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Immobilization of naringin onto chitosan substrates by using ozone activation

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ABSTRACT

Ozone oxidation can easily produce peroxides containing active free radicals that can be used for the surface modification of biomaterials. This process is highly efficient and nontoxic. In this research, naringin, an HMG-CoA reductase inhibitor that can promote bone formation, was immobilized onto a chitosan film using ozone activation. First, a chitosan film was treated by ozone to produce peroxides; these peroxides were then quantified and their amount was optimized by an iodide assay.

For the *in vitro* delivery of naringin, a chitosan–naringin substrate was immersed in phosphate-buffered saline to quantify the released amount of naringin. It was found that the immobilized naringin was slowly released over the course of two weeks, where its concentration in the medium was controlled by this delivery process. The results of cell culture showed that cell viability and early osteogenic differentiation, as measured by alkaline phosphatase expression, were promoted with the immobilized naringin on chitosan substrates. The expression of osteogenic proteins, including type-I collagen, bone siloprotein, and osteocalcin, were also enhanced. According to the results of Smad1 and Smad6 phosphorylation, immobilized naringin on ozonated chitosan substrates would be able to initiate bone morphogenetic protein-Smad signaling by activating receptor Smad and by suppressing inhibitory Smad. The results in this research demonstrated that the naringin–chitosan substrate produced by biocompatible ozone activation was highly osteoconductive without cytotoxicity.

1. Introduction

Naringin, a bioeffective compound derived from traditional Chinese herbal medicine, can promote proliferation and alkaline phosphatase (ALPase) expression in osteoblasts [1]. Naringin induces the synthesis of bone morphogenetic protein-2 (BMP-2) [2,3], which in turn is able to stimulate bone formation [4]. With BMP-2, bone mineralized density is increased by suppressing the activity of HMG-CoA reductase [5], promoting bone regeneration [6]. However, BMPs are very expensive and easily denatured. Thus, naringin, an inducer for BMP-2 synthesis *in vivo*, has recently attracted interest as an economical and highly stable pharmaceutical alternative to BMP.

Although naringin has the potential to accelerate bone regeneration, naringin in high concentration is cytotoxic [4,7,8], which is revealed by an increase in apoptosis [9]. In addition, naringin is unstable and easily oxidized after oral administration [10]. Controlled delivery from encapsulated naringin has been often applied to decrease its cytotoxicity while maintaining its effectiveness *in vivo* [11]. Another possibility would be to immobilize naringin on biomaterials, which may make naringin sustainably effective in osteoinduction without serious cytotoxicity [12]. Unfortunately, few studies have explored the immobilization of naringin on substrates derived from tissue engineering, especially on biocompatible and biodegradable biopolymers.

Many surface-modification processes have been reported for biopolymers. Creating highly active peroxides as surface modifications have been used previously to graft biomolecules [13–15]. On polymers' surfaces, ozone oxidation can easily produce peroxides, such as hydroperoxides, which can generate free radicals. By introducing these highly active groups, bioactive molecules can be further immobilized onto materials' surfaces. For example, type-I collagen (COL1) and RGD peptides were grafted onto ozonated poly-lactide scaffolds, thereby enhancing the material's cell affinity

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[16]. Compared with other methods for surface modification, ozone activation is efficient and simple. Moreover, ozone modification greatly reduces the need for nonbiocompatible organic solvents in the preparation process. Thus, ozone treatment was applied in this research to immobilize naringin onto chitosan, a biodegradable polymer that usually serves as substrate in cell culture and scaffolds in tissue engineering.

2. Materials and methods

2.1. Materials

Chemicals used in this study were sourced from several reputable firms: chitosan was purchased from Sigma–Aldrich with a deacetyl degree of 85%; acetic acid, sodium hydroxide (NaOH), ethanol, 3-(4,5-dimethythiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), and phosphate-buffered saline (PBS) were also purchased from Sigma–Aldrich; DPPH used in the study was sourced from Sigma-Aldrich; 1-methyl-2-pyrrolidinone (NMP) was purchased from Tedia Company, Inc.; ethanol was acquired from Seoul Chem. Ind. For cell cultures, α-minimum essential medium (αMEM), sodium β-glycerophosphate, ascorbic acid, and dexamethasone were purchased from Sigma–Aldrich. Fetal bovine serum (FBS), penicillin–streptomycin–amphotercin, and trypsin–EDTA solution were purchased from Gibco-BRL. Finally, reagents for ALPase, including sodium carbonate, p-nitrophenylphosphate, $MgCl_2$, ALPase, DNA quantification kit, and silver nitrate, were purchased from Sigma–Aldrich. All solvents used in this study were of analytical quality; distilled and deionized water was used throughout this study.

2.2. Preparation of chitosan substrates

Chitosan substrates were fabricated by a solvent-casting process. Chitosan powder was dissolved in an acetic acid aqueous solution (1 M) to form a 2 wt% chitosan solution. After the chitosan solution was cast on a culture dish and dried at 50 °C for 2 days, the chitosan substrates were immersed in a NaOH/ethanol aqueous solution, which was prepared by dissolving 40 g NaOH powder in 1000 mL of a 50 vol% ethanol aqueous solution. The chitosan film was then washed with PBS several times and dried at room temperature to remove the remaining liquid. This solvent-casting process allowed us to obtain dense chitosan substrates. The thickness and roughness of the chitosan films used in this research were 1 ± 0.18 mm and 12 ± 2 nm, as measured by atomic force microscope (Nanoscope III, Bruker Inc., USA).

2.2.1. Ozone treatment

The chitosan films were placed in a flask, through which a stream of O_3 was continuously bubbled, for a certain period. A modified iodide assay was used to measure peroxides produced on the surface of ozone-treated films [16].

2.2.2. Naringin immobilization and controlled release

After ozone activation, thermal induction was employed to graft naringin onto the ozonated chitosan substrate. The ozone-activated chitosan substrate and naringin solutions (0, 0.1, 5, 10, and 25 wt% naringin in NMP) were placed into a flask saturated with nitrogen, and then placed into an isothermal oil bath at 60 °C for thermal induction. The chitosan substrates with grafted naringin were subsequently washed with deionized water three times and sterilized with UV before release profiles or cell cultures were evaluated. For comparison, some chitosan substrates were also immersed into a naringin solution for 12 h without any ozone activation, where naringin was adsorbed onto chitosan surfaces directly.

Chitosan substrates with naringin immobilization were immersed in PBS at 37 °C. After immersions for 2, 4, 6, 8, 12, 24, 72, and 120 h, the buffer solution was analyzed by high-performance liquid chromatography (HPLC) to measure the concentration of released naringin. HPLC was performed with a Shimadzu (Kyoto, Japan) DGU-14A system equipped with a model LC-10AT-VP liquid chromatography pump, an autoinjector, and a diode-array detector. Shimadzu software was used to calculate peak areas. Compounds were separated on a Spherisorb ODS1 column from Waters Instruments (MA, USA). The detection wavelength was 285 nm.

2.3. Cell culture and analysis

UMR-106, osteoblast-like cells originally isolated from a rat osteosarcoma, were used for this study. The cells were cultured in αMEM, supplemented with 10% FBS and 100 U/mL penicillin–streptomycin–amphotercin, at 37 °C in 5% CO_2. UMR cells suspended in the culture medium (5×10^5 cells/ml) were added to Petri dishes containing the modified or unmodified chitosan films. The culture medium was renewed every two days during cell culturing. After various periods of incubation, the dishes were rinsed with PBS buffer in preparation for the analysis of cell viability, ALPase activity, and expressions of osteogenic proteins.

2.3.1. Analysis of cell viability

Cell viability was evaluated by MTT assay, in which yellow MTT was reduced to yield a purple formazan product by mitochondrial succinate dehydrogenase. Spectrophotometric measurement of MTT-formazan at 570 nm allowed the quantitation of cell viability. The substrates with cultured cells were transferred into new 24-well plates containing 1 mL of a working solution. After 6 h of incubation, the working solutions were replaced by DMSO. An ELISA plate reader (MQX200R, Bio-Tek Instrument Inc., USA) was used at a wavelength of 570 nm to determine absorbance.

2.3.2. Analysis of osteogenic differentiation

Early cell differentiation was determined by ALPase activity. An ALPase buffer containing 1 mg/mL PNPP was prepared by dissolving $MgCl_2$ (1 mM), $ZnCl_2$ (1 mM), and glycine (0.1 M) in deionized water. After removing the culture medium, the scaffolds with UMR-106 cells were washed twice with PBS, immersed into 0.4 mL of a lysis buffer for 15 min, and finally put into 1.2 mL of an ALPase buffer for 30 min. The reaction was stopped by the addition of 300 mL of 3 N NaOH. Absorbance was read at 405 nm using an ELISA plate reader.

After the cells cultured to 90% confluence, total RNA was extracted using Trizol reagent, and single strand cDNA synthesis was performed by using ReverTra qPCR RT kit. Real-time quantative polymerase chain reaction (PCR) was conducted using an SYBR green kit. Tested genes' expression levels were normalized to GAPDH levels. The PCR primer sequences were as follows: bone siloprotein (BSP), sense: 5′-AATGAAAA CGAAGAAAGCGAAG-3′, antisense: 5′-ATCATAGCCATCGTAGCC-TTGT-3′, osteocalcin (OCN), sense: 5′-ATGAGAGCCCTCACACTCCTC-3′, antisense: 5′-GCCGTAGAAGCG CCGATAGGC-3′, COL1, sense: 5′-ACAGCCGCTTCACCTACAGC-3′, antisense: 5′-TGCACTTTT-GGTTTTTGGTCAT-3′.

For Smad1 and Smad6 analysis, protein extractions of lysed cells were fractionated by 10% SDS-PAGE, electroblotted onto a Hybond-P membrane, which was probed with antibodies to Smad1 and P-Smad1, or with antibodies to Smad6 and P-Smad6. Then, samples were developed using an ECF Western blotting kit (Amersham Pharmacia Biotech) and visualized using a Typhoon 9410 Imager (Amersham Biosciences, Piscataway, NJ).