# Temporal-controlled Dexamethasone Releasing Chitosan Nanoparticle System Enhances Odontogenic Differentiation of Stem Cells from Apical Papilla

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## Abstract

**Introduction:** The spatial and temporal control of stem cell differentiation into odontoblast-like cells remains one of the major challenges in regenerative endodontic procedures. The current study aims to synthesize and compare the effect of dexamethasone (Dex) release from 2 variants of Dex-loaded chitosan nanoparticles (CSnp) on the odontogenic differentiation of stem cells from apical papilla (SCAP). **Methods:** Two variants of Dex-loaded CSnp were synthesized by encapsulation (Dex-CSnpI) and adsorption (Dex-CSnpII) methods. The physicochemical characterization of Dex-CSnpI and Dex-CSnpII was assessed by transmission electron microscopy, Zetasizer, and Fourier transform infrared spectroscopy, whereas the Dex release kinetics was assessed by spectrophotometry. A previously characterized SCAP cell line was cultured onto CSnp, Dex-CSnpI, or Dex-CSnpII. The biomineralization potential was determined by alizarin red staining. Alkaline phosphatase, dentin sialophosphoprotein, and dentin matrix protein-1 gene expressions were analyzed by real-time reverse-transcription polymerase chain reaction. **Results:** Dex-CSnpI resulted in slower release of Dex compared with Dex-CSnpII, but both demonstrated sustained release of Dex for 4 weeks. Biomineralization of SCAP was significantly higher ($P < .05$) in presence of Dex-CSnpII compared with that in Dex-CSnpI at 3 weeks. Alkaline phosphatase gene expression was significantly higher in the presence of Dex-CSnpII compared with Dex-CSnpI, with peak expression seen at 2 weeks ($P < .05$). The expression of odontogenic specific marker dentin matrix protein-1 was significantly higher in presence of Dex-CSnpII compared with Dex-CSnpI at 3 weeks ($P < .05$). **Conclusions:** Collectively, these data suggest that sustained release of Dex results in enhanced odontogenic differentiation of SCAP. These findings highlight the potential of temporal-controlled delivery of bioactive molecules to direct the spatial- and temporal-controlled odontogenic differentiation of dental stem cells. *(J Endod 2015;41:1253–1258)*

## Key Words

Chitosan nanoparticles, dexamethasone, odontogenic differentiation, stem cells from the apical papilla, temporal-controlled release

Physiological tooth formation involves the gradual differentiation of dental papilla cells in close proximity to epithelial cells into postmitotic odontoblasts. Odontoblasts remain active throughout the life of the tooth and secrete primary and secondary dentin (1). After dental injury and irreversible odontoblast damage, dental pulp produces reparative dentin matrix secreted by a second generation of odontoblast-like cells. Odontoblast-like cells originate from stem/progenitor cells that reside in the perivascular niche of the dental pulp tissue (2, 3). Stem cells from apical papilla (SCAP) have been demonstrated to differentiate into odontoblast-like cells and have the potential to undergo chondrogenic, adipogenic, and neurogenic differentiation, depending on the induction condition (4, 5).

Dexamethasone (Dex) is a potent synthetic corticosteroid commonly used as an anti-inflammatory drug. In addition to its common clinical use, it is routinely used to stimulate the differentiation of mesenchymal stem cells (6, 7). A typical concentration of Dex used for inducing osteogenic differentiation is from 10 to 100 nmol/L (6, 7). Several groups have used Dex to induce mineralization of dental-derived stem cells, including dental pulp stem cells and SCAP *in vitro* (8–10). Dex at 10 nmol/L concentration has shown to regulate the commitment of progenitors derived from dental pulp cells to form odontoblast-like cells (10). Interestingly, patients undergoing long-term treatment with corticosteroids often present with substantial pulpal calcifications (11). This Food and Drug Administration–approved drug when used in large concentration (5 mg) in clinical study produced adverse changes on tissue mineralizing potential (11). Thus their application as a morphogen is explored in this study. However, appropriate concentration of morphogen at a suitable time may not be available after a single application. Therefore, controlled delivery of morphogen is crucial for their clinical applications.

In recent years, polymeric nanoparticles have been evaluated extensively as one of the most promising delivery systems for bioactive molecules (12). Among many polymers, chitosan (CS) attracts considerable attention because of its physicochemical and biological properties (13). CS is a linear aminopolysaccharide composed of 2-amino-2-deoxy-$\beta$-D-glucan joined by glycosidic linkages. CS can be developed as nanoparticles, is biocompatible and biodegradable, and possesses broad range of antimicrobial

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activity (14, 15). Compared with other polymers, CS molecule contains a number of reactive amine groups, which makes it applicable for functionalization of bioactive molecules (16). These polymeric nanoparticles can be used to provide controlled release of effective levels of bioactive molecules, which can increase treatment efficacy in regenerative endodontics. Temporal-controlled release of Dex from a polymeric nanoparticles system on the odontogenic differentiation of SCAP has not been studied in detail yet. The purpose of the current study was to (1) synthesize and characterize 2 variants of Dex-loaded CS nanoparticles (CSnp) and (2) to determine the effect of 2 controlled release profiles of Dex from CSnp on odontoblast-like differentiation of SCAP.

## Materials and Methods

CS, sodium tripolyphosphate, Dex, and $\alpha$-minimum essential medium were purchased from Sigma-Aldrich Inc (St Louis, MO). Fetal bovine serum, L-glutamine solution, and antibiotic:antimycotic solution were obtained from Gibco (Carlsbad, CA). All other chemicals were of analytical grade (purity $\geq$ 95%).

### Preparation of Dexamethasone-loaded Chitosan Nanoparticles

CSnp was synthesized as previously described (17) on the basis of an ionotropic gelation technique. Dex-loaded CSnp (Dex-CSnp) were prepared by 2 different methods, encapsulation (18) and adsorption (19). Encapsulation of Dex in CSnp was carried out by ionotropic gelation with slight modification and designated as Dex-CSnpI (encapsulation method). One hundred milliliter aqueous solution of tripolyphosphate (2 mg/mL) and 100 mL ethanolic solution of Dex (1.5 mg/mL) were mixed together. The resulting solution was added to 500 mL CS solution (1.2 mg/mL) under stirring for 10 minutes, followed by centrifugation. For adsorption, however, previously synthesized CSnp (200 mg) was dispersed in ethanol/deionized water (1:1, 50 mL) by sonication for 15 minutes, and 100 mg Dex dissolved in ethanol/deionized water (1:1, 100 mL) was added. After overnight stirring at room temperature, the resultant mixture was centrifuged, and nanoparticles obtained were designated as Dex-CSnpII (adsorption method). The supernatant in both the methods was collected to estimate the free Dex. The residue was washed with deionized water and lyophilized. The initial concentration of the loaded Dex was quantified by using ultraviolet spectrophotometer at 242 nm. This was done by analyzing the free Dex in the supernatant of 5 mg Dex-CSnp suspended in ethanol/deionized water (1:1, 0.5 mL) for up to 24 hours until there was no release of Dex. Non-loaded CSnp was dissolved in the same solution and analyzed at the same time points to correct for the background absorbance.

### Physicochemical Characterization of Nanoparticles

Zeta potential ($\zeta$-potential) and size of the nanoparticles were determined by laser Doppler anemometry by using a Zetasizer (Nano series, Nano-ZS90, Malvern, UK) and transmission electron microscopy (CM12 Philips, Eindhoven, The Netherlands) as described elsewhere (20). Chemical characterization of nanoparticles was carried out by Fourier transform infrared (FTIR) spectroscopy (Perkin-Elmer spectrophotometer; Spectrum One, Waltham, MA) in the spectral range of 4000–450 $cm^{-1}$.

### Release Profile of Nanoparticles

*In vitro* release of Dex from Dex-CSnp was analyzed for 4 weeks by incubating 5.5 mg nanoparticles in 0.5 mL phosphate-buffered saline (PBS) at 37°C. At appropriate time intervals, an aliquot (0.3 mL) was withdrawn and centrifuged. The initial volume of release medium was maintained by refilling 0.3 mL of the same medium after each withdrawal. The supernatant was used to quantify the Dex content by using ultraviolet spectrophotometer at 242 nm. The ratio of the cumulative release in percentage was calculated on the basis of the total amount of Dex obtained from the predetermined weight of nanoparticle. The release experiment was performed in triplicate and repeated 3 times.

### SCAP Culture

A previously characterized SCAP cell line was used for all experiments in this study (21). SCAP were cultured and expanded by adding single-cell suspensions ($1 \times 10^5$ cells) in media composed of $\alpha$-minimum essential medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 100 U/mL antibiotics to 75-$cm^2$ cell culture flasks. Cells were allowed to expand in cultures to 70%–80% confluency, followed by treatment with 0.05% trypsin (Gibco, Carlsbad, CA) for 3 minutes at 37°C before passing the cells into culture flasks or used in experiments. SCAP from third to fifth passage were used in all experiments.

### SCAP Viability in Presence of Nanoparticles

SCAP viability in the presence of CSnp, Dex-CSnpI, or Dex-CSnpII was quantitatively assessed on the basis of the reduction of tetrazolium salts to colored formazan products via mitochondrial activity (22). Approximately $1 \times 10^5$ SCAP were seeded into 24-well plates with standard cell culture medium and incubated at 37°C for 3 days in 5% $CO_2$ humidified incubator (Thermo Scientific, Waltham, MA) to achieve cell confluence. For the cell viability test, cells were treated with respective nanoparticle suspension (300 $\mu$g/mL) in a standard culture medium and cultured for 24 hours. SCAP cultured in the absence of nanoparticles were treated as a control. The supernatant medium 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 (22). In addition, SCAP morphology after 24 hours of interaction with/without nanoparticles was determined by calcein AM staining, followed by fluorescent microscopy (Vert.A1; Carl Zeiss, Oberkochen, Germany).

### Quantitative Analysis of Alizarin Red Staining in Presence of Nanoparticles

SCAP ($5 \times 10^4$ cells/well) were grown to confluence in 6-well culture plates in standard culture medium. The medium was exchanged to mineralizing medium constituting 50 $\mu$g/mL ascorbic acid, 10 mmol/L $\beta$-glycerolphosphate, and 1.8 mmol/L $KH_2PO_4$ in standard culture medium (20). After SCAP were cultured with nanoparticles in mineralizing medium for 1 and 3 weeks, biomineralization potential was assessed by staining with alizarin red stain (ARS). In brief, the cells were washed 3 times with PBS and fixed in 10% normal buffered formalin for 30 minutes at room temperature. After additional washing in water, they were incubated in 2% ARS (pH 4.2) in water for 20 minutes at room temperature with gentle agitation, followed by 3 washes with water and allowed to air-dry. The mineralized nodules were subsequently quantified through a colorimetric assay. The ARS was incubated with 10% cetylpyridinium chloride in 10 mmol/L sodium phosphate buffer for 30 minutes to elute all calcium-bound stain. The supernatant was collected, and the optical density was determined at 560 nm. Mineralized nodule formation was represented as optical density per microgram total cellular protein, determined by micro bicinchoninic acid protein assay kit (Sigma-Aldrich). Because ARS stains CS, the basal staining caused by the CS without cells was quantified, and this value was then subtracted from the experimental data.