Hydrophilized poly(lactide-co-glycolide) nanoparticles with core/shell structure for protein delivery

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Abstract

A novel preparation method for core/shell nanoparticles with hydrophilic polymeric shell was designed and characterized. The core is composed of poly(lactide-co-glycolide) and polymeric shell is composed of pluronic (poly (ethylene oxide)-poly (propylene oxide)-poly(ethylene oxide) triblock copolymer, F-127) and hyaluronic acid (HA). The role of core is to provide the nucleus for the stable formation of hydrophilic polymeric shell by physical adsorption and that of polymeric shell is to provide the hydrophilic network for protein loading. Lysozyme, which was used as a model drug and protonated in the physiological pH, was successfully loaded into polymeric shell up to 7 wt% via ionic interaction between HA and lysozyme and the sustained release pattern was observed, which was due to the stable immobilization of lysozyme in the polymeric shell.

Keywords: Core/shell nanoparticles; Poly(lactide-co-glycolide); Pluronics (F-127); Hyaluronic acid; Lysozyme; Sustained release

1. Introduction

With the advances of biotechnology, significant numbers of therapeutically active proteins have been produced. Although a wide variety of delivery systems have been designed and characterized, the parenteral route was most widely selected for the efficient delivery of protein. However, the problems still remain in the parenteral delivery of protein because most proteins have the short half life in the physiological condition. A solution to the problem that allows the preservation of protein activity in the physiological condition is to design the proper polymeric micro/nanoparticles [1–6]. For this purpose, poly(lactide-co-glycolide)(PLGA) micro/nanoparticles have been designed and characterized for the delivery of a couple of proteins. Biodegradable microspheres containing recombinant human Erythropoietin (EPO) were prepared from ABA triblock copolymers, consisting of hydrophobic PLGA A blocks and polyethylene oxide (PEO) B blocks [7]. Using excipients with known protein stabilizing properties, such as bovine serum albumin (BSA), poly-L-histidine, poly-L-arginine or a combination of poly-L-arginine and dextran, the EPO aggregate content was significantly reduced to <5% of the encapsulated EPO. Especially, the microspheres composed of ABA triblock copolymer (35 mol% PEO; 30 kDa) in combination with 5% BSA yielded both an acceptable level of EPO aggregate and a continuous release profiles under in vitro conditions up to 14 days. The pharmacokinetic characterization of vascular endothelial growth factor controlled release microspheres composed of PLGA was performed using rat model [8]. The microshperes were administered subcutaneously to rat and the pharmacokinetic parameters were compared with those of protein (Vascular Endothelial Growth Factor (VEGF)) solution. The subcutaneous administration of protein solution resulted in rapid clearance from the tissue, with high plasma concentrations as expressed by rapid absorption and elimination. However, the subcutaneous administration of VEGF microspheres produced low plasma concentrations and high subcutaneous concentrations over a period of 7 weeks.

However, the hydrophobic character of PLGA, the loading of protein into PLGA micro/nanoparticles with
a minimal decrease of protein activity still remains challenging [9,10].

In this study, a novel preparation method for core/shell nanoparticles with hydrophilic polymeric shell was designed and characterized. The core is composed of poly(lactide-co-glycolide) and polymeric shell is composed of pluronic (poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer, F-127) and hyaluronic acid (HA). The role of core is to provide the nucleus for the stable formation of hydrophilic polymeric shell by physical adsorption and that of polymeric shell is to provide the hydrophilic network for protein loading. Especially, HA was used as one of components for polymeric shell to induce the ionic interaction between protein and HA for the efficient loading and sustained release of ionized and hydrophilic protein. For the characterization of core/shell nanoparticles as a drug carrier, lysozyme, which was used as a model protein drug, was loaded into the nanoparticles and loading amount and release pattern was observed with the variation of the composition of polymeric shell.

2. Materials and methods

2.1. Materials

F-127 was obtained as a gift from BASF corp., Korea and used as received. F-127 can be represented by the formula (EO)_{100}(PO)_{65}(EO)_{100} on the basis of its nominal molecular weight of 12,600 and 75% PEO content. DL-Poly(glycolide-co-lactide)(PLGA) (75 mol% of lactide, Molecular weight: 90,000) was purchased from Boelinger Ingelheim (Germany). Sodium hyaluronate (M_w:150,000–250,000), Tween 80, hen’s egg white lysozyme and Micrococcus Lysodeikticus (Micrococcus luteus) were purchased from Sigma Co. (USA). Sodium hyaluronate was converted to HA (acid form) via hydrolysis with HCl.

2.2. Preparation of PLGA nanoparticles

F-127 is in a flaky state and PLGA is in a powdery state at room temperature. The mixtures were prepared individually by weighing 85 mg of polymers with 0.2 of weight ratio of (PLGA/F-127), and 3 mg of Tween 80 into 20 ml vials, which were immediately put into the vacuum oven of 60 °C. Within 30 min, both polymers were liquidized completely to form transparent polymer solution. The melted mixtures were transferred to vacuum oven at 25 °C to induce the phase transition (the solidification of liquidized polymer mixture). With the phase transition, PLGA formed the phase-separated domain with spherical form and F-127 formed the continuous phase [11]. Following the equilibration period for 3 h, the solidified mixture was withdrawn from the vacuum oven and immersed in distilled-deionized water for 3 days to solubilize F-127 using dialysis bag (molecular weight cut-off range: 40,000–50,000, Fisher Scientific, USA), replacing the distilled-deionized water every hour. This aqueous solution containing PLGA nanoparticles was centrifuged for 20 min and filtered through the 0.45 μm filter membrane. The obtained PLGA nanoparticles were freeze-dried. Fig. 1 describes the preparation method of nanoparticles schematically.

2.3. Preparation of core/shell nanoparticles

Firstly, PLGA nanoparticles was suspended in the HA/F-127 aqueous solution with the variation of weight ratio of HA/F-127 as presented in Table 1. PLGA nanoparticles prepared in this study were coated with F-127 and are easily suspended in the aqueous media. Subsequently, this aqueous solution mixture was subjected to freeze-drying to induce the formation of polymeric shell. The amount of HA/F-127 coated to the PLGA nanoparticles was measured as follows: 100 mg of HA/F-127-coated PLGA nanospheres were dispersed in 50 ml of water and 10 ml of chloroform was added to extract PLGA. Obtained chloroform/water mixture was transferred into separatory funnel and agitated vigorously for 1 h using shaker. HA/F-127 mixture in the water phase was extracted by freeze-drying and PLGA in the organic phase was extracted by evaporating chloroform.

2.4. Scanning electron microscope (SEM) measurements

PLGA nanoparticles before and after the formation polymeric shell were examined by SEM (Model:2250N, Hitachi, Japan). The samples were gold deposited in vacuum and examined with a tilt angle of 45°.

2.5. Particle size distribution and zeta potential measurements

Solutions of 30 mg of dried core/shell nanoparticles in 30 ml of phosphate buffered solution (PBS, pH 7.4) were prepared for the measurement of particle size distribution and zeta potential. Experiments were performed using electrophoretic light scattering 8000 (Otsuka Electronics) and repeated three times. For measuring the change of zeta potential as a function of time for equilibrium in the PBS, solutions of 30 mg of dried core/shell nanoparticles in 30 ml of PBS was centrifuged for 20 min to collect the core/shell nanoparticles from the solution at given time interval. Subsequently, the collected core/shell nanoparticle was resuspended into PBS and zeta potential was measured.

2.6. Lysozyme loading and release pattern

Lysozyme was loaded into core/shell nanoparticles by sorption method. 100 mg of dried core/shell nanoparticles were suspended in 100 ml of PBS containing 10 mg of...
lysozyme and stored at 25 °C to induce the ionic interaction between lysozyme and HA in the polymeric shell. After the equilibrium for 12 h, this aqueous solution was centrifuged for 20 min and filtered through the 0.45 μm filter membrane. The obtained lysozyme-loaded core/shell nanoparticles were freeze-dried. The amount of unloaded lysozyme in the aqueous media was analyzed by HPLC to measure the drug loading amount. Lysozyme was determined by reversed-phase HPLC using a Shodex RSpak RP 18-415 column and trifluroacetic acid/acetonitrile (0.1/99.9 v/v %) mobile phase at a flow rate of 1 ml/min. The elute was monitored by UV absorption at 220 nm. Drug loading amount is defined as the ratio of the amount of drug in the nanoparticles/the total weight of nanoparticles.

For measuring the release pattern of lysozyme from the nanoparticles, 5 g of freeze-dried core/shell nanoparticles were put into 500 ml of phosphate buffered solution (PBS, pH 7.4). Temperature was maintained at 37 °C and stirring was maintained at 600 rpm. At given time intervals, 5 ml of aliquots were withdrawn from release medium (PBS). HPLC analysis can then be done as previously described. To maintain the sink condition in the release medium, 5 ml of fresh PBS was added to release medium after sampling.

### 2.7. Lysozyme activity measurement

For lysozyme activity assay, a suspension of 0.1 mg/ml *M. lysodeikticus* was prepared in PBS (37 °C). To 2.95 ml *M. lysodeikticus* suspension, 0.05 ml lysozyme solution was added and mixed immediately. The turbidity of the suspension was measured at 450 nm by a Shimadzu UV-1601 spectrophotometer. One unit activity corresponds to decrease in turbidity of 0.001 per min at 450 nm [6].

| Weight ratio of (HA/F-127) | Wt% of adhered HA/F-127 to the total core/shell nanoparticles | Diameter (nm) |
|----------------------------|-------------------------------------------------------------|---------------|
| 0                          | 35.4±3.5                                                    | 312.3         |
| 0.1                        | 32.1±5.7                                                    | 593.4         |
| 0.2                        | 33.5±4.1                                                    | 771.6         |
| 0.3                        | 30.1±7.2                                                    | 802.2         |
| 0.4                        | 22.3±6.4                                                    | 562.3         |

5 wt % polymer solution was used to induce the formation of polymeric shell with the variation of weight ratio of (HA/F-127).
3. Results and discussion

In our previous study, PLGA nanoparticles were prepared by the temperature-induced phase transition of PLGA and F-127 mixture in the absence of solvent. In the final stage of preparation of PLGA nanoparticles based on the previous study, the removal of F-127 was performed to obtain the nanoparticles as shown in Fig. 1 [11]. However, the complete removal of F-127 was not accomplished resulting in F-127-coated PLGA nanoparticles because of the hydrophobic interaction between PLGA and propylene domain of F-127 [12,13]. With the presence of F-127 on the surface, PLGA nanoparticles in this study showed an excellent stability in the aqueous media [11].

Based on this, the adsorption of F-127 and HA on the surface of PLGA nanoparticles was induced in the aqueous media by freeze-drying as shown schematically in Fig. 1. F-127 forms a spherical micelle in the low concentration. In this low micellar concentration regime, micelles are well-separated and solution is an isotropic fluid. At higher concentrations, the micelles approach close packing and the ordered domains, consisting of cubic packing of spherical micelle, are induced resulting in the gelation [14]. With the freeze-drying process in this study, the concentration of F-127 aqueous solution was increased with the evaporation of water and the gelation was induced to form a polymeric shell. With the evaporation of water by freeze-drying, F-127 was adsorbed (gelled) into F-127-coated PLGA nanoparticles and HA might be adsorbed into F-127-coated PLGA nanoparticles with F-127 resulting in the formation of polymeric shell composed of F-127/HA composite.

The shape of core/shell nanoparticles formed at different concentrations of F-127 in the absence of HA was examined by SEM to observe the optimum concentration of F-127 for the stable formation of polymeric shell. As shown in Fig. 2, the aggregation of nanoparticles was observed at core/shell nanoparticles formed as 10 wt% F-127 aqueous solution. This is due to the formation of polymer network between free F-127 (which was not adsorbed on the surface of PLGA nanoparticle). However, the aggregation was not observed at the core/shell nanoparticles formed at 5 wt% indicating that most F-127 in the aqueous media was adsorbed on the surface of PLGA nanoparticles. Therefore, the concentration of polymeric shell material was fixed at 5 wt% throughout the experiment. Note that fibrous structure at the background of Figs. 2(a) and 3(a) is from the filter paper.

For the efficient loading of cationic lysozyme (Isoelectric point: 11.1 [15]) in the physiological condition, anionic HA was incorporated into polymeric shell by co-adsorption with F-127. Core/shell nanoparticles were prepared with the variation of (HA/F-127) weight ratio. As shown in Fig. 3, the aggregation of nanoparticles was observed at 0.4 of weight ratio of (HA/F-127). This is due to the formation of polymer network between free HA (which was not

![Fig. 2.](image-url)
incorporated into polymeric shell). In the adsorption of F-127 and HA on the surface of F-127-coated nanoparticles, the main driving force to form the polymeric shell is the hydrophobic interaction between F-127. Because HA does not have intermolecular interactions with F-127 or PLGA nanoparticles, it is embedded physically in the F-127 network within the range of 0–0.3 of weight ratio of (HA/F-127). However, the formation of HA/lysozyme complex without PLGA core could be possible due to the unstable embedding of HA in the polymeric shell. This property should be improved for the exact manipulation of loading amount of lysozyme (or negatively charged proteins in the physiological condition).

Fig. 4 shows the variation of zeta potential of core/shell nanoparticles with the variation of weight ratio of (HA/F-127). The negative value was observed with the increase of weight ratio of (HA/F-127) indicating that anionic HA is embedded in the polymeric shell and polymeric shell is suitable for the immobilization of cationic lysozyme.

Fig. 5 shows the change of loading amount with the variation of weight ratio of (HA/F-127). The loading amount was increased up to 7 wt% with the increase of HA content in the polymeric shell. Because of the ionic interaction between cationic lysozyme and anionic HA in the PBS (pH 7.4), the stable immobilization of lysozyme into the polymeric shell was induced and this led to the increase of loading amount.

Fig. 6 shows the release pattern of lysozyme from the core/shell nanoparticles formed as a function of weight ratio of (HA/F-127). Without HA in the polymeric shell, significant burst effect was observed. With the formation of polymeric shell composed of F-127 and HA, the release...
rate was reduced significantly and almost zero-order release pattern was observed at nanoparticles with 0.2 of weight ratio of (HA/F-127). Although HA is embedded in the F-127 network within the range of 0–0.3 of weight ratio of (HA/F-127) as shown in Fig. 3, it does not have intermolecular interactions with F-127 or PLGA nanoparticles and this leads to the release of HA from the polymeric shell. Because lysozyme is ionically bound to HA, lysozyme and HA release together in the form of ion complex. To verify the release of HA from the polymeric shell, the variation of zeta potential was measured as a function of time for equilibrium in the release media. Fig. 7 shows that the zeta potential of core-shell nanoparticles with 0.2 of weight ratio of HA/F-127 was increased with the increase of equilibrium time. This indicates that HA is released from the polymeric shell during the release of lysozyme and the sustained release of lysozyme is due to the release of ionic complex composed of HA and lysozyme, which has a higher molecular weight comparing with free lysozyme.

Table 2 provides data on the specific enzyme activity of lysozyme in the 1-week released sample.

| Formulation                                | Specific enzyme activity (EU/mg) |
|--------------------------------------------|---------------------------------|
| Control                                    | $17.4 \pm 1.5$ (mean ± SD, n = 3) |
| Lysozyme released from the nanoparticle    | $14.1 \pm 0.5$                  |

Control: Lysozyme (0.25% w/v in release media) kept at 37 °C in oscillating water bath for 1 week.

Core/shell nanoparticle with 0.2 of weight ratio of HA/F-127 was increased with the increase of equilibrium time. This indicates that HA is released from the polymeric shell during the release of lysozyme and the sustained release of lysozyme is due to the release of ionic complex composed of HA and lysozyme, which has a higher molecular weight comparing with free lysozyme.

Table 2 provides data on the specific enzyme activity of lysozyme in the release media after the 1-week release experiment. In comparison to the activity of native lysozyme, 81.3% activity was preserved and this indicates that the activity of lysozyme was preserved during the freeze-drying process.

4. Conclusions

Core/shell nanoparticles with composite polymeric shell composed of F-127 and HA have been prepared based on the hydrophobic interaction between PLGA and PPO domain of F-127. Although HA does not have intermolecular interaction in the polymeric shell, the formation of the polymeric shell composed of F-127 and HA within the range of 0.3 of weight ratio of (HA/F-127) was observed.
Because of ionic interaction of cationic lysozyme and anionic HA in the physiological condition, the efficient loading and sustained release of lysozyme were accomplished. This enables us to prepare for the charged nanoparticles with core/shell structure for the delivery of protein drug.

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