Colloids and Surfaces B: Biointerfaces 150 (2017) 153–158

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Protocols

Improved rhBMP-2 function on MBG incorporated TiO_2 nanorod films

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ARTICLE INFO

Article history:
Received 29 July 2016
Received in revised form
11 November 2016
Accepted 23 November 2016
Available online 24 November 2016

Keywords:
Nanorods
Mesoporous bioactive glass
rhBMP-2
BMSCs

ABSTRACT

In the process of biomaterials mediated bone regeneration, rhBMP-2 delivery at efficient dose in sustained kinetics is crucial for promoting cell osteogenic differentiation. Meanwhile, surface morphology of the biomaterials could regulate cellular responses as well as strengthen the rhBMP-2 interaction with cells for better bone induction. Herein, TiO_2 nanorod films with varied mesoporous bioactive glass (MBG) incorporation amount were designed to strengthen the efficacy of rhBMP-2, basing on optimized loading/release behaviors and surface nanostructure cooperatively. The MBG incorporation improved rhBMP-2 loading amount and regulated its release behavior. Consequently, the osteogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) on the incorporated films was extremely enhanced, and the incorporated nanorod film with 200 nm MBG thickness exhibited the best osteoinduction effect. However, MBG film and the incorporated nanorod film had the same loading amount of rhBMP-2, the latter showed a much higher expression of 7-day osteogenic differentiation index than the former, which could be attributed to the synergistic effect of optimized rhBMP-2 release behavior and surface morphology. The MBG incorporated TiO_2 nanorod films here presents a promising strategy for enhancing osteoinduction through optimized rhBMP-2 release behavior.

1. Introduction

Currently, most orthopedic implants are made of metals such as titanium alloy, stainless steel or tantalum alloy [1,2]. However, the "native" surfaces of these metals are generally bioinert which is unfavorable to osteointegration. Various coatings have been developed to promote osteointegration [3], and these coatings involve biological components for fundamental bioactivity [4,5], topological (micro/nano) structures for biological microenvironment [6,7], and biomolecules for biological responses [8,9].

Among bioactive materials, TiO_2 has demonstrated good biocompatibility and high osteogenic responsiveness due to its versatility in micro/nano structural forms [10–13]. TiO_2 nanonodules and nanotubes were reported to enhance the attachment, spread, adhesion, proliferation, and differentiation of cells effectively [14–16], and TiO_2 nanorods as another TiO_2 nanostructure offer a unique characteristic of a pseudo three-dimension structure which could provide a more appropriate microenvironment for cell growth. The TiO_2 nanorod arrays were proved to promote initial adhesion and osteogenic differentiation of MSCs [17–19]. In spite of the fundamental bioactivity and nanostructure establishment, they have no biomolecules which can communicate with proteins or cells.

For biomolecules, recombinant human bone morphogenetic protein-2 (rhBMP-2) is a most important growth factor to accelerate bone formation [20–22], and its loading/release behavior is crucial for effective function. Abundant researches have been carried out to optimize the delivery of rhBMP-2. Among those, strategies for rhBMP-2 loading include chemical and physical immobilization. It was reported that PAH and PSS coating carrying about 500 ng rhBMP-2 with several micrometers in thickness could retain its activity toward enhanced early osteogenic differentiation of MSCs [23,24]. It is theoretically measured that a BMP-2 concentration of ≈700 ng/cm^2 in the films is exceeding to saturation of BMP-2 receptors [25]. For another hand, quick BMP-2 release was revealed to cause ectopic ossification and limited its later BMP-2 efficiency in HA layer [26]. Whereas, chemical immobilizations like polymers and HA often lead to protein denaturation and delivery unsatisfactory loading/releasing capacities. Thus, mesoporous bioactive glass (MBG) has turned out to be a promising material for rhBMP-2 loading through entrapping in mesopore [27–29]. More-

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http://dx.doi.org/10.1016/j.colsurfb.2016.11.030
0927-7765/

over, MBG has shown attractive properties of good bioactivity and tissue compatibility [30,31].

As demonstrated in our previous work [32,33], TiO_2 nanorod films with MBG incorporated could obviously increase drug loading capacity and optimize its release behavior, as well as enhance biological responses. Hence, the MBG incorporation presents a facile and effective way to improve biological performances of the nanorod films. In this work, we focused on further increase in the capacity to improve the capacity of inducing osteoinduction of TiO_2 nanorod films through loading rhBMP-2. The roles of MBG in tailoring rhBMP-2 loading and release behaviors of TiO_2 nanorod films were investigated, and the biological functions of the loaded rhBMP-2 in the films were evaluated through culturing bone mesenchymal stem cells (BMSCs) and assessing cellular osteogenic differentiation by ALP activity, real-time PCR analysis and alizarin red staining assays, respectively. The relations of loaded rhBMP-2 function and MBG were discussed.

2. Experiment

2.1. Preparation of TiO_2 nanorod films with MBG incorporation

Firstly, TiO_2 nanorod films on tantalum substrates (1 cm × 1 cm) were prepared by two steps, the formation of TiO_2 nanodots as crystalline seed layers through phase-separation-induced self-assembly method [34], and then a hydrothermal treatment to grow nanorods [35]. Finally, MBG incorporation into TiO_2 nanorod films were carried out via sol-gel technique.

For MBG precursor sol, the sol with a molar ratio of $SiO_2/CaO/P_2O_5/TiO_2 = 80/5/5/10$ was prepared by tetraethyl orthosilicate (Aladdin, AR), calcium nitrate tetrahydrate (Aladdin, AR), and triethyl phosphate (Aladdin, AR), tetrabutyl titanate (TBOT, Aladdin, AR), and amphiphilic triblock copolymer poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (P123, $M_n = 5800$, Sigma-Aldrich) as the template. The sol was formed in ethanol with adding HCl (molar ratio of P123/HCl/ethanol = 0.014/1/30), stirred at room temperature for 24 h, and aged at 4 °C for 24 h. The sol was designed to different concentration by ethanol dilution.

For MBG incorporation, 20 μL of the precursor sol was dropped onto TiO_2 nanorod films, and spin-coating at 7000 rpm for 40 s was followed. The as-spinning-coated films were placed at 4 °C and in the humidity of 75.7% for 24 h, afterwards heated at a rate of 1 °C min^{-1} – 400 °C and kept at 400 °C for 5 h. After taking out from furnace, the films were rinsed extensively with deionized water, and allowed to dry in air.

2.2. Material characterization

Field-emission scanning electron microscopy (FE-SEM, Hitachi, operating voltage at 3 kV) was employed to observe the morphological structures of the obtained films.

2.3. Loading and in vitro release of rhBMP-2

Solution of 2 ug/mL recombinant human bone morphogenetic protein-2 (rhBMP-2) (Shanghai Rebone Biomaterials Co., Ltd) was prepared by dissolving the protein in deionized water. RhBMP-2 adsorption was carried out respectively by soaking and dropping methods. The loading amount on each film was assessed by measuring the reduction of rhBMP-2 comparing original solution with soaked solution.

For loading rhBMP-2 by soaking, films were immersed into 1 mL rhBMP-2 solution for 24 h; for loading rhBMP-2 by dropping, a 100 uL aliquot of rhBMP-2 solution was added dropwise onto the films. Then, the films with loading rhBMP-2 were dried at 37 °C.

The measurement of rhBMP-2 release was carried out by immersing the films into a 5 mL PBS at 37 °C. At each designated time points, the rhBMP-2-loaded films were transferred to fresh PBS. Measurements were taken at short intervals during the first days to monitor the initial release, followed by testing every some days to observe the sustained release, until all the rhBMP-2 was released into the surrounding PBS. Ultimately, the release profiles of each experimental set were expressed in a plot of release amount/percentage vs. time.

The amount of rhBMP-2 was monitored by a BMP-2 Quantikine ELISA Kit (Shanghai Yanjin Co., Ltd). The absorbancy was measured on a microplate reader (Thermo, Multiskan MK3) at 450 nm and the corresponding rhBMP-2 concentration was calculated based on the calibration curve obtained. The amount of rhBMP-2 could be accurate to nanogramme (ng) level and per unit loading capacity was calculated by dividing the area of each film (1cm × 1 cm). The in vitro loading/release of rhBMP-2 study was undertaken in triplicate for each preparation.

2.4. Culture of cells

Bone marrow-derived mesenchymal stem cells (BMSCs) were harvested from the femur and tibias of 4-week-old male Sprague-Dawley rats following an established protocol [36]. The third passage of BMSCs was used to examine in vitro cellular responses on different films. The first passage of mesenchymal stem cells (MSCs) of rat bone marrow was used to examine the in vitro cellular responses on different films with or without loading rhBMP-2. BMSCs were cultured in various alpha-modified Minimum Essential Medium (Alpha-MEM, Gibco) supplemented with 1% antibiotic solution containing 10,000 units/mL penicillin and 10 mg/mL streptomycin (Gibco), 1% sodium pyruvate (Gibco), and 1% MEM non-essential amino acids (Gibco), 10% fetal bovine serum (FBS, PAA, Australia) under a humidified 5% CO_2 atmosphere at 37 °C. Sub-confluent MSCs growing on tissue culture polystyrene (TCPS) were trypsinized with 0.25% trypsin containing 1 mM Ethylene Diamine Tetraacetic Acid (EDTA) (Gibco), and were subcultured on different films. The protocol and all experimentation were performed in accordance with the guidelines for animal care established by the Animal Research Committee of Zhejiang University, Hangzhou, China.

2.5. Alkaline phosphatase (ALP) activity

The BMSC cells with a density of 2.0×10^4 cells/mL (for 7 days) or 1.0×10^4 cells/mL (for 14 days) were seeded on various films (three replicates) under a humidified atmosphere of 5% CO_2 at 37 °C. After incubation for 7 or 14 days, culture medium was removed, the samples were rinsed with PBS for three times. The cells cultured on samples were removed in new 24-well culture plates and then lysed with CelLytic Buffer (Sigma). ALP (ab65834,abcam) expression was assessed by the LabAssay™ ALP Kit (Beyotime Institute of Biotechnologe, Shanghai, China) at a wavelength of 405 nm and total protein contents tested in a BCA protein assay.

2.6. Western blot analysis

The Smad 1/5/8 phosphorylation degree of BMSCs after cell culture on different samples surfaces for 7 days was assayed. Cells on each type of specimen were treated with cell lysis buffer (Cell Signaling). The proteins were collected and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 5% Tris-HCl reducing gels, after transferring, the PVDF membranes were incubated with phosphorylated Smad 1/5/8 (Santa Cruz Biotechnology) at 4 °C overnight. Then HRP-antibody (KPL) was used as second antibody. Band densities on the Western blots