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Chondroitin sulfate-capped gold nanoparticles for the oral delivery of insulin

Hyun-Jong Cho [a], Jongsuk Oh [b], Moon-Ki Choo [b], Jong-In Ha [b], Youmie Park [b,*], Han-Joo Maeng [b,**]

[a] College of Pharmacy, Kangwon National University, Chuncheon 200-701, Republic of Korea
[b] College of Pharmacy, Inje University, Gimhae 621-749, Republic of Korea

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ABSTRACT

Chondroitin sulfate (CS)-capped gold nanoparticles (AuNPs) were synthesized and its feasibility for oral insulin (INS) delivery was investigated *in vivo*. CS was used as both reducing and stabilizing agent in the synthesis of AuNPs with around 48 nm mean diameter, narrow size distribution, and negative zeta potential. After loading INS into CS-capped AuNPs structure, NPs with about 123 nm mean diameter, narrow size distribution, and negative zeta potential were successfully fabricated. By surface plasmon resonance (SPR) measurement, 0.5% (w/v) CS was chosen for the synthesis of AuNPs. Stability of AuNPs and AuNPs/INS was maintained for 7 weeks according to SPR study. Cytotoxicity of AuNPs/INS in Caco-2 cells was measured and no significant cytotoxicity was observed in tested AuNPs concentration range. In the streptozotocin-induced diabetic rat model, the oral administration of AuNPs/INS exhibited an efficient regulation of glucose level, compared to INS solution-treated group. The mean INS concentration in plasma at 120 min after oral administration of AuNPs/INS was 6.61-fold higher than that of INS solution-administered group. All of these findings indicate the successful application of CS-capped AuNPs for oral delivery of INS to the therapy of diabetes.

1. Introduction

Recently, gold nanoparticles (AuNPs) have been widely employed in various biomedical areas. The versatility of AuNPs can provide several applications in the fields of biosensing, drug delivery and/or therapy, and diagnosis of diseases [1–5]. The redox activity, surface plasmon resonance (SPR), surface-enhanced Raman scattering (SERS), and fluorescence quenching effects of AuNPs can be used in the detection of signals [6–9]. The surface of AuNPs can be modified variously and conveniently, thus therapeutics could be attached onto the AuNPs [10,11]. The optical and electrochemical characteristics of AuNPs may be employed in several imaging techniques, such as computed tomography (CT), dark-field light scattering, and Raman spectroscopy [12,13]. These properties of AuNPs can endow an important role in the therapy and diagnosis of diseases.

AuNPs can be synthesized by various methods and their synthetic approaches can determine the size, shape, and surface functionality [14]. Since AuNPs were created with hydrogen tetrachloroaurate ($HAuCl_4$) and citrate (used as both reducing and stabilizing agents) in 1951 [15], various substances have been used for the synthesis of AuNPs [16]. Among them, biocompatible polymers were also adopted for a reducing agent in the synthesis of AuNPs [8,17]. It seems that biofunctional polymers can exert their own functionalities as well as a reducing capability for AuNPs synthesis.

In this investigation, chondroitin sulfate (CS) was used as a reducing agent for AuNPs synthesis. CS is a sulfated glycosaminoglycan (GAG) composed of N-acetylgalactosamine and glucuronic acid. It is a major component of extracellular matrix (ECM), thus it is very crucial for maintaining the structure of tissue. It can also interact with proteins in ECM due to its negative charge and can influence on the diverse cellular activities. It is known that it possesses several biological functions including anti-atherogenic, anti-inflammatory, anti-thrombogenic, and anti-coagulant activities [18]. Due to its biocompatibility and interaction with biological components, it has been used in the development of drug delivery vehicles [19,20]. In this study, CS, as both reducing and stabilizing agent, was introduced to AuNPs for the oral delivery of insulin (INS). Oral delivery of proteins/peptides, such as INS, has several intrinsic obstacles to attain a sufficient bioavailability as follows; an acidic environment in the stomach, digestive enzymes in the GI tract, high molecular weight (MW) of protein/peptide, and biological and structural stability of protein/peptide [21]. Because of extremely

* Corresponding author. Tel.: +82 55 320 3884; fax: +82 55 320 3940.
** Corresponding author. Tel.: +82 55 320 3459; fax: +82 55 320 3940.
E-mail addresses: youmiep@inje.ac.kr (Y. Park), maenghj@inje.ac.kr (H.-J. Maeng).

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low oral bioavailability of protein or peptide, several approaches have been introduced to improve oral absorption and consequent pharmacological efficacies. Among them, nano-sized delivery vehicles have been extensively investigated to overcome low mucosal absorption problem of protein and peptide therapeutics [22,23].

Herein, we report about the synthesis of CS-capped AuNPs for improving oral absorption of INS. Physicochemical properties, *in vitro* stability, and *in vitro* cytotoxicity of developed AuNPs formulation were investigated. *In vivo* efficacy of CS-capped AuNPs with INS (AuNPs/INS) was also assessed in streptozotocin(STZ)-induced diabetic rat model.

2. Materials and methods

2.1. Materials

Chondroitin sulfate A (CS) sodium salt from bovine trachea, gold chloride trihydrate ($HAuCl_4{\cdot}3H_2O$), insulin (INS; recombinant, human), and streptozotocin (STZ) were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and heat-inactivated fetal bovine serum (FBS) were obtained from Gibco Life Technologies, Inc. (Grand Island, NY, USA). All other reagents were of analytical grade.

2.2. Preparation of AuNPs/INS

AuNPs were synthesized according to the reported method with slight modification [24]. In the present investigation, CS-capped AuNPs were prepared for INS delivery. CS (50 mg) dissolved in distilled water (DW, 10 ml) and $HAuCl_4{\cdot}3H_2O$ solubilized in DW (0.5 mM, 1.97 mg) were mixed. This mixture was heated at 80 °C for 5 h and cooled to room temperature to make ruby-red AuNPs dispersion. AuNPs/INS dispersion was prepared by adding INS solution into AuNPs dispersion. INS (1.1 mg) dissolved in PBS (pH 7.4) was added to AuNPs dispersion and incubated. AuNPs and AuNPs/INS dispersion were stored at 4 °C before their use.

2.3. Surface plasmon resonance (SPR) study

SPR of AuNPs was measured according to the concentration of CS (0.5, 1.0, and 2.0%). The absorbance was monitored in the range of 400–700 nm by UV/vis spectrophotometer (UV-1800, Shimadzu Corp., Japan). To investigate the stability of AuNPs and AuNPs/INS, UV/vis spectra (400–700 nm wavelengths) of AuNPs and AuNPs/INS were also measured for 7 weeks.

2.4. Characterization of AuNP/INS

The characteristics of developed AuNPs (mean diameter, polydispersity index, and zeta potential) were investigated by a light-scattering spectrophotometer (ELS-Z; Otsuka Electronics, Tokyo, Japan). The viscosity of AuNPs and AuNPs/INS was measured by viscometer (Brookfield DV-E, Brookfield Engineering Laboratories. Inc., Middleboro, MA, USA). The morphological shapes of AuNPs and AuNPs/INS were observed by transmission electron microscopy (TEM). NP dispersions were stained with 2% (w/v) phosphotungstic acid. Species were loaded on copper grids with films, dried for 10 min, and observed by TEM (JEM 1010; JEOL, Tokyo, Japan). The length of the scale bar in images is 100 nm.

The encapsulation efficiency (EE) of INS was measured by ELISA assay (human insulin ELISA kit, EMD Millipore, St. Charles, MO, USA), according to the manufacturer's instructions. AuNPs/INS dispersion was centrifuged at 15,000 rpm for 30 min, and the amount of INS in the supernatant was calculated by following formula [24]:

$$\mathrm{EE}(\%) = \frac{\text{input amount of INS-the amount of INS in the supernatant}}{\text{input amount of INS}} \times 100 \quad (1)$$

The absorbance of final resultant was measured at 405 nm wavelength by UV/vis spectrophotometer (UV-1800, Shimadzu Corp., Japan) to calculate INS concentration.

2.5. In vitro cytotoxicity test

Caco-2 cells were acquired from the Korean Cell Line Bank (KCLB, Seoul, Korea). DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin was used as a cell culture medium in a 5% CO_2 atmosphere and 95% relative humidity at 37 °C. The cytotoxicity of AuNP and AuNP/INS was assessed by MTS-based assay in Caco-2 cells. Caco-2 cells were seeded in 96-well plates at a density of 1.0×10^4 cells per well and incubated for 24 h. Cell culture media were eliminated and determined concentrations of AuNPs and AuNPs/INS (0–10 μg/ml, the concentration of AuNP) were added. They incubated for 24, 48 and 72 h at 37 °C under a 5% CO_2 atmosphere and 95% relative humidity, respectively. Cells were treated with MTS-based CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay Reagent (Promega Corp., WI, USA) and incubated at 37 °C for 4 h according to the manufacturer's protocol. The absorbance was measured at a wavelength of 490 nm with an EMax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) and Caco-2 cell viability (%) was calculated.

2.6. In vivo studies in diabetic rats

The *in vivo* efficacy studies of INS solution and AuNPs/INS dispersion were investigated in STZ-induced diabetic rat model. All animal experiments were undergone according to the Guidelines for Animal Care and Use, Inje University. Male Sprague–Dawley rats (Orient Bio, Seongnam, Korea) with 220–230 g (*i.e.*, 7 weeks of age) of body weight were brought up in a temperature- (20–23 °C) and humidity- (50–60%) controlled room under a constant 12 h light/dark cycle. The SD rats were given water and standard rat Chow *ad libitum*. Diabetic rats were prepared by the administration of STZ according to the reported method [25]. Briefly, STZ solution (60 mg/ml concentration in a citrate buffer of pH 4.5) was administered once *via* intraperitoneal injection to the fasted rat at a dose of 60 mg/kg. Blood samples (50 μl) were collected *via* tail vein for the measurement of blood glucose level on a day 3. The rats with > 300 mg/dl of blood glucose level, determined by Accu-Chek glucometer (Boehringer Mannheim Corp., Indianapolis, IN, USA), were chosen as experimental diabetic rat models.

For *in vivo* studies in 1-week diabetic rats, the femoral vein was cannulated with a polyethylene tube (PE-50; Clay Adams, Parsippany, NJ) under anesthesia with ethyl ether. INS solution and AuNPs/INS dispersion were administered orally at a dose of 50 IU/kg INS to the rats, respectively. Blood samples (300 μl) were collected *via* the femoral artery at 0 (control), 30, 60, 120, 180, and 240 min after oral administration of INS solution and AuNP/INS dispersion. About 0.3 ml of heparinized 0.9% NaCl solution (20 U/ml) was added to flush the cannula immediately after collecting each blood sample to prevent blood clotting. For the determination of glucose level, 20 μl blood samples were analyzed by Accu-Chek glucometer. Blood samples, collected at 120 min post-injection of INS solution and AuNPs/INS dispersion, were centrifuged at 3000 rpm for 10 min, and plasma was separated for the determination of INS concentration. INS concentration in plasma was obtained by above described human insulin ELISA assay.