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Note

In vitro release study of mono-PEGylated growth hormone-releasing peptide-6 from PLGA microspheres

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Abstract

The purpose of this study was to investigate *in vitro* release property of mono-PEGylated growth hormone-releasing peptide-6 (GHRP-6) microspheres. The microspheres encapsulating native GHRP-6 or mono-PEG-GHRP-6 were prepared using the single oil-in-water emulsion solvent evaporation method. *In vitro* release study was performed in 0.1 M phosphate buffer, pH 7.4, containing 0.02% Tween 80 and sodium azide at 37 or 55 °C. The mono-PEG-GHRP-6 microspheres showed a lower initial burst compared with native GHRP-6 microspheres and zero-order release profile for a 1-month period. The release period was dependent on the PEG size attached to the GHRP-6 with more rapid drug release being observed with the smaller PEG size. This study suggests that PEGylated peptide has good potential as a source for a sustained release microsphere delivery system.

Keywords: PEGylation; Peptide; Microsphere; Controlled release

Biodegradable microspheres using poly(D,L-lactide-*co*-glycolide) (PLGA) as a polymer have been successfully used to achieve the sustained (preferably zero-order) release of small peptide drugs with molecular weight (MW) around 1000, such as leuprolide and octreotide, over a prolonged period of time, typically weeks or months (D'Souza and DeLuca, 2006). However, some problems associated with the microsphere release system, such as the chemical instability of the peptide and the non-linear release profile, are still major challenges to the successful commercialization (Yeo and Park, 2004; Bilati et al., 2005).

Recently, the covalent attachment of PEG to the peptide (PEGylation) was demonstrated to be effective approach for stabilizing peptide inside degrading microspheres by inhibiting the peptide acylation by PLGA polymer (Na et al., 2003a,b; Na and DeLuca, 2005). In addition, mono-PEGylation at the N-terminus of octreotide preserved the biological activity of native peptide (Na et al., 2005). Although the release properties of PEGylated proteins (MW > 5000) have been reported in some literatures (Kim et al., 2002; Diwan and Park, 2003; Hinds et al., 2005; Castellanos et al., 2005), the release profiles of microspheres encapsulating PEGylated peptide have not been reported.

The objective of this study was to investigate the *in vitro* release property of microspheres prepared with mono-PEGylated peptide. As a model peptide, growth hormone-releasing peptide-6 (GHRP-6, His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2, MW 872.4) was PEGylated and the mono-PEGylated species were incorporated into PLGA microspheres. The *in vitro* release properties of the native GHRP-6 or mono-PEG-GHRP-6 conjugates from PLGA microspheres were examined.

The GHRP-6 and succinimidyl propionate-monomethoxy PEGs (SPA-mPEG) were acquired from Bachem (Torrence, CA, USA) and Nektar Therapeutics (Huntsville, AL, USA), respectively. The hydrophilic PLGA 5010 (PLGA 50:50, MW 10,000) was obtained from Wako Pure Chemical (Tokyo, Japan). All other chemicals were of analytical grade and were used as obtained commercially.

The PEGylation of GHRP-6 was performed with different molecular weights of SPA-mPEG (MW 1000, 2000, and 5000 Da) in a 0.1 M phosphate buffer solution (pH 7.5) with a molar ratios 1:2 (GHRP-6:SPA-mPEG) for 1 h at room temperature. Mono-PEG-GHRP-6 was isolated by cation-exchange

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chromatography using a TSK-SP-5PW ion-exchange column (Tosohaas, Montgomeryville, PA, USA). The purified fraction was evaluated by MALDI-TOF mass spectrometry performed using a Voyager-RP Biospectrometry Workstation (Applied Biosystems, Cambridge, MA, USA) in a linear mode. A saturated solution of alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA), as the final concentration, was used as the matrix solution.

Microspheres were prepared using oil-in-water (o/w) emulsion solvent evaporation method. The native GHRP-6-loaded microspheres were prepared by dissolving 5 mg of GHRP-6 in 0.2 ml of methanol and mixing the resulting solution with 100 mg of PLGA 5010 in 0.8 ml methylene chloride. In contrast, mono-PEG-GHRP-6 was co-dissolved with the PLGA polymer in methylene chloride. The amount of mono-PEG-GHRP-6 equivalent to 5 mg of native GHRP-6 was dissolved in 1 ml methylene chloride with 100 mg of PLGA 5010. The resulting solution (disperse phase, DP) was then injected rapidly into 100 ml of a 1% aqueous polyvinyl alcohol solution (continuous phase, CP) during mixing with a Silverson L4R mixer (Silverson Machines, England) at 3000 rpm at below 4 °C. The resulting dispersion was agitated for 5 min and then evaporated from the CP by gradually increasing the temperature to 40 °C over a 30 min period, which was maintained for 60 min. The resulting microspheres were centrifuged, washed three times with deionized water, and freeze-dried. The size distribution of the microspheres was obtained using a Malvern laser diffraction particle size analyzer (Malvern Instruments, England). The surface morphology was examined using Hitachi model MIS-2400 scanning electron microscopy. The drug content was determined by reversed phase HPLC using a LiChrospher RP-18 column (4.0 mm × 125 mm, Merck, Germany). A gradient elution condition was 80:20 to 40:60 (0.1% TFA in water:0.1% TFA in acetonitrile) over a 10 min period with a flow rate of 1.0 ml/min. The UV absorbance was measured at 215 nm.

In vitro release study of microspheres was performed in a 0.1 M phosphate buffer (pH 7.4) containing 0.02% Tween 80 and sodium azide at 37 or 55 °C. At specific time points, the tubes were removed and centrifuged at 10,000 rpm for 3 min. The peptide content from the supernatant was directly analyzed by HPLC. After freeze-drying the microspheres, the amount of residual peptide inside the microspheres was also determined by HPLC after dissolving them in dimethylsulfoxide and subsequent diluting in 50% acetonitrile.

Mono-PEG-GHRP-6 conjugates prepared with SPA-mPEG-1000, -2000, and -5000 showed average molecular weights of 1757, 3100, and 6316, respectively, in the MALDI-TOF MS analysis. The mono-PEG-GHRP-6 conjugates were soluble in methylene chloride, which is used as a solvent for PLGA in the preparation of microspheres, unlike the native GHRP-6. This may be attributed to the solubility of PEG in methylene chloride. Fig. 1 shows the comparison of release profiles between the mono-PEG-2K-GHRP-6 microspheres prepared using methanol and methylene chloride as a drug solvent. Their *in vitro* release profiles were observed under accelerated release conditions at 55 °C to predict drug release in a short period (D'Souza et al., 2005). In the accelerated release study with GHRP-6 microspheres, the complete release at 37 °C was shortened from 30 days to 6–7 days at 45 °C, 4 days at 55 °C, and 2 days at 65 °C. Among short-term release conditions, the shape of the release profiles at 55 °C was the most similar to that at 37 °C (data not shown). Compared with the microspheres prepared using methanol as the drug solvent, the microspheres prepared with the mono-PEG-2K-GHRP-6 dissolved in methylene chloride exhibited a greatly reduced initial burst release (18.4% versus 37.6% at 2 h) and zero-order release from 4 to 60 h. The high initial burst release in the PLGA microspheres is often attributed to molecules at or near the outside surface and particularly facile diffusion to the surface through internal pores and micro-channels formed during manufacture (Yeo and Park, 2004). The reduced burst release of the PEGylated peptide from the microspheres can be attributed to the more even distribution of the PEGylated peptide within the matrix of the microspheres. In particular, the PEGylated peptide co-dissolved in the PLGA solution is expected to be loaded more homogeneously within the PLGA matrix.

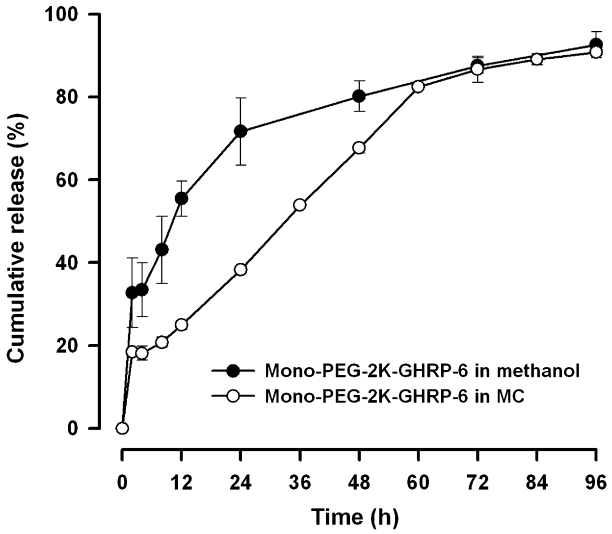

Fig. 1. Comparison of the release profiles between the mono-PEG-2K-GHRP-6 microspheres prepared using methanol and methylene chloride (MC) as a peptide solvent in the o/w method. The studies were performed in the release test medium (0.1 M phosphate buffer, pH 7.4, containing 0.02% Tween 80 and sodium azide) at 55 °C. Each point represents the mean ± S.D. of three experiments.

Fig. 2 shows the release profiles of the PLGA microspheres encapsulating the native GHRP-6 or mono-PEG-GHRP-6 conjugates with different sizes at 37 °C. All the mono-PEG-GHRP-6 microspheres were prepared using methylene chloride as the drug solvent, whereas the native GHRP-6 microsphere used methanol as the drug solvent. The size of the microspheres containing each mono-PEG-GHRP-6 (24.84–26.24 μm) was similar to that of native GHRP-6 (24.65 μm). In SEM analysis, all the microspheres were spherical with a non-porous surface and showed more than 90% encapsulation efficiency. Native GHRP-6 microsphere exhibited a tri-phasic release behavior. In 1 day, approximately 23% of the initial burst was observed and the second phase release of the peptide increased to 73% for up to 8 days. This was followed by a third phase release and the total release amount reached 98% for 30 days. All the mono-

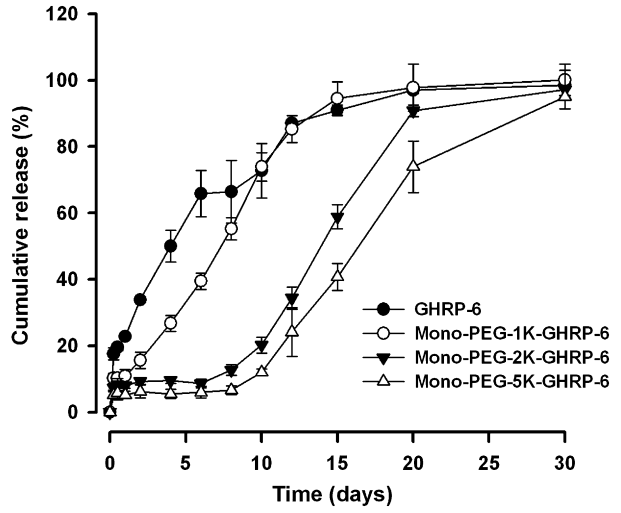

Fig. 2. The release profiles of the native GHRP-6, mono-PEG-1K-, -2K-, and -5K-GHRP-6 microspheres. The studies were performed in the release test medium at 37 °C and each point represents the mean ± S.D. of three experiments.

PEG-GHRP-6 microspheres showed a relatively lower initial burst release than the native GHRP-6 microsphere. After 1 day of incubation, the microspheres encapsulating the mono-PEG-1K-, -2K-, and 5K-GHRP-6 showed drug release of 10.8%, 8.0%, and 5.2%, respectively. Among the PEGylated GHRP-6 microspheres, mono-PEG-1K-GHRP-6 microspheres showed the most rapid and favorable profile with a linear drug release pattern for 15 days after the initial release. Both mono-PEG-2K- and -5K-GHRP-6 microspheres showed a lag period for 6–8 days after the initial release and subsequent linear drug release pattern for approximately 15–20 days. The drug release period was dependent on the PEG size attached to the GHRP-6. The time points for reaching more than 90% release of the mono-PEG-1K-, -2K-, and -5K-GHRP-6 microspheres were 15, 20, and 30 days, respectively. This means that the rate of drug release from the microspheres decreases with increasing size of the PEG attached to GHRP-6. The lag time and slower release indicates that GHRP-6 attached with a higher molecular weight PEG diffused more slowly from the microsphere matrix.

In conclusion, this study suggests that PEGylated peptide has good potential as a source for a microsphere delivery system with a low initial burst and zero-order release profile. The PEG size attached to the peptide can be a factor for controlling the peptide release period.

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