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Synthetic Carriers for Vascular Delivery of Protein Therapeutics

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Introduction: a need for improvement in the means of vascular protein delivery

Due to high potency and specificity of their activities, protein drugs are rapidly gaining clinical use. Examples of clinically exploited proteins include anticoagulants and fibrinolytic plasminogen activators (Harker *et al.*, 1997; Wieland *et al.*, 2003), interferons and cytokines (Burke, 1999; Younes and Amsden, 2002; Barnes, 2003), proteases and their inhibitors, such as serpins (Schimmoller *et al.*, 2002; Rosenblum and Kozarich, 2003; Barnes and Hansel, 2004), growth factors and hormones (Bremer *et al.*, 1997; Rosier *et al.*, 1998; Chen and Mooney, 2003; Peppas *et al.*, 2004), enzymes (e.g. for replacement therapies) (Layer *et al.*, 2001; Meikle and Hopwood, 2003; Mignani and Cagnoli, 2004), as well as an extensive and rapidly growing list of antibodies, antibody fragments and their conjugates with toxins and other biologically active agents (Foster, 1996; Muzykantov, 2001; Thorpe, 2004). The rate of FDA approvals of proteins for therapeutic applications has accelerated with exponential speed in the past decade.

Large-scale synthesis of a great variety of recombinant therapeutic proteins has become available in recent years. Nevertheless, protein drugs are typically expensive (e.g. the cost of a recombinant plasminogen activator for fibrinolytic therapy is ~US$1000). Therefore, both economic and biological reasons (e.g. immune

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Abbreviations: BSA, bovine serum albumin; BSR, bovine seminal ribonuclease; DCM, dichloromethane; DLS, dynamic light scattering; EM, electron microscopy; EPR, enhanced permeation and retention; GOX, glucose oxidase; hGCSF, human granulocyte stimulating factor; HPMA, poly(n(2-hydroxypropyl)methacrylamide); HSA, human serum albumin; PACA, poly(alkylcyanoacrylate); PCL, poly(caprolactone); PCS, photon correlation spectroscopy; PEG, poly(ethylene glycol); PEG-PPS-PEG, poly(ethylene glycol)-b-poly(propylsulfide)-b-poly(ethylene glycol); PEI, poly(ethylamide); PLA, poly(lactic acid); PLGA, poly(lactic acid-co-glycolic acid); POE, poly(orthoester); QELS, quasi-elastic light scattering; RES, reticuloendothelial system; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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response) restrict the practicality of multiple or continuous i.v. injections. Even at a single injection, the higher the dose of protein therapeutics, the higher probability of side effects and cost. This is especially true in cases where pharmacokinetics is unfavourable, due to rapid elimination via renal clearance and hepatic uptake. Furthermore, factors such as inactivation by inhibitors and proteases also limit activity and duration of protein therapeutics.

In addition, due to the specific nature of their effects (such as enzymatic conversion of selected substrates or binding to selected ligands) many, if not all, protein drugs require localization in specific areas of the body – an organ, tissue, cell, or even sub-cellular compartment. However, only a few protein drugs have a natural affinity to their desired site of therapeutic action, and the major fraction of injected dose represents a potentially harmful waste.

Therefore, the optimization of protein delivery is an extremely important goal. The vascular system, which is designed as the main physiological transport pathway, provides a delivery route for drugs to all organs, tissues, and cells. In theory, vascular protein delivery will improve treatment of disease conditions, including cancer, cardiovascular and metabolic (diabetes) diseases, inflammation and pathologies of diverse organs and tissues. This article briefly overviews modern approaches utilizing synthetic carriers in order to achieve this goal.

Roles for nanocarriers in vascular delivery of protein therapeutics

Through the addition of a carrier or vehicle, therapeutic protein delivery can be optimized in several ways. First, loading into and, in some cases, even coupling to the surface of the carriers can protect proteins from inactivation by proteases, inhibitors, and other aggressive components of blood and other compartments in the body (Rao, 1995). Second, by creating a barrier between a drug and blood and non-target tissues, a carrier minimizes potential side effects of and immune response to a protein drug. Third, loading into long-circulating carriers decelerate blood clearance of drugs that otherwise are rapidly excreted (Moghimi and Szebeni, 2003). Fourth, some types of carriers permit targeted delivery of their cargoes to therapeutic sites by either passive mechanisms (e.g. utilizing mechanical retention in capillaries or the effect of enhanced permeability and retention in areas of inflammation and tumours) (Maeda *et al.*, 2000; Torchilin, 2000) or active mechanisms (e.g. utilizing affinity moieties on the surface of carriers that bind to specific determinants in targets) (Torchilin, 1994; Moghimi *et al.*, 2001; Muzykantov, 2001). Fifth, by varying size, mechanical properties, and surface decoration of carriers, one can achieve more adequate sub-cellular addressing of cargoes, utilizing specific endocytotic pathways and intracellular sorting and traffic machinery (Muro *et al.*, 2004). Finally, slowly degrading carriers produce a prolonged and more stable profile of a drug activity in the site of accumulation (Dziubla *et al.*, 2005).

Yet, in order to realize these advantages, these carriers must not induce adverse side effects either systemically (e.g. activation of complement or platelets in blood) or locally (e.g. inflammation or formation of fibrous tissue in the site of their accumulation). They should undergo safe and effective processes of biodegradation and excretion. Also, a carrier formulation should be homogeneous in terms of mechanical and structural properties and size.

Furthermore, the carrier's size must serve delivery purposes. For example, in the case of delivery to a selected organ utilizing mechanical retention in afferent pre-capillary arterioles, optimal size is larger than 5 micron (Yoon, 2004). In contrast, to achieve a prolonged circulation in the bloodstream permitting systemic delivery, carriers must be within 50–500 nm size constraints (Moghimi and Szebeni, 2003). Carriers within 30–100 nm size that can utilize pathways for transcellular and pericellular transport through the endothelial barrier are optimal to achieve delivery to extravascular targets (Florence and Hussain, 2001; Kreuter, 2001). Larger particles, within 200–800 nm diameter, are useful for delivery into sinusoidal cells in the liver and spleen (key organs of the reticuloendothelial system, RES) (Ulrich, 2002). Finally, the effectiveness of internalization by many cell types also depends on carrier size and, in most cases, is maximally effective within nano-scale, from 30 to 300 nm.

Nanoparticle formulation must permit loading of sufficient amounts of therapeutically active proteins, most of which are sensitive to inactivation in unfavourable temperatures, media (e.g. organic solvents), and pH. Optimally, protein loading should be effective in order to reduce the cost of proteins. However, in the case of enzyme delivery, even relatively modest loading of carriers that protect cargoes from inactivation and allow for accumulation in targets might provide therapeutic effects. Enzymes can decompose or produce multiple copies of biomolecules; thus, even with only a couple of hundred copies of enzyme per particle in volume loading, the benefits of prolonging activity may far outweigh the low overall loading limits presented.

TYPES OF NANOCARRIERS

Nanocarriers are broadly defined as any submicron structure capable of being loaded with drugs, such as proteins, polynucleotides, polypeptides, and small molecules. Due to practical considerations (e.g. loading limitations, internalization potentials), this definition, which is accepted by the NIH, is more inclusive than the 1–100 nm size range for nanotechnology set by NSF (Whitesides, 2003). The structural materials, plasticity, morphological features, size, biological sensitivity, and biodegradability represent, among others, key characteristics of nanocarriers. A representation of the structural morphology of a few typical protein nanocarrier designs is presented in *Figure 13.1*; more explanations and specific references to these and other types of nanocarriers are given below.

Table 13.1 provides a general outline of nanocarriers that are being considered for drug delivery. Nanocarriers utilizing natural biomaterials or structures (e.g. liposomes consisting of natural phospholipids found in cellular plasma membranes) were the first to be explored for drug delivery (Mainardes and Silva, 2004). Arguably, liposomes represent the most extensively studied nanocarriers in this class. It should be stressed, however, that liposomes, extensively reviewed in the literature (Torchilin, 1994; Moghimi *et al.*, 2001; Ulrich, 2002; Moghimi and Szebeni, 2003), are not the subject of this article. However, biological nanocarriers, such as viral particles, tend to have short shelf lives, short *in vivo* kinetics, and are prone to eliciting immune reactions.

Synthetic materials, especially polymeric materials, offer a greater degree of

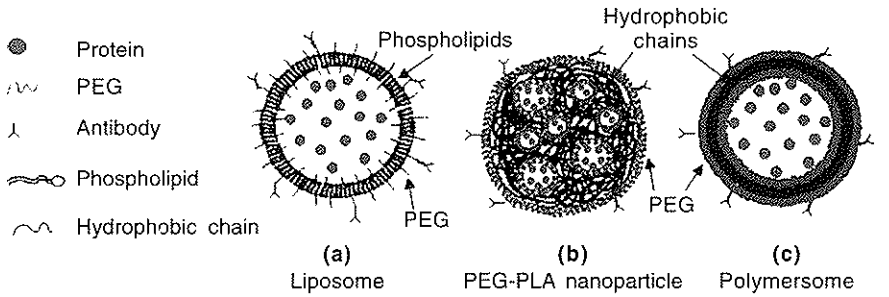


Figure 13.1. Common structural features of nanocarriers. Liposomes (a) represent the structural ideal for protein carriage, since their large internal aqueous volume permits a theoretically high loading capacity. However, due to the thermodynamic nature of liposome formation, partitioning of macromolecular proteins within this compartment represents a significant challenge. Double emulsion particles (b) illustrated by PEG-PLA nanoparticles, are the most common design for synthetic biodegradable nanocarriers. Their formation is inherently heterogeneous, with random, small aqueous inclusions within a solidified polymer meshwork, in which loaded protein theoretically resides. The advantage of nanoparticles is that protein loading is not wholly dependent on thermodynamic partitioning, allowing for an enhancement in protein load. Polymersomes (c) are an exact synthetic analogue to liposomes. Both double emulsion nanoparticles and polymersomes are more stable than liposomes, but this enhanced stability requires more rigorous formation conditions, which are typically too harsh for proteins.

freedom in that they can be designed to enhance circulation, reduce immunogenicity, provide environmentally responsive elements, and possess biologically derived properties, also known as biomimetic properties, such as adhesion response elements and receptor ligands. Due to the relatively greater body of work done on degradable polymer nanoparticles, this review will focus on emulsion-based nanoparticles for protein delivery. The authors refer readers to other sources for a description of alternative strategies for polymer-based carriers, including pluronic micelles, shell cross-linked nanoparticles, dendrimers, and worm micelles (Florence and Hussain, 2001; Kabanov and Alakhov, 2002; Kabanov *et al.*, 2002; Sadler and Tam, 2002; Becker *et al.*, 2004a,b; Joralemon *et al.*, 2004; Qi *et al.*, 2004).

Polymer selection, biodegradation, and biocompatibility

The first requirement for materials that can be used for nanoparticle synthesis is biocompatibility, meaning that they can be injected intravenously without toxicity and overt side effects including, but not limited to, activation of white blood cells, platelets, and cascades of complement, coagulation, and kinines (Anderson and Langone, 1999).

On the other hand, clearance mechanisms for inert materials in the nanoscale range (e.g. elimination via urine or bile excretion within an acceptable period of time) have not been characterized; hence, all materials used for nanoparticles must be both biocompatible and biodegradable. Biodegradation, sometimes referred to as bioerosion, is the chemical cleavage of polymer into smaller residues that are water soluble, non-toxic, and biocompatible (Shive and Anderson, 1997; Siepmann and Gopferich, 2001; Gopferich and Tessmar, 2002; Howard, 2002). Ideally, the byproducts produced during the course of biodegradation would then be

Table 13.1. An overview of nanocarriers

Source of material	Material	Morphological structure	Type
Natural	Lipid	Solid	Solid lipid nanoparticle
	Phospholipid	Aqueous core, shell	Liposomes
	Protein	Solid aggregates	Immunoconjugates, albumin nanoparticles
	Viral	Aqueous core, shell	Viral nanoparticles
Synthetic	Hydrophobic polymer	Solid	Nanoparticles
		Heterogeneous aqueous	Double emulsion nanoparticles
	Hydrophilic polymer	Solid	Hydrogels, dendrimers
		Conjugates	Protein–polymer conjugates
	Amphiphilic polymer	Micelles	Micelles (spherical and cylindrical)
		Aqueous core, shell	Polymersomes
	Metal	Solid	Colloidal gold, TO_2
		Hollow	Gold

Nanocarriers based on natural materials, in particular liposomes consisting of phospholipids, were among the very first vascular drug carrier developed. Solid lipid nanoparticles possess greater utility in topical drug delivery, such as cortisone creams. Immunoconjugates are highly effective for endothelial drug targeting, yet inherently possess a short duration of therapy due to lysosomal degradation. Synthetic materials possess a reduced immunogenicity and can be designed with very specific properties, which makes them attractive carriers for vascular protein delivery. This review will focus upon the work with degradable hydrophobic polymers.

metabolized or excreted (they could also consist of short chain polymers that can be cleared via renal or a hepatic excretion). If effective, this pathway helps to circumvent concerns over toxicity due to deposition of nanocarriers, or products of their degradation, in tissues. Two main mechanisms of biodegradation have been described, surface erosion and bulk degradation (Siepmann and Gopferich, 2001).

Surface erosion occurs when the diffusion of water determines the rate of degradation. Under this regime, only a fraction of polymer at the aqueous interface degrades, while the bulk material does not decrease in molecule weight, resulting in a carrier that can maintain structural integrity while simultaneously shrinking. Moreover, since diffusion is typically greatly limited, erosion also controls drug release. Through careful design of carrier geometry, drug release rates can be controlled.

Bulk degradation occurs when chain cleavage is slower than aqueous penetration. Unlike surface erosion, polymers undergoing bulk degradation maintain overall size and shape for the majority of the sample life, yet the molecular weight of the polymers decreases steadily. The polymer carrier will slowly lose structural strength until a critical point where sample integrity is no longer maintained and the carrier disintegrates.

For both bulk degradation and surface erosion, the rate of degradation of many types of nanocarriers depends on the pH of the milieu and in the carrier interior. In most cases, the lower the pH, the faster the rate of hydrolysis, and thereby degradation. Therefore, rational design and application of nanocarriers includes an analysis of the prospective 'unloading' or degradation site. For example, lysosomes, acidic intracellular vesicles filled with hydrolases, represent a natural final destination point of many intracellular pathways involving nanocarrier delivery (Muro *et al.*, 2003a; Panyam and Labhasetwar, 2003); hence, one can expect accelerated degradation in this compartment. On the other hand, ischemic and inflamed tissues usually have acidic pH; this factor either can be utilized as an acceleration

mechanism for controlled local drug unloading or must be accounted for as a potential 'non-target' site for drug release.

Furthermore, the degradation of some polymers in large devices (e.g. microspheres and implants) further acidifies the device interior, thus accelerating the process (Schwendeman *et al.*, 1996; Shive and Anderson, 1997). While it is not clear if this process will occur in submicron structures like nanocarriers, where clearance of acid groups is expected to be faster, it may lead to the burst release of nanocarrier content. The following is a brief list of the known materials that fall within the broad requirements of biodegradation and biocompatibility (see *Table 13.2* for a summary of degradable polymers).

POLYESTERS

Polyesters are the most extensively studied biodegradable materials for biomedical applications (Shive and Anderson, 1997; Kumar *et al.*, 2001; Siepmann and Gopferich, 2001). The backbone of these materials is cleaved through a hydrolysis reaction, which is controlled by pH, temperature, and the accessibility of water to the ester bond. Hence, factors such as the degree of crystallinity, glassy state, hydrophobicity, and the pKa of the leaving group, play a significant role in the degradation rate of these materials.

The rate of hydrolysis of the ester bond is relatively slow; therefore, polyester carriers undergo bulk degradation. Efflux of acid residues and acidic oligomers formed in the course of hydrolysis causes internal acidification of polyester microspheres, which has been implicated in the destabilization of loaded proteins (Schwendeman *et al.*, 1996; Zhu *et al.*, 2000).

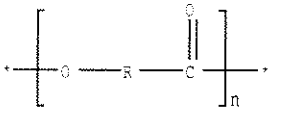
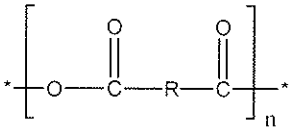
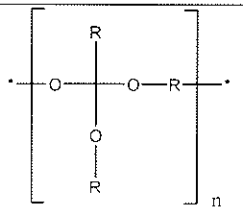
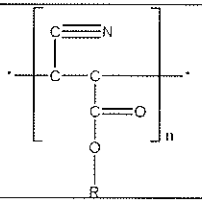
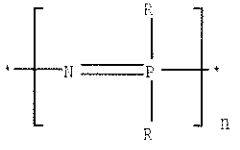
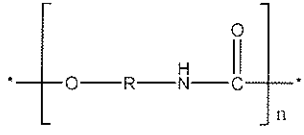
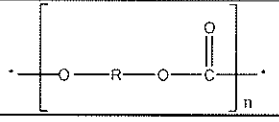
Polyesters are most commonly prepared by ring opening polymerizations of ester-based rings (e.g. lactones and lactide) (Albertsson and Varma, 2003). Of these, poly(lactic acid-co-glycolic acid) (PLGA), sometimes referred to as poly(lactide glycolide) due to the monomers used for synthesis, is the most widely acclaimed of all the polyesters. This stems from the well-tolerated byproducts of hydrolysis, lactic acid and glycolic acid, which are fully metabolizable and excretable. While this material is well accepted, the range of degradation times is rather long (a couple of weeks to months).

Another commonly used material is poly(caprolactone) (PCL), whose degradation product is gamma-hydroxyhexanoic acid. Due to a high degree of crystallinity, the pure state of this polymer possesses an extremely long degradation time (Gunatillake and Adhikari, 2003). However, this polymer has shown to possess a greater degradation rate than PLGA in the presence of acidic milieu and hydrolases, allowing lifespans more applicable to a short-term carrier (Gan *et al.*, 1999; Hayashi *et al.*, 2001). Many other lactone-based materials are available; however, their use is typically precluded due to the toxicity of the resulting products, limiting the design of polymers.

POLYANHYDRIDES

While polyesters are the conjugation of a hydroxyl group with a carboxylic acid, polyanhydrides are the result of the conjugation of two carboxylic acids. Some of

Table 13.2. Classes of degradable polymers with the products, approximate times of degradation, and examples

Polymer	Repeating unit	*In vivo* degradation times	Degradation residues	Reviews
Poly(ester)		Weeks–years	Carboxylic acid and alcohols	(Shive and Anderson, 1997; Kumar *et al.*, 2001; Siepmann and Gopferich, 2001)
Poly (anhydride)		Hours–days	Carboxylic acids	(Tamada and Langer, 1992; Siepmann and Gopferich, 2001; Gopferich and Tessmar, 2002; Katti *et al.*, 2002)
POE		Days–months	Carboxylic acid, alcohols and pentaerythritol	(Leadley *et al.*, 1998; Siepmann and Gopferich, 2001; Heller *et al.*, 2002)
PACA		Months	Alcohols and poly(cyano-acrylates)	(Siepmann and Gopferich, 2001; Vauthier *et al.*, 2003; Sullivan and Birkinshaw, 2004)
Poly (phos-phazene)		Hours–months	Phosphate, ammonia, amines and alcohols	(Vandorpe *et al.*, 1996; Siepmann and Gopferich, 2001; Lakshmi *et al.*, 2003)
Poly (urethane)		Months–years	Alcohols, amines and carbon dioxide	(Kawai, 1995; Labow *et al.*, 1999; Siepmann and Gopferich, 2001; Howard, 2002)
Poly (carbonate)		Years	Alcohols and carbon dioxide	(Labow *et al.*, 1999; Siepmann *et al.*, 2001)

the classic examples of polyanhydrides include poly(sebacic acid) and poly(fumaric acid) (Gopferich and Tessmar, 2002; Katti *et al.*, 2002). Typically, these bonds are much more unstable and readily cleaved by water than esters; thus, the degradation rate of poly(anhydrides) is faster than that of poly(esters).

Instead of degrading over many months, poly(anhydrides) typically degrade over several days to a couple of weeks or months, depending on the amount of surface area in contact with water and the hydrophobicity of the polymer (Siepmann and Gopferich, 2001; Gopferich and Tessmar, 2002). Since this exposed surface area directly determines the degradation rate of polyanhydrides, the extreme high surface-to-volume ratio of nanoparticles results in degradation times of only a few hours. Indeed, microparticles smaller than 10 μm composed of poly(fumaric co-sebacic acid) degraded in only 5 hours. In an attempt to prolong particle degradation, equal-sized particles, prepared using the more hydrophobic poly(1,3-bis(p-carboxyphenoxy)propane co-sebacic acid), were stable for up to 18 hours at physiological conditions (Mathiowitz *et al.*, 1992). However, protein loaded inside microspheres (bovine serum albumin, BSA) was shown to further accelerate particle degradation, presumably through increased aqueous diffusion and pH effects (Sandor *et al.*, 2001). These rather short degradation times are the primary reason for the lack of studies that have been conducted using these materials (Fu and Wu, 2001). While this is considered typically to be short for long-term delivery, there may be acute to sub-acute settings where such a release profile would be ideal. Yet these limitations may be overcome through the proper copolymer selection.

POLY(ORTHOESTERS)

Alza Corporation was the first to describe the use of polyorthoesters (POE) as a biodegradable biomaterial, and it is currently being developed by A.P. Pharma as a bioerodible drug depot system (Leadley *et al.*, 1998; Heller *et al.*, 2002). Like polyanhydrides, these materials possess a degradation rate faster than that of linear polyesters. Furthermore, degradation only occurs upon protonation of the alkoxide bond. This results in an accelerated degradation rate under acidic pH. Yet, unlike linear poly(esters), degradation is inhibited at basic conditions. For this reason, they may serve as an effective burst release agent in lysosomal delivery by using the acidic conditions as a trigger for release. In spite of these results, there has not been that much work done in developing nanoparticles composed of these materials.

POLY(ALKYLCYANOACRYLATES)

Poly(alkylcyanoacrylates) (PACA) are not fully biodegradable. Rather, this polymer is composed of an acrylate backbone with a pendant ester group. As in the poly-esters, this pendent ester is susceptible to hydrolytic degradation, with longer alkyl chains reducing the rate of degradation. This results in the byproducts, alkyl alcohol and poly(cyanoacrylic acid) (Gibaud *et al.*, 1998; Li *et al.*, 2001b; Sullivan and Birkinshaw, 2004). Since this process converts the polymer from hydrophobic to hydrophilic, the polymer can be excreted renally, provided the molecular weight is not too large (<40 kDa). Some groups have alluded to a depolymerization reaction that can cleave the backbones of these polymers into shorter fragments, allowing for

the degradation rate to be determined by both polymer molecular weight and alkyl length (Vauthier *et al.*, 2003). However, at best, this process has been shown to result in ~5% of the total degradation in *in vitro* physiological conditions, and is not believed to be a significantly contributing factor.

POLY(PHOSPHAZENES)

These materials represent a unique class of degradable polymer. Their synthesis has been described for over 100 years, yet only over the past 20–30 years has research been placed into developing their functional properties (Vandorpe *et al.*, 1996; Lakshmi *et al.*, 2003). By selecting proper phosphate pendant chains, the material degradation rate can be controlled (from hours to months to years). It is assumed that, since these materials break down into simple molecules, the byproducts would be well tolerated. Like POE, degradation of these materials is also acid catalysed. Moreover, since the degradation does not result in a continual build-up of acid groups, protein stability is not as likely to be impinged upon. In spite of these advantages, there have only been a few studies using poly(phosphazenes) for nanoparticles (Vandorpe *et al.*, 1996, 1997).

POLY(URETHANES) AND POLY(CARBONATES)

The urethane and carbonate bonds can be cleaved by a hydrolysis reaction. However, the rate of this cleavage under physiological conditions is typically so slow as to be considered fully stable (Howard, 2002). For this reason, it is not expected that these materials would make ideal nanocarriers. Nevertheless, some groups have demonstrated sensitivity of these polymers to various enzymes. For instance, urease, papain, and so called 'urethanases' have all been used to accelerate degradation of bulk poly(urethanes) in a physiologically meaningful time span (Kawai, 1995; Labow *et al.*, 1999). Since nanocarriers can be internalized into the lysosomes, they are subjected to a host of degrading enzymes. As such, these materials may still find a functional use.

Loading mechanisms

There are four main mechanisms for loading proteins into nanoparticles: surface absorption, aqueous inclusion, solid-phase immobilization, and complexation aggregates (see *Figure 13.2*).

Surface absorption is the easiest and most prevalent method for nanocarrier protein loading (Bousquet *et al.*, 1999; Constancis *et al.*, 1999; Michaelis *et al.*, 2000; Lvov and Caruso, 2001; Sakuma *et al.*, 2002; Muro *et al.*, 2003a, 2005; Sweitzer *et al.*, 2003). The loading of latex (poly(styrene)) beads, which exemplifies this mechanism, has been used as a model nanocarrier for the evaluation of nanoparticle internalization mechanisms (Muro *et al.*, 2004). Most likely, this mechanism also accounts for a portion of loaded protein in all other currently tested nanoparticle preparation techniques.

Typically, surface absorption occurs through hydrophobic interactions between the particle surface and the hydrophobic protein residues (e.g. tryptophan, tyrosine,

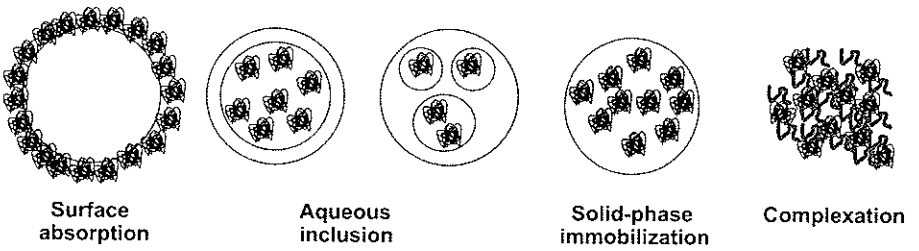


Figure 13.2. Methods of nanocarrier protein loading. In the nanoscale range, surface absorption offers the greatest protein/particle loading, and most likely accounts for a fraction of protein loaded in all reported nanoparticles. However, protection from environmental conditions is most effective with inclusion mechanisms of loading. Currently, aqueous inclusion methods are most extensively explored for protein loading into polymer nanocarriers. Therapeutic activity may be achieved via either release of cargoes or diffusion of their substrates via polymer. Only very few specific stable proteins are expected to be amendable of solid phase inclusion. Complexation relies upon the interaction of protein and polymer for particle formation, which permits formation of size-controlled loaded vehicles, yet release from these carriers is difficult to control.

phenylalanine). Besides hydrophobic interactions, surface absorption could also occur through charge–charge interactions, which would allow for pH sensitivity (Sakuma *et al.*, 2002). Upon changes of pH, the surface and/or protein charge can be neutralized, thereby initiating release. However, surface adsorption is not effective for coating stealth (e.g. poly(ethylene glycol) (PEG)-carrying) nanoparticles, due to the nature of the stealth mechanism (see below). Furthermore, since there is no barrier between the external milieu and the protein loaded by surface absorption, this mode of incorporation does not provide any environmental protection, and is not expected to greatly extend therapeutic function upon arrival at the site. However, it may prolong circulation time, alter the mode of tissue targeting, and subsequently alter sub-cellular addressing of the drugs (Muro *et al.*, 2005).

Aqueous inclusion is the loading mechanism exhibited by liposomes (Ceh *et al.*, 1997; Moghimi *et al.* 2001). In an ideal setting, a large internal aqueous environment is sequestered from external solution by a physical barrier. In liposomes, the bilayer membrane provides this barrier. However, a polymer layer can provide this barrier either through self-assembly mechanisms (as in polymersomes) (Discher *et al.*, 1999), double emulsion formation mechanism (Dziubla *et al.*, 2005), or in nanoscale hydrogel synthesis techniques (Huang *et al.*, 2004; Peppas *et al.*, 2004). By isolating the protein from the external environment, it becomes protected from deactivating mechanisms commonly found in systemic circulation. Furthermore, since the protein remains in an aqueous environment, molecular mobility remains intact, and hence activity is not compromised.

Solid-phase immobilization is an alternative strategy where crystallized or lyophilized protein is loaded as a suspension within the solid core of an organic, hydrophobic nanoparticle. For some proteins, the organic environment (when unfolding does not occur) restricts protein mobility that may paradoxically, yet simultaneously, reduce activity and extend functional use (Klibanov, 1997, 2001). Moreover, since the protein is not in a soluble state, loading is not constrained by aqueous solubility

limits. Also, the entire particle core could support inclusion. For these reasons, this mechanism may result in a much higher degree of loading.

The final class of loading is based upon the complexation of proteins with the carrier material. This class of loading is sometimes referred to as polymer therapeutics, and represents a whole field in itself (Harada and Kataoka, 2001; Kopecek *et al.*, 2001; Duncan, 2003). Common approaches to complexation include inter-ionic associating mechanisms, the biotin–streptavidin cross-linking system, or covalent bonding. For instance, regular polymeric micelles of poly(ethylene glycol)-b-poly(aspartic acid) were formed when in the presence of the positively-charged lysozyme (Harada and Kataoka, 2001). Complexes can also take the form of polyplexes (e.g. poly(ethylimide) (PEI) and DNA) or in single polymer chain coupling multiple proteins (Godbey *et al.*, 1999; Duncan, 2003). This latter form has been popularized by the use of hydrophilic polymers, such as poly(n(2-hydroxypropyl)methacrylamide) (HPMA), which uses amide linkages to covalently attach proteins and small molecules onto the polymer backbone (Kopecek *et al.*, 2001).

Yet, even hydrophobic associations, disulfide linkages, streptavidin–biotin or antibody–antigen pairs can be used to form protein–polymer complexes. By controlling feed conditions, the complexation mechanism can result in nano-sized aggregates with a relatively high degree of protein inclusion (Shuvaev *et al.*, 2004). However, these conjugates (polyplexes) are characterized by significant heterogeneity, both in molecular composition and size. Due to the nature of the complexing mechanism, release from these systems is typically poor, and mainly controlled by degradation of the components. Thus, in the case of enzyme therapies, conjugates of this type function effectively, typically only if enzyme substrates are small and diffusible enough to be accessible within the aggregate core, such as H_2O_2 in the case of catalase delivery (Dziubla *et al.*, 2005).

Limits of protein loading

Protein sizes range from a couple of angstrom to 10–15 nm. These larger proteins pose a significant challenge in nanocarrier loading. To illustrate this, consider the loading limits of IgG (10 nm diameter) in a surface absorption v. volume loading mechanism. To compare the theoretical loading limits, the volume lost to loading by the mass occupied by the carrier will be ignored. The following equation is used to determine the number of IgGs that can fit onto the surface spherical particle:

$$N_s = a4\left(\frac{r_p}{r_{IgG}}\right)^2 \tag{13.1}$$

where N_s is the number of IgG molecules per particle surface, a is the circular packing factor (0.78), r_p is the particle radius, and r_{IgG} is the radius of the IgG molecule. Interestingly, experimental data for mouse IgG absorption onto 100 NM latex beads is in good agreement with the equation, 220 molecules/particle v. 280 molecules/particle, respectively (Wiewrodt *et al.*, 2002).

In order to estimate the number of protein molecules that can fill a nanosphere volume, the following equation is used:

$$N_p = C_{IgG}\frac{N_a}{MW_{IgG}}\frac{4}{3}\pi r_p \tag{13.2}$$

where C_{IgG} is the protein mass concentration, N_a is Avogadro's number, and MW_{IgG} is IgG molecule weight (assumed to be 150 000). Even for maximal aqueous concentrations of IgG (10 mg/ml), significant loading starts at particle sizes greater than 200 nm (see *Figure 13.3*). However, surface absorption results in significant IgG loading for particles as small as 50 nm. Yet, since volume loading increases with the cube of the particle radius, there exists a critical size in which sizes greater than this value possess a higher volume loading than surface loading. If this critical size is plotted as a function of protein concentration, it becomes apparent that very high protein concentrations (>11 mg/ml) are required before submicron particles have an efficient protein loading. Hence, solid phase inclusion, where solubility limits do not play a major role, may provide a very effective means of loading.

In spite of the overall loading advantages of surface absorption, the targeting and subcellular localization abilities of nanocarriers may result in therapeutic effects with relatively low loading, such as highly active therapeutic enzymes that decompose multiple copies of their substrates.

Nanoparticles

Nanoparticle synthesis was developed as an offshoot of the more established delivery technology, microspheres. Microsphere research began in the early 1970s as an easy-to-formulate controlled release depot of small organic drugs (Crotts and Park, 1998; Hans and Lowman, 2002). They possessed several advantages over competing depot technologies (e.g. solid implant, gels), such as easily controlled drug release profile and injectability. Yet, protein formulations were not pursued until the 1990s. In spite of the relatively late development period, already there has been some commercial success of depot systems, Lupron and Zoladex (polypeptide-based gonadotropin-releasing agonists) and Nutropin (human growth hormone therapy) (Crotts and Park, 1998).

With the success of microspheres, it is logical to expect that nanoparticles are poised to be one of the first synthetic nanocarriers to result in clinical use. Indeed, the majority of studies on protein-loaded synthetic nanocarriers have been based upon these strategies (see *Table 13.3*). Yet, it would be wrong to assume that the transition into the submicron domain is trivial.

Loading occurs simultaneously with nanocarrier synthesis in all strategies, except surface absorption. Therefore, the formation process must effectively generate carriers in the submicron range, but not disrupt the activity and/or structure of the protein being loaded. Due to the wide variability of protein properties and their general sensitivity to denaturation, such a constraint is not a trivial matter, and must be handled on a case-by-case basis. Synthesis conditions must be individualized in order to balance between loading of protein, its activity, yield of nanoparticles, their size and homogeneity. This section deals with strategies that have been employed for protein loading, and highlights the areas that still pose a significant challenge.

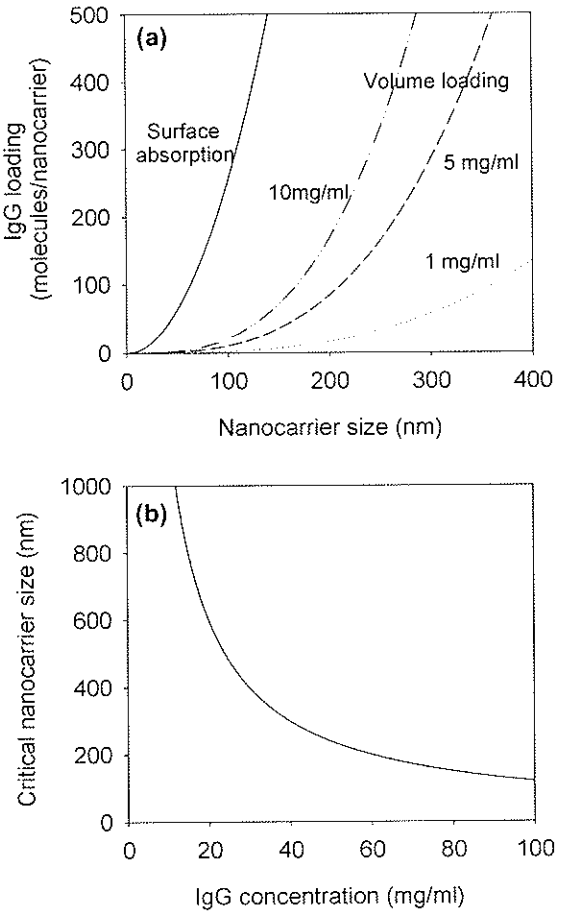


Figure 13.3. Theoretical loading limits of proteins in nanocarriers. a) For typical protein concentrations, surface loading provides the greatest potential for protein carriage. Loading of proteins inside nanocarriers becomes significant at sizes greater than 200 nm. b) Critical nanocarrier size is defined as the size at which volume loading and surface loading is equivalent. This value decreases as IgG concentration increases. As shown, volume loading concentrations must be exceedingly high in order to equal surface absorption limits.

NANOPARTICLE SIZE: CHARACTERIZATION AND CONTROL

Size represents a key determinate in blood clearance, delivery and targeting characteristics, sub-cellular addressing, degradation rate, function, and therapeutic utility of the nanocarriers (see below). The balancing act between production of nanoparticles within optimal size range and loading of sufficient amounts of active protein cargo typically produces preparations that are not ideal in either parameter. For example, mechanical homogenization protocols that avoid inactivation of incorporated proteins produce nanocarriers heterogeneous in size and morphology.

Particles with wide size distributions would likely possess populations that exhibit the clearance, biodistribution, cellular uptake, and degradation profiles of

Table 13.3. Summary of reported nanocarrier protein loading

Polymer	Protein	Protein MW	Carrier size	Loading efficiency	Comments	References
			DOUBLE EMULSION NANOPARTICLES			
PLA and/or PLGA	Bet V1 allergen	17 000	270–750 nm	1.6 wt/wt%	Sonication-based emulsion, immunization more effective when coinjected with basic solution	(Scholl *et al.*, 2004)
	BSA	67 000	110–5000 nm	25%	Sonication-based emulsion, 90% efficiency loading, long-term aggregation of protein	(Panyam *et al.*, 2003)
	Cystatin	11 000	300–350 nm	12–57%	Low energy mechanical emulsification used to preserve activity (up to 85%)	(Cegnar *et al.*, 2004)
	Haemo-globin	68 000	70–390 mm	13–19%	Not clear if loading is surface or internal aqueous inclusion	(Yu and Chang, 1994, 1996)
	HSA	67 000	200 nm	1.3 wt/wt%	Sonication-based emulsion, 33% loading efficiency, surfactant (poly(vinyl alcohol) irreversibly bound to surface	Zambaux *et al.*, 1999a,b)
	Insulin	6700	100–180 nm	8–12%	Solvent extraction emulsification, pH buffer dependent loading. Variations of surface content dependent on buffer	(Barichello *et al.*, 1999)
	Inter-feron α	22 000	280 nm–40 μm	65–80%	Sonication-based emulsion, microspheres possessed better long-term activity. Low nano-particle activity may be due to low loading, small particles prevented acid build-up	(Sanchez *et al.*, 2003)
	l-aspara-ginase	180 000	370–400 nm	0.39–0.45 wt/wt%	Sonication-based emulsion, glycerol protected activity	(Wolf *et al.*, 2003)
			200 nm	4.86, 2.65 wt/wt%	Sonication-based emulsion, carboxy terminated polymer had slower release, yet overall lower activity (activity was still present after 14 days *in vitro*)	(Gasper *et al.*, 1998)
PCL	BSA	67 000	260–300 nm	80%	Pressure induced emulsification, high loading, low burst release	(Lamprecht *et al.*, 1999, 2000)
PACA	hGCSF	20 000	130–230 nm	40–80%	Solvent extraction emulsification, activity maintained during loading mechanism	(Gibaud *et al.*, 1998)
Dextran-PCL	BSA and lectins	67 000 33 000 47 000	180 nm	30% BSA, 30–60% lectins	Sonication-based emulsion, lectin binding domains on polymer enhanced lectin loading	(Rodrigues *et al.*, 2003)
PEG-PLA, PEG-PLGA	BSA	67 000	200 nm	40–60%	Mechanical emulsification, *in vivo* biodistribution studies performed	(Li *et al.*, 2001a)

Table 13.3. *continued*

Polymer	Protein	Protein MW	Carrier size	Loading efficiency	Comments	References
	Catalase	242 000	250–400 nm	0.5–2 wt/wt%	Mechanical emulsification reduced enzyme activity loss, freeze–thaw enhanced active protein loading, loaded enzyme protected from proteolytic degradation	(Dziubla *et al.*, 2005)
	HSA	67 000	200–450 nm	2–6 wt/wt%	Sonication-based emulsification, demonstrated internal v. external loading	(Quellec *et al.*, 1998, 1999)
	Protein C	62 000	200–300 nm	33–45%	Sonication-based emulsification, activity of release protein was greatly enhanced by acetone/dichloromethane solvent mixture	(Zambaux *et al.*, 1999b, 2001)
PEG-PACA	TNF-α	17 000	150 nm	0.5–3 w/v%	Sonication-based emulsification, activity maintained (~85%)	(Li *et al.*, 2001b)
				SURFACE ABSORPTION		
Poly (methylidene malonate)	HSA	60 000		~900 molecules/particle	Protein absorption decreased with increasing protein electronegativity	(Bousquet *et al.*, 1999)
PLGA and PCL	BSR	14 000	200–250 nm	20–25 wt/wt%	Anti-tumour activity was well maintained in *in vitro* cell experiments	(Michaelis *et al.*, 2000)
Poly (leucine-b-glutamate)	Insulin	6600	250–450 nm	0.05 mg/mg reversible,	Concentration dependent surface absorption was not stable	(Constancis *et al.*, 1999)
Poly (styrene n-isopropyl acrylamide)	Calcitonin	3400	150–900 mm	15–100%	Absorption increased with presence of cationic comonomer (vinylamine)	(Sakuma *et al.*, 2002)
Poly (styrene)	Catalase, IgG	242 000 160 000	100–1000 nm	~25%	High surface absorption dependent upon surface chemistry and pH	(Muro *et al.*, 2003a, 2005; Wiewrodt *et al.*, 2002)
	Urease	69 000	200–1000 nm	20%	Enzyme activity stabilized by smaller (12–40 nm) inorganic spheres for enzyme stability	(Lvov and Caruso, 2001)
				COMPLEXATION		
Dextran-PEI	Insulin	6600	250–500 nm	90%	Complexing did not disrupt insulin's structural conformation	(Tiyaboonchai *et al.*, 2003)
PEG-poly (aspartic acid)	Lysozyme	14 300	50 nm	Not reported	No activity measured	(Harada and Kataoka, 2001, 2003)

Table 13.3. *continued*

Polymer	Protein	Protein MW	Carrier size	Loading efficiency	Comments	References
Cholesterol-pullulan	Insulin	6600	20–30 nm	30–50%	Complexes protected insulin from enzymatic degradation, rapid release in BSA solution	(Akiyoshi *et al.*, 1998)
PEG-cyclodextrin	Transferrin	80 000	70–200 nm	40%	Conjugates destabilized with 5% salt concentration	(Bellocq *et al.*, 2003)
2-Hydroxypropyl-chitosan choloride	BSA	67 000 180 nm	110–	50–90%	High burst release 2003)	(Xu *et al.*,
			HYDROGEL			
Gelatin	BSA	68 000	800 nm	93%	Gelatin nanoparticles maintained long-term stability of BSA inside PLGA microspheres	(Li *et al.*, 1997, 1998)
			SOLID PHASE INCLUSION			
PBCA	Insulin	6600	240 nm	77%	Activity not determined	(Sullivan and Birkinshaw, 2004)
			POLYMERSOME			
PEG-PPS-PEG	GOX	154 000	~200 nm	Not reported	Freeze–thaw, membrane extrusion polymersome formation, GOX was capable of oxidizing polymer, enhancing release of substrate	(Napoli *et al.*, 2004)

both small and large particles. This would result in erratic pharmacokinetics and effects, impeding the therapeutic needs. Therefore, effective and reliable methods of size determination and control are crucial in the development of nanocarriers. The following section describes some of the commonly used methods in nanocarrier sizing.

Estimation of nanocarrier size: dynamic light scattering (DLS)

The most widely accepted method for size determination of nanocarriers or any colloidal particles in the 20–1000 nm size range is dynamic light scattering. DLS, sometimes called quasi-elastic light scattering (QELS) or photon correlation spectroscopy (PCS), is a fast, reliable, absolute method that does not require preliminary calibration or standards. However, DLS is based on the principle of light scattering of moving objects that implies specific limitations on preparation of the sample and its reading. There are a number of important issues that should always be taken into consideration to obtain meaningful values and avoid abuses in its interpretation.

First of all, it is important to possess a rudimentary knowledge of the theory in order to effectively use the instrument. Briefly (for a more detailed description, please refer to the following: Brown, 1993; Mishenko *et al.*, 2000), at a moment in time, particles in solution will scatter light with a particular intensity at a set angle (at 90° in the case of a Brookhaven 90Plus Particle Sizer). If we wait for some time (Δt) and then check the scattering intensity, the intensity will change as a result of the change in particle orientation. If Δt is very small, then the intensity will not change very much, since the particles have not had enough time to move around in solution. However, as Δt increases, the chances of the intensity being the same (a.k.a. autocorrelating) will decrease dramatically. This dependence of intensity autocorrelation on time is directly related to the ability of particles to randomly move. If we assume the particles move according to the rules of Brownian motion, we can obtain equations that describe the speed of particle motion as a function of particle size. Hence, we can relate the decay in the autocorrelation directly to particle size.

When the assumptions built into the math equations are accurate (e.g. particle size is monodispersed, particle is a hard sphere, no particle–particle collisions), then the DLS provides a rapid, reliable means of measurement. Monodisperse samples result in the exponential decay of the autocorrelation function. However, in practical solutions of polydisperse sample, this decay typically will fit a more complex, multiple exponential function. In order to back-calculate a size and distribution, many fitting algorithms have been designed to best describe the data (Johnsen and Brown, 1992).

For most situations, the average size obtained is a reasonably accurate account of particle size, and the distributions presented by the analysis are more of a weak gauge in demonstrating heterogeneity. Yet, often times it is equally important to visualize nanocarriers using electron microscopy (EM). From scanning electron microscopy (SEM) and tunnelling electron microscopy (TEM), information about actual particle morphology and distribution can be obtained. Indeed, EM has been used as a tool for visualizing the structural effects of degradation on microspheres and, more recently, nanoparticles (Giunchedi *et al.*, 1998; Leach and Mathiowitz, 1998; Leach *et al.*, 1998; Avgoustakis, 2002). When used in conjunction, DLS and EM provide a robust and reliable characterization of synthesized nanocarriers. Other useful techniques for sizing nanocarriers include atomic force microscopy (Deacon *et al.*, 2000) and the analytical ultracentrifuge (Harding, 1994).

Nanocarrier size control: fractionating techniques

A need to purify protein-loaded nanoparticles from a larger, undesired population is a typically under-emphasized issue in the literature. While research into developing nanocarrier synthesis strategies providing simultaneous high protein loading and size control is ongoing, it is important that the currently used preparations be tested *in vivo* for their ultimate therapeutic use. Too often, this ultimate test reveals sub-optimal, and even unacceptable, pharmacokinetics of candidate carriers; in many cases, it is due to the inadequate or heterogeneous size of the carriers.

Currently, filtration is one of the most common methods of size-dependent separation of particles. Yet, filtration results in a significant loss of carriers,

especially when overall yield in the nanoscale range is sub-optimal. Also, filtration is only capable of removing larger particles. Hence, if there is a population of nanocarriers that are below the desired size range, filtration provides a poor means of isolation.

An alternate strategy for nanocarrier purification is serial centrifugation (Dziubla *et al.*, 2005). This strategy is best used when synthesized particles possess two distinct population sizes. Here, the synthesized particles are centrifuged at low speed (typically 1000 g). The centrifugation time can be determined by calculating the settling velocity for the larger particle sizes using the following equation:

$$V = 2ga^2 \frac{\rho_1 - \rho_2}{9\eta} \tag{13.3}$$

where V is the settling velocity, g the relative centrifugal force, a the particle diameter, ρ_1 and ρ_2 is the particle density and buffer density, respectively, and η is the buffer viscosity. After centrifugation, the supernatant can be collected and respun at a greater speed to collect the nanoparticle fraction. This procedure is an efficient and rapid method of both size selection and washing residual surfactant from the nanocarrier surface. However, care must be taken in selecting the rate of centrifugation, since too high a force may result in the irreversible aggregation of nanoparticles in the pellet. Finally, this method may also include the use of density gradients for even greater control in nanocarrier size selection (Yamanishi *et al.*, 2002; Behlke and Ristau, 2003). As yet, however, this approach remains to be explored for nanocarrier preparations.

NANOPARTICLE SYNTHESIS: DOUBLE EMULSION STRATEGY FOR PROTEIN LOADING

Double emulsion strategy is currently the most common synthesis/protein loading technique (Hans and Lowman, 2002). Theoretically, double emulsion nanoparticles possess both an inner and external aqueous phase, resulting in an enhanced energy penalty that makes the loading of aqueous drug inside nanoparticle pockets unlikely. Since entropy works against loading, nanocarrier loading is not determined by equilibrium partition of the drug, but rather by the kinetic effects of polymer gelation that reduces the inner aqueous domain and releases into the outer aqueous compartment. This transition of the polymer into the gel state is predominantly determined by the rate of solvent loss, which can occur through either extraction or evaporation methods, and which require different levels of mechanical energy input for the formation of nano-scale particles.

Solvent extraction approach

Solvent extraction occurs when the polymer solvent used is fully miscible with water. Hence, during the emulsion phase, the solvent rapidly leaves the polymer phase. As such, the polymer hardens rapidly, thereby minimizing coalescence (Crotts and Park, 1998; Panyam *et al.*, 2003). Furthermore, with a dilute polymer solution and high surfactant content, it is possible to ensure nanoscale nucleation sites.

Under these conditions, mechanical energy is not typically needed to form the nanoparticles, which favours loading of proteins without inactivation. However, examples of such loading are still relatively rare, especially with documented proof that functional activity of loaded proteins remained. Still, nanoparticles have been loaded with insulin (functional activity for insulin was not reported) and the human recombinant colony stimulating factor, with activity of the colony stimulating factor being maintained during the loading procedure (Gibaud *et al.*, 1998; Barichello *et al.*, 1999). Nevertheless, since extraction mechanisms typically result in a solid core particle, it is not clear whether the proteins were loaded inside the particles or were just present on the surface.

Solvent evaporation approach

More frequently, solvent evaporation is used as the forming mechanism for double emulsion nanoparticles. Under these conditions, the organic solvent is immiscible with the aqueous phase, and the hardening of the polymer phase occurs as solvent evaporates from the system. Due to the relatively slow process of evaporation, several strategies have been used to enhance protein loading. For example, a successful protein loading has been achieved with the use of low vacuum for solvent evaporation (Quellec *et al.*, 1998, 1999).

Panyam and colleagues found that larger molecular weight polymers possessed a greater protein loading capacity (Panyam and Labhasetwar, 2003; Panyam *et al.*, 2003). It was theorized that the higher molecular weight provided a greater viscosity and a more rapid transition into a gel/solid state of the polymer phase. In support of this notion, we have demonstrated improved loading of amphiphilic PEG-PLGA polymer nanoparticles with a large, labile enzyme (catalase) using a freeze–thaw cycle (Dziubla *et al.*, 2005). Freeze–thaw mechanisms have been used previously to induce hydrophobic association/crystallizations (Peppas and Merrill, 1977; Mallapragada *et al.*, 1997), and presumably these associations during homogenization improve the transition of the polymers into a pseudo-gel state.

Mechanical energy input for nanoparticle formation

Unlike solvent extraction, solvent evaporation requires the addition of mechanical energy to form the two emulsion phases. Ultrasound sonication and homogenization represent two main choices, the former providing higher energy inputs (Zambaux *et al.*, 1998; Barichello *et al.*, 1999; Avgoustakis *et al.*, 2003; Dziubla *et al.*, 2005). However, both methods may inactivate protein cargoes during loading by such factors as increase in local temperature and cavitations.

Due to the high energy input required for the production of particles with a diameter below 0.5 micron, the most commonly used method has been ultrasound probe sonication. While many studies have used this mechanism, only a few were able to maintain the enzyme activity of the loaded cargo proteins under these intense energy conditions by the cost of a great reduction in energy input, which may lead to sub-optimal sizing of the resulting carriers (Cegnar *et al.*, 2004; Dziubla *et al.*, 2005).

Zambaux and colleagues reported that not only a reduction in energy was required

to maintain protein C activity, but also the selection of organic solvents was very important (Zambaux *et al.*, 1999b, 2001). When pure dichloromethane (DCM) was used, no measurable activity was found. However, activity greatly increased when a solvent pair of acetone/DCM was used. It was believed that the hydrophobic DCM interface was the source of protein C deactivation, and that acetone helped reduce this hydrophobicity. In a separate study, sonication did not denature catalase unless the hydrophobic interface of DCM was present. However, unlike the protein C results, acetone mixtures actually exacerbated the denaturing effects of sonication on catalase, likely due to its precipitation by the addition of acetone (Dziubla *et al.*, 2005). These diverse outcomes illustrate individuality of every protein in terms of resistance to the inactivating conditions of loading to nanocarriers.

When sonication conditions are incompatible with maintaining the structural and functional integrity of the loaded protein, mechanical homogenization can be used. Mechanical homogenization uses a high speed turbine (1000–20 000 rpm) contained within a shaft. The narrow aperture between the shaft and turbine, coupled with the high mixing speeds, results in extremely high shear rates that are capable of forming nanoscale-sized droplets. Compared to sonication, the amount of heat generation and energy input is greatly reduced, and there is less concern about secondary complications (e.g. radical formation, cavitations, probe tip contamination). However, one drawback to using mechanical homogenization is the large size distributions that result (Crotts and Park, 1998; Dziubla *et al.*, 2005). Typically, a dual mode population will exist, a microparticle (1–5 μm) and nanoparticle (100–700 nm) population and additional separation steps described above may be needed to isolate particle fractions within a relatively narrow size range.

POLYMERSOMES

Another synthetic nanocarrier strategy theoretically applicable for protein delivery is polymersomes, which are polymer-derived analogues of liposomes (see *Figure 13.1*). Like liposomes, these structures have a large internal aqueous domain, which can be utilized for protein carriage (Discher *et al.*, 1999, 2000; Discher and Eisenberg, 2002). Furthermore, the membrane bilayer of polymersomes is thicker than liposomes (~8 nm, compared to ~3 nm), which has been shown to provide a more durable carrier possessing a greater circulation half-life.

Preparation and loading of polymersomes follow the same patterns of liposomes. Small solutes, such as sucrose and glucose, are encapsulated at concentrations comparable to their bulk concentrations. From a MW series of dextrans, hydrophilic drugs, with sizes up to 500 kD (~20 nm), can be encapsulated, although the efficiency of encapsulation generally decreases with increasing MW (Ahmed and Discher, 2004). Furthermore, one study has shown the successful encapsulation of rhodamine-myoglobin. However, the aforementioned stability of polymersomes possesses one significant drawback; encapsulation conditions required to overcome the more durable nature of these materials (e.g. high temperature, pressure) are more likely to affect protein stability and activity.

The polymers used to form polymersomes possess an exaggerated hydrophilic/hydrophobic character, and are significantly larger than common surfactants and phospholipids (Discher *et al.*, 2000). Some of the polymers used for synthesis of

stable polymersomes include the block copolymers, PEG-poly(butadiene), PEG-poly(ethylethylene) and PEG-poly(propylene sulfide)-PEG (PEG-PPS-PEG).

PEG-PPS-PEG is of unique interest in that the sulfide group of the polymer is sensitive to oxidative stress (Napoli *et al.*, 2004). This hydrophobic block can be converted into the more hydrophilic poly(propylene sulfoxide), resulting in the destabilization of the polymer vesicle. Such a mechanism would allow for the triggered release of its cargo when in the presence of an oxidative environment, such as in the sites of inflammation. However, unlike PEG-PLA, these materials possess a questionable biological future due to their yet unknown biocompatibility and clearance mechanisms.

COMPLEXATION LOADING

One of the examples of more recent charge-based associations is called the layer-by-layer particle synthesis (Balabushevitch *et al.*, 2001). In these systems, proteins are immersed in a dilute solution of poly(allylamine), resulting in an aggregation of polymer and the negatively charged protein. These aggregates are then reciprocally immersed into a poly(anionic) polymer solution (e.g. poly(acrylic acid). Repeated several times, this procedure yields highly stable particles that have alternating positive–negative layers (Hong *et al.*, 1993; Balabushevitch *et al.*, 2001).

The majority of publications in the area have involved micron scale spherical shells. Yet, there has been at least one study that has successfully encapsulated urease by first surface absorption of protein onto a latex bead, and the subsequent layer-by-layer formation (Lvov and Caruso, 2001). This approach is extremely interesting in its ability to load large amounts of protein onto a nanoparticle and ensure a covering around the drug. However, due to the rather favourable loss of counter ions from this system, it is extremely hard to release protein loaded in this fashion.

Vascular delivery of synthetic nanocarriers

CIRCULATION OF NANOCARRIERS IN THE BLOODSTREAM

In contrast to elastic blood cells, which can circulate for prolonged periods of time (e.g. months for erythrocytes), micron-size carriers display very limited lifespan in the bloodstream: particles larger than 5 micron are typically mechanically entrapped instantly in the pre-capillary arterioles downstream of the injection site, while smaller microparticles are taken up by reticuloendothelial system (RES, mainly liver and spleen in rodents) within a few minutes after injection. Circulation of nanocarriers is also, at least in part, regulated by their size. Generally, the smaller the particle, the longer is their half-life in the bloodstream.

However, RES clearance of nanocarriers in the range 100–500 nm is extremely rapid (typically, 90% after 5 mins in mice), mainly to resident macrophages in hepatic synuses (Kuppfer cells) (Roser *et al.*, 1998; Torchilin, 2000; Li *et al.*, 2001a). In addition, decoration of nanoparticles by specific sugar moieties, such as mannose, that has abundant receptors on hepatocytes, facilitates delivery to these cells of

nanoparticles within 50–250 nm diameter amenable diffusion through fenestrations in hepatic blood vessels. This mechanism is being used for directing protein therapies to hepatic cells (such as drugs for the treatment of leushmania localized in Kuppfer cells).

Nevertheless, in terms of delivery of therapeutics to organs and targets other than the liver, this pathway represents a major obstacle. However, it can be delayed greatly with the use of 'stealth' technology. When linear PEG polymers are attached to proteins or incorporated onto lipid or polymer surfaces (e.g. nanoparticles formulated from biocompatible poly(lactic acid), PLA), they form a hydrophilic shell, or brush, that repels plasma opsonins, complement, and phagocytes (Abuchowski *et al.*, 1977; Discher *et al.*, 1999). This enhanced circulation time can vary from a few hours to days for nanosphere structures (Photos *et al.*, 2003).

In many cases, blood components represent the drug delivery target. Delivery of haemoglobin substitutes, anti-thrombotic proteins, or any enzymes, of which activity must be restricted to the blood compartment (e.g. pro-coagulant factors for the treatment of haemophilia), provides examples of such medical contexts. Functions of nanocarriers in these cases include prolongation of circulation time and limitation of extravascular effects of proteins, which otherwise can diffuse into tissues and cause side effects.

Delivery of materials to immune cells, such as dendritic cells or lymphocytes, also represents a promising area of application of nanocarriers for stimulation or suppression of immune response to a selected antigen. First, these cells are relatively accessible to circulation (e.g. immune cells in blood, RES, and bone marrow). Second, by presenting multiple copies of antigens to the target immune cells, nanocarriers work as adjuvant.

Nanocarrier-mediated delivery of protein cargoes to extravascular targets, such as cardiomyocytes, neuronal cells, or tumour cells, represents a formidable challenge. In this context, their size works against the therapeutic objective, at least in part due to the necessity to permeate the monolayer of the endothelial cells that form a barrier separating blood from tissues. However, under pathological conditions, including inflammation, trauma, oedema, and tumour growth, vascular permeability increases, permitting more effective deposition of nanocarriers in the affected area.

For example, a form of 'passive' extravascular drug delivery available to nanoparticles is through the enhanced permeation and retention effect (EPR) described in some solid tumours (Maeda *et al.*, 2000; Moghimi *et al.*, 2001). The enhanced permeability, erratic flow patterns characteristic of tumour vasculature, and poor lymphatic drainage form 'dead zones' within the tumour interstitium, allowing the build-up of nanocarriers. However, in order to utilize EPR to accumulate in tumours, nanocarriers must circulate for a sufficient time; hence, the use of stealth carriers.

TARGETED DELIVERY OF NANOCARRIERS IN THE VASCULATURE

In addition to the above listed paradigms for 'passive targeting', nanocarriers are amenable to 'active' targeting that can be achieved via coupling affinity moieties on the carrier's surface, providing selective binding to characteristic target determinants. Surface absorption, biotin–steptavidin cross-linking, or protein

conjugation chemistries all can be utilized for such coupling (Torchilin, 1994; Muzykantov, 2001; Muro *et al.*, 2004).

One of the most commonly employed conjugation strategies is that of maleimide sulfhydryl chemistry (Hermanson, 1996). The maleimide group is more hydrolytically stable than other protein conjugation means, such as the amine directed n-hydroxysuccinate esters. Maleimide reacts with free thiol to create a non-reducible sulfide linkage. Since most proteins do not contain a free thiol group, competition between the therapeutic protein and targeting moiety for available binding sites can be eliminated.

Maleimide can be included onto the distal end of a PEG group in a PEG diblock copolymer, where the hydrophobic block can be any of the degradable materials mentioned (Olivier *et al.*, 2002; Tessmar *et al.*, 2003). Upon nanoparticle synthesis, the PEG chain will extend out into the hydrophilic solution, ensuring the exposure of the maleimide group for subsequent conjugation. This allows for the separation of loading and nanoparticle formation, from target group addition. However, while maleimide hydrolysis is relatively slow, at typical nanoparticle synthesis temperatures, it may still occur to a significant extent, thereby limiting the overall capacity for target group addition. To our knowledge, to date, there have been no examples of *in vivo* studies of protein-loaded synthetic nanoparticles coated with targeting groups.

Bioengineering difficulties associated with the design of such nanocarriers possessing active protein cargo inside and affinity moiety on the surface represent one of the reasons for lack of success in this area. On the other hand, many important targets are very challenging in terms of defining specific affinity moieties and accessibility to circulating agents. Targeted drug delivery as a field has been established mainly in the context of anti-cancer therapies, with lesser side effects and higher potency. However, effective delivery of macromolecules and nanocarriers to tumour cells is impeded by many factors. Poor perfusion of tumour interstitium at distances as short as 100–200 micron from an arteriole represents a difficult diffusional barrier. On the other hand, diffusion of high-affinity macromolecules and nanocarriers is even further impeded by their binding to the first accessible target cells; hence, distribution throughout the tumour (and, hence, effect) is very heterogeneous.

In this context, targeting of the vascular endothelium, the cellular monolayer lining the luminal surface of blood vessels, is a relatively less challenging, yet important, goal. By virtue of their anatomical position and spectrum of their vital role in cardiovascular, pulmonary, neural, and many other physiological systems, endothelial cells represent an important target for many protein therapeutics, including antioxidant, anti-inflammatory, and anti-thrombotic compounds. Loading these proteins into nanocarriers coated with ligands that bind preferentially to endothelial surface determinants permits their delivery to the endothelium.

For example, constitutively expressed endothelial cell adhesion molecules, ICAM-1 and PECAM-1 (Murciano *et al.*, 2003; Muzykantov, 2003), as well as endothelium-specific ectoenzymes, angiotensin-converting enzymes (Danilov *et al.*, 1991; Reynolds *et al.* 2001), and caveoli-associated antigens (Schnitzer, 1998) are plausible targets for delivery of nanocarriers. After systemic intravenous administration, nanoparticles directed to these targets tend to accumulate in the pulmonary

vasculature, since it represents ~30% of endothelial surface in the body, receives whole cardiac venous output, and is perfused by blood at a relatively slow rate, favouring binding to endothelium. Local injection in afferent arteries permits the use of these vascular immunotargeting means for pursuing other vascular areas.

Pathologically altered endothelium, such as the areas involved in inflammation or tumour growth, represents a unique target for the delivery of diagnostic and therapeutic agents. For example, tumour endothelium expresses specific surface determinants, including endoglins, receptors for angiogenic factors, and cell adhesion molecules (Huang *et al.*, 1997; Rajotte *et al.*, 1998). These molecular targets can be utilized for selective inhibition of angiogenesis or inflicting damage in tumour vasculature, leading to tumour infarction.

By passing the endothelial barrier separating blood from underlying tissues using pericellular or trans-cellular pathways, endothelial targeting may permit delivery of nanocarriers directly to tumour cells and other extravascular targets. In this context, delivery to specific endothelial surface domains involved in endocytotic pathways and trans-cellular traffic, such as caveoli, represents an area of intense interest. Furthermore, coupling of some non-internalizable ligands (i.e. that bind to cellular surface molecules that normally do not undergo endocytosis) to nanocarriers markedly stimulates internalization (Wiewrodt *et al.*, 2002; Muro *et al.*, 2003b). Although molecular mechanisms of this endocytotic process induced by multivalent binding to non-internalizable molecules remain to be better understood, apparently it represents a significant advantage of using nanocarriers for intracellular drug delivery (Muro *et al.*, 2005).

Conclusions

Nanocarriers present an exciting avenue to capitalize on the potential of protein-based drug therapies. Speaking in terms of translation into practical medical applications, the field is still in its infancy. It relies upon the expertise of many fields from polymer chemistry, carrier loading/synthesis, to targeting design, and carrier inclusion. Further, due to a wide diversity of features and functions, each protein drug represents an individual research object. Therefore, progress in the field depends on the extent of our understanding of both individual features of specific protein drugs and the biomedical context in which we plan to intervene using these drugs loaded into polymer nanocarriers.

However, since initiation of active experimental investigations in this field, including *in vivo* studies, an impressive progress in formulation and characterization of diverse types of prospective nanocarriers has been achieved. Important parameters of nanocarriers, including loading, structure, size, homogeneity, pharmacokinetics, tissue deposition, biocompatibility, biodegradation, and controlled drug release, are being defined and optimized in order to adequately serve therapeutic goals. Challenges, such as production of nano-scale polymer particles without inactivation of protein cargoes, sub-optimal circulation time of nanocarriers, and limited access to extravascular targets, are being tackled. Recent years have produced numerous fresh ideas and approaches promising to circumvent these and other hurdles. Animal studies provide more and more experimental data that both address important mechanistic issues related to

these drug delivery systems and represent, in essence, initial phases of toxicity and effectiveness studies.

Looking into technical difficulties and expenses associated with protein delivery by polymer nanocarriers, one can ask a fair question – is it worth it? We believe the answer is 'yes'. Paradoxically, a marriage of an expensive and difficult to manage means of delivery with expensive and difficult to manage therapies can minimize their common downsides. Effective and precise delivery via the vascular system of proteins, specific and powerful effectors designed by nature, will allow them to exert their full therapeutic and prophylactic potential. Taken together with revolutionary progress in molecular design and modifications of protein therapeutics, this outcome will mark the beginning of a new era in medicine, pharmacology, and pharmaceutics.

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