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Review Article

Protein Instability in Poly(Lactic-co-Glycolic Acid) Microparticles

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In this review the current knowledge of protein degradation during preparation, storage and release from poly(lactic-co-glycolic acid) (PLGA) microparticles is described, as well as stabilization approaches. Although we have focussed on PLGA microparticles, the degradation processes and mechanisms described here are valid for many other polymeric release systems. Optimized process conditions as well as stabilizing excipients need to be used to counteract several stress factors that compromise the integrity of protein structure during preparation, storage, and release. The use of various stabilization approaches has rendered some success in increasing protein stability, but, still, full preservation of the native protein structure remains a major challenge in the formulation of protein-loaded PLGA microparticles.

KEY WORDS: controlled release; microparticles; PLGA; protein stability; stabilization.

INTRODUCTION

Biodegradable polymers, especially in the form of injectable microparticles, have been investigated extensively for their capability of releasing therapeutically useful proteins in a controlled way (1–3). Microparticulate protein release systems, such as microspheres, microcapsules, as well as nanospheres, are generally designed to achieve either sustained (preferably zero-order) or pulsed release over a prolonged period of time, typically weeks or months. The purpose of sustained release is to achieve sustained therapeutic plasma levels of protein drugs with a short half-life, such as human growth hormone (4–10) and cytokines (4,6,11–13). To mimic conventional repetitive vaccination schedules with a single injection, pulsed release is aimed at for vaccine components, such as bacterial toxoids (14–19) and viral antigens (6,20,21).

Among the biodegradable polymers suitable for preparing injectable protein-loaded microparticles, most experience has been gained with copolymers of D,L-lactic and glycolic acid (poly(D,L-lactic-co-glycolic acid), PLGA). These copolymers degrade by bulk erosion through hydrolysis of the ester bonds, the rate of which depends, amongst others, on the lactic acid/glycolic acid ratio, molecular weight, and microparticle porosity (3). Thus, the degradation rate can be tailored according to the desired release pattern of the protein to be incorporated. The popularity of these biocompatible copolymers can be ascribed in part to their approval by the FDA for use in humans and their success as biodegradable sutures.

In the past decade, numerous papers have appeared on the successful incorporation of proteins in PLGA microparticles with respect to loading and encapsulation efficiency as well as microparticle size and morphology. However, in the past few years incomplete release of native protein as a result of protein instability has become recognized as a major problem. Denatured or aggregated protein species will not only be therapeutically inactive, but also may cause unpredictable side effects, such as immunogenicity or toxicity (22). Even when the recovery of native protein is high, it is the (low) amount of degraded protein that may pose problems. Thus, optimization of protein-loaded PLGA microparticle production processes should be primarily focussed on full preservation of the native protein structure during preparation, storage, and release. The present article reviews the recent literature regarding the identification of protein degradation mechanisms and approaches to minimize protein degradation during these stages.

PROTEIN INSTABILITY DURING MANUFACTURE

Figure 1 summarizes critical steps in the most common preparation protocols for protein-loaded PLGA microparticles. The process can be divided into three steps: protein loading, microparticle formation, and drying. The starting

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ABREVIATIONS: BSA, bovine serum albumin; CA, carbonic anhydrase; DMSO, dimethyl sulfoxide; DTd, diphtheria toxoid; ESCA, electron spectroscopy for chemical analysis; Etac, ethyl acetate; FTIR, Fourier transform infrared spectroscopy; GdnHCl, guanidinium hydrochloride; HPβCD, hydroxypropyl β-cyclodextrin; HSA, human serum albumin; MC, methylene chloride; NMR, nuclear magnetic resonance; PLGA, poly(D,L-lactic-co-glycolic acid); rhEPO, recombinant human erythropoietin; rhGH, recombinant human growth hormone; rhIFNγ, recombinant human interferon-gamma; SDS, sodium dodecyl sulfate; TNF-α, tumor necrosis factor-alpha; TTd, tetanus toxoid.

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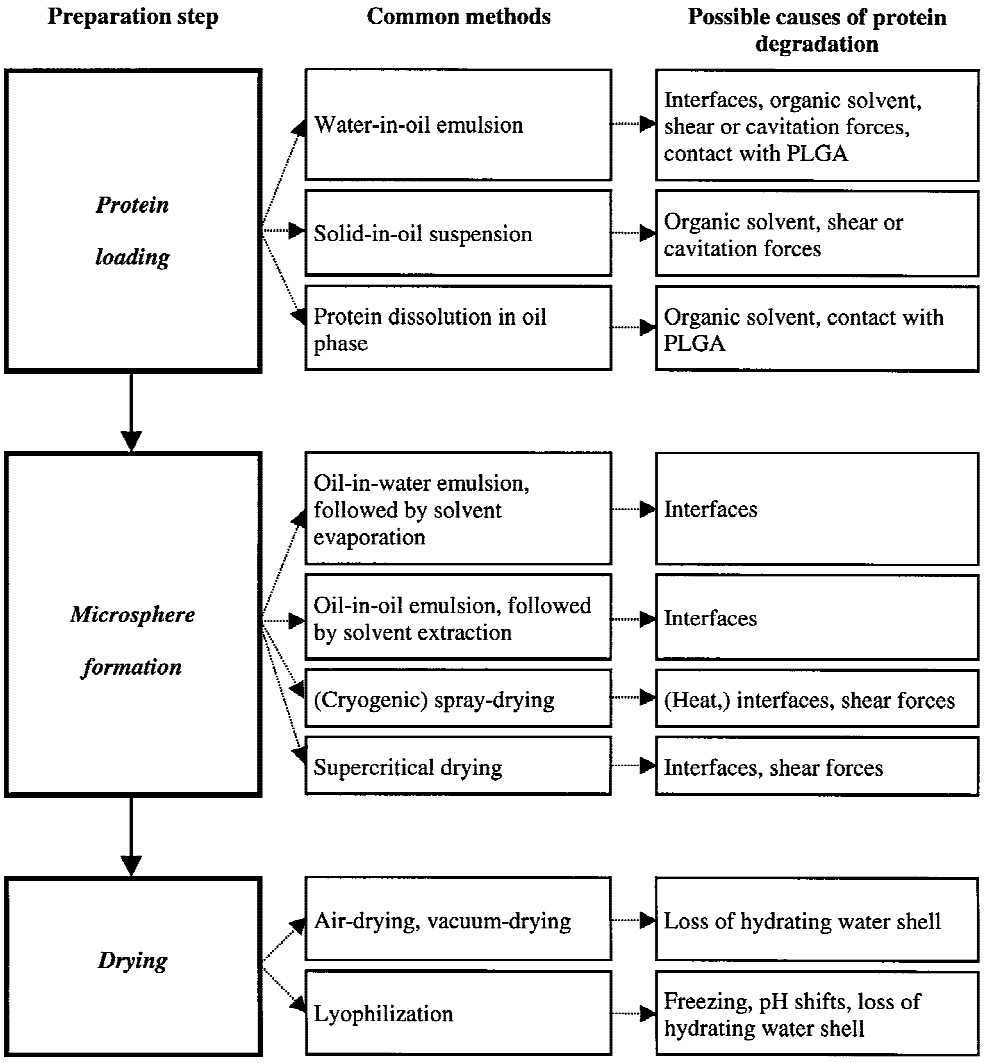

Fig. 1. Critical steps in common preparation processes for protein-loaded PLGA microspheres and possible causes of protein degratdation at each step.

step in all cases is dissolution of PLGA in an organic solvent, usually methylene chloride (MC) or ethyl acetate (EtAc).

Protein Loading

The protein can be entrapped in the organic PLGA solution by creating a water-in-oil emulsion (emulsion method), a solid-in-oil suspension (suspension method), or by dissolving the protein in the organic solvent (dissolution method). So far, a number of denaturing stresses has been identified in each of these processes (Figure 1).

Emulsion Method

The presence of water/organic solvent interfaces is an important disadvantage of the emulsion method, and has been identified as a major cause of protein denaturation and aggregation (4,8,11,14,21,23–28). The formation of interfaces is a common destabilizing factor for proteins and generally results in interfacial adsorption followed by unfolding and aggregation (22). The way emulsions are created plays an important role, as this will determine not only the size of the interfacial area the proteins are subjected to, but also the extent of shear and cavitation stress as well as the transport rate of proteins to the interfaces. Particularly sonication can provoke cavitation stress that may destroy proteins because of local temperature extremes (29) and resulting free radical formation (30). Morlock *et al.* showed that shear can play a role during emulsification, because the use of sonicators and vortex mixers in the first emulsification step produced more aggregation of recombinant human erythropoietin (rhEPO) than homogenizers did (26). Also, reducing the sonication time during PLGA nanoparticle preparation limited the activity loss of protein C (25). However, for several proteins it has been shown that the presence of a large interface between aqueous and organic phase, rather than shear stresses, is primarily responsible for protein instability during emulsification (21,23,24,31).

The type of organic solvent can also be of influence. As compared to the more hydrophobic MC, EtAc usually induces less emulsification-induced denaturation of proteins (4,14,24). So far, only protein C has been found more sensitive to EtAc than to MC (25).

In addition to protein/organic solvent contacts, hydrophobic contacts between the protein and PLGA are a concern

in the emulsification method, and may lead to protein unfolding and aggregation (2,27,28,32–36). Enhanced degradation of proteins upon emulsification with PLGA in the organic phase has been observed (27,36).

Suspension Method

An approach to circumvent emulsification-related protein instability is protein loading by suspension of solid proteins in the organic phase (4,7,9,19,27,37). A drawback of the suspension method is that prior to encapsulation the protein must be (freeze-)dried, usually in the presence of a lyoprotectant. Among twelve tested lyoprotectants, only trehalose provided both protection and a sufficiently fine suspension of BSA in the organic PLGA phase for the purpose of microencapsulation (37). Trehalose and mannitol were found to be efficient lyoprotectants for rhGH (4).

Klibanov and co-workers have shown that the conformation of solid proteins in organic solvents is kinetically trapped (38). Thus, protein unfolding is extremely slow under these preparation conditions. Problems may arise, however, when the protein (partially) dissolves in the organic phase. The latter often results in unfolding (39,40). The preservation of the native protein structure using the suspension method has been reported for three different proteins (4,7,27,37).

Dissolution Method

Park *et al.* directly dissolved lysozyme and PLGA in a mixture of dimethyl sulfoxide (DMSO) and MC, which was emulsified into an aqueous phase to prepare microparticles (41). It is not clear whether the partial aggregation and adsorption observed during release were due to the preparation method or to the release phase. This method is unlikely to be generally applicable for all proteins, and others have suggested hydrophobic ion pairing or formulation of the solid protein at its isoelectric point to increase protein solubility in organic solvents (4). Primary concerns for the dissolution method are protein unfolding upon dissolution in the organic solvent (39,40) and the intimate PLGA/protein contacts, as discussed earlier.

Microparticle Formation

After distribution of the protein in the PLGA solution, microparticles need to be prepared. In many cases one creates a (new) emulsion for this purpose by adding a non-solvent for the PLGA, usually water (with an emulsifier, mostly partially hydrolyzed polyvinyl alcohol) or silicone oil. While the mixture is stirred, the microparticles are formed. During and after this emulsification step, the organic solvent in which the PLGA was dissolved is either extracted or allowed to evaporate. This causes the PLGA to precipitate as spherical particles entrapping the protein. Little is known about the potential denaturing stresses generated during the particle formation using these solvent extraction or evaporation methods, but interfacial stress may be detrimental to protein molecules located at the outside of the nascent microparticles. In addition, in the water-in-oil-in-water preparation method high ionic strengths are sometimes used in the outer aqueous phase to obtain smooth particles (42,43). This may lead to extraction of water from the microparticles and thus an increase in protein concentration. The latter may cause protein aggregation. The use of an oil-in-oil emulsion may reduce the potential denaturation stress when compared to the use of aqueous solutions as the outer phase.

An alternative method to produce protein-loaded microparticles is spray-drying (7,9,17,18,44–46). The protein/PLGA dispersion is sprayed through a (heated) nozzle, and the organic solvent is rapidly evaporated by a hot gas flow. It is obvious that the high temperatures required for spray-drying may have an adverse impact on protein integrity. On the other hand, the duration of exposure to these elevated temperatures is often very short. A variation of the conventional spray-drying method is a cryogenic method in which a protein/PLGA dispersion is ultrasonically sprayed into liquid nitrogen over solid ethanol. During evaporation of the liquid nitrogen the ethanol melts, resulting in extraction of the organic solvent from the microparticles formed by the spraying process. This method has been used to prepare PLGA microparticles that release bioactive rhGH over a one-month period (7,44) and has resulted in the only protein-containing PLGA microparticle formulation currently on the market (Nutropin Depot).

A novel method for the preparation of PLGA microparticles is precipitation with a supercritical fluid, usually carbon dioxide (47,48). A mixture of protein suspended or dissolved in an organic PLGA solution is sprayed in supercritical carbon dioxide, which results in dissolution of the organic solvent in the supercritical phase and precipitation of protein-loaded PLGA microparticles. This non-aqueous method was capable of encapsulating lysozyme, but no stability data were reported (48).

In general, the particle formation step seems to be less detrimental to proteins than the loading step. For example, emulsification in an aqueous phase or spray-drying of rhEPO/PLGA-emulsions were mild compared to the first emulsification step (26,45). Also, variation of the particle formation step (spray-drying or coacervation) had a minor impact on diphtheria toxoid (DTd) antigenicity when compared to other process variables (46).

Drying

The final stage in the manufacture of microparticles is the drying step, during which residual solvents are removed. Depending on the previous process steps, drying can be achieved by air-drying, vacuum-drying, or lyophilization. The latter is most commonly used when water was used in one or more previous stages of the preparation process. The effects of lyophilization on protein integrity have been investigated thoroughly, and denaturation and aggregation are common phenomena during freezing and subsequent dehydration (22,49).

Remarkably, for PLGA-encapsulated proteins there is no published evidence of drying-induced protein degradation. From several papers it is clear that drying-induced protein degradation is often insignificant in PLGA microparticle preparation processes (4,7,9,14,44,50). The amorphous character of PLGA may help in preventing drying-induced damage to encapsulated proteins. Conversely, hydrophobic contacts may become more pronounced during or after drying and be partly responsible for incomplete protein release (see below).

A complicating factor in the analysis of proteins encap-

sulated in dried microparticles is that many analytical techniques require preceding extraction of the protein. Among the non-invasive techniques of potential use for the analysis of proteins in the solid state (e.g., ESCA, FTIR, solid-state NMR, dielectric spectroscopy), only FTIR has become common practice in the analysis of PLGA-encapsulated proteins (10,37,51–53). Using this method, some proteins were observed to be in a non-native state (52) and evidence of encapsulated protein aggregates has been reported (53). When extraction is required, one must be alert to artifacts caused by the extraction method (1,13,17,18,54–57). For instance, some methods may not be quantitative (18,54–57) and preferentially extract entrapped monomers or those protein molecules that most easily diffuse out of the particle. The extent of aggregation can be under- or overestimated when the extraction medium dissolves aggregates or induces aggregate formation, respectively. Furthermore, irreversible conformational or chemical changes can be induced by commonly used extraction media (including MC, DMSO, SDS, and sodium hydroxide (18,54,55)), which is a serious problem when one wishes to study the conformation or activity of the encapsulated protein.

PROTEIN INSTABILITY DURING STORAGE

Published information on the long-term stability of proteins within dried PLGA matrices is scarce. Although the storage stability may be a minor issue when compared to the stability problems encountered during preparation and release stages, it has become clear that proteins are not necessarily stable in the solid state (58,59). In particular, several chemical degradation reactions can occur and interactions between PLGA and incorporated proteins leading to accelerated protein degradation have been reported (59). One of such reactions may be the formation of amide bonds between carboxyl groups of the (degrading) polymer and primary amines of proteins (60). Generally, degradation of lyophilized protein formulations is avoided by the addition of lyoprotectants and by controlling the storage conditions with respect to temperature and humidity (22,49). The maintenance of low residual moisture levels may be particularly critical for protein-loaded PLGA matrices, because moisture can induce premature polymer hydrolysis. This will not only unintentionally affect the release profile, but can also result in a pH drop and thus may compromise protein stability. Moreover, acid environments are reported to accelerate hydrolysis of non-reducing sugars commonly used as lyoprotectants, yielding reducing sugars, which may form covalent bonds with lysine residues of the protein (19). Finally, formalinized antigens such as DTd and TTd are especially sensitive to moisture-induced aggregation through formaldehyde-induced cross-linking (61).

In one of the few papers showing data on the storage stability of PLGA-encapsulated proteins, Tracy reported that zinc:rhGH complex encapsulated in PLGA microparticles was fully intact after storage for 6 months at 4 °C as assessed by size exclusion, reversed-phase, and ion-exchange chromatography (9). Morlock *et al.* showed that the number of aggregates in rhEPO-loaded PLGA microparticles containing hydroxypropyl β-cyclodextrin (HPβCD) did not increase when stored for 8 weeks at –20 °C, 8 °C or room temperature, and only slightly increased at 37 °C (26). Moisture-induced non-release of BSA after storage could be partly related to disulfide-mediated aggregation (1). Shao and Bailey showed in an accelerated stability test (1 month at 40 °C, 75% relative humidity) that the amount of covalent dimers and deamidated species in insulin-containing PLGA microparticles gradually increased with time (62). Since co-encapsulated pH-sensitive dyes indicated a progressive pH drop within the microparticles (pH 3.8 after three weeks), these acid-catalyzed degradation reactions could be ascribed to acidification of the microparticles.

PROTEIN INSTABILITY DURING RELEASE

Protein release from PLGA microparticles is often characterized by an initial diffusion-controlled burst release phase within the first day, followed by little and incomplete release that does not match the polymer degradation rate (1,5,13,33,41–43,45,56,63,64). The amount of initially released protein primarily depends on the preparation conditions of the microparticles and on the size of the encapsulated protein. If the protein fraction released during the first day is partly degraded, the degradation products are likely to be formed during the preparation process or during storage, if the formulation was stored. On the other hand, incomplete release subsequent to the initial burst can be due to protein-polymer interactions and/or protein aggregation during preparation, storage (if applicable), or release. In addition to incomplete release, it has often been observed that the release of bioactive protein falls behind that of total protein (11,15,17,19,25), indicating partial degradation of the released protein.

Incomplete release of active protein is often associated with one or more of the following phenomena: covalent or non-covalent aggregation, hydrolysis, or non-specific adsorption to the PLGA matrix. The main stress factors involved are acidification of the microenvironment and protein-PLGA interactions, and are discussed below. In addition to PLGA-related degradation, one should be conscious of the various protein degradation routes that may occur in the aqueous state also in the absence of PLGA, as recently reviewed by Wang (65). The encapsulated protein should be stable in aqueous milieu at 37 °C during the intended release period to make microencapsulation beneficial at all. Furthermore, it has been documented that extrapolation of *in vitro* release studies to the situation *in vivo* is not always straightforward (1,5,11,21,28,57,66).

Analogous to dry protein-loaded microparticles, the determination of protein integrity of non-released proteins is hampered by the possible influence of the extraction procedure on the protein structure. In addition to the artifacts discussed before, the amount of released protein can be overestimated when applying too high centrifugation forces to separate the protein-containing release medium from the microparticles, thereby squeezing entrapped proteins out of the microparticles (5,13).

Acidification

Protein instability during release has been attributed to a local pH drop inside the microparticles due to trapped acid PLGA degradation products (11,15–17,21,28,66–68). For instance, Park and co-workers showed that the pH in buffered release medium dropped to values as low as 3 if the medium

was not exchanged, resulting in severe hydrolysis of BSA and CA (28,66). Igartua *et al.* also ascribed hydrolysis of BSA released from PLGA microparticles to acidification of the medium (68). Others used circular dichroism and fluorescence spectroscopy to show that TTd released from PLGA microparticles had undergone conformational changes and chemical decomposition of Trp residues (15,16). Similar changes were observed in TTd when incubated at pH 2.5.

Evidence for acidification within degrading microparticles has been provided only recently and local pH values between 1.5 and 4.7 have been reported (62,69–73). This has been demonstrated by probing the intra-microparticle environment with pH-sensitive dyes (62,69,71,72) or spin labels (70,73), and by pH measurement after extraction (72). Ongoing acidification of the microparticle interior was shown to induce deamidation and covalent dimerization of non-released insulin (62).

Despite the evidence of acidification mentioned above, there still is controversy on this subject. It has been pointed out that the sampling scheme has a significant impact on the degree of acidification; frequent replenishment of the release medium or the use of a dialysis bag can effectively prevent the acidification of the medium with subsequent reduced protein degradation (66,74). It is unsure, however, whether this also reflects the situation *in vivo,* in which the PLGA microparticles are often surrounded by a fibrous capsule that may reduce efflux of acidic degradation products from the PLGA matrix (66). On the other hand, studies on rhGH-loaded PLGA microspheres showed a reasonable *in vitro-in vivo* correlation only when a strong high capacity was used, which effectively minimized the pH drop (5).

Polymer-Protein Interactions

Interactions between PLGA and encapsulated proteins are an important cause of incomplete protein release *in vitro*. Hydrophobic contacts are regarded to play a major role in protein adsorption to PLGA (2,25,28,32,35), but electrostatic interactions have also been reported (33,41,56). It is unknown whether such interactions will also play an important role during the release *in vivo,* as the components of biological fluids may compete with the protein.

The PLGA type can influence the extent of polymer-protein interactions. PLGA with capped carboxyl end groups has been compared with PLGA containing free carboxyl end groups (uncapped). The release rate of BSA was slower from nanoparticles composed of uncapped PLGA when compared to capped PLGA, despite the higher hydrolysis rate of the latter polymers (56), indicating that electrostatic polymer-protein interactions interfered with BSA release kinetics. Gaspar *et al.* reported a reduced release rate of L-asparaginase from uncapped PLGA nanoparticles (33). Moreover, they showed that L-asparaginase activity loss was complete after 2 weeks of release from capped PLGA nanoparticles, whereas from uncapped particles 50% activity was recovered after 3 weeks. The mechanism of the partial protection by uncapped PLGA remained unclear. No effect of the end group hydrophobicity was found on the integrity of encapsulated and released rhGH (7).

Park and co-workers have investigated the mechanism of incomplete release by extraction of non-released protein with three media interfering with different types of interactions (8,36,41). Solutions of sodium chloride can be used to extract protein molecules that electrostatically interact with uncapped PLGA end groups. The denaturant guanidinium hydrochloride (GdnHCl) will dissolve non-covalent aggregates, and sodium dodecyl sulfate (SDS) will release both non-covalent aggregates and proteins adsorbed to PLGA by hydrophobic interactions. The difference in protein amount extracted by SDS and GdnHCl is a measure for the contribution of non-specific adsorption to the non-release. Using this approach, Park *et al.* showed that the initial release of (positively charged) lysozyme from uncapped PLGA microparticles was mainly controlled by electrostatic interactions between lysozyme and free carboxyl end groups of PLGA chains, whereas non-covalent aggregation and hydrophobic PLGA-protein contacts were responsible for the incomplete release later on (41). Similarly, comparison of the extractability of non-released rhGH by SDS or GdnHCl showed non-covalent aggregation to be the main cause of non-release after the burst (8). At a later stage, non-specific adsorption also contributed to the non-release of rhGH. Incomplete release observed for carboxymethylated BSA and BSA was largely due to adsorption, as GdnHCl was not able to extract these proteins from PLGA-microparticles, whereas SDS was (36).

APPROACHES TO PREVENT PROTEIN DEGRADATION

During Preparation

Adding excipients to the inner aqueous phase that compete with the water/organic solvent interface can prevent emulsification-induced denaturation and aggregation. This approach may be particularly useful when PLGA microparticles are loaded with low amounts of therapeutically potent proteins. A self-protecting effect of proteins by increasing their concentration during emulsification has been observed for CA (28) and rhGH (4). On the other hand, increasing the protein concentration during emulsification was shown to result in higher absolute amounts of aggregated protein at the interface, indicating that multi-layer interfacial adsorption can occur (24,28).

BSA has been shown to limit emulsification-induced aggregation of CA (28), ovalbumin and lysozyme (31), and inactivation of TTd (17). The protective effect of BSA can probably be ascribed to accumulation at the water/organic solvent interface (75), thereby shielding the interface from the protein of interest. Especially when using proteinaceous stabilizers, their integrity should be guaranteed in order not to compromise product safety. Since albumins are relatively resistant against process-induced degradation (23,24,76,77), the use of HSA as stabilizer may be a realistic option. Other stabilizing interface-active excipients include phosphatidylcholine (11), PEG 2000 and gelatin (21), and PEG 400 (43).

Common surfactants have not been very successful as stabilizer in the emulsification step. For instance, Tweens are able to protect rhGH and recombinant human interferon-gamma (rhIFNγ) against shear-induced inactivation, but promoted rather than prevented extensive aggregation of these proteins during emulsification and microencapsulation (4). Pluronic F68 prevented TTd aggregation during homogenization in aqueous medium, but not during emulsification with MC (14). The poor protection by surfactants may be ex-

plained by insufficient competition with the protein for the water/organic solvent interface, or promotion of organic solvent/protein contacts through hydrophobic contacts with both components.

Stabilizing excipients for rhGH during emulsification are mannitol, trehalose and PEG 3350, but of these only trehalose was effective for rhIFNγ (4). Encapsulation of these proteins by either the emulsion method or a non-aqueous spray-drying method induced similar degradation patterns, which were in both cases prevented by the addition of trehalose. The authors suggested that in the emulsion method trehalose shields the proteins from the organic solvent by preferential hydration. For solid proteins dispersed in organic PLGA solutions, trehalose (and other sugars or polyalcohols) can act as a water substitute (22), thereby preventing organic solvent-protein contacts. In contrast to these findings, trehalose and mannitol did not prevent emulsification-induced aggregation of rhEPO, whereas HPβCD, BSA and arginine did (26). The stabilizing capability of these additives was ascribed to shielding of hydrophobic amino acid residues on the protein surface (HPβCD), surface active properties (BSA), or electrostatic interactions (arginine). HPβCD also protected lysozyme and ovalbumin against emulsification-induced aggregation, whereas sucrose and trehalose did not (31).

Another approach to protect proteins against degradation during emulsification has been pre-entrapment in a hydrophilic core, which is subsequently encapsulated in PLGA microparticles (78–81). Although this approach generally improved release characteristics, in none of these studies data on the protein structure were reported.

Anhydrous preparation methods have shown to be advantageous for the preservation of the native protein structure. For instance, when water was used in the inner or outer phase of double emulsions, tumor necrosis factor-alpha (TNF-α) was inactivated to a large extent (27). When a solid-in-oil-in-oil dispersion was prepared instead, the activity of TNF-α was fully preserved. Hydrophobic ion pairing with negatively charged surfactants has been proposed for protecting positively charged solid proteins against organic solvents (4), but the feasibility of this approach has yet to be established.

During Storage

Excipients known to protect freeze-dried proteins (lyoprotectants) have been incorporated in protein-loaded PLGA microparticles, such as non-reducing sugars and polyols (4,10,17,19,37) and cyclodextrins (17,26,37). The protective effect of these compounds may in part be ascribed to their amorphous, glass-forming character and their role as water substitute in the solid state (22,49). However, systematic studies on the stabilizing effect of these and other excipients during storage of dried PLGA-encapsulated proteins are lacking. Assurance of low residual organic and aqueous solvent levels as well as storage in appropriate containers at 4 °C (well below the glass transition temperature of the dried matrix) are also likely to be crucial for the long-term stability of these systems.

During Release

In addition to general procedures to improve protein stability in the aqueous state (65), specific stabilization approaches to minimize protein degradation associated with the direct environment of (degrading) PLGA are often needed.

To inhibit acid-induced protein degradation inside the microparticles during release (if occurring at all *in vivo*), co-incorporation of poorly water-soluble basic inorganic salts, such as magnesium hydroxide (82–84), calcium carbonate (17,85), and sodium bicarbonate (71,85) has been proposed. The buffering capacity of zinc carbonate, which was added to improve release kinetics by decreasing the solubility of rhGH (7), may contribute to the complete release of fully active rhGH from PLGA microparticles. In most cases this buffering approach markedly increased the stability of the released protein *in vitro*. However, co-encapsulation of a too strong base, calcium hydroxide, induced the formation of covalent BSA aggregates as a result of alkali-induced thiol-disulfide exchange (83).

The buffering effect of the encapsulated protein itself at high loading may counteract a local pH drop. Alternatively, BSA was suggested to act as a proton scavenger during release of TTd from PLGA microparticles (17). However, Zhu *et al.* revealed that even 15% BSA in a PLGA matrix did not significantly alter the acidic microenvironment during release (82). Thus, the protective effect of BSA may be ascribed in part to other mechanisms, such as competitive adsorption to PLGA (28). Chen *et al.* added BSA to recombinant human interleukin-1 alpha-loaded PLGA microparticles to improve the release characteristics of the cytokine (11). Several stabilizers that improved protein stability during emulsification could not eliminate non-release of aggregated rhEPO, possibly because of their rapid diffusion from the microparticles (26).

Interactions between BSA and carboxyl end groups of uncapped PLGA were reduced by the addition of Pluronic F68, as evidenced by an increased release rate (56). As poloxamers are neutral molecules, hydrophobic interactions probably played a role in addition to the electrostatic interactions reported. The stability of TTd during release was improved by co-encapsulating carbohydrates (dextran, trehalose, or heparin), but the stabilization mechanism was not investigated (19).

CONCLUDING REMARKS

Over the last few years it has become clear that the encapsulation of proteins in PLGA microparticles is hampered by significant protein degradation. Although we focussed on PLGA microparticles in this review, many of the destabilization mechanisms and stabilization approaches described can be valid for other polymeric delivery systems, too. The main stress factors relevant for protein-loaded PLGA microparticles, as well as stabilization approaches and mechanisms are summarized in Table I.

Up to now, complete release of fully intact protein mostly has not been achieved, even in cases where significant improvement of the stability of the encapsulated protein through optimized process and formulation protocols was reported (4,7,17,19,21,26,43,71,79,82). Unfortunately, large differences exist between individual proteins in sensitivity to stress factors. For a rational optimization of protein stability, one must first establish the stage at which degradation occurs and identify the stress factors compromizing the stability of the particular protein. Then, a rational stabilization approach

Table 1. Approaches to Protect Microencapsulated Proteins Against Stress Factors During Preparation, Storage, and Release

Stage	Stress factor	Stabilization approach	Stabilization mechanism
Preparation	Water/organic solvent interfaces	Add sugars, polyols, PEG	Increase of Gibbs free energy of unfolding, shielding from interfaces by preferential hydration
		Increase protein loading	Reduction of interface/protein ratio
		Add other proteins	Competition for interfaces
		Avoid emulsification, use non-aqueous process	Absence of water/organic solvent interfaces
		Pre-encapsulate protein in hydrophilic core	Shielding from interfaces
Preparation	Protein-PLGA contacts	Add other proteins	Competition for PLGA
		Pre-encapsulate protein in hydrophilic core	Shielding from PLGA
		Hydrophobic ion pairing	Shielding from PLGA
Preparation	Shear	Add surfactants	Competition for interfaces
		Reduce homogenization time	Minimized exposure to shear
		Avoid sonication, use other homogenization method	Absence of cavitation stress
Preparation	Drying	Add lyoprotectants	Increase of Gibbs free energy of unfolding, water substitution
		Avoid lyophilization, use other drying method	Absence of freezing step
Storage	Moisture Dehydration	Reduce residual solvent level	Minimized mobility and water-induced degradation
		Add lyoprotectants	Increase of Gibbs free energy of unfolding, water substitution
Release	Acidification	Add basic compounds	Buffering
	Protein-PLGA contacts	Add other proteins	Competition for PLGA
		Add surfactants	Shielding from PLGA
		Add sugars	Increase of Gibbs free energy of unfolding, shielding from PLGA by preferential hydration

can be followed to ensure the safety and efficacy of these protein-loaded PLGA microparticles.

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