a novel approach for the treatment of endrocine-related cancer". Endocrine-Related Cancer 2005;12(Supplement 1):S189-S199.

11. Chiellini F., Bartoli C., Dinucci D., Piras AM., Anderson R., Croucher T. "Bioeliminable polymeric nanoparticles for proteic drug delivery". International Journal of Pharmaceutics 2007 Oct 1;343(1-2):90-97.

12. Janmey PA., Winer JP., Weisel JW. "Fibrin gels and their clinical and bioengineering applications". Journal of Royal Society Interface 2009 Jan 6;6(30):1-10.

13. Zisch AH., Schenk U., Schense JC., Sakiyama-Elbert SE., Hubbell JA. "Covalently conjugated VEGF-fibrin matrices for endothelialization". Journal of Controlled Release 2001;72(1-3):101-113.

14. Giannoni P., Hunziker EB. "Release kinetics of transforming growth factor-β1 from fibrin clots". Biotechnology and Bioengineering 2003;83(1):121-123.

15. Park J., Yoon J., Li H., Moon D., Han K. "Buccal mucosal ulcer healing effect of rhEGF/Eudispert hv hydrogel". Archives of Pharmacal Research 2003;26(8):659-665.

16. Zhou W., Zhao M., Zhao Y., Mou Y. "A fibrin gel loaded with chitosan nanoparticles for local delivery of rhEGF: preparation and in vitro release studies". Journal of Materials Science: Materials in Medicine 2011;22(5):1221-1230.

17. Munoz EM., Linhardt RJ. "Heparin-binding domains in vascular biology". Arteriosclerosis, Thrombosis, and Vascular Biology 2004;24(9):1549-1557.

18. Cardin AD., Weintraub HJ. "Molecular modeling of protein-glycosaminoglycan interactions". Arteriosclerosis, Thrombosis, and Vascular Biology 1989;9(1):21-32.

19. Sommer A., Rifkin DB. "Interaction of heparin with human basic fibroblast growth factor: Protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan". Journal of Cellular Physiology 1989;138(1):215-220.

20. Rolny C., Spillmann D., Lindahl U., Claesson-Welsh L. "Heparin amplifies platelet-derived growth factor (PDGF)- BB-induced PDGF α-Receptor but not PDGF β-receptor tyrosine phosphorylation in heparan sulfate-deficient cells". Journal of Biological Chemistry 2002 May 31, 2002;277(22):19315-19321.

21. Govender T., Stolnik S., Garnett MC., Illum L., Davis SS. "PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug". Journal of Controlled Release 1999;57(2):171-185.

22. Solaro R., Chiellini F., Signori F., Fiumi C., Bizzarri R., Chiellini E. "Nanoparticle systems for the targeted release of active principles of proteic nature". Journal of Materials Science: Materials in Medicine 2003;14(8):705-711.

23. Bawa R. "Nanopharmaceuticals: nanopharmaceuticals". European Journal of Nanomedicine 2010;3(1):34-40.

24.   Garnett MC., Kallinteri P. "Nanomedicines and nanotoxicology: some physiological principles". Occupational Medicine 2006;56(5):307-311.

25.   He C., Hu Y., Yin L., Tang C., Yin C. "Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles". Biomaterials 2010;31(13):3657-3666.

26.   Capila I., Linhardt RJ. "Heparin–protein interactions". Angewandte Chemie International Edition 2002;41(3):390-412.

27.   Tang DW., Yu SH., Ho YC., Mi FL., Kuo PL., Sung HW. "Heparinized chitosan/poly(gamma-glutamic acid) nanoparticles for multi-functional delivery of fibroblast growth factor and heparin". Biomaterials 2010;31(35):9320-9332.

28.   Zhou W., Zhao M., Zhao Y., Mou Y. "A fibrin gel loaded with chitosan nanoparticles for local delivery of rhEGF: preparation and in vitro release studies". Journal of Materials Science: Materials in Medicine 2011;22(5):1221-1230.

29.   Jeon O., Kang S-W., Lim H-W., Hyung Chung J., Kim B-S. "Long-term and zero-order release of basic fibroblast growth factor from heparin-conjugated poly(l-lactide-*co*-glycolide) nanospheres and fibrin gel". Biomaterials 2006;27(8):1598-1607.

30.   Chiellini F. "Perspectives on: *In vitro* evaluation of biomedical polymers". Journal of Bioactive and Compatible Polymers 2006;21(3):257-271.

31.   Crespo-Diaz R., Behfar A., Butler GW., Padley DJ., Sarr MG., Bartunek J. "Platelet lysate consisting of a natural repair proteome supports human mesenchymal stem cell proliferation and chromosomal stability". Cell Transplantation 2011;20(6):797-811.

32.   Naaijkens BA., Niessen HWM., Prins HJ., Krijnen PAJ., Kokhuis TJA., Jong N. "Human platelet lysate as a fetal bovine serum substitute improves human adipose-derived stromal cell culture for future cardiac repair applications". Cell and Tissue Research 2012;348(1):119-130.

5. <u>Chapter V</u>: Preparation of magnetic core and polymer shell nanoparticles using an innovative protein biosurfactant

5.1. Introduction

Drug delivery technology has emerged as a truly interdisciplinary science aiming at improving human health. The basic goal of a controlled drug delivery system (DDS) is to deliver a biologically active molecule at a desired rate for a desired duration, to maintain the drug level in the site of action within the therapeutic window [1]. The current challenges associated with DDS include the specific targeting of active agents at the pathological site, in order to avoid large amount of drug administration, non-specific toxicity and undesired side-effects [2]. Among the current schemes of drug delivery, a promising one employs polymeric nanoparticles (NPs) endowed with magnetic proprieties [3]. This technology is based on the co-encapsulation of drugs with magnetite (Fe_3O_4) inside a polymer shell, in order to prepare carriers that can be magnetically guided to the desired tissues thus releasing the drug at the target site [4].

Polyhydroxyalkanoates (PHAs) are natural aliphatic polyesters, produced by a wide range of microorganisms. These biodegradable polyesters are stored intracellularly and act as carbon and energy reserve for the cells [5]. PHAs are characterized by good biocompatibility and biodegradability [6]. Due to these features, PHAs have been investigated as matrices for drug delivery applications and tissue engineering [7]. Over the past years, over 150 different types of PHA have attracted medical interest and particularly poly-3-hydroxybutyrate (PHB), poly-4-hydroxybutyrate (P4HB), copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) and poly-3-hydroxyoctanoate (PHO) [8]. PHBHHx is a good matrix for the preparation of carries loaded with hydrophobic drugs since it is basically constituted by an hydrophobic ester backbone and alkyl side chains (Figure 1). It is also characterized by good biodegradability and its products of degradation are not cytotoxic [9].

PHAs granules have a phospholipids membrane with embedded and attached proteins with regulatory or structural functions. One of the most abundant classes of structural proteins in PHAs granules is represented by phasins. This class of proteins is characterized by molecular weight ranging between 14 and 28 kDa [10], amphiphilic proprieties and the capability to non-specifically bind hydrophobic polymers. Their function is to promote PHA biosynthesis, influencing the morphology, number and size of PHA granules [11]. Phasins, in several bacterial strains, have been shown to increase the production and accumulation of PHAs granules in cells and to provide an interface between the hydrophobic granule surface and the aqueous cell cytoplasm [12, 13]. In

recent years, phasins have been widely used as stabilizing agent to develop methods for protein purification, drug delivery and tissue engineering [14-18].

Phasin P (PhaP) was successfully employed as a biosurfactant to stabilize emulsions of water with lubricating oil, diesel and soybean oil. The results showed that PhaP displayed in the emulsions the same stabilizing effects as the other commonly used surfactants [19].

In this study we report some preliminary results on the preparation of magnetite loaded NPs by using PhaP as stabilizing agent. Two polymeric materials, PHBHHx and poly (lactide-co-glycolide) (PLGA), were employed for the NPs preparation, in order to investigate the capability of PhaP to stabilize NPs based on the two employed polymers. The prepared nanocarriers were characterized in term of size and Z-potential. The presence of magnetite and the morphology of prepared NPs were evaluated by Transmission Electron Microscopy (TEM).
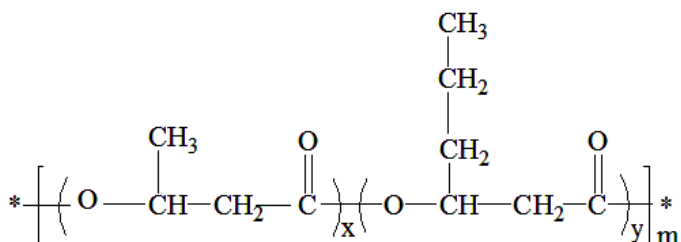
Figure 1: Chemical structure of PHBHHx.

5.2. Material

PLGA$_{85:15}$ [lactide:glycolide (85:15); inherent viscosity midpoint of 3.1 dl/g] was obtained from Purac-Biochem (Amsterdam, NL). PHBHHx$_{80:20}$ (Mw 80 KDa) and PhaP were kindly provided by Laboratory of Microbiology, Tsinghua University (Beijing, China). Magnetite was purchased from Sigma Aldrich (St. Louis, MO). All solvents used were commercially available (Beijing Chemical Works, Beijing, China).

5.3. Methods

5.3.1. Nanoparticles formulations

Preparation of PLGA based nanoparticles by emulsification diffusion technique

Unloaded and magnetite loaded PLGA based NPs were prepared by an emulsification diffusion technique [20]. In brief, 15 mg of PLGA were dissolved in 1 ml of DCM and then, 5 mg of magnetite were added. This organic phase was sonicated (SCINTZ JY92-2D ultrasonic cell crusher) (300W/5minutes) to prepare a homogeneous dispersion of magnetite; 2 ml of water solution, containing 200 µg of PhaP, were added to the organic

phase and the subsequent mixture was emulsified by sonication (300W/5minutes). An excess amount of water (5 ml) was added to the o/w emulsion under sonication. After solvent evaporation under vacuum at 30˚C NPs were concentrated in aqueous medium.

<u>Preparation of PLGA based nanoparticles by emulsification solvent-evaporation process</u>

PLGA based NPs were prepared by an emulsification solvent-evaporation process. Briefly, 10 mg of PLGA were dissolved in 1 ml of chloroform to prepare the organic phase. The aqueous phase was prepared by dispersing 5mg of magnetite in 10 ml of water; the resulting suspension was homogenized by sonication (SCINTZ JY92-2D ultrasonic cell crusher) (600W/3min). Following 1.3 mg of PhaP was added, and the resulting dispersion was further sonicated (200W/3min). The polymer solution was added dropwise with an 18G needle into the aqueous phase. The resulting emulsion was sonicated (400W/3min) to reduce the droplet size of the organic phase and stirred at room temperature to allow the organic solvent evaporation. The obtained NPs suspension was then filtered through a 1μm filter. Unloaded NPs were obtained without including the magnetite in the aqueous phase preparation.

<u>Preparation of PHBHHx based nanoparticles by emulsification solvent-evaporation process</u>

PHBHHx based NPs were prepared by an emulsification solvent-evaporation process. Briefly, 10 mg of PHBHHx were dissolved in 1 ml of chloroform to prepare the organic phase. The aqueous phase was prepared by dispersing 5mg of magnetite in 10 ml of water; the resulting suspension was homogenized by sonication (SCINTZ JY92-2D ultrasonic cell crusher) (600W/3min). Following 1.3 mg of PhaP was added, and the resulting dispersion was further sonicated (200W/3min). The polymer solution was added dropwise with an 18G needle into the aqueous phase. The resulting emulsion was sonicated (400W/3min) to reduce the droplet size of the organic phase and stirred at room temperature to allow the organic solvent evaporation. The obtained NPs suspension was then filtered through a 1μm filter. Unloaded NPs were obtained without including the magnetite in the aqueous phase preparation.

5.3.2. Nanoparticles characterization

<u>Purification of nanoparticles</u>

NPs suspensions were purified by using an Eppendorf centrifuge (Model 5424, Hamburg, Germany). NPs were centrifuged at 10000g for 15 minutes at 4˚C. The obtained pellets of purified NPs were freeze-dried for further analysis.

<u>Dimensional and Z-potential analysis</u>

Dynamic light scattering (DLS) was used for size, polydispersity index and Z-potential measurements (Zetasizer Nano ZS90, Malvern Instruments Inc., Southborough, MA).

The measurements were performed at 25˚C. The viscosity and refraction index of the continuous phase were set equal to those specific to water. Z-potential measurements were made with a disposable capillary cell with a volume of 1 ml. The values of the size and Z- potential were calculated as the mean value of 3 replicates for each NPs formulation.

Morphological analysis

NPs morphology was evaluated by Transmission Electron Microscopy (TEM, Hitachi H-7650B; Hitachi, Japan) at an accelerating voltage of 80kV. A drop of sample was deposited on the copper grid and air dried before the analysis.

5.4. Results and Discussion

In this work we investigated the possibility to prepare magnetite loaded PLGA or PHBHHx based NPs by using PhaP as biosurfactant. Preliminary formulation studies revealed that the emulsification diffusion technique was not suitable to prepare magnetite loaded PLGA based NPs. Indeed, as it is shown in the following results magnetite was not entrapped into their matrix. Based on these results, further formulation studies were performed by using emulsification solvent-evaporation process. This method turned out to be suitable for the preparation of NPs by employing both PLGA and PHBHHx as polymer matrix. We also attempted to prepare unloaded and magnetite loaded NPs without the addiction of PhaP. However, in absence of this stabilizing agent the formation of NPs did not occur.

Preparation of PLGA based nanoparticles by emulsification diffusion technique

NPs were prepared with the addition of PhaP as a stabilizing agent. The formation of stable colloidal suspensions confirmed that the presence of PhaP was essential for the NPs preparation. The prepared nanosystems were characterized by monomodal distribution of diameters and negative Z-potential (Figure 2). The Z-potentials of the prepared NPs were in the range of -20 mV, and there were no significant difference in surface charge between the unloaded and magnetite loaded NPs. Table 1shows the NPs diameter size, polydispersity index (PI) and Z-potential values. The PI of prepared NPs confirmed that the nanosystems were monodispersed. In fact, PI is a parameter to define the particle size distribution, and NPs characterized by PI less than 0.7 displays narrow size distribution [21]. The addition of magnetite to the system increased the size of the PLGA prepared NPs. However, this increase in size cannot be attributed to an effective encapsulation of magnetite into the NPs since, as highlighted by the TEM image (figure 3/B), magnetite (black dots) was just surrounding the NPs but not entrapped into their matrix. Figure 3 shows unloaded NPs that were characterized by spherical morphology.

Based on these results, further formulation studies were carried out in order to efficiently encapsulate magnetite inside the NPs.
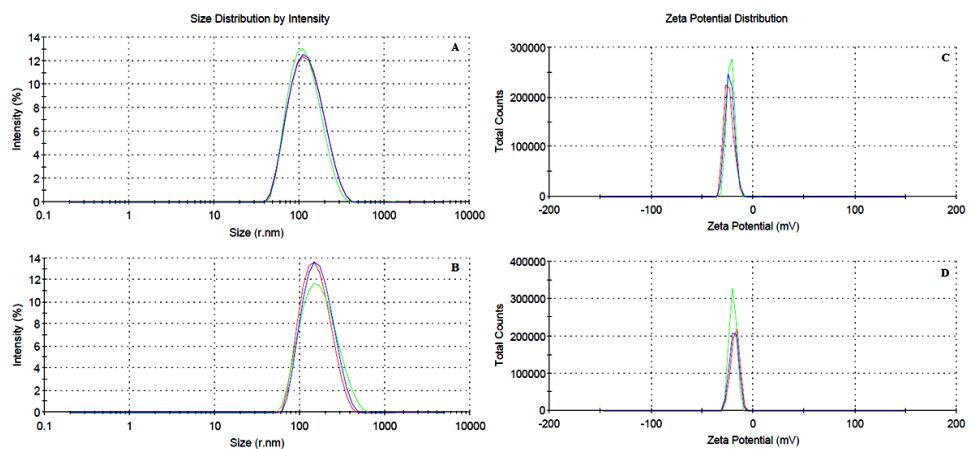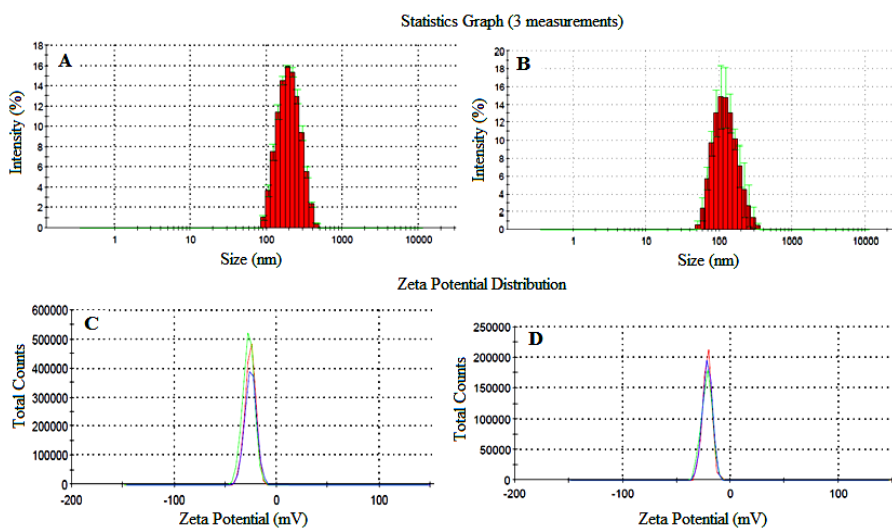
Figure 2: Characterization of PLGA based NPs. Diameter distributions of unloaded NPs (A) and magnetite loaded NPs (B). Z-potential of unloaded NPs (C) and magnetite loaded NPs (D).

Table 1	Particle average size			Z-Potential average
	Distribution	Size ± S.D. (nm)	Polydispersity Index (PI ± S.D.)	(mV) ± Zeta Deviation
Unloaded PLGA based NPs	Monomodal	220 ± 0.5	0.160 ± 0.01	-19 ± 4.14
Fe_3O_4 loaded PLGA based NPs	Monomodal	311 ± 3.8	0.203 ± 0.01	-23 ± 4.26

Table 1: Size analysis, polydispersity index and Z-potential of PLGA based NPs.
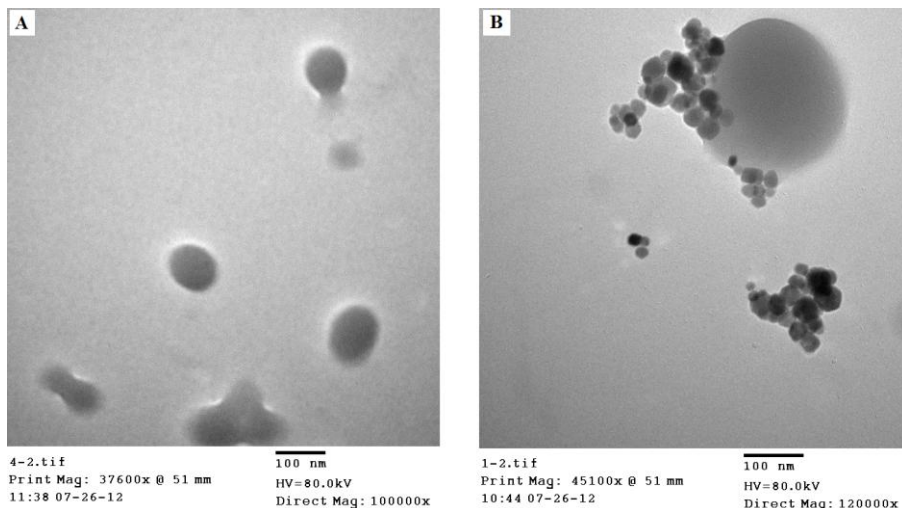
Figure 3: Morphological analyses (TEM micrograph) of PLGA based NPs. Unloaded NPs (A). Magnetite loaded NPs, the black dots represent the magnetite (B).

Preparation of PLGA based nanoparticles by emulsification solvent-evaporation process

The physical-chemical characterizations of the prepared NPs are reported in Table 1. The prepared nanosystems, both unloaded and loaded with magnetite, were characterized by monomodal distribution of diameters and negative Z-potential values (Figure 4). The presence of magnetite did not affect the size and Z-potential of prepared NPs and their size was around of 200 nm with a good PI. Likely, the negativity of Z-potential (below -20 mV) was due to the presence of terminal carboxylic groups on the PLGA chains [22]. PhaP probably did not contribute to the NPs surface charge since the pI of the protein is 5.6 [23] and it is not charged at the pH (~ 5.5) of the NPs formulation.

Table 2	Particle average size			Z-Potential average
	Distribution	Size ± S.D. (nm)	Polydispersity Index (PI ± S.D.)	(mV) ± Zeta Deviation
Unloaded PLGA based NPs	Monomodal	186 ± 8.8	0.101 ± 0.003	-25 ± 5.56
Fe_3O_4 loaded PLGA based NPs	Monomodal	147 ± 46	0.340 ± 0.133	-21 ± 4.55

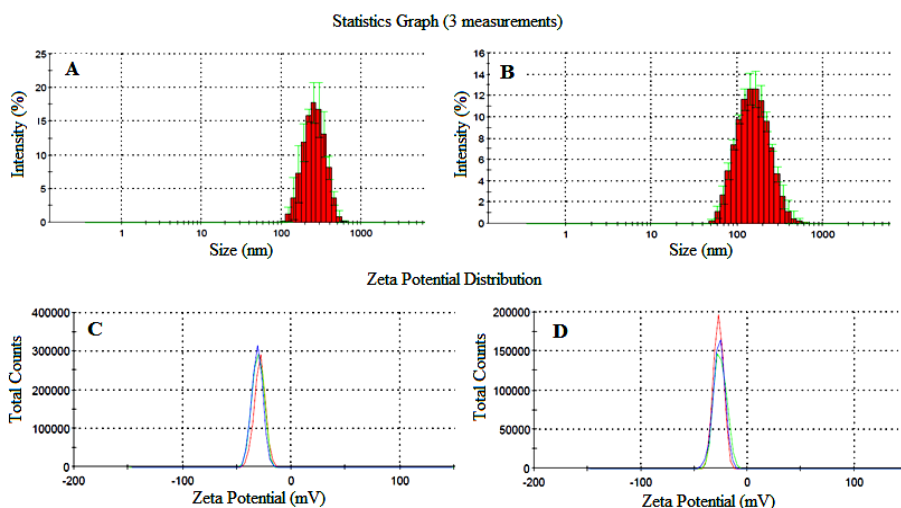**Table 2**: Size analysis, polydispersity index and Z-potential of PLGA based NPs.

Figure 4: Characterization of PLGA based NPs. Diameter distributions of unloaded NPs (A) and magnetite loaded NPs (B). Z-potential of unloaded NPs (C) and magnetite loaded NPs (D).

TEM was used to investigate the morphology of the prepared nanosystems and to gain qualitative information on the encapsulation of magnetite into NPs. Figure 5 shows that the NPs were characterized by spherical morphology and confirmed the presence of magnetite into the core of the particles. Likely, the black dots (Figure 5/B) correspond to the magnetite embedded into the polymer matrix.
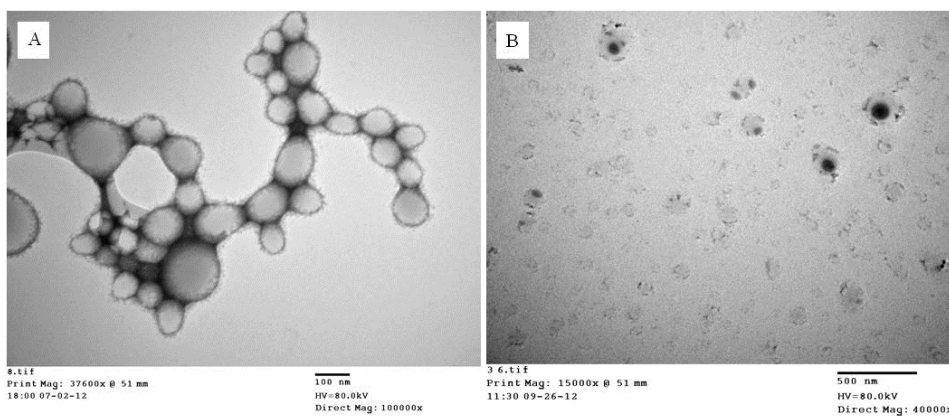
Figure 5: TEM images of PLGA based NPs. Unloaded NPs (A) and magnetite (black dots) loaded NPs (B).

<u>Preparation of PHBHHx based nanoparticles by emulsification solvent-evaporation process</u>

Based on the results obtained by using PLGA, the emulsification solvent-evaporation process was employed for the preparation of PHBHHx based NPs. Table 1 shows the physical-chemical features of prepared NPs. The unloaded and magnetite loaded NPs were characterized by monomodal distribution of diameters (Figure 6A/B) with an average diameter size of 150 nm. The narrow diameter distributions were confirmed by the PI, below to 0.2 in both NPs formulations. The prepared NPs displayed a negative Z-potential value (Figure 6), around -30 mV, which was not affected by the presence of magnetite and PhaP. This confirmed the same trend observed for PLGA based NPs where the presence of PhaP did not contribute to the particles surface charge.

Table 3	**Particle average size**			**Z-Potential average**
	Distribution	Size ± S.D. (nm)	Polydispersity Index (PI ± S.D.)	(mV) ± Zeta Deviation
Unloaded PHBHHx based NPs	Monomodal	140 ± 0.9	0.156 ± 0.02	-30.3 ± 5.03
Fe_3O_4 loaded PHBHHx based NPs	Monomodal	155 ± 1.2	0.185 ± 0.01	-26.7 ± 5.50

Table 3: Size analysis, polydispersity index and Z-potential of PHBHHx based NPs.

Figure 6: Characterization of PHBHHx based NPs. Diameter distributions of unloaded NPs (A) and magnetite loaded NPs (B). Z-potential of unloaded NPs (C) and magnetite loaded NPs (D).

The morphology of PHBHHx based NPs was determined by TEM. Figure 7 shows that the NPs were characterized by spherical shape. In figure 7/B, the presence of black dots could be attributed to the presence of magnetite, that appeared to be homogeneously distributed within the NPs.

Preliminary investigations of the magnetic property of the NPs was carried out by applying an external magnetic field outside of vial containing a suspension of PHBHHx based NPs loaded with magnetite. Figure 8/A shows the suspended NPs in water and Figure 8/B the NPs attracted aside by a magnet.
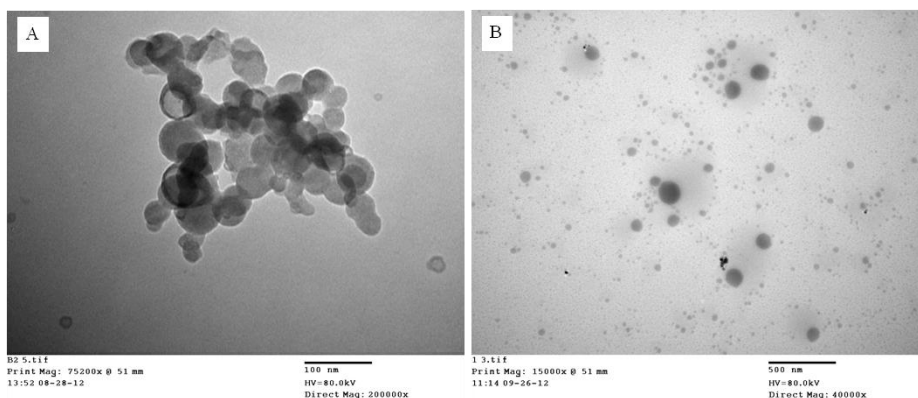
Figure 7: TEM micrographs of PHBHHx based NPs unloaded (A) and loaded with magnetite (B).
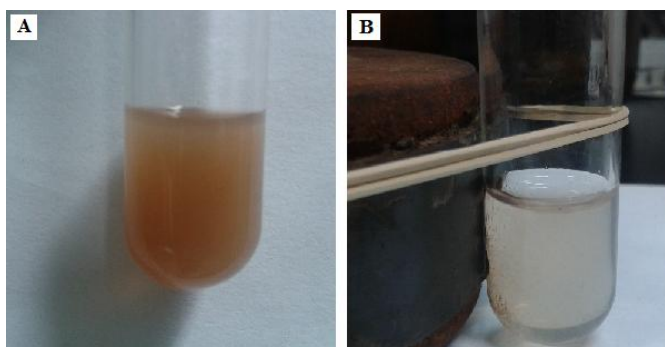
Figure 8: Picture showing the magnetite loaded PHBHHx based NPs dispersed in water (A) and NPs separated from suspension in response to an external magnetic field (B).

5.5. Conclusions

This work focused on the preparation of NPs loaded with magnetite by using the small bacterial protein PhaP as biosurfactant. PLGA and PHBHHx were selected as polymer matrices for NPs preparations. These preliminary results showed that PhaP was essential for NPs preparation. In fact, the formation of NPs did not occur in absence of PhaP. Moreover, PhaP was able to stabilize the NPs based on either PLGA or PHBHHx,

confirming that PhaP binds unspecifically hydrophobic polymers. The emulsification diffusion technique was employed for the preparation of PLGA based NPs. The prepared NPs were characterized by submicron size and good spherical shape. However, by using this method, the magnetite was not encapsulated into the PLGA NPs. Thus an emulsification solvent-evaporation process was investigated for the preparation of colloidal systems based on PLGA or PHBHHx. The developed NPs were preliminary characterized in term of size, Z-potential and morphology. The prepared PLGA or PHBHHx NPs displayed submicron size (below to 200 nm), narrow monomodal diameter distributions and negative Z-potential values. The NPs surface charge was not due to the presence of PhaP, which is not charged at the NPs formulation pH. The presence of magnetite did not affect the physical-chemical characteristics of both NPs formulations. TEM analysis showed that the NPs were characterized by spherical morphology. In addition, the black dots, visible in the TEM images, indicated that magnetite might be loaded into the core of the PLGA and PHBHHx based NPs.

These preliminary results suggest that PhaP can be used as biosurfactant for NPs preparation, reducing the costs of production, since it is easily and cost effectively produced by recombinant microorganisms.

5.6. References

[1] Mora-Huertas CE., Fessi H., Elaissari A. "Polymer-based nanocapsules for drug delivery". International journal of pharmaceutics. 2010;385:113-42.

[2] Colombo M., Carregal-Romero S., Casula MF., Gutierrez L., Morales MP., Bohm IB. "Biological applications of magnetic nanoparticles". Chemical Society reviews. 2012;41:4306-34.

[3] Chomoucka J., Drbohlavova J., Huska D., Adam V., Kizek R., Hubalek J. "Magnetic nanoparticles and targeted drug delivering". Pharmacological Research. 2010;62:144-9.

[4] Bootdee K., Nithitanakul M., Grady B. "Synthesis and encapsulation of magnetite nanoparticles in PLGA: effect of amount of PLGA on characteristics of encapsulated nanoparticles". Polymer Bulletin. 2012;69:795-806.

[5] Nair L., Laurencin C. "Polymers as biomaterials for tissue engineering and controlled drug delivery". Advances in Biochemical Engineering/Biotechnology. 2006;102:47-90.

[6] Chen GQ. "A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry". Chemical Society reviews. 2009;38:2434-46.

[7] Peng Q., Zhang Z-R., Gong T., Chen G-Q., Sun X. "A rapid-acting, long-acting insulin formulation based on a phospholipid complex loaded PHBHHx nanoparticles". Biomaterials. 2012;33:1583-8.

[8] Rezwan K., Chen QZ., Blaker JJ., Boccaccini AR. "Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering". Biomaterials. 2006;27:3413-31.

[9] Kılıçay E., Demirbilek M., Türk M., Güven E., Hazer B., Denkbas EB. "Preparation and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHX) based nanoparticles for targeted cancer therapy". European Journal of Pharmaceutical Sciences. 2011;44:310-20.

[10] Pötter M., Madkour MH., Mayer F., Steinbüchel A. "Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in Ralstonia eutropha H16". Microbiology. 2002;148:2413-26.

[11] Handrick R., Reinhardt S., Schultheiss D., Reichart T., Schüler D., Jendrossek V. "Unraveling the Function of the Rhodospirillum rubrum Activator of Polyhydroxybutyrate (PHB) Degradation: the Activator Is a PHB-Granule-Bound Protein (Phasin)". Journal of Bacteriology. 2004;186:2466-75.

[12] Hanley SZ., Pappin DJC., Rahman D., White AJ., Elborough KM., Slabas AR. "Re-evaluation of the primary structure of Ralstonia eutropha phasin and implications for polyhydroxyalkanoic acid granule binding". Federation of European Biochemical Societies Letters. 1999;447:99-105.

[13] Pfeiffer D., Jendrossek D. "Interaction between poly(3-hydroxybutyrate) granule-associated proteins as revealed by two-hybrid analysis and identification of a new phasin in Ralstonia eutropha H16". Microbiology. 2011;157:2795-807.

[14] Banki MR., Gerngross TU., Wood DW. "Novel and economical purification of recombinant proteins: Intein-mediated protein purification using in vivo polyhydroxybutyrate (PHB) matrix association". Protein Science. 2005;14:1387-95.

[15] Wang Z., Wu H., Chen J., Zhang J., Yao Y., Chen G-Q. "A novel self-cleaving phasin tag for purification of recombinant proteins based on hydrophobic polyhydroxyalkanoate nanoparticles". Lab on a chip. 2008;8:1957-62.

[16] Yao Y-C., Zhan X-Y., Zhang J., Zou X-H., Wang Z-H., Xiong Y-C. "A specific drug targeting system based on polyhydroxyalkanoate granule binding protein PhaP fused with targeted cell ligands". Biomaterials. 2008;29:4823-30.

[17] Backstrom BT., Brockelbank J., Rehm B. "Recombinant *Escherichia Coli* produces tailor-made biopolyester granules for applications in fluorescence activated cell sorting: functional display of the mouse interleukin-2 and myelin oligodendrocyte glycoprotein". BMC biotechnology. 2007;7:3.

[18] Dong Y., Li P., Chen CB., Wang ZH., Ma P., Chen G-Q. "The improvement of fibroblast growth on hydrophobic biopolyesters by coating with polyhydroxyalkanoate granule binding protein PhaP fused with cell adhesion motif RGD". Biomaterials. 2010;31:8921-30.

[19] Wei D-X., Chen C-B., Fang G., Li S-Y., Chen G-Q. "Application of polyhydroxyalkanoate binding protein PhaP as a bio-surfactant". Applied Microbiology and Biotechnology. 2011;91:1037-47.

[20] Lee S-J., Jeong J-R., Shin S-C., Kim J-C., Chang Y-H., Lee K-H. "Magnetic enhancement of iron oxide nanoparticles encapsulated with poly(d,l-latide-*co*-glycolide)". Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2005;255:19-25.

[21] Nidhin M., Indumathy R., Sreeram KJ., Nair B. "Synthesis of iron oxide nanoparticles of narrow size distribution on polysaccharide templates". Bulletin of Materials Science. 2008;31:93-6.

[22] Esmaeili F., Ghahremani MH., Esmaeili B., Khoshayand MR., Atyabi F., Dinarvand R. "PLGA nanoparticles of different surface properties: Preparation and evaluation of their body distribution". International Journal of Pharmaceutics. 2008;349:249-55.

[23] Cai SF., Cai L., Liu HL., Liu XQ., Han J., Zhou J. "Identification of the Haloarchaeal Phasin (PhaP) That Functions in Polyhydroxyalkanoate Accumulation and Granule Formation in Haloferax mediterranei". Applied and Environmental Microbiology. 2012;78:1946-52.

6. <u>Chapter VI: Overall Conclusive Remarks</u>

The present PhD thesis comprises the implementation of different experimental activities performed at the Laboratory of Bioactive Polymer Materials for Biomedical and Environmental Applications (BIOLab) of the Department of Chemistry and Industrial Chemistry of the University of Pisa and those developed during a six months period spent at the Laboratory of Microbiology, School of Life Sciences, Tsinghua University (Beijing, China).

6.1. PLGA based Nanoparticles Loaded with Growth Factors

During this research activity we developed growth factors (GFs) loaded NPs in order to obtain new delivery systems for the treatment of ischemic diseases. In particular, bFGF and VEGF were selected for their essential role in the improvement of new blood vessels formation, and PLGA was selected as a polymer matrix due to its good biodegradability, biocompatibility and low toxicity. Heparin (Hp) and human serum albumin (HSA) were used as stabilizing agents for the NPs preparation. As reported by the literature, GFs present in their architecture an heparin-binding domain and the interaction of Hp with GFs has shown to potentiate its activity and to protect them from heat and enzymatic degradation. HSA was selected as a second stabilizing agent due to its amphiphilic properties. Indeed, the presence of HSA avoids the interaction of GFs with the organic solvent during the NPs formulations, thus protecting them against degradation.

The prepared NPs were characterized by size, morphology and Z-potential values suitable for their future applications. No appreciable differences, in term of the above mentioned features, were observed between the unloaded and GFs loaded NPs. As exception, the Z-potential value of the GFs loaded NPs was significantly decreased confirming the interaction between Hp and GFs. ELISA assays were performed to determine the encapsulation efficiency of bFGF and VEGF into NPs. The results showed that a high encapsulation efficiency, corresponding to 75% and 95% for bFGF and VEGF respectively, was achieved. The successful co-encapsulation of both bFGF and VEGF might result in a synergic proangiogenic activity, important for the future application of these NPs in the treatment of ischemic conditions.

6.2. PLGA based Nanoparticles Loaded with Platelet Lysate

Platelet lysate (PL) is a haemoderivative rich in GFs deriving from the lysis of platelets and it is currently investigated in clinical practice for the treatment of several ischemic conditions. In this research study, we designed and developed PLGA based NPs loaded with PL. The advantage of using PL, instead of recombinant GFs, is that it provides a mixture of autologous GFs in their biological concentration, able to promote vascularization with greater similarity to the natural process. NPs were successfully prepared by a modified solvent diffusion technique, and Hp was used as stabilizing agent for their preparation. The developed nanosystems were characterized by monomodal

distribution of the diameters, submicron size (below to 200 nm), negative Z-potential values and good spherical shape. The results showed a PL encapsulation efficiency and loading of about 75% and 2% respectively. In order to specifically evaluate the effect of Hp on the GF encapsulation efficiency, we selected PDGF-AB as reference. PDGF-AB encapsulation efficiency (EE %) was investigated by ELISA assay, and we observed an increase of EE% from 14% to 30%. The prepared nanosystems displayed good stability in water medium within 4 weeks. The *in vitro* release study, performed by using HSA-FITC as model protein, revealed that under physiological conditions the developed NPs release their proteic content in 13 days. Taking into account all these features, the developed nanosystems can be considered very promising PL delivery systems for the treatment of wound healing. The prepared nanosystems were successfully incorporated into a fibrin matrix in order to prepare an active wound dressing with proangiogenic properties for topical treatments.

6.3. Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based Nanoparticles Loaded with Platelet Lysate

In this study PL loaded Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs were successfully prepared by *co*-precipitation technique. The *co*-precipitation technique is an original procedure developed by the research group involved in the present study. NPs displayed a monomodal distribution of diameters with an average size below 200 nm and good spherical shape. Hp was used as stabilizing agent for the NPs preparation, in order to obtain an improvement of PL encapsulation and stabilization of the dispersions. In fact, NPs showed a PL encapsulation efficiency of 55 %, which was raised to 68 % by adding Hp. The addiction of Hp also improved the PL loading % that rose from 5.6 % to 7.2 %. *In vitro* release studies were carried out by using HSA-FITC as model protein, under physiological conditions, and the results showed that NPs release kinetic was characterized by an initial burst followed by a sustained release of 3 days. PL loaded Poly (Gly$_{25}$-*co*-HPMA$_{75}$) NPs were characterized by excellent proprieties: they were stable in aqueous medium within 4 weeks, and displayed a good cytocompatibility with an IC$_{50}$ higher than 10 mg/ml. Overall the obtained results suggest that PL loaded Poly (Gly$_{25}$-*co*-HPMA$_{75}$) based NPs could be a very promising injectable system for the treatment of ischemic diseases. The developed NPs were successfully incorporated into fibrin matrices in order to obtain subcutaneously injectable systems for the treatment of ischemic conditions, such as peripheral vascular diseases.

6.4. Preparation of magnetic core and polymer shell nanoparticles using an innovative protein biosurfactant

Polyhydroxyalkanoates (PHAs), a class of biodegradable polyesters produced by many bacteria grown under unbalanced conditions, have been proposed in the last years as polymer matrices for the preparation of drug delivery systems due to their excellent biocompatibility. Surrounding the PHAs granules, many proteins with different functions

were found. The function of one of these proteins, phasin P (PhaP), consists in the stabilization of the polymer granules, providing an interface between the hydrophobic granules surface and the aqueous cell environment. Based on this knowledge, in this study we reported some preliminary results on the preparation of magnetite loaded NPs by using PhaP as stabilizing agent. The developed NPs, prepared with two different polymeric materials, PLGA and Poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx) showed that the presence of PhaP was essential for NPs preparation. In fact, the formation of NPs did not occur in absence of PhaP. Both PLGA and PHBHHx based NPs were characterized by submicron size (below to 200 nm), narrow monomodal diameter distributions and negative Z-potential values. TEM analysis showed that the NPs were characterized by spherical morphology and indicated that magnetite might be loaded into the core of the PLGA and PHBHHx based NPs. These preliminary results suggest that PhaP can be effectively used as biosurfactant for NPs preparation.

ACKNOWLEDGEMENTS

APPENDIX

Publications

Paola Losi, Enrica Briganti, Cesare Errico, <u>Antonella Lisella</u>, Elena Sanguinetti, Federica Chiellini, Giorgio Soldani *"Fibrin-based scaffold incorporating VEGF and bFGF loaded nanoparticles stimulates wound healing in diabetic mice".* Submitted to Acta Biomaterialia, 2012

Contributions in National and International Conferences and Workshops

<u>Antonella Lisella</u>, Cesare Errico, Maria Chiara Barsotti, Paola Losi, Angela Magera, Claudio Farina, Rossella Di Stefano, Giorgio Soldani, Federica Chiellini "*Polymeric Nanoparticles for the Controlled Administration of Platelet Lysate"* Workshop on: Nanotechnologies for HealthCare, Trento, Italy, May 25-26, 2012

Maria Chiara Barsotti, Federica Chiellini, <u>Antonella Lisella</u>, Cesare Errico, Silvia Burchielli, Giorgio Soldani , Rossella Di Stefano *"Polymeric Nanoparticles For Controlled Delivery Of Platelet Lysate In Ischemic Tissue"* 73rd National Congress of the Italian Society of Cardiology Rome, December 15-17, 2012