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Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers

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Abstract

Sustained release of pharmaceutical proteins from biocompatible polymers offers new opportunities in the treatment and prevention of disease. The manufacturing of such sustained-release dosage forms, and also the release from them, can impose substantial stresses on the chemical integrity and native, three-dimensional structure of proteins. Recently, novel strategies have been developed towards elucidation and amelioration of these stresses. Non-invasive technologies have been implemented to investigate the complex destabilization pathways that can occur. Such insights allow for rational approaches to protect proteins upon encapsulation and release from bioerodible systems. Stabilization of proteins when utilizing the most commonly employed procedure, the water-in-oil-in-water (w/o/w) double emulsion technique, requires approaches that are based mainly on either increasing the thermodynamic stability of the protein or preventing contact of the protein with the destabilizing agent (e.g. the water/oil interface) by use of various additives. However, protein stability is still often problematic when using the w/o/w technique, and thus alternative methods have become increasingly popular. These methods, such as the solid-in-oil-in-oil (s/o/o) and solid-in-oil-in-water (s/o/w) techniques, are based on the suspension of dry protein powders in an anhydrous organic solvent. It has become apparent that protein structure in the organic phase is stabilized because the protein is ''rigidified'' and therefore unfolding and large protein structural perturbations are kinetically prohibited. This review focuses on strategies leading to the stabilization of protein structure when employing these different encapsulation procedures.

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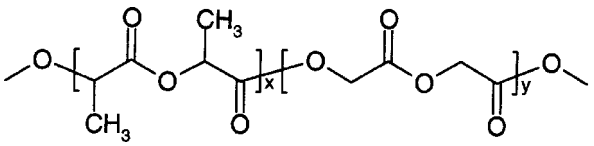
Introduction

Sustained-release of pharmaceutical proteins encapsulated in biodegradable polymers is a promising technology to prevent and cure disease (Johnson & Tracy 1999; Langer 2000). Among the variety of biodegradable polymers evaluated for this purpose, poly(lactide-co-glycolide) (PLG) is the most widely employed, and thus will be the focus herein. The current review outlines recently developed strategies to stabilize protein structure upon encapsulation in PLG and delivery from such polymer devices. These strategies rely on non-invasive monitoring of protein structure by Fourier-transform infrared (FTIR) spectroscopy. The utility of FTIR investigation of protein structure in PLG delivery devices has been extensively covered in recent reviews (Griebenow et al 1999a; Van de Weert et al 2000a).



Scheme 1 Structure of poly(lactide-co-glycolide): x, lactide; and y, glycolide copolymer.

Protein drugs

Understanding disease at the molecular level, notably due to major advances in molecular and cell biology, has made available many new drug candidates and targets. Advances in recombinant DNA technology now allow for large-scale production of pharmaceutical proteins. This facilitates drug development in broad applications not limited to small target groups. Numerous protein and peptide pharmaceuticals have already received approval from regulatory authorities worldwide, and several hundred more are in clinical trials.

Even so, there are substantial difficulties in employing protein drugs. Despite initiatives to deliver proteins non-invasively (Brange 1997), the use of protein pharmaceuticals is restricted by the fact that patient-convenient routes of administration (e.g. oral) result in substantial degradation and poor bioavailability (Wang 1999). Therefore, parenteral delivery is required. Proteins often exhibit short half-lives in serum, thus requiring frequent administration to maintain therapeutic levels. Compliance with such inconvenient dosing can be an issue, particularly when a prolonged treatment regimen is required.

Formulation of protein pharmaceuticals for long-term storage is challenging, largely due to their inherent physical (i.e. conformational) and chemical instability (Wang 1999). Unlike low-molecular-weight compounds, proteins have a three-dimensional structure required to maintain their activity. In addition, many of the amino acid residues comprising the protein backbone are susceptible to chemical degradation (Lai & Topp 1999; Wang 1999). These multiple degradation pathways must be minimized during processing to commercially develop a protein formulation with suitable integrity and shelf-life (Tracy 1998; Wang 1999, 2000).

Advantages and challenges of protein delivery from biocompatible polymers

A possible solution to some of the aforementioned problems is the controlled delivery of proteins from biodegradable and biocompatible polymers (Cleland & Langer 1994; Langer 1995, 2000; Kuo & Saltzman 1996), such as the PLG family of polymers (Jain et al 1998) (Scheme 1). For decades, PLGs have been used widely in medical devices such as sutures. Encapsulation in these materials for sustained release provides advantages over conventional therapeutic approaches e.g. frequent injections. They have the potential to: deliver their active component for a sustained period; target specific tissues; extend the half-life of the drug; and enhance its in-vivo stability (Langer 1995). In addition, patient compliance and comfort may be improved with the development of sustained-release protein injectables, since regular invasive doses can be avoided. Development of "one shot" immunization, e.g. sustained release of tetanus toxoid (Hanes et al 1997), would be particularly valuable. Success in this endeavour would boost efforts by the World Health Organization (WHO) to achieve high levels of vaccination in countries where frequent medical attendance is still a very serious problem.

Despite the promise discussed above, the success of protein delivery from bioerodible polymers has been limited thus far. This is primarily due to the relatively fragile physicochemical nature of proteins. For instance, consider protein encapsulation into PLG microspheres by the most commonly employed method, the water-in-oil-in-water (w/o/w, or double emulsion technique) (Figure 1). An aqueous protein solution is introduced into an organic solvent containing the dissolved polymer. After sonication or homogenization, the resulting emulsion is quickly transferred into a second aqueous phase containing an emulsifier, followed by polymer hardening, microsphere collection, washing, and lyophilization.

Many of the steps in this process are reported to cause detrimental protein structural perturbations and, in particular, the formation of the first emulsion is suspicious in this context (Sah 1999a, b, c; Van de Weert et al 2000b; Pérez & Griebenow 2001). These works have shown that because proteins are surface active they tend to adsorb at water/organic solvent interfaces. This adsorption causes protein unfolding, inactivation, and irreversible aggregation in the first emulsion step. Mechanical forces employed in the creation of the emulsion might also cause protein structural perturbations, which often result in irreversible aggregation (Meinel et al 2001). Also, many proteins adsorb to hydrophobic polymers, such as PLG, with similar consequences as the absorption to hydrophobic interfaces (for details, see below). Lastly, microspheres are usually dehydrated to prolong storage stability, commonly achieved by freeze-drying (Wang 2000). This is important since it has been shown that dehydration may cause reversible or irreversible change in protein structure (Prestrelski et

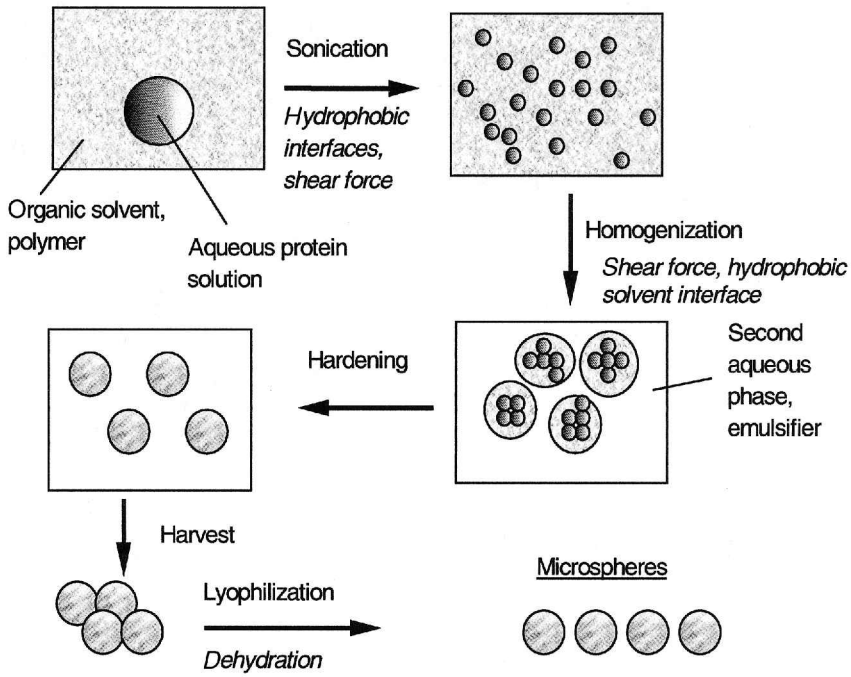


Figure 1 Schematic presentation of the encapsulation of a protein into PLG microspheres following a typical w/o/w protocol.

al 1993; Desai & Klibanov 1995; Dong et al 1995; Griebenow & Klibanov 1995; Carpenter et al 1998). Even for proteins where these structural perturbations are completely reversible upon re-dissolving the powders in aqueous buffer (Griebenow & Klibanov 1995), irreversible solid-phase aggregation frequently occurs under pharmaceutically relevant conditions (Costantino et al 1994a, b). Procedure-induced adsorption or aggregation has been implicated in incomplete delivery of proteins from polymeric delivery systems (Morlock et al 1997, 1998; Crotts & Park 1998; Kim & Park 1999; Van de Weert et al 2000a, b).

Interface-induced protein inactivation and aggregation in the w/o/w technique

The w/o/w technique has been widely tested for the encapsulation of therapeutic proteins and peptides and vaccine antigens. The main reason for this is that this technique is straightforward, unlike the non-aqueous procedures discussed later. However, protein inactivation and aggregation at the water/organic (e.g. water/CH_2Cl_2) interface (first emulsification step) is one of the most detrimental events hindering the encapsulation of structurally unperturbed proteins into PLG microspheres (Morlock et al 1997, 1998; Crotts & Park 1998; Kim & Park 1999; Sah 1999a, b, c; Van de Weert et al 2000b; Pérez & Griebenow 2001). The formation of hydrophobic interfaces (an important disadvantage of this technique) generally results in interfacial adsorption followed by protein unfolding and aggregation. A schematic of these events is presented in Figure 2.

It has been shown for various proteins such as lysozyme, ovalbumin, and bovine serum albumin (BSA) that exposure to the water/CH_2Cl_2 interface caused aggregation. For lysozyme, the aggregates formed are non-covalent in nature and have intermolecular β-sheet structure (Van de Weert et al 2000b), compatible with the notion of unfolding at the interface followed by aggregation. In addition, activity loss occurred for soluble, non-aggregated lysozyme (Van de Weert et al 2000b; Pérez & Griebenow 2001). FTIR spectroscopic data suggested that this was due to irreversible formation of an unfolded lysozyme species (denoted U* in Figure 2) that was able to dissociate from the interface and accumulate in the aqueous phase (Pérez & Griebenow 2001).

Amelioration of protein unfolding and aggregation at the o/w interface

Since proteins may unfold and aggregate at the o/w interface, a straightforward strategy towards stabilization is to minimize exposure to this interface. One approach is to increase protein concentration while

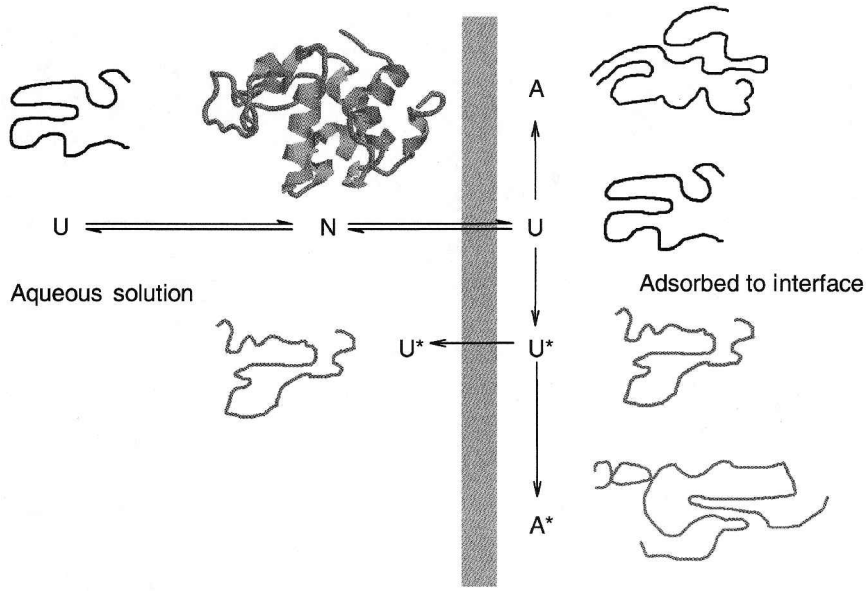


Figure 2 Schematic of conformational pathways of protein in the presence of interfaces. Native lysozyme (N) adsorbs to the interface and unfolds (U). The unfolded species either aggregates (A) or is transformed into a stable unfolded and inactive form (U*) that is able to leave the interface. U* can also aggregate (modified after Pérez & Griebenow (2001)).

maintaining the interfacial area. Upon increasing the protein concentration during emulsification, a self-protecting effect has been observed for carbonic anhydrase and BSA (Lu & Park 1995), hen egg-white lysozyme (Pérez & Griebenow 2001), and recombinant human growth hormone (rhGH) (Cleland & Jones 1996). For insulin-like growth factor encapsulation, protein concentration was increased to form a gel, which resulted in good stabilization upon encapsulation in and release from PLG microspheres made using a w/o/w method (Singh et al 2001). However, such high concentrations may promote protein–protein processes such as aggregation. Also, it is difficult to completely prevent aggregation using this strategy.

A similar approach would be to use an amphipathic excipient, for instance, another protein, which would compete with the therapeutic protein for the interface. For example, interface-induced aggregation has been minimized by adding BSA to carbonic anhydrase (Lu & Park 1995), ovalbumin, and lysozyme (Sah 1999c). However, the "excipient protein" itself might aggregate under these conditions perhaps making this approach unappealing in medical applications where the delivery of any type of potentially immunogenic protein aggregates to patients is best avoided.

Surfactants represent another amphipathic excipient with the potential to compete for interfacial binding and thus decrease protein–interface interactions. In practice, this approach has not been very successful thus far for encapsulation. For instance, Tweens have been show to promote extensive aggregation of rhGH and recombinant human interferon-gamma (rhIFN-γ) during emulsification and microencapsulation (Cleland & Jones 1996). Also, Pluronic F68 did not prevent tetanus toxoid aggregation during emulsification with methylene chloride.

Yet another approach to prevent interface-induced protein denaturation and aggregation is addition of polyol or sugar excipients in the aqueous phase (Cleland & Jones 1996; Pérez & Griebenow 2001). For instance, rhGH was stabilized against interface-induced protein aggregation by mannitol and trehalose, although for rhIFN-γ only trehalose was useful in this regard (Cleland & Jones 1996). It was hypothesized by the authors of that report that trehalose shielded the proteins from the organic solvent via preferential hydration of their surface, thus preventing protein–interface contacts.

It should be noted that the preferential hydration mechanism has been described for relatively high excipient concentrations ($\sim$ 1 M) resulting in improved solution thermostability (for a scholarly discussion of the mechanism of preferential hydration, see Timasheff (1998)). It is uncertain whether this mechanism is also relevant for lower excipient concentrations in the presence of the o/w interface. Recently, it was shown that

excipient conditions found to improve lysozyme unfolding and aggregation at the o/w interface did not improve thermostability, as would be expected from the preferential exclusion hypothesis (Pérez & Griebenow 2001).

An alternative explanation currently favoured by this laboratory relies upon a concept known as protein hydrophilization (Mozhaev et al 1998). According to this scenario, polyols non-covalently interact with proteins in aqueous solution, as reported for lysozyme in the presence of sorbitol (Wimmer et al 1997). This shell of non-covalently bound polyol should make protein–interface contacts less favourable.

It is interesting to note that polyols that are efficient in preventing lysozyme interfacial unfolding and aggregation are also effective lyoprotectants (Pérez & Griebenow, unpublished data). It has been proposed that such lyoprotection is also linked to protein–sugar interactions (i.e. hydrogen bonding) in the solid state. The existence of protein–sugar interactions in the solid state is supported by moisture sorption of solid protein–sugar systems (Sarciaux & Hageman 1997; Costantino et al 1998b). Therefore, it is possible that the mechanisms of preventing lyophilization- and interface-induced denaturation are related.

In addition to polyols, simple inorganic salts represent another excipient class that has been reported to stabilize proteins upon emulsification (Pistel & Kissel 2000). The mechanism of how the salts prevent unfolding and aggregation still has to be elucidated. Preliminary data obtained in our laboratory indicate that it is unlikely that this effect is mediated via preferential exclusion.

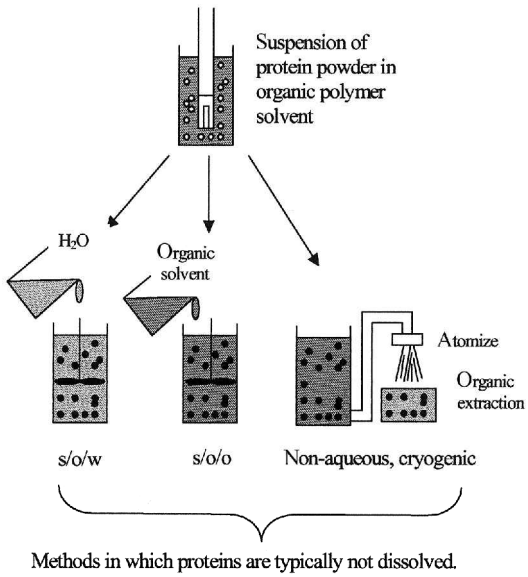


Figure 3 Main methods used to encapsulate solid protein powder formulation into PLG microspheres (adapted from Castellanos et al (2001a)).

Use of non-aqueous encapsulation procedures

Rationale

The various studies discussed above show that some progress has been made in formulation optimization to improve protein stability during classical emulsion-based processing (e.g. w/o/w). Even so, complete retention of native protein following this type of processing remains a formidable challenge. Additional strategies need to be explored to develop commercially viable, sustained-release protein formulations.

To this end, non-aqueous encapsulation approaches have been explored, where the protein is processed as a solid to avoid any oil–water interface. An example is the solid-in-oil-in-oil (s/o/o) encapsulation method (Figure 3). The rationale behind this strategy is that dehydrated protein powders in organic solvents are very "rigid" or restricted in their conformational mobility (Affleck et al 1992; Griebenow & Klibanov 1996; Barron et al 1997; Klibanov 1997). For example, when dehydrated enzyme powders were suspended in organic solvents incapable of protein dissolution, enzymatic activity remained viable for prolonged periods at elevated temperatures (Zaks & Klibanov 1984) because the thermal denaturation temperature was much higher than in aqueous solution (Volkin et al 1991; Griebenow et al 1999b, 2001; Santos et al 2001). Hence, protein conformation is kinetically trapped for solids in organic suspension (Griebenow & Klibanov 1996; Partridge et al 1999). Despite a thermodynamic situation favouring protein unfolding, native structure is maintained since reduced conformational flexibility prohibits large structural alterations.

Encapsulation in the absence of a primary water/methylene chloride interface holds promise for stabilizing proteins. For example, tetanus toxoid was released from PLG microspheres prepared by a s/o/o protocol having a high fraction of antigenically active protein (Tobio & Alonso 1998; Tobio et al 1999a; Schwendeman et al 1998).

Minimization of encapsulation-induced protein structural perturbations and aggregation

Conceptual and technical advances in Fourier-transform infrared (FTIR) analysis of protein powders allows

for monitoring of (secondary) structure under conditions in aqueous solutions, organic suspensions, and in dried solids. It is now established that many dehydration procedures (including lyophilization) cause significant structural alterations in most proteins (Prestrelski et al 1993; Dong et al 1995; Griebenow & Klibanov 1995; Tzannis & Prestrelski 1999). In numerous studies, FTIR spectroscopy has been used to screen and identify excipients capable of preventing such lyophilization-induced structural changes (Prestrelski et al 1993; Griebenow & Klibanov 1995; Carrasquillo et al 1998; Costantino et al 1998a; Arakawa et al 2001). Furthermore, this reduction of structural perturbations by excipients has been shown in cases to improve the storage stability of pharmaceutical proteins, e.g. of rhGH (Costantino et al 1998a).

Similarly, FTIR spectroscopy has also been employed to monitor protein secondary structure at various processing steps in s/o/o encapsulation. For instance, when rhGH and BSA were co-lyophilized with appropriate excipients, native-like secondary structure was maintained upon lyophilization and subsequent suspension and dispersion (i.e. homogenization) of powder in organic solvent containing dissolved PLG (Carrasquillo et al 1998, 1999). Furthermore, it was shown for BSA that co-lyophilization with excipients to minimize structural perturbations resulted in native-like structure throughout s/o/o processing and also reduced the formation of water-soluble aggregates upon encapsulation and initial in-vitro release (Carrasquillo et al 2001a, b). To our knowledge, this level of structural preservation has not been demonstrated thus far for proteins encapsulated using a primary w/o emulsion (e.g. see Fu et al (1999)).

Non-aqueous, cryogenic encapsulation

A non-aqueous, cryogenic methodology has been employed in developing a sustained-release formulation of zinc-complexed rhGH in PLG microspheres (Johnson et al 1997; Bartus et al 1998; Tracy 1998). In this procedure, protein powder was suspended in organic solvent containing dissolved PLG, and microspheres were formed by atomization to form a droplet, followed by freezing in liquid nitrogen, and extraction of organic solvent in a cold organic co-solvent (i.e. ethanol) to remove the polymer solvent. This process resulted in the first microencapsulated protein formulation to realize commercialization.

Interestingly, FTIR spectroscopic studies suggested that protein structure was perturbed for zinc-complexed rhGH in microspheres (Yang et al 1999). This indicated that the mechanism of stabilization of rhGH upon microencapsulation and release was not related to protein structure in the solid state. In this case, it was likely that stabilization was facilitated by the extremely cold processing temperatures, lack of any potentially destabilizing interface during encapsulation, and restriction in protein mobility due to interaction with the zinc salt. Data has suggested that zinc-complexed BSA had a perturbed structure, even though it was stabilized upon non-aqueous, cryogenic encapsulation (Costantino et al 2000). It is important to note that the dehydration-induced structural perturbation for rhGH and BSA (and many other proteins as well) is reversible in nature.

Recently, this non-aqueous, cryogenic encapsulation process has been reported to stabilize other pharmaceutical proteins such as recombinant human insulin-like growth factor-I (rhIGF-I) (Lam et al 2000), vascular endothelial growth factor (rhVEGF) (Cleland et al 2001), and nerve growth factor (rhNGF) (Lam et al 2001). rhIGF-I formulated without excipients in succinate buffer was stabilized upon encapsulation (and release). The optimal stabilization of rhVEGF upon encapsulation and release was reported at a low level of trehalose in the formulation i.e. 1:10 sugar:protein (w:w) (Cleland et al 2001). For rhNGF, trehalose was also used for stabilization, at a weight ratio of 1:1 sugar:protein (w:w). Although this approach was successful in stabilizing rhNGF upon processing for encapsulation, during in-vitro release from the PLG microspheres, there was a loss in native protein (native dimer) and a substantial loss in biological activity. When rhNGF was complexed with zinc before spray-freeze drying, there was only slight loss in native protein upon encapsulation and release. The same strategy of zinc-complexation was successfully employed for encapsulation of rhGH (discussed above). For rhGH and rhNGF, binding with zinc did not substantially alter the protein structure (Yang et al 2000).

Current issues in non-aqueous encapsulation procedures

Until recently, there were few studies exploring non-aqueous procedures for encapsulating proteins in PLG microspheres or other delivery systems. Thomasin et al (1998) highlighted the issues remaining to be explored in this area: "Further, the unique physicochemical and stability properties of peptides and proteins and especially their interactions with polymer solvents and coacervating and hardening agents will remain a critical parameter to be clarified in all coacervation processes".

One issue encountered when encapsulating proteins into microspheres by s/o/o techniques is the significant initial release of protein (Schwendeman et al 1998; Tobio

et al 1999b; Carrasquillo et al 2001a). This initial release is due to protein particles that have access to the solvent upon hydration, such as those on the microsphere surface, or with ability to diffuse there via pores.

One solution to minimize the initial release is to decrease the size of the protein powder particles relative to the microsphere diameter to reduce surface-exposed protein. A technique ideally suited to produce small powder particles is spray freeze-drying (Costantino et al 2000). This procedure results in very porous, structurally fragile protein powders that are capable of disintegration into sub-micron-sized particles upon sonication or homogenization. Employment of such an approach has been demonstrated to reduce initial release following microencapsulation (Costantino et al 2000).

Reduction in protein particle size alone is not sufficient to ensure a lowered initial release. For example, this approach alone was not successful in yielding a low initial release for encapsulation using PLG with low M_w (Carrasquillo et al 2001b). This was likely due to the very small microsphere diameter obtained for such conditions. Carrasquillo et al (2001b) found that addition of a poloxamer to the PLG was efficient in reducing initial release (this approach also has utility in preparing protein microspheres by the w/o/w technique (Yeh et al 1996)). In this case, there was an increase in microsphere diameter such that the ratio of protein particle size to the microsphere decreased, which promoted reduction in initial release as discussed above.

Another issue in s/o/o processing (and to various extents for other encapsulation methods) is the encapsulation of unwanted materials: the polymer solvent (e.g. methylene chloride), the hardening agent (e.g. silicon oil), and the quench solvent (e.g. heptane). For instance, methylene chloride is denoted according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines as a Class 2 solvent with a permitted daily exposure of 6 mg in a pharmaceutical product. Also, there are Occupational Safety Hazard Agency (OSHA) guidelines for exposure to workers upon processing (Sherman et al 1998). The use of alternative solvents in s/o/o procedures has thus been a focus of some research (Thomasin et al 1996).

Protein encapsulation by solid-in-oil-in-water (s/o/w) emulsion techniques

Rationale

A "compromise" between completely non-aqueous methods and the w/o/w technique exists in the s/o/w technique. In this method, the dehydrated protein powder is suspended in an organic solvent containing the dissolved polymer. This suspension is emulsified in an aqueous solution containing an emulsifier agent. Microspheres are then hardened by dissolving or evaporating the organic solvent, after which they are finally washed and lyophilized.

While the initial step still employs protein powders suspended in the organic solvent, the microsphere formation and hardening is performed in aqueous emulsion. This method may be suitable for protein drug encapsulation since there is no potentially destabilizing primary w/o emulsion. This technique also avoids the presence of additional hardening agents and quench solvents during processing.

Cleland & Jones (1996) reported that rhGH lyophilized with either trehalose or mannitol was released from PLG microspheres fully bioactive when it was encapsulated by a s/o/w technique. Even though the protein encounters an aqueous environment in the presence of the organic solvent during the o/w emulsion step, the time for polymer hardening is rapid and does not allow for dissolution or denaturation. In agreement with this view, Castellanos et al (2001a) showed that the encapsulation-induced structural perturbations for non-stabilized BSA using the s/o/w method were reduced compared with those observed when employing the w/o/w technique (Fu et al 1999). King & Patrick (2000) reported the successful development of microspheres loaded with biologically active vascular endothelial growth factor using the s/o/w technique while the w/o/w method yielded inactive hormone. Finally, Morita et al (2000) reported the encapsulation of horseradish peroxidase without significant loss of activity using this method.

Current issues in s/o/w processing

A major problem in s/o/w process is the low efficiencies normally observed when hydrophilic drugs are encapsulated (Atkins 1997; King & Patrick 2000; Pérez et al 2000; Castellanos et al 2001a). This results from partitioning of the protein from the inner oil phase suspension to dissolution in the outer aqueous phase in the o/w emulsion step.

It has been shown that optimizing the process conditions can minimize this loss of protein (Maa & Hsu 1997; Morita et al 2000; Castellanos et al 2001a, b). In particular, reduction in the protein powder particle size has been shown to be critical. This can occur before encapsulation (Maa & Hsu 1997), during the o/w emulsification step (Castellanos et al 2001a), or by the

use of excipients that dissolve in the organic solvent and in this manner afford micronization of the protein powder in the suspension step (Morita et al 2000). These strategies can dramatically improve the protein loading efficiency from less than 20% to over 80%.

Another issue in s/o/w processing is the loss of excipients from the protein-containing solid phase. These excipients are typically of low molecular weight (e.g. trehalose) and hydrophilic. They can be lost to the outer aqueous phase (as can occur for the protein as discussed above), or can be stripped from the protein-containing solid phase and later trapped in the polymer matrix during the hardening process. In this way, the excipient may no longer be available to afford protein stabilization during processing, particularly the final drying step.

There is experimental evidence showing this excipient loss and its consequences for s/o/w processing. For example, Castellanos et al (2001b) have investigated a dried BSA/trehalose formulation encapsulated using the s/o/w process. It was shown that trehalose was largely lost into the aqueous phase in the o/w step during encapsulation of BSA using this method. Consequently, there were structural perturbations of the protein upon encapsulation due to its exposure to water in the o/w step followed by dehydration. Two strategies were tested to prevent the loss of excipient during s/o/w processing. The first was saturation of the aqueous phase in the o/w step with the excipient. The second was choosing a solvent with low water solubility (namely chloroform) that could minimize the contacts of the protein and excipient with water. These strategies resulted in encapsulation of BSA without procedure-induced structural perturbations and reduction in soluble aggregate formation upon in-vitro release.

Protein degradation during release

Preservation of protein structure during encapsulation alone is not sufficient to develop a successful sustained-release formulation. It is required that the protein retains its native form upon release from the system. Most studies in this area to date have focused on chemical stability, and relatively few studies have probed the question of protein structure during release from polymeric matrices.

Changes of pH in the microspheres during release

The degradation products of PLG are acidic in nature, which can lead to a decrease in the pH-value within the microsphere and/or the release medium. This drop in pH is a potential source for unfolding and irreversible inactivation of encapsulated proteins. The work thus far (as discussed below) has largely focused on the chemical, rather than conformational, consequences.

In an early study, Lu & Park (1995) showed that the pH-value of the release medium dropped from 7.4 to around 3 after two months of in-vitro incubation of PLG microspheres obtained using a w/o/w method, resulting in hydrolysis of BSA and carbonic anhydrase. Later studies have supported this effect. Igartua et al (1998) fabricated BSA-loaded microspheres using the w/o/w process and showed that after one week of incubation the released protein started to hydrolyse into smaller fragments. Chen et al (1997) encapsulated interleukin-1 alpha using different PLGs with different molecular weights. They found that in slowly degrading microspheres (higher polymer molecular weight) the accumulation of acidic degradation products was slower, allowing for their diffusion out of the microspheres before a significant drop in pH occurred. Zhu & Schwendeman (2000) and Zhu et al (2000) investigated BSA instability when encapsulated in PLG microspheres and rods. Their results suggested that an acidic pH (< 3) triggered unfolding of encapsulated BSA, resulting in peptide bond hydrolysis and non-covalent aggregation.

Recently, studies have been focused in the development and application of methods that allow for the non-invasive assessment of the pH environment inside PLG microspheres during release. For example, the development of pH-sensitive nitroxides has permitted the non-invasive pH-measurement by electron paramagnetic resonance (EPR). Brunner et al (1999) reported that the pH in PLG microspheres was less than 4.7 after 14 days even though the release buffer was replaced daily. Shenderova et al (1999) assessed the pH in microspheres during release using two techniques. The first was measurement of H^+ content of the dissolved microspheres in an 80:20 acetonitrile/H_2O mixture. The second was confocal microscopy of an encapsulated pH-sensitive dye, fluorescein. The results showed that the interior of the microspheres had a pH of 2, even though the buffer was changed every three to four days. Similarly, Fu et al (2000) showed with pH-sensitive fluorescence dyes bound to dextrans that the pH-value in PLG microspheres obtained by a w/o/w method can drop to a comparable extent.

One strategy to ameliorate pH decrease within microspheres upon release is to co-encapsulate a basic salt. To this end, inorganic salt hydroxides have been explored for stabilization of BSA released from PLG microspheres. For instance, it was shown that co-encapsulation of $Mg(OH)_2$ and BSA in PLG matrices

was successful in imparting stabilization (Zhu & Schwendeman 2000; Zhu et al 2000).

Another approach to ameliorate the potential pH decrease within microspheres is to create porosity within the microsphere. This would allow for acidic biodegradation products to diffuse out of the microspheres, and also allow for buffer components in the release media to permeate within. For example, Burke (1996) has shown for microspheres made using a cryogenic, non-aqueous process that there was not a significant pH decrease within PLG microspheres, as determined by ^{31}P NMR spectroscopy. In this case, it was hypothesized that this particular microsphere manufacturing method yielded a more porous microsphere compared with techniques relying on emulsion formation.

Moisture-induced protein aggregation in microspheres

Another factor leading to potential deterioration of proteins upon release from microspheres is exposure to moisture. Moisture in solid proteins can lead to irreversible protein aggregation (Costantino et al 1994b, 1998c). The presence of water increases protein molecular mobility, and thus accelerates other deleterious events in solid proteins, such as deamidation (Lai & Topp 1999).

A typical mechanism for moisture-induced aggregation of solid proteins is thiol–disulphide interchange, requiring the accessibility of free thiols and disulphide groups (Liu et al 1991; Costantino 1995). The accessibility of these groups may be increased by encapsulation-induced protein structural perturbations. If conditions within the microsphere were acidic (see above), then thiol groups would likely be protonated, prohibiting thiol–disulphide interchange. However, process-related protein unfolding and formation of non-covalent aggregates might still occur in the wetted state.

If the mechanism of moisture-induced aggregation is known, then one can devise a rational strategy to promote stabilization (Costantino et al 1994b, 1998c). Perhaps a general strategy would be to increase polymer hydrophobicity, which limits the amount of moisture sorbed in the microsphere, thus promoting stabilization. Surface-erosion (as opposed to bulk erosion) might have an advantage in that water would theoretically be excluded from the encapsulated protein until the moment of release. Poly(ortho esters) are a potential biodegradable matrix in this regard (Heller 1985; Heller & Gurny 1999; Ng et al 2000).

Adsorption of proteins to polymers

Incomplete release of proteins from PLG microspheres in-vitro has been reported to be associated with non-specific adsorption of proteins on the polymer by hydrophobic (Tsai et al 1996; Park et al 1998; Butler et al 1999; Kim & Park 1999) and ionic interactions (Blanco & Alonso 1997; Park et al 1998). Still, it is unknown whether such interaction between protein and PLG affect the release profile in-vivo, as the component of biological fluids may compete with the protein.

Park et al (1998), Kim & Park (1999) and Nam & Park (1999) showed that the main cause for non-release behaviour of lysozyme from PLG microspheres was non-specific adsorption onto the polymer matrix by hydrophobic and ionic interactions. When they incubated blank microspheres with native lysozyme, a decrease in protein concentration was observed suggesting lysozyme adsorption to the microspheres. Three different media were used to differentiate the types of interactions of protein with PLG. They showed that lysozyme underwent non-specific adsorption and non-covalent aggregation and suggested that it was mainly controlled by electrostatic interactions between lysozyme and free carboxyl end groups of PLG chains. Similarly, incomplete release of BSA and rhGH were associated with protein adsorption to PLG, in addition to formation of non-covalent aggregates.

Butler et al (1999) measured the kinetics of BSA adsorption to films of PLG with different end groups (hydrophilic carboxylic and hydrophobic ester). BSA adsorbed readily to both. Ageing of the films in water, thus decreasing the hydrophobicity, correlated with a significant decrease in the initial BSA adsorption. This is consistent with the expected effect of surface hydrophobicity on protein adsorption (Bouillot et al 1999). Thus, the PLG type used can influence the extent of polymer–protein interactions. However, no effect of the PLG end group hydrophobicity was found for the integrity of encapsulated and released rhGH, likely due to the fact that in this case rhGH was encapsulated as a zinc-complex (Johnson et al 1997).

Johansen et al (1998) showed that incomplete in-vitro release of tetanus toxoid from microspheres was partly due to adsorption to PLG, and they also found some adsorption to the glass vials used for in-vitro release testing. This result illustrates the caveats in using in-vitro release for characterizing sustained-release protein formulations. Specifically, the result depends not only on the formulation, but also on how the in-vitro release test is conducted. Interestingly, it was shown that addition of BSA to the release media significantly reduced adsorption of tetanus toxoid to the polymer and/or glass surface, and resulted in a more complete tetanus toxoid release in-vitro. Similarly, co-encapsulation of tetanus toxoid and BSA in the same microsphere

formulation resulted in a more complete tetanus toxoid release in-vitro.

Encapsulation of protein–zinc complex

Finally, there have been some encouraging reports regarding stabilizing of zinc-complexed proteins released from microspheres. As discussed above, a non-aqueous, cryogenic process has been developed for the encapsulation of zinc-complexed rhGH (Johnson et al 1997; Bartus et al 1998; Tracy 1998). For this system, the protein is stabilized upon encapsulation and released in its native form. Furthermore, Lam et al (2001) have reported that rhNGF is stabilized upon release from PLG microspheres when the protein was encapsulated as a zinc-complex. This effect was apparently not related to conformation stabilization (see discussion above). In this case, stabilization may be linked to reduced interface and mobility. Even in cases where binding does not occur, there may be an advantage since zinc salts have been hypothesized to slow the hydration of PLG microspheres (Johnson et al 1997; Lam et al 2000).

Summary and future directions

This review has documented recent advances in the stabilization of proteins microencapsulated within biodegradable (e.g. PLG) microspheres. A particular focus has been placed on structural investigations related to destabilization pathways relevant for encapsulation and release. Particular success has been achieved by using encapsulation techniques that employ dehydrated protein powders in initial stages of the encapsulation procedures, thus avoiding the main draw-back of the w/o/w method, the exposure of dissolved protein to an aqueous–organic interface. As a result, some proteins can be successfully encapsulated and delivered with a native structure.

Continued work is necessary to improve our understanding of protein stabilization upon encapsulation. For instance, studies are required to generalize the positive effects of using excipients to stabilize proteins upon encapsulation using the s/o/o and s/o/w techniques. Particular emphasis should be given to addressing long-term storage stability, demonstrating the applicability to other, therapeutic proteins, and establishing benefits in biological activity when protein structural perturbations are minimized. The employment of alternative polymer solvent systems should be given more attention as well.

The effect of release conditions on protein stability within microspheres deserves further scrutiny also. These studies are ideally conducted with protein already stabilized upon encapsulation. Acid hydrolysis products due to PLG degradation may result in protein deterioration. Combining the structure-guided encapsulation approach (Carrasquillo et al 1998, 1999, 2001a, b; Castellanos et al 2001a, b) with the stabilization approach introduced by Zhu et al (2000), Shenderova et al (1999) and Zhu & Schwendeman (2000) should remedy this problem, but such investigations are still not performed. Another interesting direction would be to study the encapsulation and release of proteins that have been chemically modified, e.g. PEGylated (Diwan & Park 2001). In addition, polymers producing non-acidic breakdown products (e.g. poly ε-caprolactone (Lin et al 2001)) may prove useful and it would be interesting to extend the structure-guided encapsulation approach to these materials. The stability of dry protein powders in PLG microspheres exposed to humidity (to mimic release conditions) has not been well reported so far. In addition, in-vitro–in-vivo correlation development would be helpful in devising systems to overcome in-vitro assay limitations. Lastly, studies geared towards elucidating the mechanism of protein adsorption onto PLG surfaces would be helpful.

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