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Recent Advances in the Stabilization of Proteins Encapsulated in Injectable PLGA Delivery Systems

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ABSTRACT: Injectable controlled-release systems based on biodegradable copolymers of lactic and glycolic acids (PLGAs) have become widely used for delivery of protein therapeutics and vaccine antigens. Over the last five years, great strides have been made toward overcoming the difficulty of stabilizing PLGA-encapsulated proteins. In addition to stabilizing proteins during encapsulation with anhydrous methods, two approaches have proven highly effective to stabilize proteins during 1-month release incubation under physiological conditions: protein complexation with zinc and control of PLGA microclimate pH with antacid excipients. Described here are recent advances in the stabilization of proteins encapsulated in PLGA delivery systems.

KEYWORDS: protein stability, poly(lactide-co-glycolide), encapsulation, vaccine stability, antigen

I. INTRODUCTION

Chances are that for every important protein that has undergone pharmaceutical development, the polymer-controlled release option, at the very least, has been considered seriously and, in many cases, aggressively pursued. Unfortunately, successful controlled release of proteins has been a daunting task and, until recently, there has been significant doubt whether a significant number of therapeutic proteins could be slowly and completely released in a native state from the biodegradable polymer-of-choice for general biomedical applications, copolymers from lactic and glycolic acids (commonly referred to as PLGAs, PLGs, or PLAGAs). The PLGAs are one of only a few biodegradable polymers used in pharmaceutical products or medical devices approved by the United States food and drug administration (US FDA). Thus, the most significant obstacle in the development of controlled-release injectable depots for proteins has emerged as the instability of the protein during encapsulation and release in vivo.[1] Several examples of the reported instability of proteins encapsulated in PLGA delivery systems are shown in Table 1. The basic concepts of this field have been carefully described before[2] and thoroughly elaborated with many useful examples.[3,4] These reviews and others[5–9] will not be duplicated here. Instead, the present focus is on the

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TABLE 1
Some Examples of the Instability of Proteins Encapsulated in PLGA Delivery Systems

Protein	Report of instability	Reference
Bovine serum albumin	Peptide-bond fragmentation during release	9, 15
	Noncovalent aggregation during release (with negligible to minor disulfide-bonded component in aggregate)	15, 70
Hen egg-white lysozyme	Noncovalent aggregation during encapsulation by solvent evaporation	101
	Covalent dimerization and formation of unknown product during release	9
Ribonuclease A	Noncovalent aggregation during encapsulation by solvent evaporation	25
Growth hormone	Soluble aggregation in the absence of zinc acetate and zinc carbonate during release	14
	Deamidation, oxidation, and aggregation observed at rates similar to those in solution during release	102
	Aggregation during encapsulation and release	103
Tetanus toxoid	Incomplete release[a]; losses in immunoreactive antigen during release	104–109
Erythropoietin	Covalent aggregation during solvent evaporation; aggregation during release	110
Insulin-like growth factor-I	Incomplete protein release over 25 days in the absence of zinc carbonate[a]	41
Vascular endothelial growth factor	Heparin affinity decreased by 13% after 8 days of release	63
Bone morphogenetic protein	Incomplete protein release; 30% immunoreactive protein recovered after 28 days	15
Basic fibroblast growth factor	Incomplete protein release; 38% immunoreactive protein recovered after 28 days with heparin stabilizer	15

[a] Incomplete release appears to involve insoluble aggregation, but encapsulated aggregates not always verified.

forefront of this research area, that is, what is ambiguous and what is not, and what significant obstacles remain.

Although therapeutic proteins have been in existence for many years, because of their short serum half-lives and generally poor bioavailability when administered by noninvasive routes, routine parenteral administration with a syringe needle is still the most common method to deliver protein (e.g., insulin, erythropoietin, and growth hormone).[10] Reducing injection frequency of protein drugs offers the potential of eliminating serious problems

with patient comfort, compliance, and mental stress that accompany frequent injections.[11] Therefore, with the recent increase in protein drugs under development, one of the most important goals in drug delivery is to find new and better methods to deliver proteins. Several promising alternatives are being actively pursued, including pulmonary[12] and percutaneous[13] strategies, and injectable depots.[14,15] The injectable controlled-release option has been the first to successfully complete clinical trials and to be approved by the US FDA (i.e., the Nutropin Depot™, which controls the release of human growth hormone from PLGA microspheres developed by Alkermes and Genentech[14]).

The first injectable PLGA formulations to reach the market for small peptides demonstrated that a therapeutic agent could be released slowly and continuously for 1, 3, or even 4 months, depending on the implant size and polymer degradation rate.[16–18] These dosage forms, which can be injected through a syringe or trocar, established two injectable configurations: spherical particles approximately 1 to 100 μm diameter, commonly referred to as "microspheres," and single cylindrical implants approximately 0.8 to 1.5 mm in diameter (sometimes termed "millicylinders"[15]). Recently, an experimental in-situ–forming implant strategy, which uses a biocompatible solvent to dissolve the polymer (e.g., water-miscible *N*-methyl-2-pyrrolidone [NMP]), has been examined for delivery of leuprolide acetate.[19] In this approach, the peptide is dissolved in the polymer/NMP solution just before injection. Once injected in the body, the polymer rapidly hardens in-situ as water penetrates the implant. This and other in-situ–forming delivery systems are very new and little information is available regarding their capability to stabilize and control the release of proteins.

In addition to delivery of protein drugs, many vaccine antigens are protein-based and require adjuvants to improve the immune response. Poorly soluble aluminum salts, such as aluminum phosphate, aluminum hydroxide, and alum, are the only vaccine adjuvants currently licensed in the United States. Although generally quite effective for many antigens, aluminum adjuvants have several limitations, including the induction of infrequent local reactions, poor adjuvanticity for all antigens, the induction of IgE antibody responses, an inability to induce cell-mediated immunity, and, importantly, they typically require at least two or three doses for protection even when highly immunogenic antigens (e.g., tetanus toxoid) are used (see O'Hagan et al.[8]). Although diseases such as tetanus and diphtheria are not a problem in the United States, in many developing countries poor compliance for receiving booster vaccinations has resulted in severe mortality from these diseases.[20–22] In addition to reducing the number of doses for injection for older bacterial vaccines, smaller synthetic and subunit vaccines are poorly adsorbed to aluminum salts and require improved adjuvants to be protective against infection.[8,23,24]

Perhaps the most promising replacement or adjunct to the aluminum salts is the injectable PLGA microspheres.[21,22] In contrast to alum, PLGA microspheres cause no significant adverse reactions, induce cell-mediated immunity, and are capable of controlled release, allowing for the potential reduction in booster doses required for protection against infection (i.e., the single-dose vaccine concept).[8] However, it has been suggested that the major obstacle in the development of PLGA microspheres as single-dose vaccines, as with protein pharmaceuticals, is the instability of the vaccine antigen during encapsulation and protein release.[8]

Therefore, two separate but related fields, the delivery of protein pharmaceuticals and the delivery of protein antigens, await advancement of the stabilization of encapsulated therapeutics or biologicals, particularly for a month or more during release incubation. Described below is the most recent progress toward this goal.

This review is organized as follows. First, a summary of several general rules in this developing research area are enumerated, including a general description of stability issues during encapsulation and analysis of protein loading. The next sections concern protein stability during release. The principal stresses causing instability of encapsulated proteins are re-examined based on new findings since a previous review of this subject.[2] Then, experimental approaches to simplify the formulation problem of stabilizing encapsulated proteins and the assembly of these approaches into an overall mechanistic experimental paradigm are described. Finally, several examples of how proteins have been stabilized during release from the polymer are discussed, before some concluding remarks.

II. GENERAL RULES AND DESCRIPTION

Although numerous factors controlling the stability of proteins encapsulated in PLGA delivery systems remain ambiguous, some very useful principles have emerged to help those pursuing stabilized formulations. Some of these general rules are listed in Table 2. It is important to note throughout this discussion that, although general statements and attempts to organize/simplify this research are useful, each polymer formulation and specific encapsulated protein is unique. Therefore, it is essentially impossible to write one set of rules that governs all PLGA formulations encapsulating proteins, although the important details described in Table 2 should provide a good starting point.

Physical–chemical analyses of the timeline of deleterious events occurring during encapsulation, storage, and release of the protein have been described.[2,5] During encapsulation, for the protein to be finely dispersed (i.e., system size such as diameter > 5–10 times protein particle size) within the polymer matrix, the protein must be micronized either as a microparticulate (for nonaqueous encapsulation) or as an emulsion. This creates a very large surface, which proteins with their amphipathic character like to occupy and where protein unfolding and other deleterious processes can occur.[25–27] This mechanism has been shown to occur for ribonuclease A when encapsulated in PLGA microspheres by the water-in-oil-in-water (w/o/w)–solvent evaporation method, which initiates aggregation of the enzyme.[25] Bovine serum albumin (BSA), in contrast, does not aggregate significantly under these conditions and can be used to inhibit ribonuclease aggregation.[25] Although intense mixing has not been implicated for model proteins, as would be expected for various proteins such as insulin,[28] in certain cases the addition of a sugar such as trehalose to promote preferential hydration of the protein has been shown to be useful (e.g., for growth hormone and interferon-γ[29]). This success in inhibiting protein aggregation with trehalose also suggests that for various proteins with poor conformational stability, the presence of the organic solvent denaturant in the aqueous phase could become problematic.

Microparticles of protein for anhydrous encapsulation can be prepared by a variety of techniques, including spray freeze-drying, spray drying, freeze-drying, grinding, jet milling, liquid-phase antisolvent precipitation, and supercritical CO_2-based methods.[14,27,30,31] Most of these procedures can micronize the particles to just a few microns or even to the submicron range. However, the smaller the particle size, the greater the specific surface area of the powder, which can correlate directly with increased protein damage.[27,31] It is also desirable if the dried protein can be retained in its native conformation (dried proteins without excipients are generally unfolded to varying degrees[32,33]) before hydration in the polymer

TABLE 2
Some General Rules for Stability of Proteins Encapsulated in PLGA Delivery Systems

Instability can occur during encapsulation, storage, or release from the polymer; stability during release is generally most significant if anhydrous encapsulation is used.

During release the primary stresses on the protein are (1) the presence of moisture at physiological temperature, and (2) the acidic microclimate that commonly occurs due to the built-up water-soluble acidic degradation products in the polymer. The hydrophobic polymer surface has been hypothesized to be important, although definitive evidence remains elusive.

During encapsulation, damage to proteins can occur from (1) the presence of water, which makes the protein flexible and reactive; (2) dispersing the protein in the form of a microparticulate or an emulsion, which generally requires formation of a large hydrophobic surface area; (3) the presence of denaturants (e.g., organic solvents, salting out salts) in the protein phase (particularly if the water is present); and (4) drying. The large hydrophobic surface area has been shown to be particularly important.

During drying and storage, as with any protein formulation, a structurally native state is desired, which has been commonly accomplished by the co-encapsulation of disaccharides such as trehalose and sucrose, or complexation with multivalent cations (e.g., zinc).

Primary anticipated mechanisms of instability resulting from deleterious conditions are protein unfolding, soluble and insoluble aggregation, hydrolysis, deamidation, and oxidation.

Analysis of loading, protein release, and protein instability mechanisms is generally made more complicated by the presence of the polymer, and sometimes very low concentrations of protein. Some useful techniques have been developed both to extract the protein from the polymer and to monitor the structure of the protein directly within the polymer matrix or at low levels in the release medium.

Protein instability (particularly, common insoluble aggregation) often alters release kinetics; this alteration complicates studies focused on nonstability issues, indicating that stability should be addressed before attempting to manipulate release kinetics or other aspects of the polymer formulation.

Two approaches are commonly used to stabilize proteins encapsulated in injectable PLGAs: formulation screening and mechanistic paradigms.

Mechanistic paradigms may involve one or more of the following: (1) examination of the denatured state of the protein in the polymer; (2) characterization of the deleterious stress(es) responsible for the instability mechanism; (3) simulating the instability of the protein in the polymer matrix; (4) use of a model protein to isolate one deleterious stress or mechanism of interest, (5) use of a simpler polymer matrix; (6) use of anhydrous protein encapsulation (to eliminate significant protein instability during encapsulation); (7) use of combinations of points (1) to (6) to elucidate the deleterious cause and mechanism of protein instability; and (8) use of the elucidated instability pathway to develop rational approaches to protein stabilization.

Adding adsorption competitors (e.g., albumin) and/or substances to promote preferential hydration of the protein (e.g., trehalose) are two approaches shown useful to minimize instability during preparation by the w/o/w emulsion-solvent evaporation method.

During release incubation, maintaining the protein immobilized in the solid state is often preferable to simply allowing the protein to dissolve in the aqueous pores within the polymer matrix.

Microclimate pH and water content in the polymer matrix can be controlled to some degree by (1) the co-incorporation of antacid excipients (e.g., $MgCO_3$, $Mg(OH)_2$, and $ZnCO_3$); (2) adjusting loading of either the protein or other water-soluble agents, (3) controlling permeability of the polymer matrix; and 4) changing the polymer degradation rate.

(see below). These studies[14,27] indicate that micronization can be accomplished with typical losses in the neighborhood of < 5% to 10% of native protein when low-temperature processes are used.

For anhydrous encapsulation methods that do not dissolve the protein, once the microparticulate is formed, no further instability typically occurs during encapsulation.[3] To encapsulate the protein, the protein/excipient microparticulate is generally suspended in an organic solvent, and the protein is quite stable owing to the kinetic trap in the solid state, which immobilizes the protein and strongly inhibits reactivity.[2,3,34,35] The "kinetic trap" describes the inability of the protein to unfold and reach an energetically more favorable free-energy state because of the very high activation energy barrier caused by the solid state. Therefore, because there is little protein damage during preparation of the microparticulate and anhydrous encapsulation, this two-step combination has become steadily more popular for protein encapsulation and is the way the Nutropin Depot® is prepared.[14,36]

Apart from the normal difficulties in protein analysis,[37,38] encapsulation and release of proteins from the PLGAs poses a unique set of analytical challenges. Methods are required to examine the protein integrity postencapsulation and to determine the protein loading for dosing and quality control purposes. Two general approaches are used: extraction[14,15] and destructive (usually hydrolytic) techniques.[39–41] During extraction the protein is physically separated from the polymer matrix before analysis. For the same reason that the protein is typically more stable for anhydrous encapsulation, extraction usually should be performed in the absence of water. The advantage of the extraction technique is that the extent and type of damage to the protein that occurred during encapsulation can be assessed, as well as the protein's structure and activity. The disadvantage of using extraction followed by most total protein assays is that if insoluble aggregation has occurred during encapsulation, the protein recovered after extraction may not be easily quantified. This limitation could possibly explain the difficulty of determining tetanus toxoid loading by this method.[42] If the aggregation is of the type that can be solubilized, then denaturing and/or reducing solvents can be used to dissolve any aggregate before performing an appropriate protein assay.[15]

One of the most robust techniques for determining total protein, short of radiolabeling with ^{125}I,[112] is the seldom-used hydrolytic technique of acid hydrolysis followed by amino acid analysis,[39] which can even break up formaldehyde-mediated aggregates.[43] In this method, both the polymer and protein are hydrolyzed in concentrated HCl at an elevated temperature. One only needs to make certain that the distribution of amino acids in the hydrolysate, which are stable to acid hydrolysis (e.g., leucine and alanine), matches that of the standard to be certain that the hydrolysis was complete. The more commonly used hydrolytic technique involves the hydrolysis of the polymer and protein in base (e.g., 1 N NaOH for 1 day at room temperature[40,41]) followed by an appropriate protein assay. A potential difficulty with this approach is that the base hydrolysis of the protein is incomplete, and then the question of an appropriate standard curve requires some validation. A final useful destructive technique for protein loading determination is based on analysis of nitrogen and/or sulfur content, which is compromised only if additional nitrogen or sulfur excipients are present in the formulation. Unlike extraction, both hydrolytic and elemental techniques do not permit quantification of sample purity.

Protein instability, particularly when insoluble aggregation is involved, will alter release kinetics. Therefore, it is generally best to find conditions to stabilize the protein before other aspects of the formulation, such as the controlled-release characteristics, are optimized. For example, a sugar, amino acid, or antacid excipient may be required to stabilize the protein,

each of which can increase water uptake in the polymer matrix leading to an increase in release rate. In the scenario in which controlled-release conditions are optimized before such a stabilizer has been identified, it is likely that upon addition of the new stabilizer the release kinetics may change enough to require reformulation. Certainly, there is a sharp contrast between encapsulating a highly water-soluble protein[15] or a poorly soluble zinc-protein complex.[14,36] Switching between these two cases would be expected to alter the requirements in the formulation necessary to attain the controlled-release function (e.g., low vs. high polymer matrix permeability for the protein, respectively) because protein solubility in water may be important for any diffusion component of release.[7,44]

III. RE-EVALUATION OF PRINCIPAL SOURCES (OR STRESSES) RESPONSIBLE FOR PROTEIN INSTABILITY WHILE ENCAPSULATED IN THE POLYMER DURING RELEASE INCUBATION

During release incubation, the polymer is exposed to either a simulated or a real physiological environment. The key features of the physiological environment include temperature, pH, osmotic pressure, ionic strength, buffer capacity, buffering species, the presence of surface-active substances, the polymer/solution and release-vessel/solution interfaces, frequency of release-medium replacement, and several others. Generally, physiological conditions are simulated by using a phosphate-buffered saline with or without a nonionic surfactant. In instances in which a very low amount of protein is liberated by the polymer into the release medium, albumin can be added to the release medium to strongly inhibit adsorption of the released protein onto the release vessel or to the polymer[15] (such as is commonly done during enzyme-linked immunosorbent assays), although the added bulk protein prohibits the use of routine protein assays.

Once the polymer is placed in the release environment, it takes up water rapidly in accordance with the polymer's inherent physical–chemical properties (e.g., molecular weight distribution, including initial monomer content, end-capping, glycolide content, degree of lactide isomerization, and catalyst type[45]), excipient type (e.g., water-soluble and microclimate pH modifying), and content. This amount of water is often in the neighborhood of 20% to 100% relative to the initial weight of the polymer matrix (i.e., polymer + protein + excipients). During this initial period, the polymer will generally swell slightly and the polymer chains are sufficiently mobile to alter the morphology of the polymer (e.g., pores have been shown to open or close[46]). Sufficiently high internal surface area of the polymer is typically present because the standard encapsulation methods typically remove organic solvents and/or water, leaving initially evacuated pores behind. Various measurements of the pH within the aqueous pores of the polymer (i.e., microclimate pH) have shown that this value can vary over a wide range (e.g., ~1.5–7.4), and even within the spatial distribution of a single microsphere, depending on several important factors (see below).

Therefore, the three principal sources of protein stability during release are expected to be moisture, microclimate pH, and the polymer surface. Each of these sources is re-examined below and the most recent advances from the previous treatment are considered.[2] Additional sources identified previously include water-soluble oligomers, a dialysis effect, and direct chemical reactivity between the protein and the polymer.[2] These effects have not yet been examined in detail and likely play a relatively minor destabilizing role.

III.A. Moisture

As with most unstable pharmaceuticals, an important question when examining stability of the protein is whether the protein exists in the solid or solution state.[47] The importance of this question stems from the principle that most instability pathways either will not happen or are far less likely when the protein is immobilized within the solid state, as described above.[2,3,35,48] When more water is added to a solid protein sample, at some stage (e.g., glass transition water content[49]) the protein molecule becomes flexible and reactive. Several examples of the solution–solid distinction are illustrated elsewhere.[3] Moreover, the transitions from solution to solid (e.g., freeze-drying) and solid to solution (e.g., rehydration) are well known to destabilize proteins.[35,50]

A relatively simple but useful expression can be used to estimate whether the encapsulated protein is dissolved by defining a nondimensional concentration of the protein in aqueous pores within the polymer matrix, Θ:

$$\Theta \equiv \frac{C}{S} = \frac{l/(SR-1)}{S} \tag{1}$$

where C, S, l, and SR are the aqueous pore concentration (w/w), aqueous solubility of the protein (w/w), loading of the protein (w/w), and swelling ratio of the polymer matrix (w/w) (≡ ratio of wet to dry mass of the matrix), respectively. Note that although polymer matrix porosity does not directly appear in (Eq. 1), the effect of this parameter is weighted in the SR term. For $\Theta \gg 1$, the encapsulated protein is expected to be present in the solid state, whereas for $\Theta \ll 1$, the protein is dissolved. For the estimate used in (Eq. 1) it must be assumed that (1) all water imbibed in the polymer is accessible to dissolve the protein, and (2) the solubility of the protein in the polymer is unaltered by the microclimate and excipients therein. Also note that the value of Θ will be time-dependent because the amount of protein encapsulated (affecting l), water imbibed (affecting SR), and polymer mass remaining (affecting SR) will change with time of incubation under physiological conditions.

Figure 1 illustrates a plot of $\Theta(l, S)$ for a PLGA specimen encapsulating a protein with a typical water content of 40% (w/w) (i.e., SR = 1.4). As one would expect, favoring the solid state ($\Theta \gg 1$) are low solubility and high protein loading. Similarly, high solubility and low loading favor the solution state ($\Theta \ll 1$). Thus, peptides such as octreotide acetate with $S \gg 0.5$, at reasonable loading values (e.g., 1% to 10%), will always be expected to be in solution in the polymer pores. Very water-soluble proteins such as BSA with $S \sim 0.5$ will be near saturation ($S \sim 1$) and very poorly soluble proteins (e.g., $S \ll 0.01$) such as zinc precipitated growth hormone are expected to always be in the wetted-solid state. If it is established that the protein is in the solid state, it is traditional to examine stability as a function of water content in the solid protein phase.[48,51] If a significant fraction of the protein is dissolved or sufficient water is present to allow refolding or aggregation, then the next obvious question is at which pH (see below).

When assessing the influence of moisture independent of alternative deleterious conditions occurring in the polymer, it is generally desirable to examine a range of water contents in the neighborhood of an estimate such as the one taken from Figure 1. Proteins are exceptionally prone to aggregation at moisture levels intermediate between the solid and solution states,[43,51,52] the behavior of which has been rationalized mechanistically.[51] Several examples of a bell-shaped dependence, which consists of low aggregation rates at both low

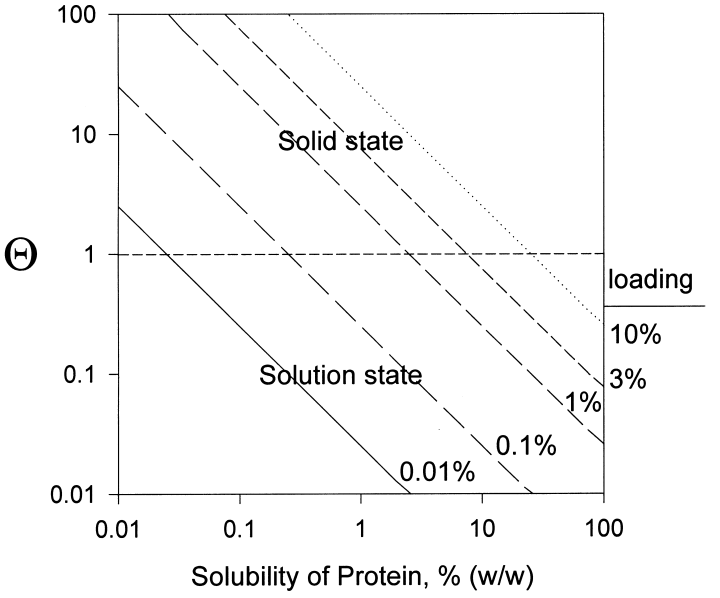

FIGURE 1. Nondimensional concentration (Θ) of a protein encapsulated in a PLGA delivery system as a function of aqueous protein solubility for constant protein loadings of 0.01% to 10% according to (Eq. 1). A typical value of 1.4 for the swelling ratio was assumed. If $\Theta \gg 1$ the protein is expected to exist mostly in the solid state, and for $\Theta \ll 1$ the protein is completely dissolved.

and high water contents and a maximal aggregation rate at intermediate moisture levels, have been shown for several proteins, including BSA[52,53] and tetanus toxoid.[43] An important point is that the width of the water content window in which aggregation is rapid can vary substantially and is dependent on pH. For example, BSA undergoes aggregation according to thiol-disulfide interchange when lyophilized from neutral pH with a very narrow moisture window (~20–50 g water/100 g protein).[51] In contrast, BSA undergoes noncovalent aggregation triggered by acid-induced unfolding when lyophilized from pH 2[15] with a very broad moisture window (~20–500 g water/100 g protein).[54] Obviously, if the water content can be manipulated in the protein phase within the polymer matrix (e.g., via the use of excipients) to be outside the rapidly aggregating moisture window (which is obviously simpler with a narrow window), aggregation can be ameliorated.

III.B. Microclimate pH

For many years a lowering of pH within the aqueous pores of PLGA delivery systems has been a prime suspect for the instability of encapsulated proteins. In 1993, several research teams gathered at a meeting sponsored by the World Health Organization on the development of a single-dose tetanus vaccine. Scientists present were perplexed by poor release of total and antigenically active (e.g., antigen recognizable by immunoassay) protein antigen, tetanus toxoid, from PLGA microspheres.[55] At this time there was already a strong recog-

nition of the possible effects that moisture and pH might have on the encapsulated antigen, which loses the ability to combine with neutralizing antibodies below pH 4–5.[56]

In addition to the problem of insufficient methods for monitoring microclimate pH in the polymer, confusion arose from the conclusion by one group that the polymer surface was primarily responsible for protein instability in the PLGAs.[9] Then, surprisingly, an early ^{31}P NMR-measurement suggested that the microclimate pH inside a formulation of PLGA 50/50 microspheres prepared by the ProLease® method was in the neutral range.[57] After publication of this report, several groups reported that the pH in PLGA microspheres prepared by solvent evaporation (particularly the 50/50) was in fact acidic.[58–62] Other investigators continue to find no acidity in their formulations,[63] although antacid additives (frequently referred to as release enhancers or modifiers because of their ability to sometimes increase release rate when coencapsulated with the protein) such as $ZnCO_3$[41] and strong buffering excipients[64] were often used. Therefore, because of the varying methods of microsphere preparation, polymer type, protein loading, presence or absence of encapsulated buffering species, microsphere size, and techniques of microclimate pH measurement, a significant controversy has arisen concerning the exact value of the microclimate pH during release incubation and the extent to which this value can change as a function of time and spatial position within the individual microspheres themselves.

From direct evidence, such as potentiometric,[65] fluorescent,[60,62] electron paramagnetic resonance (EPR),[58,59,61] and NMR[57] techniques, and indirect evidence, such as observing pH-dependent products,[15,56,60,66–70] one can make the following conclusions regarding the microclimate pH in PLGA specimens when incubated in vitro under physiological conditions:

1. The microclimate pH averaged throughout the polymer can be either neutral (pH 6–7.5) or acidic (pH 1.5–6), depending on the specific polymer formulation. However, it appears that the most common techniques (e.g., solvent evaporation) and conditions (e.g., conditions to slowly release a very water-soluble protein over 1 month) used to prepare PLGA microspheres larger than ~10 μm generate acidic regions in the polymer for at least some period during the release incubation. Neutral pH values have been reported for PLGA 50/50 microspheres prepared by the ProLease method (Alkermes)[57] and for thin films coated on glass electrodes.[65] Favorable conditions for the latter were PLGAs with higher lactide content, increased porosity (e.g., by encapsulating NaCl), and reduced thickness (e.g., < 10 μm).[71] It is noted that the ProLease method for microsphere preparation is expected to favor formation of microspheres with higher permeability than those prepared by solvent evaporation because in the former, the polymer solution is frozen in liquid nitrogen with little time to form a dense polymer film at the particle surface,[36] as is known to occur commonly during the solvent evaporation method.[72]
2. The microclimate pH can vary as a function of time and position within the polymer. When the microclimate has been found acidic, pH gradients separating the neutral bathing solution and the acidic polymer pores have been observed to be as small as a few microns.[60,62]
3. The developing picture of microclimate pH points to several important determinants (see below for stabilization approaches), including (a) rate of acid production (or polyester hydrolysis), with the fastest degrading PLGA 50/50 tending to be most acidic; (b) polymer permeability to the transport of water-soluble acid by-products,

with the more permeable polymer specimens being least acidic to neutral pH; and (c) the presence of encapsulated buffering species (including the protein itself).

4. When microclimate acidity develops, it is possible to control this value over a reasonable range (e.g., 1–2 pH units), at least for a reasonable duration (e.g., 1 month).

III.C. The Polymer Surface

From what is known in the areas of protein adsorption to biocompatible materials and protein interactions with solid surfaces in chromatography,[73–75] it is reasonable to anticipate that some proteins could be susceptible to instability when placed in contact with the solid PLGA surface. Proteins adsorb to polymer surfaces and PLGA is no exception.[76] Adsorption to polymers can be either reversible or irreversible and is often associated with a conformational change of the adsorbed and desorbed protein.[73–75] Although several different mechanisms of instability of proteins in contact with the PLGA surface can be envisaged, the adsorption phenomenon has most commonly been identified as a possible source to prevent the encapsulated protein from being released out of the polymer.[9,77] For example, Crotts and Park[77] have examined the ability of sodium dodecyl sulfate to enhance the release of BSA from PLGA microspheres when the surfactant is introduced into the release media at the later stages of release. The SDS was shown to cause otherwise unreleasable protein to be released from the polymer, which was explained by an induction of protein desorption from the internal PLGA surface by the surfactant.[77]

In contrast, Zhu et al.[15] demonstrated that BSA steadily forms noncovalent insoluble aggregates in the acidic microclimate of the polymer, which is initiated by the well-known unfolding of the protein at highly acidic pH.[78] Moreover, the SDS-induced BSA release could be explained by the finding that SDS was able to dissolve the aggregates formed in the polymer.[15]

In general, both surfaces and proteins are grouped into two types: hydrophilic (e.g., silica) and hydrophobic (e.g., polystyrene) surfaces, and proteins having either high ("hard," e.g., lysozyme) or low conformational stability ("soft," e.g., albumin).[74] It appears that the potential for either irreversible adsorption or irreversible changes in structure on protein desorption from the surface follow the trend: hydrophobic > hydrophilic and soft > hard (see Norde for details[74]). It is interesting to note that although low molecular-weight PLGA 50/50 has been termed a moderately hydrophobic polymer irrespective of endcapping,[76] the soft BSA was recoverable in its native form in excess of 90% after a 1-month release duration when the acidic microclimate in PLGA 50/50 was neutralized.[15] Thus, any deleterious contribution of adsorption for BSA under these conditions must have been less than 10% of the original encapsulated protein.

IV. MECHANISTIC APPROACHES TO SIMPLIFYING THE PROBLEM

In formulation, one often searches for ways to reduce the number of variables and/or to isolate the problem of interest. One way to accomplish this is to define and answer more simple and attainable problems that, taken together, can define all the conditions necessary for a stabilized formulation. Several techniques to accomplish this task have demonstrated that this stepwise pathway is a very powerful alternative or companion to formulation screening.

IV.A. Use of Model Proteins

It is important to recognize that therapeutic proteins often have multiple pathways of instability during encapsulation and release. Therefore, if only the therapeutic protein of interest is used, one cannot usually examine these mechanisms separately. Instead, instability data will reflect multiple instability mechanisms, making definitive conclusions difficult or impossible. On the other hand, a model protein (or peptide), when used correctly, can be selected to characterize a specific instability pathway that the therapeutic protein is likely to incur. That is, the model protein will ideally be unstable by one single dominant pathway. The mechanistic work with ribonuclease A during encapsulation, as described above, serves as a good example.

IV.B. Use of Simulations and Monitoring Structure of Protein in the Polymer

One of the particularly difficult tasks of examining protein stability mechanisms is the presence of the polymer, which causes two basic problems. First, technical problems arise when the protein is encapsulated. For example, most biochemical assays of protein structure and biological activity cannot be performed without removing the protein from the polymer, which in some cases can damage the protein, as described above. In addition, polymer degradation products can interfere with some protein assays. Second, the physical–chemical characteristics of the polymer microclimate that the protein experiences are not as well defined. Simulating the various deleterious stresses that the protein may experience in the polymer is a method by which both the stress(es) responsible for instability (e.g., extremes of pH and/or moisture) and mechanism(s) (e.g., protein unfolding followed by noncovalent aggregation) can be elucidated without potential artifacts that protein extraction can impose. It should be noted, however, that if the protein is removed from the polymer while the protein is in the solid state, a good mass balance between initial protein loaded and the sum of released and residual encapsulated protein has been obtained routinely (i.e., typically > 80% recovery for stabilized preparations[15,69,70,79]).

A recently developed method for monitoring the protein noninvasively involves FTIR spectroscopy.[80–82] These studies have largely confirmed that under anhydrous conditions of encapsulation little further structural damage occurs, and a native-like protein conformation can be encapsulated in PLGA delivery systems. Moreover, in the future these techniques may help to determine under which conditions protein extraction is or is not appropriate.

IV.C. Use of an Alternative Polymer Matrix

As a general rule, when the polymer matrix becomes smaller, it becomes more difficult to characterize and, in many instances, to manufacture. Therefore, it can be more efficient to assess certain stability issues with microspheres by using a single larger polymer matrix. It has been demonstrated, for example, that it is more difficult to stabilize BSA in PLGA 50/50 microspheres prepared by solvent evaporation as compared with larger millicylinders prepared by solvent extrusion.[15] The potential danger here is obviously that an instability mechanism can be affected when changing between one polymer matrix configuration and another. There-

fore, as with any simulation (i.e., simulating protein stability in many smaller polymer matrices with a single larger one), the instability mechanism elucidated in the simulation needs to be verified in the polymer matrix of interest. The following considerations motivate the use of larger matrix geometries to simplify mechanistic evaluation of stability of encapsulated proteins: (1) encapsulation in millicylinders is easily performed under anhydrous conditions; (2) deleterious micronization of the protein powder[27,30,31] typically need not be performed; (3) usually higher loading values, which have been shown to be desirable for controlling microclimate pH,[69] can be accomplished in millicylinders relative to microspheres without loss of controlled-release function; and (4) microencapsulation of proteins in millicylinders is simple and nearly 100% efficient, which is not always the case with microspheres.

V. ASSEMBLING SIMPLIFYING TECHNIQUES INTO AN EXPERIMENTAL PARADIGM

General mechanistic paradigms have been described to elucidate the instability mechanism(s) and stress(es) of proteins.[35] Once this information has been gathered, techniques to inhibit the mechanism or to bypass it by removing the stress can be performed.[35] When the protein is encapsulated, these paradigms generally require some modification and can become more elaborate. This is in part due to the numerous potential stresses involved and, until more recently, the polymer microclimate has been a "black box." As an example of a mechanistic paradigm for stabilizing proteins encapsulated in polymers, several of the tools to simplify the problem described in the previous section were used to elucidate the instability pathway of BSA encapsulated in millicylindrical implants and then to stabilize the protein.[15]

As described in Figure 2, the first step in the paradigm was to encapsulate BSA by an anhydrous technique in large millicylindrical PLGA 50/50 implants. The morphology of these delivery systems before and after release incubation is shown in Figure 3. The kinetics of insoluble aggregation of the encapsulated BSA were monitored after protein extraction from the polymer during in vitro release incubation, as seen in Figure 4.[15] The denatured BSA extract was found to consist of peptide fragments and insoluble aggregate soluble in 6-M urea, indicating that the instability mechanisms involved peptide bond hydrolysis and physical aggregation of BSA.[15] To identify the deleterious stress responsible for the mechanism of BSA instability, various stresses on the protein were simulated.[15] Among those simulations, BSA incubated in the presence of moisture and an acidic pH of 2, but not more than pH 3 (controlled by pH before lyophilization), was required to match the denatured state of BSA with that of encapsulated BSA. As described above, the pH required for BSA aggregation coincided with the lowest pH unfolding transition of the protein to the expanded form at pH 2.7.[78]

As shown in Table 3, the simulated instability of BSA at the low pH was matched with the instability when encapsulated in PLGA, in terms of time to 50% aggregation, aggregation type, and distribution of peptide-bond fragmentation. These data proved that the acidic and moist microclimate inside the PLGA pores was responsible for BSA instability during the release experiment. Next in the paradigm was the removal of the acidic stress. This was accomplished by adding to the polymer an antacid excipient, $Mg(OH)_2$.[15] After base addition, the aggregation and hydrolysis of BSA was virtually eliminated, and intact protein was slowly released (Fig. 4) and recoverable after a 1-month release duration.[15] The generality

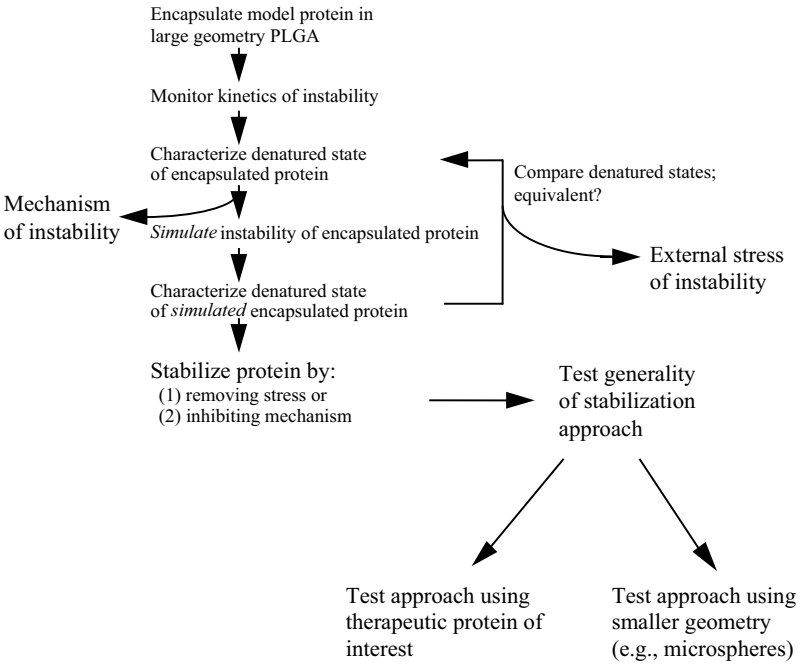

FIGURE 2. An example of a mechanistic experimental paradigm that was used to elucidate the instability of BSA in PLGA delivery systems and to develop general approaches toward minimizing acid-instability of encapsulated proteins. See Zhu et al.[15]

of the acid-induced instability and the antacid stabilization approach was demonstrated by using antacid excipients to improve both the stability of BSA in microspheres and the stability of therapeutic proteins, basic fibroblast growth factor, and bone morphogenetic protein-2 in millicylinders (see below).[15]

VI. STABILIZATION OF PROTEINS WHEN ENCAPSULATED IN INJECTABLE PLGA

During release incubation, the two deleterious stresses most commonly identified as responsible for protein instability are moisture and the build-up of acid in the PLGA pores. The anticipated minor role of adsorption processes are expected to become more noticeable as steadily more proteins are formulated under conditions that prevent moisture- and acid-induced instability. Techniques developed to inhibit these two dominant stresses are described below. For general techniques for minimizing unfolding and chemical protein instability mechanisms (not specific for encapsulated proteins), see Volkin and Klibanov,[35] Timasheff,[83] and Cleland and Langer.[6]

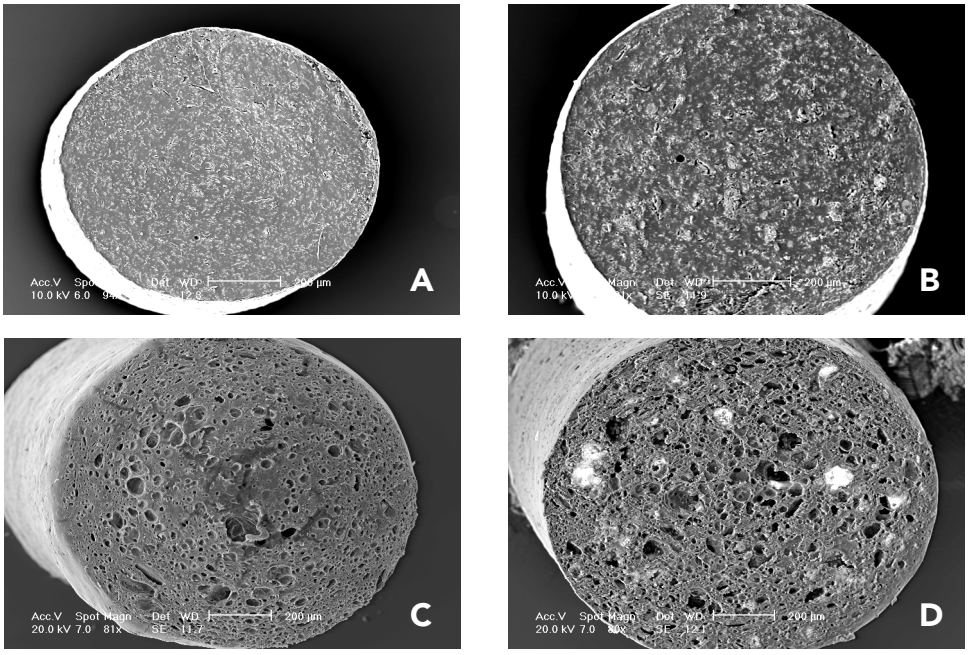

FIGURE 3. Scanning electron micrographs of 15% BSA-loaded PLGA 50/50 millicylinders before **(A, B)** and after **(C, D)** a 7-day incubation in physiological buffer at 37 °C. Polymers were prepared in the presence **(B, D)** or absence **(A, C)** of 3% $Mg(OH)_2$. The polymer formulation containing $Mg(OH)_2$ was among the first examples of protecting a protein from microclimate acidity in PLGAs during release by coencapsulating an antacid. Reproduced from Zhu and Schwendeman,[69] with permission.

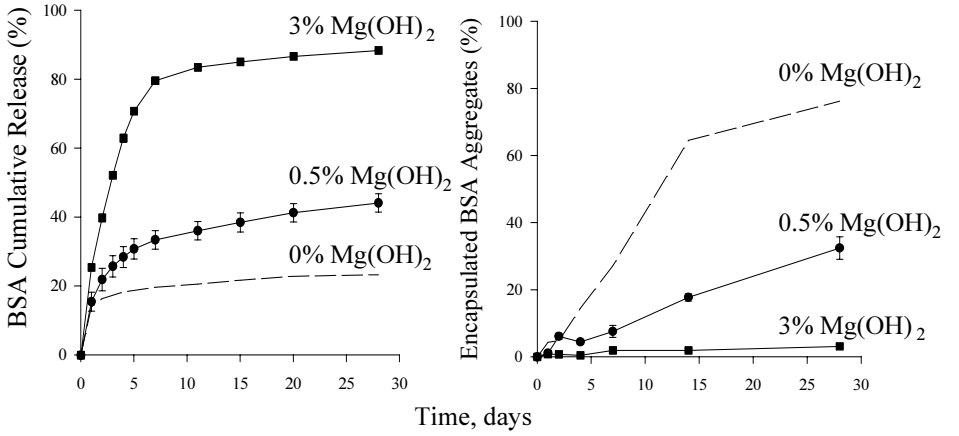

FIGURE 4. Stabilization of BSA encapsulated in PLGA 50/50 millicylinders during in vitro release in a physiological buffer at 37 °C by coencapsulating $Mg(OH)_2$. Incomplete release (left) and noncovalent aggregation (right) were observed in the absence of base, whereas BSA was released nearly completely with negligible aggregation when 3% $Mg(OH)_2$ was added. Data from Zhu et al.[15]

TABLE 3
Matching Simulated with Encapsulated BSA Instability in PLGA Millicylinders[a]

Matching feature	Encapsulated[b]	Simulated[c]
Time to 50% aggregation of BSA	12 days	7 days
BSA aggregates soluble in 6-M urea + 1-mM EDTA	> 98%	> 94%
Peptide fragments observed by reducing SDS-PAGE	25, 40, and 55 kDa	25, 40, and 55 kDa

[a] Data from Zhu et al.[15]
[b] 15% w/w BSA was encapsulated PLGA 50/50 millicylinders; BSA release and encapsulated aggregation was evaluated in a physiological buffer.
[c] Encapsulated BSA instability was simulated by lyophilizing BSA at pH 2 and incubating the solid protein at 86% RH and 37 °C.

VI.A. Inhibition of Moisture-Induced Instability

As discussed previously, if the protein is expected to exist in the solid state within the polymer (see Fig. 1), the protein is remarkably prone to aggregation at intermediate moisture levels, as well as to numerous other deleterious reactions. Several techniques have been developed to successfully bypass the destabilizing stress either by altering the role of water in the solid or immobilizing the protein or, alternatively, by directly inhibiting the mechanism (e.g., aggregation). Clearly, one of the most significant findings in the field of protein stability in polymers is the success of the immobilization strategy of Zn^{2+} precipitation, as performed with human growth hormone (hGH).[14,36,84,85] The 2:1 mole ratio Zn-protein complex, which immobilizes the hGH as a solid precipitate in a near-native state,[80] has been shown to confer superior stability on the protein encapsulated in PLGA for a 1-month release incubation (see Table 4). Since then, other proteins, such as interferon-α[86] and nerve growth factor,[87] have also been stabilized in PLGA by this approach. Another interesting approach originating in the patent literature is the precipitation of erythropoietin with the salting-in salt, ammonium sulfate,[88] which is a technique commonly used in protein processing.

Other methods to alter the role of water in the reaction involve the addition of agents that alter the amount of water sorbed in the polymer and/or the activity of the water present. For example, both water-soluble salts (e.g., NaCl) and antacid excipients (e.g., $Mg(OH)_2$) are known to dramatically increase the amount of water sorbed in PLGAs, with the former due to osmosis and the latter to a complex effect of neutralizing acidic degradation products and end-groups of the polymer (which also involves an osmotic component).[15,69,89] In contrast, for a given moisture content, humectants such as sorbitol, which dissolve in water bound to the protein, reduce available free water necessary to mobilize the protein or perform other roles in deleterious reactions.[48]

The alternative to bypassing the deleterious role of moisture is to inhibit the aggregation mechanism directly. Several examples of ways to accomplish this have been reported, particularly in the solid state and in the absence of the polymer.[43,48,51,90,91] Aggregation frequently accompanies an initial unfolding event,[35] and proteins without additives will most commonly

TABLE 4
Examples of Controlling Moisture Effects and Acidic Microclimate pH to Stabilize Proteins Encapsulated in PLGA Delivery Systems During Release

Approach/protein	Report of stabilization	Reference
Inhibit effects of moisture via complexation of Zn^{2+}		
Growth hormone	98% structure and bioactivity remaining after 28-day release from PLGA 50/50 (uncapped) microspheres[a]	14
Nerve growth factor	95% total protein released over ~21 days; 93% monomer retained after 10 days release from PLGA 50/50 (uncapped) microspheres[a]	87
Inhibit formation of acidic microclimate with antacids		
BSA	Peptide-bond fragmentation and noncovalent aggregation inhibited; > 90% structure retained during 28-day release in PLGA 50/50 millicylinders with 3% $Mg(OH)_2$	15, 69
	Aggregation reduced to 2% to 13% in PLGA 50/50 microspheres with 3% $MgCO_3$ during 28- to 51-day release	15
Hen egg-white lysozyme	74% to 81% enzyme released without activity loss over 52 days from PLGA 50/50/3% $Mg(OH)_2$ millicylinders	111
Ribonuclease A	86% to 88% enzyme released without activity loss over 52 days from PLGA 50/50/3% $Mg(OH)_2$ millicylinders	111
Basic fibroblast growth factor	92% immunoreactive protein recoverable from heparin/ BSA/$Mg(OH)_2$-containing PLGA50/50 millicylinders over 28 day-release; 60% to 70% bioactivity retained over same interval	15
Bone morphogenetic protein	80% to 83% immunoreactive protein recoverable from gum arabic/ or BSA/$Mg(OH)_2$-containing PLGA50/50 millicylinders over 28 day-release	15
Tissue plasminogen activator	100% active protein released after 28 days from millicylindrical PLGA 50/50/3% $Mg(OH)_2$-containing excipients from the manufacturer (i.e., arginine, phosphoric acid, polysorbate 80)	79
Inhibit formation of acidic microclimate by decreasing glycolide content and adding pore-forming agents		
BSA	Full recovery of protein structure without aggregation for 35-day release from 80/20 PLA/PEG microsphere blends.	70

exist in a partially denatured form in the solid-state.[32,33] In this instance, the protein monomer may have insufficient time to refold if aggregation kinetics is rapid. Therefore, lyoprotectants such as sucrose and trehalose are frequently used to help maintain the protein in the native state during the freezing and drying stages of encapsulation.[29,32,33,50,81,92]

The two covalent aggregation mechanisms commonly described during exposure of the solid protein to moisture are disulfide interchange/exchange[52,53,93] and the formaldehyde-mediated aggregation pathway (FMAP), which is operative for protein antigens that have been detoxified with formaldehyde exposure.[43,91] In the former pathway, the reaction is typically initiated by a thiolate ion on the protein or free thiolate ions that accompany β-elimination of an intact disulfide.[7,51] Decreasing the concentration of the reactive species (e.g., lowering pH to favor the nonionized thiol, covalently blocking the thiol group, or oxidizing free thiols as they appear with divalent copper ion) has been shown to block this mechanism.[51] To inhibit the FMAP, strongly formaldehyde-interacting amino acids (e.g., histidine and lysine)[94] have been colyophilized with the formalinized protein antigen. On exposure to moisture, the amino acids appear to bind with the reactive Schiff base (or equivalent electrophile[43]) in the protein before a neighboring protein nucleophile can react to form an intermolecular cross-link.[91] Sorbitol has also been identified to inhibit the FMAP of tetanus toxoid at the maximal aggregating water content of the antigen (~30 g H_2O/g protein),[95] although whether this is a humectant effect[48] or a possible covalent reaction with the highly reactive electrophile in the antigen has not been determined.

VI.B. Bypassing Acid-Induced Protein Instability

The most straightforward method for decreasing acid-induced instability of encapsulated proteins in PLGAs that display an acidic microclimate is to adjust the formulation to raise microclimate pH. Because it is difficult to measure microclimate pH directly in PLGA microspheres,[57–62] some of the most useful conceptual information on how to accomplish a homogeneous microclimate pH increase has been obtained by indirect measurements (e.g., monitoring acid-induced aggregation of BSA[15,65,69,70]). Some additional information is now forthcoming using simple and fast direct measurements with model systems (e.g., thin films coating pH-glass electrodes).[65] For example, the stabilization against insoluble acid-induced noncovalent BSA aggregation in PLGA microspheres afforded by a series of antacid excipients has been shown to correlate with the ability of the antacid to neutralize acidic pores in films of the same lot of PLGA-coating pH glass electrodes.[65] These data and comparisons between simulated acid-induced instability and that occurring in the polymer (Table 3) indicate that the noncovalent aggregation of encapsulated BSA can be used as a marker for the presence of highly acidic pores in the PLGA matrix.

Although the influence of several variables on microclimate pH has been examined, it must be stressed that in essentially all of these cases, in vitro conditions were used. Few in vitro/in vivo correlative studies have been performed,[64,96] therefore, potential differences between microclimate pH in vitro and in vivo remain to be evaluated. As described in Figure 5, three principal ways have been identified thus far to avoid the formation of highly acidic microclimate regions in the PLGAs during protein release, as indicated by the prevention of acid-induced physical aggregation of BSA[99]: (1) increasing the permeability of the polymer to facilitate escape of the water-soluble hydrolytic products of the PLGA polyester,[70] (2) decreasing the degradation rate of the polyester,[70] and (3) coencapsulating additives to neutralize the weak acids formed by PLGA hydrolysis.[15,69] Several examples of the success of this strategy are listed in Table 4. Additionally, elevated initial acid content in the polymer[100] and low frequency of release media exchange[62] are likely to favor a lowering of microclimate pH.

The concept of controlling polymer permeability is difficult because attempts to increase permeability can spoil the controlled-release function of the polymer and cause the encapsulated protein to be released too rapidly. For example, Jiang and Schwendeman[70] increased the permeability of slow-degrading PLA (M_w = 145 kDa) by blending in PEG (M_w = 10 or 30 kDa) at 0%, 10%, 20%, and 30%. Insoluble BSA aggregation in the PLA microspheres containing 4.5% to 5% w/w BSA was found in 0% and 10% PEG after a 1-month incubation, but not in those preparations containing 20% or 30% PEG. Structural integrity of BSA was also intact in the stabilized formulations. However, between 10% and 30% PEG, the release rate of BSA increased rapidly and by 30% PEG, 60% of the protein encapsulated was released in only 3 days.[70] In contrast, Zhu and Schwendeman[99] found that 5% BSA encapsulated in a more permeable PLA (M_w = 77 kDa), BSA formed < 2% insoluble aggregates over 1 month, strongly suggesting that in some instances the slow degradation rate of the non-glycolic-acid–containing PLA is sufficient to inhibit acid formation in the microclimate.

In instances in which it is undesirable to increase permeability and/or to decrease the hydrolytic rate of PLGAs (e.g., where a highly water-soluble protein requires release for 1 month or longer), it becomes necessary to coencapsulate a basic additive. Antacids such as $MgCO_3$, $Mg(OH)_2$, or $ZnCO_3$ have been found to be particularly potent in preventing instability of acid-labile proteins.[15,69,79] Although much of the physical chemistry of microclimate pH adjustment with these additives is unclear, the strength of the base, the base solubility, and the association of the divalent cation with the carboxylate of the degradation

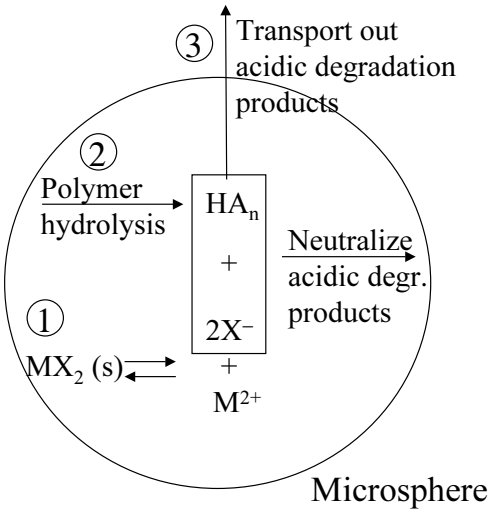

FIGURE 5. Description of formulation strategies for preventing development of highly acidic pores in PLGA microspheres: (1) addition of an antacid excipient, which dissolves in response to the liberation of acid degradation products; (2) decrease polymer hydrolysis (e.g., by using higher lactide content); and (3) increase transport of acidic degradation products by increasing the permeability of the polymer (e.g., by blending poly(ethylene glycol) in with the PLGA).

products and/or polymer endgroups appear to be important.[15,89] For example, the use of $Mg(OH)_2$ was found to be optimal for stabilization of BSA in millicylindrical PLGA 50/50 implants, whereas $Ca(OH)_2$ was found to be too strong a base (as indicated by an increase in disulfide-bonded aggregation favored at neutral to basic pH) and $ZnCO_3$ too weak (as indicated by increased noncovalent aggregation favored at acidic pH).[69] From microclimate pH measurements in PLGA films coating pH glass electrodes, $MgCO_3$ and $Mg(OH)_2$ were found to be very similar under conditions that favor homogeneous neutralization (that is, high protein loading sufficient to make pores for the base to diffuse to all regions of the polymer matrix), but $MgCO_3$ was found to increase microclimate pH higher than $Mg(OH)_2$ when no protein was added.[71] This latter result was consistent with the improved BSA stability in PLGA 50/50 microspheres when $MgCO_3$ was used in place of $Mg(OH)_2$[15]

Finally, the concept of "homogeneous" microclimate neutralization evolved from (1) confocal micrographs, indicating both neutral and highly acidic regions in PLGA microspheres that contained antacid and no protein; [60] and (2) the increase in acid-induced instability of BSA as protein loading was decreased.[69] These two pieces of evidence indicated that for "homogeneous" neutralization of acidic pores to occur with antacid additives, a percolating cluster of aqueous pores in the polymer connecting both protein and base were necessary (see Zhu and Schwendeman[69]).

VII. CONCLUDING REMARKS

Over the past several years, several important experimental findings and careful analysis of the physical–chemical events occurring in PLGA delivery systems have revealed important principles that have converted a previously ubiquitous problem of encapsulated protein instability into one that is much more manageable. Stabilizing proteins encapsulated in PLGA delivery systems has now become a realizable goal. It is now understood how to minimize instability of proteins during encapsulation, and the use of anhydrous encapsulation methods is expected in increase in the future. During release, the ability to prevent deleterious reactions by complexation with a multivalent metal ion and the role of the dynamics of moisture and microclimate pH in the PLGAs have become clearer, as have techniques evolved to monitor and control them. Currently, the ability to control the release of proteins for over a month has been attained with minimal loss of structure and/or activity. The next benchmarks will be to develop new stabilization approaches to further minimize these losses and to expand delivery time to 3 months, as has been accomplished for delivery of peptides from the PLGAs.[17,18]

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