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POLYMER-BASED NANOSYSTEMS FOR THE DELIVERY OF THERAPEUTIC PEPTIDES AND PROTEINS

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Abstract

The utilization of therapeutic proteins and peptides both in the pharmaceutical field and in the field of nanotechnology has dramatically emerged over recent years. The effective and potent action of the proteins/peptides makes them the drugs of choice for the treatment of numerous diseases including diabetes mellitus, cancer, cardiovascular, metabolic, infectious, and neurological diseases. However, they are unstable biomolecules under storage conditions and in biological milieus, they have a short half-life and fragile structure, and their high molecular weight limits permeation through the biological membrane. Hence, several strategies have been developed in order for these limitations to be overcome and finally, effective delivery through various routes of administration have been accomplished. Polymer-based nanosystems are widely utilized for eliciting an immune response and for delivering proteins/peptides therapeutic drugs to the systemic circulation with the desirable pharmacokinetics features and stability at their specific targeting sites. In this dissertation, the natural and synthetic polymeric nanoparticles, as well as the parameters which influence their ability of delivery such as the size, shape, structure, and surface are discussed. Furthermore, several production techniques which play an important role during the design of the nanoparticles are presented. Moving on, this dissertation focuses on the several polymer-based nanosystems and strategies for the delivery of therapeutic proteins and peptides with the simultaneous demonstration of examples from the literature. Finally, there are highlighted the promising up-to-date reviews and clinical trials.

Keywords: polymeric nanosystems, therapeutic protein and peptides, nanoparticles, drug delivery systems, amphiphilic block copolymers, polyelectrolytes, protein therapy, nanocarrier

1. Introduction

Proteins and peptides are the building blocks of life and are now evolving as a very promising brand of therapeutic entities. Both proteins and peptides are comprised of amino acids and held together by peptide bonds. Their basic distinguishing factors are the size and the structure, where peptides are made up of smaller chains of amino acids than proteins. Traditionally, peptides are defined as molecules consisted of between 2 and 50 amino acids, whereas proteins are made up of 50 or more amino acids. They are playing many key roles in the living systems. Proteins are engines of life that perform essential functions inside cells, such as enzyme catalysis, signal transduction, and gene regulation, and maintain a fine balance between cell survival and programmed death. Therefore, the intracellular distribution of functional proteins has important therapeutic inferences in biological applications, including disease therapies, vaccination, and imaging [1].

The foundation for the popularity of proteins as therapeutics was laid down with the regulatory approval of recombinant insulin by the US Food and Drug Administration (FDA) in 1982, which became the first commercially available recombinant protein and a source of major therapy for patients suffering from diabetes mellitus [2]. Nowadays, a variety of therapeutic proteins and peptides have been designed and developed and approved in order for various diseases to be combated and treated, such as diabetes and cancer (Table 1). These potent therapeutics are indicated for several chronic conditions such as cancer, hepatitis, diabetes, rheumatoid arthritis, and leukemia.

Table 1: Recently approved protein and peptide therapeutics [3].

Protein or peptide	Brand name, manufacturer	Year of FDA approval	Route of administration	Half-life	Target disease
Ziv-aflibercept	Zaltrap®, Regeneron and Sanofi	2012	Intravenous infusion	4-7 days	Metastatic colorectal cancer
Ocriplasmin	Jetrea®, ThromboGenics Inc.	2012	Intravitreal injection	Not available	Symptomatic vitreomacular adhesion
Raxibacumab	Abthrax®,	2012	Intravenous	16-19	Inhalational

	GlaxoSmith Kline		infusion	days	anthrax
Belimumab	Benlysta®, Human Genome Sciences, Inc.	2011	Intravenous infusion	19.4 days	Systemic lupus erythematosus
Ipilimumab	Yervoy®, E.R. Squibb & Sons, L.L.C	2011	Intravenous infusion	15.4 days	Unresectable or metastatic melanoma
Belatacept	Nulojix®, E.R. Squibb & Sons, L.L.C	2011	Intravenous infusion	8-10 days	Prophylaxis of organ rejection (kidney transplant)
Brentuximab vedotin	Adcetris®, Seattle Genetics, Inc.	2011	Intravenous infusion	4-6 days	Hodgkin lymphoma and systemic anaplastic large cell lymphoma
Asparaginase Erwiniachryanthemi	Erwinaze, Jazz Pharmaceuticals, Inc.	2011	Intramuscular injection	16 h	Acute lymphoblastic leukemia
Aflibercept	Eylea®, Regeneron Pharmaceuticals, Inc.	2011	Intravitreal injection	5-6 days	Neovascular (Wet) age-related macular degeneration (AMD), Macular edema following central retinal vein occlusion (CRVO)
Velaglucerase alfa	Vpriv®, Shire US Manufacturing Inc.	2010	Intravenous infusion	11-12 min.	Type 1 Gaucher disease
Tesamorelin	Egrifta®, EMD Serono, Inc.	2010	Subcutaneous injection	26-38 min	Lipodystrophy
Tocilizumab	Actemra®, Genen-tech, Inc.	2010	Subcutaneous injection and Intravenous infusion	6.3 days	Rheumatoid and systemic juvenile idiopathic arthritis
Collagenase clostridium histolyti-cum	Xiaflex®, Auxilium Pharmaceuticals, Inc.	2010	Intralesional injection	Not available	Dupuytren's contracture
Alglucosidase alfa	Lumizyme®, Genzyme	2010	Intravenous infusion	2.4 h	Pompe disease

	Corporation				
Denosumab	Prolia®, Amgen Inc.	2010	Subcutaneous injection	25.4 days	Postmenopausal osteoporosis
IncobotulinumtoxinA	Xeomin, Merz Aesthetics. Inc.	2010	Intramuscular injection	Not available	Cervical dystonia
Pegloticase	Krystexxa®, Savient Pharmaceuticals, Inc.	2010	Intravenous infusion	Not available	Chronic gout

However, several pharmaceutical and biopharmaceutical challenges limit their clinical application. Due to the delicate tertiary structure of proteins, they are susceptible to many attacks or neighboring changes and thus many challenges are faced during their release and administration. Proteins are difficult to be utilized as therapeutics entities because of their instability, short half-life, immune responses, and low permeability as a consequence of the, usually negative, charge at blood pH. Additionally, protein delivery to the intracellular space, which is the main site of action, is limited due to the intrinsic properties of many proteins including their large size, varying surface charges and fragile structures. Consequently, they have intrinsic sensitivity to different environmental conditions that come across within the human body including hydrolysis, oxidation, and proteolysis. When they are administered either orally or parenterally, they can be easily degraded by enzymes in the gastrointestinal tract (GIT), have difficulty permeating across gastrointestinal mucosa, and be eliminated during first-pass hepatic clearance (Figure 1). Hence, it is essential to design efficient protein delivery nanocarriers that can have improved therapeutic efficacy and also control protein release. Thus, it has been suggested that encapsulation within biocompatible matrices can protect therapeutic proteins from premature denaturation and subsequent loss of effectiveness, while simultaneously reducing their immunogenicity and systemic toxicity [4].

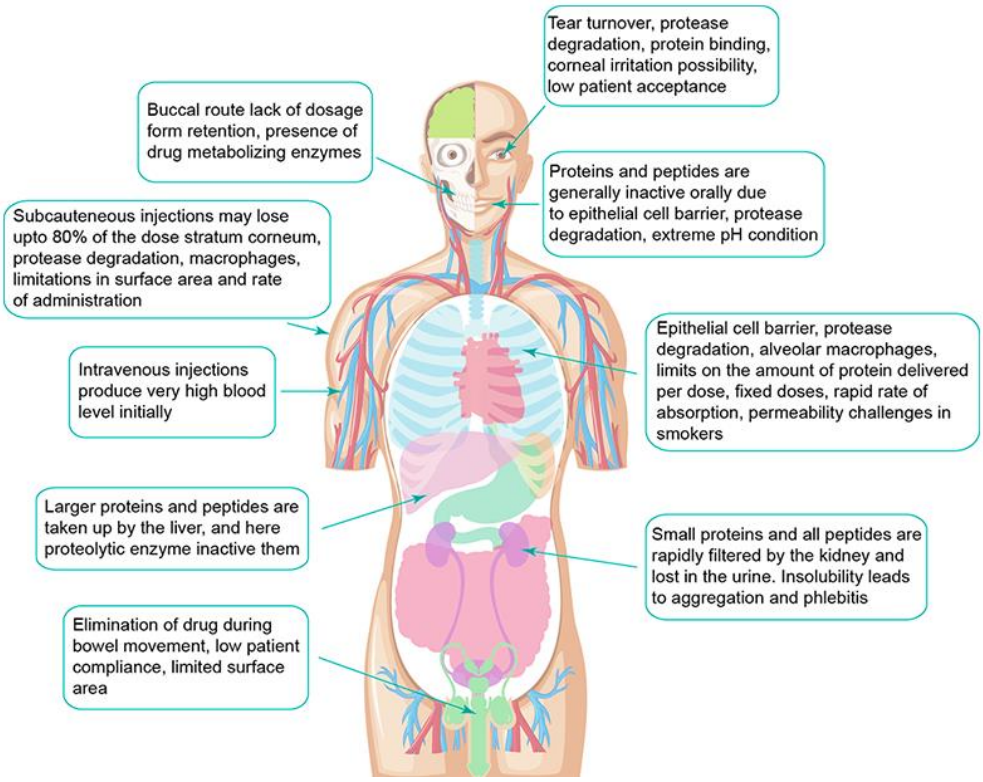

Figure 1: Challenges of invasive and non-invasive protein delivery [1].

Several strategies have been shown to improve the current limitations of therapeutic peptides and proteins. This can be accomplished either by a change in the agent itself (e.g., mutations in protein structure or covalent attachment of moieties) or by a change in formulation. Another efficacious perspective on protein structure modification contains the covalent chemical attachment of compounds such as poly(ethylene glycol) (PEG) or polysialic acid (PSA). Last but not least, synthetic, natural or composite materials at the size of nanoscale have been investigated as vehicles for the delivery of proteins due to their ability to facilitate intracellular uptake. Various desired subcellular compartments, such as the cytosol, the mitochondria and the nucleus are also some obstacles that the vehicle needs to overcome (Figure 2).

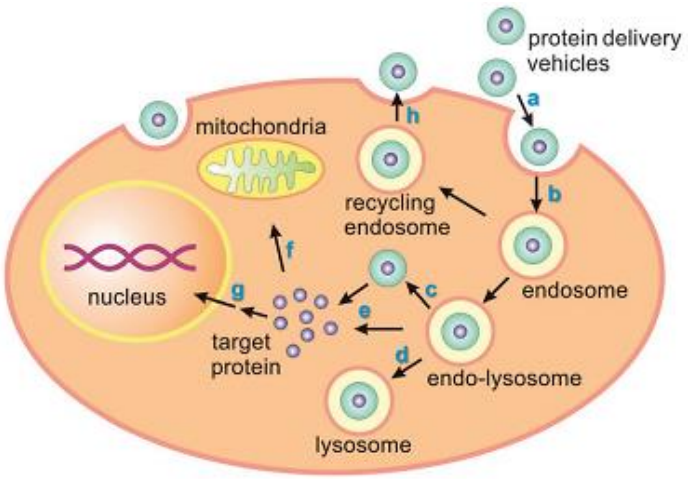

Figure 2: Schematic process of a typical endocytic pathway for delivery vehicles with protein cargoes. (a) Cell-surface attachment of protein delivery vehicles; (b) internalization of delivery vehicles via endocytosis; (c) endosomal escape of delivery vehicles or (d) lysosomal degradation; (e) target protein diffuses into cytoplasm; (f) transport of target proteins to specific organelle; (g) participation in cellular functions such as signal transduction; (h) exocytosis of delivery vehicles [5].

Therefore, the delivery vehicle must be able to escape the endosomal pathway to avoid being trafficked through endomembrane compartments and being subject to clearance and degradation under harsh lysosomal conditions. Among the novel drug delivery systems, polymeric nanoparticles and microspheres have shown a degree of success for eliciting an immune response and for delivering proteins to the systemic circulation with the desirable pharmacokinetic profile at their specific targeting sites [5], [6].

The remarkable scientific interest regarding the delivery of therapeutic proteins and peptides by utilization of polymer-based nanosystems as vehicles, lead to the development of the existing works. The aim of this study is to focus on the recently in vitro and in vivo studies of such nanosystems. Besides, an up-to-date survey of representative examples of different types of the developed polymer based nanosystems will be presented by illustrating their benefits and limitations, the administration routes of these therapeutic entities and the biological barriers that come across. Finally, existing products in the market will be also discussed.

2. Theory

2.1 Polymeric Nanoparticles

Polymers are substances of high molecular weight, and they are consisted of repeated units called monomers that are connected onto a long chain. They have unique properties depending on the type of molecules being incorporated and the way they are bonded within polymer chains. Polymer molecules can be either linear or branched, while the linear or the branched chains can be linked via covalent bonds. In addition, polymers do not form perfect crystals but have semicrystalline and amorphous domains. The crystal structure of a polymer and thus its behavior is closely connected to the melting temperature Tm and the glass transition temperature Tg (g: glass) which delimits two different behaviors for the amorphous polymer. In a temperature lower than the Tg, polymer chains are rigid-immobile, and the polymer is glassy, stiff and fragile, while in a temperature above the Tg, the polymer is soft, flexible and characterized by viscoelasticity. Polymer properties are depending on the way that monomers are connected to each other and their overall behavior that is directly related to their chemical structure, are important features for the development of pharmaceutical formulations [7].

Polymers have emerged as vehicles for the incorporation of drugs and their release to the human body because they can be characterized by biocompatibility and bioavailability while maintaining the therapeutic efficacy of the drug. For these purposes, polymers are constructed to the micro and nano scale with the aim to overcome the limitations during the administration and the protection of the encapsulated substances. The size of nanoparticle (NP) formulations for drug delivery should be considered within 10–1000 nm, since the nanoformulation contains a carrier and an active pharmaceutical ingredient. They are non-toxic colloidal particles prepared from synthetic or natural polymers via different strategies and they can form aggregates. Polymers are divided to natural and non-synthetic where the natural polymers can be completely broken down by microorganisms, thus are defined as biodegradable and biocompatible and they also display low toxicity whereas non-synthetic ones are usually hydrophobic and

chemically and mechanically stronger in nature compared to their non-synthetic counterparts. This mechanical strength reduces the degradation rate of the polymer, thereby providing the biomaterial with excellent durability (Figure 3) [8].

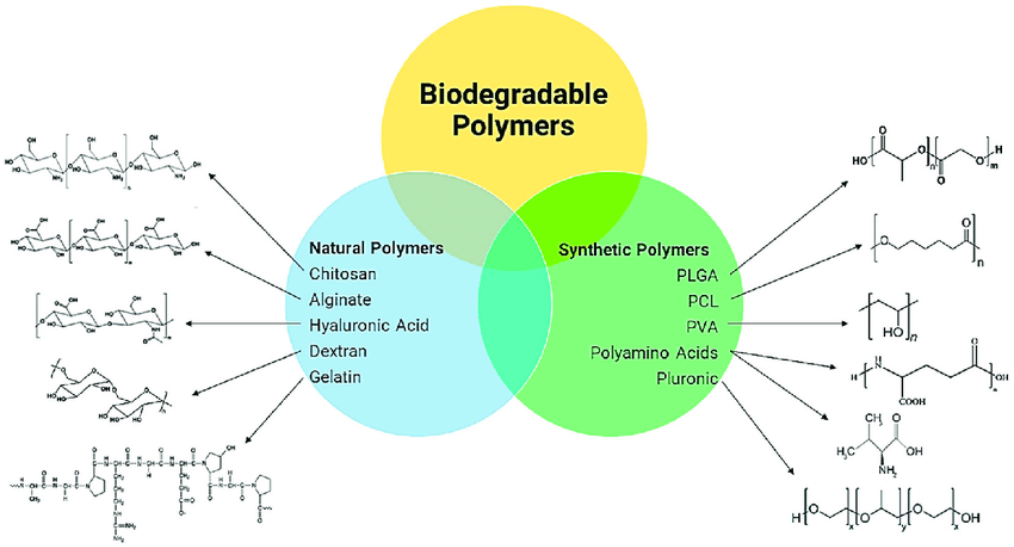

Figure 3: Biodegradable polymers employed in the delivery of nano-insulin formulations [8].

In particular, NPs containing charged polymers are preferred for many applications as they provide gentle protection to the encapsulated drug through electrostatic interactions. In addition, polymer nanosystems protect the drug from possible interactions with regular precipitation medicines which gives adverse drug-drug interactions. Consequently, fragile proteins and peptides can also be incorporated into the polymer shell, as they not only enable the targeted delivery to a specific organ or tissues but also facilitate their cellular penetration and endolysosomal escape.

2.1.1 Natural Polymers

Natural polymers have significant benefits due to their ability to deliver therapeutic proteins to the target sites and thus different types of proteins have successfully incorporated into them. The importance of their use in drug delivery systems is the presence of reactive sites which help in cross-linking, ligand conjugation, and various other modifications that make these polymers appropriate vehicles for a wide range of delivery of therapeutic proteins. Natural polymers have

many advantages in correlation with synthetic polymers due to their natural resources, being inexpensive, and having the capability of being easily modified chemically. Natural polymeric nanoparticles are commonly synthesized from chitosan, alginate, hyaluronic acid, dextran and proteins, such as gelatin which are used as vehicles. In the following sub-sections, some of the main natural polymers will be briefly discussed.

2.1.1A Chitosan (CS)

Chitosan (CS) is a naturally occurring polysaccharide that is composed of glucosamine and N-acetylglucosamine residues derived from partial deacetylation of chitin, which is a natural biopolymer derived from crustacean shells. The primary unit in the chitin polymer is 2-deoxy-2-(acetylamino) glucose. These units combined by β-(1,4) glycosidic linkages, forming a long chain linear polymer. Although chitin is insoluble in most solvents, chitosan is soluble in most organic acidic solutions at pH less than 6.5 including formic, acetic, tartaric, and citric acid. Thus, the increasing solubility under acidic conditions is useful for oral drug delivery, but the low solubility under physiological pH possesses some limitations. Chitosan is a cationic, biocompatible, and biodegradable non-toxic polymer that has many biomedical applications and is recommended as a suitable candidate for delivering genes, proteins and drugs, as it has been FDA-approved because of its enhancement of the intestinal absorption of large molecular weight therapeutic proteins by increasing paracellular permeability. Furthermore, it promotes the absorption of large molecular weight therapeutic proteins through intestinal epithelial mucosa. Generally, due to its mucoadhesive nature, CS has been used as a vehicle to deliver drugs to nasal, ocular, buccal, and pulmonary tissues [9].

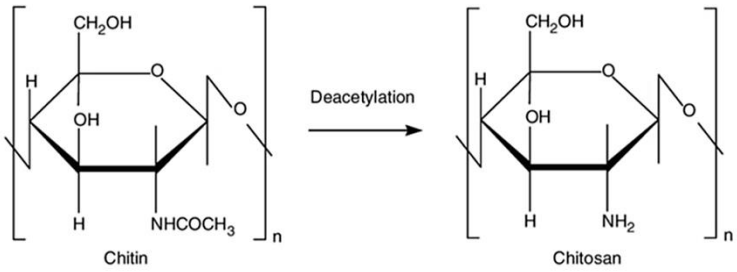

Figure 4: Chitin and Chitosan structure [10].

Chitosan-based nanoparticles have been primarily used for the delivery of low molecular weight proteins and peptides. The CS-nanoparticles are synthesized by emulsion, coacervation/precipitation, ionic gelation, reverse micellar methods etc. The problem of poor water solubility of CS under physiological conditions, which is required for efficient delivery of drugs, is usually solved by chemical modification of CS and includes quaternization, alkylation, acetylation, carboxymethylation, CS/polyol salt combinations, synthesis of N-trimethyl CS, generation of sugar-bearing CS, conjugation with polyethylene oxide, generation of glycol-CS, etc. For the encapsulation of hydrophobic substances, amphiphilic CS derivatives were synthesized.

Han et al., prepared a practical chitosan-based protein carrier, which was a novel cationic copolymer composed of methoxy poly(ethylene glycol)-chitosan-poly (L-lysine) (mPEG-CS-PLL). The mPEG-CS-PLL NPs were prepared under mild conditions by using tripolyphosphate (TPP) as the cross-linker. As a model protein bovine serum albumin (BSA) was used and encapsulated in the mPEG-CS-PLL nanoparticles by electrostatic binding. The copolymer showed very high encapsulation efficiency and loading capacity for BSA loading which was, respectively, up to 78% and 42%. In addition, cell viability tests against L929 cells showed that the mPEG-CS-PLL copolymers had very low cytotoxicity, which made these nanoparticles safe and effective carriers for protein delivery, as well as, favorable nanosystems for practical applications [11].

2.1.1B Alginate

The natural polymer, alginate, has been recently used as a material for protein and peptide drugs, it has acclaimed permission from Food and Drug Administration (FDA) for human use and has attracted increasing attention due to its excellent biocompatibility, mucoadhesive biodegradability, and mild gelation conditions. It is also known as align and/or alginic acid and is a linear anionic polysaccharide that is widely distributed in the cell walls of brown algae. It is mainly extracted from three different species of brown algae and is composed of alternating blocks of 1–4 linked a-L-guluronic and b-D-mannuronic acid residues. Alginate is a biodegradable and biocompatible copolymer of guluronic acid and mannuronic acid and due to its

requirement for mild processing conditions in aqueous media, it is the preferred material for the delivery of heat-sensitive therapeutic proteins. In addition, alginate has demonstrated the ability to protect fragile proteins and peptides from the acidic environment of the stomach to be safely delivered to the intestine. The high gel porosity which allows for high diffusion rates of macromolecules, the ability to control this porosity with simple coating procedures, and the dissolution and biodegradation of the system under normal physiological conditions are also some of the important properties of alginates that are used as matrixes for the delivery of proteins. Even though alginate has been widely used for the incorporation and protection of pH-sensitive labile proteins, it has some limitations as a protein carrier system, including drug loss during preparation of beads and/or leaching of the drug through the pores in beads [12], [13].

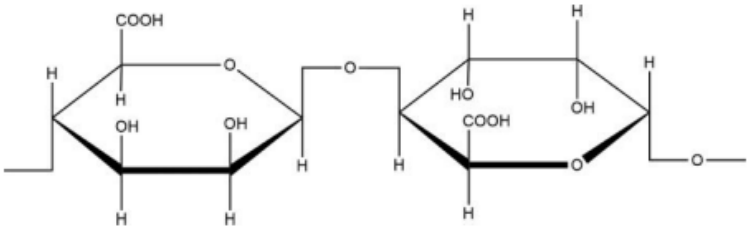

Figure 5: The chemical structure of alginate constitutes of random sequences of chains of β-D-mannuronic and a-L-guluronic acids [12].

Gombotz and Wee have reviewed the encapsulation of therapeutic proteins and peptides using alginates alone and/or with other copolymers. The degree of flexibility of alginate, which contains the chemistry and the relatively mild crosslinking conditions of it, has enabled this naturally biopolymer to be used for the encapsulation of active agents including proteins, cells and oligonucleotides. According to their publication, it is important to select the proper alginate type, the gelation conditions, added excipients, and coating agents, the appropriate matrices and pore size, the water content and the dehydration rates in order to fabricate the preferable nanoparticle system for the delivery of therapeutic peptides and proteins, with an active delivery period ranging from minutes to months [14].

2.1.1C Hyaluronic Acid

HA is a linear anionic polysaccharide that is comprised of N-acetyl-D-glucosamine and D-glucuronic acid disaccharide units, held by alternating b-(1, 3) b-(1, 4) glucosidic linkages. HA can encapsulate proteins in a well-hydrated environment, and thus can protect the structure of proteins effectively. HA is both biocompatible and biodegradable, in addition to having low immunogenicity but compared to alginate and chitosan, hyaluronic acid is negatively charged and due to its stereochemistry, it is energetically stable. In addition, it can be chemically modified by cross-linking, grafting, linking with hydrophobic substances and drugs, or through polyion complex formation with oppositely charged polysaccharides, proteins or surfactants. Its interpenetrating networks produce self-assembled aggregates, nanoparticles and gels [15].

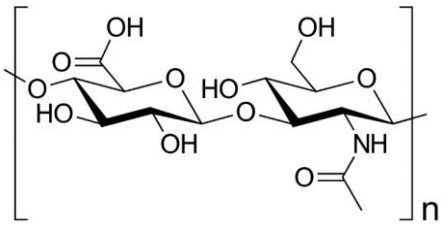

Figure 6: The chemical structure of hyaluronic acid (HA) [15].

Chen et al. have investigated polymeric nanogels for the delivery of therapeutic proteins for cancer therapy in vivo. In their experiments they utilized bioresponsive fluorescent photo-click hyaluronic acid (HA) nanogels, in which two intracellular protein drugs, cytochrome C (CC) and granzyme B (GrB) were loaded. They prepared HA nanogels (NGs) from two HA derivatives, i.e., HA-cystamine-methacrylate (HA-Cys-MA) and HA-lysine-tetrazole (HA-Lys-Tet), by combing inverse nanoprecipitation and “tetrazole-alkene” photo-click reaction. In addition, HA NGs show intrinsic targetability to CD44 positive malignant cancer cells such as human breast and lung tumor cells (MCF-7, A549), human multiple myeloma (LP1), and acute myelogenous leukemia (ALM2). According to their results, cytochrome C and granzyme B-loaded HA NGs could efficiently target and release proteins to CD44 overexpressing MCF-7 and A549 cancer cells. This means that, these protein-based HA nanogels yielded impressive antitumor effects with a half-maximal inhibitory

concentration thousands of times lower than clinical chemotherapeutics. Therefore, this nanoplatform represents a promising vehicle for efficient and safe targeted delivery of intracellular anticancer protein therapeutics [16].

2.1.1D Dextran

Dextran is a non-toxic and highly water-soluble exocellular bacterial polysaccharide. It mainly contains linear a-1,6-linked glucopyranose units with some degree of 1,3-branching. The sucrose-rich environment of Lactobacillus, Leuconostoc, and Streptococcus is the main source of its production and commercially it is available with different molecular weights. Furthermore, the physicochemical properties of dextran are affected by the degree of branching and molecular weight. Dextran is known to have a wide range of therapeutic applications, as it can be biocompatible with incorporated proteins. Nevertheless, low molecular weight dextrans have a short biological half-life, which is approximately 8 hours, and are secreted from the kidneys, while high molecular weight dextrans exhibit longer half-lives and are subsequently degraded by the reticuloendothelial system. Additionally, dextrans are metabolized by enzymes (a-1-glucosidases) in various parts of the body. Dextran-based systems can be obtained either by chemical and/or chemical cross-linking and due to the presence of -OH groups they allow for chemical manipulations to take place. Up to today, a lot of therapeutic proteins have been successfully incorporated in dextran-based carrier systems and significant therapeutic outcomes have been obtained either from in vitro or in vivo experimental studies [17].

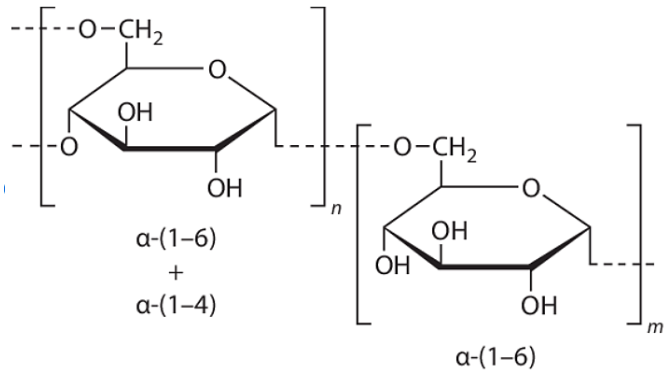

Figure 7: The chemical structure of dextran [17].

Researchers in Iran prepared a copolymer of dextran–poly-lactic-co-glycolic acid (PLGA) nanoparticles for the oral administration of insulin, which was carried out by blending an aqueous solution of insulin with the copolymer and various-sized polymersomes were formed due to the self-assembly properties of the copolymer. The results showed that at a 10:3 dextran–PLGA to insulin ratio, the NPs had an encapsulation efficiency and loading capacity of 90% and approximately 30%, respectively. The in vitro permeability of dextran– PLGA NPs was greater than that of free insulin, as well as the bioavailability of them was greater than that of free insulin, at 9.77% and 0.62%, respectively [18].

2.1.1E Gelatin

Gelatin is a protein polymer that is widely used in biomedical applications due to its biodegradable, biocompatible and nontoxic properties. Gelatin is a biopolymer that is prepared by thermal denaturalization of collagen, which is available in animal skin and bones in the presence of dilute acids. Gelatin consists of many glycine, proline, and 4-hydroxy proline residues. Furthermore, it has multiple functional groups, allowing for a lot of chemical manipulations, as well as because of being of protein nature, it allows easy modification on the amino acid level. It has hydrophilic properties, is a polyampholyte and the physical and chemical modifications to gelatin depend on the crosslinking degree. There are large numbers of functional groups on the backbone of gelatin that can be used for chemical modification or conjugation of ligands [8], [19].

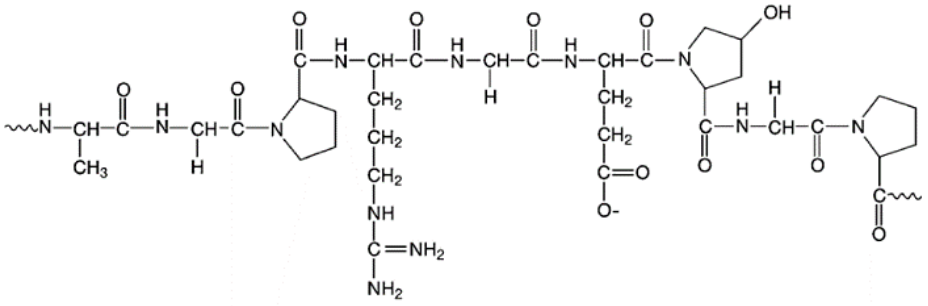

Figure 8: Basic chemical structure of gelatin [20].

Zhao et al. modified gelatin nanoparticles with D,L-glyceraldehyde and poloxamer 188 for pulmonary administration of insulin for the treatment of

diabetes. Novel water-in-water emulsion technique was used to prepare insulin-loaded nanoparticles and the negatively charged insulin was bound to them through electrostatic interaction with electro-deficient D,L-glyceraldehyde. According to the results on animal experiments, it was demonstrated that insulin-loaded NPs under gelatin–poloxamer 188 ratio at 1:1 exhibited a prolonged hypoglycemic effect and enhanced pharmacological bioavailability and reduced insulin deposition within the lung which was more favorable for lung health [19].

2.1.2 Synthetic Polymers

Synthetic polymers are generally hydrophobic and chemically and mechanically stronger in nature compared to their non-synthetic counterparts. They are characterized by excellent durability due to the reduction of the degradation rate of the polymer caused by mechanical strength. Consequently, they are mainly used for pharmaceutical applications because they have consistent quality and low immunogenicity. They can also enhance various pharmacokinetic and circulation characteristics such as prolonged half-life in blood plasma and protection from proteolytic enzymes. By combining synthetic and natural polymers, researchers are able to manipulate properties to achieve superior protein delivery systems with enhanced therapeutic efficacy. Some of the synthetic polymers that are mainly used are PLGA (Poly-Lactic-co-Glycolic Acid) copolymers, polyacrylates, poly(caprolactone)s (PCLs), polyamino acids and pluronics.

2.1.2A Poly-Lactic-co-Glycolic Acid

Poly-Lactic-co-Glycolic Acid (PLGA) is one of the most known carriers for drug encapsulation and is used for its controlled release kinetics. Additionally, when hydrolysis takes place, PLGA breaks down and generates glycolic acid and lactic acid, which are metabolized naturally by the body. PLGA and polylactide (PLA) belong to the group of FDA-approved polyesters, have excellent biocompatibility and biodegradability, and are thus frequently used for protein delivery. PLGA is usually copolymerized with other polymers due to its physical instability causing initial burst release of encapsulated proteins.

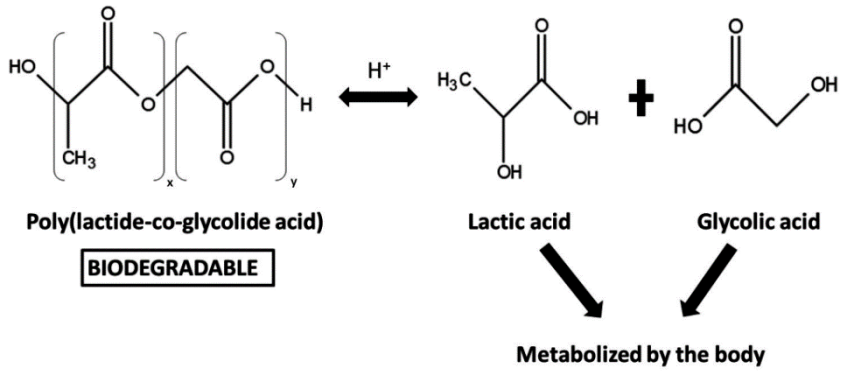

Figure 9: Biodegradability of PLGA based on the hydrolysis of the copolymer**.** [Adapted from https://www.nanovexbiotech.com/858-2/].

Wang and his colleagues prepared cationic micelles of amphiphilic copolymers in order to enhance the bioavailability of therapeutic protein and improve the stability of storage and delivery. The amphiphilic copolymer consisted of cholic acid (CA) initiated poly(D,L-lactide-co-glycolide) (CA-PLGA) and water-soluble polyethyleneimine cross-linked polyethylene glycol (PEI-PEG) denoted as CA-PLGA-b-(PEI-PEG) and prepared through the self-assembly method. The final self-assembled cationic CA-PLGA-b-(PEI-PEG) micelles were featured with a hydrophobic CA-PLGA "core" and the "shell" of water-soluble PEI-PEG. The "shell" of PEI-PEG provided the micelles with a strong positive nature, and it can interact with negatively charged proteins. The therapeutic protein investigated was insulin and was complexed with the copolymer via electrostatic interactions to obtain nanoscale micelle/insulin complexes-loaded CA-PLGA microspheres (MIC-MS). According to the results of the in vitro experiments, the cationic micelles of MC-1 (with a weight ratio of PEI/PEG equal to 0.93) can improve the insulin release kinetics and increase the bioavailability of insulin in CA-PLGA MS effectively. In animal experiments, the MIC-MS exhibited a more sustained and prolonged hypoglycemic effect on n streptozotocin-induced diabetic rats in comparison with that of INS-MS (insulin-microspheres) [21].

2.1.2.B Poly-ε-caprolactone (PCL)

Poly-ε-caprolactone (PCL) is a synthetic polymer that is degraded by hydrolysis of its ester linkages in physiological conditions, like those in the human body, and has consequently received a great deal of attention for use in drug delivery. It is a

hydrophobic, biodegradable, semi-crystalline polymer and is prepared by ring-opening polymerization of ε-caprolactone using a catalyst such as stannous octanoate. Generally, it is especially interesting for the preparation of long-term implantable devices, owing to its degradation slower than that of polylactide. The main synthesis method of PCL nanoparticles is nanoprecipitation, solvent displacement, and solvent evaporation. Except for the ability of PCL for hydrolysis under physiological conditions, the suitable solubility of this polymer, its low melting point, and amazing blend-compatibility are some other features that have encouraged studies regarding its possible uses as a vehicle for protein and drug delivery [8], [22].

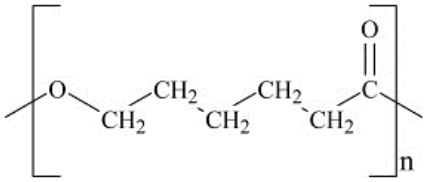

Figure 10: Structure of polycaprolactone [23].

Nomani et al., aimed to develop polymesomes using biodegradable copolymers for delivery of bovine serum albumin (BSA) as a model protein. Methoxypoly (ethylene glycol) poly (ε-caprolactone)/(mPEG-PCL) copolymers which are recyclable and biocompatible self-assembled into polymersomes and BSA was encapsulated into them and this way, BSA-loaded mPEG-PCL polymersomes were formed. The results demonstrated that factors including the ratio of drug to polymer and copolymer composition had a significant effect on the particle size, morphology, and encapsulation efficiency of the BSA-loaded polymersomes. Furthermore, the protein, BSA, was efficiently encapsulated up to 92% and its release from polymersomes showed the classic triphasic profile. Overall, mPEG-PCL polymersomes could be considered promising carriers for protein encapsulation and release [24].

2.1.2.C Polyvinyl Alcohol (PVA)

Polyvinyl alcohol (PVA or PVOH, or PVAI) is a biodegradable and biocompatible polymer which is characterized by low toxicity and thermal stability. Polyvinyl acetate can be formed from the radical vinyl polymerization of the monomer vinyl

acetate and consequently, polyvinyl Alcohol (PVOH) is synthesized by the reaction of polyvinyl acetate with NaOH and methanol. PVA has a high level of mechanical strength and is easy to prepare. Additionally, it has the ability to be blended with natural polymers, and thus novel drug delivery systems can be synthesized with improved and enhanced features than those that have each one separately. PVA is commonly synthesized and used as hydrogel by physical and chemical cross-linking and in combination with other polymeric nanoparticles. In order to avoid toxicity and residual problems of the chemical cross-linking molecules, PVA hydrogels are formed with the physical repeated freeze–draw method. The hydrogels formed by this method are stable, highly elastic, and non-degradable at room temperature [25].

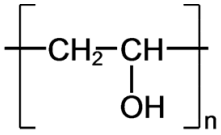

Figure 11: Chemical structure of poly(vinyl-alcohol) (PVA) [26].

Rewet et al., prepared PVA nanoparticles via solid-in-oil-in water (S/O/W) emulsification method while optimizing the surfactant in an aqueous phase medium. As far as the results of this study are concerned, high-molecular-weight PVA validated good chemical and physical properties for the stabilization and protection of insulin. Further evidence has been gained via animal studies by retaining insulin bioactivity, as well as by showing hypoglycemic effects. This study also emphasizes the importance of understanding and selecting surfactant system in optimum concentration for successful formulation development. Hence, PVA can also be utilized as a stabilizing surfactant in the manufacture of nanoparticles for oral insulin administration [27].

2.1.2.D Pluronics

Pluronic® block copolymers, which are also known with the name of “poloxamers”, are triblock copolymers that are amphiphilic in nature. Pluronic is consisted of ethylene oxide (EO) and propylene oxide (PO) blocks arranged in a basic A-B-A structure: EO_x-PO_y-EO_x. These arrangements lead to the development of an amphiphilic copolymer, in which the number of hydrophilic EO(x) and hydrophobic PO(y) units can be changed. In other words, they are made up of polypropylene

oxide with polyethylene oxide blocks on either side (PEO-PPO-PEO). Moreover, pluronic block copolymers are synthesized by sequential addition of PO and EO monomers in the presence of an alkaline catalyst, such as sodium or potassium hydroxide. Pluronics are tasteless, odorless, and waxy white granules that have thermosensitive gelling properties and are biocompatible. They are categorized based on their physical state (i.e., solid, paste, or liquid form) and their molecular weights [28].

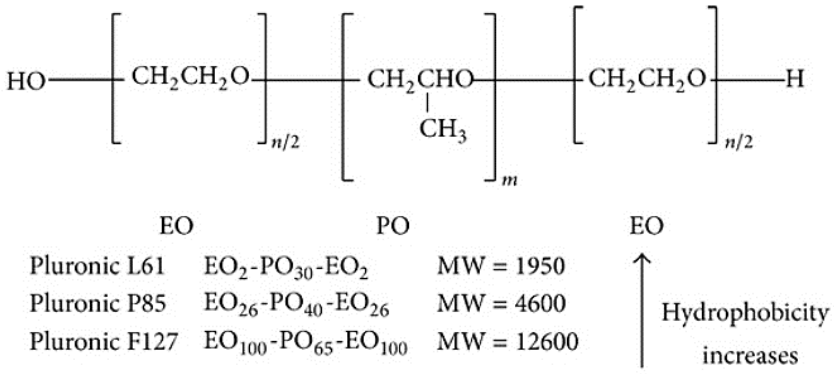

Figure 12: Pluronic block copolymers available from BASF (Wyandotte, MI, USA), contain two hydrophilic EO end blocks and a hydrophobic PO middle block [28].

Pluronic F127 block copolymer (PF127) has been recognized as an important safe biomaterial that is FDA-approved and can be used for the efficient delivery of therapeutic proteins and peptides against various diseases. Firstly, and foremost, PF127 maintains the thermostability of incorporated proteins. Due to its thermo-reversible characteristics, the aqueous solution of PF127 at and/or critical gelation concentration (CGC) remains in a liquid state at room temperature and rapidly converts into semi-solid, rigid gel at body temperature. Thus, PF127 is easily administered into the body via the parenteral route because of this sol-gel transition characteristic. PF127 has been extensively studied for the sustained release of a large number of pharmaceutical ingredients. Dasal et al., conducted a study with the aim to optimize the controlled buccal delivery of insulin loaded in PF127-based thermosensitive gel using SD-rats, as the experimental animal model. They assessed the optimized formulation with three different doses of insulin (10, 25, and 50 IU/kg)

having the potential for buccal delivery of basal insulin in SD-rats. The optimized formulation without insulin did not show any influence on the plasma levels of glucose, and thus it is referred that it is probable that the excipient had no effects on plasma levels of insulin and glucose. Nevertheless, these three doses of insulin loaded in optimized formulation produced considerable hypoglycemic effects in a dose-dependent manner that lasted for 8 hours as compared to the subcutaneously administered insulin alone. Additionally, the observed results and responses were in close agreement with the predicted values of the optimized formulation. The optimization technique in developing buccal Pluronic F-127 gel formulation could be considered as feasible for the administration to a patient with improved compliance and maintenance of basal insulin [29].

2.2 Design of polymeric nanoparticles

The ability of polymeric nanoparticles (PNPs) to achieve a targeted drug delivery is influenced by its size and shape, molecular weight, and surface charge. Generally, polymeric nanoparticles need to remain in systemic circulation in order to achieve the targeted delivery, the encapsulated drug to be absorbed through the first-pass effect, and elimination to be avoided. At the same time, nanoparticles need to maintain their surface charge. The size of polymeric nanoparticles also affects their ability to cross physical barriers and arrive at the target site. To achieve a prolonged release of the drug in the systemic circulation, a higher molecular weight of the polymer is required. To conclude with, these factors can influence the stability of the nanoparticles, targeting specificity, protein release kinetics, and thus the therapeutic efficacy and tissue distribution.

2.2.1 Size

The size of a nanoparticle, which can now be engineered to precise dimensions and high monodispersity, is an important design parameter that can be tailored for purposes of directing particle distribution in vivo. Size leads several biological phenomena with discrete cut-off size ranges that include circulation half-lives, extravasation through leaky vasculature and macrophage uptake. For instance, nanoparticles with diameters approximately less than 5 nm rapidly undergo renal clearance upon intravenous administration. Furthermore, nanoparticles between 10

and 20 nm are excreted from hepatic clearance, which is a route of excretion for those that undergo renal clearance. Therefore, it is widely known that as the size of a nanoparticle decreases, so the toxicity behavior is increased. In most published studies, spherical NPs are used with a diameter of approximately 200 nm based on the amount of cellular internalization [30]. He et al. investigated the effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. According to the review, they prepared modified chitosan NPs (150-500nm) for cellular uptake. NPs with a diameter of 150 nm tended to accumulate in tumor cells much more efficiently compared with larger diameter NPs. This is because larger NPs require stronger driving forces and more energy for cellular uptake [31].

2.2.2 Shape

The geometry of nanoparticles with distinct morphologies plays a key role in the design of their shape due to the fact that it may affect hemorheological dynamics, cellular uptake, and in vivo fate. The most typical morphologies of polymer nanoparticles are spheres, capsules, or amorphous structures. Nanocapsules are empty shells that the drug can be confined into a cavity consisting of a liquid core surrounded by a solid material shell, while nanospheres are matrixes without a cavity, the entire mass is solid in which the drug can be dispersed. Various shapes of polymeric NPs have been prepared including polymersomes, micelles, dendrimers and solid polymeric spheres (Figure 13) [22]. The shape of nanoparticles affects their in vivo intracellular delivery and thus the extracellular transport events such as circulation, extravasation, and tissue penetration. To name an example, Gang et al. have demonstrated that filamentous polymer micelles, which are also called filomicelles, have longer-circulating lifetimes of over one week from administration, compared with spherical particles with 2–3 days, owing largely to the tendency of these particles to align with blood flow [32].

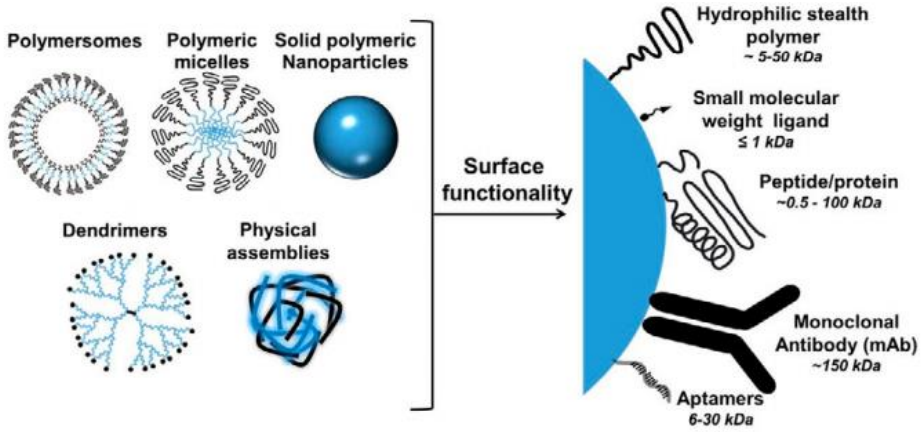

Figure 13: Different types of polymeric NPs and commonly used surface functionalities and their average molecular weights [33].

2.2.3 Internal Structure and Connectivity

Besides the size and the shape that are crucial factors for the design of a polymeric nanosystem, due to their feature of recognition as foreign particles from the RES (reticuloendothelial system) and clearance rapidly from the blood circulation, the structure has also an important role. Therapeutic proteins are fragile molecules and therefore either are usually inside the NP or within an inert shell of the NPs to protect them from extreme environmental conditions and for effective delivery. Thus, proteins can either form a core-shell structure with polymer NPs bearing surface charges or form a layer-by-layer structure (especially on a surface) by homogeneously mixing with the polymer solution. Afterward, the loaded proteins can be released when the charge is changed, or the polymer is degraded.

Proteins can be linked to polymer nanoparticles in different ways, such as direct conjugation, physical adsorption, or encapsulation (Figure 14), in which covalent and electrostatic interactions represent the majority of protein-polymer interactions. During the direct conjugation, protein bio-conjugation can be achieved through manipulation of the reaction between the side chains on polymers and functional groups of proteins. Nevertheless, this linked method could affect, and undesirable change the structure of the protein. On the other hand, through physical absorption, proteins are not at risk of damage, and also it is a method with simple preparation and application to any type of macromolecule. However, since there is no chemical bond connecting two atoms, electrostatic interactions are preferable

due to the better preservation of protein structure. Lastly, encapsulation of a protein into a polymer nanoparticle can allow a higher loading amount compared to physical adsorption [4], [5].

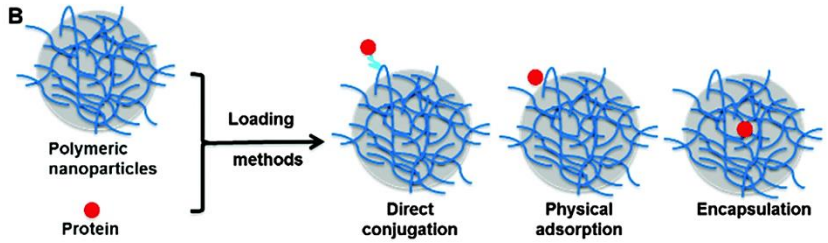

Figure 14: Drug Loading Mechanisms of NPs [4].

2.2.4 Surface

The surface properties such as surface charge, softness, porosity and hydrophobicity can significantly influence opsonization, phagocytosis, and the circulation in blood distribution of nanoparticles. Hydrophobic particles in the body are coated with immunoglobulin and other plasma proteins like albumin and then cleared by RES. Additionally, the surface properties affect the nanoparticle's stability and interaction with cells. The most frequently used method for surface modification is the coating with a hydrophilic polymer such as PEG (polyethylene glycol) (Figure 15). PEG has been approved by the Food and Drug Administration (FDA) as "generally recognized as safe". The presence of PEG can increase the hydrophilicity of NPs and can create an impermeable barrier over their surface. In this way, nanoparticles can avoid recognition and absorption by plasma proteins, defined as opsonins, within the circulation. In other words, those NPs are invisible to the RES, thus reducing the opsonization and leading to suspension of macrophage recognition. This coating is referred to as "stealth" moiety and allows the extension of NPs in blood circulation [34].

The success of a protein and a peptide to be considered as a therapeutic agent relies on the development of a formulation and its structure and activity during the preparation and delivery period. Except for modification of the nanoparticle's surface, it is also necessary for proteins to be stabilized via chemical modification with hydrophilic polymers. Because they are fragile and unstable molecules, the

attachment with polymers increases their hydrodynamic radius and/or molecular weight, which would translate into lower renal clearance and extended protein half-life. Covalent conjugation of PEG polymers, known as PEGylation, is a classic example to improve protein stability and other pharmacokinetic properties. The processes of surface adjustment of protein surface have helped over 10 (PEG)-modified therapeutic proteins to be approved by the FDA and be launched to the pharmaceutical market. Some of those are Adagen®, Somavert®, Oncaspar®, and Naloxegol. Furthermore, many other PEGylated proteins are currently in different clinical phases of development [35].

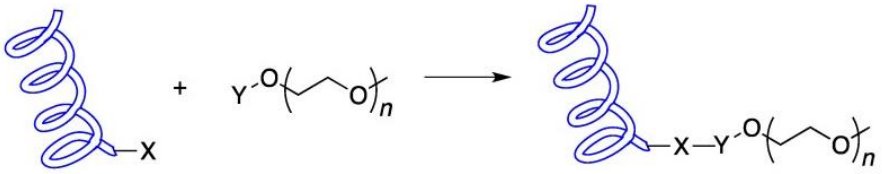

Figure 15: The general strategy for protein PEGylation: A functional group (X) on a protein is reacted with a complementary group (Y) on a poly(ethylene glycol) polymer (PEG) molecule forming a protein–PEG conjugate [35].

Another way to enable protein delivery is by manipulation of surface charge. This can be achieved by the electrostatic interactions through polymer-protein interactions and polymer-cell membrane interactions. Most proteins and protein analogs are slightly negatively charged in mammalian cell membranes because of the presence of sulfated proteoglycans. In addition, most organs exhibit a specific charge. Thus, the surface charge of NPs will determine the electrostatic interaction with cells to a certain extent. Subsequently, electrostatic attraction increases the time the NPs spend in physical contact with cells which enhances the possibility of these NPs to penetrate into cells, while electrostatic repulsion will result in less contact between NPs and cells and thus less penetration of NPs into cells.

It is worthy to be mentioned that the surface of nanoparticles influences the absorption of opsonins, leading to their recognition by macrophages and finally to their elimination with the simultaneous effect to their biodistribution. In other words, the NPs' surface charge is very important for the interaction of them with the blood proteins and for their internalization in the cell membrane. According to the

literature, it has been reported that negatively charged nanoparticles have a low phagocytic uptake, thereby contributing to the extension of blood circulation time, but they can potentially bind to available cationic sides on the cell surface. On the contrary, positively charged nanoparticles lead to the increase of phagocytosis due to their better interaction with the anionic cell membrane. Neutral particles can prevent interaction with the vascular wall, leading to less clearance and longer time. However, some researchers have demonstrated that neutral and cationic nanoparticles can have a reduced uptake by RES and are cleared less rapidly compared to negatively charged ones [36].

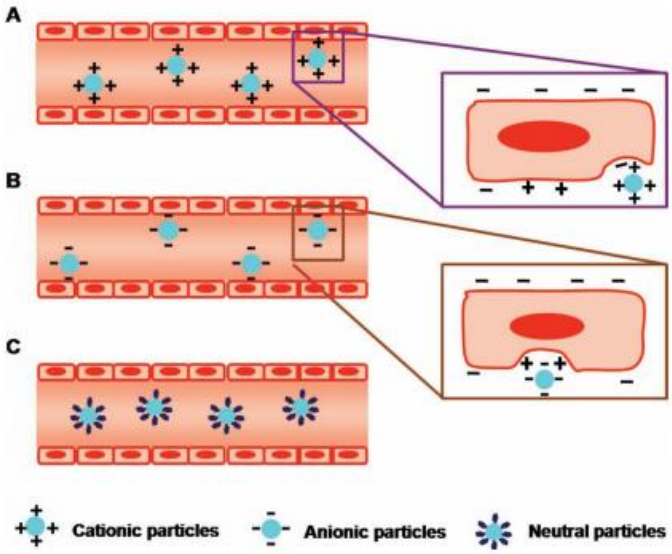

Figure 16: The clearance of particles is affected by surface properties. The charged nanoparticles can interact with the vascular wall through electrostatic interactions, thereby increasing the clearance rate. For example, positively charged nanoparticles have a high affinity for the anionic cell membrane (A), and negatively charged nanoparticles can potentially bind to available cationic sites on the cell surface (B): as a result, they are all likely to collide with blood vessel walls and are captured by macrophages. However, neutral particles (e.g., those coated with hydrophilic non-charged/non-ionic polymers) can prevent interaction with the vascular wall, leading to less clearance and a longer circulation time (C) [36].

Zwitterionic polymers, which are characterized with equal anion and cation groups on the molecular chains, have reported as candidates for protein delivery due to their ability of overcoming the obstacles that are mentioned previously. For

instance, degradable thermo-responsive amphiphilic PMPC copolymers (poly(2-methacryloyloxyethyl phosphorylcholine, in which phosphorylcholine (PC) is a zwitterionic polar side group similar to phospholipid heads on cell membranes) nanogels with acid degradable cross-linkers, were constructed for protein encapsulation and controlled release. The nanogels were composed of zwitterionic PMPC, thermo-responsive poly(methoxydiethylene glycol methacrylate) (PMeODEGM), and cationic poly(2-aminoethyl methacrylamide hydrochloride) (PAEMA). Negatively charged proteins, such as insulin, BSA, and β-galactosidase can be loaded into the nanogels. The release of protein can be accelerated at lower pH because of the acidic degradation of nanogels [37].

3. Methods – Production Techniques for Polymeric Nanoparticles

Several production techniques can be employed to produce nanoparticles and microparticles. The size comprises the most important feature for the construction of micro/nano particles, as different sizes of drugs, proteins and peptides can be incorporated into different sizes of particles. For this reason, there are different production methods that lead to different formulation performance. Natural polymers are generally more sensitive to processing conditions. Therefore, NPs with natural polymers are generated using mild techniques including ionic gelation, polyelectrolyte complexation and coacervation. NPs composed of synthetic polymers are normally prepared by more extensive techniques such as interfacial polymerization, emulsification–polymerization, emulsification-solvent evaporation, nanoprecipitation, salting out, supercritical fluids and emulsification solvent diffusion. Some of the production techniques are briefly discussed in the following section. Nevertheless, it is valuable to be mentioned that all production methods have some limitations regarding the preservation of protein and peptide integrity. The stability of proteins and peptides during manufacturing, storage and release from nanoparticles and microparticles should be maintained, as denaturation decreases activity.

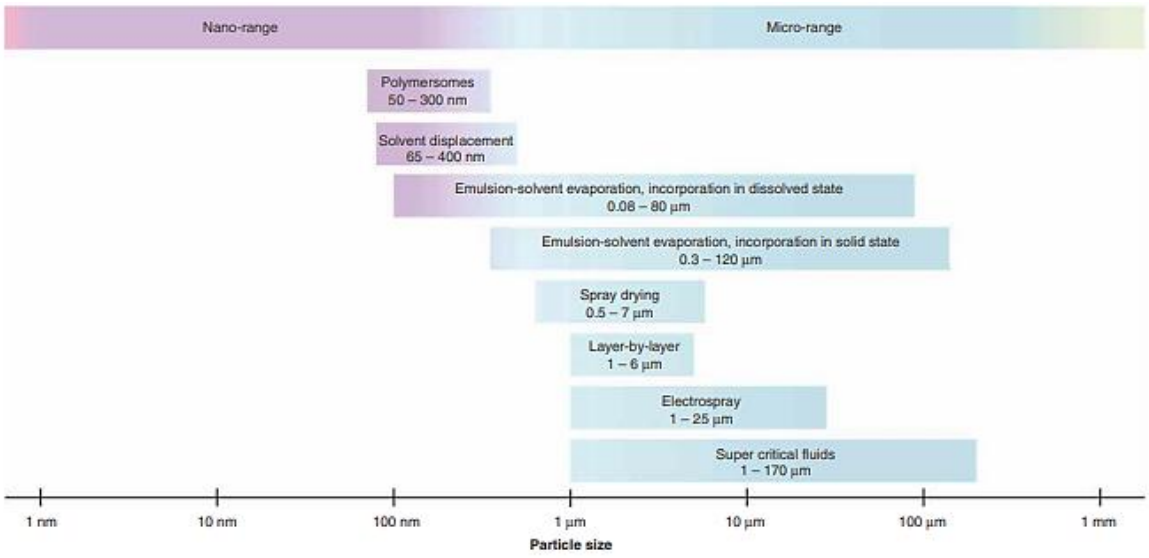

Figure 17: Overview of size range of nanoparticles and microparticles produced with different techniques [38].

3.1 Emulsification-Solvent Evaporation

Emulsification-solvent evaporation was the first method developed to prepare polymer nanoparticles and is the most frequently used process for manufacturing them containing proteins and peptides. In this method, polymer solutions are prepared in volatile organic solvents in order for the emulsions to be formulated. Ethyl acetate, which has a better toxicological profile, has replaced the dichloromethane and chloroform that were widely used in the past. This first step constitutes the primary dispersion. The peptide or protein can be added in the solid-state (S/O dispersion), as aqueous solution (W/O dispersion), as organic solution, when the used organic solvents form a single-phase (Om/O solution), as organic dispersion, when the solvent containing the protein emulsifies into the other organic solvent (O/O dispersion), as emulsion (W/O/O dispersion) or as organic suspension (S/O/O dispersion). Then, the secondary dispersion is followed. The primary dispersion or organic solution is emulsified with the external continuous phase, which is immiscible with the dispersed phase. The emulsion is converted into a nanoparticle suspension on evaporation of the solvent for the polymer, which can be diffused through the continuous phase of the emulsion. Depending on the physicochemical properties of the components and the process conditions different types of emulsions can be used. In the conventional methods two main strategies

are being used for the formation of emulsions: the preparation of single-emulsions, e.g., oil-in-water (o/w) or double-emulsions, e.g., (water-in-oil)-in-water, (w/o)/w. These methods use high-speed homogenization or ultrasonication. Afterward, the organic solvent removal follows with the evaporation of the solvent, either by continuous magnetic stirring at room temperature or under reduced pressure. Subsequently, the nanoparticles, which are solidified, can be collected via centrifugation or by filtration, washed with distilled water, with the aim of removing the additives such as surfactants, and finally, the product is dried by lyophilization or evaporation at reduced pressure. Usually, the oil phase is formed by the dissolved polymer in an organic solvent, whereas the water phase is formed by the aqueous phase containing the stabilizer. Figure 18 briefly presents the preparation method by the double-emulsion-solvent evaporation method [38], [39].

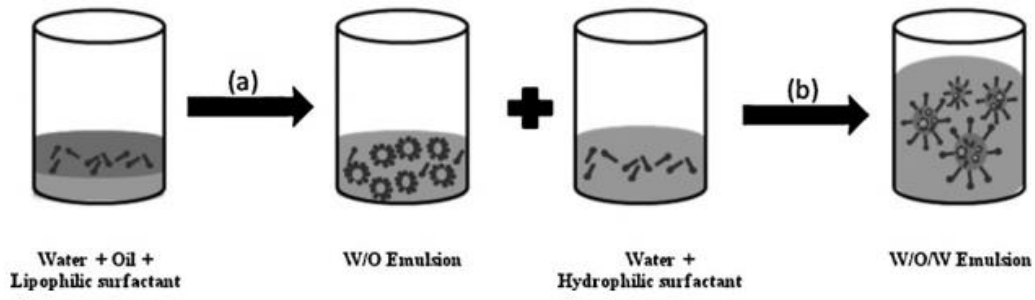

Figure 18: Preparation of w/o/w double emulsion: (a) high-shear emulsification and (b) low-shear emulsification [39].

The performance of the formulation depends on many factors, including emulsion type, type of organic solvents and cosolvents, process conditions, type of polymer carrier and inclusion, amount, and type of co-excipient. Miralem et al. aimed to prepare poly (lactic-co-glycolic acid) (PLGA)-based microparticles for entrapment of recombinant human epidermal growth factor (rhEGF) with minimum burst release. Bovine serum albumin (BSA) was chosen as the model protein due to its relatively high molecular weight and low cost, which may be used as a potential stabilizer and carrier in aqueous solutions. The protein loaded PLGA microparticles were prepared through (w/o/w) double emulsion solvent evaporation technique because rhEGF is a hydrophilic and water-soluble agent and this technique is the best for the preparation of such encapsulated drugs. The prepared rhEGF-loaded

microspheres had an average size of 6.44 ± 2.45 mm, encapsulation efficiency of 97.04 ± 1.13%, burst release of 13.06 ± 1.35% and cumulative release of 22.56 ± 2.41%. The biological activity of the released rhEGF was assessed using human skin fibroblasts cell proliferation assay. According to the results, the proliferation of the cells cultivated with rhEGF was like that of pure rhEGF, indicating thus the biological activity of released protein confirming the stability of rhEGF during microsphere preparation [40].

3.2 Spray Drying – Electrospraying

Spray drying is a process, which is based on the conversion of liquid material into dry powder by atomizing a solution, emulsion, or suspension into a hot drying gas medium, commonly air. The spray-drying process consists of four main steps. The first step is associated with the atomization of liquid feed into a spray, in which a solution of the drug is pumped through a nozzle, producing an aerosol and then, the spray droplets are mixed by a heated gas stream. Subsequently, the dry particles are formed by evaporation of the liquid and finally, the dry particles are subsequently separated from the drying gas and guided into a collection vessel, for example, using a cyclone. The size of the spray dried particles, generally between 0.5 and 5.5 μm (volume average) depends on several factors like viscosity and surface tension of the solution, nozzle type, the atomizing airflow, the flow rate during spraying, and concentration of the sprayed solution.

The spray-drying process is a simple and appealing technique to produce dry microparticles, with many benefits compared to other techniques such as emulsion/solvent evaporation, nanoprecipitation, and freeze-drying. It provides the ability of continuous and fast process, it is cost-effective, scalable, and allows the control of the morphology and the size of the particles, in which heat-sensitive drugs, such as therapeutic proteins, are incorporated. The effective encapsulation of heat-sensitive compounds is possible due to the formation of small droplets during the atomization, leading to fast solvent evaporation due to the high surface area. The droplets are exposed to high temperatures for a very short time during the drying process. The rapid solvent evaporation has a cooling effect on the formulation, despite the high temperatures during the drying process, because is an

endothermic reaction. On the other hand, one of the main disadvantages of the spray-drying technique is caused due to the requirement of hot dry air that might cause thermal stress to some proteins, thus contributing to their instability and loss of their native structure. Moreover, the dehydration process, which is involved in this technique, is feasible to cause structural modification and protein denaturation due to shear stress (e.g., by nozzle atomization), which may affect particle stability. The denaturation of proteins may be overcome by the loading of therapeutic proteins into nanoparticles. Another drawback is that the production yields directly depend on the work scale, which means that in lower scale setups like those of a conventional laboratory, the yield of production is typically low, because of the loss of product in the walls of the drying chamber [38], [41].

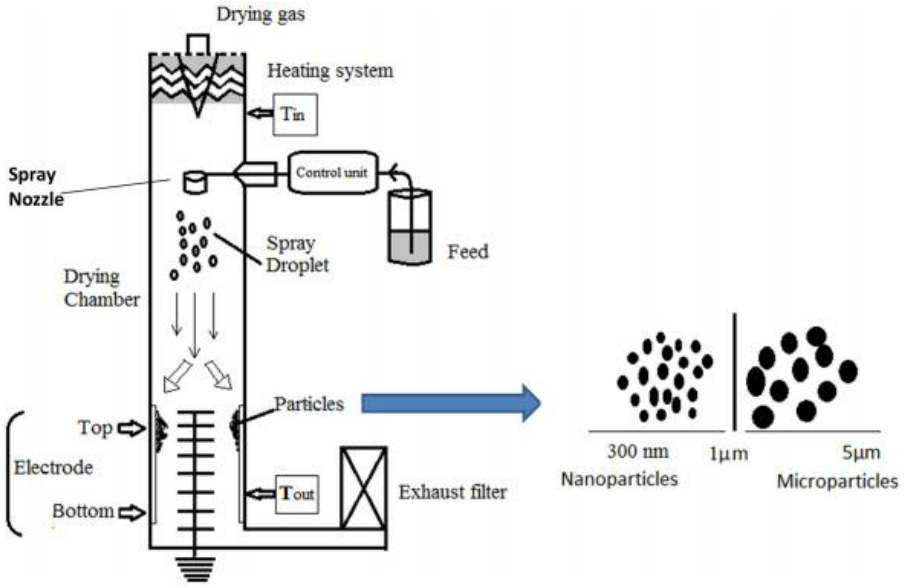

Figure 19: Schematic design of the Nano Spray Dryer B-90 and its products of nano/microparticles with an average size range of 300 nm–5 µm [42].

Spray drying has been employed to improve drug solubility and bioavailability of active ingredients, modified release, and pulmonary delivery of proteins or vaccines. Nano Spray Dryer was used by Harsha and his colleagues for high yields of vildagliptin nanospheres which were produced cost-effectively. The vildagliptin nanospheres were prepared with aminated gelatin and were designed to treat type 2 diabetic patients. These mucoadhesive nanospheres were marked by their narrow

particle size distribution of average size ca. 445 nm, prepared by the Büchi Nano Spray Dryer B-90, thus enabling them for oral administration use [42].

Another technique to produce nanoparticles and microparticles, which resembles conventional spray drying, is electrohydrodynamic spraying or electrospraying. Electrospraying is a technique in which a solution is pumped through a nozzle in order to create aerosol jets under an electrostatic field. The high voltage overcomes the surface tension at the interface of the spraying capillary, generating a Taylor cone. In the case of applying a higher voltage the break of the cone tip into small, highly charged droplets will take place, which are directed to the collection surface or nonsolvent of opposite charge. The distance to the collection surface is relevant for the size of the particles, as the particles will shrink because of the evaporation of solvent during traveling through the gas phase. Electrospraying is a recently developed process that has emerged as a cost-effective and versatile technique to produce microstructures for the delivery of therapeutic agents. One remarkable feature of electrospraying is its ability to generate monodisperse droplets whose size may vary by as much as hundreds of micrometers to as little as tens of nanometres by optimizing the processing parameters. According to Wu et al., electrospraying of emulsions is suitable for core/shell particle production (Figure 20), provided that the polymer deposits at the oil/water interface, for poly(ε-caprolactone) (PCL)-polyamino-ethyl ethylene phosphate/bovine serum albumin (BSA) microparticles [43].

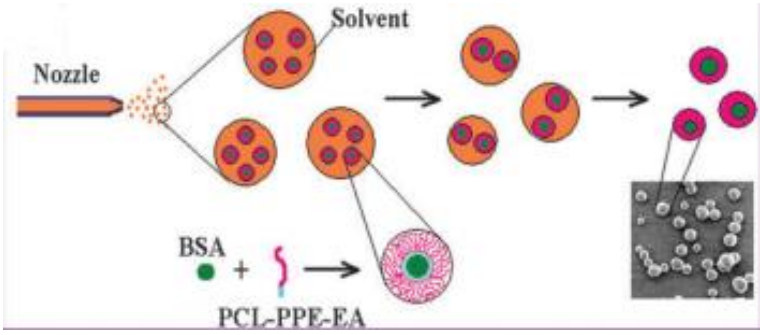

Figure 20: Schematic representation of the formation of core– shell structured particles through electrospraying an emulsion solution [43].

3.3 Nanoprecipitation - Solvent Displacement

The nanoprecipitation technique or solvent displacement method was first developed and patented by Fessi and his co-workers for the preparation of polymer nanoparticles [44]. This straightforward technique has many advantages, including the feature of rapidness and easiness to perform. The nanoparticle formation is rapid, and the entire procedure is carried out in a single step. Briefly, it requires two solvents that are missible. Both polymer and drug must be dissolved in the first one, the solvent, but not in the second system, which is the non-solvent. When the polymer solution is added to the non-solvent with the simultaneous rapid desolvation, then the nanoprecipitation occurs. As soon as the polymer-containing solvent has diffused into the dispersing medium, the polymer precipitates, involving immediate drug entrapment. The nanoprecipitation enables the production of small nanoparticles, with sizes ca. 100–300 nm, with narrow unimodal distribution. Some of the polymers which can be utilized with this technique are poly(D, L-lactic-co-glycolic acids), cellulose derivatives, or poly ε-caprolactones. This method does not require extended shearing/stirring rates, sonication, or very high temperatures, and is characterized by the absence of oily-aqueous interfaces. The lack of those conditions that might damage a protein structure leads to the maintenance of its biological activity. Furthermore, surfactants are not always needed, and unacceptable toxic organic solvents are generally excluded from this procedure. Nevertheless, except from the advantages that this technique is characterized, it has also some drawbacks. Nanoprecipitation is mostly used in correlation with compounds having a hydrophobic nature such as indomethacin, which is soluble in ethanol or acetone, but displays very limited solubility in water. Consequently, reduced or even zero drug leakage toward the outer medium led to nanoparticles with entrapment efficiency values reaching 100%.

Lince et al. indicated that the process of particle formation using nanoprecipitation method includes three steps: nucleation, growth and aggregation. The rate of each stage defines the particle size and the ratio of polymer concentration over the solubility of the polymer in the solvent mixture is related with the driving force of these phenomena. The separation among the nucleation

and the growth stages, is the key factor for uniform particle formation. Preferably, operating conditions should allow a high nucleation rate strongly dependent on supersaturation and low growth rate. [45]. Lee et al. prepared gelatin-based NPs by nanoprecipitation, in which water and ethanol were used as solvent and non-solvent, respectively. According to the review, it was shown that the non-crosslinked particles have an irregular shape due to particle aggregation. However, the cross-linked particles have a unimodal size of 251 nm, low polydispersity index (0.096), and uniformly round shape. The results indicated that nanoprecipitation is a suitable method for the preparation of gelatin NPs [46].

3.4 Ionic Gelation and Coacervation Techniques

Ionic gelation is also used to promote the formation of nanoparticles and microparticles. This kind of process is based on the reversible physical cross-linking by electrostatic interactions, instead of chemical cross-linking to reduce the toxicity of reagents. The resulting particles present some defects such as poor mechanical strength, low thermal stability and improper surface morphology, limiting their usage in controlled release. Ionic gelation and coacervation are techniques mainly employed for preparing NPs composed of natural polymers such as CS, gelatin and sodium alginate. The preparation conditions are mild, and proteins can be encapsulated without utilization of organic solvents or elevated temperature. Nevertheless, long-term controlled release is difficult to be achieved due to solubility of polymers. Some of the factors affecting protein encapsulation by ionic gelation technique are molecular weight of the polymer, initial protein concentration and polymer concentration. On the other hand, the complex coacervation has few drawbacks since it involves the use of toxic chemicals for crosslinking, and the complete removal of the un-reacted agent may be difficult. Additionally, the loading agent efficiency is poor, offering less stability to the particles [47].

According to the literature, ionic gelation is extensively used for the preparation of chitosan nanoparticles in mild conditions by reversible electrostatic interactions between positively charged chitosan chains and polyanions which are employed as cross-linkers, mainly pentasodium tripolysphophate (TPP). Vandana & Sahoo prepared Bovine Serum Albumin-loaded chitosan nanoparticles based on the

method of ionic gelation and TPP, with the concentration adjusted to get a chitosan/TPP ratio 3:1. Using the technique of ionic gelation, BSA loading was optimized, where the analysis included the efficiency entrapment of different MW-chitosan NPs loaded with different concentrations of BSA. Thus, an increase in the entrapment of BSA was observed with the increase of MW of chitosan and concentration of BSA [48].

3.5 Self-Assembled Systems

Self-assembled systems are developed by weak non-covalent interactions between molecules of the carrier matrix such as block copolymers. These interactions lead to a specific structural organization. Amphiphilic block copolymers, as analyzed previously, are mainly utilized for the formation of such systems by assembling into a more organized structure spontaneously or in response to an exogenous stimulus, such as temperature or pH. From the self-assembly technique different systems with different sizes can be produced. These include the production of polymersomes with a size range between 50 to 300 nm and the layer-by-layer capsules with sizes between 1-6 μm.

3.5.1 Self-assembled Systems: Micelles and Polymersomes

Micelles and polymersomes are conventional structures formed by a self-assembly process. Micelles are structures consisting of a hydrophobic inner core surrounded by a hydrophilic outer shell, while polymersomes are spherical hollow vesicles that have a polymeric lamellar structure, which is a bilayer membrane, surrounding an aqueous core. The bilayer membrane is composed from hydrated hydrophilic coronas both at inside and outside of the hydrophobic middle part of the membrane. In doing so, the fluid core is protected from the outside medium [24]. Amphiphilic block copolymers are utilized for the construction of such systems and due to their amphiphilic features, hydrophilic compounds such as proteins can be covalently attached to the outer surface of polymersomes. In addition, during self-assembly by solvent-switching techniques or by rehydration of a polymer film with the protein/peptide solution, proteins can be directly encapsulated within the polymersome. Nevertheless, a noticeable drawback of polymersomes constructed by layer-by-layer technique is the low encapsulation efficiency. This happens because

the peptide/protein in the inner core of the polymersome has low diffusion or because these molecules have poor entrapment efficiency during self-assembly [38]. Qiao et al. took advantage of the characteristic of polymersomes for the co-encapsulation of active compounds with different hydrophilicity and prepared an acid-responsive nanosystem in which hydrophobic doxorubicin (DOX), and water-soluble fluorescein isothiocyanate-lysozyme (FITC-Lys) were successfully entrapped. The system exhibited pH-dependent drug release profiles, while the DOX-loaded aggregates showed concentration-dependent cytotoxicity to tumor cells, but the copolymers are nontoxic. This type of formulation needs further investigation and can lead to producing innovative nanosystems for the therapy of a pharmacological effect using simultaneously two different compounds [49].

3.5.2 Self-assembled Systems: Layer-by-layer method

Layer-by-Layer (LbL) method is a technique based on the electrostatic interaction between charged materials such as polyelectrolytes, nanoparticles and oppositely charged blocks in general. This means that, if, for instance, onto a positively charged surface an aqueous solution of an anionic polymer (polyanion) is introduced, then the polyanions will adsorb and thus, the surface charge is reversed from positive to negative. Hence, as the surface is now negatively charged, a cationic polymer (polycation) can be introduced to the surface in the same fashion. The layer-by-layer process is a nanoscale coating and surface functionalization technique which can be repeated as many times as required for the final product to be performed. Compared to the traditional drug nano-device procedures, the layer-by-layer technique displays the advantages of simplicity and chemical mildness due to its ability to be achieved just by immersing a substrate into a solution containing an oppositely charged substance, without the requirement of special conditions. Furthermore, it is a widely known technique due to its ability to control the surface properties of the systems based on the layering of different polymers or other building materials. The hydrophilicity/hydrophobicity, the permeability related to the porous of the network and the bio- and chemical reactivity are some features that can be controlled via this method. In addition, to surface modification, hollow capsules can be created by LbL, which is important for the entrapment of several

drugs. However, except from drugs or synthetic charged polymers such as polyelectrolytes, other charged biomacromolecular entities such as DNA and proteins can also be deposited on surfaces with this method. [38], [50].

3.6 Supercritical Fluids Methods

The methods described previously involve organic solvents, and the need to develop environmentally safer methods to produce polymer nanoparticles is rather urgent. The need for utilization of supercritical fluids led research for the investigation of those fluids as more environmentally friendly solvents, with the potential to produce polymer NPs with high purity and without any trace of organic solvent. In this technique, drug and polymer are first dissolved in supercritical fluid and the solution is expanded through a nozzle. The supercritical fluid is evaporated using the spraying process which eventually leads to precipitation of solute particles. Two principal processes have been developed to produce nanoparticles using supercritical fluids: 1. Rapid expansion of supercritical solution (RESS); and 2. Rapid expansion of supercritical solution into liquid solvent (RESOLV).

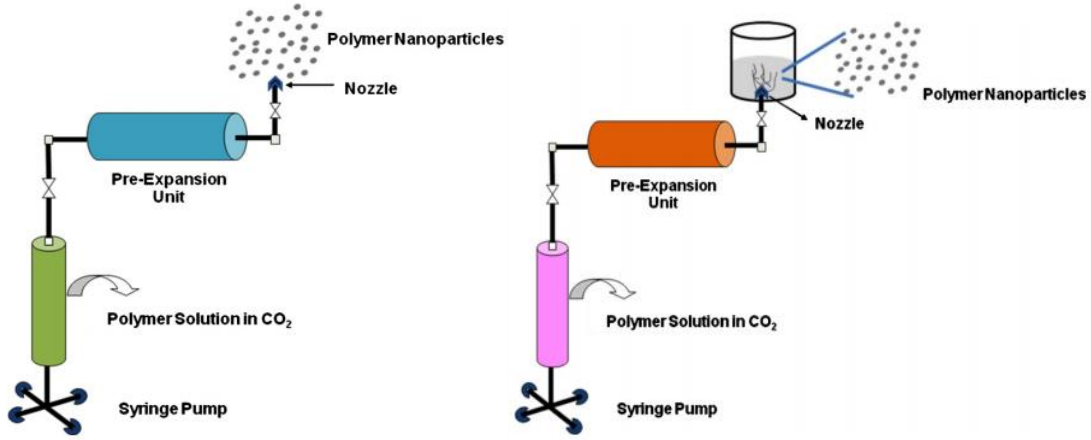

Figure 21: **Left image**: Experimental set-up for the preparation of polymer nanoparticles by rapid expansion of supercritical fluid solution. **Right image**: Scheme showing the experimental set-up for the rapid expansion of supercritical fluid solution into liquid solvent process [39].

Advantages of this method include processing of biolabile pharmaceuticals under mild operating conditions, flexibility in procedures, and elimination of organic solvent in the final product. However, the major obstacles observed with this

method are the requirement for special equipment, poor solubility of high molecular mass (10,000) polymers and strong polar substances in supercritical CO_2 [3], [39].

4. Classification of polymer-based nanosystems as vehicles

The recent advancements in the combination of nanotechnology with pharmacology and biotechnology have resulted in the development of novel therapeutically active proteins and peptides, which are indicated for the combat of several chronic diseases, such as diabetes, cancer, hepatitis, rheumatoid arthritis, and leukemia. Nevertheless, there are many challenges and limits must be overcome for their effective administration. For this reason, numerous protein carrier platforms have been developed for better therapeutic performance. For instance, microspheres and hydrogels have been utilized to solve the sustained-release issue, but due to their large size, have limited applications for intracellular protein delivery. Other systems including liposomes, polymer conjugates, nanotubes, nanogels and nanoparticles (NPs) are being extensively investigated for effective intracellular delivery. Among them, biodegradable polymeric NPs have provoked great interest as potentially protein carriers as a result of their biocompatibility, biodegradability, amenability to formulation, and capability with intracellular and systemic delivery.

Table 2: Barriers to protein and peptide delivery and advantages of polymeric NPs [3].

Route of Administration	Advantages	Barriers	Advantages of Polymeric NPs
Oral	High patient compliance	Low permeability through GIT epithelia Degradation by proteolytic enzymes Instability at acidic pH in stomach	Enhancement of oral absorption Improved bioavailability Prolonged residence time in the intestine Sustained drug release Enhanced stability in the GIT
Ocular	Ease of administration Avoidance of first-pass metabolism	Poor permeability Enzymatic degradation Nasolacrimal drainage	Protection from enzymatic degradation Prolonged residence time in cul-desac Lower drug loss due to tear turnover Sustained drug release
Transdermal	Avoidance of first-pass metabolism Large surface area Ease of application	Poor permeability across stratum corneum	Accumulation in the hair follicles creating high local concentrations of loaded drugs
Parental	High bioavailability	Rapid plasma	Protection from

	and rapid onset of action	degradation and generally poor patient compliance	enzymatic degradation Enhanced residence time in the plasma Site specific delivery via targeting ligands
Pulmonary	Large surface area Highly vascularized mucosa Porous endothelial membrane Lower enzymatic activity No first-pass metabolism	Pattern of deposition and size distribution depending on delivery device. Mucociliary clearance mechanisms	Improved absorption Sustained release Minimal enzymatic degradation of encapsulated proteins Target specificity via surface modifications
Nasal	Highly vascularized mucosa Porous endothelial membrane Lower enzymatic activity Direct brain delivery Avoidance of first-pass metabolism	Mucociliary clearance	Improved systemic and brain absorption. Sustained release Minimal enzymatic degradation of encapsulated proteins

In conclusion, for effective protein and peptide delivery, the ideal polymer nanoplatform should show 1) high protein loading which means that NP mass is low in order to achieve therapeutic dose; 2) “green” protein encapsulation, where little or no organic solvents are used to prevent protein denaturation; 3) minimal contact with carriers to avoid the low local pH value caused by polymer degradation; and 4) sustainable and controllable protein release with low initial burst. In the following section, several polymer-based nanosystems for delivery of therapeutic proteins will be discussed extensively with the simultaneous presentation of many recent reports from the scientific literature [51].

4.1 Amphiphilic Block Copolymers

Nanomedicine research has been focused on amphiphilic block copolymers (ABCs) for sustained release of drugs, proteins, and genes due to their unique features and numerous potential applications. Block copolymers are comprised of two or more different polymers with diverse chemical groups (blocks) and can be self-assembled into a solution. Depending on the hydrophilic and hydrophobic

polymer, the amphiphilic block copolymers can be synthesized and self-assembled to different morphologies such as spherical micelles, cylindrical micelles, lamellas, and vesicles, among others (Figure 22). The utility of ABCs for the delivery of therapeutic agents results from their unique chemical composition, which is characterized by a hydrophilic block that is chemically connected to a hydrophobic block. The reason why amphiphilic block copolymers have self-assembly behavior is to decrease the interfacial area of insoluble blocks for lowering interfacial free energy. In contrast, the increase in the number of assembling block copolymers related to the increase in insoluble core size, leads to the stretching of the blocks forming the core. Furthermore, the association of the block copolymers also increases the density of shell-forming segments directed toward a stretched conformation.

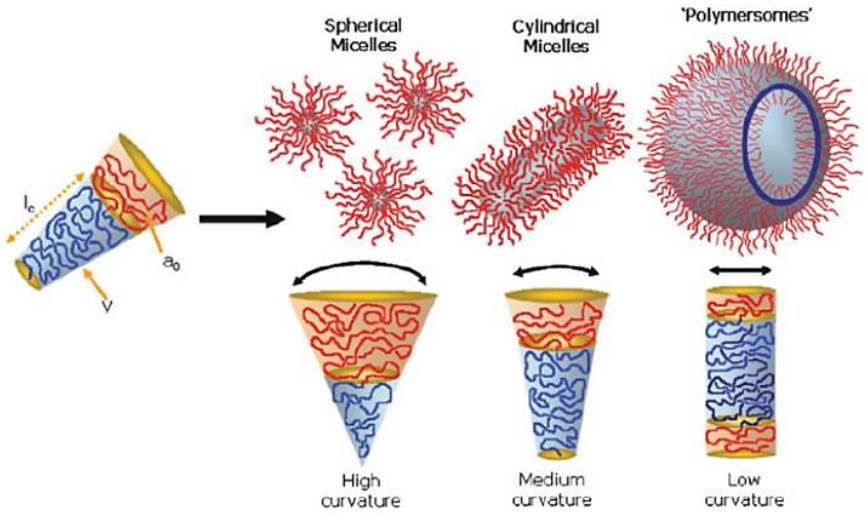

Figure 22: Various self-assembled structures formed by amphiphilic block copolymers in a block selective solvent. The type of structure formed is due to the inherent curvature of the molecular interface, which can be estimated through calculation of its dimensionless packing parameter [52].

The available block copolymer architectures include linear block copolymers, graft copolymers, dendritic polymers, starlike polymers, cyclic polymers and others, which can self-organized into aggregates of diverse morphologies under certain conditions. Among those, linear block polymers are the most studied and used systems and are defined as linear macromolecules containing long sequences of repeating units. In addition, by changing factors such as pH and processing temperature ABCs with different functions can be formed. These block copolymers

have different interactions and functions and can thus behave as stimuli-responsive polymeric nanostructures. To conclude with, some of the main applications of such polymeric nanostructures in nanomedicine contain the delivery of therapeutic proteins through these vehicles and their utilization in bioimaging, hyperthermia and in photodynamic therapy. Some of those examples will be presented including the encapsulation of hydrophobic proteins in block copolymer micelles and block polyelectrolyte complexes with proteins [52].

4.1.1 Amphiphilic Block Copolymer Micelles as Nanocarriers

Polymeric micelles belong to the most known architecture which can be constructed from the synthesis and self-assembly of synthetic polymers and natural macromolecules. In addition, they can combine the processability and adaptability of the former with the ability of the latter to program assembling mechanisms and control the structure and function. When an amphiphilic block copolymer is dissolved in a selective solvent at a constant temperature, above a specific concentration called the critical micelle concentration (cmc), micellization occurs. However, molecularly dissolved copolymer chains, called unimers, are present in the solution and below the critical micelle concentration, while above the cmc multimolecular micelles are in thermodynamic equilibrium with the unimers. The size of block copolymer micelles ranges from 10 to 100 nm and if the critical micelle concentration is low, then the micelles are stable at high dilution. Nevertheless, micelles with lower cmc are generally more stable compared to low-MW surfactant micelles due to the greater interfacial free energy derived from the larger insoluble segments. Hence, the segregation of the core-forming segments in the micellar core can generate a variety of intermolecular forces leading to micelles with lower critical micelle concentration.

The assembly process, the arrangement of the polymers and the cargo, and the stability of the nanoassemblies, as well as the performance in biological environments, are some features that are controlled by the components of the block copolymers. It is worthy to be mentioned that the selection of the polymers should not only be related to the structural and functional roles in the final micellar assembly but also the safety of these segments has to be taken under consideration

because of a repeated administration of micelles is needed. Hence, besides being biocompatible and nontoxic, as indicated by the FDA guidelines for biomedical polymers, it is desirable to reduce the number of polymers in the body after the drug delivery is accomplished. The big number of polymers in the body after the administration may cause side effects, such as activation of immune responses. Therefore, block copolymers used for forming micelles should be designed to be biodegradable and safe. The aim during their choice and thereafter their construction should be correlated with the complete disintegration of the polymers into the forming monomers, and to be safely excreted from the body without causing accumulation and avoiding any long-term toxicity [52], [53].

A block copolymer micelle is constructed with the combination of a ligand-installed block copolymer comprised of a hydrophilic block and a drug-loading block and of a small molecule which behaves as a pilot one (it can be a protein, an antibody, a peptide, a pDNA, or a mRNA) and is connected with the hydrophilic block. As it is presented in Figure 22, after the self-assembly process, a polymeric micelle is formed which is consisted of a core that is responsible for the loading of bioactive molecules and for cargo's protection. The outer shell is hydrophilic and aims to protect the cargo from degradation, to reduce the interactions with the serum proteins and the uptake by macrophages and to extend the blood half-life. The ligand on the surface of the hydrophilic shell is the pilot molecule and acts as a receptor/recognition site (i.e., a targeting moiety).

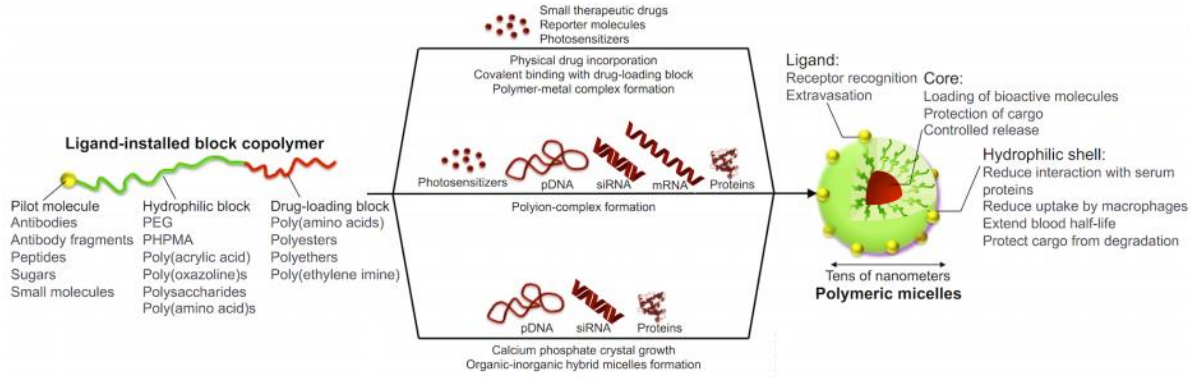

Figure 22: Self-assembled polymeric micelles of block copolymers constitute a versatile platform for loading bioactive molecules by controlling the interaction of the payloads with

the segments forming the core. The hydrophilic shell, high loading efficiency, ability to introduce ligands, and their relatively small size are substantial advantages of polymeric micelles for acting at the biological interface [53].

Self-assembled polymeric micelles of block copolymers represent a versatile nanoplatform for the loading of active molecules such as proteins and peptides by controlling the interaction of the payloads with the parts of the core. The hydrophilic shell that provides the ability to introduce ligands and the high loading efficiency with the relatively small size of the whole nanocarrier are some of the main advantages. However, self-assembled micelles with different morphologies and stability can be prepared, depending on the block copolymer composition and structure. Thus, polymeric micelles are widely used due to their ability to stabilize the drugs in aqueous conditions, protecting these agents within their core from outer environments, stably circulating in the bloodstream, and selectively accumulating in solid tumors, in cases of administration of hydrophobic anticancer drugs, where they can release the loaded drugs in a programmed manner. The drugs can be incorporated into the core of micelles through physical interactions. In other words, they are taking advantage of the interaction of the drug with the hydrophobic core-forming segment, or through conjugation of the drugs to the core-forming backbone via labile bonds, which can be cleaved at specific conditions to recover the active drug. The unimers of copolymers play an important role in drug absorption through the GI mucosa, either by increasing membrane permeability to the drug and/or the carrier or by inhibiting drug efflux transporters or first-pass metabolism. Moreover, as it is mentioned before, polymeric micelles are characterized by low toxicity and high safety related to the polymers chosen and the clearance and the metabolism of the agents [54], [55].

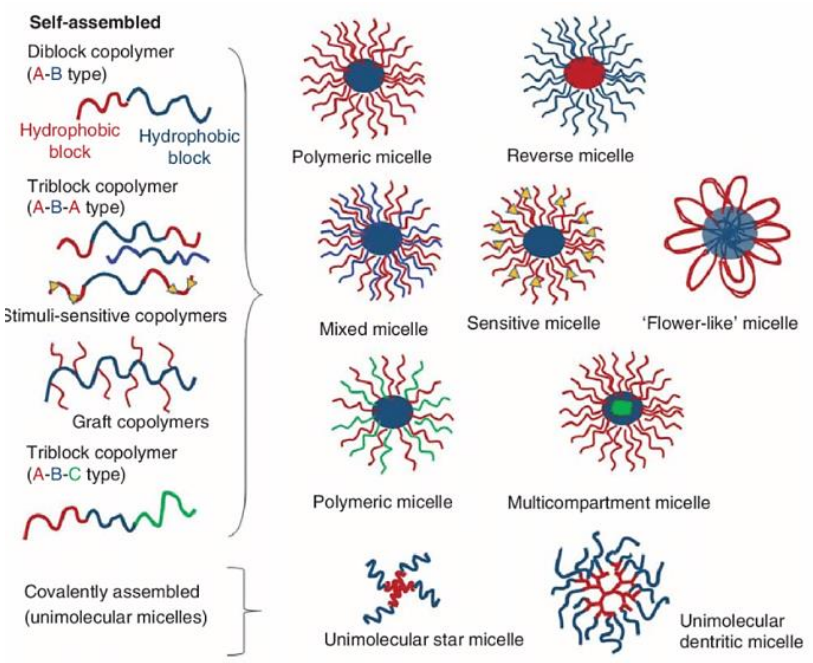

Figure 23: Types of polymeric micelles that can be formed depending on the copolymer architecture and the intermolecular forces [54].

Protein drugs, including cytokines, enzymes, and antibodies, are mainly used in recent pharmaceuticals. The main obstacle during the administration of proteins is their low stability and their enzymatic degradation. For this reason, in order their degradation to be avoided, hydrophobic proteins are encapsulated into the core of the polymeric micelles. Generally, drug release from block copolymer micelles depends on the design and method used for their preparation, the structure of the micelle-forming block copolymer and the drug, their physicochemical properties, as well as the localization of the drug in the polymeric micelles. As it is illustrated in Figure 24 there are different ways of drug-protein release. There is the drug release from polymer-drug conjugates which contains two mechanisms, the dissociation of micelles followed by drug cleavage from the polymeric unimers or the drug cleavage inside the micellar structure followed by diffusion out of the carrier (Figure 24A). The second way refers to the procession of diffusion in which there is a drug release from drug-loaded micellar carriers (Figure 24B). On the other hand, the drug release from polyion complex micelles is triggered via ion exchange in physiological media (Figure 24C).

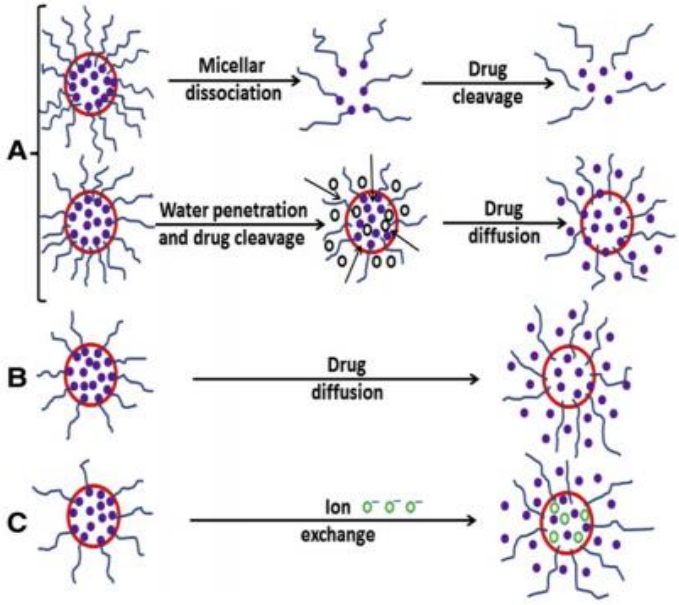

Figure 24: Modes of drug release from polymeric micelles. (A) Drug release from block copolymer-drug conjugates, (B) Drug release from drug encapsulated micellar carriers and (C) Drug release from polyion complex micelles [56].

In the article of Wang et al. a method is reported to produce a PBA-based block copolymer poly(ethylene glycol)-block-poly(acrylic acid-co-acrylamidophenylboronic acid) (PEG-b-P(AA-co-AAPBA)) nanocarrier that could respond to glucose at physiological conditions for insulin delivery and controlled release. The amphiphilic block copolymer was transformed after the AA segments modification, which could self-assemble into core-shell micelles but dissociate in response to glucose at a suitable concentration at neutral pH. Insulin was loaded via hydrophobic interaction during self-assembly and could be released at a faster rate in the solution with a higher concentration of glucose. The experimental results show that the insoluble insulin can be entrapped in the hydrophobic core which is composed of the PAAPBA segment of PEG-b-(PAA-co-PAAPBA) when the aqueous solution has pH 6.0 (Figure 25). Conversely, in the aqueous solution of glucose at pH 7.4, the insulin-loaded micelles disaggregate and thus, the insulin loaded in the micelles is released during this process. This was caused by the combination of PAAPBA segments with glucose resulting in an increase in hydrophilicity of the PAAPBA core. The next goal is the reduction of glucose-responding concentration, for example, adjusting the content

of PAAPBA segments in the polymers, to optimize this system for the glucose-responsive release of insulin in physiological conditions [55].

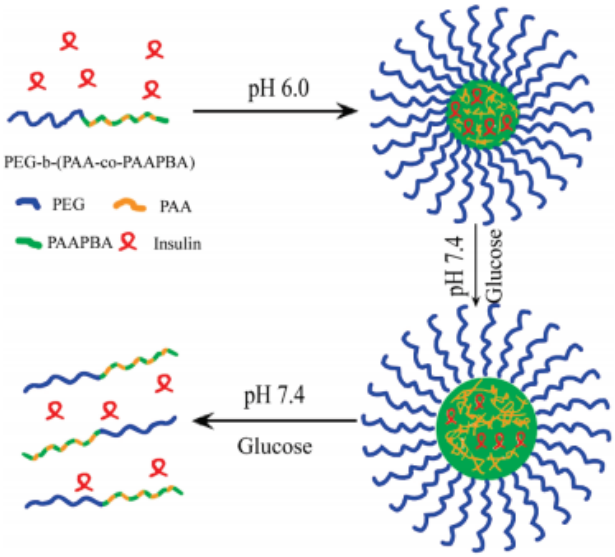

Figure 25: Schematic Illustration of the formation, swelling, and disaggregation of insulin-loaded micelle and release of insulin from the micelle according to glucose responses [55].

Liu et al. aimed to produce a complex polymeric micelle in order to control the blood glucose concentration via insulin delivery. They prepared a phenylboronic acid (PBA)-functionalized glucose-responsive complex polymeric micelle (CPM), which was synthesized containing two types of diblock copolymers, poly(ethylene glycol)-b-poly(aspartic acid-co-aspartamidophenylboronic acid) (PEG-b-P(Asp-co-AspPBA)) and poly(N-isopropylacrylamide)-b-poly(aspartic acid-co-aspartamidophenylboronic acid) (PNIPAM-b-P(Asp-co-AspPBA)). When the weight ratio between PNIPAM and PEG was 6/4, the CPM formed complex micelles with a novel core–shell–corona structure and exhibited a sensitive reversible swelling in response to the changes in glucose concentration. This was a result from the repeated on-off release of insulin regulated by glucose level. Likewise, the CPM could also effectively protect the encapsulated insulin against proteolytic and hydrolytic degradation, thus improving the delivery efficiency [57].

4.1.2 Block Polyelectrolytes and Protein Complexes

Polyelectrolyte block copolymers belong to a class of macromolecules which combine the structural properties of amphiphilic block copolymers, polyelectrolytes

and surfactants and provide various possibilities for use as delivery nanosystems of proteins and peptides through electrostatic interactions. Polyelectrolytes are polymers whose repeating units have an electrolyte group. Polyanions and polycations are both polyelectrolytes. The electrolyte group produces electrically conducting solution when dissolved in a polar solvent such as the water. The dissolved electrolyte is separated into cations and ions and thus the polymers are charged. Moreover, proteins belong to a particular class of natural weak polyelectrolytes with both positive and negative residues on the solvent-accessible surface, as well as, polypeptides, glycosaminoglycans, and DNA are considered as polyelectrolytes. According to protein complexation with polyelectrolyte different separation phases can be observed.

Protein complexation with a polyelectrolyte can lead to macrophase separation when the polyelectrolyte is a homopolymer or microphase separation when the polyelectrolyte is a block copolymer. For macrophase polyelectrolyte complexes, this means tuning protein or polymer charge to impact protein partitioning and coacervation or precipitation. On the other hand, in microphase separated polyelectrolyte micelles, polymer architecture and external stimuli are the factors that control micelle size and morphology, as well as incorporating responsive elements that allow polyelectrolyte micelles to be optimized for specific applications, such as protein delivery [58], [59].

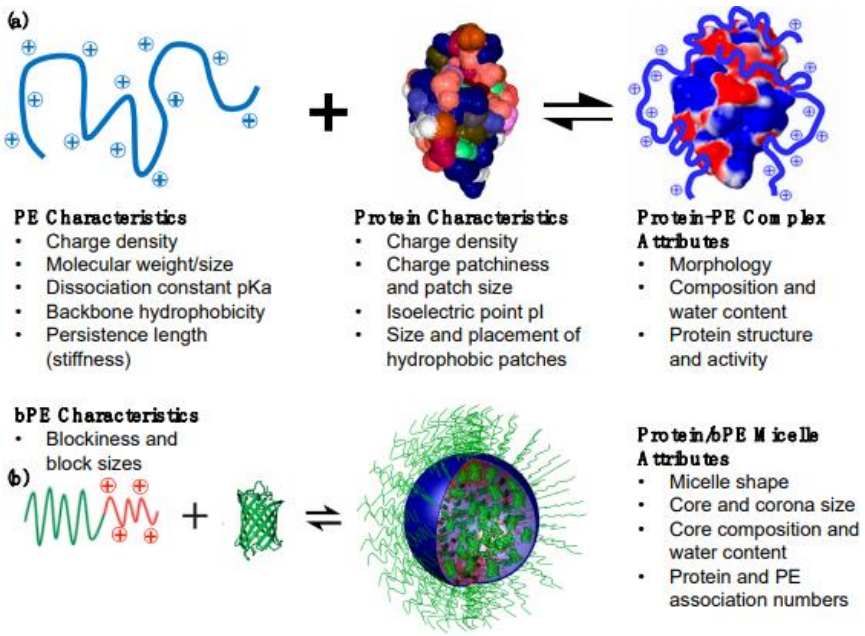

Figure 26: Schematics depicting complexation of proteins with **(a)** polyelectrolytes and **(b)** block polyelectrolytes to form protein–polyelectrolyte complexes and protein–block polyelectrolyte micelles, respectively. The key characteristics of the (block) polyelectrolytes and the proteins that regulate the properties of the complexes and the micelles are listed. The proteins depicted in the schematics are **(a)** chicken egg white lysozyme and **(b)** enhanced green fluorescent protein. The three-dimensional rendering of lysozyme globules also depicts the diversity of the amino acid residues, colored differently, present on the globular surface [58].

When a polyelectrolyte is conjugated with a neutral hydrophilic polymer then bulk phase separation upon complexation of the block polyelectrolyte with proteins is prevented, thus leading to nanoscale colloidal assemblies with core–corona micellar architectures. These colloidal assemblies usually have a compact core comprising the proteins and the charged blocks surrounded by a dilute corona composed of the neutral blocks, and, according to the literature, are sometimes referred as protein/bPE micelles. Moreover, these micellar colloids, can be considered to belong to a class of self-assemblies known as polyelectrolyte complex (PEC) micelles. The first demonstration of self-assembled PEC micelles was from Kataoka and coworkers in 1995. According to their pioneering work, they mixed a pair of oppositely charged block copolymers poly(ethylene glycol)–block-poly(L-lysine) (PEG–b-P(Lys)] and poly(ethylene glycol)–block-poly(α,β-aspartic acid) [PEG–b-P(Asp)) [60]. This was the initiatory research on PEC micelles and since then, lots of studies have been carried out on their fundamental properties and applications as nanocarriers for delivery of biologically active charged macromolecules with therapeutic efficacies, including nucleic acids and proteins/enzymes.

Nevertheless, it is worthy to mention that during the complexation of proteins with block polyelectrolytes not only conventional micelles with the spherical architecture are constructed, but also other morphologies have been reported, including worm-like micelles vesicles and lamellae. Micelle morphology is influenced by a variety of aspects, depending on the polymers, such as salt concentration, mixing ratio, temperature and relative size of each component of the system. In the case of micelle morphology influence, depending on the salt concentration,

morphological changes result from two factors which are the swelling of the core of the micelle and the change in solubility of the hydrophilic block. The increased screening of electrostatic interactions causes solvation of the core and thus micellar swelling. Generally, in order a complex-polyelectrolyte core micelle to be created, a diblock copolymer with a charged block and a neutral, hydrophilic block is necessary to be combined with an oppositely charged polyelectrolyte (or neutral-charged block copolymer), as it is also mentioned previously. The electrostatic interactions and the counterion release are the driving forces for the first form of soluble polyelectrolyte complexes. Moving on, the concentration of these soluble complexes is adequate to cause aggregation into a microphase-separated micellar structure. This microphase-separated micelle is stabilized and protected from solvent interactions by the neutral block, resulting in microphase separation of a consistent morphology and size. Like micelle morphology, micelle size is also affected by properties of the constitutive molecules and environmental conditions such as salt concentration, pH, and temperature. In most systems, micelle size is primarily determined by the length of the charged block of the block copolymer [59].

Among the morphologies that block polyelectrolyte and protein complexes can have, the application of microphase separated protein PECs involved the PEC micelles as nanocarriers are mainly studied and investigated due to their ability to protect the therapeutic proteins from degradation for a succeeding delivery intracellularly via endocytosis. Because of the choice of macromolecular compounds, the PEC micelles can be made to be responded to changes in pH, temperature, or the presence of reducing agents. Such changes allow the micelle for acquiring a controlled disassembly upon delivery into the cell. Consequently, they represent reasonable colloidal stability in vivo and a versatile platform for controlled delivery of many different therapeutic proteins with control of the point of release. Pippa et al. studied the electrostatic complexation process between a cationic-neutral polyelectrolyte and a protein. The polyelectrolyte was the block polyelectrolyte quaternized poly [3,5-bis(dimethylaminomethylene)hydroxystyrene]-b-poly(ethylene oxide) (QNPHOSEO) and the protein was the insulin. According to the results, it is indicated that the size, the structure, the distribution and the z-potential of the

nanocarriers in aqueous and biological media of the formed complexes depend on the ratio of the two components, on the pH and the ionic strength of the solution during complex preparation, whereas the in vitro release profiles of the entrapped protein are found to depend on the ratio of the components and the solution conditions used during preparation of the complexes. Furthermore, the electrostatic interactions and the charge screening are correlated with the ionic strength. As the ionic strength increases in the solutions, the electrostatic interactions become weak which leads to different structures of the complexes [61].

4.1.2.A. Applications of polyelectrolyte-protein complexes

Polyelectrolyte complexes, as mentioned before, are nanoparticles stabilized by electrostatic interactions between oppositely charged polymers. The formation of a PEC incorporates three main steps, in which the first one is the assembly process of the primary complex driven by electrostatic interaction and the second one includes the formation of hydrogen bonds which result in conformational changes of PE chains. The final step is the aggregation of secondary complexes forced by hydrophobic interactions. Polyelectrolyte complexes are utilized for the delivery of drugs and therapeutic proteins and peptides through different administration routes, including oral, intranasal, intravenous, and intramuscular injection. Nevertheless, oral administration is the most convenient option, but it is particularly challenging, especially for peptide/protein-based drugs, because of the harsh conditions in the gastrointestinal tract and fast enzymatic degradation of therapeutic agents. Jeong and his co-workers investigated a novel oral insulin delivery system by combining two different artificial polypeptides with this protein, thus producing polyelectrolyte complexes (PCs) by using negatively charged poly(L-glutamate-co-N-3-L-glutamylsulfanilic acid) (PLGS), cationic alpha helical peptide poly-L-lysine (PLL), and insulin. According to the results, PCs achieved to protect insulin in the acidic stomach condition while releasing it in the small intestine due to the releasing amount of the loaded FITC insulin in the intestinal condition. Furthermore, through an in vivo hypoglycemic effect study, the feasibility of the PCs was confirmed, in which, the PCs showed an improved hypoglycemic effect. Thus, it was a shred of evidence that they can be successfully suggested as potential nanocarriers for the

delivery and penetration of the loaded insulin. In addition, the blood glucose level was lowered to 80% of its initial value after the oral administration of the PCs. Because of the long-lasting hypoglycemia, which was lasted for more than 14 h, it was concluded that the use of PCs leads to reduce the number of administrations, and it will contribute to improving the quality of patients' lives [61].

On the other hand, there are lots of studies referring to the use PEC micelles for the delivery of proteins. The types of PEC micelles formed by the utilization of block copolymers with a neutral block and a charged block when mixed with the oppositely charged protein, are also known with the names of polyion complex (PIC) micelles, interpolyelectrolyte complex, complex coacervate core micelles, and block ionomer complexes. These nanoparticles, which are formed when the neutral block stabilizes the charged protein–polymer core, have been indicated to be able to deliver therapeutic proteins. For instance, the protein Sprouty 1, which can act as an endogenous angiogenesis inhibitor, was encapsulated, and delivered using a PEC micelle composed from albumin as the charged part and a pegylated polymer as the stabilizing neutral block. The goal of this study was to create a system for the efficient delivery of the particular therapeutic protein in order to treat breast cancer [62].

Moreover, PEC micelles' utilization is studied for the combat against brain or neurological diseases such as Parkinson's disease. PEI-PEG was used to form PIC micelles with catalase. The resulting 60–100 nm-sized PIC micelles, which according to the authors are called nanozymes, were non-toxic, and protected the enzyme, catalase, from hydrolytic degradation. The produced nanoparticle was loaded into bone marrow macrophages where the uptake of the nanozymes by cells was complete within 1 hour while the enzyme was slowly released again from the cell within several days. In addition, it was important that the loading of nanozymes did not affect a4-integrin levels of the macrophages, making these vehicles possibly suitable for the treatment of Parkinson's disease [63]. Subsequently, brain tissue damages are related to the levels of oxidative stress, which have to be reduced in order to treat diseases that affect the brain and the central nervous system. A major obstacle that nanoparticles face is the Blood Brain Barrier (BBB) which protect

against circulating toxins or pathogens that could cause brain infections, while at the same time allowing vital nutrients to reach the brain. D. S. Manickam et al., tested crosslinked CuZnSOD-loaded PIC micelles, in which copper/zinc superoxide dismutase (CuZnSOD) was the enzyme which stabilized. This system was tested in a rat middle cerebral artery occlusion (MCAO) model. The delivered enzyme resulted in a reduction of oxidative damage, thus proving that the crosslinked CuZnSOD-loaded PIC micelle was able to reduce infarct size and improve motor function in rat models. To understand how the crosslinked PIC micelle was able to demonstrate this enhanced therapeutic effect, the rat brain was examined in more detail [64]. The nanoparticles were found to be in the lumen of the blood vessel, but they did not cross the blood–brain barrier, and hence, their therapeutic activity was connected with the accumulation in the infarct region [65].

4.2 Hydrogels

Hydrogels are networks composed of cross-linked hydrophilic and biocompatible polymers that can absorb huge amounts of water within their three-dimensional structures. Their ability for swelling in aqueous media is also correlated with the exhibition of thermodynamic compatibility with water. In addition, hydrogels can be made to undergo sol-gel transition by various stimuli such as temperature and pH. In the hydrated state, they have a mechanical behavior and water content like soft tissue, and as a result, they exhibit excellent biocompatibility. Their main unique property is to be undergoing abrupt volume changes from their collapsed to swollen state in response to environmental changes. The hydrogels are also defined as “intelligent” materials and stimuli-responsive materials and display both sensor and effector functions. Due to their features, hydrogels are widely used for various applications such as contact lenses, biosensors, biomaterials for tissue engineering, and drug delivery carriers, in which they have exhibited extended interest due to their effective and convenient way to administer the drugs. Furthermore, hydrogels are characterized by amphiphilic properties, thus making them potential candidates as implanted delivery systems for the long-term delivery of hydrophilic small molecules, protein or nucleic acid drugs that rapidly degrade in the presence of proteolytic enzymes. Among the natural and synthetic polymers, 2-

hydroxyethyl methacrylate, ethylene glycol dimethylacrylate, N-isopropyl acrylamide, acrylic acid, methacrylic acid (MAA), poly (ethylene glycol) (PEG), and poly (vinyl alcohol) (PVA) are commonly utilized in hydrogels for protein delivery.

These polymeric hydrogels have to protect the proteins until their release at target sites, and so they must maintain their integrity. Subsequently, the mechanical properties of hydrogels are also important during their construction for pharmaceutical applications. The mechanical properties are closely related to the crosslinking process because according to this, the final hydrogel can have different structures, different interactions with the proteins-drugs, different properties such as mechanical resistance and flexibility. Hence, the degree of hydrogel crosslinking must be altered to obtain the desired mechanical properties of the final product. For instance, a higher degree of crosslinking results in a stronger but at the same time more fragile structure. Besides crosslinking, copolymerization can also be utilized to acquire relatively strong and simultaneous elastic hydrogel. Moreover, after the administration of a hydrogel conjugated or incorporated with a protein, it is affected by the hydrolysis or enzymatic digestion; so, chemically cross-linked polymer gels can be degraded and thus, in these delivery systems, protein is released at a rate that is dependent on the rate of polymer degradation. The extend of crosslinking and the degree of swelling are also determining the protein release rate, as well as the size of the pores located in the structure. Generally, the macromolecules incorporated in the hydrogels, after their swelling and contact with water are experiencing diffusion throughout the entire matrix [1], [66].

The hydrogels are designed with the aim of responding under specific conditions of the human body and certain physiological stimuli such as pH, temperature, and ionic strength. To achieve this, they are able to change their swelling behavior, the network structure, and mechanical and chemical characteristics such as strength and permeability. Generally, pH-triggered drug release systems are mainly adopted for more effective oral delivery of protein drugs, due to their ability of proteins' protection from the different environments and mostly the harsh gastric environments. The pH-responsive hydrogels are ionic hydrogels containing pendant groups ionized in response to environmental pH

changes; this causes the hydrogel network to swell [1]. These smart polymeric nanosystems are applied in oral insulin delivery because, during the administration of the protein, it is necessary to be protected from the stomach and release in the intestine. Both acidic functional groups (e.g., sulfuric and carboxylic acids) or basic functional groups (e.g., amine, ammonium salts) can interact with H^+ and show shrinking or swelling behavior, respectively. The shrinking or swelling in response to pH regulates the insulin release from polymeric delivery systems. Hence, the protein absorption on charged hydrogels and the simultaneously counter-ion release lead to the reduction of osmotic pressure (osmotic pressure is defined as the pressure that is applied to the solution side in order to stop the fluid movement; for example when a cell placed in a hypertonic solution, then the water flows out of the cell and into surrounding solution, causing the shrinkage of the cell), and then the shrinkage of the charged hydrogel. (Figure 27) [67].

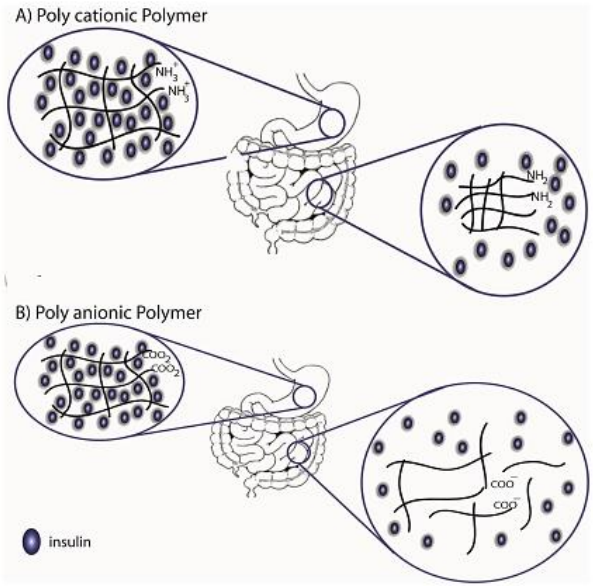

Figure 27: A) Poly cationic polymer contains numerous cationic groups with positive charge that preserve insulin at low pH in the stomach and shrink at the high pH in the intestine due to neutralization of positive charges and thus insulin is released in the intestine. B) Poly anionic polymer contains numerous anionic groups that are in neutral status in the acidic environment of the stomach and the polymer can protect insulin there, but charge of these groups changes to negative as a consequence of alkaline environment of the intestine and therefore the polymer swells and insulin is released in the intestine [67].

In the work of Lima et al. an alginate-based hydrogel loaded with BSA as a model protein was fabricated and in order to evaluate the applicability of that protein delivery system, they studied the cytotoxicity, the drug release profile, and the swelling performance in basic and acidic environments. According to the results, the hydrogel exhibited pH-dependent swelling performance with a higher value at pH 7.4 and enhanced pharmacological activity, thus indicating that the protein release mechanism was dependent on pH and composition [68]. Likewise, Sabaa et al. prepared a xanthan gum/poly (N-vinyl imidazole) hydrogel system loaded with BSA in order to study this system as a candidate protein delivery system. They also obtained that % Drug (BSA) loading (% DL) and Encapsulation Efficiency (% EE) increased with increasing both gelation time and loaded BSA concentration, while they decreased with increasing polymer concentration. To conclude, the particular hydrogels exhibited high loading efficiency and encapsulation efficiency [69].

4.2.1 Injectable Hydrogels

The development of injectable hydrogels has gained attention for the loading of therapeutic proteins through simple mixing, due to their ability to be administered in a minimally invasive manner. These hydrogels exist as flowing liquids prior to administration, and rapidly turn into viscoelastic gels upon administration and they are responsive to various stimuli including temperature, pH, and enzymes. Beyond these stimulating actions, temperature is the most widely used stimulus in injectable hydrogels. The stimuli-responsive injectable hydrogels, also named smart hydrogels, can change their sol-to-gel phase transitions in response to various stimuli. It is noteworthy that hydrogels are polymers and as a result of the exhibition of lower critical solution temperatures (LCSTs) with ideal phase transitions between room and body temperature are candidate materials for injectable hydrogel construction. These polymers form hydrogels after the administration, but at the previous phase, they are soluble at room temperature. Pluronic, poly(phosphazene), and poly(N-isopropyl acrylamide) are polymers that are switching their structure after exposure to different temperatures. More specifically, they exist in a sol state at room temperature and transform into gel at body temperature. Compared to those polymers which have limited utilization because of the non-biodegradability and

biocompatibility, poly(ε-caprolactone-co-lactide)-b-poly(ethylene glycol)-b-poly(ε-caprolactone-co-lactide) (PCLA)-based copolymer and thermosensitive injective hydrogels based on poly(ethylene glycol)-b-poly(ε-caprolactone)-b-poly(ethylene glycol) (PEG-b-PCL-b-PEG) have been developed for biomedical applications. Sim et al. developed a series of heparin-based injectable hydrogels composed of PCLA-conjugated heparin (Hep-PCLA) which are ionic and biodegradable for the sustained delivery of a positively charged model protein, namely lysozyme. The Hep-PCLA copolymer exhibited sol-to-gel transitions in aqueous solutions as temperature increased, where it formed a stable gel at 37 ^{o}C and subsequently, it rapidly formed in situ gels after a single subcutaneous injection of the copolymer solution into the backs of SD rats. The lysozyme-loaded Hep-PCLA injectable hydrogels had shown a reduction at the initial in vivo burst release, and maintained a sustained release of lysozyme, thus making them potential carriers for protein delivery [70]. Similarly, Turabee et al. indicated that polypeptide-based pH- and temperature-responsive injectable hydrogels can be used as candidates for the sustained delivery of various therapeutic proteins such as the positively charged protein lysozyme, with improvements in the stability and inhibition of the burst release [71].

4.2.2 Glucose Sensitive Hydrogels

Diabetes mellitus (DM) is a metabolic disease in which there is a high blood glucose level over a prolonged period of time. The traditional treatment of diabetes includes injection with the appropriate amounts of insulin, but it is a painful and inconvenient for the patients, method. Thus, as an alternative route of administration and delivery of insulin, researchers have offered the utilization of glucose-responsive hydrogels in which insulin has been incorporated. The glucose sensitive hydrogels can respond in a smart way to high blood glucose levels through releasing specific amounts of insulin in a controlled way instead of the continuous invasive insulin administration The hydrogels are able to sense a biomolecule e.g., the glucose and respond to it by a release of the hormone e.g., the insulin. For the release of insulin from the hydrogels there are two possible pathways. The first pathway is referred to the hydrogel which can be utilized as a membrane with a controlled permeability and is triggered by glucose; and the second one is the

separation of a reservoir full of insulin from the outside by the membrane. However, in both pathways, when the hydrogel swells then insulin is released.

The initial glucose-responsive materials were made from the combination of a sensing element, the glucose oxidase (GOD), with a pH-sensitive hydrogel. The glucose oxidase reacts with glucose and converts it into gluconic acid, while inside the hydrogel the pH is lowered. The capture of protons from the constituting element of the gel leads it to respond to pH and thus modify the charge density of the polymer. To attain insulin release, hydrogel swelling is needed when glucose concentration increases, that is, when the pH decreases because of the production of gluconic acid (Figure 28) [1].

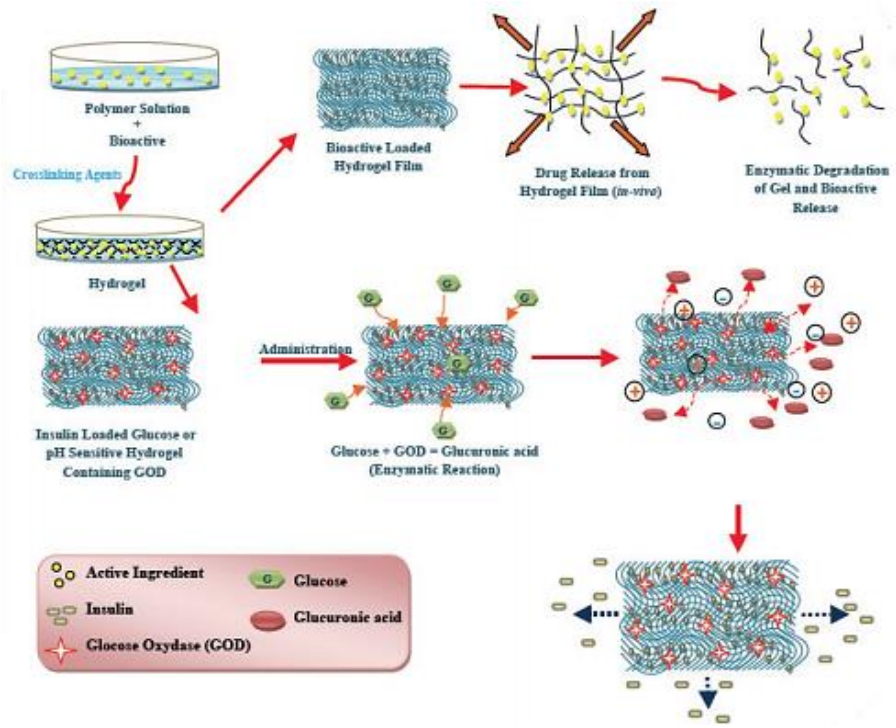

Figure 28: Hydrogel matrix system: pH and glucose level dependent bioactive release [1].

Gu et al. developed injectable nanocapsule-containing microgels for controlled glucose-responsive release of insulin. The monodisperse microgels consisted of a pH-responsive chitosan matrix, enzyme nanocapsules, and recombinant human insulin. Into the nanocapsule, glucose-specific enzymes were covalently connected with the aim to improve enzymatic stability by protecting them from denaturation and immunogenicity, including the minimization of loss due to diffusion from the matrix.

During hyperglycemia, glucose oxidase will convert glucose into gluconic acid and then, the resulting acidic environment causes protonation of the chitosan amino groups leading to matrix swelling followed by insulin release. According to the in vivo studies, the incorporation of enzymes into microgels facilitated the release of insulin and improved control of blood glucose levels. However, further, development is necessary with the aim to optimize the glucose response sensitivity and sustain long-term release in order to achieve dynamic regulation of blood glucose levels under in vivo conditions [72].

4.2.3 Nanogels

Nanogels are hydrogels constructed at the nano scale and composed of hydrophilic or amphiphilic swellable polymer chains that are able to retain large amounts of water without being solubilized. They are promising drug and protein delivery systems due to their high loading capacity, high stability and responsiveness to environmental stimuli such as pH, temperature and ionic strength causing a stimuli-responsive sustained release of the drug. In addition, they are able to both transport drugs and incorporate bioactive molecules through the formation of salt bonds, hydrogen bonds and/or hydrophobic interactions. The size of the networks and thus of the nanogels are closely connected to the concentration of polymers used for their construction, as well as to the physiological stimuli because each designed vehicle has different behavior through the administration in the human body and under different conditions in each organ. For example, Asada et al. prepared protein nanogels by temperature induced gelation of oppositely charged proteins, such as ovalbumin and lysozyme or ovotransferrin [73]. Similarly, nanogels were obtained by pH- and temperature-induced gelation of chitosan and ovalbumin [74].

From the different polymeric complexes produced, polyelectrolyte nanogels are utilized due to their ability of incorporation of oppositely charged, low-molecular-mass drugs and biomacromolecules such as oligo- and polynucleotides (siRNA, DNA) and proteins. Furthermore, the nanogel has a strong interaction with cells, and proteins compared to other carriers. An important benefit of nanogels is that they can form a colloidally stable complex with protein, with an overall complex

size of about 50 nm, which is suitable for effective intracellular uptake. They can also assist the protein in refolding and protect it against the aggregation or denaturation [1]. Zhao et al. investigated the encapsulation and sustained release of insulin into/out of nanogels which are sensitive and dependent to pH and temperature. The nanogels synthesized was from hydroxypropyl methylcellulose (HPMC) and the was process characterized as eco-friendly method as there was no need for a surfactant and thus no organic solvent. The results show that the drug loading was as high as 21.3%, while the entrapment efficiency was 95.7% [75].

4.3 Layer-by-layer films

Layer-by-layer (LbL) assembly has emerged as a versatile and simple method for immobilization of functional molecules in an easily controllable thin film morphology. The layer-by-layer films produced from the self-assembly offer huge freedom in material selection and flexibility of structural design, which are fully matched with the fabrication needs of drug delivery materials requiring complicated designs. The description of this technique has been illustrated in the previous unit. However, as it was mentioned, the layer-by-layer assemblies occur between cationic and anionic polyelectrolytes (Figure 29A). The cationic polyelectrolyte is usually over-absorbed in the negatively charged surface of a solid, thus causing reversal in surface charge under appropriate conditions. Then, the anionic polyelectrolyte is absorbed resulting again in a reversal of the surface charge so that the alteration of the surface charge permits continuous fabrication of the layered structure. This procedure can also be utilized to assemble cationic polyelectrolyte and anionic particles such as protein molecules (Figure 29B). In addition, it is noteworthy, that not only electrostatic interactions can be used as driving forces but also hydrogen bonding, which allows for expanded options for materials and deposition conditions for film construction with different film properties [76]. For instance, Anandhakumar and Raichur demonstrated a nanoparticle loading protocol for the construction of a multifunctional polyelectrolyte multilayer film for externally activated drug and protein delivery. Bovine serum albumin (BSA) was utilized as the model protein and it was immobilized into the polymeric network of the film using electrostatic interactions, hydrogen bonds, and hydrophobic interactions. After BSA adsorption,

two bilayers of PAH/DS (poly(allylamine hydrochloride)/PAH, dextran sulfate/DS) were added to the polyelectrolyte multilayers (PEMs) to maintain the negative surface charge of the film. The negative charge would favor the incorporation of the low molecular weight water-soluble drug ciprofloxacin hydrochloride (CH) into the film via electrostatic interactions [77].

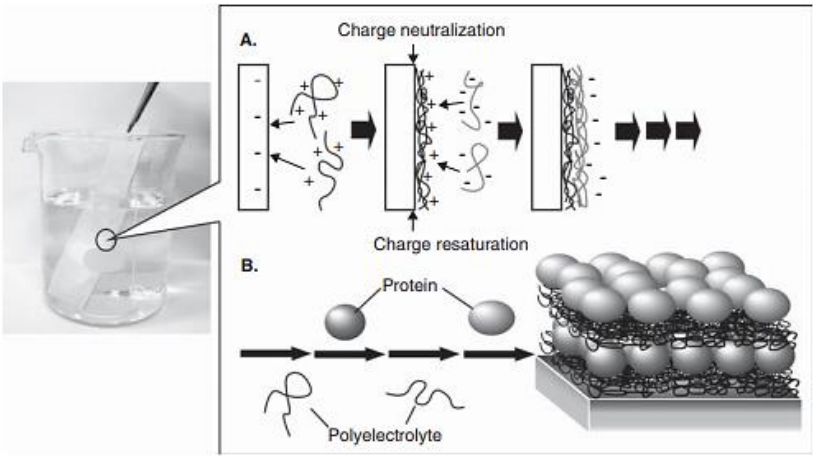

Figure 29: An example of a layer-by-layer film from (A) polyelectrolytes and (B) polyelectrolyte/protein [76].

Proteins and nucleic acids are fragile biomolecules, so the mild aqueous conditions for the encapsulation of those molecules into multilayer films preserve their bioactivity. By employing degradable polyelectrolytes as building blocks, the ability to tune the degradation kinetics of multilayer assemblies has been demonstrated and used to control the release kinetics of compounds embedded in these films [78]. Mehrotra et al. studied the time-controlled protein release from layer-by-layer assembled multilayer functionalized agarose hydrogels, because these hydrogels can be utilized into the arrays of uniaxial channel for the support of linear axial growth into spinal cord lesion sites. They showed that there was sustained release of protein under physiological conditions for more than four weeks from the pH-responsive H-bonded poly(ethylene glycol)(PEG)/poly(acrylic acid)(PAA)/protein hybrid layer-by-layer (LbL) thin films, when prepared over agarose. Lysozyme, a protein similar in size and isoelectric point to BDNF (brain-derived neurotrophic factor), is released from the multilayers on the agarose. The protein was loaded after the agarose hydrogel fabrication rather than pre-loaded directly into the hydrogel,

avoiding the caustic conditions used in the templated agarose scaffold fabrication [79].

Other pharmaceutical studies using layer-by-layer film for the encapsulation of proteins are related to insulin for better control over the blood glucose level in diabetic patients. Ding and co-workers prepared layer-by-layer multilayer films which were fabricated from poly(vinylalcohol) (PVA) and poly[acrylamide-co-3-(acrylamido)phenylboronic acid] [P(AAm-AAPBA)] using covalent phenylboronate ester bonding as driving force. The disassembly of the film is accelerated by the addition of glucose and thus, the PVA/P(AAm-AAPBA) film presents glucose-sensitive behavior even under physiological conditions. The enhanced glucose-sensitive behavior at physiological pH may originate from the stabilization of phenylboronate ester by the adjacent amide group [80]. Kentaro prepared layer-by-layer thin films composed of insulin and negatively-charged polymers such as poly(acrylic acid) (PAA), poly(vinylsulfate) (PVS), and dextran sulfate (DS). According to the results, these layer-by-layer film-insulin complexes were stable in acidic solutions, such as in the environment of stomach with pH 1.4, while they decomposed under physiological conditions because there was a change in the net charge of insulin from positive to negative. On the other hand, the LBL film consisting of insulin and composed of positively charged polymers such as poly(allylamine hydrochloride) (PAH) were affected by the acidic environment and decomposed because in acidic media positive charges are generated on insulin. Moving on, Kentaro prepared insulin-containing microcapsules by coating LbL films on the surface of insulin-doped calcium carbonate ($CaCO_3$) microparticles to investigate the release of insulin. The release of insulin from the microcapsules was enhanced at pH 7.4, while it was suppressed in acidic solutions, thus suggesting insulin-containing microcapsules as candidates for oral delivery of insulin [81].

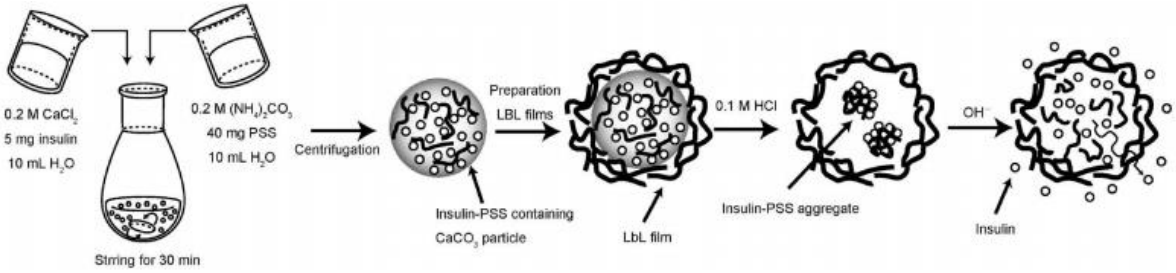

Figure 30: A schematic Illustration of the construction of an insulin-containing LbL film on PLA microbeads and the release of insulin at neutral pH [81].

4.4 Nanocapsules

The traditional delivery of proteins has been related to their conjugation with polymers, liposomes or inorganic nanoparticles. However, a new approach for the delivery has emerged and it includes the in-situ formation of polymeric coatings around the protein, that is nanocapsules. Nanocapsules are hollow spherical structures (heterogenous systems) with dimensions in the sub-micrometer region. They are typically polymer empty shells with a hollow inner space that the drug can be entrapped inside. Depending on the physiochemical features and composition of the nanocapsules, the drug can be either absorbed onto the surface or being included at the central core. They can be characterized as a "reservoir" system in which the core may be aqueous or composed of a lipophilic solvent such as oil. The main difference between nanocapsules and nanospheres is that the nanospheres are matrixes (homogenous systems) without a cavity in which the drug can be dispersed and not entrapped as happens with the nanocapsules. One of the advantages of nanocapsules over nanospheres is that the drug loading as a percentage of polymer content can be increased if the core is composed from a material that is a good solvent for the drug. Subsequently, because of the structure of the nanocapsules, the incorporated drugs will not directly encounter the surrounding tissues, thus enabling the reduction of the irritation at the site of administration with the simultaneous protection of the drug both during storage and after the administration.

The methodologies for the preparation of nanocapsules has been already mentioned before and include the emulsions either oil/water (O/W) emulsions, which lead to the production of nanocapsules with an oily core suspended in water, or water/oil (W/O) emulsions, in which the nanocapsules have an aqueous core suspended in oil. In addition, in order for the limitations of each emulsion to be overcome, i.e. inability of oil-based nanocapsules to encapsulate water-soluble compounds and the inability of intravenous administration of nanocapsules with aqueous core in an oily phase, nanocapsules with an aqueous core suspended in an

aqueous medium have been designed [82]. The encapsulation process comprises three steps in which the first step includes the functionalization of protein with acryl groups to introduce polymerizable groups on the protein surface facilitating the polymer coating around the macromolecule. In the second step, the acrylated protein produced is mixed with the monomers in a deoxygenated buffer. This buffer without oxygen is utilized because the presence of this molecule stops the polymerization due to the radical scavenger behavior of triplet oxygen. Additionally, in this step, the protein surface has absorbed monomers through electrostatic interactions thus forming a dynamic monomer layer around it. Finally, the third step is the in situ polymerization which is initiated by the addition of radical initiators such as ammonium persulfate (APS) and N,N,N' ,N' -tetramethylenediamine (TMEDA) (Figure 31) [83].

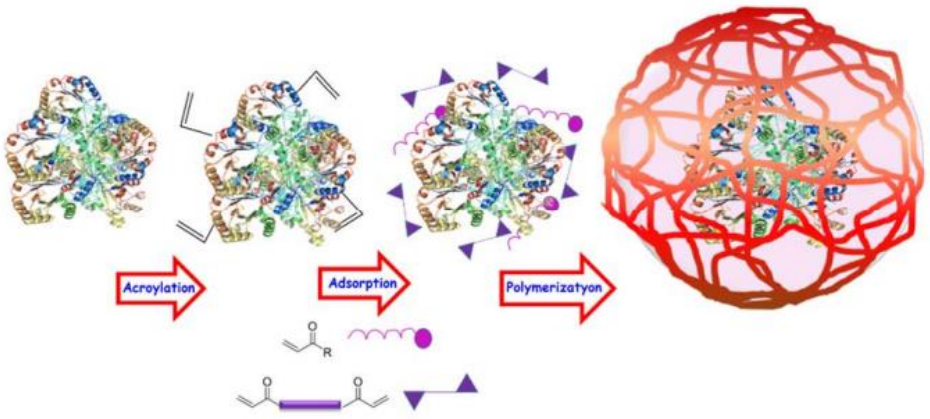

Figure 31: Scheme of the encapsulation process [83].

4.4.1 Degradable Nanocapsules

Before the construction of a nanocapsule that is intended for the transportation of a therapeutic protein, it is necessary to evaluate the stimuli that may affect the host protein release. For this reason, degradable nanocapsules are selected due to their ability to allow an on-demand release of the housed protein and, therefore, precise control of the protein administration. Different stimuli have been explored to trigger the nanocapsules disassembly as a function of the pursued purpose and therefore, different crosslinkers have been explored [83].

Nanocapsules are designed according to the effect of specific stimuli such as pH, because by changing this, the entrapped protein can be released. Shu et. al.

demonstrated a nanocapsule entrapping Bovine serum albumin (BSA) with the aim to avoid the burst release, enhance the encapsulation efficiency and allow the release of the protein at a pH 5.4. The biodegradable hollow polyelectrolyte capsules were synthesized by layer-by-layer assembly of water-soluble chitosan and dextran sulfate on protein-entrapping amino-functionalized silica particles and the subsequent removal of the silica. The burst release was decreased to less than 10% in phosphate-buffered saline within 2 hours and the cell viability study suggested that the nanocapsules had good biocompatibility. Because of the demonstration of good capacity for the encapsulation and loading of BSA, these nanocapsules can be candidates for the delivery of ionic protein and peptide drugs such as insulin [84]. pH-responsive nanocapsules are also designed for the release of protein drug to combat tumor tissues. In solid tumors, there are acidic conditions, which present a mild-acidic environment because of their accelerated metabolism and hypoxic conditions. Therefore, when the polymeric nanocapsules reached the tumor tissue it is possible to release the enzyme. Use of the degradable nanocapsules is related with the degradation within the extracellular matrix and the allowance of the drug-loaded nanocarrier for a homogenous distribution inside the tumoral mass. However, due to the presence of a denser extracellular mass of tumoral tissues compared to healthy tissues, the penetration of nano-devices faces difficulties, therefore, limiting the therapeutic efficacy of drugs carried to a peripheral effect. Besides the conditions of solid tumors, endosomes also present acidic pH values, and thus, a pH-responsive polymeric nanocapsule can be degraded in them, upon intracellular entrance, releasing the protein into the cytoplasm. This strategy has been employed to avoid the degradation of proteins into the lysosomes. Min et al. designed a novel delivery platform based on nanocapsules consisting of a protein core and a thin permeable polymeric shell that can be engineered to either degrade or remain stable at different pH. These pH-sensitive nanocapsules were able to escape from lysosome (as pH-responsive crosslinker glycerol dimethacrylate was employed). The endosomal escape was examined by the incubation of rhodamine labeled HRP nanocapsules with HeLa cells and posterior labeling of early endosomes and late lysosomes [83], [85].

Polymeric nanocapsules can also be designed to be redox-responsive. Zhao et al. prepared redox-responsive single-protein nanocapsules for intracellular protein delivery. The protein is non-covalently encapsulated into a thin positively charged polymeric shell, which serves as a protective layer through in situ interfacial polymerization. Cell-free assays in the presence of glutathione (GSH) were used in order to evaluate the dissociation of the polymeric shell under reducing conditions and the subsequent release of protein. The GSH is found in millimolar concentrations inside the cell and due to the high concentration of reduced GSH, the cell cytosol exhibits lower redox potential compared to the external intracellular media. According to the results, the nanocapsules were efficiently internalized into cells and successfully achieve release of the protein in the reducing cytosol. In conclusion, it was demonstrated that using that platform active caspase 3 (CP-3) can be delivered and can induce apoptosis in a variety of human cancer cell lines, including HeLa, MCF-7, and U-87 MG [83], [86].

4.5 Polymer-based scaffolds

The field of tissue engineering has advanced drastically in the last 10 years, as it offers the ability to regenerate almost every tissue and organ of the human body. The goal of tissue engineering is to achieve restoration, maintenance, or improvement of tissue functions that are defective or have been lost by different pathological conditions, either by developing biological substitutes or by reconstructing tissues. The general strategies to accomplish this goal can be classified into three groups. The first group is associated with the implantation of isolated cells or cell substitutes into the human body. The second one aims to deliver tissue-inducing substances such as growth factors, and the third is related to the placement of cells on or within different matrices.

In tissue engineering, scaffolds belong to the most important tool for repair and restoration of tissues. Scaffolds are defined as three-dimensional porous solid biomaterials which aim to provoke the minimum toxicity or inflammation in vivo with the simultaneous permission for transport of nutrients and regulatory factors to allow cell survival, proliferation, and differentiation. Furthermore, the scaffold has to be designed from the appropriate biomaterial with biodegradable abilities and

bioactivity in order not to be rejected but to be integrated within the human body. The developing scaffolds need to be characterized with the optimal features such as strength, rate of degradation, porosity, and microstructure, as well as their shapes and sizes. These features are more readily and reproducibly controlled in polymeric scaffolds either from synthetic or natural materials. Polymeric scaffolds have high surface-to-volume ratio, high porosity with very small pore size, biodegradation, and mechanical property, as well as they offer distinct advantages of biocompatibility, versatility of chemistry, and the biological properties which are extremely important in tissue engineering applications. There are different types of scaffolds including porous scaffold, microsphere scaffold, hydrogel scaffold, fibrous scaffold, polymer-bioceramic composite scaffold and acellular scaffolds, but the scaffold structure is closely connected with the methods utilized for their process. The bulk and surface properties of the material and the proposed function of the scaffold are entirely influenced by the fabrication technique for tissue engineering scaffolds. Most techniques involve the application of heat and/or pressure to the polymer or dissolving it in an organic solvent to mold the material into its desired shape [87].

4.5.1 Growth Factors

Growth factors are naturally occurring substances capable of stimulating cell proliferation, wound healing, and occasionally cellular differentiation. They are usually proteins/peptides that are important for regulating a variety of cellular processes such as the increase of production of connective tissue, the promotion of remodeling, as well as the creation of a new supply of blood vessels. Growth factors generally behave as signaling molecules between cells. From those, cytokines and hormones are well-known, and they bind to specific receptors on the surface of their target cells. They often promote cell differentiation and maturation, which varies between growth factors. For instance, epidermal growth factor (EGF) boosts osteogenic differentiation while fibroblast growth factors and vascular endothelial growth factors stimulate blood vessel differentiation (angiogenesis) [88].

In tissue engineering the number of active proteins utilized in the applications are limited. Cytokines and growth factors are responsible for specific processes, such as the growth and development of tissues (Table 3). However, due to their ability to

act upon multiple tissue types, there is need for localized delivery systems instead of systemic application. In repair and regeneration of damaged bone tissue osteoinductive and angiogenic growth factors, such as transforming growth factor-β (TGF-β) superfamily, bone morphogenetic proteins (BMPs), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) play an essential role. [89], [90].

Table 3: Growth factors commonly used in bone regeneration [90].

Growth Factor	Abbreviation	Relevant known activities
Transforming growth factor-β	TGF-β	Proliferation and differentiation of bone
Bone morphogenetic protein	BMP	Differentiation of bone forming cells
Insulin-like growth factor	IGF-1	Stimulates proliferation of osteoblasts and the synthesis of bone matrix
Fibroblast growth factor-2	FGF-2	Proliferation of osteoblasts
Platelet-derived growth factor	PDGF	Proliferation of osteoblasts

4.5.1.A. Transforming growth factor-β (TGF-β)

Transforming growth factor-β is a dimer which belongs to the TGF-β superfamily. The TGF-β superfamily is comprised of more than 30 closely related polypeptides, mainly including typical TGF-βs, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and activin/inhibin, which regulate multiple cell functions from early development to regulating homeostasis throughout adulthood. TGF-β is one of the main initiators of chondrogenesis of mesenchymal precursor cells, and the differentiation of mesenchymal stem cells (MSC) into chondrocytes. Among its three isoforms (TGF-β1, TGF-β2, TGF-β3), TGF-β1 and TGF-β3 have been utilized in many studies to explore the effect of TGF-β on the repair of cartilage. However, there are still some studies that do not support the role of TGF-β in cartilage repair in vivo [91]. Guo et al. developed a rabbit osteochondral defect model in which oligo polyethene glycol (PEG) fumarate (OPF) hydrogel composites containing gelatin microparticles (GMPs) loaded with MSCs with or without TGF-β1 did not improve cartilage morphology [92].

4.5.1.B. Bone Morphogenetic Proteins (BMPs)

BMPs are members of the TGF-β superfamily. They have two active forms of homodimer and heterodimer, which can also induce differentiation of MSCs. They are utilized in reconstruction and restoration of damaged bone tissue because of their ability of osteoinductive effects and of stimulating the formation of new bone tissue due to the differentiation of mesenchymal stem cells (MSCs) into osteoblasts. At least 15 different BMPs have been identified, of which BMP-2, BMP-4, BMP-6, and BMP-7 have been the most widely studied in the field of cartilage tissue engineering. U.S. FDA has approved some of those, such as BMP-2 and BMP-7, and they are already being used in clinical practice. Particularly, BMP-2 is highly expressed throughout the chondrogenic process. That is the reason why it has been commonly applied to improve cartilage regeneration in vitro and in vivo. Even though, recombinant BMPs are characterized by high efficiency, they have short life and thus, some problems during their clinical use do exist [89], [91],

4.5.1.C. Insulin-Like Growth Factor

Both insulin-like growth factor (IGF) isoforms, IGF-1 and IGF-2, according to in in vitro and in vivo studies, have been shown to promote the proliferation of chondrocytes, stimulate the synthesis of the extracellular matrix of cartilage, and prevent the activity of extracellular matrix-degrading enzymes, which is beneficial to the cartilage repair. More specifically, IGF-1 induces chondrogenic differentiation of MSCs independently, and its functions were enhanced when combined with other growth factors [89].

4.5.2 Applications of scaffold-based protein delivery

The scaffolds can be considered as special types of drug delivery matrices which additionally possess pores or accessible regions for cell penetration. The incorporated active substances such as drugs, therapeutic proteins and peptides have to be released over an extended time and in a controlled fashion. However, the design of controlled release systems for proteins and peptides for tissue engineering applications presents several challenges, primarily related to the chemical structures of the drug substances as well as the varied properties of the scaffolds used for each application. For that reason, several strategies were developed to design different

scaffold types for their long-term release under difficult circumstances. For instance, functional sites can be introduced into a polymer for the covalent immobilization of growth factors or hydrogel systems can be designed with attachment sites for bioactive proteins providing enzymatically cleavable linkers, allowing the release of growth factors to be triggered by the presence of enzymes [90].

Polymeric Nanoparticles (PNPs) are promising delivery vehicle in cartilage tissue engineering as they have high versatility and are able to release growth factors outside and inside of target cell, as well as they have good bioavailability, distribution and pharmacokinetic profiles. Furthermore, they show good performance in their affinity to growth factors, allowing for the accumulation of encapsulated growth factors due their property of having a greater surface area to volume ratio [91]. Kim et al. developed fibrous PLGA scaffold integrated with BMP-7 loaded PLGA NPs, and subsequently combined them with synovial-derived mesenchymal stem cells (MSCs) to investigate the influence on full-thickness osteochondral defects in rabbits. The results have shown that BMP-7 effectively enhanced the chondrogenic potential of synovial-derived MSCs supporting the high collagen type II and proteoglycan production and thick hyaline cartilage formation [93]. Moreover, Deepthi and his colleagues investigated the effect of prolonged release of Transforming growth factor-β (TGF-β) by encapsulating it in chondroitin sulphate nanoparticles (nCS) incorporated in chitin and polycaprolactone (PLC) scaffold. A prolonged release of TGF-β over 4 weeks was observed which improved the attachment, proliferation and chondrogenic differentiation of rabbit adipose-derived mesenchymal stem cells (rASC)s [94]. In conclusion, even though, the high surface area to volume ratio increases the NPs loading efficiency of growth factors, their intrinsic properties may influence the advantages, for instance by reducing the stability of the NPs during the preparation process and thus, the overall behavior of these vehicles.

5. Conclusions and future outlook

Therapeutic protein/peptide molecules have attracted considerable attention in the treatment of various chronic diseases such as diabetes mellitus, cancer, or neurodegenerative diseases due to their high potency and specificity. However, the physicochemical properties of proteins and peptides, such as fragile structure and high molecular weight, with the combination of their low-stability during the administration and the connection with different environmental conditions, are some of the main obstacles for their delivery and administration. Moreover, when they are administered either orally or parenterally, they can be easily degraded by enzymes in the gastrointestinal tract (GIT), have difficulty permeating across gastrointestinal mucosa, and can be eliminated during first-pass hepatic clearance. Thus, low delivery efficiencies have motivated research in nanostructured drug carriers with enhanced targeting. Structures that are very small (< 5 nm) can be removed by renal clearance, while larger ones (hundreds of nanometers) can be filtered out by the liver. The ideal nanoplatform should provide some fundamental features in order to achieve an efficient protein delivery: 1) the functional structure of protein transportation must be constant during the encapsulation process; 2) the carrier must present high loading capacity; 3) the protein should be protected against enzyme degradation, proteolysis or thermic denaturalization, among others; 4) the nanoplatform must protect the activity of the protein and not allow the release until the nanodevice reaches the target site, as well as to be designed to respond to different stimuli that enable the release of the protein and 5) nanoplatforms should avoid opsonization and increase the half-life of the protein.

Polymer-based nanosystems made up from alginate, chitosan or PLGA, PCL provoked the interest of the scientists and have been the most-known candidate vehicles for protein/peptide delivery, as they are characterized by biocompatibility, biodegradability, amenability to formulation, and capability with intracellular and systemic delivery. Especially, charged polymer NPs have many of the features required for an ideal carrier system because they demonstrate high encapsulation efficiency with retention of protein bioactivity, targeting ability into intracellular

compartments, and sustained release kinetics. Nevertheless, size, shape, and structure with the distinctive feature of PEGylated modification are also significant parameters that have to be taken under consideration during the construction of polymeric nanoparticles because they can influence their stability, targeting specificity, and thus the therapeutic efficacy.

As it has been extensively described along this dissertation, amphiphilic block copolymers which self-assemble into different morphologies, such as polymeric micelles or polymersomes, in which hydrophobic proteins can be encapsulated into the core or hydrophilic proteins or can be covalently attached to the outer surface of the polymersome respectively, are utilized for the delivery of therapeutic proteins because they protect them and prevent their degradation. At the same time, polyelectrolyte-protein complexes have emerged in combination with the layer-by-layer technique as challenging candidates especially for oral delivery of therapeutic proteins because of the acidic conditions in the gastrointestinal tract and fast enzymatic degradation. Especially, PEC micelles can be made to be responsive to changes in pH and temperature, and thus, they represent reasonable colloidal stability in vivo and a versatile platform for controlled delivery and release. In addition, PEC micelles have been investigated for the delivery of several therapeutic proteins such as oral insulin and the enzyme catalase to combat Parkinson's disease. pH-triggered hydrogels are those that are mainly implemented for more effective oral or injectable delivery of protein drugs, due to their ability of protection from the different environments, especially the harsh gastric ones, while they can change their swelling behavior, the network structure, and mechanical and chemical characteristics under different conditions, beneficially affecting the total response of the system. Subsequently, the utilization of polymeric nanocapsules consists of polymerization in situ around the protein-making a polymeric coating and as a strategy can be used for many proteins. Finally, another strategy includes the polymer-based scaffolds from which proteins such as growth factors are released. However, the release of growth factors from the delivery system in humans needs extensive clinical validation.

Taking everything into account, many advances have been made in the delivery of therapeutic protein/peptides by using polymeric NP delivery vehicles. Barring certain obstacles which certainly require stringent evaluation, the future of such carrier devices is envisioned to be promising. The development of novel polymeric NPs to enhance the bioavailability of proteins and peptides remains an active field of research. Further studies are still required to develop novel targeted NPs formulation for site-specific and sustained release of proteins and peptides in a non-invasive patient complaint manner.

6. References

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