

MINIREVIEW

Chemical Degradation of Peptides and Proteins in PLGA: A Review of Reactions and Mechanisms

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ABSTRACT: Biodegradable poly(lactide-co-glycolide) (PLGA) polymers have been studied extensively for the controlled release of peptide and protein drugs. In addition to polymer biodegradation, chemical degradation of the incorporated peptide/protein has also been reported in PLGA devices, and the role of the polymer in promoting these reactions has been debated. This review summarizes the peptide/protein chemical degradation reactions that have been reported in PLGA systems and their mechanisms. Reported methods for stabilizing peptides and proteins in PLGA devices are also discussed. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 97:2395–2404, 2008

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INTRODUCTION

Peptide and protein drugs are the most rapidly growing class of pharmaceuticals due to their increased specificity, lower toxicity, and decreased risk of drug–drug interactions compared to traditional small molecule drugs.¹ However, the stability and delivery challenges associated with these drugs have limited the number of marketed products. Maintaining adequate shelf-life of peptide and protein drugs often requires solid-state formulation to limit hydrolytic degradation reactions.² Drug delivery requires parenteral formulation to avoid degradation in the digestive tract and first pass metabolism, while the short circulating half-lives of peptides and proteins contribute to the need for parenteral formulations that will reduce dosing frequency.³ These stability

requirements, together with the desire for patient comfort and compliance, often lead formulators to develop long acting or controlled release products.

Poly(lactide-co-glycolide) (PLGA) is the most commonly used FDA approved polymer for biodegradable and biocompatible controlled release devices. The polyester undergoes hydrolysis to produce lactic and glycolic acid monomers, cleared by the Krebs cycle. Yet the same characteristics that make PLGA amenable to controlled release formulations can lead to peptide and protein instability. The accumulation of carboxylic acid oligomers and monomers formed upon hydrolysis produce an acidic microclimate with in the matrix, initiating acid catalyzed peptide degradation. The hydrolyzable PLGA ester bonds are also subject to nucleophilic interactions with incorporated drugs.

This review summarizes the chemical degradation reactions that have been reported in protein/PLGA systems. These include the hydrolysis of PLGA itself, together with the chemical degradation reactions of peptides and proteins that occur in PLGA during storage and drug release. The

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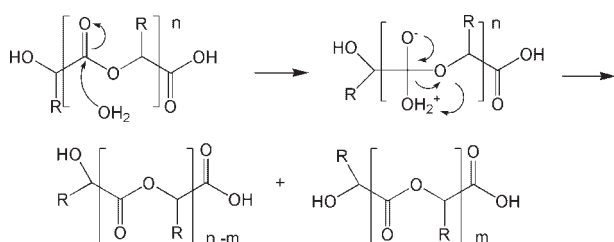
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field has advanced from an initial concern with the reactions of PLGA (i.e., polymer biodegradation), to an awareness that proteins can degrade *in* PLGA (e.g., hydrolytic degradation reactions), and more recently to the realization that proteins can react *with* PLGA (e.g., acylation). The review begins with a brief review of the mechanisms of PLGA hydrolysis, and then presents the peptide/protein degradation reactions that have been reported in PLGA systems. Methods for controlling peptide/protein degradation in PLGA are also discussed. This review is complementary to earlier reviews on protein drug stability in PLGA systems, which emphasized methods of stabilization,⁴ protein instability in PLGA microparticles during manufacturing and storage,⁵ and the formulation of stable protein antigens in PLGA.⁶ The interested reader is referred to these reviews for additional information on these topics.

PLGA Hydrolysis

PLGA is initially a hydrophobic polymer, necessitating the use of organic solvents for formulation. Upon exposure to either aqueous solution or vapor,⁷ the ester carbon is subject to nucleophilic attack by water, as shown in Scheme 1. Hydrolysis proceeds via a tetrahedral intermediate, producing both a primary alcohol and a carboxylic acid. Esters are susceptible to hydrolysis because the electron withdrawing effects of the attached oxygen atoms result in a partially positive ester carbon. Delocalization of electrons between the two oxygen atoms provides some stabilization of the ester carbon relative to anhydrides and acyl halides; however this delocalization is not as effective as that observed in amide bonds. The accumulation of carboxylic acid hydrolysis pro-



Scheme 1. PLGA ester hydrolysis mechanism. R groups represent CH_3 for lactide and H for glycolide. The resulting alcohol can abstract a proton from the carboxylic acid during the chain cleavage step as shown, or the protonation of the alcohol and deprotonation of carboxylic acid can be facilitated by water.

ducts in PLGA matrices results in autocatalysis of the hydrolysis reaction.⁸ This proceeds by carbonyl oxygen protonation, which facilitates the reaction by stabilizing the tetrahedral intermediate. In addition to autocatalysis, other factors can affect the rate of PLGA hydrolysis, and can therefore be modified to change the rate of drug release from the matrix.

One of the simplest ways to adjust the rate of PLGA hydrolysis is to alter the ratio of lactic to glycolic acid. Glycolic acid is slightly more hydrophilic than lactic acid, leading to increased hydrolysis rates with increased glycolic acid content.^{9–12} Typical ratios of lactide to glycolide range from 50:50 to 100:0, respectively. This ratio, as well as the lactic acid enantiomer composition, may also affect hydrolysis rates by altering the degree of polymer crystallinity.¹³ The effects of both the average molecular weight (M_w , weight average or M_n , number average) and polydispersity (PD, M_w/M_n) on PLGA hydrolysis are well known. Molecular weights used typically range from 10 to 100 kDa. Because PLGA degrades by bulk erosion, the higher molecular weight, larger particle size formulations allow acidic monomer and oligomer hydrolysis products to accumulate.^{14,15} Thus, high molecular weight PLGA experiences more rapid hydrolysis after an initial lag phase due to autocatalysis.^{14,16} The effect of PLGA polydispersity (PD) on hydrolysis rates is also related to autocatalysis. Most manufactured PLGAs have PD values from 2 to 3. As PD decreases, fewer PLGA monomers and oligomers are expected in the initial formulation, lowering the propensity for autocatalysis.^{13,17} In practice, however, since the PD value may be relatively insensitive to small amounts of low molecular weight oligomers that may have a much greater effect on acidity, PD values may be poorly correlated to autocatalysis.¹⁸

PLGA is available with either carboxylic acid or ester-capped end groups. The hydrolysis rate of PLGA depends upon end group chemistry for two reasons.¹⁹ First, carboxylic acid end groups make the polymer more hydrophilic, leading to an increase in water uptake, and therefore, hydrolysis. Second, carboxylic acids within the PLGA matrix can increase autocatalysis of the ester bond degradation. Thus, esterified end groups decrease PLGA hydrolysis rates.

The microclimate pH of PLGA devices is intrinsically related to the concentration of water soluble carboxylic acid end groups present in the degrading matrix.^{20,21} During the initial stage of

PLGA degradation, only water soluble monomers and dimers have been detected in the aqueous pores of PLGA microspheres.²¹ As degradation progresses to bulk erosion, larger water soluble oligomers have also been identified,²¹ and a lower core pH measured.²² The internal pH of PLGA matrices has been studied extensively, and many methods have been developed to measure the pH of degrading matrices, including the use of pH sensitive dyes,^{22,23} analysis of pH sensitive spin probes by electron paramagnetic resonance,²⁰ potentiometric μpH detection,^{8,21} and the use of ³¹P and ¹³C NMR probes.^{24,25} The microclimate pH has been estimated in the range of 1.5–3, leading to acid catalysis of PLGA hydrolysis.^{8,22} Though the minimum pH has been a topic of debate, ultimately the microclimate pH depends upon the ability of the monomer and oligomer hydrolysis products to diffuse from the matrix.¹⁴ A pH-rate profile developed for the hydrolysis of lactic acid oligomers shows that the pH of maximum ester stability is ~ 4 , and that ester hydrolysis is subject to both acid and base catalysis.¹¹

Chemical reactions in PLGA matrices are complicated by physical heterogeneity, and by the potential for phase separation and nonuniform distribution of solutes among phases or domains. Though the polymer itself is hydrophobic, aqueous domains or “pores” are created on exposure to water, in part by the leaching of residual solvent.²⁶ Hydrolysis of the polymer produces hydrophilic oligomers which distribute preferentially into the aqueous region.²¹ Incorporated peptide and protein drugs may also be distributed nonuniformly between hydrophobic (i.e., polymer rich) and hydrophilic (i.e., aqueous) regions. As polymer biodegradation proceeds, polymer hydrophilicity increases, affecting the distribution of solutes and the hydrophobic/hydrophilic domains themselves. A consequence of the heterogeneity of PLGA systems is a lack of definition regarding the local chemical environment for both polymer and protein degradation reactions, though recent modeling efforts have begun to address this.²⁶

Peptide and Protein Chemical Stability in PLGA Matrices

Several possible sources of peptide and protein drug instability in PLGA have been proposed, including the acidic microclimate pH produced by the accumulation of acidic monomers and

oligomers during PLGA degradation,^{22,27,28} the lability of electropositive PLGA ester carbons,^{29,30} the use of organic solvents in formulation development,^{5,31} high protein mobility in the solid associated with elevated moisture levels,²⁸ and protein adsorption to the polymer surface.²⁸ While these factors may influence both the chemical and physical stability of incorporated proteins, their effects on chemical degradation reactions are the focus of this review. The peptide and protein degradation reactions that have been reported in PLGA systems include acid-catalyzed reactions, such as deamidation and chain cleavage, drug/polymer acylation reactions and protein denaturation and aggregation. Chemical degradation is residue specific and therefore controlled by the primary sequence of peptides and proteins and by the higher order structure.

Deamidation

Deamidation is acid-catalyzed in aqueous solution³² and in solids composed of hydrated poly(vinyl pyrrolidone),³³ a hydrophilic polymer that is not subject to hydrolytic degradation. Thus, it is reasonable to expect that deamidation will contribute to peptide and protein chemical instability in PLGA matrices due to the acidic microclimate pH. The established acid and base catalyzed solution deamidation mechanisms are shown in Scheme 2. In acidic conditions, deamidation proceeds via direct hydrolysis of the side-chain amide bond, with the nucleophilic attack of water on the amide carbon as shown in Scheme 2a. An acidic environment facilitates this reaction by protonating the amide nitrogen, making it a better leaving group. The reaction is similar to the ester hydrolysis of PLGA discussed earlier, in that it involves the attack of water on a carbonyl. In aqueous solution, the rate of ester hydrolysis is two orders of magnitude greater than that of amide bonds.³⁴ This suggests that at later stages of PLGA degradation, once polymer oligomers are solubilized in the aqueous pores or domains,²¹ the hydrolysis of PLGA ester bonds is kinetically favored over peptide deamidation in acidic PLGA matrices. The mechanism of deamidation in neutral to basic solutions, shown in Scheme 2b, involves the intramolecular nucleophilic attack of the backbone amide nitrogen on the side-chain amide carbon to produce a tetrahedral intermediate, which degrades to form a cyclic imide. While the cyclic imide is acid stable, in basic solution it is hydrolyzed to iso-aspartic acid and aspartic

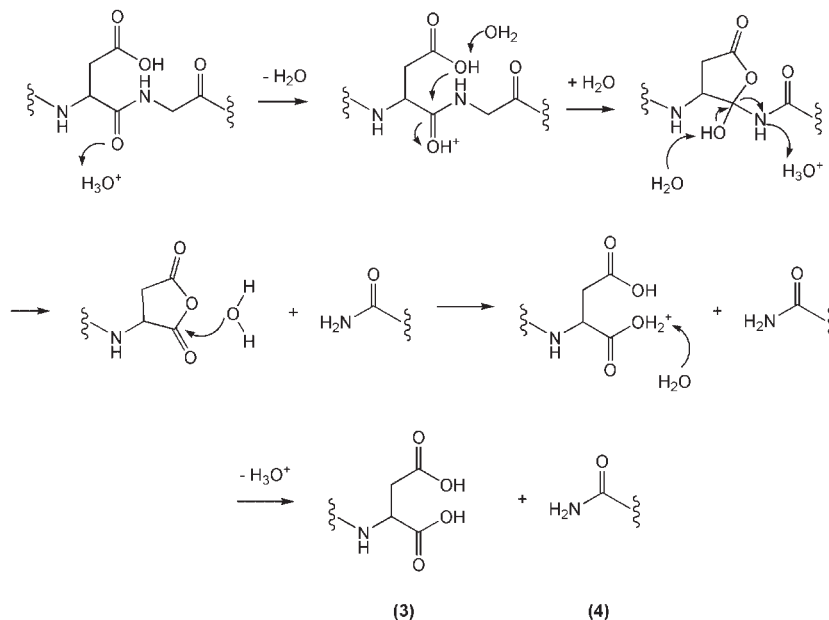
PLGA hydrolysis, as controlled by RH,³⁵ but attempts to correlate deamidation to PLGA hydrolysis controlled by other means (i.e., monomer ratios, molecular weight, end group modification) have not been reported.

Amide Bond Hydrolysis

Acid catalyzed amide bond cleavage is also a common source of peptide chemical instability in PLGA formulations.^{35,39–41} Amide bond cleavage is often observed after aspartic acid (Asp) residues. The mechanism of Asp-Xxx hydrolysis has been established in both solution and polymeric solids as the acid catalyzed intramolecular attack of the Asp side chain on the C-terminal amide nitrogen^{32,42} as shown in Scheme 3. The reaction is acid catalyzed by the protonation of the backbone carbonyl oxygen, which acts to stabilize the tetrahedral intermediate. The cyclic anhydride formed upon chain cleaving rearrangement is unstable. Although the center oxygen can stabilize the anhydride through delocalization of its *p*-orbital electrons, this effect is shared by both acyl oxygens, resulting in less stabilization than is experienced by ester or amide groups. Hydrolysis of either acyl bond produces a C-terminal aspartic acid residue. As in the PLGA hydrolysis and peptide/protein deamidation reactions, the rate-limiting step involves nucleophilic attack on a

carbonyl carbon. Due to the similarities with the deamidation reaction, the rate of chain cleavage is expected to be similar to that of deamidation in PLGA systems. This relationship between deamidation and cleavage reactions has been observed for a model peptide in PLGA films.³⁵ In this report, the concentrations of products produced by these two degradation reactions were comparable for all accelerated storage conditions.

The extent of peptide and protein chain cleavage in PLGA matrices depends on the microclimate pH of the PLGA matrix, as the reaction is not observed above the pK_a of the aspartic acid side-chain ($pK_a = 3.9$).⁴² Thus, chain cleavage at Asp in PLGA requires the accumulation of PLGA hydrolysis products.^{40,43} Chain cleavage at Asp has been observed in carbonic anhydrase upon encapsulation in PLGA microspheres.^{40,41,43} This protein contains two Asp-Pro sequences, which are particularly susceptible to cleavage due to the increased basicity of the proline nitrogen relative to other amino acids.³² During a release study at 37°C and pH 7.4, ~25% of the initial carbonic anhydrase experienced chain cleavage after 1 week.⁴¹ The deamidated model peptide VYPDGA also experienced cleavage at the Asp-Gly bond, reaching a maximum of ~40% of the initial peptide concentration after storage at 95% RH and 70°C for 5 days.³⁵ A decrease in peptide chain cleavage was



Scheme 3. Asp-catalyzed amide chain cleavage mechanism, as determined in solution³² and solid-states.⁴² (3)—N-terminal cleavage product (4)—C-terminal cleavage product.

In controlled released studies conducted at 37°C in pH 7.4 release buffer, ~45% of atrial natriuretic peptide (ANP) and ~10% of salmon calcitonin (sCT) encapsulated in 50/50 PLGA microspheres were acylated after 28 days.²⁹ Octreotide experienced ~24% acylation under similar conditions after 56 days.³⁰ The specificity for either lactide or glycolide adduct formation^{10,29,35,48} as well as the specificity for reaction with PLGA monomers²⁹ *versus* larger oligomers³⁵ are still matters of debate. While some reports have shown strong preference for lactoyl addition,^{29,35} others show preference for glycoloyl addition.^{10,48} Steric hindrance of the lactide methyl group¹⁰ or steric hindrance of the N-terminal amino acid side chain³⁵ have been proposed as causes of this selectivity. Like PLGA hydrolysis, increased PLGA molecular weight and glycolide content have been associated with increased acylation.¹⁰

Other Reactions

Oxidative reactions have also been documented in PLGA formulations, though the rates and mechanisms of these reactions have not been correlated to chemical characteristics of the polymer. Methionine oxidation of rhGH thiol ether to sulfoxide was observed during release in pH 7.4 buffer at 37°C. The amount of oxidation correlated to that observed in a pH 7.4 solution study, which the authors interpreted as an indication of neutral pH in the microspheres.³⁸ Previous reports suggest that methionine oxidation is favored at acidic pH.⁵⁰ However, a recent report showed that methionine oxidation dependency in proteins is actually the result of secondary structure changes that occur upon changes in formulation pH.⁵¹ Therefore, oxidation rates may increase if the PLGA acidic microclimate pH induces protein unfolding, resulting in greater solvent exposure of reactive methionine groups. Disulfide bond exchange has also been observed in PLGA matrices. This reaction has led to covalent aggregation of formalinized BSA (f-BSA)⁵² and BSA in PLGA microspheres.^{39,53} The role of PLGA in this process was proposed to result from nonspecific adsorption of the protein to the polymer,³⁹ however, more recent studies have shown that BSA aggregation is the result of a reduced microclimate pH in PLGA microspheres. Degradation products identical to those extracted from PLGA can be produced in pH 2 aqueous solution.⁴⁵

Controlling Peptide and Protein Degradation

Much of the recent PLGA literature has been devoted to modifications of PLGA formulations to improve the stability of incorporated peptides and proteins. The types of modifications can be separated into two categories: pH modifiers and covalently linked modifiers. The most commonly used PLGA pH modifiers have been weakly basic salts, such as calcium carbonate,^{54,55} magnesium hydroxide,^{28,55-57} and zinc carbonate.^{55,56} In general, these salts serve to buffer the PLGA matrix as lactic and glycolic acid monomers and oligomers accumulate. The degree of pH control depends on many factors, including the concentration of salt, its dissociation constant, and its relative solubility in either the polymer or aqueous phase.^{54,55} Concentration is particularly important because use of pH sensitive fluorescent probes has shown that the buffering effect is heterogeneous,⁵⁷ probably due to poor salt solubility in the hydrophobic polymer.⁵⁵ Inclusion of basic salts has two notable and seemingly counterbalancing effects on the polymer formulations. First, PLGA hydrolysis is reduced because buffering reduces acid catalysis in this reaction^{28,57} and buffering of the aqueous pores reduces autocatalysis as well.^{54,55} It has also been noted that ionic complexes between the polyesters and metal counter ions could reduce the solubility of the PLGA oligomers, further reducing the rate of hydrolysis.⁵⁵ Second, the presence of salts (basic or neutral, such as sodium chloride) increases the water absorption of PLGA matrices,^{57,58} which could promote hydrolysis. However, because the increased water uptake is localized in the aqueous pores, little effect on the degradation of the polymer has been observed.⁵⁸

The effect of pH buffering salts on peptide stability is also highly dependant upon salt concentration and dissociation constant. Low salt concentrations are ineffective at preventing acid catalyzed degradation once the concentration of lactic and glycolic acid monomers exceeds salt concentration⁵⁶ or can allow for regions of acidic pH to persist due to heterogeneous buffering.⁵⁷ Highly dissociative hydroxide salts (i.e., magnesium hydroxide and calcium hydroxide) can also create an initially basic environment, leading to base catalyzed peptide degradation.^{28,56} This effect is especially important when attempting to stabilize deamidation reactions, as the rate of base catalyzed deamidation is even greater than that of acid catalyzed deamidation.³³ An increase

in deamidation of a model peptide was observed in PLGA films containing 3% w/w magnesium hydroxide, resulting in the deamidation of ~80% of the peptide *versus* only ~25% deamidation in unmodified PLGA films.²⁵ Base catalysis was confirmed by the production of iso-aspartic acid, a product not observed in unbuffered control studies.

An alternate method of pH stabilization that has received little attention is the incorporation of proton scavengers or "sponges." Proton sponges typically contain basic amines, and have the potential to prevent the drop in pH associated with PLGA hydrolysis products without increasing the pH of the formulation. Proteins with a pI lower than that of the drug have been employed as proton scavengers to reduce acid catalyzed peptide degradation by ~40%.⁵⁹ A highly substituted basic amine has also been used to preferentially absorb protons resulting from PLGA hydrolysis, limiting both deamidation and chain cleavage reactions.²⁵ Regardless of the effectiveness of pH modifiers, these additives do not prevent acylation reactions. In fact, they have been observed to increase acylation by improving the nucleophilicity of primary amines at neutral to basic pH,²⁵ since the neutral amine is a more effective nucleophile than the protonated form.

Recent work has focused on preventing acylation by PEGylation of a peptide drug to prevent covalent interaction.^{48,60} Although the octreotide used in these studies contained a lysine group, PEGylation at the N-terminus alone was sufficient to prevent acylation at both amines. This result was attributed to the higher propensity of the N-terminal amine to act as a nucleophile due to differences in pK_a , as well as increased steric hindrance of peptide/polymer interactions upon PEGylation.^{48,60} The PEGylation of this peptide did not reduce its biological activity.⁶⁰ Other possible benefits of PEGylation include increased circulation times upon delivery and reduced immunogenicity.⁶¹ Like PEGylation, N-terminal acetylation has been used to prevent nucleophilic reactions between peptides and polymers in other systems.⁶² Further research in this area is needed to determine whether other chemical modifications can prevent acylation in PLGA.

SUMMARY AND CONCLUSIONS

PLGA is attractive for peptide and protein drug delivery because it is biodegradable and the rates

of hydrolysis can be tailored to control drug release rates. The susceptibility of PLGA ester bonds to nucleophilic attack and the accumulation of polymer hydrolysis products can lead to peptide and protein chemical instability, however. PLGA hydrolysis, deamidation at Asn residues, peptide bond hydrolysis and acylation of protein primary amines (i.e., N-terminus, Lys) occur for peptides and proteins in degrading PLGA systems. These reactions all involve labile ester and amide bonds and the nucleophilic attack of water or primary amines. There have been few reports of oxidative degradation of peptides and proteins in PLGA. Incorporation of pH modifying salts and covalent protection of peptide and protein reactive functional groups has been effective in controlling degradation.

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