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G-CSF loaded biodegradable PLGA nanoparticles prepared by a single oil-in-water emulsion method

Seung Ho Choi, Tae Gwan Park *

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, South Korea

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Abstract

A new formulation method was developed for preparing poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles loaded with recombinant human granulocyte colony-stimulating factor (rhG-CSF). Lyophilized rhG-CSF powder and PLGA polymer were directly co-dissolved in a single organic phase, and the resulting solution was dispersed into an aqueous solution. PLGA nanoparticles encapsulating rhG-CSF were produced by a spontaneous emulsion/solvent diffusion method. In this manner, rhG-CSF was molecularly dissolved in the polymer phase. Release profile of rhG-CSF from PLGA nanoparticles was compared with those from two kinds of PLGA microparticles which were separately prepared by either single oil-in-water (O/W) or double water-in-oil-in-water (W/O/W) emulsion technique. The sizes of rhG-CSF loaded nanoparticles, O/W microparticles, and W/O/W microparticles were about 257 nm, 4.7 μm, and 4.3 μm, respectively. For rhG-CSF nanoparticles, about 90% of encapsulated rhG-CSF was released out in a sustained manner from PLGA nanoparticles over a 1 week period, but for rhG-CSF microparticles, only about 20% of rhG-CSF could be released out during the same period. Reversed phase and size exclusion chromatograms revealed that the structural integrity of released rhG-CSF from nanoparticles was nearly intact, compared to that of native rhG-CSF.

Keywords: rhG-CSF; Biodegradable; PLGA; Nanoparticles; Sustained release

1. Introduction

There have been continuous interests in sustained delivery of therapeutic proteins using various biodegradable polymeric microparticular formulations (Langer, 1990; Cohen et al., 1991; Cleland and Langer, 1994). In many cases, proteins have been encapsulated into biodegradable polymeric microparticles by a double emulsion technique (W/O/W) due to their limited solubility in organic solvents (Ogawa et al., 1988). However, due to unstable nature of therapeutic proteins, it has been difficult to achieve a desirable sustained release profile over an extended period (Morlock et al., 1997; Weert et al., 2000). One primary concern with encapsulation of proteins into PLGA microparticles is that their structural integrities are severely damaged through chemical and physical degradation reactions occurring during formulation process and release period. Inactivation of encapsulated proteins can take place via two different mechanisms. In the formulation stage, proteins are exposed to organic solvents during an emulsion formation process in the double emulsion technique, resulting in protein adsorption/aggregation at the water/organic solvent interface (Crott and Park, 1997; Kim and Park, 1999). Secondly, during the release period, an acidic environment generated through the degradation of PLGA phase produces acidic monomers and oligomers that cause the encapsulated proteins to denature and aggregate to a greater extent (Fu et al., 2000; Kim and Park, 2004). More importantly, the acidic microenvironment developed within the interior of degrading PLGA microparticles might alter the chemical structure of the encapsulated proteins by an acylation reaction. It was recently reported that degradation products of PLGA, lactic and glycolic acid monomers and their oligomers, were chemically conjugated to free amino groups of the encapsulated peptides and proteins under acidic conditions (Murty et al., 2003; Na et al., 2003). The chemical modification of encapsulated proteins within the degrading PLGA microparticles was clearly demonstrated by employing a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) technique. This might elicit serious concerns for sustained delivery systems of

* Corresponding author. Tel.: +82 42 869 2621; fax: +82 42 868 2610.
E-mail address: tgpark@kaist.ac.kr (T.G. Park).

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therapeutic peptides and proteins using biodegradable PLGA microspheres.

In contrast to PLGA microparticles, PLGA nanoparticles are submicron-sized polymeric colloidal particles encapsulating therapeutic drugs within their polymeric matrix (Brigger et al., 2002). They were mainly used for target-specific drug delivery of various anti-cancer drugs including poorly water soluble drugs such as paclitaxel (Dong and Feng, 2004). Therapeutic proteins such as vaccines can also be encapsulated in PLGA nanoparticles by the double emulsion solvent evaporation technique, similar to that of PLGA microparticles (Desai et al., 1996; Sanchez et al., 2003). However, they exhibited extremely low protein loading efficiency and high burst releases at an early incubation stage. This rapid protein release was primarily related to the small size of nanoparticles with a short diffusion path-length, and to uneven and surface localized distribution of hydrophilic protein molecules within hydrophobic polymeric nanoparticles. Thus sustained protein delivery systems using PLGA nanoparticles were scarcely reported in literature. Nevertheless, PLGA nanoparticles are expected to minimize the acidic microenvironment problem to a greater extent than PLGA microparticles because of their nano-sized dimension. The acidic degradation products would diffuse out more readily to the outer aqueous medium from the PLGA nanoparticles, resulting in little chance of accumulation inside. To this end, a new sustained release formulation based on PLGA nanoparticles that can avoid the chemical modification of proteins by the acidic degradation products is demanded.

Recombinant human granulocyte colony-stimulating factor (rhG-CSF) can induce predominantly the differentiation and proliferation of neutrophilic granulocyte progenitor cells and has been administered for treatment of neutropenia occurring during chemotherapy (Souza et al., 1986). Because rhG-CSF has a very short half-life and goes through rapid clearance during blood circulation, even if high doses administered, various studies have been attempted to increase the efficacy of rhG-CSF treatment (Kinstler et al., 1996; Gibaud et al., 1998; Choi et al., 2003).

In this study, rhG-CSF was molecularly encapsulated into PLGA nanoparticles to achieve a sustained release profile from PLGA nanoparticles. Lyophilized rhG-CSF powder was directly dissolved in a mixture of organic solvents to prepare a single emulsion system. rhG-CSF loaded PLGA nanoparticles were prepared by a spontaneous emulsion/solvent diffusion method. The release profile was compared with conventional PLGA microparticular formulations prepared by a single or double emulsion technique. Structural stabilities of rhG-CSF dissolved in an organic solvent and released from PLGA nanoparticles were comparatively examined.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-co-glycolide) with a lactide/glycolide molar ratio of 50/50 with a molecular weight of 8300 as determined by gel permeation chromatography was obtained from Boehringer Ingelheim (Resomer RG502H). Recombinant human granulocyte-colony-stimulating factor (rhG-CSF) was obtained from Mogam Biotechnology Research Institute (Gyeonggido, South Korea). Pluronic F-127 was purchased from BASF (Parsippany, NJ). All other chemicals and reagents were of analytical grade.

2.2. Methods

2.2.1. Dissolution of rhG-CSF in organic solvents

rhG-CSF was dialyzed in deionized water to remove salts, and then the pH value of rhG-CSF solution was adjusted to pH 4.0 and freeze dried. The lyophilized rhG-CSF (120 μg) was dissolved in 1 ml of a mixture of dimethyl sulfoxide (DMSO) and methylene chloride in varying volume ratios. The molecular dissolution of rhG-CSF in the mixture of organic solvents was judged by measuring the change in transmittance at 600 nm.

2.2.2. Preparation of rhG-CSF encapsulated nanoparticles

For encapsulating rhG-CSF into PLGA nanoparticles, 47.5 mg of PLGA (RG502H) and 2.5 mg of rhG-CSF dissolved in 1 ml of DMSO was slowly added into 50 ml of 1% (w/v) Pluronic F-127 solution and then homogenized at 4000 rpm. After 2 min, the suspended solution was dialyzed in deionized water to remove residual DMSO. The suspended nanoparticles were stored in a frozen state (−20 °C) until use. To determine the loading amount, a known amount of freeze-dried nanoparticles was dissolved in a lysis buffer solution (0.1 N NaOH, 0.5% (w/v) sodium dodecyl sulfate) for 2 days, and the amount of extracted rhG-CSF was determined by micro-BCA assay (Pierce, Rockford, IL).

2.2.3. Preparation of rhG-CSF encapsulated microparticles

For encapsulating rhG-CSF into microparticles by the double (W/O/W) emulsion and solvent evaporation method, 0.3 ml of 10 mM sodium acetate buffer solution dissolved with 20 mg of rhG-CSF was emulsified in 1.2 ml of methylene chloride phase containing 180 mg of PLGA (RG502H). The primary emulsion solution was homogenized at 3000 rpm (PowerGene, Fisher, Pittsburgh, PA), and added to 25 ml of 0.5% (w/v) PVA solution with subsequent homogenization at 1000 rpm briefly. The double emulsion solution was incubated for 3 h with continuous homogenization at 200 rpm. The hardened microparticles were collected, washed three times with deionized water, and then freeze-dried. For encapsulating rhG-CSF into microparticles by a single emulsion and solvent evaporation method, PLGA (190 mg) and rhG-CSF (10 mg) were co-dissolved in 2 ml of DMSO/methylene chloride mixture (1:1 volume ratio). The above solution was emulsified in 25 ml of 0.5% (w/v) PVA solution with homogenization at 1000 rpm for 2 min, and followed by incubation for 3 h with further stirring at 200 rpm. The resultant microparticles were recovered and freeze-dried.

2.2.4. Characterization of PLGA nanoparticles and microparticles

The size of PLGA nanoparticles was analyzed by using a dynamic light scattering method (ZetaPlus, Brookhaven

Instruments Corporation, NY). The average size of PLGA microparticles was determined by analyzing the diameter of microparticles appeared on a field emission scanning electron microscope (FE-SEM, XL30SFEG, Philips, The Netherlands) ($n > 100$).

2.2.5. Release of rhG-CSF from nanoparticles and microparticles

In vitro releases of rhG-CSF from PLGA nanoparticles and microparticles were performed in pH 7.4 phosphate buffered saline (PBS) solution containing 0.01% NaN_3 (w/v) and 0.02% Tween 20 (w/v). Five milligrams of dried PLGA nanoparticles was suspended in 5 ml of PBS solution. For PLGA microparticles, 5 mg of dried PLGA microparticles was suspended in 5 ml of PBS solution. The suspended solution was continuously shaken at 37 °C. At predetermined time intervals, the supernatant was collected after centrifugation for determining the amount of released rhG-CSF by micro-BCA assay.

2.2.6. Structural integrity of rhG-CSF dissolved in organic solvent and released from nanoparticles and microparticles

Structural integrities of rhG-CSF either dissolved in organic solvents or released from nanoparticles were examined after reconstituting rhG-CSF in an aqueous PBS buffer solution. The C18 reversed phase HPLC was operated with a flow rate of 1 ml/min and a linear gradient of 0–70% acetonitrile for 35 min. Size exclusion HPLC was performed with an isocratic mobile phase of 0.1 M sodium phosphate buffer, pH 7.0, on a Shodex protein KW-802.5 column (Tokyo, Japan). The circular dichroism (CD) spectra were obtained from a CD spectropolarimeter (J715, Jasco, Japan) at 200 μg/ml of rhG-CSF solution. The spectra were taken at room temperature using a 0.1 cm cylindrical quartz cell.

3. Results and discussion

It is known that some proteins can be molecularly solubilized in various polar organic solvents with or without additives (Chin et al., 1994; Meyer and Manning, 1998; Knubovets et al., 1999). The solubility of a specific protein in organic solvents can be acquired by controlling the pH of an aqueous protein solution before lyophilization. In our previous study using lysozyme as a model protein, it was demonstrated that when lysozyme was lyophilized from an aqueous solution of pH 3, far away from its isoelectric point of pH 11.0, lyophilized lysozyme powder exhibited a dramatic enhanced solubility in a mixture of DMSO and methylene chloride (Park et al., 1998; Yoo et al., 2001). The direct dissolution of proteins in organic solvents was applied to prepare protein loaded PLGA microparticles by a single O/W emulsion technique. Because proteins and biodegradable polymers could be co-dissolved in the same organic solvent, the resultant PLGA microparticles could encapsulate the protein molecules within the polymer bulk phase in a molecularly dissolved state. More recently, we reported that salmon calcitonin (sCT) loaded PLGA nanoparticles were successfully prepared for oral delivery by a spontaneous emulsion and solvent evaporation method (Yoo and Park, 2004). In order to directly dissolve sCT in a polar organic solvent, sCT was hydrophobically ion-paired with sodium oleate to form sCT–oleate complexes that could be dissolved in DMSO. The sCT loaded PLGA nanoparticles had high sCT loading efficiency and exhibited potential for oral sCT delivery.

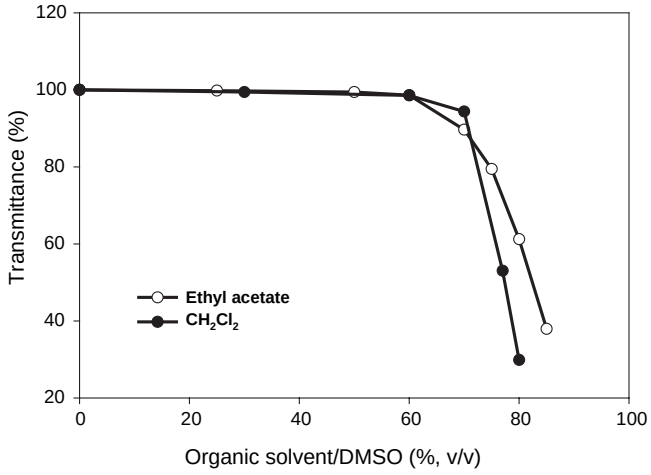

Fig. 1. Transmittance change of freeze-dried rhG-CSF in DMSO/CH_2Cl_2 and DMSO/ethyl acetate co-solvent systems at varying volume ratios.

rhG-CSF is a protein with 174 amino acid residues, an apparent molecular weight of 18,800 and its isoelectric point (p*I*) around 6.1. When rhG-CSF was lyophilized at pH 4, rhG-CSF was completely soluble in DMSO, a polar organic solvent. DMSO was employed to co-dissolve rhG-CSF and PLGA in a single organic phase. DMSO is a water miscible solvent with low toxicity which can also dissolve low molecular weight PLGA polymers. As shown in Fig. 1, rhG-CSF dissolved in DMSO exhibits almost 100% transmittance and no precipitates were observed. This means that rhG-CSF was molecularly dissolved in the DMSO phase. To test the solubility limit of rhG-GCF according to gradual decrease in solvent polarity, rhG-CSF was dissolved in a mixture of organic solvents composed of methylene chloride and DMSO in varying volume ratios. Methylene chloride is a less polar solvent than DMSO. rhG-CSF was completely dissolved in the organic solvent mixture up to the 60% methylene chloride, but its solubility sharply decreased thereafter.

To evaluate the structural stability of rhG-CSF after DMSO treatment, C18 reversed phase and size exclusion HPLC were used. Fig. 2A shows reversed phase HPLC chromatograms of native and DMSO treated rhG-CSF. DMSO treated rhG-CSF was eluted as a single peak, meaning that rhG-CSF was not chemically altered by the DMSO treatment. Fig. 3B shows size exclusion chromatograms of native and DMSO treated rhG-CSF. In the case of DMSO treated rhG-CSF, there is no apparent difference in the elution profile, indicating that rhG-CSF was not physically aggregated even after exposure to DMSO. Fig. 2C shows circular dichroism spectra of native and DMSO treated rhG-CSF. There is no significant change in conformation of rhG-CSF after molecularly dissolving rhG-CSF in the DMSO phase and subsequently reconstituting in an aqueous buffer solution. This suggests that rhG-CSF regains its original conformation in the aqueous solution even after exposure to DMSO.

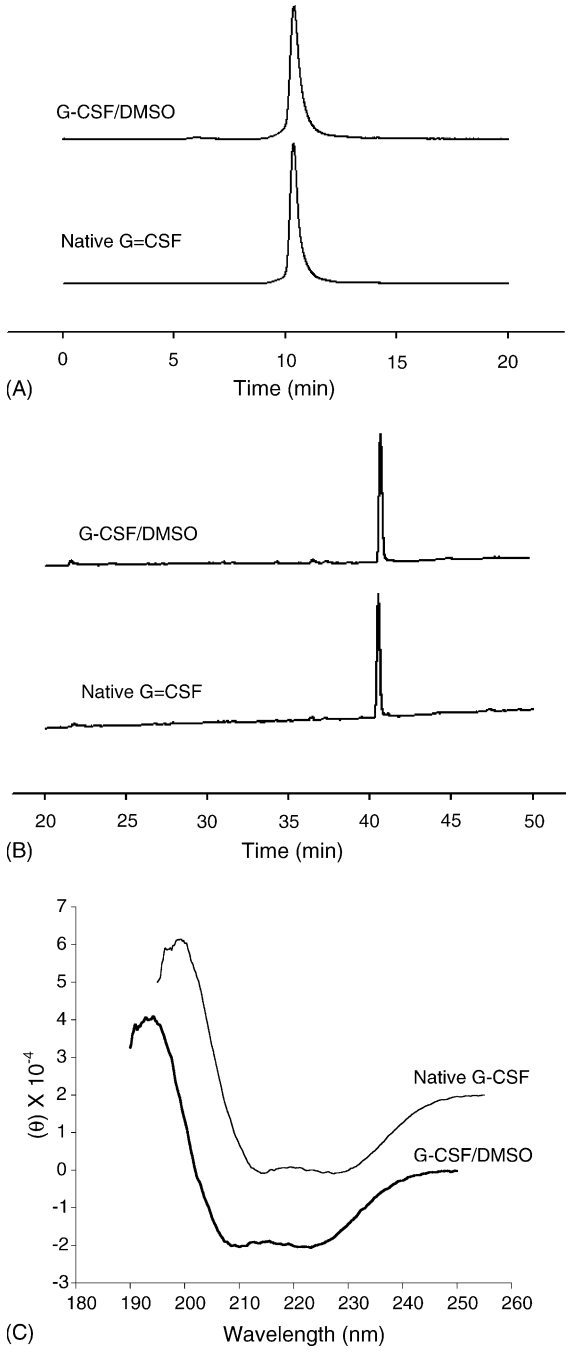

Fig. 2. (A) C18-reversed phase HPLC, (B) size exclusion HPLC, and (C) circular dichroism spectra for native rhG-CSF and DMSO treated rhG-CSF.

PLGA nanoparticles encapsulating rhG-CSF were prepared as follows. When a DMSO phase containing rhG-CSF and PLGA was dropped into an aqueous phase, DMSO immediately diffused out to the aqueous phase and rhG-CSF loaded

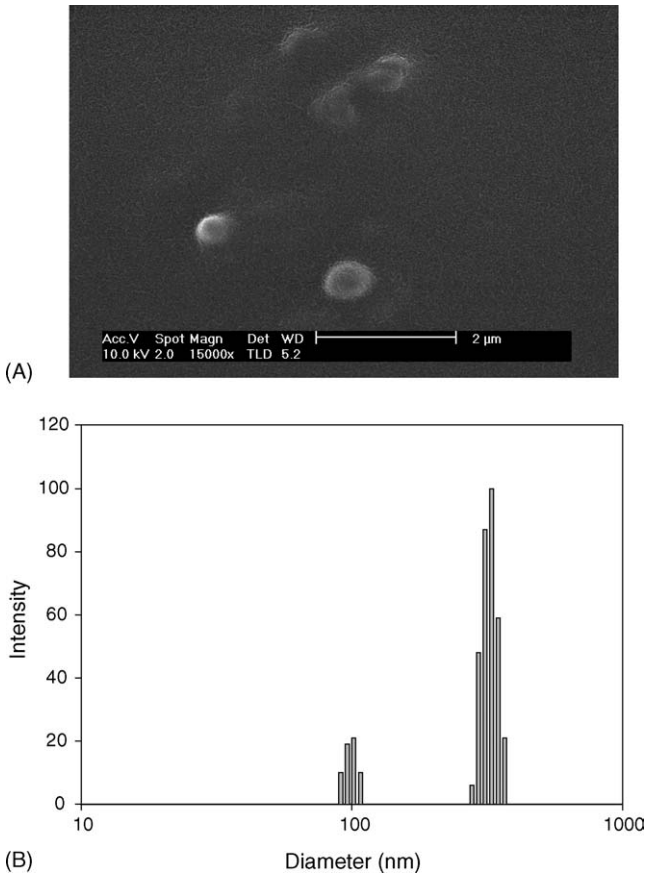

Fig. 3. The size distribution of rhG-CSF loaded PLGA nanoparticles as determined by (A) SEM image and (B) dynamic light scattering.

PLGA nanoparticles were spontaneously formed by a solvent diffusion/self-emulsification process. After dialysis, rhG-CSF loaded nanoparticles were produced. PLGA microparticles encapsulating rhG-CSF were prepared by a single or a double emulsion method. To prepare rhG-CSF loaded microparticles by the single emulsion method, methylene chloride must be included in the formulation process because of its limited water solubility (2%, v/v) which is required for the generation of temporal emulsion droplets larger than 1 μm. As judged from Fig. 1, a volume ratio of 1:1 methylene chloride/DMSO was selected to co-dissolve rhG-CSF and PLGA to prepare the microparticles. Upon emulsification in aqueous phase, DMSO preferentially diffused out to the aqueous phase while methylene chloride emulsion droplets were produced. During solvent evaporation of methylene chloride, the dissolved rhG-CSF was kinetically entrapped within semi-solidified PLGA microparticles. To produce rhG-CSF loaded microparticles by the conventional double emulsion method, rhG-CSF must be dissolved in an aqueous phase, which was emulsified in a pure methylene chloride phase

Table 1
Characteristics of rhG-CSF loaded PLGA nanoparticles and microparticles

	Target loading (%)	Yield (%)	Loading (%)	Encapsulation efficiency (%)	Size
G-CSF loaded NP	5.0	103.4	1.89	37.8	257 ± 6.1 nm
G-CSF loaded MP (O/W)	5.0	52.0	4.53	90.6	4.69 ± 4.1 μm
G-CSF loaded MP (W/O/W)	10.8	72.9	9.42	86.9	4.26 ± 3.1 μm

to make a primary emulsion. Table 1 shows the characteristics of rhG-CSF loaded nanoparticles and the two microparticles prepared by O/W and W/O/W emulsion methods. The average size of rhG-CSF loaded nanoparticles was 257.0 ± 6.1 nm and those of O/W and W/O/W microparticles were 4.7 ± 4.1 and 4.3 ± 3.1 μm, respectively. Fig. 3A and B are the FE-SEM image and the results of dynamic light scattering (DLS) of rhG-CSF loaded nanoparticles. The nanoparticles had a spherical shape and were about 300 nm in diameter with a narrow size distribution. It is also noted that rhG-CSF loaded microparticles had a much higher rhG-CSF loading amount than rhG-CSF loaded nanoparticles. Based on the loading efficiency, nearly 90% of rhG-CSF could be loaded into microparticles, whereas only 37.8% of rhG-CSF was encapsulated in the nanoparticles. The lower encapsulation efficiency for the PLGA nanoparticles could be attributed to rapid diffusion of rhG-CSF from DMSO phase to water phase during the process of spontaneous emulsion and solvent diffusion. Additionally, a large surface area surrounding the tiny nanoparticles provided more chance for loss of rhG-CSF. However, the loading amount of 1.89% (w/w) for PLGA nanoparticles is high enough for sustained release.

Fig. 4 shows in vitro release profiles of rhG-CSF from nanoparticles and microparticles (O/W and W/O/W). The nanoparticles showed initial burst releases of 27.6% at 12 h and 43.6% at day 1, followed by a sustained release period up to over 90% for a 1 week. In contrast, O/W microparticles only showed 22.0% release and W/O/W microparticles exhibited 14.4% release with minimal bursts during the same period. The difference in release profiles might be attributed to the size of the particles. Owing to the smaller diameter of nanoparticles, the rhG-CSF within them can diffuse out to the aqueous phase more rapidly than that within the microparticles. The slower and incomplete releases observed for the two microparticles could also be attributed to the structural instability of rhG-CSF occurring in the formulation stage and the release period. Physical degradation of rhG-CSF such as denaturation, aggregation, and non-specific adsorption might take place within the degrading microparticles, which are likely to be responsible for the very slow and incomplete slow releases (Crott and Park, 1997; Kim and Park, 1999). PLGA nanoparticles encapsulating molecularly dissolved rhG-CSF in the polymer bulk phase would have a shorter diffusion path-length and a higher surface area for release of rhG-CSF through a diffusion mechanism. Fast elimination of acidic degradation products from nanoparticles, resulting in a little chance of accumulation in the interior region was likely to be an additional factor that minimized the protein instability problems. Thus rhG-CSF was released out continuously from the nanoparticles in a diffusion controlled manner.

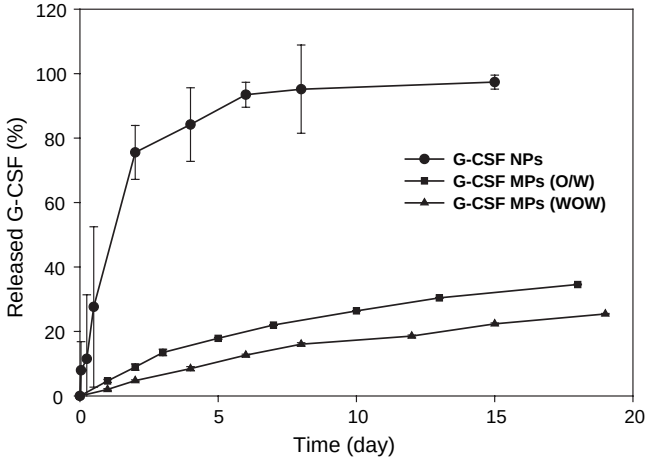

Fig. 4. In vitro release profiles of rhG-CSF loaded PLGA nanoparticles, O/W, and W/O/W microparticles.

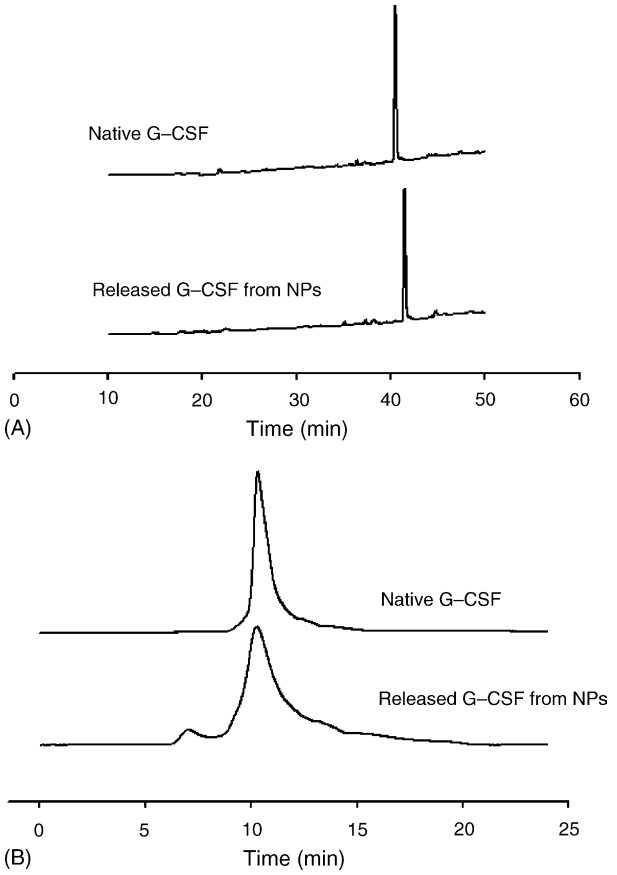

Fig. 5. The stability of native rhG-CSF and released rhG-CSF from PLGA nanoparticles as determined by (A) C18-reversed phase HPLC analysis and (B) size exclusion HPLC analysis.

Fig. 5 shows reversed phase and SEC HPLC results of native and released rhG-CSF from nanoparticles at day 6. In the reversed phase HPLC chromatogram, the rhG-CSF fraction released from nanoparticles showed the same retention time to that of native rhG-CSF. The released rhG-CSF from nanoparticles had a nearly intact structure compared to that of native rhG-CSF, suggesting that no adverse chemical degradation reactions occurred in rhG-CSF during the release period. In the SEC chromatogram of the released rhG-CSF fraction, a small, early eluting peak can be seen before a main rhG-CSF peak. This small peak was presumably caused by aggregated species of rhG-CSF. The extent of aggregation was 6.1%. To further validate that the released G-CSF from nanoparticles has comparable biological activity to that of native G-CSF, an in vivo animal study would be definitely necessary. This study is under progress and will be reported in the near future.

4. Conclusions

The present study investigated a PLGA polymeric nanoparticulate formulation of rhG-CSF, and compared the release behaviors and structural stability of the protein with those of the microparticulate formulation. rhG-CSF could be solubilized in an organic solvent phase without using any additives, which resulted in the formation of rhG-CSF encapsulated PLGA nanoparticles. By using an O/W single emulsion method, rhG-CSF was successfully encapsulated within the bulk phase of PLGA in a molecularly dissolved state. rhG-CSF loaded PLGA nanoparticles exhibited a sustained release profile over an extended period without showing any significant alterations in structure. Protein encapsulated PLGA nanoparticulate formulation, based on the direct dissolution of proteins in organic solvents, will be a new promising strategy for delivering various therapeutic protein drugs in a sustained manner.

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