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Effect of Peptide Charge on the Formation of Acylated Peptide Impurities in PLGA Formulations

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ABSTRACT − The purpose of this study was to investigate the effect of peptide charge on the interaction between peptide and poly(D,L-lactide-co-glycolide) (PLGA) for evaluating mechanism of acylated peptide formation in PLGA matrix. As a model peptide, octreotide, a synthetic somatostatin analogue and active ingredient of commercial PLGA product, was used. The disulfide group of octreotide was reduced with dithiothreitol and the sulfhydryl groups were modified with N-β-maleimidopropionic acid (BMPA) to neutralize octreotide with positive charge in physiological conditions. The BMPA-conjugated octreotide was identified by measuring the molecular mass with liquid chromatography-mass spectrometry. In the interaction study with PLGA, native octreotide showed initial adsorption to PLGA and substantial production of acylated peptides (56% of overall peptide), whereas BMPA-conjugated octreotide showed minimal adsorption to PLGA and no acylation products for 42 days. Consequently, the neutralization of octreotide completely inhibited the peptide acylation by preventing interaction of peptide with PLGA. In conclusion, this study demonstrates that the initial polymer interaction of peptide is important step for peptide acylation in PLGA matrix and suggests the modulation of peptide charge as strategy for inhibiting the formation of acylated peptide impurities.

Key words − Drug stability, Peptide acylation, Peptide charge, Octreotide, Poly(D,L-lactide-co-glycolide)

Poly(D,L-lactide-co-glycolide) (PLGA) is the most commonly used biopolymer for the controlled release delivery of peptides and proteins owing to its good biodegradability and biocompatibility (Wischke and Schwendeman, 2008). Several peptide drugs, such as the Lupron Depot® (leuprolide), Zoladex® implant (goserelin) and Sandostatin LAR® (octreotide), have been developed by using PLGA. However, instability of therapeutic peptides encapsulated in the PLGA formulations has emerged as one of the significant obstacles in the development of PLGA delivery systems (Bilati et al., 2005; Houchin and Topp, 2008). Among several peptide instability issues, the formation of acylated peptide impurities in PLGA matrix was recently found by mass spectrometric techniques (Lucke et al., 2002; Na et al., 2003a). The acylated peptide impurities may result in the incomplete drug release from the PLGA matrix, lower pharmacological effect and higher toxicity (Na et al., 2007).

In the previous studies, we demonstrated that the acylated peptides are formed as a result of reaction of primary amino groups of peptide (N-terminal and lysine's amines) with ester backbone of PLGA (Na et al., 2005). The mechanism of acylated peptide formation has been proposed to involve an initial ionic interaction between cationic peptide and carboxylate end-group of PLGA, followed by a nucleophilic attack of peptide's amines to ester groups of PLGA and subsequent release of acylated peptides by PLGA hydrolysis. The peptide acylation reaction has been shown to be affected by several factors, such as polymer structure and medium pH (Murty et al., 2005a and 2005b; Na, 2010). Several strategies for minimizing and preventing the peptide acylation in PLGA formulations have been proposed, including incorporation of pH modifying excipients or divalent cationic salts into PLGA formulations and chemical modification of peptide with polyethylene glycol (PEG) (Houchin et al., 2007; Na et al., 2003b and 2005; Park et al., 2010; Sophocleous et al., 2009; Zhang et al., 2009).

The purpose of this study was to evaluate mechanism of acylated peptide formation in PLGA matrix by investigating effect of peptide charge on peptide acylation. As a model peptide, octreotide, a synthetic somatostatin analogue and active ingredient of commercial product Sandostatin LAR® depot (Novartis Pharma, Switzerland), was used. In a previous study, the octreotide in Sandostatin LAR has been also shown to form acylated peptide impurities after *in vitro* incubation in phosphate buffer saline (Murty et al., 2003). In this study, disulfide group of octreotide was reduced with dithiothreitol (DTT) and the sulfhydryl groups were modified with N-β-

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maleimidopropionic acid (BMPA) to neutralize octreotide, which is positively charged in physiological conditions. The BMPA-conjugated octreotide was identified by measuring the molecular mass with liquid chromatography-mass spectrometry (LC-MS). The stability of the neutralized octreotide against peptide acylation by PLGA was studied to investigate the effect of peptide charge on the formation of acylated peptide impurities in PLGA formulations.

Materials and Methods

Materials

Octreotide (H_2N-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol, molecular weight 1019.26) manufactured from Bachem (Torrance, CA, USA) was a gift from Oakwood Laboratories, LLC (Oakwood, OH, USA). PLGA with free carboxylic end groups (molar ratio of D,L-lactide:glycolide=50:50, viscosity: 0.16-0.24 dL/g, product name: RESOMER® RG502H) was supplied by Boehringer Ingelheim (Ingelheim, Germany). N-β-maleimidopropionic acid (BMPA, MW 169.13) was purchased from Pierce (Rockford, IL, USA). Dithiothreitol (DTT) was obtained from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA) were obtained from J.T. Baker (Philipsburg, NJ, USA) and Acros Organic (Pittsburgh, PA, USA), respectively. All other chemicals were of analytical grade and were used as obtained commercially.

Conjugation of BMPA to octreotide

Octreotide was treated with DTT for 30 min at 37°C to produce free sulfhydryl groups by disulfide bond cleavage. BMPA was added to octreotide in 0.1 M phosphate buffer (pH 7.0) with molar ratio of 1:10 (octreotide:BMPA). The reaction mixture was shaken at 700 rpm with EYELA cube mixer CM-1000 (Rikakikai Co. Ltd., Tokyo, Japan) for 2 hrs at room temperature and the BMPA-octreotide conjugate was separated by HPLC.

Separation of BMPA-conjugated octreotide

The reaction mixture between BMPA and octreotide was loaded onto a Dionex Ultimate-3000 VWD model (Dionex Corp., Sunnyvale, CA, USA) with Acclaim C-18 column (4.6×250 mm, 5 μm particle size, Dionex Corp.). Isocratic elution was performed with the mobile phase consisted of deionized water, acetonitrile, and TFA (water:acetonitrile:TFA= 65:35:0.1). A flow rate was 1 mL/min and UV absorbance was monitored at 215 nm. The BMPA-conjugated octreotide fraction was collected and the organic solvents were evaporated by Speed-Vac Apparatus (Eppendorf, Hamburg, Germany). The molecular weights of the separated MA-octreotides were determined by LC-MS.

LC-MS analysis

LC/MS was carried out using an Agilent Technologies Series 1100 LC/MSD VL system (Palo Alto, CA, USA). The column, mobile phase, and elution conditions were the same as those used in HPLC method described above. The mass spectrometer was operated in the positive ion mode using the following conditions: drying gas (N_2) flow of 10 L/min, drying gas at 350°C, nebulizer pressure of 45 psi, and capillary voltage of 3 kV. The fragmentor voltage was 100 V. Ions were detected by scan mode and mass range was set from *m/z* 400 to 1400.

Polymer interaction study with PLGA

The polymer interaction of octreotide or BMPA-octreotide with PLGA was investigated by adding 10 mg of PLGA polymer to 1 mL of octreotide or BMPA-octreotide (peptide concentration of 200 μg/mL) in 10 mM phosphate buffer (pH 7.4) at 37°C. Samples were collected at 3, 7, 14, 21, 28, 35, and 42 days. After centrifuge at 5000 rpm, supernatants filtered with 0.45 μm membrane filter were loaded onto HPLC system. The HPLC analysis was carried out on a Prosphere C-18 column (4.6×250 mm, 5 μm particle size, Alltech, Deerfield, IL, USA). A gradient elution was performed with mobile phase A (0.1% TFA in water) and mobile phase B (0.1% TFA in acetonitrile). A linear gradient from 80:20 to 65:35 (mobile phase A:B) for 20 min was performed and total run time was 30 min. The flow rate of 1.0 mL/min and sample injection volume was 20 μL. UV absorbance was monitored at 215 nm.

Results and Discussion

Octreotide has two positively charged residues, N-terminus (Phe^1) and Lys^5, which are reactive sites for ionic interaction with PLGA. The ionic interaction between peptide and PLGA has been regarded as an important initial step for the formation of acylated peptide impurities in PLGA matrix. To investigate the effect of positive charge of peptide on the formation of acylated peptide impurities, the neutralized octreotide was prepared by reducing disulfide group of octreotide with DTT followed by conjugation with BMPA (Figure 1).

Figure 2 shows the HPLC chromatograms of octreotide, DTT-treated and BMPA-conjugated octreotides, which were eluted with the retention times of 3.56, 4.67 and 3.84 min, respectively. The HPLC results show that the BMPA-conju-

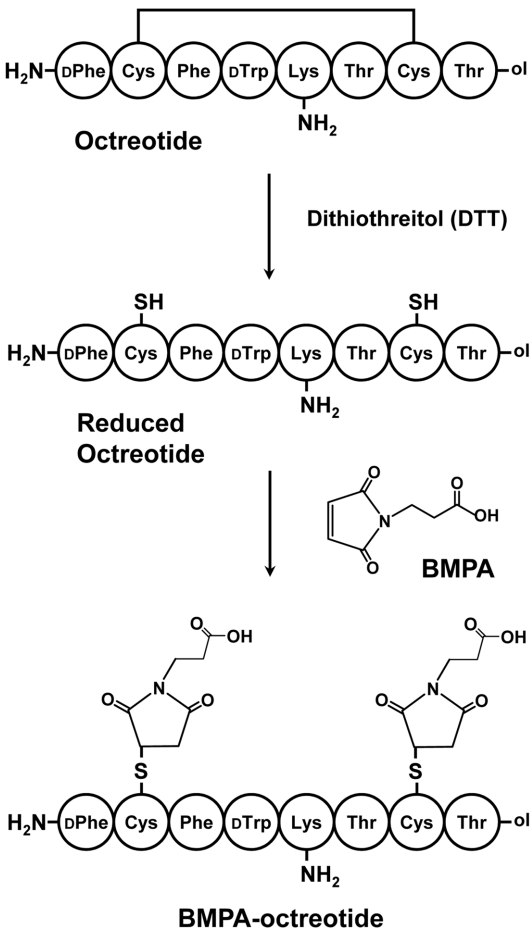

Figure 1. Conjugation of BMPA to cysteine residues of octreotide following reduction of disulfide bond with DTT.

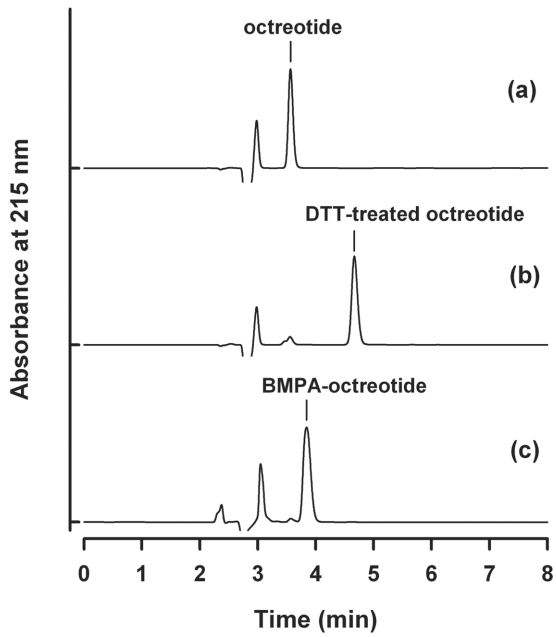

Figure 2. HPLC chromatograms of native octreotide (a), DTT-treated octreotide (b), and BMPA-conjugated octreotide (c).

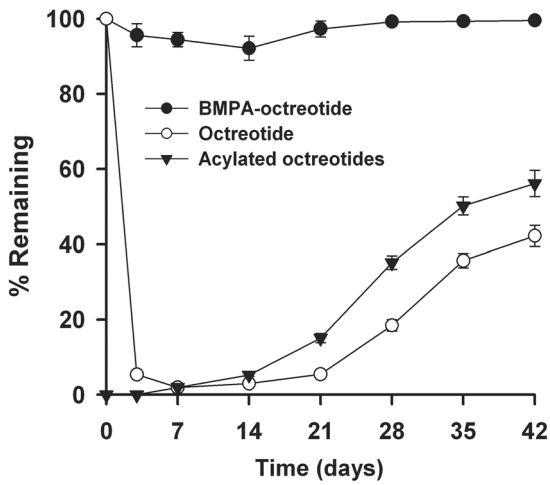

Figure 3. Polymer interaction profiles of octreotide and BMPA-octreotide with PLGA in 10 mM phosphate buffer (pH 7.4) at 37°C.

gated octreotide was successfully prepared with high purity. Each peak was identified by measuring their molecular masses with LC-MS (Table I). The doubly charged molecular ion masses ($[M+2H]^{2+}$) of octreotide, DTT-treated and BMPA-conjugated octreotides were measured to be *m/z* 510.3, 511.4, and 680.4, respectively, which were almost identical to the calculated masses.

The stability of native octreotide and BMPA-octreotide against peptide acylation by PLGA was studied by incubating with PLGA in 10 mM phosphate buffer (pH 7.4) at 37°C. Figure 3 shows the polymer interaction profiles of native octreotide and BMPA-octreotide for 42 days. The polymer interaction of native octreotide involved an initial adsorption of the peptide to PLGA, peptide acylation reaction with PLGA, and the release of intact and acylated octreotides. After incubation for 7 days, over 98% of octreotide was adsorbed to PLGA and then the amount of intact octreotide in the supernatant increased to 42.2% after 42 days. The acylated octreotides were 5.2% at day 14 and then the amount increased to 56.1% for 42 days. After incubation for 42 days, the sum of intact and acylated octreotides reached 98.3%. The BMPA-conjugated octreotide showed much lower polymer interaction and the acylation products were not observed for 42 days. Although the adsorptions of 4.4% for 3 days and 7.8% for 14 days were observed, the BMPA-octreotide was entirely recovered after 28 days. At day 42, the remaining amount of BMPA-octreotide in the supernatant was 99.5% of the added amount. Therefore, the neutralization could completely inhibit peptide acylation by PLGA.

Table I. *LC/MS of DTT-treated and BMPA-conjugated octreotides*

Peptides	Retention time (min)	Measured $[M+2H]^{2+}$	Calculated $[M+2H]^{2+}$
Octreotide	3.56	510.3	510.5
DTT-treated octreotide	4.67	511.4	511.5
BMPA-octreotide	3.84	680.4	679.8

Conclusions

The present study evaluates that the ionic interaction between positively charged peptide and PLGA plays an important role in the formation of acylated peptide impurities in the formulations prepared with PLGA. Based on this mechanism, the incorporation of divalent cations, such as Ca^{2+} and Mn^{2+}, into PLGA microparticles was recently proposed for blocking the carboxylic acid end-groups of PLGA (Sophocleous et al., 2009; Zhang et al., 2009). The blocking of positively charged groups of peptide with biocompatible materials can be another strategy for preventing peptide acylation. As the charge of peptide is dependent on its isoelectric point (pI), the formation of acylated peptides in PLGA matrix would vary according to the peptide species. Based on the result of this study, the acidic peptide with low pI value would show lower chance for peptide acylation compared with the basic peptide with high pI value.

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