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Preparation and Cytotoxicity Comparison of Type A Gelatin Nanoparticles with Recombinant Human Gelatin Nanoparticles

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Abstract: Gelatin nanoparticles derived from bovine or porcine have been developed as various types of drug delivery system, and they need to be cross-linked to maintain their physicochemical properties in aqueous environments. Although gelatin is a widely used material in pharmaceutical industries, the safety issue of animal-origin gelatins, such as transmissible mad cow disease and anaphylaxis, remains to be solved. The purpose of this study was to prepare type A gelatin (GA) nanoparticles by modified, two-step, desolvation method and compare the toxicity of the resulting GA nanoparticles with recombinant human gelatin (rHG) nanoparticles. The GA nanoparticles were characterized, and drug loading and release pattern were measured. FITC-BSA, a model protein, was efficiently loaded in the nanoparticles and then released in a biphasic and sustained release pattern without an initial burst. In particular, the cell viability of the GA nanoparticles was less than that of the rHG nanoparticles. This finding suggests that rHG nanoparticles should be considered as an alternative to animal-origin gelatin nanoparticles in order to minimize the safety problems.

Keywords: nanoparticles, gelatin, recombinant human gelatin, protein drug, drug delivery.

Introduction

Polymer-based nanoparticles have emerged as a peptide, protein drug and nucleic acid carrier because its advantages such as sustained-release of drug and sub-cellular size.[1,2] In particular, gelatin nanoparticles have the potential to protein drug delivery system in terms of simple preparation,[3,4] biocompatibility and biodegradability because the gelatin derived from bovine or porcine widely used in pharmaceutical industry as capsule materials and stabilizers.[5-7] However, animal origin gelatin might cause transmissible mad cow disease and anaphylaxis. Recombinant human gelatin (rHG) derived from human collagen have been considered as an alternative to conventional animal origin gelatins. The safety test with rHG on human volunteers at similar and greater doses with animal-derived gelatin in market vaccines showed that no significant changes with rHG was observed in the standard serum chemistry parameters.[8] Gelatin nanoparticles are very rapidly dissolved and disappeared in water due to its highly water-soluble property.[9] Cross-linking is, therefore, necessary in order to protect the gelatin nanoparticles from dissolution in water. Glutaraldehyde known as cross-linker is generally used for preparation of gelatin nanoparticle in two step desolvation method; however, it has high toxicity. In order to avoid the use of the toxic substance, naturally occurred cross-linker, genipin, has been used for cross-linking of gelatin nanoparticles.[10] It was reported that the cytotoxicity and the cell proliferation with genipin are greatly lower and higher than glutaraldehyde, respectively.[11]

We hypothesized that the previously reported rHG nanoparticles may have low toxicity compared to type A gelatin (GA) nanoparticles prepared with modified two step desolvation method. In this study, GA nanoparticles cross-linked with genipin was prepared as described in previous research.[12] GA nanoparticles were optimized and characterized in order to compare the cytotoxicity and physicochemical properties with rHG nanoparticles. FITC-BSA, fluorescein isothiocyanate bovine serum albumin, was loaded in GA nanoparticles, and the loading efficiency and *in vitro* release studies showed high loading and sustained release of the model protein. The cell viability of both rHG and GA nanoparticles were, finally, measured and compared with 293 cell lines.

Experimental

Materials. Picrylsulfonic acid (TNBS), collagenase, type A gelatin and FITC-BSA were purchased from Sigma (St. Louis, MO). Genipin was obtained from Challenge Bioproducts (Taichung, Taiwan). rHG (MW: 100 kDa) was purchased from Fibrogen (San Francisco, CA). A dialysis

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membrane (MWCO: 100000) was obtained from Spectrum Laboratories (Rancho Dominguez, CA). Human embryonic kidney 293 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). All other reagents were of analytical grade.

Preparation of rHG Nanoparticles. We have reported the preparation and characterization of FITC-BSA loaded rHG nanoparticles.[12] In order to prepare FITC-BSA loaded rHG nanoparticles, 2.5 mg FITC-BSA was added to 40 mL of 0.05% rHG aqueous solution and the mixture was incubated at 37 °C for three hours. The mixture was desolvated with 59 mL of ethanol containing genipin at a final genipin concentration of 0.05%. The cross-linked rHG nanoparticles were purified by the centrifugation at 12,000×g for 20 min.

Preparation of FITC-BSA Loaded Gelatin Nanoparticles. GA nanoparticles were prepared by the modified two step desolvation method, previously described by Won and Kim.[12] In order to prepare FITC-BSA loaded gelatin nanoparticles, 1.25 g of GA was dissolved in 25 mL of water under heating and stirring. To separate the high-molecular weight gelatin from the solution, 25 mL of ethanol was added to the solution. The sediment was collected and redissolved with 100, 200 and 400 mL of water. Prior to the second desolvation step, 2.5 mg of FITC-BSA was dissolved in the 40 mL of gelatin for 3 h at 37 °C. After the incubation, various volumes of ethanol containing genipin ranging from 57-62% were added into the 40 mL of gelatin/FITC-BSA solution. The genipin concentration was fixed at a final weight/volume percent of 0.05. GA nanoparticles were incubated for cross-linking in 37 °C water bath for 72 h, and then ethanol was evaporated by rotary evaporator (N-1000, EYELA, Japan) under heating and rotation. The nanoparticles were purified by centrifugation at 12,000 g for 20 min and then the purified nanoparticles were redispersed in water by sonication and lyophilized for long-term storage.

Characterization of FITC-BSA Loaded Gelatin Nanoparticles. The particle size and surface zeta potential were measured by the dynamic light scattering, Zetasizer, Nano ZS (Malvern, UK). The particle size and surface zeta potential was measured in water at a concentration of 1 mg/mL with three replicates. The morphology of GA nanoparticles was observed using conventional transmission electron microscope (TEM, Philips CM 30). The lyophilized nanoparticles were dispersed in water by sonication in order to prepare TEM samples.

Quantification of free amino groups of FITC-BSA loaded gelatin nanoparticles was evaluated by the TNBS (picrylsulfonic acid) reaction.[13] The lyophilized nanoparticles were dispersed in water at a concentration of 0.36 mg/mL and 1 mL of 4% sodium hydrogen carbonate solution (pH 8.5) and 1 mL of aqueous 0.1% TNBS solution were added to the nanoparticle dispersion. After stirring at 500 rpm for 2 h at 40 °C, the mixture was centrifuged at 13,000 g for 30 min to separate the nanoparticles from the nanoparticle dispersion. The resulting supernatant was diluted with water prior to an assay of un-reacted TNBS using an UV/VIS spectrophotometer at 349 nm (DU 730, Beckman, Fullerton, CA). The amino group contents of the nanoparticle were calculated relative to a TNBS reference containing water and non-cross-linked gelatin.

The efficiency of the FITC-BSA loading in the nanoparticles was evaluated by enzymatic degradation of the nanoparticles. The dispersion of FITC-BSA loaded nanoparticles was digested in PBS with collagenase (20 U/mL) in a water bath under heating and shaking for 24 h. The end of degradation after the treatment of collagenase was examined by size measurement followed by the quantification of released FITC-BSA from the nanoparticles.

***In vitro* Release of FITC-BSA from the Nanoparticles.** Lyophilized FITC-BSA loaded GA nanoparticles were dispersed in PBS and loaded into a dialysis membrane. The membrane bag was placed in a vial containing PBS at 37 °C in a shaking water bath. At a predetermined point in time, the PBS solutions were collected for measurement of absorbance and replaced in the respective vial containing nanoparticles to maintain the constant volume. The loading efficiency and the cumulative released amount of FITC-BSA were measured using UV/VIS spectrophotometer at 495 nm and then calculated from the appropriate calibration curves.

***In vitro* Cell Viability Test.** 293FT cells were seeded on 96 well plates at a density of 1.0×10^4 cells/well and incubated for 24 h. The different concentrations of FITC-BSA loaded GA nanoparticles were added to the each well and then the cells were cultured. After 24 h of incubation, the cells were washed three-times with PBS and all of the media was replaced by fresh media followed by an addition of MTT-reagent to the each well. The cells were cultured additionally for 4 h and then all of media was replaced with dimethyl sulfoxide. After 30 min of incubation, the 96 well plates were shaken vigorously, and the absorbance was measured in a microplate reader (SpectraMax M2^e, Molecular Devices) at 540 nm.

Statistical Analysis. The data were expressed as mean± SD. Differences in cell viability between the groups were analyzed using an unpaired *t*-test. $P<0.05$ was considered significant.

Results and Discussion

The conventional two step desolvation method is one of well-known methods to prepare animal origin gelatin nanoparticles using organic solvents and cross-linker. The modified two step desolvation method had emerged as a simple and reproducible method for the preparation of rHG nanoparticles using natural origin cross-linker.[12] In order to compare cytotoxicity of rHG nanoparticles with GA nanoparticles, GA and rHG nanoparticles were prepared by the

modified two step desolvation method.

Preparation and Characterization of FITC-BSA Loaded Type A Gelatin Nanoparticles. The influence of the gelatin and ethanol concentration on the particle size was investigated with three different concentrations of the gelatin solution and various volumes of ethanol. Genipin concentration and cross-linking time were fixed at a final concentration of 0.05% and 72 h, respectively, as previously described.[12] Three concentrations of GA and various volumes of ethanol were examined in order to prepare the nanoparticles. As shown in Figure 1(A), the increase in concentration of gelatin and volume of ethanol led to an increase in the particle size. The optimum concentrations of ethanol and gelatin were 59% and 1.5 μg/mL, respectively. These conditions resulted in lowest polydispersity index (PDI) and, reproducible and stable nanoparticle preparation. The PDI and the particle size was 0.005±0.004 and 304± 5.40 nm at the optimized condition, respectively. These data were examined three-times with three replicates, and the stability of the optimized particles was tested at all of the preparation steps by measuring of the PDI and the particle size. Significant differences were not observed at all of the steps, suggesting that the optimized particles were stable during the preparation (data not shown). Loading of FITC-BSA into nanoparticles is an easy way to measure the loading efficiency and *in vitro* release using a spectrophotometer. As amount of loaded FITC-BSA in the nanoparticles increased, the size of FITC-BSA loaded nanoparticles increased (Figure 2). Optimum amount of loaded FITC-BSA was determined by comparing of PDI and reproducibility for the preparation of FITC-BSA loaded gelatin nanoparticles, and the loading amount of FITC-BSA was fixed at 2.5 mg for all experiments.

As shown in Figure 1(B), the size of FITC-BSA loaded GA nanoparticles increased with increasing volume of ethanol ranging from 56 to 59%. The size distribution, PDI and reproducibility were poor both above 59% and below 56% (data not shown). The size of FITC-BSA loaded GA nanoparticles was compared with unloaded GA nanoparticles at an ethanol concentration of 59%. The increase in size was observed at this concentration by loading of FITC-BSA in the nanopaticles, suggesting that FITC-BSA was successfully loaded in the GA nanoparticles. The zeta potentials of FITC-BSA loaded gelatin nanoparticles were 18.1±6.74 and 17.4±4.70 mV at 57 and 59% ethanol concentrations, respectively. FITC-BSA loaded gelatin nanoparticles, therefore, were prepared at the optimum conditions, and FITC-BSA loading efficiency in the gelatin nanoparticles was measured to be 70±5%. The morphological characteristics of FITC-BSA loaded nanoparticles were observed using conventional transmission electron microscopy (TEM) in Figure 3. The morphology and size distribution of nanoparticles in Figure 3 were spherical and homogeneous, suggesting that the nanoparticles were well dispersed in water without aggregation. Determination of remaining free amino groups

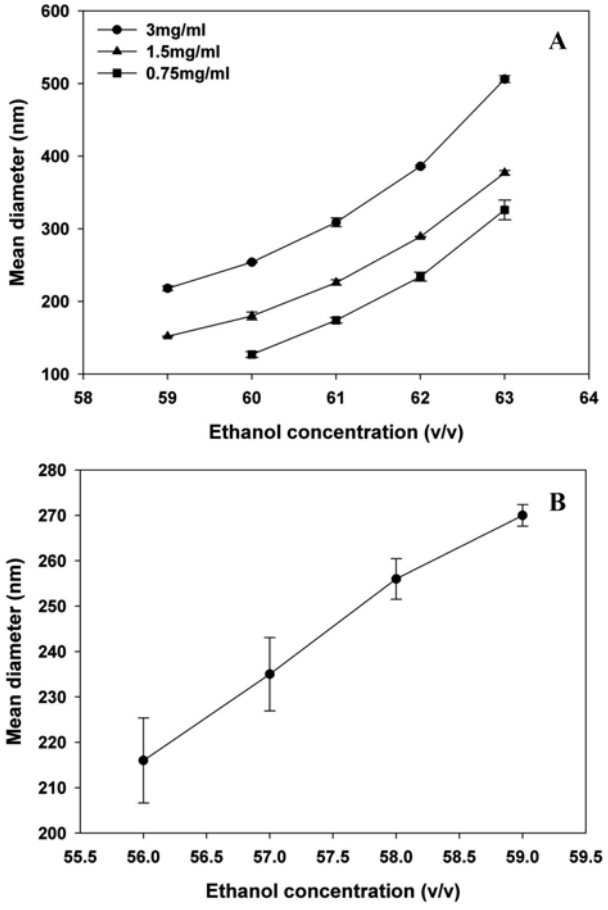

Figure 1. Size variation of unloaded (A) and FITC-BSA loaded (B) GA nanoparticles. The samples were prepared at the gelatin concentration of 1.5 mg/mL and FITC-BSA concentration of 2.5% (w/v).

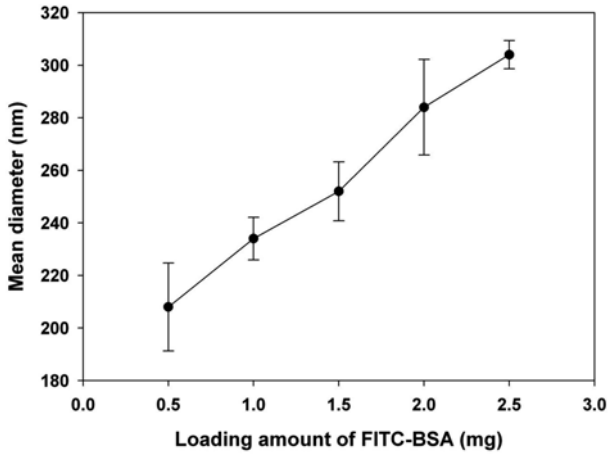

Figure 2. Size variation of FITC-BSA loaded GA nanoparticles at various FITC-BSA concentrations. The samples were prepared at the gelatin concentration of 1.5 mg/mL and the ethanol concentration of 59%.

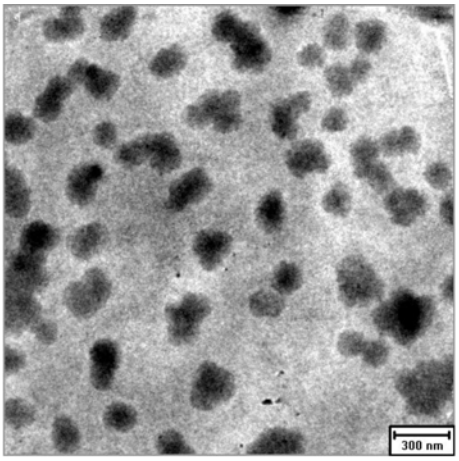

Figure 3. TEM picture of FITC-BSA loaded GA nanoparticles. The FITC-BSA loaded GA nanoparticles were prepared with optimal condition, 1.5 mg/mL for gelatin, 59% for ethanol.

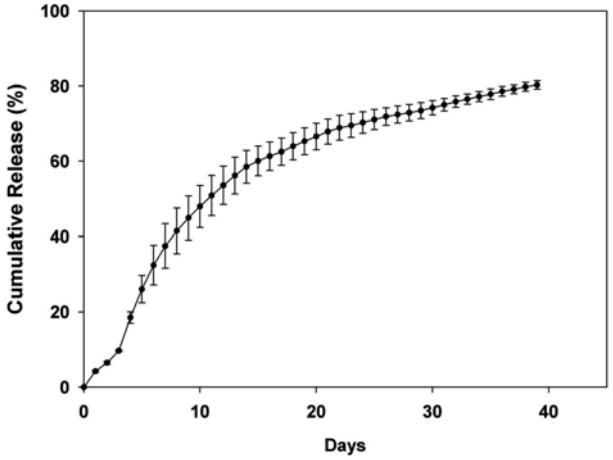

Figure 4. FITC-BSA release profile from GA nanoparticles. The FITC-BSA loaded GA nanoparticles were prepared with optimal condition, 1.5 mg/mL for gelatin, 59% for ethanol, and FITC-BSA released from the nanoparticles in PBS (pH 7.4), 37 °C under shaking.

is necessary to understand the cross-linking degree because amide bond is one of powerful tools for a conjugation of bio-active molecules to the nanoparticles.[14,15] In addition, the cross-linking degree may affect the nanoparticles' physicochemical properties *in vivo* such as a degradation rate and half-life in blood stream. The remaining content of free amino groups of FITC-BSA loaded GA nanoparticles was determined as 30%.

***In vitro* Release of FITC-BSA from Nanoparticles.** As shown in Figure 4, FITC-BSA released from the GA nanoparticles in biphasic and sustained release pattern without significant initial burst over 40 days. Gelatin, water-soluble substance, is dissolved rapidly when added in hot water; however, these nanoparticles were not dissolved in hot water and showed sustained release of FITC-BSA, suggesting that cross-linking with genipin prevented redissolution of the nanoparticles in water. The swelling of gelatin matrix in aqueous environment results in the release of drug from the nanopartcles. The uptake of water and degree of cross-linking, therefore, affects the drug release kinetic by changing of gelatin glass-rubbery transition and matrix erosion/degradation rate. The loose structure of gelatin chain due to the swelling of nanoparticles and the replacement of loaded drug with release media induces the crossing of swollen gelatin layer and the release of drug.[12] Although, *in vitro* release study showed sustained release over 40 days, *in vivo* release kinetic may be different from *in vitro* profile because of enzymatic hydrolysis of gelatin nanoparticles in the body.[16,17] Gelatinases or collagenases may affect the degree of enzymatic degradation of gelatin nanoparticles; however, the type, concentration and location are diverse, suggesting no direct correlation between *in vitro* and *in vivo* profiles.[18]

***In vitro* Cell Toxicity.** The cell viability of 293 cells was measured by MTT assay after 24 h of incubation with various concentrations of rHG and GA nanoparticles. As the concentration of nanoparticle increased, the viability of 293 cells decreased in Figure 5. Considerable toxicity increase was not observed between rHG and GA nanoparticles; however, rHG nanoparticles showed high level of cell viability compared to GA nanoparticles. Animal origin gelatin is a well-known material in pharmaceutical industries due to its biocompatibility and biodegradability. Therefore, animal origin gelatin nanoparticles have been developed as a delivery vehicle for various drug candidates, such as protein, DNA and RNA. As shown in this study, the cell viability of rHG nanoparticles was higher than that of GA nanoparticles, suggesting that rHG nanoparticles may be more com-

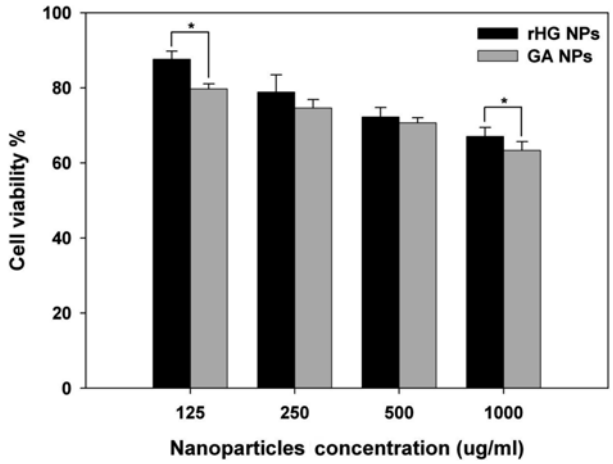

Figure 5. Cell viability at various concentrations of rHG and GA nanoparticles. RHG nanoparticles were prepared with 0.05% of rHG, 59% of ethanol and 2.5 mg of loading amount of FITC-BSA (*P<0.05).

patible as a drug delivery system for human applications than GA nanoparticles.

Conclusions

This study presents a preparation of GA nanoparticles by modified two step desolvation method and a safety comparison of GA nanoparticles with rHG nanoparticles. Release profiles of GA nanoparticles showed sustained release of FITC-BSA in a biphasic release pattern without initial burst. Although, both GA and rHG nanoparticles showed almost similar characteristics, the cell viability of GA was slightly less than that of rHG, suggesting that rHG nanoparticles are more compatible as drug delivery systems for human applications. RHG nanoparticles constitute a promising tool as a drug delivery system because they showed not only the overcoming of safety issue but also the decrease in cytotoxicity. These results lead us to conclude that rHG nanoparticles should be considered as an alternative to animal origin gelatin nanoparticles in order to increase the biocompatibility when gelatin-based drug delivery systems are developed.

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