Temporal-controlled Release of Bovine Serum Albumin from Chitosan Nanoparticles: Effect on the Regulation of Alkaline Phosphatase Activity in Stem Cells from Apical Papilla

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Abstract

Introduction: The controlled delivery of bioactive molecules is crucial for the regulation of stem cell differentiation. In this study, we examined the effects of temporal-controlled release of bovine serum albumin (BSA) from chitosan nanoparticles (CSnp) to regulate the alkaline phosphatase activity (ALP) in stem cells from apical papilla (SCAP). **Methods:** BSA-loaded CSnp were synthesized by 2 methods to achieve the variant temporal-controlled release: (1) the encapsulation technique (BSA-CSnpI) and (2) the adsorption technique (BSA-CSnpII). After characterization of the size, charge, and release kinetics, SCAP were cultured in the presence of these bioactive molecule–loaded nanoparticles. SCAP viability was analyzed at 1, 7, 14, 21, and 28 days, and ALP activity was analyzed every 7 days until 21 days to determine the effect of these bioactive molecule–releasing nanoparticles on the cytotoxicity and differentiation potential, respectively. **Results:** BSA-CSnpI and BSA-CSnpII presented distinct *in vitro* release profiles of BSA in a time-controlled manner. Cell viability was significantly enhanced over time in the presence of BSA-CSnpI and BSA-CSnpII ($P < .01$), when compared with BSA nonloaded CSnp. ALP activity was significantly higher ($P < .01$) in the presence of BSA-CSnpI after 3 weeks than in BSA-CSnpII. **Conclusions:** BSA-loaded CSnps were synthesized and characterized in this study. Based on the physical/chemical interaction of BSA with CSnp (encapsulation or surface adsorption), different time-controlled release profiles were observed that influenced the ALP activity of SCAP *in vitro*. This study highlighted the potential of temporal-controlled bioactive molecule release technology in the differentiation of stem cells in dentin pulp regeneration. *(J Endod 2014;40:1349–1354)*

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Key Words

Alkaline phosphatase activity, bovine serum albumin, chitosan nanoparticles, stem cells from apical papilla, temporal-controlled release

Tissue repair or regeneration is a complex cascade of multistep events that involves an intricate interplay among stem cells, scaffolds, and growth factors. Stem cell proliferation and differentiation into a specific lineage *in vivo* and *in vitro* are tightly regulated by growth factors (1, 2). The bioavailability of those growth factors is particularly important for the ideal temporal and spatial differentiation of stem cells (3, 4). Thus, a well-controlled release system of specific growth factors is a pivotal strategy in dentin pulp tissue engineering (5). The analysis of the expression and regulation of genes at different stages of cellular differentiation could reveal the importance of specific bioactive molecules at a specific stage of cellular differentiation (6). Numerous studies have focused on creating appropriate delivery systems such as nanoparticles, microspheres, hydrogels, and scaffolds for tissue regeneration (7, 8). Although the evaluated delivery systems have different designs and compositions, they share the same goal, which is the release of bioactive molecules in a time-controlled manner.

The incorporation of various drugs or proteins in a biodegradable polymer has long been recognized as an effective way to control the release profile of a retained substance (9, 10). The increase in efficacy, bioavailability, and cell/tissue targeting of such molecules can be obtained by incorporating them in a polymer. Among a variety of candidates, biodegradable polymeric chitosan nanoparticles (CSnp) have been widely used as a favorable vehicle in delivering various nucleic acids and proteins (11–13). Bovine serum albumin (BSA) has been extensively studied as a model protein for drug delivery studies (14, 15). BSA incorporated in the CSnp when dispersed in collagen hydrogels and subsequently distributed in poly (lactide-co-glycolide) scaffolds resulted in controlled release maintaining the bioactivity of the released BSA (14). However, despite the application of diverse approaches, temporal-controlled growth factor release for stem cell differentiation, especially under *in vivo* conditions, is still enormously challenging. This is mainly because of the issues associated with the monitoring of released growth factors over a period of time. In addition, to date, there is no report on either the interaction of growth factor–loaded CSnp with stem cells from apical papilla (SCAP) or the effect of temporal-controlled growth factor release on SCAP differentiation.

The main factor that governs the association of the protein to polycationic polysaccharide nanoparticles is the protein-polysaccharide electrostatic interaction. The following diverse approaches are used to incorporate bioactive molecules in polycationic polysaccharide nanoparticles:

1. The encapsulation technique involves embedding of bioactive molecules in nanoparticles. In this technique, most of the bioactive molecules are entrapped/embedded in the polysaccharide-protein nano-matrix (16).
2. In the adsorption technique, the bioactive molecules are solely adsorbed to the surface of nanoparticles (16). In this case, the interaction between nanoparticles and bioactive molecules is relatively weak. Because of the variance in the incorporation techniques, difference release patterns of the incorporated bioactive molecules can be anticipated. In this study, BSA-encapsulated nanoparticles (BSA-CSnpI) and

BSA-adsorbed nanoparticles (BSA-CSnpII) were synthesized, and the effect of temporal-controlled release of BSA on cellular differentiation of SCAP was investigated. We speculated that the unique release profiles of BSA might affect the regulation of alkaline phosphatase (ALP) activity in SCAP.

Materials and Methods

The polymeric chitosan, polyanionic sodium tripolyphosphate, BSA, chitosanase from *Streptomyces griseus* with an activity of 50 U/mg, and alpha minimum essential medium were purchased from Sigma-Aldrich Inc (St Louis, MO). Fetal bovine serum (FBS), L-glutamine solution, and antibiotic:antimycotic solution were obtained from Gibco (Carlsbad, CA). All other chemicals were of analytic grade (purity $\geq$ 95%).

BSA-loaded Chitosan Nanoparticle Preparation

The polymeric CSnp were synthesized as previously described (17) based on an ionotropic gelation technique (18). In brief, chitosan was dissolved in 1% (v/v) acetic acid solution at a concentration of 1.2 mg/mL, and the pH was raised to 5 with 1 mol/L sodium hydroxide (NaOH). CSnp were formed by adding polyanionic tripolyphosphate (1 mg/mL) to chitosan solution in a ratio of 3:1 under magnetic stirring at a speed of 1000 rpm for 10 minutes followed by centrifugation (20,000 rpm, 1 hour [J2-21M; Beckman, Palo Alto, CA]). The supernatant was collected, and nanoparticles were extensively rinsed with deionized water to remove any residual NaOH and then freeze-dried before further use.

BSA-loaded CSnp (BSA-CSnp) were prepared by 2 different methods: encapsulation (19) and adsorption (20). In the encapsulation method, 1% BSA was included in the polyanionic phase in the same process as in the BSA nonloaded CSnp. However, for adsorption, previously synthesized CSnp (500 mg) were stirred in 50 mL BSA (1.1 mg/mL) for 7 hours at 4°C. After rinsing with deionized water, nanoparticles were freeze-dried.

Nanoparticle Physicochemical Characterization

The zeta potential of nanoparticles was determined by laser Doppler anemometry using a Zetasizer (Nano-ZS90; Malvern, Malvern, UK). For this, a 5-mg sample was suspended in 0.7 mL Milli-Q water (Direct-Q UV3, Millipore, France) and sonicated for 1 minute. The size of the nanoparticles was determined from morphologic images by transmission electron microscopy (CM12 Philips; Philips, Amsterdam, The Netherlands). Copper grids with Formvar films (300 mesh; Electron Microscopy Sciences, Hatfield, PA) were made hydrophilic by glow discharge followed by immediate treatment with the nanoparticle solution and observed using transmission electron microscopy after air-drying. Chemical characterization of nanoparticles was performed by Fourier transform infrared spectroscopy (FTIR) spectroscopy (Perkin-Elmer Spectrophotometer; Spectrum One, Waltham, MA) in the spectral range of 450–4000 cm^{-1}.

The amount of free BSA in the clear supernatant after centrifugation of the nanoparticle suspension was measured using a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich Inc) at 562 nm after appropriate dilution to determine the BSA-loading capacity of the nanoparticles (15).

Nanoparticle Release Profile

In vitro release of BSA from loaded CSnp was performed for 30 days in 5% trehalose solution. Freshly prepared nanoparticles (50 mg) in centrifuge tubes (n = 3) were suspended in 6 mL trehalose and incubated at 37°C under agitation. At appropriate time intervals, an aliquot (4 mL) was withdrawn and centrifuged (20,000 rpm for 10 minutes). The initial volume of release medium was maintained by refilling 4 mL of the same medium after each withdrawal. The supernatant was analyzed for BSA content using the BCA kit. BSA nonloaded CSnp were dissolved in the same solution and analyzed at the same time points in order to correct the absorbance because of the chitosan. The ratio of the cumulative release was calculated based on the total amount of BSA obtained from the predetermined weight of nanoparticles (21).

After the release period, BSA-CSnpI (30 days) and BSA-CSnpII (14 days) nanoparticles were degraded with chitosanase (1 mg/100 mL in phosphate buffered saline [PBS]) for 1 day. The supernatant was analyzed to quantify the amount of BSA that was not released (19). The fresh BSA loaded and BSA nonloaded CSnp were also allowed for direct degradation by chitosanase for 1 day to compare the amount of total BSA release from the release experiment with that of total BSA actually incorporated in the same quantity of nanoparticles and deduct the absorbance caused by chitosan itself. The release experiment was performed in triplicate and repeated 3 times.

SCAP Culture

A previously characterized SCAP cell line was used in all experiments (22). Cells were cultured and expanded by adding single-cell suspensions (1×10^5 cells) in media composed of alpha minimum essential medium supplemented with 10% FBS, 2 mmol/L L-glutamine, and 100 U/mL antibiotics to 75-cm^2 cell culture flasks. Cells were allowed to expand in culture to 70%–80% confluency followed by treatment with 0.05% trypsin (Gibco) and passing the culture to subsequent culture flasks or used in experiments. SCAP from the third to fifth passages were used in all experiments.

SCAP Viability in the Presence of Nanoparticles

SCAP viability in the presence of BSA loaded or BSA nonloaded CSnp was quantitatively assessed based on the reduction of tetrazolium salts to colored formazan products via mitochondrial activity (23). Approximately 1×10^5 SCAP were seeded into 24-well plates in a cell culture medium and incubated for 3 days in 5% CO_2 incubator (Thermo Fisher Scientific, Waltham, MA) for confluency. For the cell viability test, the cell medium was exchanged with BSA nonloaded and BSA loaded CSnp suspension in 3 different concentrations (0.1, 0.3, and 1 mg/mL) in standard culture medium and cultured for 28 days. At predetermined times (1, 7, 14, 21, and 28 days), the supernatant media was removed without disturbing the cells and washed with 1 mL PBS. Cell survival was determined by the standard 3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (23). MTT was added at a concentration of 1 mg/mL in PBS and incubated for 4 hours. After the incubation period, MTT solution was removed, and 1 mL dimethyl sulfoxide was added to dissolve the insoluble formazan crystals. A reading was then taken at 540 nm using an ultraviolet-visible Synergy microplate reader (BioTek Instruments, Winooski, VT). The percentage survival of cells was calculated based on the control samples without any treatment as 100% viable. All analyses were performed in triplicate, and the experiments were repeated 3 times.

Cell morphology after 24 hours of interaction with or without nanoparticles was determined by a trypan blue exclusion test (24); however, nanoparticles may interfere. Therefore, to better understand the morphology of cells after nanoparticle treatment, cells were incubated for 20 minutes with 200 μL calcein AM/ethidium homodimer dye, and cells were observed using a fluorescent microscope (Vert.A1; Carl Zeiss, Jena, Germany).