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Preparation and Stability of Poly(Ethylene Glycol) (PEG)ylated Octreotide for Application to Microsphere Delivery

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ABSTRACT

The purpose of this study was to prepare poly(ethylene glycol) (PEG)ylated octreotide and investigate the stability against acylation by polyester polymers such as poly(lactic acid) and poly(lactic-co-glycolic acid). Octreotide was modified by reaction with monomethoxy PEG-propionaldehyde (molecular weight 5,000) in the presence of sodium cyanoborohydride. The mono-PEGylated fraction was isolated by reversed-phase high-performance liquid chromatography (HPLC) and characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Circular dichroism demonstrated no significant secondary structural differences between mono-PEGylated octreotide (mono-PEG-octreotide) and intact octreotide. As a test system for the stability study against acylation reaction, lactic acid (LA) solutions with various concentrations and pH values were prepared with water dilution and subsequent accelerated equilibration at 90°C for 24 hours. Native octreotide was found to be acylated in all the diluted LA solutions with different concentrations (42.5%, 21.3%, and 8.5%, wt/wt) and pH values (2.25, 1.47, and 1.85, respectively). The remaining amounts of intact octreotide continuously decreased to 50% through 30 days of incubation at 37°C. MALDI-TOF MS identified the octreotide to be acylated by LA units. However, acylation reaction of mono-PEG-octreotide in LA solutions was negligible, and the remaining amounts of intact one through 30 days of incubation in LA solutions were also comparable to the initial concentration. These data suggest that mono-PEG-octreotide may prevent the acylation reaction in degrading PLA microspheres and possibly serve as a new source for somatostatin microsphere formulation.

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INTRODUCTION

One of the critical issues in the development of controlled-release systems is the stability of peptides and proteins incorporated into a biodegradable polymer matrix.[1,2] The acidic microenvironment inside the matrix due to degradation of poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) polymers is known as the major source of instability of incorporated peptide and protein.[3,4] Recently, it was demonstrated that degradation of PLA and PLGA microspheres can lead to covalent modifications of incorporated peptides by acylation with lactic and glycolic acid units.[5,6] The acylation of peptide drugs inside degrading PLA and PLGA microspheres is regarded as one of the obstacles to be overcome for the successful delivery of bioactive materials.

Several strategies have been proposed for preventing acylation reaction of peptide in degrading PLGA and PLA microspheres. Avoidance of the acidic microclimate that is usually present inside degrading PLA and PLGA is one approach, either by enhancing the drainage of polymer degradation products using hydrophilic additives, such as poly(ethylene glycol) (PEG) or by neutralizing the acidic pH with basic additives, such as magnesium hydroxide.[3] Lucke et al investigated whether the block copolymerization of PLA with PEG prevents peptide acylation inside degrading microspheres.[7] The results showed that the combination of PEG with PLA does not necessarily display a favorable effect on peptide acylation reaction inside degrading microspheres.

The covalent attachment of PEG, PEGylation, is a technique widely used to provide functional bioconjugates with improved chemical and biological stability.[8-10]

The effects of PEGylation also include better physical and thermal stability, increased circulation half-life, reduced immunogenicity and antigenicity, and decreased toxic-

ity.[11,12] Some PEGylated proteins, such as lysozyme, epidermal growth factor, and interferon alpha, were incorporated into PLGA microspheres.[13-15] PEGylated proteins showed much better stability than native proteins against exposure to organic solvents during homogenization and reduced adsorption onto the surface of blank PLGA microspheres. Microspheres of PEGylated proteins also exhibited different drug release profiles with a reduced initial burst compared with those of unPEGylated proteins. These results provide a new possibility for the use of PEGylated proteins as a drug source for microsphere formulation. However, these approaches have been limited to protein drugs with molecular weight (MW) of over 10 kDa. In addition, the focus of stabilization was to improve physical stability of proteins, such as aggregation and denaturation, during encapsulation. Peptide drugs with MW of below 5 kDa have shown chemical instability, such as acylation reaction, in the PLA and PLGA microspheres.[16]

In a previous study, octreotide, an octapeptide analog of somatostatin, was identified to be acylated by glycolic and lactic acid (LA) in both PLA and PLGA microspheres.[17] To provide insight into what is happening in the microenvironment of the degrading microspheres, this study was performed to investigate whether PEGylation of the octreotide can prevent or minimize peptide acylation. As a test system for peptide acylation, aqueous LA solutions with various LA concentrations and pH values were prepared[18] and the acylation reaction in solutions of octreotide and PEGylated octreotide was investigated.

MATERIALS AND METHODS

Materials

Octreotide acetate and monomethoxy PEG-propionaldehyde (ALD-mPEG, MW 5000) were obtained from Bachem (Torrence, CA) and Shearwater Polymers (Huntsville, AL), respectively. Alpha-cyano-4-hydroxycinnamic acid (α-CHCA) and sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) were purchased from Sigma (St Louis, MO). Concentrated LA (85%, wt/wt) and acetonitrile (High-performance liquid chromatography [HPLC] grade) were supplied from Fisher Scientific (Fair Lawn, NJ). Trifluoroacetic acid (TFA) and Micro BCA protein assay kit were obtained from Pierce (Rockford, IL). All other chemicals were of analytical grade and used as obtained commercially.

Preparation of PEGylated Octreotide

A solution of octreotide (10 mg/mL in 0.1M phosphate buffer, pH 5.5) was added to a vial containing equimolar amount of ALD-mPEG in the presence of 20mM sodium cyanoborohydride ($NaCNBH_3$) in water. The reaction was stirred at 4°C for 12 hours. The PEGylation reaction mixture was loaded onto reversed-phase HPLC. Mono-PEGylated octreotide (mono-PEG-octreotide) was isolated and freeze dried following evaporation by Speed-Vac (Eppendorf, Hamburg, Germany). The purified mono-PEG-octreotide was characterized by MALDI-TOF MS. Reversed-phase HPLC of PEGylated octreotide was performed with Prosphere C-18 column (4.6 × 250 mm, Alltech, Deerfield, IL). A linear gradient of 30% to 60% (vol/vol) acetonitrile in water containing 0.1% (vol/vol) TFA for 20 minutes served as mobile phase at a flow rate of 1.0 mL/min. Chromatograms were recorded by UV detection at 215 nm.

Circular Dichroism

Circular dichroism (CD) spectra were recorded in the range of 190 to 250 nm with a Jasco J-710 spectropolarimeter (Jasco, Easton, MD). CD cell of 0.1-cm path length was used at a band width of 1 nm. Sample concentrations were precisely determined by BCA assay[19] and set to 200 μg/mL in 0.1M phosphate buffer (pH 7.4).

Preparation of Lactic Acid Solutions

LA solutions were obtained by dilution of concentrated LA (85%, wt/wt) with deionized water and subsequent accelerated equilibration at 90°C for 24 hours to reach a stable amount of LA oligomers. To obtain LA solutions of pH 2, 3, 4, and 6, the pH values were adjusted by adding sodium hydroxide prior to equilibration. The relative amounts of LA monomers and oligomers were determined by reversed-phase HPLC described previously.[18] The samples were analyzed on a Prosphere C-4 (4.6 × 250 mm, Alltech) at 40°C with the mobile phase consisting of 20% (vol/vol) acetonitrile and 0.1% (vol/vol) TFA in water. The flow rate was 1.0 mL/min. Chromatograms were recorded by UV detection at 220 nm. To calculate absolute amounts of monomers and oligomers from HPLC data, the total LA content was divided by the relative peak areas of the monomers and oligomers, respectively.

Stability Study in Lactic Acid Solutions

Octreotide and mono-PEG-octreotide were dissolved in LA solutions with a peptide concentration of 100 μg/mL. The solutions were incubated at 37°C and samples collected at the scheduled time were analyzed by reversed-phase HPLC with Prosphere C-18 column (4.6 × 250 mm). For the analysis of octreotide in LA solutions, a linear gra-

dient of 27.5% to 37.5% (vol/vol) acetonitrile in water containing 0.1% (vol/vol) TFA for 20 minutes served as mobile phase at a flow rate of 1.0 µL/min. Chromatograms were recorded by UV detection at 215 nm. HPLC conditions of mono-PEG-octreotide in LA solution were as described earlier in the "Preparation of PEGylated Octreotide" section. Native octreotide and the acylated products were determined using a calibration curve prepared with 12.5 to 200 µg/mL octreotide in each LA solution.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

The mono-PEG-octreotide and acylated octreotides were characterized by MALDI-TOF MS. Spectra were obtained on a Kratos Kompact SEQ time-of-flight mass spectrometer (Kratos Analytical, Manchester, UK). Each of the peak fractions from HPLC analysis of octreotide incubated in LA solution were collected and mixed with the matrix solution, a saturated solution of α-CHCA in 50% acetonitrile in water with 0.1% TFA. For the characterization of mono-PEG-octreotide, sinapinic acid in 70% acetonitrile in water with 0.1% TFA was used as matrix. Data for 2-nanosecond 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

As octreotide has 2 PEGylation sites of Phe (N terminus) and Lys residues, PEGylation of octreotide can produce 3 species of PEGylated octreotides (ie, 2 mono-PEG-octreotides [Phe or Lys] and 1 di-PEG-octreotide). PEGylation with ALD-mPEG at low pH produces selectively an N-terminal PEGylated molecule (Figure 1).[20] The N-terminal PEGylation occurs because of the difference in reactivity of a- and e-amino groups at low pH. The pK_a value of the ε-amino group is 7.8, whereas the pK_a value of the e-amino group is 10.1 and is therefore largely protonated. Figure 2A shows the reversed-phase HPLC chromatogram of the reaction mixture of octreotide and ALD-mPEG at pH 5.5 in the presence of sodium borohydride. This PEGylation resulted in production of mono-PEG-octreotide with high yield (80.7%) at molar ratio of 1:1 (ALD-mPEG:octreotide). In this reaction condition, di-PEG-octreotide was not observed. However, when 2- and 3-fold molar excess of ALD-mPEG reagent was added, 15.3% and 30.1% of di-PEG-octreotide were produced, respectively. As the presence of di-PEG-octreotide causes difficulty in separating the mono-PEG-octreotide, the molar ratio of 1:1 was selected for PEGylation of octreotide. The mono-PEG-octreotide isolated by reversed-phase HPLC was characterized by MALDI-TOF MS (Figure 2B). The average mass of mono-PEG-octreotide was observed to be m/z 6550. The difference in the masses of octreotide (1019.5 Da) and mono-PEG-octreotide was ~5500 Da, which is almost consistent to the mass of ALD-mPEG. In the CD analysis, the spectra of intact octreotide and mono-PEG-octreotide were nearly superimposed in the range of 190 to 250 nm, suggesting that PEGylation had no significant effect on the secondary structure of the octreotide (Figure 3).

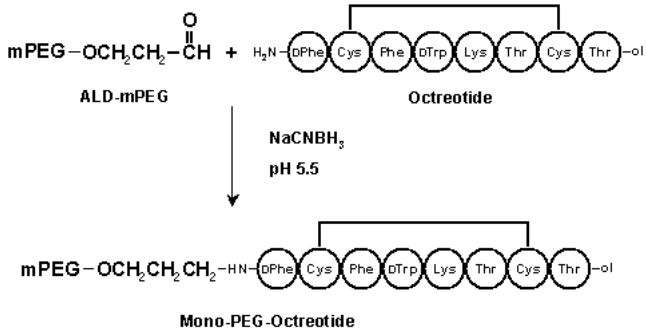

Figure 1. Schematic diagram for bioconjugation of octreotide with ALD-mPEG for preparation of N-terminally PEGylated octreotide.

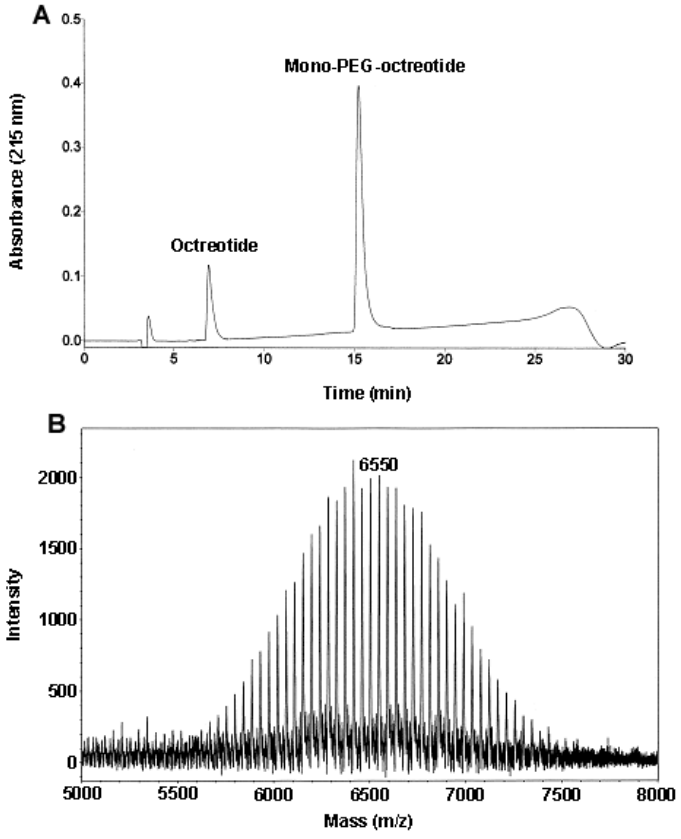

Figure 2. Preparation and characterization of PEGylated octreotide: (A) Reversed-phase HPLC chromatogram of PEGylation reaction mixture of octreotide, (B) MALDI-TOF mass spectrum of purified mono-PEG-octreotide.

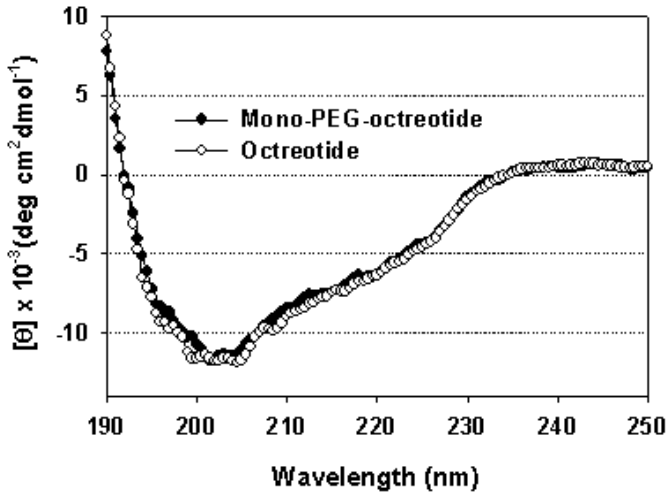

Figure 3. Circular dichroism of octreotide and mono-PEG-octreotide.

The pH of diluted LA solutions (1%-50%, wt/wt) ranges from pH 1.0 to 2.3 because of the negative inductive effect of α-hydroxy group of LA, which has a pK_a value of 3.86. The pH values inside degrading polymer matrices have been reported as low as 1.5.[21] Therefore, LA solutions could be a model system for observing the peptide acylation as the solutions provide the low pH and the oligomers that are characteristic for the microclimate inside degrading PLA.[18] The LA solutions with different concentration and pH were prepared at 90°C and the amounts of LA monomers (n = 1) and all oligomers (n > 1) in the solutions were determined by reversed-phase HPLC (Table 1). Three LA solutions were prepared by dilution and subsequent heating without pH adjustment. Different LA concentration ranging from 42.5% to 8.5% yielded different oligomer composition and pH values. In the 42.5% LA solution, the relative amount of dimer and oligomers (n > 1) was 12.15%, whereas the presence of dimer and oligomers was not observed in 8.5% LA solution. The pH values were higher in solutions with lower LA concentration. Four LA solutions were prepared by dilution and pH adjustment prior to heat treatment. LA solutions with the same LA concentration and different pH resulted in different oligomer compositions. In LA solutions with low pH below 4, oligomer contents were measured from 8% to 5.7%, whereas in LA solutions with high pH above 4, only a monomer peak was observed and the contents were less than those of low pH LA solutions. In addition, the LA solutions with high pH were significantly more viscous compared with LA solutions with low pH. This may be related to pK_a value 3.86 of LA. It is probable that polymerization of LA may occur in LA solutions with high pH. Since a 42.5% solution at pH 2.25 has a sufficient amount of LA oligomers and resembles the acidic microclimate inside degrading PLA microspheres, it was thought be a model system for investigating peptide acylation. Although a 42.5% solution at pH 0.89 has sufficient LA oligomers, the very low pH might be problematic as a model system.

Acylation reaction of octreotide was investigated in 3 LA solutions with different concentrations (42.5%, 21.3%, and 8.5%, wt/wt) and pH values (2.25, 1.47, and 1.85, respectively) at 37°C. Figure 4 shows HPLC chromatograms of octreotides before and after incubation in the LA solutions (42.5%, pH 2.25) at 37°C for 30 days. After incubation, 6 additional peaks were observed and identified by MALDI-TOF MS (Table 2). The additional peaks of octreotide (peaks 2-7) shown in HPLC chromatogram after incubation were shown to be acylation products by mass difference of LA unit (72 Da). During the incubation in the LA solutions of various concentrations, the acylation products of octreotide were observed in a time-dependent as well as concentration-dependent manner (Figure 5). In a 42.5% LA solution (pH 2.25), the remaining amounts of intact octreotide steadily decreased to 51.0% through 30 days of incubation, as the acylation products increased. However, acylation reaction of mono-PEG-octreotide in LA solution was significantly reduced compared with that of native octreotide. Even after 30 days of incubation in LA solutions, the remaining amounts of mono-PEG-octreotide in 42.5% LA solution (pH 2.25) were comparable to the initial concentration. The acylation products of mono-PEG-octreotide in 42.5% LA solution were less than those of native octreotide in 8.5% LA solution. Figure 6 shows the HPLC chromatograms of mono-PEG-octreotides before and after incubation in the LA solution (42.5%, pH 2.25) for 30 days. In the chromatogram of 30-day incubation, one small additional peak was observed but the amount was below the quantification threshold. These data suggest that mono-PEGylation may prevent the acylation reaction of octreotide in degrading PLA microspheres. The stability of mono-PEG-octreotide may be attributable to the steric hindrance of PEG strand. The steric hindrance of PEG molecules might prevent LA units from approaching other acylation site. In the previous study with PLA microspheres containing octreotide, the acylation products of 9.7% were found after 50 days in phosphate buffered saline (pH 7.4), whereas PLGA microspheres showed the acylation products ranging from 66.4% to 25.6% according to the PLGA MW and LA:glycolic acid (GA) composition.[17] In a 42.5% LA solution, an amount of ~50% acylation products is comparable to that produced from degrading PLGA 50:50 microspheres. From this point of view, the stability results of LA solutions could be enough to expect that mono-PEG-octreotide could be also stable in PLGA as well as PLA microspheres.

Table 1. Contents of Lactic Acid Monomers and Oligomers in Solutions With Different Lactic Acid Concentration and pH Determined by High-Performance Liquid Chromatography Analysis*

LA Solution	LA Concentration	pH	Monomer (n = 1)	Dimer (n = 2)	Oligomer (n > 2)
†LA Solution 1	42.5%	0.89	87.9%	11.5%	0.61%
†LA Solution 2	21.3%	1.47	96.4%	3.56%	ND
†LA Solution 3	8.5%	1.85	100%	ND	ND
‡LA Solution 4	42.5%	2.25	92.1%	7.74%	0.21%
‡LA Solution 5	42.5%	3.11	94.3%	5.74%	ND
‡LA Solution 6	42.5%	4.23	100%	ND	ND
‡LA Solution 7	42.5%	6.09	100%	ND	ND

*LA indicates lactic acid; ND, not detected.

†pH not adjusted.

‡pH adjusted with NaOH prior to heating treatment.

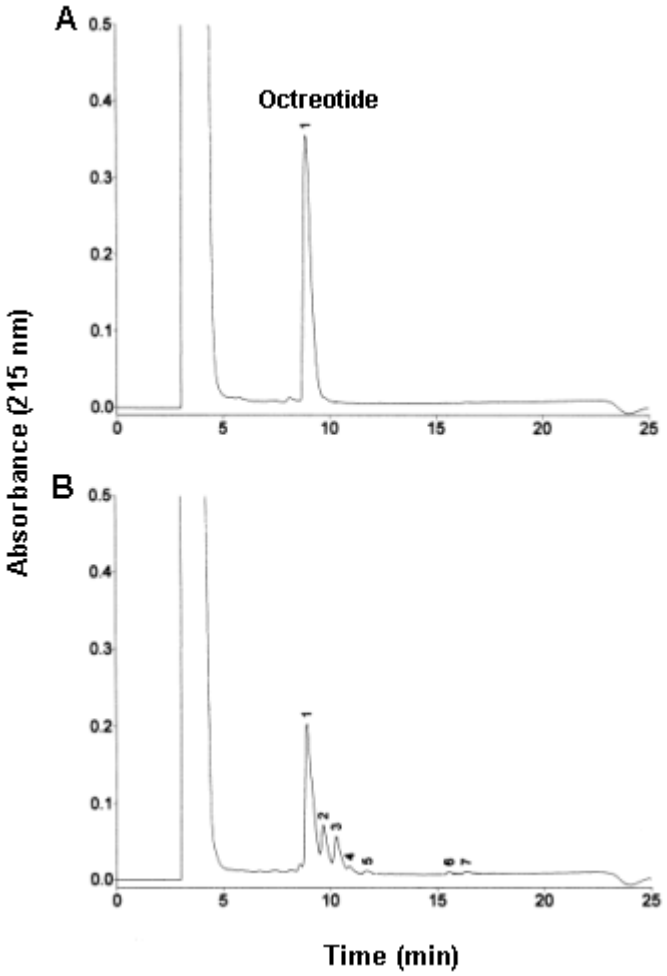

Figure 4. HPLC chromatograms of octreotide before (A) and after (B) incubation at 37°C in 42.5% LA solution (pH 2.25) for 30 days. Masses of each peak are presented in Table 2.

CONCLUSION

Mono-PEG-octreotide prepared by N-terminal site-specific PEGylation preserved the secondary structure of native octreotide and was stable against acylation reaction in LA solutions. Incorporation of PEGylated octreotide into biodegradable microspheres would result in a controlled-release system with different release pattern, such as reduced initial burst and prolonged drug release, as well as increased peptide stability inside degrading microspheres.

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Table 2. Characterization of Acylation Products of Octreotide by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry*

HPLC Peak†	Observed Mass (m/z)	Expected Structure
1	1019, 1041	Octreotide, Octreotide-Na
2	1091, 1113	Octreotide-1LA, Octreotide-Na-1LA
3	1113, 1129	Octreotide-Na-1LA, Octreotide-Na-OH-1LA
4	1091, 1113, 1129	Octreotide-1LA, Octreotide-Na-1LA, Octreotide-Na-OH-1LA
5	1091, 1113, 1129	Octreotide-1LA, Octreotide-Na-1LA, Octreotide-Na-OH-1LA
6	1091, 1162	Octreotide-1LA, Octreotide-2LA
7	1091, 1162	Octreotide-1LA, Octreotide-2LA

*HPLC indicate high-performance liquid chromatography.

†See Figure 4.

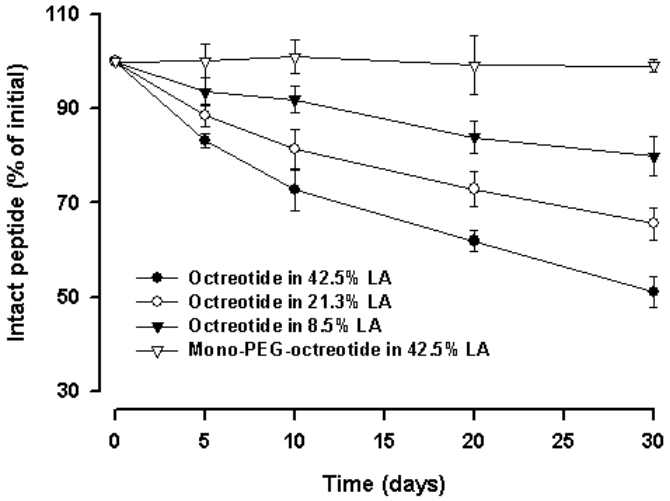

Figure 5. Stability of octreotide and mono-PEG-octreotide in various LA solutions.

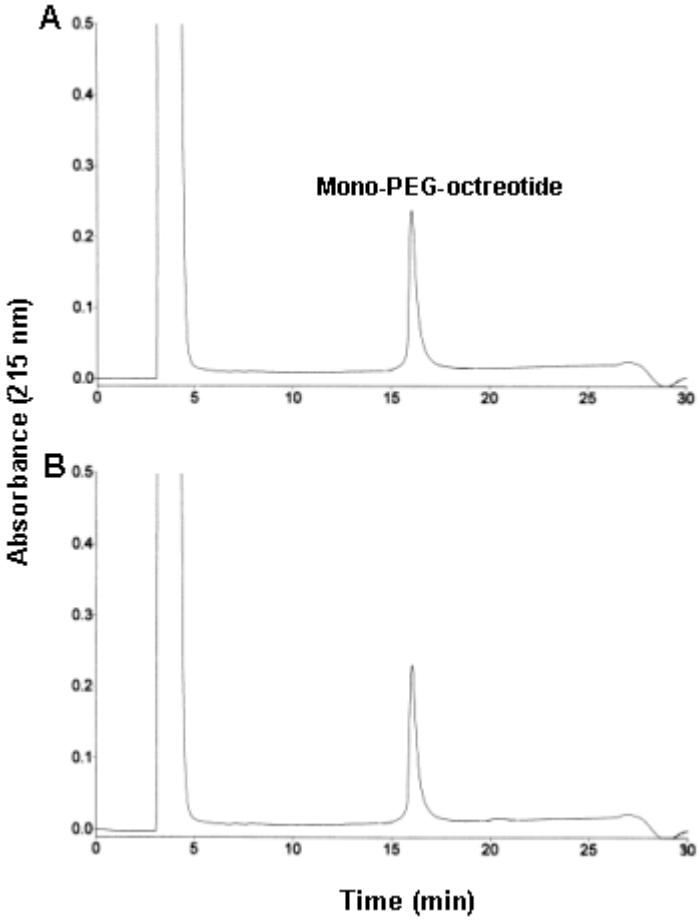

Figure 6. HPLC chromatograms of mono-PEG-octreotide before (A) and after (B) incubation at 37°C in 42.5% LA solution (pH 2.25) for 30 days.

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