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Deamidation, acylation and proteolysis of a model peptide in PLGA films

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Abstract

The relative rates of deamidation, acylation and proteolysis (i.e. amide bond cleavage) were determined for a model peptide (VYPNGA) in poly (DL-lactide-co-glycolide) films. Films were stored at 70 °C and either 95%, 75%, 60%, 45%, 28%, or ~0% relative humidity and at 37 °C and 95% relative humidity. Peptide degradation products were identified by ESI+MS/MS and quantitated by LC/MS/MS. Extensive overlap of degradation mechanisms occurred, producing a complex mixture of products. Acylation was the dominant peptide degradation reaction (10–20% of total peptide) at early stages of PLGA hydrolysis and at intermediate relative humidity (60–45% RH). Deamidation and proteolysis were dominant (25–50% and 20–40% of total peptide, respectively) at later stages and at high relative humidity (95–75% RH). Understanding the relative rates of each peptide degradation reaction will allow for improved design of PLGA formulations that preserve the stability of peptide and protein drugs.

Keywords: PLGA; Deamidation; Acylation; Proteolysis; Peptide stability

1. Introduction

Peptide and protein drugs have had the highest rate of sales growth of any drug class in the last decade and more than forty new protein drugs have entered the market in the last twenty years [1,2]. This rate of growth is expected to continue, with the number of peptide active pharmaceutical ingredients (APIs) projected to increase at nearly double the rate of traditional, small molecule APIs [2]. Parenteral routes of administration, including polymeric controlled release devices, provide an attractive means of delivering intact protein drug by circumventing degradation in the G.I. tract and first pass metabolism by the liver [3]. Poly (lactide-co-glycolide) (PLGA), one of the few polymers approved by the FDA, has many desirable properties for protein controlled release. PLGA, a polyester, is both biodegradable and biocompatible. Cleavage of the ester bonds produces lactic and glycolic acid, which are easily metabolized. Polymer degradation and release rates can be tailored by altering the lactide to glycolide ratios and the polymer molecular weight [4,5]. These properties have made PLGA an attractive vehicle for protein and peptide delivery, leading to extensive research into PLGA encapsulation of protein drugs in a wide variety of delivery systems [5–12].

Despite these advantages, incorporation of protein drugs in PLGA often results in incomplete release from controlled release devices, as well as physical and chemical degradation of the drug [5,10,13]. These instabilities have been attributed to the production of free carboxylate groups upon the hydrolysis of PLGA, producing a reduced pH microclimate within the device [13,14]. This acidic environment accelerates acid-catalyzed degradation reactions such as deamidation and the hydrolysis of peptide bonds [15,16]. Physical and chemical interactions between PLGA and incorporated proteins have also been proposed as contributors to incomplete drug release [17–19]. Reaction of protein primary amines with ester or carboxylate groups via acylation has been identified as a specific source of these protein–polymer interactions [20–22].

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Attempts have been made to control pH within PLGA formulations by including buffers and basic salts [5,23,24], but these techniques may not prevent degradation reactions that are both acid and base labile, such as deamidation. Other additives and peptide modifications have been implemented to prevent physical interactions between the PLGA and encapsulated protein drugs, with moderate success [8,9,18,25,26].

The studies reported here address the chemical stability of a model hexapeptide (VYPNGA) in PLGA films during storage. The peptide is subject to deamidation at the Asn (N) residue in acidic solution and in rubbery and glassy amorphous solids [27–31]. Our studies show that cleavage of the peptide bond and acylation also occur, and that the appearance of the products of the three reactions (deamidation, acylation, peptide bond hydrolysis) depends on time and storage conditions.

2. Materials and methods

2.1. Materials

Val–Tyr–Pro–Asn–Gly–Ala (VYPNGA) (Asn-hexapeptide) was synthesized by American Peptide Company, Inc. (Sunnyvale, CA). All amino acids, except Gly, were in the L-configuration. Peptide purity was determined by RP-HPLC to be >95.0%. 50/50 Poly (DL-lactide-co-glycolide) (PLGA) was purchased from Absorbable Polymers International (Pelham, AL). The inherent viscosity of PLGA was 0.58 dL/g and the molecular weight was 75.4 kDa. Acetonitrile, acetone, and buffer salts were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium acetate, formic acid, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). All water was deionized and purified using a Millipore MILLI-Q™ water system.

2.2. Film preparation and storage

Films containing VYPNGA in PLGA were prepared by dissolving 100 mg/mL polymer and 300 μg/mL peptide in acetone and sonicating for 30 min to enhance dissolution. A 0.5 mL aliquot of the solution was then pipetted onto 22×22 mm glass coverslips and allowed to dry for approximately 18 h. Accelerated degradation studies were conducted at 70 °C and either 95%, 75%, 60%, 45%, 28%, or ~0% relative humidity (% RH) achieved using K_2SO_4, NaCl, KI, NaBr, $MgCl_2$ saturated salt solutions and anhydrous calcium sulfate, respectively. An additional study was conducted at 37 °C and 95% RH. Triplicate samples were removed from storage at appropriate times based upon the expected degradation rate of the parent peptide. Upon removal, films were dissolved in 2 mL of a solution containing 25% (v/v) acetonitrile, 0.05 M ammonium acetate, and 0.1% (v/v) TFA (pH=4.5). To remove any PLGA precipitate, 0.5 mL aliquots were centrifuged for 3 min using Gelman Sciences Nanosep GHP 0.45 μm filters (Pall Corp., East Hills, NY). Prior to analysis, 100 μL aliquots of the sample mixture were spiked with 10 μL of a 200 μL/mL stock solution of internal standard, VYPAGA.

2.3. Sample Analysis

2.3.1. ESI MS/MS

Product identification was accomplished by precursor and product ion scans of the mass of the VYPNGA and its known degradation product masses. Prior to MS/MS analysis, the sample films were dissolved in 0.5 mL acetonitrile. To remove high molecular weight polymer fragments, 0.4 mL aliquots were centrifuged for 30 min using Ultrafree-MC 5K NMWL filters prior to injection (Millipore, Billerica, MA). The spectra were acquired using positive mode electrospray ionization (ESI+) tandem mass spectroscopy on a Micromass Quatro Micro Mass Spectrometer (Manchester, UK) using Mass Lynx software (version 4.0). The sample was infused at a flow rate of 20 μL/min, with a capillary voltage of 3.5 kV, a source temperature of 150 °C and a cone voltage of 20 V. The MS/MS spectrum of ESI-MS ions was produced by collision-induced decomposition (CID), using argon as the collision gas with a collision energy of 45 eV. Both precursor and product scans were performed at an average rate of 1 sec per 100 m/z.

2.3.2. LC/MS/MS

Chromatographic separations were performed using a Waters 2690 HPLC system (Milford, MA) with an Econosphere C18 reversed-phase column (4.6×250 mm, 5-μm particle size; Alltech Associates, Inc., Deerfield, IL). The column was maintained at 25 °C and the autosampler at 15 °C. 50 μL volumes were injected. The total flow rate was 1.0 mL/min. Mobile phase A consisted of 0.05 M ammonium acetate, 1% (v/v) acetonitrile and 0.1% (v/v) formic acid. Mobile phase B consisted of 0.05 M ammonium acetate, 30% (v/v) acetonitrile, and 0.1% (v/v) formic acid. Both mobile phases were filtered using 0.45 μm nylon filters (GE Osmonics, Inc., Minnetonka, MN) and sonicated prior to use. The gradient used for separation was 100% A from 0 to 5 min, followed by a linear gradient that increased the concentration of B from 0% to 100% B from 5 to 20 min. The concentration of B was maintained for 1 min, then returned from 100% to 0% B over the next 5 min. The column was allowed to reequilibrate for 10 min prior to the next sample injection. The HPLC elution stream was split, sending 0.35 μL/min to the mass spectrometer and the rest to waste.

Detection was performed using ESI+ tandem mass spectroscopy. Ionization parameters were as described above, with the exception of collision energy, which was reduced to 30 eV. Multiple reaction monitoring (MRM) experiments were performed with a dwell time of 200 ms per compound. The following transitions were monitored: VYPNGA (Asn-hexapeptide), m/z 620.3>358.2; VYPDGA (Asp-hexapeptide), m/z 621.3>359.2; VYPUGA (Asu-hexapeptide), m/z 603.3>341.1; VYPD (tetrapeptide), m/z 493.3>231.1; Lactoyl–VYPUGA (Lac–Asu), m/z 675.15>603.35; Lactoyl–VYPDGA (Lac–Asp), m/z 693.3>621.3; Glycoloyl-VYPUGA (Gly–Asu), m/z 661.2>603.35; Dilactoyl–VYPUGA (Lac-2-Asu), m/z 747.15>603.35; Glycine (G), m/z 76.0>30.0; Alanine (A), m/z 90.0>44.0; Glycine–Alanine (GA), m/z 147.4>90.0; Proline (P), m/z 116.02>70.0.

2.3.3. Mass spectroscopy calibration

Data were analyzed using MassLynx 4.0 software (Waters, Milford, MA). VYPAGA was used as an internal standard in all separations (m/z 577.2>315.2). Standard curves were determined using a linear regression analysis of the peak area ratios of VYPNGA, VYPDGA, GA, glycine, alanine, and proline to the internal standard, VYPAGA. The limits of detection for these standards are 0.8, 1.4, 12, 3.9, 1.2, and 4.9 μg/mL, respectively.

Calibration standards were not available for the cyclic imide hexapeptide, the tetrapeptide, and the acylated degradation products. Quantitation of the Asu-hexapeptide and tetrapeptide with UV detection allowed for indirect mass spectroscopy calibration. The concentrations of the Asn-hexapeptide, Asu-hexapeptide and tetrapeptide are proportional to peak area when detected by UV absorption at 214 nm [27,28,30]. Using UV detection, the concentrations of the Asu-hexapeptide and tetrapeptide were calculated at 5 separate time points (samples run in triplicate). Concentration (μg/mL) versus LC/MS/MS peak area was plotted for the Asn-hexapeptide, Asu-hexapeptide and tetrapeptide. Using Origin 7.0 (Microcal Software, Inc., MA) an F-test was performed, which showed that with a linear fit, the cyclic imide and tetrapeptide datasets were not statistically different from the Asn dataset at the 0.05 significance level. Thus, the Asu-hexapeptide and tetrapeptide concentrations were calculated based upon Asn-hexapeptide LC/MS/MS calibration for all subsequent separations.

N-acetylated Asn-hexapeptide was initially tested as a calibration standard for the acylated degradation products, but rejected due to low ionization efficiency. Concentrations of all acylated products were estimated based upon Asn-hexapeptide calibration. It is assumed that this data analysis results in an underestimation of acylated product concentration due to the presumed decreased ionization efficiency of the more hydrophobic acylation products relative to the Asn-hexapeptide.

2.4. Data analysis

Plots of % Asn-hexapeptide remaining versus time were fit to a first-order rate equation. The following equation was used to calculate the observed degradation rate constant (k_{obs}):

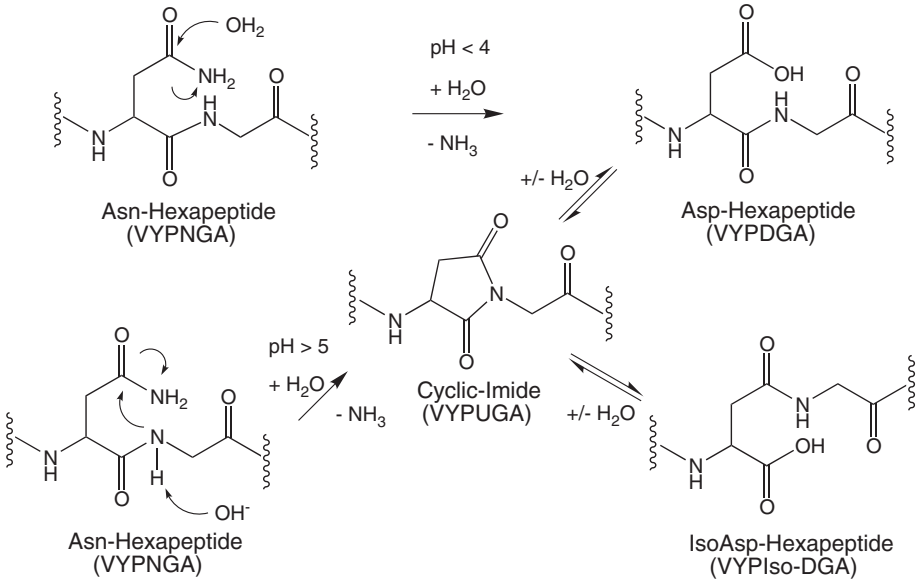
$$A = A_o e^{(-k_{obs}t)} \quad (1)$$

where A is the concentration of the parent peptide (VYPNGA) at time t, and A_o is the initial concentration of the peptide. Nonlinear regression was performed using Origin 7.0 with instrumental error weighting (Microcal Software, Inc., MA). All data was normalized as a percent of the initial Asn-hexapeptide concentration.

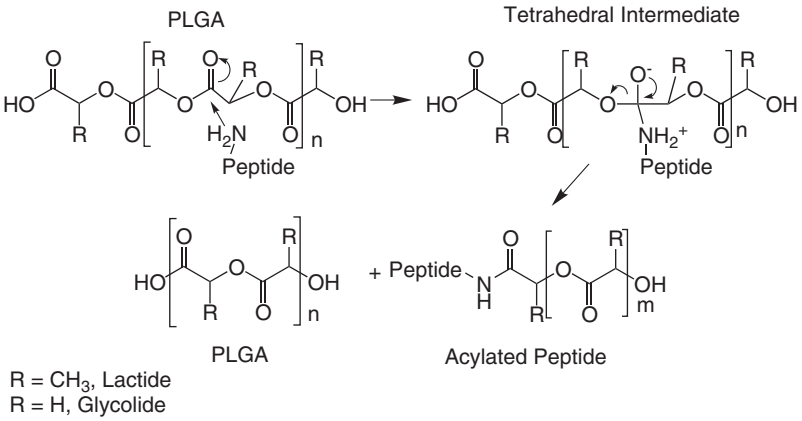
3. Results and discussion

3.1. Identification of degradation products

VYPNGA is subject to three types of chemical degradation when encapsulated in PLGA: deamidation, acylation, and amide bond cleavage. Products of each of the three reactions were identified in peptide-loaded films. Cyclic imide and Asp-containing deamidation products (see Scheme 1) were identified by RP-HPLC elution times [30] and by mass spectrometry product and precursor scans. The Asp-containing product (VYPDGA) eluted immediately before the Asn-hexapeptide (14.4 vs. 15.4 min), and mass spectroscopy confirmed a m/z of 621. Product scans contained major peaks at 341 and 358 amu, consistent with y*4 and y''4 ionization fragments, respectively. The cyclic imide product eluted after the Asn-hexapeptide (16.8 min), and mass spectroscopy confirmed a mass of 603 amu, indicating the loss of water from the original peptide mass. Control samples subjected to deamidation in basic solution and assayed using this LC-MS method confirmed that the *iso*-Asp deamidation product can be separated and



Scheme 1. Asn-hexapeptide deamidation pathways at acidic (pH<4) and neutral to basic (pH>5) pH ranges.



Scheme 2. Acylation reaction: nucleophilic attack of the N-terminal primary amine of the peptide on an ester carbon in PLGA.

elutes prior to the Asp product (13.6 min). *iso*-Asp-containing peptides were not detected in PLGA samples. It is noteworthy that the deamidation products include the cyclic imide and Asp-containing hexapeptides, but not the *iso*-Asp-containing hexapeptide. In solution, the *iso*-Asp product dominates at neutral to basic pH, typically in 3- to 5- fold greater concentration than the Asp product. The absence of the *iso*-Asp product is consistent with an acidic environment in the solid matrix. The cyclic imide is typically formed at neutral to basic pH (see Scheme 1), but is stable under acidic conditions [31,32]. This suggests that the cyclic imide is produced in the initially neutral matrix, and persists in the increasingly acidic environment that results from PLGA hydrolysis.

VYPNGA also undergoes acylation in PLGA films, in which the N-terminus of the peptide reacts with the polymer or its degradation products (Scheme 2). Acylation products were identified by precursor scans of the Asn-hexapeptide m/z and of known deamidation products. All identified acylation products were also confirmed by product scans of their respective m/z values to verify that fragmentation patterns matched those of the peptide. In Fig. 1, the precursor scan of the cyclic imide ($m/z=603$) contains two sets of acylation products. The smaller peaks (m/z of 661 and 732) correspond to the mass of the Asu-hexapeptide plus one and two glycoloyl groups, respectively; each glycoloyl unit added results in the addition of 58 Da. The larger peaks (m/z of 675 and 747) are equivalent to the Asu-hexapeptide plus one and two lactoyl units, respectively ($X+72$ per lactoyl). A product scan of the m/z ratio of 747 confirmed the loss of each of the lactoyl groups ($m/z=72$) from the cyclic imide upon further ionization (data not shown). Since the lactoyl and glycoloyl addition products are expected to have similar ionization efficiencies, the results shown in Fig. 1 suggest that the lactoyl products are present in a higher concentration. This was confirmed by LC/MS/MS (see Section 3.3).

The predominant acylation products identified by LC/MS/MS corresponded to the addition of one or two lactoyl or glycoloyl groups to the N-terminus of either the Asp- or Asu-hexapeptide, a finding consistent with previous reports [20,22]. However, product scans of m/z values corresponding to Asu-hexapeptide+3, 4, and 5 lactoyl units indicate that these products also exist. Precursor ion scans of $m/z=819$, 891, and 963, respectively, contain peaks consistent with the removal of each lactoyl group via ionization ($m/z=x-72$), as well as the cyclic imide peptide fragment ($m/z=603$). These products were not detectable by LC/MS/MS using MRMs of appropriate m/z's. This discrepancy may be caused by ester bond hydrolysis in the aqueous/organic solvent mixture used for LC/MS/MS separation, since the mass spectroscopy infusion experiments were performed in pure acetonitrile. Alternatively, since the addition of lactoyl groups increases peptide hydrophobicity, the higher order adducts may be retained on the C18 column or may have limited ESI+ ionization efficiency, and so may not be detectable.

In addition to deamidation and acylation, VYPNGA undergoes peptide bond hydrolysis in PLGA films. Two types of amide bond cleavage are likely. First, aspartic acid side chain

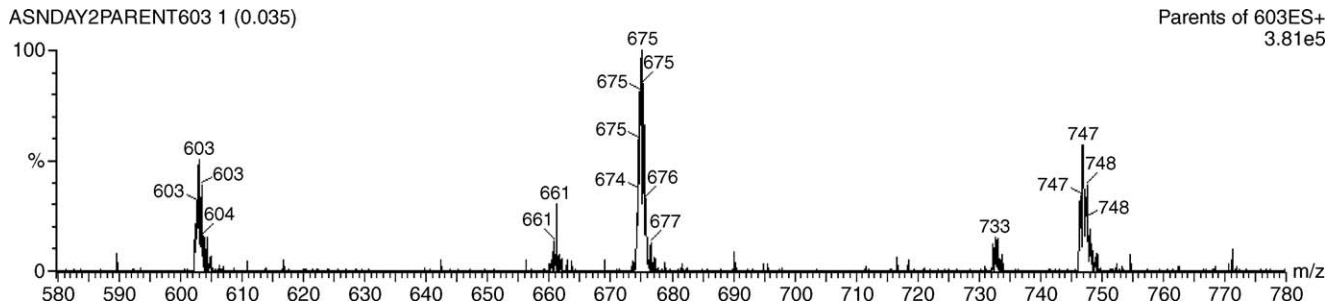


Fig. 1. Precursor (parent) ion scan of the Asu-hexapeptide (b).

catalysis of amide bond hydrolysis to produce the tetrapeptide, VYPD, is a common degradation product in acidic (pH < 3) solution and solids (Scheme 3) [32,33]. The presence of the VYPD tetrapeptide was confirmed by LC retention time (13.9 min) and by mass spectroscopy, with a mass of 493 amu and a $y''2$ ionization fragment at 231 amu.

More extensive amide bond cleavage, presumably catalyzed by PLGA degradation products, also occurred in this study. Intermolecular catalysis by either lactic or glycolic acid could produce an anhydride intermediate that is further hydrolyzed by water [34]. In mass spectrometry, the appearance of fragments with the complete amino acid mass confirms the presence of free amino acids in the sample, since fragmentation of peptides only produces immonium ions of the constituent amino acids ($m/z = x - 45$). Product ion scans of the m/z's of the amino acids present in VYPNGA were performed, as well as precursor scans of their respective immonium ion masses. Glycine and alanine were identified by ESI+MS after 48 h of storage at 70 °C and 95% RH. Valine, tyrosine, VY, proline and aspartic acid were identified after 96 h of storage at 70 °C and 95% RH. The dipeptide, GA, produced by Asp-catalyzed cleavage may be more susceptible to cleavage than other peptide bonds, allowing glycine and alanine to be detected at earlier time points. The extent of peptide bond cleavage observed here is influenced by the accelerated stability testing conditions employed, in which films were stored at high temperature and relative humidity but without the removal of degradation products. Extensive peptide bond cleavage has only been reported in PLGA degradation studies where polymer degradation products were allowed to accumulate, rather than being removed by dialysis [35].

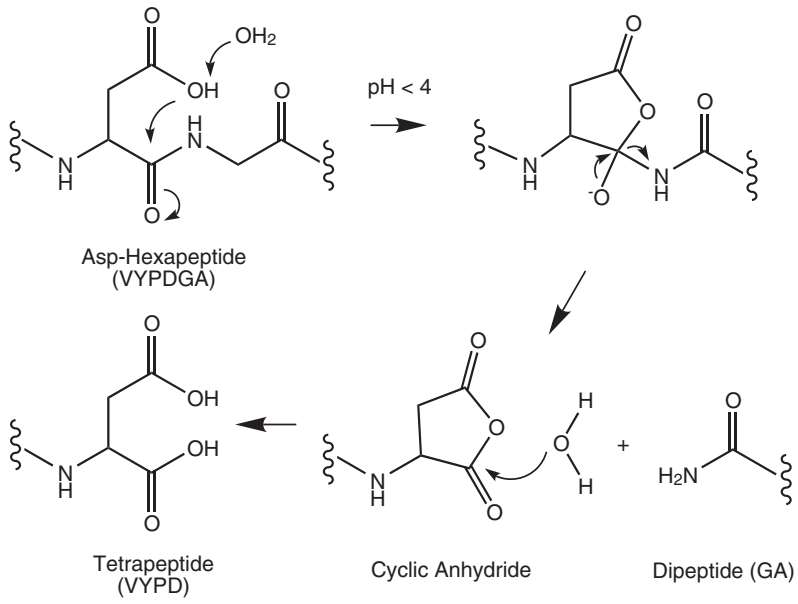
3.2. Asn-hexapeptide degradation kinetics

The kinetics of VYPNGA loss during storage in PLGA films were fit as pseudo first-order for all samples (see Fig. 2a–f). Rate constants (k_{obs}) were determined by nonlinear regression; values at various RH storage conditions are presented in Table 1. Films stored at 70 °C and ~ 0% RH showed no Asn-hexapeptide degradation after one year of storage (data not shown).

There is no obvious relationship between the k_{obs} value and the RH of storage. Previous studies of VYPNGA deamidation in lyophilized PVP formulations have shown an increase in parent peptide loss with increasing relative humidity [28], attributed to both an increase in mobility and moisture [27]. Here, the greatest rate of loss occurred at an intermediate RH (45% RH). The apparent loss kinetics are complicated by the presence of multiple peptide degradation pathways (i.e., deamidation, acylation, peptide bond cleavage) and by changes in properties of the PLGA matrix (e.g., moisture content, acidity) as the polymer degrades. Both deamidation and peptide bond cleavage are acid catalyzed hydrolytic reactions (Schemes 1 and 3), favored by high moisture content and by the acidic environment produced by PLGA degradation products. In the acylation reaction, however, the peptide primary amine competes with water for nucleophilic attack on the ester bonds of the polymer, and so is favored by low moisture content (Scheme 2). The complex relationship between peptide loss kinetics and relative humidity may reflect these competing dependencies.

3.3. Product formation kinetics

The kinetics of formation and relative amounts of the deamidation, acylation and peptide bond hydrolysis products provide mechanistic information on these concurrent reactions. In this system, the concurrent reactions produce a complex mixture of degradation products, many of which are the result of more than one of these degradation processes. In the following discussion, products that are the result of deamidation only (i.e., cyclic imide and Asp-hexapeptide) are classified as "Deamidation" products, those that have undergone any lactoyl



Scheme 3. Aspartic acid catalyzed chain cleavage at the Asp–Gly amide bond.

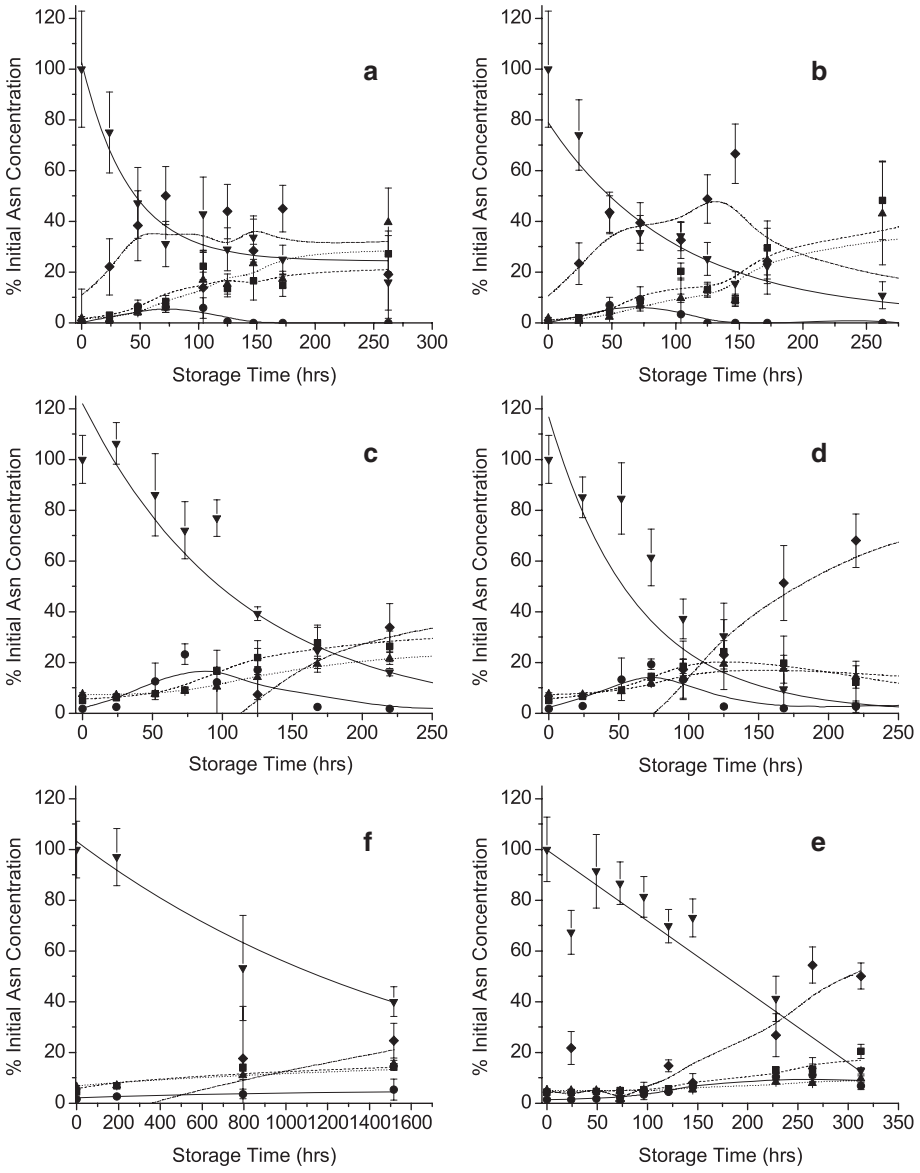


Fig. 2. Degradation profile of the PLGA incorporated Asn-hexapeptide upon storage at 70 °C and 95% RH (a), 70 °C and 75% RH (b), 70 °C and 60% RH (c), 70 °C and 45% RH (d), 70 °C and 28% RH (e), 37 °C and 95% RH (f). Deamidation (■), acylation (●), cleavage (▲), Asn (▼), and other (◆). Lines for Asn-hexapeptide loss represent nonlinear regression fits to a first order rate loss (see text). Other lines represent adjacent averaging and are included to show product formation trends.

or glycoloyl addition as "Acylation" products, and those that exhibit peptide bond hydrolysis as "Cleavage" products. These categories and the specific identifiable products associated with each of them are summarized in Table 2. Note that all Acylation products and some Cleavage products have previously undergone deamidation. The difference between the initial peptide mass and the total products assignable to these groups was classified as "Other". This category includes products that were present in limited amounts, retained on the LC column, poorly ionized and/or not identified. Mass loss during sample handling is also absorbed into the "Other" category.

Fig. 2 shows the kinetic profiles for product formation under various conditions; Fig. 3 summarizes these results in terms of the maxima of the product profiles in each product category. Product kinetics at low RH (28% RH) and low temperature (37 °C) are not included in the following discussion due to the slow rate of peptide degradation relative to the other studies. However, the same pathways of peptide degradation exist, as evidenced by the products identified (Fig. 2e and f).

Table 1
Observed pseudo first-order rate constants for the loss of VYPNGA in PLGA films stored at various conditions

Storage conditions	k_{obs} (hr^{-1})[a]	n[b]
95% RH, 70 °C	$9.2\ (\pm 3.5)\times 10^{-3}$	9
75% RH, 70 °C	$9.9\ (\pm 2.2)\times 10^{-3}$	12
60% RH, 70 °C	$9.0\ (\pm 0.5)\times 10^{-3}$	12
45% RH, 70 °C	$1.6\ (\pm 0.04)\times 10^{-2}$	12
28% RH, 70 °C	$1.8\ (\pm 25)\times 10^{-8}$	10
95% RH, 37 °C	$5.7\ (\pm 16)\times 10^{-4}$	4

[a] Values in parentheses represent standard errors of the k_{obs} values as determined by nonlinear regression.

[b] n = number of time points in regression analysis.

Table 2
Classification of quantifiable degradation products according to degradation pathway

Deamidation	Acylation	Cleavage
Asu-hexapeptide (VYPUGA)	Lac–Asu Lactoyl–(VYPUGA)	Tetrapeptide (VYPD)
Asp-hexapeptide (VYPDGA)	Lac–Asp Lactoyl–(VYPDGA)	Alanine (A)
	Gly-Asu (Glycoloyl-VYPUGA)	Dipeptide (GA)
	Lac-2-Asu (Dilactoyl–VYPUGA)	Proline (P)

At 70 °C and at high (95–75% RH) and intermediate (60–45% RH) relative humidity, acylation is the dominant degradation pathway early in the study, with the concentration of acylated products reaching a maximum around 72 h of storage (Figs. 2 and 3). Conditions are most favorable for acylation at early stages of degradation. The primary amine at the peptide N-terminus is the dominant nucleophile in this initially solid, neutral matrix. The pK_a of the N-terminal amine is 9.72 [36], so as the water content of the formulation increases and the effective pH decreases, the amine becomes protonated. The protonated primary amine is a poorer nucleophile than the deprotonated form, which has a lone pair of electrons available for the Sn_2 reaction. Competition by water for nucleophilic attack on the polymer ester bond also serves to limit acylation at later time points. Thus, the inverse relationship between the concentration of acylation products and relative humidity (Fig. 3) can be attributed to competition by sorbed moisture for reaction with the PLGA ester bonds and to matrix acidity resulting from ester hydrolysis. It should be noted that residues with primary or secondary amines (i.e. Lys and Arg, respectively) are also suspected to experience acylation reactions with PLGA [8,9,20]. However, due to the higher pK_a's (10.69 and 12.48, respectively) [36] of these amines relative to that of the N-terminus, reaction rates are expected to be slower [8,9].

The addition of one lactoyl unit to the Asu-hexapeptide (Lac–Asu) was the predominant quantifiable acylation product regardless of storage condition. Precursor ion scans indicated that Asu-hexapeptide acylation with lactoyl groups was greater than with glycoloyl groups, assuming similar ionization efficiency for lactoyl and glycoyl addition products. Quantitative analysis showed that the percentage of acylation products resulting from glycoloyl addition rather than lactoyl addition ranged from 0% at high RH (i.e. 95 and 75% RH) to 2–6% at intermediate RH (i.e. 60% and 45% RH). Preference for lactoyl addition has been reported for acylation involving PLGA and atrial naturetic peptide (ANP) or salmon calcitonin (sCT) [20]; however, octreotide reaction with PLGA resulted in greater glycoloyl addition [21,22,37]. N-terminal amino acids with bulky side-chains, such as the phenylalanine of octreotide, may favor reaction with glycoyl groups due to steric hindrance [22,37]. Amino acids with less bulky side-chains, such as the N-terminal valine in this study, or serine (in ANP) and cysteine (in sCT) [20] should experience less steric hindrance, able to react with the slightly more electrophilic lactoyl groups in PLGA. The pK_a's of lactic acid and glycolic acid are 3.86 and 3.83, respectively; this slight difference in pK_a does not fully explain the dominance of lactoyl addition in these studies.

Figs. 2 and 3 also show a parallel increase in chain cleavage and deamidation with storage time. Since both reactions are hydrolytic and acid catalyzed, their rates are expected to increase with increasing matrix water content and with the increasing acidity that results from PLGA hydrolysis. At early storage times, the cyclic intermediates required for both neutral pH deamidation (cyclic imide)(Scheme 1) and Asp–Gly amide bond cleavage (cyclic anhydride)(Scheme 3) are formed in an environment of relatively low moisture. As the moisture content and acidity increase, direct hydrolysis of the Asn side chain to produce the Asp-containing peptide can occur, with subsequent cleavage of the labile Asp–Gly peptide bond. Therefore, the concentration of deamidation and chain cleavage products would be expected to increase with increasing relative humidity of storage, as shown in Fig. 3.

Figs. 2 and 3 also show the increase in unidentified "Other" products with increasing storage time. The concentration of "Other" products and the storage time required for these products to reach their maximum concentration increases with decreasing relative humidity at 70 °C (Figs. 2 and 3). This category of reaction products probably reflects further degradation of the identifiable products, products produced in low concentration, and mass loss during sample handling and analysis. While mass loss in PLGA formulations has been attributed to adsorption to the polymer caused by hydrophobic [38–40]or ionic interactions [8,40,41] this is unlikely here. Hydrophobic interactions are unlikely at late stages of PLGA degradation because polymer hydrolysis increases PLGA hydrophilicity. Ionic interactions between PLGA and encapsulated peptides at acidic pH are only problematic for peptides containing basic residues, such as Arg or Lys, which are not present in this model peptide [17,40,41].

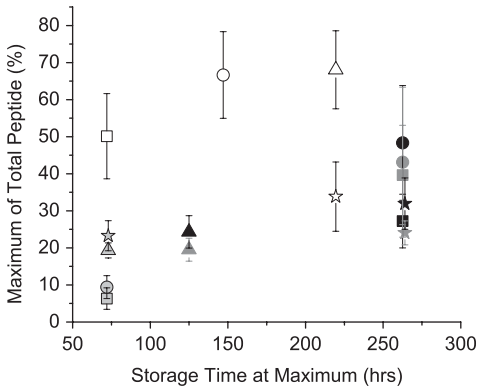


Fig. 3. Comparison of degradation maximums. Colors indicate degradation pathway: Deamidation (black), acylation (black outline, gray fill), chain cleavage (gray), and other (black outline, white fill). Shapes indicate storage relative humidity at 70 °C: 95% RH (square), 75% RH (circle), 60% RH (star), and 45% RH (triangle).

4. Conclusions

Peptides and proteins encapsulated in PLGA are subject to acid catalyzed hydrolytic chemical degradation reactions such as deamidation and peptide bond chain cleavage, as well as acylation reactions between the peptide and the polymer. For the model peptide studied here, the results demonstrate that hydrolytic reactions are favored late in PLGA matrix degradation when moisture content and acidity are higher. In contrast, acylation reactions are favored under neutral conditions and low moisture content, conditions typical of the earlier stages of PLGA matrix degradation. A more thorough analysis of the relationship between PLGA degradation and the peptide degradation reactions discussed here will be presented in a subsequent article. Optimal formulation is required to preserve the chemical stability of peptide and protein drugs in PLGA matrices.

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