Chapter 11

Polymer-based protein delivery systems for loco-regional administration

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Abstract

With the advent of recombinant technology, a wide variety of biocompatible therapeutic proteins can be produced with relative ease. These proteins are formulated and subsequently administered in patients to treat various of diseases in a more effective and targeted manner. At the level of formulation development, protein molecules can be physically and/or chemically-conjugated to a wide array of naturally-occurring, semi-synthetic and synthetic biomaterials to form different types of protein delivery systems. Depending on their architecture and the extent of protein-scaffold interactions, these delivery systems can modify the pharmacokinetic and pharmacodynamic properties of the proteins. The versatility of polymer-based protein delivery systems such as micro/nanoparticles, hydrogels, porous scaffolds and fibrous scaffolds means it is possible to alter the spatial distribution of the protein load within the system as well as the protein release kinetics. These can then influence the ability of the protein molecules to exert their effects in their immediate microenvironments, be it to kill cancer cells or to recruit stem/progenitor cells. In this Chapter we discuss the production of protein therapeutics and the application of polymer-based biodegradable delivery systems for these proteins which include nanoparticles and scaffolds. We also include discussion of 'green synthesis' methods for production of these delivery systems.

1. Introduction

The previous two decades have seen a remarkable progress in biotechnology that enables production of many proteins for use in biomedical research. To improve their therapeutic values, much attention has been dedicated to prolonging the biological activity of these proteins after administration in patients. Due to the challenges faced by proteins in crossing biological barriers and target disease sites a variety of organic and inorganic biomaterials have been developed. It is ranging from basic drug first delivery system such as polymer system for delivery of biomacromolecules, described in 1976 to smarter systems with capabilities of stimulating therapeutic release and local biological action in response to interactions with the surrounding environment (1,2). Proof of concept has already been obtained in the field of regenerative medicine (3,4) and in cancer therapy (5,6). A common approach involves incorporating the protein molecules into an appropriate matrix that permits gradual release of the protein load. In doing so, the matrix limits the exposure of proteins from proteases and neutralizing antibodies that may be present in the immediate physiological environment, thus preventing them from undergoing rapid degradation. Polymers have been widely-used to produce protein-loaded matrices due to the high versatility of this material group. By changing the type of monomers, controlling the polymerization conditions or functionalizing the polymer chains with chemical groups of interest, the physicochemical and biological properties of the polymer matrix, including surface charge, hydrophobicity, biodegradability and biocompatibility can be regulated.

2. Protein Therapeutics

2.1 A brief history and rationale

Proteins have the most dynamic and diverse role of any macromolecule in the body. They act as catalysts to biochemical reactions by forming receptors and channels in the membranes. Furthermore, proteins provide intracellular and extracellular scaffolding support and they transport molecules within the cells or from one organ to another (7). It has been estimated that there are approximately 25,000–40,000 different genes in the human genome and with alternative splicing of genes and post-translational modification of proteins the number of distinct functional proteins is likely to be much higher (8,9). The great number of functional proteins could pose vast challenges to modern medicine, as disease may result when any one of these proteins contains mutations or other abnormalities, or is present in abnormally high or low concentration. These proteins however may also present immense possibilities in terms of development of protein therapeutics to alleviate disease. It is for this reason that they have progressively become the forerunners in biopharmaceutics. Protein therapeutics can be grouped into molecular types that include: antibody-based drugs, anticoagulants, blood factors, bone morphogenetic proteins (BMPs), enzymes, fusion proteins, growth factors, hormones, interferons, interleukins, and thrombolytics (10,11). Protein therapeutics have also been classified based on their pharmacologic activity as drugs that: i) replace a protein that is deficient or abnormal, ii) supplement an existing pathway, iii) provide a novel function or activity, iv) interfere with a molecule or organism, or iv) deliver a payload such as a radionuclide, cytotoxic drug, or protein effector (7).

Proteins first emerged as a major class of pharmaceuticals in the 1980s, with a majority of them mainly developed for therapeutics and a small number for diagnostics and vaccines (10). More than three decades later, a better understanding of the molecular biology and biochemistry behind these macromolecules and their role in various body functions and pathological conditions has led to the realisation of enormous therapeutic applications for proteins (12). Advances in the development of

protein therapeutics has demonstrated that these molecules offer several advantages over the more conventional small molecule drugs (Fig. 11.1).

The first reported use of protein therapeutics was in the 1920s when Insulin that was purified from bovine and porcine pancreas was used as a life-saving daily injection for patients suffering from type 1 diabetes mellitus (13). The low availability of animal pancreases for purification of insulin, the high cost of the purification and the immunological reaction of some patients to animal insulin hindered the widespread use of this protein (14). In 1982 Insulin became the first FDA approved human protein therapeutic derived from recombinant DNA technology (https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/recombinant-dna-technology) and has since become the major therapy for type 1 and type 2 diabetes mellitus (14,15). Soon after, other recombinant human proteins were developed as therapeutics to replace the natural proteins deficient in some patients (e.g., growth hormone) or boost existing pathways (e.g., interferon-α, tissue plasminogen activator, and erythropoietin) (7, 16).

Recombinant production of proteins is highly favoured over purification of proteins from their native source. A small number of non-recombinant proteins purified from their native source have been reported, such as pancreatic enzymes from hog and pig pancreas (17) and alpha-1 proteinase inhibitor from pooled human plasma (18). This strategy however has proven to be rather challenging and expensive. In this regard, the production of therapeutic proteins by genetic engineering using recombinant DNA technology has presented great opportunities towards overcoming the challenges faced with conventional non-recombinant proteins. In addition to availability in sufficient quantities and the reduced risk of immunological rejection, recombinant technology allows the modification of proteins or the selection of particular gene variants to improve function or specificity and enables the production of proteins that provide novel functions or activity (7). Thus modern therapeutic proteins are largely produced by recombinant technology.

In the field of cancer treatment, it has been shown that a synergic effect with ionizing radiation could occur upon exposure to BMPs. HrBMP4, for instance, is expressed in the embryonic cortex, indicating its role in the formation of mesoderm and neurogenesis, e.g. morphological differentiation of neural stem cells (19,20). A recent clinical trial carried out on the brain tumor glioblastoma (GBM) is in course after resection or biopsy of the tumor, using Convection Enhanced Delivery (CED) allowing increasing amounts of HrBMP4 solutions combined with Gd-DTPA and determining the extent of intra-tumor and interstitial drug delivery (21). HrBMP4, can indeed inhibit the proliferation of brain tumor stem cells, induce their morphological changes to a more differentiated phenotype and reduce their invasiveness (22,23). The possibility to abolish the tumor's self-renewal potential by depleting the tumor stem cell compartment with a differentiating, non-toxic compound such as BMP4 is attractive because it could be used to render the stem cells more vulnerable to conventional post-surgery therapies. The differentiated cancer stem cells then could be better eliminated by external beam radiation or internal radiotherapy after loco-regional implantation (24). This is well illustrated by Stupp et al. (25) differentiating strategy: GBM is a heterogeneous tumor that can be initiated and maintained by a minority of $CD133^+$ cancer stem-like cells that have a high tumorigenic potential and a low proliferation rate. Exposure to BMPs can force these $CD133^+$ tumor cells into a more differentiated phenotype characteristic of the $CD133^-$ tumor bulk, abolishing their self-renewal potential and increasing their sensitivity to radiotherapy. Hence, BMP originally influences multiple signalling pathways originally involved organogenesis and lineage-specific differentiation but also in cancer stem-like cell maintenance.

In the last decades, tissue engineering and regenerative medicine have emerged as promising strategies for bone reconstitution, with the ambition to circumvent the complications associated with traditional techniques. Bone tissue engineering aims to induce new functional bone regeneration *via* the synergistic combination of biomaterial scaffolds, cells, and signal factor therapy. Engineered bone tissues are considered as a potential alternative to the conventional use of

bone grafts, due to their limitless supply and no disease transmission. Bone scaffolds can be defined as an artificial temporary 3D matrix with micro- and nanostructures exhibiting biomimetic properties that provide a specific environment and architecture for bone growth and development (26,27). Scaffolds can be combined with different types of cells able to promote bone formation *in vivo* either by differentiating towards the osteogenic lineage or by releasing specific soluble cytokines. A challenge within scaffolds association with drugs and/or growth factors (e.g. BMPs) is that they can deliver those cytokines in the environment and exerting their therapeutic/regenerative effects (e.g. proliferation, differentiation). Interestingly, clinicians have demonstrated that such polymer-based systems can be injected or implanted locally thereby adverse effects. These two examples show that recombinant proteins of the same class may exert therapeutic effects against different diseases depending on their ways of administration and their interactions with the biological microenvironment.

2.2 Limitations and challenges

Tremendous effort has been invested in cellular engineering to optimise various hosts for protein production and there are many examples in which proteins have been used in therapy successfully. However, this kind of therapy has also presented various challenges.

The use of protein therapeutics is often limited by their instability, solubility, distribution, method of administration and side effects (28,29). The stability of protein therapeutics is a critical issue. These molecules can suffer loss of activity in response to environmental triggers such as moisture or temperature, which can occur during storage or even when administered *in vivo* (30). Nevertheless, several reports have shown that optimized processing protocols allow that proteins encapsulated into polymer matrices or grafted to polymer scaffolds may preserve their native conformation and thus their bioactivity for several months (31-32). The permeability of protein therapeutics through barriers such as the skin, mucosal membranes and cellular membranes is substantially reduced due to high molecular mass, which leads to injection being the primary mode

of administration (33). As many protein molecules have their therapeutic targets inside cells, challenges arise in transporting these molecules into the target cells without them breaking down while in the blood stream (28). The half-life of the therapeutic proteins can also be considerably reduced by proteases, protein-modifying chemicals or other clearance mechanisms in the body (34-36). Stromal cell-Derived Factor 1 (SDF-1α), for example, can be cleaved by matrix metalloproteinase-2 and 9 (MMP-2/9) released during a traumatic event such as tumor resection in the case of GBM, resulting in loss of its chemotactic activity (37).

BMP-2 has been previously isolated directly from bone. However, the limited yield and potential health risks associated with its isolation from allogeneic donor bone limited its clinical application (38). The expression of the recombinant BMP-2 in mammalian cell culture such as Chinese Hamster Ovarian (CHO) cells also generates low yields of protein and the procedure is relatively expensive (39,40). Eukaryotic systems such as yeast and animal cells were initially considered to produce recombinant BMP-2 to ensure adequate post translational modifications. Indeed, BMP-2 is a naturally glycosylated protein, but it has been discovered that glycosylation is not required for its function (41). Recently several authors have reported the production of biologically active BMP-2 expressed in *E. coli*. Although, the expressed BMP-2 was insoluble and formed inclusion bodies, active BMP-2 could be successfully refolded *in vitro* using specific refolding solutions and protocols (42-44). BMP-2 is biologically active only in a dimeric form, which is stabilised by an inter-molecular disulphide bridge that connects two cysteines: Cys 114 and Cys 228 from two different BMP-2 protein molecules (45). The disulphide bridge that stabilises BMP-2 dimer ensures the interaction of the BMP-2 dimer with transmembrane serine/threonine kinase receptors on osteogenic cells; which activates proliferation and differentiation of osteoblast cells (42).

Another challenge with protein therapy is the immune response that the body may build against the proteins. Virtually all therapeutic proteins generate some level of antibody response (46). There are cases where the immune response can neutralise the protein and can even cause a harmful reaction

in the patient. An example of such an immune response is the activation of B cells, which produce antibodies that bind to the proteins and reduce and possibly eliminate their therapeutic effects. Such antibodies can cause complications that can be life threatening. Thus the immune response of therapeutic proteins is a concern for researchers, manufacturers and clinicians (47). The uses of protein therapeutics in clinical animal trials usually do not effectively predict the response in humans. It is thus critical to evaluate the safety and efficacy of protein therapeutics and their probability to trigger antibody formation during development.

Another shortcoming associated with protein therapeutics is the high production costs. Protein therapeutics are expensive and this may limit clinical applications as well as patient access. This high cost issue is further aggravated for protein therapeutics where multi-gram doses are needed for a treatment course, as is the case for some antibodies (48).

Nano and micro-sized engineered materials have received considerable attention in modern pharmaceutics due to their potential to address the challenges encountered with conventional therapeutics. These materials can address issues associated with current pharmaceuticals such as extending product life, or can add to their performance and acceptability, either by increasing efficacy or improving safety and patient compliance (49,50). Targeted delivery and specific release can be achieved with these delivery systems *via* electrostatic interaction and pH or temperature dependent responses to controlled stimuli *in vivo* (51, 52).

Current research is focused especially on developing biodegradable polymer materials that have shown significant therapeutic potential. Biodegradable polymers are natural or synthetic polymers that are able to degrade in vivo into biocompatible and toxicologically safe by-products that are subsequently resorbed or excreted by the body. Naturally occurring biodegradable polymers are widely explored because of their abundance in nature, biocompatibility and lower toxicity. Chitosan (53), hyaluronic acid (54), silk fibroin (55), cellulose (56) or collagen (57, 58) have been among the most investigated natural biodegradable polymers for protein delivery applications. However, their

use is challenging because of significant molecular weight distributions and batch-to-batch variability and the necessity to collaborate with companies that are able to purchase materials following clinical Good Manufacturing Practices (cGMP). On the other hand, cGMP synthetic biodegradable or bioeliminable polymers are commercially available with different and well-defined compositions, molecular weights and degradation times. Aliphatic polyesters such as poly(lactic-co-glycolic acid) (PLGA) and polycaprolactone (PCL) have been among the most successfully used synthetic biodegradable polymers so far (59) .

3. Common forms of polymer-based protein delivery systems

Common examples of polymer-based systems that have been utilized in recent years to deliver various drug molecules, including therapeutic proteins, include micro/nanoparticles, hydrogels and porous scaffolds (Fig. 11.2).

3.1 Micro/nanoparticles

Micro/nanoparticles are injectable drug carriers that are usually prepared from hydrophobic polymers using straightforward processes such as solvent evaporation, phase separation and spray-drying (60). In the solvent evaporation method, an organic phase is first formed by dissolving a hydrophobic polymer and the drug molecules to be encapsulated in a water-immiscible, volatile organic solvent. This phase is then dispersed in an aqueous phase containing stabilizers such as polyvinyl alcohol under continuous mechanical agitation to form an oil-in-water (O/W) emulsion. Drug-loaded particles are formed upon evaporation of the organic solvent from the inner phase at reduced or atmospheric pressure. The particles can then be collected by filtration or centrifugation, washed to remove the stabilizing molecules adsorbed to the particle surface and lyophilized to minimize hydrolytic degradation of the particles during long-term storage. However, the single emulsion technique may not be suitable for encapsulating hydrophilic drugs such as proteins as they tend to diffuse into the external aqueous phase during the emulsification step. Therefore, protein

molecules are first solubilized in an aqueous solvent, and then dispersed in a polymer-containing organic phase to form a primary water-in-oil (W/O) emulsion, followed by dispersion in another aqueous solvent to form the secondary O/W emulsion (61). The preparation of the primary W/O emulsion is also relevant to the phase separation method (Fig.11.3). Following this step, instead of adding an aqueous solvent, an organic solvent that is non-solvent to the dissolved polymer is gradually introduced to extract the solvent of the polymer and decrease its solubility. The phase separation of the polymer from its solution contributes to the formation of polymer-rich liquid phase (coacervate) that surrounds the inner drug-containing aqueous phase. Upon completion of the phase separation process, the coacervate solidifies to produce drug-loaded particles (62). An obvious drawback of this method is the requirement for a large volume of organic solvent. Recent work proposed the use of water-miscible organic solvents to dissolve the polymer. This replaces the need for organic solvents to induce phase separation as aqueous media can be used to extract the polymer solvent (63). Finally, in the spray-drying method, the W/O emulsion is sprayed into a heated chamber that leads to a spontaneous production of drug-loaded particles. This method is more rapid and convenient and has fewer processing parameters than the other two but is limited by the adhesion of the formed particles to the inner surfaces of the drying chamber (60).

Due to their small size, micro/nanoparticles can be administered either directly to the intended site of action or into the systemic circulation to reach a desired location by passive or active targeting mechanisms (64) Several peptide-loaded polymer-based microparticle formulations have been approved by the FDA for clinical use. The first is Lupron Depot®, which received approval in 1989 to provide sustained release of leuprolide acetate for prostate cancer treatment (65). A more recent example is Bydureon® that was approved in 2012, which releases exenatide to improve glycemic control in type 2 diabetes patients (66).

In general, drug release from the particles is dependent upon the diffusion rate of the drug molecules and the degradation rate of the polymer-based matrix (60,65). However, as significant

proportion of the drug load can be weakly-adsorbed onto the large surface area of the micro/nanoparticles rather than incorporated in the polymer-based matrix, the drug release profile of this system is usually characterized by a huge initial burst that is followed by relatively short duration of release of the remaining drug load (67). Another disadvantage of this system is that the particles can move away from the targeted drug release site. The gradual translocation of the particles can become more prominent as the size of the particle decreases (64).

3.2 Hydrogels

Hydrogels are three-dimensional networks of cross-linked hydrophilic polymers. The cross-linking can be mediated by the physical interactions (e.g. hydrogen bonds, electrostatic interactions) between the polymer chains (68,69) or the covalent bonds resulting from the use of chemical crosslinkers (e.g. carbodiimide, glutaraldehyde) (70-73). Most hydrogels are characterized by highly-porous structure. The pore size can range from 10 to 500 μm and is dependent upon the degree of cross-linking in the hydrogel matrix (74,75). The porous structure is responsible for the deformability of hydrogels, enabling them to conform to the shape of the site to which they are applied (76). Due to their hydrophilicity, water-soluble drug molecules can be conveniently loaded into the porous structure of a pre-formed hydrogel. However, this is not always true for high molecular weight drug molecules such as proteins, which have diffusive limitations to their partitioning into the pores of the hydrogel (77). The high dependency of the drug loading process on the pore size of the hydrogel also means that the loaded drug molecules are usually released rapidly at the site of application as the release process is governed mainly by the diffusion rate of the drug molecules through the pores (65). In fact, the release of hydrophilic molecules from a hydrogel system typically lasts for only several hours or days, shorter than the release durations achieved with micro/nanoparticles made of hydrophobic polymers (65). To counter this, several strategies to enhance drug-hydrogel interactions have been proposed, including the introduction of charged moieties into the hydrogel to boost ionic interactions (78)] and the direct conjugation of the drug

molecules to the hydrogel via covalent bond formation (79). Another credible strategy to prolong drug release is to load the drug molecules directly into the hydrogel matrix during the hydrogel fabrication process instead of loading into the pores of a pre-formed hydrogel (80). Finally, several groups proposed the strategy of pre-encapsulating drug molecules into suitable micro/nanoparticles and co-formulating the particulate system into the hydrogel matrix to achieve sustained drug release (81,82).

As virtually any water-soluble polymer can be manipulated to produce this system, it is possible to obtain hydrogels with physicochemical and biological properties that are useful for a wide range of applications. Despite this, the number of hydrogel-based drug delivery systems approved for clinical use is still limited. An example of these is Regranex®, which consists of a carboxymethylcellulose gel that releases recombinant human platelet-derived growth factor (becaplermin) for the treatment of diabetic foot ulcers (76).

In addition to the rapid drug release issue mentioned above, hydrogels possess several drawbacks that could limit its use for applications. Their poor mechanical strengths make them susceptible to premature dissolution (77), limiting the time window for acting in the micro-environment. In addition, in the absence of cell adhesive proteins, hydrogels tend to have low capacity for cell adhesion and attachment due to their low stiffness (83-85).

3.3 Porous scaffolds

Porous scaffolds refer to three-dimensional solid polymer matrices characterized by interconnected pores. Generally, they are formed by removing the solvent from a polymer solution that leads to the precipitation of the polymer molecules. Methods that have been employed to produce porous scaffolds include freeze-drying (86), particulate leaching (87) and gas foaming (88). In the first method, a polymer solution is initially frozen at a sub-zero temperature inside an airtight chamber. The pressure is then gradually decreased to vaporize the frozen liquid. As more and more solvent

evaporates, the polymer molecules precipitate and solidify to form a porous scaffold (86). In the particulate leaching method, a polymer solution is first mixed with salt particles of well-defined size. The solvent is subsequently removed under vacuum, leaving behind a solid polymer matrix loaded with salt particles. The subsequent leaching of the salt particles in distilled water results in the formation of a porous scaffold (87). Gas foaming is another common method used to make porous scaffolds. It relies on the nucleation and growth of gas bubbles in a polymer phase. Traditionally, the gas bubbles can be formed *in situ* by adding into the polymer phase a foaming agent such as ammonium bicarbonate, which generates inert gas such as CO_2 when the pH of the system is decreased. A porous scaffold is formed upon removal of the dispersed gas bubbles from the polymer phase (88). Recently, supercritical fluids have been used as an alternative foaming agent. A supercritical fluid is any substance existing at a temperature and pressure above its critical point with an intermediate behavior between that of a liquid and a gas. The use of supercritical fluids is useful especially in making porous scaffolds from hydrophobic polymers as it circumvents the need for organic solvents during the preparation of the polymer phase. CO_2 is widely-used as a supercritical fluid due to its minimal toxicity and low cost. Initially, polymers can be dissolved or plasticized in supercritical CO_2. Upon depressurization of the system, the rapid expansion of the polymer phase as a result of the escape of CO_2 gas leads to the formation of a porous scaffold (88-90)].

Similar to hydrogels, the use of porous scaffolds as a drug delivery system can be achieved by loading drug molecules into the pores of a pre-formed scaffold or incorporating them directly into the polymer phase before the scaffold fabrication process. A notable example of clinically-used porous scaffold-based drug delivery systems is Infuse®, which consists of a porous collagen scaffold that can be conveniently loaded with recombinant human bone morphogenetic protein-2 (BMP-2) prior to administration in patients undergoing bone reconstruction procedure (91). Interestingly, the osteoinductive effect of this treatment relies on the chemotactic effect of BMP-2

that induces the infiltration of mesenchymal stem cells (MSCs) into the pores of the collagen scaffold (92). The considerable mechanical strength of the scaffold means that it can withstand the traction forces generated during cell attachment and migration, thus sustaining the cell infiltration process. After initial proliferation, the MSCs are further stimulated by BMP-2 to undergo differentiation into bone-forming osteoblasts to enable new bone formation (93). Considering its huge clinical success, Infuse® presents a working example to the idea of using a chemotactic agent and a suitable scaffold to recruit a certain cell population. During a bone reconstruction surgery, the site of bone defect can be accessed and filled with the Infuse® bone graft consisting of BMP-2-loaded porous collagen scaffolds to recruit mesenchymal stem cells (MSC) by chemotaxis. Upon infiltration into the injury site, the MSCs proliferate to increase their number before undergoing differentiation into the bone-forming osteoblasts, which secrete collagen and calcium-binding proteins to support the formation of mineralized bone tissues.

3.4: Fibrous scaffolds as a polymer-based protein delivery system

Fibrous scaffolds refer to scaffolds made of fibers with diameters on the order of several micrometers down to the tens of nanometers that are stacked layer-by-layer to form a three-dimensional non-woven mesh (Fig. 11.4). Compared to micro/nanoparticles, hydrogels and porous scaffolds, the use of fibrous scaffolds as a delivery vehicle for therapeutic proteins is less common despite the multiple advantages offered by this system. This being said, the amount of research conducted to investigate the value of fibrous scaffolds in this field of application has increased steadily over the last two decades and multiple strategies for loading protein molecules into fibrous scaffolds have been proposed.

Depending on the scaffold preparation technique, protein molecules can be embedded randomly in the fibers or partitioned into a specific fiber compartment as in the case of core-shell fibers (Fig.11.4). Chew *et al.* incorporated β-nerve growth factor (NGF) into fibers made of poly(ε-caprolactone-ethyl ethylene phosphate) (PCLEEP) and examined the release profile. They observed

that the fibrous scaffold was able to sustain NGF release over a period of 90 days. They claimed that the slow degradation of PCLEEP contributed to the sustained release profile as NGF molecules could only be released by diffusion through the hydrophobic matrix of the fiber (94). On the other hand, Zhang *et al.* produced fibers with a core-shell structure as a vehicle to deliver bovine serum albumin (BSA). The outer shell was made of the hydrophobic PCL while the core compartment dispersed with the BSA molecules was made of the hydrophilic bioeliminable poly(ethylene glycol) (PEG). They reported that the core-shell system produced lower initial burst and longer duration of BSA release than fibers made of a single blend of PCL, PEG and BSA (95). Jiang *et al.* further explored the possibility of tuning the kinetics of protein release from core-shell fibers. They showed that by varying the mass ratio of PCL and PEG in the outer shell, the time to achieve complete release of BSA from the inner dextran core could be varied from one week to approximately one month. BSA release was accelerated with increasing PEG mass in the outer shell as its water-solubility resulted in formation of pores through which BSA molecules could escape from the dextran core (96).

Protein molecules may also be encapsulated into micro/nanoparticles prior to incorporation into fibrous scaffolds. Liu *et al.* prepared dextran-based nanoparticles loaded with basic fibroblast growth factor (βFGF) that were subsequently embedded in poly(L-lactic acid) (PLLA) nanofibers. The duration of βFGF release provided by the nanoparticle-nanofiber composite scaffold was 10 days longer compared to what was achieved with nanofibers with directly embedded βFGF (28 vs. 18 days). In addition, the encapsulation of βFGF into the dextran-based nanoparticles was also useful in reducing βFGF structural changes during the fiber-making process (97). Qi *et al.* also adopted a similar approach. They incorporated BSA-loaded alginate microparticles into PLLA fibers and observed that the composite scaffold produced a longer duration of BSA release compared to the naked alginate microparticles (98).

3.5 Fibrous scaffolds with surface-bound protein molecules

Alternatively, protein molecules can be loaded onto the surface of a pre-fabricated fibrous scaffold. This is especially useful when preparing protein-loaded fibrous scaffold using a hydrophobic polymer and there is a need to reduce the exposure of the protein molecules to organic solvents that are needed to solubilize the polymer prior to the scaffold fabrication step. The nano/micro dimension of the fibers confer a large surface area for adsorption of protein molecules (Fig.11.5). In fact, the amount of protein that can be adsorbed by a fibrous scaffold is generally four times greater than that afforded by a porous scaffold of equal volume (99).

Immobilization of protein molecules to the surface of the fibers can be mediated by non-covalent interactions including hydrophobic interaction, van der Waals interaction, hydrogen bonding and electrostatic interaction. Heparin, a naturally-occurring polysaccharide, is known to have strong binding affinity for various growth factors (e.g. VEGF, transforming growth factor-β (TGF-β), fibroblast growth factor (FGF)), morphogens (e.g. BMP-2, BMP-7, BMP-14) (100) and ECM proteins (e.g. laminin) (101) due to its ability to form non-covalent interactions with these proteins. Therefore, heparin-functionalized fibrous scaffolds can be conveniently loaded with these proteins for local delivery applications. Casper *et al.* prepared PEG and poly(lactic-co-glycolic acid) (PLGA) nanofibers functionalized with low molecular weight heparin (LMWH) that were adsorbed with βFGF. To slow down the dissociation of LMWH from the fibrous scaffold and thus prolong the duration of βFGF release, LMWH was conjugated to PEG prior to its incorporation into the nanofibers. Although the βFGF release profile was not assessed in their study, they reported that LMWH was retained in the fibrous scaffolds for at least 14 days (102). Furthermore, Patel *et al.* prepared heparin-functionalized PLLA nanofibers as a delivery vehicle for βFGF and laminin. The adsorption of βFGF and laminin to the surface of PLLA nanofibers was very stable, with less than 0.1% of the total amount of immobilized protein molecules released into the surrounding solution

after 20 days. The slow release of the adsorbed protein molecules could be useful in certain neuroregenerative applications as the immobilized βFGF was found to be as effective as its soluble counterpart in inducing neurite outgrowths from dorsal root ganglion tissues (103). Fiber surfaces can also be adsorbed with protein-loaded nanoparticles. Wei *et al.* prepared BMP-7-loaded PLGA nanoparticles that were subsequently immobilized onto PLLA nanofibers. They reported that the release kinetics of BMP-7 could be controlled by varying the degradation rate of the PLGA nanoparticles. However, as the nanoparticle surfaces were exposed to the surrounding solutions, a characteristic burst release could be observed with each formulation of BMP-7-loaded PLGA nanoparticles prepared in their study (104).

Another widely-used method for functionalizing fiber surfaces with proteins is by chemical immobilization. This approach results in formation of covalent bonds between the fiber surfaces and the protein molecules. As the covalently-attached protein molecules cannot be easily desorbed from the fibers, this functionalization method is especially useful in many regenerative applications, where long-term immobilization of protein molecules in the fibrous scaffold is often necessary for the reparative actions to take place. Primary amine and carboxyl groups are the most common example of functional groups utilized in covalent conjugation of fibers and protein molecules. Many groups have prepared polymer-based nanofibers functionalized with carboxyl groups that can be activated by a combination of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) for subsequent conjugation with primary amine groups present in protein molecules. Ye *et al.* prepared nanofibers from poly(acrylonitrile-co-maleic acid) (PANCMA) that were subsequently functionalized with lipase. However, the immobilized lipase molecules were found to have lower enzymatic activities than their soluble counterparts (105). A similar loss in activity was also reported by a group that functionalized polystyrene (PS) nanofibers with α-chymotrypsin (106). There are two possible explanations for the partial inactivation of the immobilized enzymes. First, the immobilization process may introduce covalent alterations to the

active sites of the enzyme. The other is that direct conjugation of protein molecules to the fiber surfaces may cause certain parts of the immobilized molecules to be sterically inaccessible to their corresponding ligands (107). To address the latter issue, several polymer-based linkers have been utilized to introduce a physical gap between the immobilized molecules and the fiber surfaces. To obtain these linkers, primary amine-terminated hydrophilic polymers such as PEG-diamine can be chemically-conjugated to a hydrophobic polymer such as PCL and PLGA. The linker can then be mixed with an unconjugated hydrophobic polymer to prepare fibers displaying primary amine groups on their surface that can be conjugated with protein molecules. Choi *et al.* immobilized EGF on the surface of fibers composed of PCL and PCL-PEG-NH_2 for wound healing applications. They showed that the EGF-functionalized fibers were able to induce differentiation of keratinocytes to a greater extent than fibers supplemented with EGF solution. The enhanced activity of the former could potentially be attributed to the fact that covalently-immobilized EGF could be better retained at the wound site and thus was able to induce more durable pro-differentiation signals in the locally-residing keratinocytes (108). Kim *et al.* also utilized a polymer-based linker to conjugate lysozyme to the surface of PLGA nanofibers. The immobilized lysozyme displayed comparable activity to its soluble counterpart (109). This is opposite to the significant loss of enzymatic activities observed with direct conjugation of enzyme molecules to the fiber surfaces as discussed above.

4. Conclusions

With the advent of recombinant technology, a wide variety of biocompatible therapeutic proteins can be manufactured with relative ease. These proteins would then be carefully formulated and subsequently administered in patients to address different types of diseases more effectively and selectively. At the level of formulation development, protein molecules can be physically and/or chemically-conjugated to a wide array of naturally-occuring, semi-synthetic and synthetic biomaterials to form different types of protein delivery systems. Depending on their architecture and the extent of protein-scaffold interactions, these delivery systems can modify the

pharmacokinetic (PK) and pharmacodynamic (PD) properties of the protein molecules. The versatility of polymer-based protein delivery systems such as micro/nanoparticles, hydrogels, porous scaffolds and fibrous scaffolds means it is possible to alter the spatial distribution of the protein load within the system as well as the protein release kinetics. These can then influence the ability of the protein molecules to exert the intended effects in their immediate microenvironments, be it to kill cancer cells or to recruit stem/progenitor cells. From a pharmaceutical development perspective, the design of a protein delivery system should be commenced only after the attainment of an in-depth understanding of the PK/PD profile of the protein of interest for a given medical application. Therefore, a close communication between formulation scientists, molecular biologists, PK/PD scientists and clinicians are crucial to ensure successful development of protein delivery systems that are fit for pre-clinical proof-of-concept and subsequently clinical studies.

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Figures