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# Electrophoretic deposition of dexamethasone-loaded gelatin nanospheres/chitosan coating and its dual function in anti-inflammation and osteogenesis

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ABSTRACT

Surface modification of metallic implants with bioactive and biodegradable coatings could be a promising approach for bone regeneration. The objective of this study was to prepare chitosan/gelatin nanospheres (GNs) composite coating for the delivery of dexamethasone (DEX). GNs with narrow size distribution and negative surface charge were firstly prepared by a two-step desolvation method. Homogeneous and stable gelatin nanospheres/chitosan (GNs/CTS) composite coatings were formed by electrophoretic deposition (EPD). Drug loading, encapsulation efficiency and *in vitro* release of DEX were estimated using high performance liquid chromatography (HPLC). The anti-inflammatory effect of DEX-loaded coatings on macrophage RAW 264.7 cells was assessed by the secretion of tumour necrosis factor (TNF) and inducible nitric oxide synthase (iNOS). Osteogenic differentiation of MC3T3-E1 osteoblasts on DEX-loaded coatings was investigated by osteogenic gene expression and mineralization. The DEX in GNs/CTS composite coating showed a two-stage release pattern could not only suppress inflammation during the burst release period, but also promote osteogenic differentiation in the sustained release period. This study might offer a feasible method for modifying the surface of metallic implants in bone regeneration.

## 1. Introduction

Traditional therapies for bone defect such as autograft and allograft have many limitations including the shortage of organ resource, side effects, etc [1]. Titanium (Ti), one of metallic implants, has become a versatile and economical alternative to treat bone defect. Nonetheless, the bioinert character of titanium and subsequent immunoreactions are huge challenges during the healing progress, which may lead to various complications and even implant failure [2,3]. A well-known approach is to activate the surface of titanium by fabricating functional coatings incorporated with biomolecules including antibiotics, proteins/peptides, and growth factors [4,5]. Some researchers have used therapeutic ions such as copper, magnesium or strontium to improve the osteogenic capability of titanium implants [5–8]. But how to inhibit inflammation during osseointegration, and realize the long effect of osteogenesis and anti-inflammation still need further study.

Dexamethasone (DEX), a typical synthetic glucocorticoid used for anti-inflammation, can also promote osteogenic differentiation at a relative lower concentration [9–11]. The anti-inflammatory function of DEX is often realized at the initial inflammatory stage by down-regulating the expression of pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6 and TNF [9]. In the case of osteogenesis effect, DEX was reported to drive progenitor cells to the osteoblasts phenotype [12]. Other reports suggested that it may facilitate osteogenesis and enhance alkaline phosphatase (ALP) activity and bone mineralization at a concentration range of $10^{-8}$ to $10^{-7}$ M [11–15]. However, systemic dosage of glucocorticoids may lead to side effects such as osteoporosis and nontraumatic osteonecrosis [12,16,17]. Hence, by incorporating DEX during the coating process, these functional molecules could release locally to accelerate bone healing with lower side effects but higher efficiencies. In addition, with proper control of DEX release profile from the coating, e.g. a rapid release rate at the initial stage and substantially minimized release rate afterwards, it was expected that such DEX-loaded coatings could prevent inflammation issues upon metallic implant and simultaneously promote osteogenesis [10,12].

In recent years, nanoparticles have been increasingly used as drug carriers for sustained release. According to our previous investigation, gelatin nanospheres (GNs) could be formed through two-step desolvation method [3]. Gelatin has been widely used in food, pharmaceutical and biomedical materials because of its excellent biocompatibility, biodegradability and non-immunogenicity [18–21]. GNs could be

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either positively or negatively charged by balancing the relative amount of carboxyl and amine groups [22]. GNs have been developed as delivery vehicles for different kinds of biomolecules such as growth factors, antibiotics and other drugs through covalent [23] or physical bonds (hydrogen bond, hydrophobic and electrostatic interactions) [19,24,22,25]. In addition, the degradation products of gelatin contained mainly arginine-glycine-aspartic acid (RGD) sequences, which were usually applied to promote osteoblasts adhesion [19,26].

Various coating techniques have been applied to fabricate bioactive coatings on titanium implants, such as plasma spraying, dip coating, microarc oxidation, solution casting, electrophoretic deposition (EPD), etc. [3,27–29]. In order to avoid the damage or decomposition of biomolecules in the coating, a mild coating process should be taken into account. EPD was a rapid, efficient and versatile approach which could form uniform and stable coatings with tunable thickness at room temperature [3,28,30,31].

In our previous study, chitosan (CTS) was used to prepare coatings on metallic substrates by EPD [3]. CTS, a cationic natural polymer, has features such as nontoxicity, biodegradability and antimicrobial activity [20,28,32]. Therefore, the objective of this study was to prepare a biodegradable DEX-releasing coating on titanium substrate which could inhibit inflammation and promote osteogenesis at the same time. To this end, GNs with different crosslinking density were firstly fabricated to load DEX. Based on this we developed DEX-loaded coatings by EPD. The properties and morphology of the GNs and EPD coatings were tested. The release kinetics of DEX from GNs and EPD coatings were also studied *in vitro*. The effects of anti-inflammatory and osteogenic differentiation of DEX releasing from the coatings were examined in relation to the possible application of metallic implant surface for preventing the initial inflammation but promote osteogenesis.

## 2. Methods

### 2.1. Preparation and characterization of GNs

GNs were prepared by a two-step desolvation method. Firstly, 1.25 g gelatin B was dissolved into deionized water at 50 °C under stirring to obtain 25 mL of a 5% (w/v) gelatin solution. Equal volume of acetone was added to precipitate the high molecular weight gelatin. After 1 h's standing at room temperature, the supernatant with small molecule gelatin was poured away. Secondly, the sedimented gelatin was redissolved in water at 50 °C under stirring, followed by the adjustment of pH to 2.5 after the gelatin solution cooled down. Then the gelatin solution was re-heated to 40 °C and 80 mL of acetone was added dropwise (~2 mL/min) under vigorous stirring. At this time GNs have been initially formed, then different amounts of 25 wt% glutaraldehyde (GA) as crosslinker was added dropwise to strengthen the structure of GNs. After crosslinking reaction overnight, equal volume of glycine solution (100 mM) was added to get rid of the unreacted GA, keeping it stirring for 1 h. The suspension was centrifuged at 10,000 Reactive Centrifugal Force (RCF) for 10 min. The nanospheres were resuspended in deionized water, and washed by water at least three times. Then GNs were lyophilized in acetone/water (1:3 v/v) for 2 days. The lyophilized GNs with different crosslinking density were sealed for further use [22,33].

The morphology of GNs was characterized by scanning electron microscope (SEM) (ZEISS, Germany) with an accelerating voltage of 1.0 kV after coating with gold. The size of lyophilized GNs was calculated by selecting 100 nanospheres per SEM image randomly. Dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern Instruments Ltd.) was used to analyze the average size, size distribution and zeta potential of GNs in water.

There were two different crosslinking GNs, the low crosslinking GNs (L-GNs) with a fixed molar ratio of GA/$[NH_2]_{gelatin}$ equal to 1:1 and the high crosslinking GNs (H-GNs) with the ratio equal to 4:1. The degradation of GNs was detected as the supplementary data mentioned.

### 2.2. DEX-loaded GNs and its properties

A diffusional post-loading method was used to load DEX onto lyophilized GNs [24]. In brief, lyophilized L-GNs or H-GNs were mixed with DEX solution (30 mg/mL) in 4 mL tubes and were incubated at 4 °C overnight for completely swelling. 1 mL PBS was added into the each tube and mixed by vortex mixer, and they were centrifuged to get the supernatant. DEX in the supernatant was detected by HPLC, which was the drug unloaded in GNs. The drug loading and encapsulation efficiency were calculated by the formula (1) and (2):

$$\text{Drug loading}\left(\frac{\mu g}{mg}\right) = \frac{\text{Amount of DEX in GNs}(\mu g)}{\text{Amount of GNs}(mg)} \quad (1)$$

$$\text{Encapsulation efficiency}(\%) = \frac{\text{Actual DEX in GNs}(\mu g)}{\text{Theoretical drug loading}(\mu g)} \times 100\% \quad (2)$$

The same volume PBS was added, and then the tubes were incubated at 37 °C on a rotating plate. At the selected time point, the samples were centrifuged at 10,000 RCF for 5 min, and the supernatant was collected for detection. Meanwhile the same volume of fresh PBS was added [19]. The DEX in collected release medium was determined by HPLC [34]. The detailed method of HPLC for DEX was mentioned in supplementary data.

### 2.3. EPD process and characterization of the EPD coating

Ti substrate ($3 \times 1\ cm^2$, 0.5 mm in thickness) was used as the cathode electrode. A parallel 316L stainless steel plate was used as anode, with 10 mm between them. All electrodes were deposited with area of $2\ cm^2$. During EPD process, two electrodes were connected with a direct current about 0.005 A past. 5 min were needed to finish EPD process. After EPD, the Ti substrates were removed and air-dried overnight [35].

The morphology of EPD coatings was characterized by SEM. The wettability of the coating surfaces was measured (DSA30 Kruess GmbH, Germany) using deionized water at room temperature. Five drops of 3 μL volume were placed in the center of coating surface, and the contact angle was measured after attaining equilibrium [1,3,29].

### 2.4. Swelling and degradation of EPD coatings

The dry weight of each Ti substrate ($W_0$) was measured before the experiment. Ti substrates with coating were placed vertically in 15 mL tubes, 10 mL PBS (pH = 7.4) were added in each tube, and incubated at 37 °C for 1, 4, 8, 24, 48, and 72 h (n = 3). The swelling ratio was expressed as the percentage water uptake (W.U.) [36], and determined as formula (3):

$$W.U. = (W_1 - W_2)/(W_2 - W_0) \times 100\% \quad (3)$$

where $W_1$ was the weight of samples removed at different time after the surface residual PBS absorbed by filter paper. $W_2$ was the dry weight of samples after rinsing with deionized water, and dried in vacuum at room temperature.

The samples for degradation were taken out at 4 and 8 weeks. At each time point, Ti substrates with coating were rinsed with deionized water for at least 5 times, and air-dried overnight. The degradation of coating *in vitro* was observed by SEM.

### 2.5. In vitro release of DEX-loaded coating

The DEX release behavior of DEX-loaded coating was investigated in PBS at pH 7.4. Briefly, each sample was remained uprightly in 3 mL of PBS in 4 mL tubes, and the tubes were incubated at 37 °C on a rotating plate. At intervals, 0.5 mL of the release medium was taken out and