Expert Opinion

Formulating protein therapeutics into particulate forms

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This review is aimed at providing critical comments on selected approaches to formulating protein drugs into particulate forms feasible as practical pharmaceutical dosage forms. From a practical point of view, the need to formulate protein therapeutics into particulate forms includes inhalation and sustained-release delivery proteins, stabilizing and incorporating proteins into tissue engineering scaffolds and medical devices, as well as protecting and targeting protein therapeutics in an *in vivo* environment. For either of the applications, a common challenge is that proteins are easily denatured during particle-forming processes in which water–oil or water–air interfaces, multivalent ions or polyelectrolytes, strong shear stress and/or reactive crosslinking agents are often involved. Moreover, methods to protect proteins during the particle-forming processes must not compromise their pharmaceutical objectives, such as encapsulation efficiency, burst-free controlled release and storage convenience. Although numerous methods have been reported to formulate proteins into particulate systems, few of them meet the criteria above. To stimulate critical and interactive readings of the vast and booming information, the authors also provide their analysis regarding the feasibility of the formulation strategies summarized in this review.

Keywords: protein delivery, protein microparticles, protein nanoparticles, protein stability

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1. Introduction

Protein therapeutics, including blood products, antibodies, vaccines and cell growth factors, are the most rapidly growing category of drugs, and have shown an annual rate of global market growth of 10.9% in the last few years [1]. However, the advances in delivery technologies for these types of therapeutics are far behind the pace at which the drugs are created. As a result, most protein drugs at present are given by frequent injections, an administrative route compromised by patient compliance and, sometimes, therapeutic efficacy. Strategies to reduce injection frequency and improve protein administration include non-invasive dosage, sustained-release injection, and extension of *in vivo* contact and circulation. To achieve these strategies, formulating proteins of native state into particles resistant to organic solvents, moisture, acidic environment and/or enzymatic degradation is widely accepted as a practical approach. However, loading proteins into particulate systems is especially challenging owing to these macromolecules' susceptible tertiary structures, which are missing in peptides and small chemical drugs. Therefore, a particle forming process, which does not cause protein denaturing, must be designed. In this review, the discussions and comments are focused on the needs, challenges and approaches in formulating proteins (rather than peptides) into particulate forms.

The formulation of proteins into particles for different applications encounters different difficulties; thus, different criteria are required for the particle-forming process. For inhalation delivery of proteins, for example, the protein should be loaded in particles 1 – 5 μm in diameter and be able to dissolve immediately at the site

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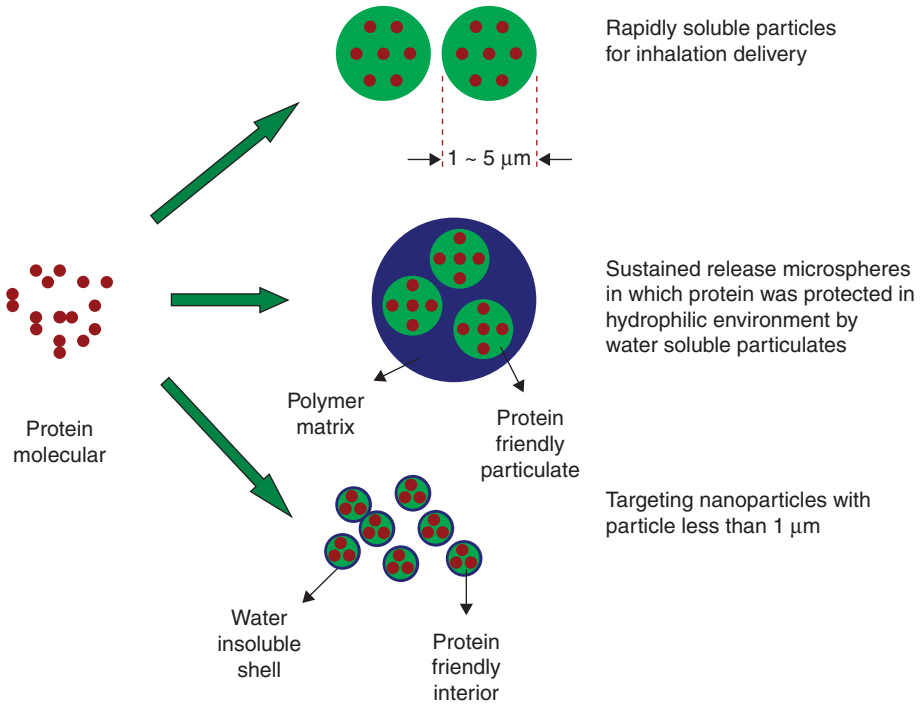

Figure 1. Particle-forming process of rapidly soluble particles, sustained release microspheres and targeting nanoparticles.

of administration. The challenge of loading proteins into the particles of water-soluble excipients is that the particle-forming process should be free of water–oil or water–air interfaces, factors known to cause protein denaturing [2]. For sustained-release delivery purposes, proteins have to be encapsulated in a water-insoluble matrix to achieve prolonged *in vivo* action. In this case, protecting the proteins in a hydrophobic environment becomes an issue in addition to water–oil or water–air interfacial tension. For *in vivo* targeting, protein drugs need to be encapsulated in particles of nanometer size to ensure a sufficient period of circulation and to be protected from enzymatic degradation. However, creating a protein-friendly interior within the nanoparticles and trapping proteins with high efficiency is a great challenge. To comprehend better the challenges and approaches in formulating protein particles for different purposes, the discussions are divided into the three categories accordingly. **Figure 1** describes the three particle-forming criteria schematically.

2. The need to formulate proteins into particulate forms

2.1 Delivering protein drugs by pulmonary inhalation

Although the lung may not be the right organ to accept solid particles repeatedly for multiyear or lifelong treatments, pulmonary inhalation is a promising route for non-invasive delivery of protein drugs in local treatment and systematic drug administration for limited periods of time. To ensure that therapeutics reach pulmonary alveoli (which represent sufficient area of mucosa), protein-loading particles have to be formulated in the range of 1 – 5 μm in diameter so that they can be carried in by the airflow of respiration to the destination without earlier deposition and exhalation [3,4]. On the other hand, as particles deposited on the mucosal surface of alveoli will be recognized and cleaned by alveolar macrophages, which leads to degradation of the proteins and incites immune responses, the inhalation dosage forms should be formulated with water-soluble excipients so that the particles can be dissolved before macrophage recognition and phagocytosis [5]. In general, formulating proteins of native form into particles of water-soluble excipients is easier than loading the delicate molecules into particles of hydrophobic matrix. However, a physicochemical driving force, such as hydrophilic/hydrophobic surface tension or intra-droplet crosslinking, is still indispensable for water-soluble materials to form particles. Therefore, a particle-forming process suitable for encapsulating proteins must be mild enough by avoiding high temperature, strong shear stress, water–oil or water–air interfaces, and covalent or ionic crosslinking agents.

2.2 Loading proteins into controlled-release dosage forms

Particulate injectables have long been proposed as controlled- and sustained-release delivery of proteins [2]. Sustained-release microspheres form a drug depot at the injection site from which proteins may be released gradually for weeks. Although

Table 1. Some systems/materials and biological applications of the described delivery systems.

Long-acting protein dosage forms	Microspheres of degradable hydrophobic polymers	Microspheres of hydrophilic polymers	Temperature-sensitive *in situ* gelling systems	Structural modification
Long-acting mechanisms of proteins	Slow release from gradually degrading polymer depot	Slow release from hydrated polymer depot	Slow release from depot formed by *in situ* gelling	*In vivo* life being extended by structure modification
Protein stability	Native state is maintained in microspheres by polysaccharide dispersed phase	Proteins are loaded in hydrophilic network	The PEG phase may denature very delicate proteins	Stable with an *in vivo* half-life
Profile and period of extended protein action	May act for weeks with minimal initial burst	May act for a week with considerable initial burst	May act for weeks with considerable initial burst	Blood concentration decay exponentially
Formulation or preparation	Complicated and need to use organic solvent	Easy and no need to use organic solvent	Easy and no need to use organic solvent	Complicated for reaction and purification
Tissue-specific administration	Yes	Yes	Yes	No
Cell-targeted delivery (for APCs)	Yes, but has to be < 5 μm in diameter	No	No	No
Load in scaffolds or devices	Yes	No	No	No
Safety of materials to form delivery systems	PLGA polymer has been used for surgical string for decades	Hyaluronic acid is regarded as a biocompatible material	PLGA polymer has been used for surgical string for decades	PEG, < 30,000 Daltons in molecular mass can be eliminated

there is not a product available at present in the market, since the drop-off of Nutropin Depot (sustained-release microspheres of growth hormone), some reported microsphere systems have been shown to be promising [6]. The particulate systems feasible for protein controlled or sustained release may be divided into three categories: pure protein particles [7]; microspheres made of hydrophilic polymers; and composite microspheres formed of hydrophobic polymers as the controlled-release matrix in which proteins are protected in the hydrophilic dispersed phase for its protein-friendly microenvironment [6,8]. Hydrophilic microsphere systems and crystalline protein particles have been reported to hold protein therapeutics for a maximum of 1 week with a considerable initial burst owing to their intrinsic water solubility and swellability [9,10]. Proteins loaded in microspheres of water-insoluble polymers are expected by theory to be released in a sustained manner for a prolonged period of time and with reduced initial burst [11]. However, stabilizer agents required for protecting proteins in formulation processes and inside the hydrophobic matrix of microspheres often cause intense osmotic pressure, which leads to severe burst release. To meet the criteria of protein stabilization and controlled-release kinetics simultaneously, a composite microsphere system consisting of a controlled-release matrix of degradable polymer and hydrophilic dispersed phase in which proteins are loaded was proposed [12]. It is best that the hydrophilic dispersed phase be formed by high-molecular-mass polysaccharide to avoid rapid dissolution, intense osmotic pressure and immediate release from the microspheres.

For sustained release delivery of native proteins, a system called reversed thermal gel should be taken into consideration. Reversed thermal gel is an aqueous solution of a triblock copolymer that adsorbs water by hydrogen bonding at room temperature, but becomes water insoluble (i.e., the gel state) at body temperature by desorbing the hydrogen-bonded water. Proteins may be added in the aqueous solution of the polymer at room temperature and form a sustained-release depot with the polymer after injection to the body [13]. This system is superior to microspheres in that complicated formulation steps and use of organic solvents can be avoided completely. However, as the thermal gelling process is initiated by hydrophobic shrinking of the polymer solution in the body, the protein solution loaded in the system may be squeezed out and result in a severe burst. In addition, the thermal gelling requires a lag time during which proteins, in solution form, may be released rapidly before the gel-like depot is formed. In summary, microsphere systems are preferred for the applications in which burst-free long-term release is required, whereas reversed thermal gels are the choice if a certain level of initial burst is acceptable.

2.3 Incorporating delicate proteins into polymeric medical devices and tissue-repair scaffolds

Sustained and localized release of cell growth factor proteins is an essential function for tissue engineering scaffolds and

functional medical devices. Tissue engineering scaffolds may be formed from hydrophilic polymers (as hydrogel network [14]) and degradable hydrophobic polymers (in the forms of porous matrix, sheets and fibers [15,16]). For hydrophilic scaffolds, cell growth factors may be added directly to the hydrogel network in molecular form for formulation simplicity or added in the form of sustained-release microspheres for better control of the drug concentration. For the latter, however, the difficulties for protein microencapsulation discussed above will be encountered again.

Degradable hydrophobic scaffolds are able to offer prolonged action of cell growth factors for their degradation-controlled release mechanism. However, loading the proteins into the polymeric matrix becomes an issue of concern. Although some researchers claimed positive results [17,18], the process of adding proteins into the matrix of hydrophobic polymers in molecular form has been proved to denature proteins by the decades-long efforts in developing sustained-release microspheres. Similarly, loading proteins onto medical devices as surface coatings or pore structure fillings faces protein stability issues as well. Therefore, a common issue in loading proteins into hydrophobic scaffolds and device coatings is how to preserve the proteins' native conformation, and pre-formulating proteins into protective hydrophilic fine particles seems to be a reasonable strategy [2].

2.4 Loading proteins into nanoparticles

The need to formulate proteins into particles of nanometer sizes includes extending *in vivo* circulation time of proteins, targeting proteins to therapeutic tissues or cells, and possibly improving cross-membrane transportation and absorption of proteins [19]. For example, the preferred particle diameters for dendritic cells (the most popular antigen presenting cells (APCs)) to engulf are in the range 100 – 400 nm [20]. Nanoencapsulation is well reported as a strategy to improve bioavailability through oral, intranasal and transdermal administration [21-23]. Various mechanistic pathways have been suggested for nanoparticle-facilitated protein absorption, such as particle migration through tight junctions of intestine wall [24], particle engulfing by intestine M cells [25], phagocytosis by ordinary tissue membrane cells for nanoparticles attached with transmembrane peptides [26], and enhancing epidermis permeability to surfactant-rich protein particles suspended in oil [27]. So far, however, none of the approaches could increase protein bioavailability with practical significance.

Moreover, in most of the studies above, bio-inactive model proteins of insulin, a small protein without tertiary structure and conformation susceptibility, were encapsulated or precipitated to solid nanoparticles. Most therapeutic proteins possess susceptible conformational structure and are easily denatured within a hydrophobic environment or by ionic crosslinking. To load proteins into nanometer-sized particulate systems with preserved native state and acceptable loading efficiency is more difficult than microencapsulating them into micrometer-sized spheres. Although some researchers claimed that they could effectively nanoencapsulate proteins within nanometer-sized particles (polymer and solid lipid nanoparticles, micelles, solid-in-oil suspensions) [21,28,29], few of the nanoparticulate systems possess a hydrophilic and protein-friendly interior. Liposomes may be used to encapsulate water-soluble proteins in their aqueous interior, but the mechanical instability limits their applications. Polymersomes, bilayer vesicles formed of hydrophilic–hydrophobic block copolymers, seem to meet the criteria with increased mechanical stability and hydrophilic interior; but reported polymersomes were formed by block polymers with polyethylene glycol (PEG) as the hydrophilic block. The hydrodynamic PEG drives proteins away from the vesicle interior (which is why PEG-conjugation, i.e., PEGylation, is used to prevent proteins from enzymatic degradation), leading to low encapsulation efficiencies.

3. The challenges to formulate proteins into particulate forms

3.1 Tissue impermeability and structure instability of protein molecules

Most protein drugs are hydrophilic macromolecules highly impermeable to lipophilic tissue membranes. For proteins to reach therapeutic sites, physical, chemical and/or biological approaches to enable these macromolecules to overcome the permeability barriers are essential. However, owing to delicate higher structures, proteins are often susceptible to the physical, chemical and biological conditions applied. For example, for proteins to enter target cells, the proteins have to be loaded in particles of nanometer size and engulfed by the cells. Encapsulating proteins in or adsorbing proteins on water-insoluble nanoparticles may denature proteins owing to the hydrophobic environment. In addition, the phagocytosed proteins may be degraded by the acidity inside the vesicle of phagocytosis. During the formulation processes of controlled-release systems, proteins will encounter water/oil or water/air interfaces where the shear stress may easily overcome the energy barrier for protein unfolding (5 – 20 kcal/mol) [30,31]. For some proteins, an aggregated form may be more stable than native monomer so that an energy-adding process may facilitate proteins crossing the energy barrier from monomer state to aggregated state [32]. These processes may involve elevated temperature and shear stress, as well as the presence of multivalent ions. For example, using multivalent metal ions to precipitate proteins to particles may lead to irreversible aggregates of some proteins (such as EPO [33]).

3.2 Low encapsulation efficiency

Low encapsulation efficiency is another long-standing problem for loading proteins in polymeric particulate systems, especially those of nanometers in diameter. For example, the encapsulation efficiency for proteins in particulate systems by the so-called water-in-oil-in-water (W/O/W)

double-emulsion method is in general considerably low and poorly reproducible, especially for those with nanometer sizes [34,35]. This is partially because most therapeutic proteins are water-soluble and most pharmaceutical particulate systems are water-insoluble that are formed in a water-based continuous phase. The kinetic barrier to prevent water-soluble proteins leaking into the water-based continuous phase is the water-insoluble matrix of the particles themselves. For small particles, this barrier is thin and is formed in a dynamic condition within the water-based continuous phase. Therefore, improving protein loading efficiency still based on this type of particle-forming processes is a daunting task.

3.3 Burst release and incomplete release

Controlling initial burst release is one of the primary objectives for developing long-term (multi-weeks or months-long) sustained-release systems of proteins. For example, releasing 20% of total loadings in the first day may be perfect for a weekly dose, but may lead to a dosage form index close to or > 100 for a monthly sustained-release injection. For the causes of burst release of microspheres, most related reports attribute it to surface-rich distribution of proteins due to microencapsulation processes [36,37]. Some researchers believe that proteins microencapsulated by the so-called W/O/W double-emulsion method may be stored in the pores and channels formed in the polymer matrix during the microsphere forming process [38,39]. These mechanistic illustrations may be reasonable in many double-emulsion-based processes but not for some 'particle-in-particle' composite microspheres. For example, when protein particles precipitated by ammonium sulfate were encapsulated to form composite PLGA microspheres, up to 40% of total loadings were released in the first day [33]. However, when pure protein particles formed by phase separation from a PEG solution were encapsulated in PLGA microspheres, the first day release was < 10%, but incomplete release of 30% of the total loadings became a problem [8]. It may be far-fetched to attribute the discrepancy between the two composite microspheres systems to whether the proteins were located more or less in the surface region of microspheres or whether cavities were formed inside microsphere matrix. The likelihood may be that osmotic pressure generated by dissolved salt functioned as the driving force of burst release [40].

The above examples regarding burst and incomplete release of proteins from composite microspheres also suggest that burst and incomplete release may be the two sides of an objective and hard to get rid of at the same time. The aggregation between protein molecules and adsorption of proteins onto polymer matrix, both involving interaction with protein's interior hydrophobic chains, are believed to be responsible [41]. A protein's stabilizing method, which effectively prevents protein aggregation and adsorption on PLGA matrix without compromising burst release, is highly desired.

4. Reported approaches to preventing protein denaturing in formulation process and their pros and cons

Pre-formulating proteins to fine solid particles has been reported to be an effective approach to endow proteins with immobility and resistance to organic solvents before incorporating them into a variety of polymer-based dosage forms and medical devices for controlled-release purposes [42-44]. However, the particle-forming process itself often associates with hazardous conditions to proteins. To summarize better the techniques developed for formulating protein particulates, the protein particles formed directly in a hydrophilic environment will be classified as 'primary particles' and those encapsulating primary particles for controlled-release or other applications as 'polymer-based particles' in the discussions below.

4.1 Crosslinking proteins with multivalent ions

As most proteins possess multiple carboxylic groups, using multivalent ions to precipitate proteins is a convenient method to form protein primary particles in aqueous solutions. The growth hormone particles loaded in sustained-release microspheres (a once-commercialized product, Nutropin Depot) are prepared by means of interaction between growth hormone and Zn [2,45]. This is a thermodynamic process by which the Zn^{2+} ions bridge growth hormone together at high concentration but release the protein at low concentration. It is interesting that zinc precipitation was also reported to facilitate protein aggregation when applied to erythropoietin [33]. This protein-dependent effect in Zn^{2+}-induced protein precipitation may be explained in that the ionic complexation leads to a thermodynamically favored state for some proteins but raises structural constraints for others, leading to irreversible aggregation. In addition to protein dependency, irreversible protein aggregation may also be induced depending on types of multivalent ion interacting with a protein. For example, polycationic chitosan may also precipitate proteins but induce deactivation [46].

In addition to protein and ion dependency, the morphology of Zn^{2+}-precipitated protein particles relies strongly on the process of preparation. Directly adding Zn^{2+} ions into an aqueous protein solution leads to flake-like particles of irregular shapes and sizes [47]. Size and shape may in turn affect the performance of the final dosage form in which these particles are encapsulated [48]. To resolve this problem, the authors added Zn^{2+} ions to a mixture of bovine serum albumin and PEG solution, followed by freeze-drying [49]. Once Zn^{2+} ions were added, the protein was immediately precipitated out as indicated by the change of the mixed solution from transparent to cloudy. By washing the lyophilized powder with acetone to remove the PEG continuous phase, spherical protein-Zn^{2+} particles uniform in size (100 nm in diameter) were prepared (**Figure 2**).

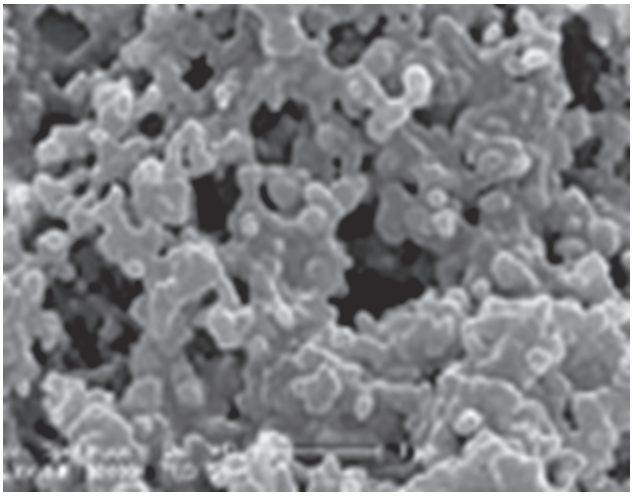

Figure 2. Scaning electron microscope image of BSA–Zn^{2+} particles formed from a solution of PEG of various molecular masses [71].

4.2 Spray drying and spray freeze-drying

Although spray drying is a routine method for preparing hydrophilic pharmaceutical powders for chemical therapeutics, it is less feasible when applied to preparing protein particles because of the exposure of proteins to high temperature, water–air interfaces, and strong shear stress at the spray nozzles [35,50,51]. In addition, the particle yield is low and particle sizes have to be > 2 μm [50]. For example, Webb had reported that spray-dried rhIFN-γ aggregated owing to its fragile conformation is susceptible to the interface of air and liquid [52]. To get rid of these hazardous conditions, an alternative method, 'spray freeze-drying', was proposed. In this process, a co-solution containing a protein and excipients is sprayed immediately into a cold continuous phase to form frozen particles [53]. The frozen particles are then lyophilized to remove water and harvested as light and porous particles for inhalation.

4.3 Dispersing protein-carrying solutions into supercritical fluids

Supercritical fluids (SCF) are liquids formed by compressing a gas to cross its critical pressure of liquidation. The advantage of using SCF as the continuous phase to disperse or dissolve proteins and solidify the so-formed protein droplets is that SCF has a mild interfacial tension with water and can easily be removed by bringing it to normal pressure. Compressed CO_2, as well, used SCF for pharmaceutical applications. Protein particles of desired sizes are formed by nucleation and particle growth of the agent during rapid evaporation of SCF [54-58]. This method has been used to produce particles of anti-CD4 antibody, α1-antitrypsin (AAT) and trypsinogen for its organic solvent-free nature [59].

4.4 Formation of protein particles in aqueous–aqueous phase separation and 'emulsification'

Aqueous solutions containing two polymeric solutes may undergo phase separation to form two immiscible phases owing to reduced entropy and free energy of mixing, ΔS_M and ΔG_M. During the phase separation process, if the dispersed phase is stabilized before fusion into a block phase, an aqueous–aqueous 'emulsion' may be formed [2,6,8]. In this case, if a protein sample is added in this aqueous–aqueous emulsion, it may partition into the dispersed phase preferentially and be dried to particles without contacting water–oil or water–air interfaces. There were three methods reported to stabilize the aqueous dispersed phases: intra-droplet crosslinking; forming a diffuse double layer around each droplet; and stabilizing the dispersed phase by reduced temperature [2,6,8].

For intra-droplet crosslinking, dextran modified with an olefin group was used to form the dispersed phase. After the protein to be encapsulated was partitioned in the dispersed phase, a linker agent reactive to the C=C double bond was added to solidify the dispersed phase with continuous stirring [60]. Although this method enables us to prepare protein solid particles without contacting water–oil or water–air interfaces, proteins with an abundance of functional groups have to be exposed to reactive crosslinking agents.

A comparably milder method is to use a polyelectrolyte (normally an anionic polysaccharide) to form a diffuse double layer to prevent fusion of the dispersed phase [2]. The diffuse double layer could help to maintain the 'emulsion' state of the system for a sufficient period of time during which proteins were partitioned in the dispersed phase and lyophilized to particles under an all-aqueous condition. Depending on the concentration of the polyelectrolyte, glassy, dense particles uniformly sized within 500 – 2000 nm may easily be prepared. However, for some proteins (especially those with ionization point below their storage pH), aggregation may be induced by the polyelectrolyte (**Figure 3**).

Using reduced temperature to stabilize the separated dispersed phase is the mildest approach to form protein particles under all aqueous conditions [61,62]. However, the particle size distribution is larger and less uniform than the polyelectrolyte-assisted method owing to some levels of fusion of the dispersed phase. To improve particle morphology, an alternative version of the temperature-assisted method was developed (temperature-induced phase separation) [2,61-63]. A mixed solution containing a protein and two potentially immiscible hydrophilic polymers was prepared in a concentration below phase separation. This co-solution was then exposed to a continuously dropping temperature to bring it to the two-phase region of its phase diagram. By selecting the polymer concentration precisely, the system may be prepared in such a way that the dispersed phase is frozen immediately after its formation.

Another important advantage of the aqueous–aqueous emulsification method is that the proteins are immobilized in the glassy matrix of hydrophilic polysaccharide and the

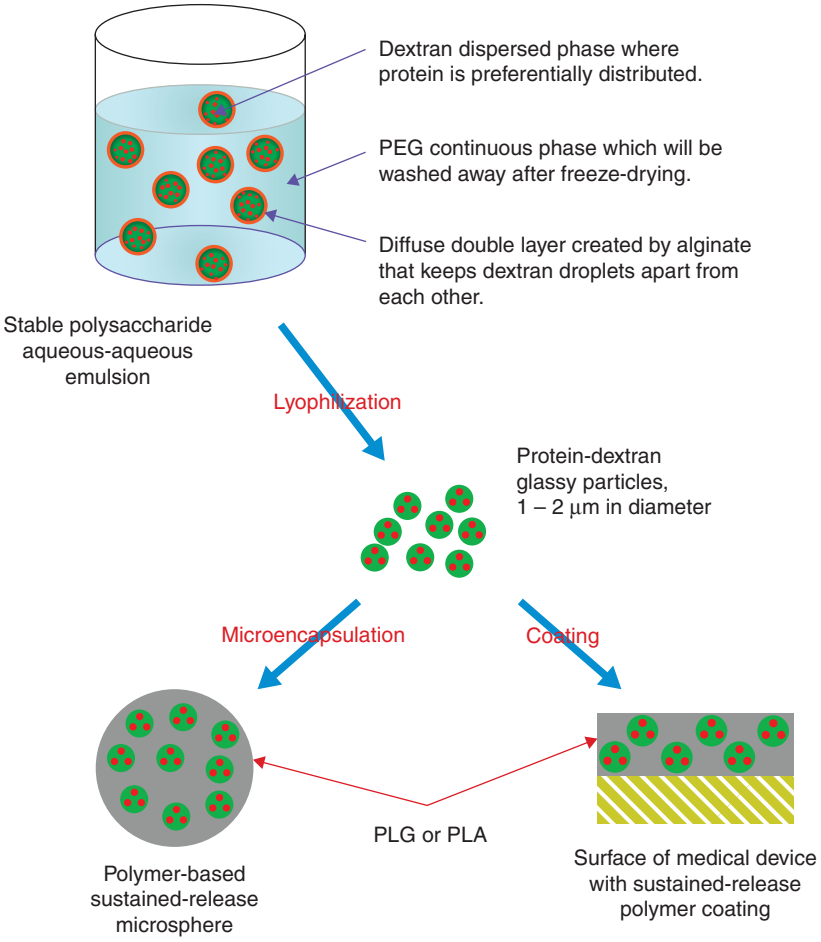

Figure 3. Protein formulation strategy using polysaccharide aqueous–aqueous emulsion.
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ratio of protein to polysaccharide is adjustable. On microencapsulation of these primary particles in composite microspheres for sustained release, the content of polysaccharide is an effective variable to modulate protein release kinetics.

The protein–polysaccharide particles formed by lyophilization of stabilized aqueous–aqueous emulsions are primary particles that are soluble in water and body fluid. These readily soluble protein particles of micrometer sizes may well be feasible for inhalation delivery of proteins, but for internal administrations, further formulation to maintain their particulate form is needed.

4.5 Microspheres of 'core–shell' structure

A method to prevent distributing proteins to the surface region of sustained-release microspheres is to prepare microspheres of 'core–shell' structure. By selecting the ratio of PLA/PLGA, microspheres with a protein-loaded core and a protein-free shell may be formed [8,64,65]. The core–shell microspheres may be formed using both W/O/W and solid-in-oil-in-water (S/O/W) processes and microencapsulation, as well as a water-free method called solid-in-oil-in-oil (S/O/O) process [8,64-66]. As examples, Langer *et al.* reported a multilayer encapsulation method to meet the impulse delivery of vaccines [67]. Pekarek *et al.* used two layers of polymer microspheres to control drug release in 1994 [68]. Yang *et al.* used the W/O/W method with polyorthoester core and PLGA shell [69]. Recently, core–shell nanoparticles have also been reported to be carriers for proteins [70].

In general, the water-free S/O/O method offers better protein loading efficiency by avoiding any protein–water contact. However, application of an oily continuous phase complicates the manufacturing process because of the need for large amounts of solvents to clean up. To improve protein loading efficiency and release kinetics without compromising the environmentally unfriendly process, the authors have tried to microencapsulate pre-formulated protein particles using a unique solid-in-oil-in-hydrophilic 'oil' (S/W/hO) approach. Instead of real oil, a glycerol-based formulation was used as the continuous phase to form core–shell-structured microspheres [71]. This continuous phase does not dissolve the protein-loaded polysaccharide particles, but it is soluble in water and environmentally friendly (**Figure 4**).

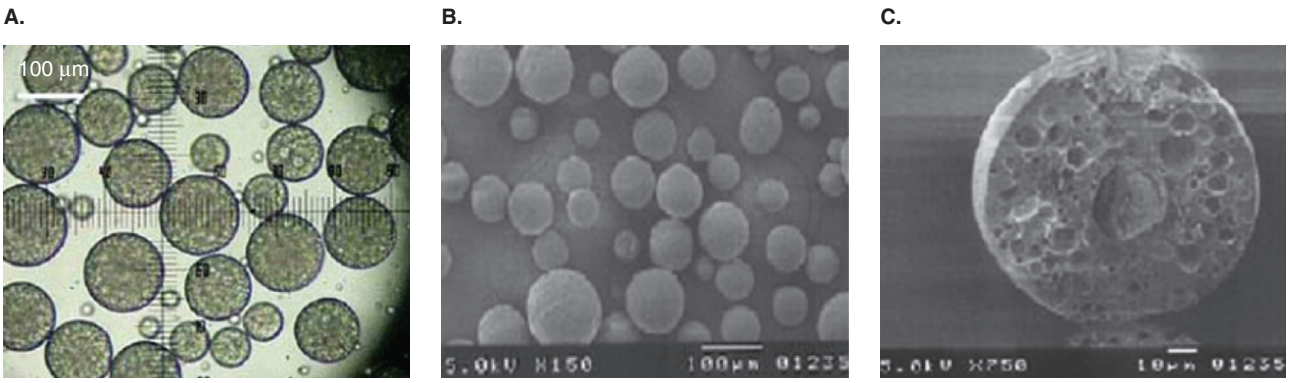

Figure 4. Core–shell microspheres. A. Optical microscope, **B,C.** Scanning electron microscope [71].

Another strategy to reduce burst release is to prepare microspheres of uniform size. By getting rid of the portion of small microspheres, burst release may be reduced substantially [72]. Ma and co-workers reported a unique method to prepare polymeric microspheres by squeezing a protein-loaded polymer solution through a porous membrane of designed pore size [72]. This method may not only regulate microsphere sizes, but also avoid the repeated shearing of the polymer solution in the emulsification-based process of microsphere preparation [72].

4.6 Polymersomes for nanoencapsulation of proteins

To nanoencapsulate proteins of native state, a nanometer-sized particulate system possessing a hydrophilic and protein-friendly interior to store the macromolecules is essential. Polymersome, a bilayer vesicle of hydrophilic/hydrophobic block copolymer, seems to meet this criterion quite well [73]. Its polymeric bilayer is thicker and mechanically stronger than that of liposomes, which allows it to be regarded as a nanoparticle rather than a vesicle. Differing from most nanoparticles consisting of a hydrophobic core, the spherical bilayer of polymersomes is a water-based interior in which proteins may be encapsulated. However, polymersomes reported so far are all formed of block copolymers having a PEG chain as the hydrophilic block. This may limit protein encapsulation into polymersome because most water-soluble proteins tend to get away from PEG (which is why protein PEGylation is used to prevent enzyme binding to the protein). Some researchers reported that protein loading efficiency into polymersomes is as low as 5% [74]. To load proteins into the core of polymersome with sufficient efficiency, an aqueous interior thermodynamically favoring protein partition is needed.

5. Expert opinion

Technologies for encapsulating protein therapeutics into particulate systems are being increasingly demanded owing to the widening of therapeutic areas involving gene recombinant products. Particulate systems for delivering proteins may be divided into three categories, readily soluble particles, slow degrading microspheres and slow degrading nanoparticles. For each of the categories, preserving the protein's native state within the particle matrix and during its forming process is of primary importance and very challenging.

Readily soluble particles are for inhalation delivery of proteins or for protective solidification of proteins in successive formulation steps, and are normally formed with sugars or polysaccharides for their protein-friendly nature. The only formulation challenge for these particles is that a particle-forming process has to be mild by avoiding water–oil or water–air interfaces, reactive agents and extreme pH, temperature or shear stress. In addition, it is better for the process to be simple to scale up and able to ensure sufficient protein encapsulation efficiency as well as good particle morphology and size control. Based on these criteria, the authors' preference for this category of protein particles is for freezing-induced phase separation, aqueous–aqueous emulsion, and PEG-mediated zinc precipitation among the particle-forming processes discussed above. For the materials encapsulating and protecting proteins in these particles, polysaccharides are better than small molecular sugars for better protective effect, propensity to be amorphous, mild dissolution rate and lower osmotic pressure generated by dissolution. As long as a polysaccharide matrix remains in the glassy state, proteins loaded in it gain considerable resistance to temperature and moisture (or even are stabilized by moisture). Although polysaccharides may not be good lyophilizing protectors for some proteins, adding small portion of sugars (such as trehalose) may help.

For slowly degrading microspheres, especially those for sustained-release purposes, composite microspheres wherein proteins are protected in readily soluble dispersed particles are a better choice for good balance between protein protection and sustained-release kinetics. A well-selected protein-stabilizing material dispersed in the microsphere matrix will not induce burst release of proteins, but might even improve the release profiles for minimal burst and incomplete release

by reducing the portion of aggregated and adsorbed (to the polymer) proteins. To improve release kinetics and protein encapsulation efficiency of composite microspheres, the diameters of the inner particles should be 20 times less than those of the microspheres. A steady and long-lasting release profile is the main advantage over the *in situ* gelling depots, which are much easier to formulate, but associated with initial burst.

For nanoencapsulation of proteins, having these macromolecules loaded in a protein-friendly interior with sufficient efficiency is essential but highly challenging. The idea of the composite microspheres is no longer feasible because the sizes of the particles to be dispersed in the matrix of a nanoparticle are comparable to the sizes of the protein molecules to be encapsulated. Therefore, the hydrophilic protein-friendly interior has to be created during the process for assembling the nanoparticle. Moreover, the nanoparticle-assembling process should not denature proteins but facilitate them to be encapsulated in the hydrophilic interior. The safety of these systems/materials and biological application of the above described delivery systems with a summary is shown in **Table 1**. A reasonable strategy to achieve this goal is to design a process that not only creates the protein-friendly interior, but also involves preferential partition for proteins to be distributed into the hydrophilic interior. The simple equation $\Delta G = -RT\ln K_p$ (where K_p is partition coefficient) suggests that a nanoparticle-forming process that facilitates protein nanoencapsulation yet stabilizes proteins at the same time is feasible and preferable.

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Declaration of interest

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