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Research Paper

PEGylation of Octreotide: I. Separation of Positional Isomers and Stability Against Acylation by Poly(D,L-lactide-*co*-glycolide)

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Purpose. To investigate the mechanism by which polyethylene glycol (PEG) conjugation (PEGylation) prevents the acylation of octreotide by poly(D,L-lactide-*co*-glycolide) (PLGA).
Methods. Octreotide was chemically modified by reaction with succinimidyl propionate-monomethoxy PEG. Each PEGylated octreotide species with different PEG number and modified position was separated by reversed-phase high-performance liquid chromatography (RP-HPLC) and characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with endoproteinase Lys-C digestion. Acylation of octreotide and PEGylated octreotides was observed with hydrophobic and hydrophilic PLGA.
Results. Two mono- and one di-PEGylated octreotides were separated by RP-HPLC. MALDI-TOF MS of the PEGylated products after Lys-C digestion at different pH revealed that the two mono-PEGylated octreotides were modified at the N-terminus and Lys^5 residue, respectively. The interaction of octreotide with PLGA involved an initial adsorption followed by acylation and the subsequent release of octreotide and acylated octreotide. The initial adsorption of octreotide was dependent on the acidity of PLGA. PEGylation of octreotide significantly inhibited the initial adsorption and acylation by PLGA. In particular, the acylation could be completely prevented by mono-PEGylation at the N-terminus of octreotide.
Conclusions. This study shows that the N-terminus of octreotide is the preferred PEGylation site to prevent acylation in degrading PLGA microspheres. The mono-N-terminally PEGylated octreotide may possibly serve as a new source for somatostatin microsphere formulation.

KEY WORDS: acylation; microsphere; octreotide; PEGylation; poly(D,L-lactide-*co*-glycolide).

INTRODUCTION

Biodegradable poly(D,L-lactide) (PLA) and poly(D,L-lactide-*co*-glycolide) (PLGA) have been widely used for the prolonged release of peptides and proteins (1–3). However, stability of these therapeutic entities in the PLA or PLGA matrix during manufacture, storage, and after administration remains as one of the major challenges to successful product development (4). The acidic microenvironment within the matrix during polymer degradation has been reported to be deleterious to the stability of peptides and proteins (5,6). In particular, impurities of peptides within degrading PLA or PLGA matrices formed as a result of acylation with lactic and glycolic acid units (7,8). Nucleophilic groups such as the primary amino groups present in the N-terminus and lysine residue were shown to be the major targets for peptide acylation (9). As a strategy for preventing acylation of peptide in degrading PLGA and PLA microspheres, Lucke *et al.* investigated whether block copolymerization of PLA with PEG would prevent peptide acylation inside degrading microspheres (10). However, the combination of PEG with PLA did not necessarily protect the peptide from acylation within degrading microspheres.

Octreotide, an octapeptide analogue of somatostatin, has been commercially formulated in PLGA microspheres (Sandostatin LAR depot, Novartis Pharma, Basel, Switzerland) as a monthly dosage form for the treatment of acromegaly (11). A previous study revealed that the commercial product in PLGA microspheres formed lactoyl and glycoyl adducts after *in vitro* incubation in phosphate buffer saline (12). PEGylation, the covalent attachment of polyethylene glycol (PEG) to the peptide, was successfully used to improve the chemical and biological stability of salmon calcitonin, a 32-amino-acid peptide (13). This finding would suggest PEGylation as a strategy for preventing the acylation of octreotide. In lactic acid solutions as a test system for the stability study against acylation reaction, PEGylated octreotide (PEG-octreotide) was more stable than native octreotide (14). The stability of PEG-octreotide may be attributed to the steric hindrance of the PEG strand.

The aim of this study was to investigate the mechanism by which PEGylation prevents the acylation of octreotide by PLGA. In general, PEGylation results in heterogeneity of the molecule with respect to distribution in both the number and position of the attached PEG molecules as well as the inherent polydispersity of PEG itself (15,16). To prepare a homogeneous PEGylated peptide, Felix *et al.* proposed the site-

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specific conjugation of PEG to the growth hormone-releasing factor 1-29 by solid-phase peptide synthesis (17). However, this procedure is likely to yield final products with unacceptable impurities and missing amino acids because of the high molecular weight and polydispersity of PEG. Recently, Morpurgo *et al.* reported a tailored chemistry for an optimized PEG conjugate of the somatostatin analogue RC160 (18). Specifically, each of the two primary amines of RC160 was modified with PEG by a selective pH-driven chemical protection of each amine, followed by conjugation of PEG to the other. This approach while efficient for selectively modifying α- and ε-primary amines, requires several complicated steps such as chemical blocking, isolation of the blocked peptide, PEGylation, deblocking, and purification of final product. In this study, following PEGylation of octreotide, each PEGylated species with different binding sites and PEG numbers (mono-PEG-Phe^1-, mono-PEG-Lys^5-, and di-PEG-octreotide) were directly separated by reversed-phase high-performance liquid chromatography (RP-HPLC), and the PEGylation sites and the attached PEG numbers were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). This approach is expedient because large-scale preparation and purification of products can be facilitated by optimization of the chromatographic method. With positional isomers of mono-PEG-octreotides, the effect of PEGylation site on the stability against acylation by degrading PLGA was investigated and compared with native octreotide.

MATERIALS AND METHODS

Materials

Octreotide acetate (MW 1019.26) and succinimidyl propionate-monomethoxy PEG (SPA-mPEG, molecular weight 1000 Da) was obtained from Bachem (Torrence, CA, USA) and Nektar Therapeutics (Huntsville, AL, USA), respectively. Hydrophilic and hydrophobic 50:50 PLGA polymers (Resomer RG502H, RG502 and RG503H) were supplied by Boehringer Ingelheim (Ingelheim, Germany). Alpha-cyano-4-hydroxycinnamic acid (α-CHCA), Endoproteinase Lys-C (from Lysobacter enzymogenes, sequencing grade), and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and tetrahydrofuran (THF) (HPLC grade) were supplied from Fisher Scientific (Fair Lawn, NJ, USA). Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, USA). All other chemicals were of analytical grade and used as obtained commercially.

Preparation and Separation of PEGylated Octreotide

A solution of octreotide (10 mg/ml in deionized water) was added to Eppendorf tubes containing a two molar excess of SPA-mPEG in 0.1 M phosphate buffer at pH 6.0, 7.4, or 8.0. The reaction mixtures were shaken gently at room temperature for 1 h and loaded onto a Prosphere C-18 column (4.6 × 250 mm, Alltech, Deerfield, IL, USA) for RP-HPLC analysis by UV detection at 215 nm. A linear gradient of 35–50% (v/v) acetonitrile in water containing 0.1% (v/v) TFA for 20 min served as the mobile phase at a flow rate of 1.0 ml/min. The PEG-octreotides corresponding to two mono-PEG-octreotides and one di-PEG-octreotide were isolated and freeze-dried following evaporation by a Speed-Vac Apparatus. (Eppendorf, Hamburg, Germany). The molecular weights of the separated PEG-octreotides were determined by MALDI-TOF MS.

Identification of PEGylation Sites

Identification of the PEGylation sites was as described previously (19,20). PEG-octreotides were treated with DTT at a final concentration of 5 mM for 4 h to reduce the disulfide bond. Thereafter, enzymatic digestion with endoproteinase Lys-C was performed in 0.1 M Tris-HCl buffer (pH 8.0) at 37°C with an enzyme to substrate ratio of 1:100 (w/w) for 4 h. The concentration of the PEG-octreotides was 200 μg/ml. The Lys-C digests were directly analyzed by MALDI-TOF MS. The PEGylation sites were identified from the molecular weights of the PEG-octreotide fragments digested with Lys-C.

Acid Content of PLGA

Acid number, a measure of the carboxylic acid content of the polymers, was determined by titration (21). Approximately 100–400 mg of polymer (depending on the acid content) were dissolved in 20–40 ml of acetone/THF (1:1) mixture. The solution was immediately titrated with 0.01 N methanolic potassium hydroxide to a stable pink end point using phenopthalein as an indicator. The acid number (mg KOH/ g PLGA) was calculated as follows:

$$\text{Acid number} = \frac{(\text{volume of sample})(N_{KOH})(Mw_{KOH})}{(\text{weight of PLGA})}$$

Interaction with Polymers

Each 100 mg of PLGA (RG502H, RG502, and RG503H) was added to 10 ml of native octreotide or PEG-octreotides (peptide concentration of 200 μg/ml) in 0.1 M phosphate buffer (pH 7.4) at 37°C (n = 3 per sample). Samples were collected at 1, 3, 7, 14, 21, 28, 35, 42, and 49 days. At each sampling time, the pH value in the medium was measured. The supernatants after centrifuge were analyzed by RP-HPLC in triplicate per sample, and the acylated products were characterized by MALDI-TOF MS. To analyze the in-

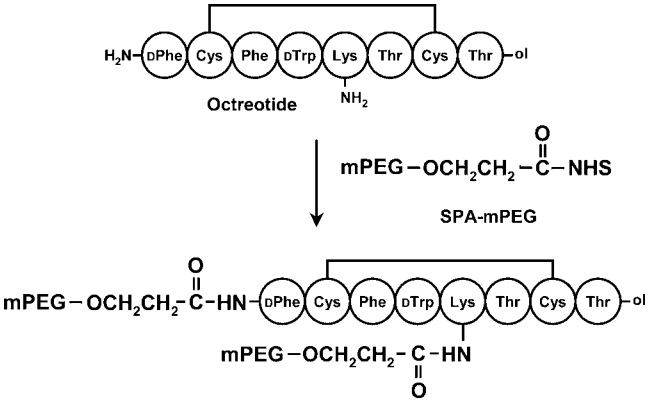

Fig. 1. Schematic diagram for bioconjugation of octreotide with SPA-mPEG. PEGylation of octreotide produces three species of PEG-octreotides; that is, two mono-PEG-octreotides (N-terminus or Lys) and one di-PEG-octreotide.

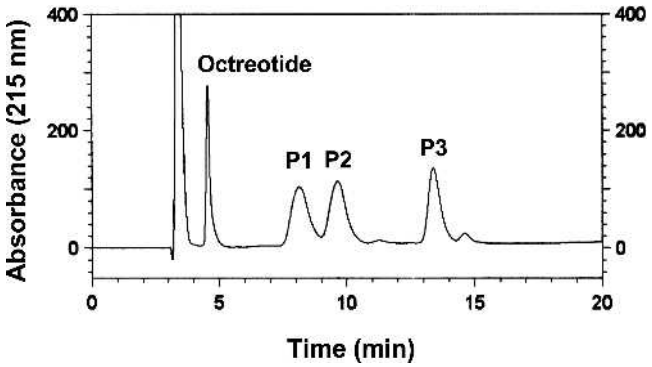

Fig. 2. RP-HPLC separation of the PEG-octreotides produced at pH 7.4 and molar ratio of 1:2 (octreotide:SPA-mPEG). P1, P2, and P3 indicate the PEG-octreotides.

teraction of native octreotide with polymer, a linear gradient of 20–35% (v/v) acetonitrile in water containing 0.1% (v/v) TFA for 20 min was used. The other conditions were the same as the RP-HPLC method for PEG-octreotide described above.

MALDI-TOF MS

The spectra of acylated octreotide and the separated PEG-octreotides were obtained on a Kratos Kompact SEQ time-of-flight mass spectrometer (Manchester, UK) as reported previously (14,22). The α-CHCA in 50% of acetonitrile in water with 0.1% TFA was used as a matrix. Data for 2-ns pulses of the 337-nm nitrogen laser were averaged for each spectrum in a linear mode, and a positive ion TOF detection was performed using an accelerating voltage of 20 kV.

RESULTS AND DISCUSSION

Preparation of PEGylated Octreotide

As octreotide has two PEGylation sites of N-terminus (Phe^1) and Lys^5, three species of PEG-octreotides can be produced; that is, two mono- and one di-PEG-octreotide (Fig. 1). The PEG-octreotides produced with SPA-mPEG were separated by RP-HPLC into three peaks (Fig. 2). Each peak was isolated, and their molecular masses were determined by MALDI-TOF MS (Table I). The molecular masses of the first (retention time = 8.1 min) and second peaks (retention time = 9.6 min) were almost the same as *m/z* 2097, which corresponds to the mono-PEG-octreotide. The third peak (retention time = 13.5 min) was identified as di-PEG-octreotide with a molecular mass of *m/z* 3153. In the MALDI-TOF MS spectra of the separated conjugates, the peaks corresponding to native octreotide or free PEG were not observed.

Table I. MALDI-TOF MS of PEG-Octreotides Separated by RP-HPLC

Sample	Mass[a] of intact molecule	Mass after Lys C digestion
P1	2097	2097
P2	2097	1787
P3	3153	3153

The molecular mass of octreotide is 1019 Da and that of SPA-mPEG-1K was determined to be *m/z* 1187.

[a] The number is centroided mass value of the polydisperse peaks.

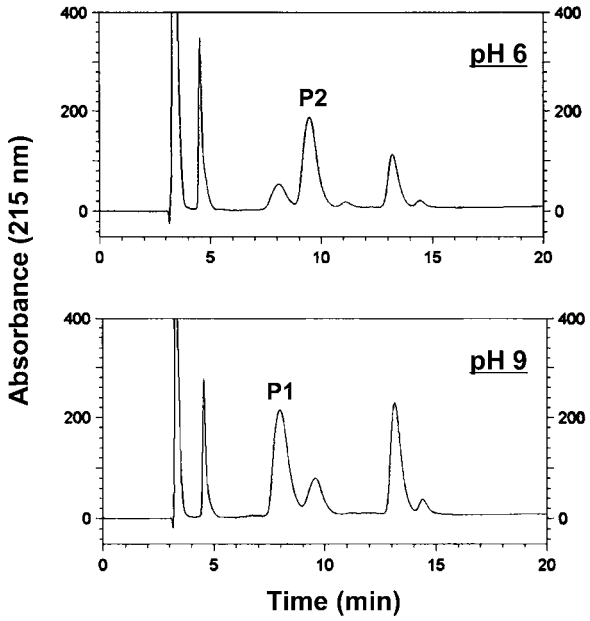

Fig. 3. Comparison of PEGylation reactivity of octreotide at pH 6 and pH 9.

Identification of PEGylation Sites

To identify the PEGylation sites of the two mono-PEG-octreotides, the difference in PEGylation reactivity of the α-

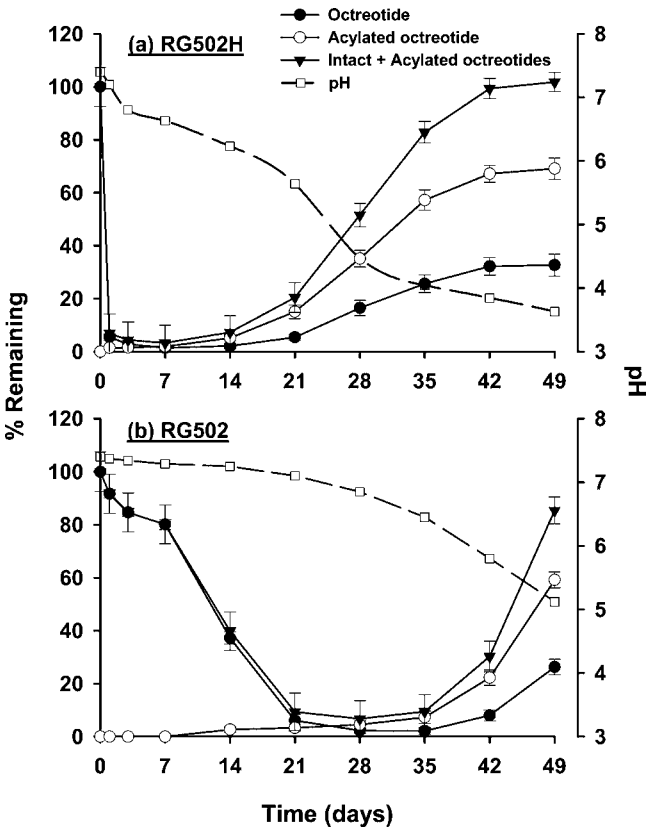

Fig. 4. Interaction of octreotide with hydrophilic PLGA RG502H (a) and hydrophobic PLGA RG502 (b) in 0.1 M phosphate buffer (pH 7.4) at 37°C. Mean ± SD, n = 3.

Table II. Relationship of Acid Number of PLGA with Initial Adsorption of Octreotide During Incubation of 1 Day

PLGA	Acid number	Octreotide:PLGA (w/w)	Adsorbed amount of octreotide (%)[a]
RG502H	14.3	1:50	94.5 ± 1.2
RG503H	4.58	1:50	50.6 ± 2.3
		1:100	69.6 ± 3.4
		1:150	81.6 ± 4.1
RG502	0.94	1:50	8.30 ± 1.6

[a] Mean value ± standard deviation.

(N-terminus) and ε-amino group (Lys) was assessed as a function of the medium pH. In general, the pK_a value of the α-amino group is 7.8, whereas that of the ε-amino group is 10.1 (23). Therefore, the α-amino group of the N-terminus would be more reactive than that of the Lys residue at low pH, whereas the ε-amino group of the Lys residue would be more susceptible to the PEGylation reaction at high pH. Figure 3 shows the RP-HPLC chromatograms of the PEGylation of octreotide at pH 6 and 9. At pH 6, the second peak (P2) was predominant, whereas at pH 9 the first peak (P1) was greater. This indicates that P1 resulted from PEGylation at the Lys residue and P2 was that at the N-terminus. Another approach to assessing the PEGylation sites was to compare the peptide mapping pattern between native and PEGylated peptide after enzymatic cleavage (19,20), in which PEGylation provides protection against proteolytic cleavage. As octreotide has only one Lys residue, PEGylation at the Lys residue would show no mass change when treated with Lys-C following DTT, whereas such treatment of the modification at the N-terminus would produce a reduced mass fragment. As expected, when the Lys-C was treated, the P1 fraction showed no mass change in the MALDI-TOF MS, whereas the P2 fraction produced mass peaks surrounding *m/z* 1787 except

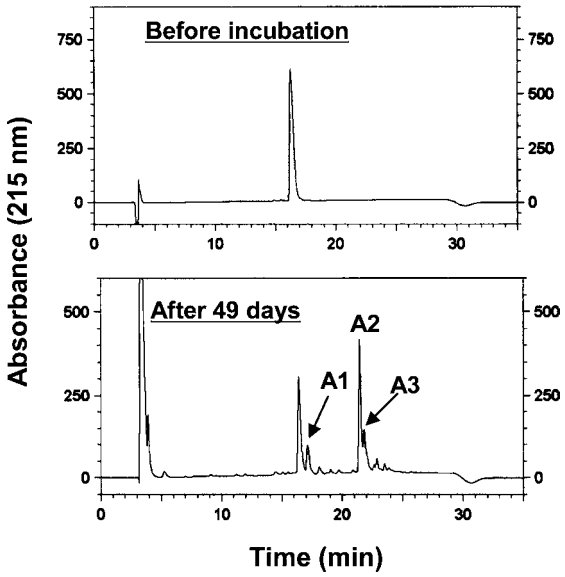

Fig. 5. RP-HPLC chromatograms of octreotide before and after incubation with RG502H in 0.1 M phosphate buffer (pH 7.4) at 37°C for 49 days (A1, mono-glycoyl-conjugated octreotide at Lys residue; A2, mono-glycoyl-conjugated octreotide at N-terminus; A3, di-glycoyl-conjugated octreotide at N-terminus and Lys residue).

Table III. MALDI-TOF MS of Octreotide Incubated with RG502H in 0.1 M Phosphate Buffer (pH 7.4) at 37°C for 49 Days

Observed mass	Expected structure	Peak intensity
1019	Octreotide	1600
1042	Octreotide-Na	3200
1077	Octreotide-1GA	2200
1091	Octreotide-1LA	250
1100	Octreotide-Na-1GA	5600
1114	Octreotide-Na-1LA	500
1135	Octreotide-2GA	500
1149	Octreotide-1GA-1LA	220
1158	Octreotide-Na-2GA	1200
1172	Octreotide-Na-1GA-1LA	400
1216	Octreotide-Na-3GA	220
1230	Octreotide-Na-2GA-1LA	220

GA, glycolic acid; LA, lactic acid; Na, sodium.

the mass peaks of the intact mono-PEG-octreotide (Table I). This confirmed the identities of positional isomers of the mono-PEG-octreotides.

Interaction of Octreotide with PLGA

The interaction of octreotide with hydrophilic and hydrophobic PLGA polymers involved an initial adsorption of the peptide to the polymer followed by an acylation reaction

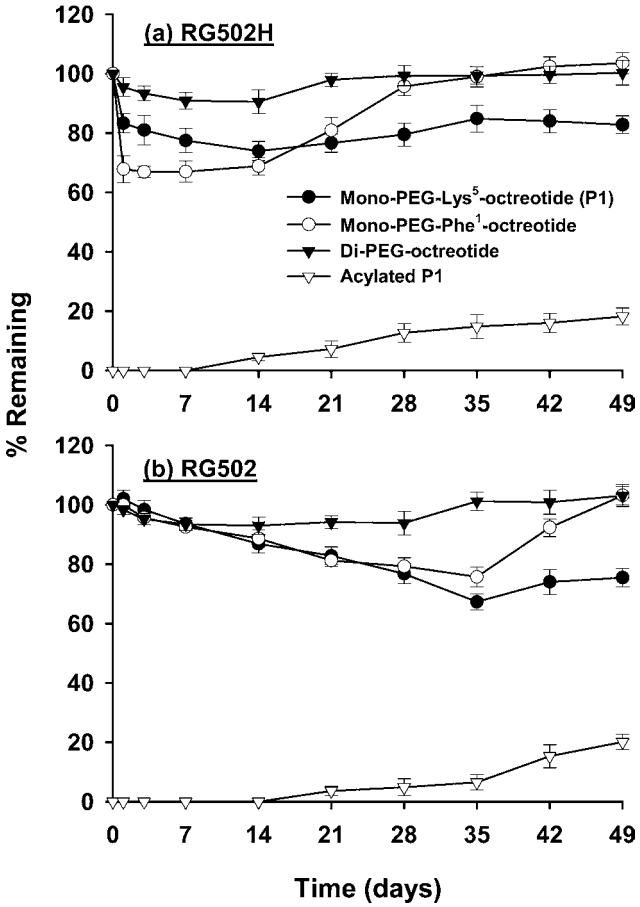

Fig. 6. Interaction of PEG-octreotides with hydrophilic PLGA RG502H (a) and hydrophobic PLGA RG502 (b) in 0.1 M phosphate buffer (pH 7.4) at 37°C. Mean ± SD, n = 3.

and subsequent release of intact and acylated octreotide. Fig. 4a shows the interaction profile of octreotide with hydrophilic PLGA RG502H in 0.1 M sodium phosphate buffer of pH 7.4. From day 1 through day 21, around 5% of octreotide remained in the supernatant, and then the amount increased to 32% by day 42. The acylated octreotide was observed from day 14 and the amount increased to 67% by day 42. At day 49, the sum of intact and acylated octreotides reached 99%. The interaction profile of octreotide with hydrophobic PLGA RG502, which carboxylic acid end groups are end-capped, is shown in Fig. 4b. The initial adsorption was low, 20% through day 7, but then increased at a higher rate. At day 21, approximately 95% of octreotide was adsorbed, where it remained through day 35. At day 49, the relative amounts of octreotide and the acylated octreotide observed in the supernatant were 26% and 59% of initial amount of octreotide, respectively. The low initial adsorption of octreotide to RG502 suggests the carboxyl end groups of PLGA are mainly involved in interaction with octreotide. The adsorption of octreotide from days 7 to 21 may be attributed to polymer degradation and the generation of carboxylic acid end groups. The initial adsorption of octreotide at day 1 was also related to the acid content of PLGA (Table II). Acid number, a measure of acid content, represents the number of free carboxylic acid functionalities for each polymer. When the octreotide was incubated with RG503H, which acid content is one-third of RG502H at the same weight, the initial adsorption proportionally increased as the amount of RG503H increases from 1:50 (octreotide:RG503H) to 1:150. This result suggests the initial adsorption being mainly attributed to an ionic interaction between the amino group of octreotide and the carboxyl group of PLGA.

HPLC analysis of octreotide incubated with RG502H at pH 7.4 shows that various acylated octreotides were formed (Fig. 5). As reported (12), the structures of three distinct peaks (A1, A2, and A3) among the acylated octreotides were as follows: A1, mono-glycoyl-conjugated octreotide at Lys residue; A2, mono-glycoyl-conjugated octreotide at N-terminus; A3, di-glycoyl-conjugated octreotide at the N-terminus and Lys residue. The most prevalent acylation product is the N-terminal glycoyl-conjugated octreotide (A2), followed by the di-glycoyl-conjugated octreotide (A3). The least favorable was the mono-glycoyl-conjugated octreotide at the Lys residue (A1). These results suggest that the greater nucleophilic activity of the N-terminus over the lysine residue is probably caused by a pK_a phenomenon. It was also reported that the N-terminal glycoyl-conjugated octreotide showed greater *in vitro* release from 50:50 PLGA microspheres (12). At physiologic pH, a greater percentage of N-terminal amine groups could be un-ionized and in the more nucleophilic $–NH_2$ form as opposed to Lys groups in the ionized $–NH_3^+$ form. Various acylated octreotides were also identified by MALDI-TOF MS (Table III).

Interaction of PEG-Octreotides with PLGA

The interaction of PEG-octreotides with the hydrophilic and hydrophobic PLGAs is shown in Fig. 6. PEGylation of octreotide significantly inhibited the initial adsorption to hydrophilic RG502H (Fig. 6a). After 24 h, 67.8% and 80.9% of mono-PEG-Phe^1-octreotide and mono-PEG-Lys^5-octreotide remained in the supernatant. Through 35 days, the mono-PEG-Phe^1-octreotide was recovered entirely intact, whereas the mono-PEG-Lys^5-octreotide remained around 80%, and

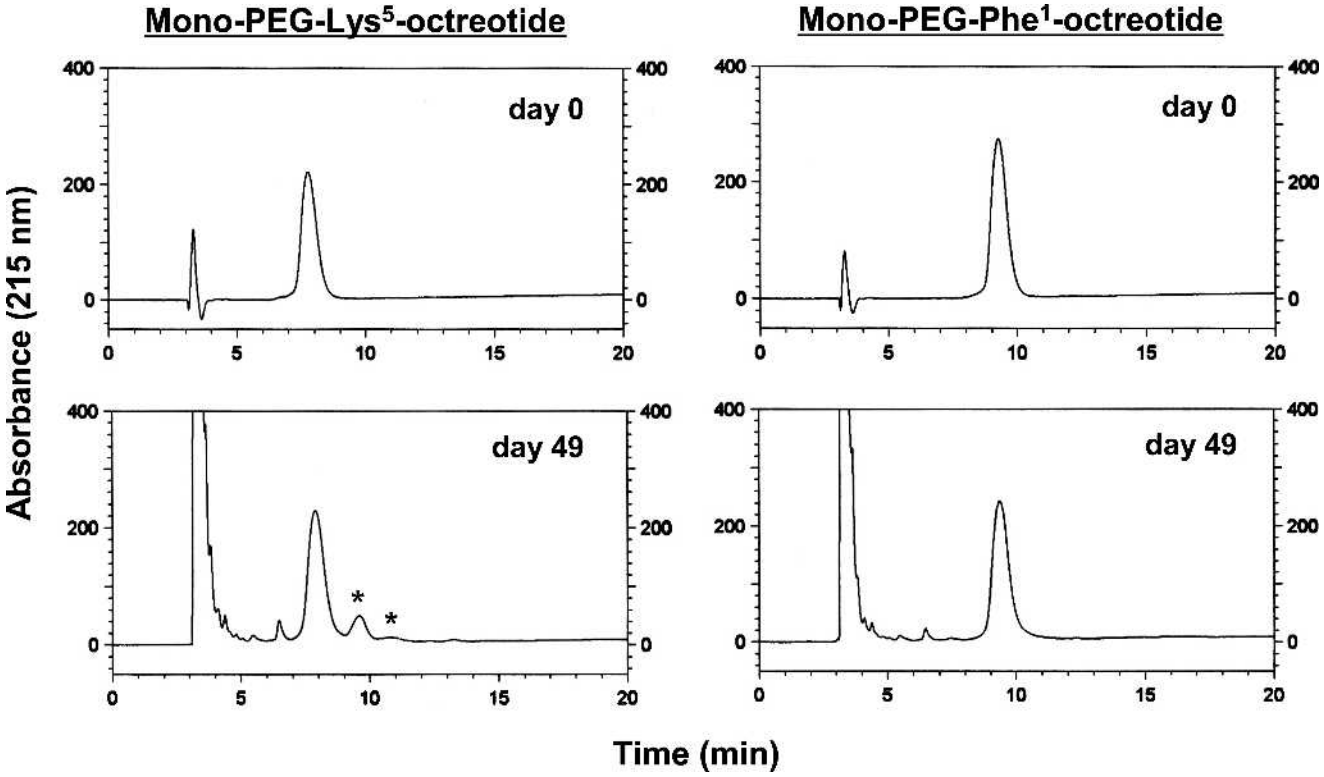

Fig. 7. RP-HPLC chromatograms of mono-PEG-octreotides incubated in 0.1 M sodium phosphate buffer (pH 7.4) at 37°C for 49 days. Asterisk (*) represents acylation products.

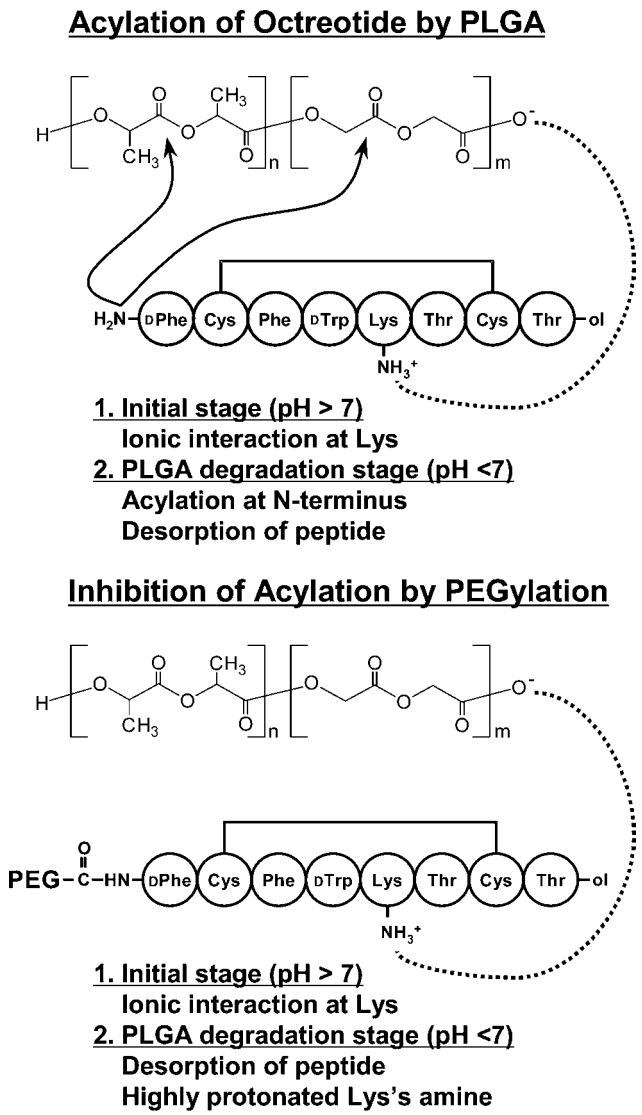

Fig. 8. Proposed mechanism for inhibition of octreotide acylation by PEGylation.

approximately 20% of the acylation products were observed at day 49. The di-PEG-octreotide showed 5–10% adsorption through 14 days with almost complete recovery at day 28. Figure 6b shows the interaction profile with hydrophobic RG502. Similar to interaction of native octreotide with RG502, the initial adsorption of PEG-octreotides was very low by day 7. At day 35, 67.3% and 75.7% of mono-PEG-Phe[1]-octreotide and mono-PEG-Lys[5]-octreotide were observed in the supernatant. At day 49, the mono-PEG-Phe[1]-octreotide was entirely recovered, whereas the mono-PEG-Lys[5]-octreotide showed about 20% of acylation products. The di-PEG-octreotide showed about 7% of adsorption through day 28 and was entirely recovered at day 35. Although the mono-PEG-octreotides showed approximately 30% of adsorption and 20% of acylation products with RG502H and RG502, the interaction and formation of acylation products were substantially lower than those of native octreotide.

HPLC chromatograms of mono-PEG-octreotides incubated with RG502H at pH 7.4 for 49 days are shown in Fig. 7. The HPLC of the mono-PEG-Lys[5]-octreotide represented two additional peaks corresponding to the acylation product, whereas that of mono-PEG-Phe[1]-octreotide was devoid of these peaks except a small peak at a retention time of 6.5 min. This suggests that N-terminal PEGylation could completely protect octreotide from acylation by PLGA. Although di-PEG-octreotide showed lower adsorption without acylation than mono-PEG-octreotides, di-PEGylation may result in significant loss of biological activity. Therefore, the mono-PEG-Phe[1]-octreotide may have the most potential as a source of microspheres.

Mechanisms of Inhibition of Octreotide Acylation by PEGylation

Figure 8 depicts the adsorption and acylation of octreotide to PLGA followed by desorption in solution and the inhibition of acylation by N-terminal PEGylation. Prior to PLGA degradation, the positive charge of the Lys residue of the peptide may interact with the carboxylic acid end group of the polymer via ionic attraction. As the polymer degrades, the medium pH decreases, and the primary amine of the N-terminus in the peptide will be predominantly acylated, and the acylated peptide will be released from the PLGA. As the primary amine of the Lys residue is highly protonated at the acidic pH, the reactivity may be significantly lowered. Therefore, the acylation of octreotide can be prevented by mono-PEGylation at the N-terminus. However, the mono-PEGylation at the Lys residue may be still susceptible for the acylation because of the primary amine of the N-terminus.

CONCLUSIONS

PEGylated isomers of octreotide were successfully separated and characterized by RP-HPLC and MALDI-TOF MS. PEGylation at the N-terminus with the amino group of lower pK_a could prevent octreotide from acylation by degrading PLGA. Therefore, the mono-N-terminally PEGylated octreotide could be effectively used for developing a stable somatostatin microsphere formulation.

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