Biomaterials 35 (2014) 5565–5571

Contents lists available at ScienceDirect

Biomaterials

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

Stimulation of bone regeneration following the controlled release of water-insoluble oxysterol from biodegradable hydrogel

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ARTICLE INFO

Article history:
Received 21 February 2014
Accepted 11 March 2014
Available online 14 April 2014

Keywords:
Hydroxycholesterol
Oxysterol
Water-insoluble drug
Controlled release
Gelatin hydrogel
Bone regeneration

ABSTRACT

Recently bone graft substitutes using bone morphogenetic proteins (BMPs) have been heralded as potential alternatives to traditional bone reconstruction procedures. BMP-based products, however, are associated with significant and potentially life-threatening side effects when used in the head and neck region and furthermore, are exorbitantly priced. Oxysterols, products of cholesterol oxidation, represent a class of molecules that are favorable alternatives or adjuncts to BMP therapy due to their low side effect profile and cost. In order to establish the optimal clinical utility of oxysterol, an optimal scaffold must be developed, one that allows the release of oxysterol in a sustained and efficient manner. In this study, we prepare a clinically applicable bone graft substitute engineered for the optimal release of oxysterol. We first solubilized oxysterol in water by making use of polymeric micelles using L-lactic acid oligomer (LAo) grafted gelatin. Then, the water-solubilized oxysterol was incorporated into a biodegradable hydrogel that was enzymatically degraded intracorporeally. In this manner, oxysterol could be released from the hydrogel in a degradation-driven manner. The water-solubilized oxysterol incorporated biodegradable hydrogel was implanted into rat calvarial defects and induced successful bone regeneration. The innovative significance of this study lies in the development of a bone graft substitute that couples the osteogenic activity of oxysterol with a scaffold designed for optimized oxysterol release kinetics, all of which lead to better repair of bone defects.

1. Introduction

Bony defects of congenital, traumatic, iatrogenic, or oncologic origins pose extraordinary challenges to reconstructive surgeons. Surgical care for these wounds accounts for hundreds of millions of dollars in healthcare expenditures per year. Traditional approaches to bone reconstruction include autologous bone grafting and the use of alloplastic implants. While it is the gold standard, autologous bone grafting requires an additional harvest procedure, which adds a significant amount of surgical time, risk, and pain. Furthermore, this technique carries the risk of graft resorption. In addition, sources of autologous bone are limited [1]. The use of alloplastic implants bypasses the need for graft harvesting but may result in infection, exposure, or extrusion. To address these concerns, tissue engineering approaches to bone regeneration based on the potent osteoinductive activity of bone morphogenetic proteins (BMPs) have been proposed. While BMP has been shown to have clinical efficacy in bone healing, its potential for widespread use is limited by its unsustainably high cost and its deleterious side effect profile [2,3]. Few alternatives to BMPs have been described for use in bone tissue engineering.

Oxysterols, also known as hydroxycholesterols, comprise a large family of 27-carbon molecules that are oxidized derivatives of cholesterol found naturally in tissues and circulatory systems of humans and other higher animals [4]. They are involved in diverse biological processes, including cholesterol homeostasis, sphingolipid metabolism, platelet aggregation, and apoptosis [5]. In particular, the naturally occurring 22(R)-, 20(S)-, and 22(S)-hydroxycholesterol analogues have been shown to induce osteogenic differentiation in the murine multipotent stem cell line as well as primary murine mesenchymal stem cells (MSCs) [5].

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http://dx.doi.org/10.1016/j.biomaterials.2014.03.018
0142-9612/

Furthermore, semi-synthetic oxysterol, Ox49, induced osteogenic differentiation in primary mesenchymal stem cells [6] and showed successful bone regeneration in a mouse spinal fusion model and rabbit cranial bone defect model when Ox49 was delivered to the defect site via collagen sponge scaffold [7,8].

As a derivative of cholesterol, oxysterols are hydrophobic, and as such when carried on a hydrophilic collagen sponge, they are rapidly released into the surrounding wound milieu. Since bone regeneration occurs over a prolonged period of time (on the order of weeks to months), any growth factor contributing to new bon regeneration should ideally exert its influence over a similar period as well. Therefore, the controlled and sustained release of oxysterol into the bone defect site should continuously stimulate bone growth over time and result in improved regenerative efficiency and capacity. To demonstrate this, we have developed a drug delivery system that will facilitate the controlled release of oxysterol, and in this study we test its efficacy in bone regeneration animal model.

2. Materials and methods

2.1. Materials

Gelatin with an isoelectric point of 9.0 was kindly supplied by Nitta Gelatin Inc. (Osaka, Japan). Disuccimidyl carbonate (DSC), 4-dimethylaminopyridine (DMAP), and Dodecanol (DoOH) were purchased from Nacalai Tesque Inc., Kyoto Japan. 20(S)-hydroxycholesterol [20(S)OHC] was purchase from Sigma–Aldrich (St. Louis, MO, USA). Other chemicals were purchased from Wako Pure Chemical Inductries, Ltd., Osaka, Japan.

2.2. Synthesis of L-lactic acid oligomers

Briefly, an LAo with the number-average molecular weight of 1000 was synthesized from L-lactide monomer by ring-opening polymerization using stannous octate as a catalyst and DoOH as an initiator. L-lactide (20 g, 138.8 mmol) was melted at 130 °C in a nitrogen atmosphere, followed by the addition of toluene (5.6 ml) containing DoOH (1.38 mmol) and stannous octate (0.56 g, 1.38 mmol). After mixing for 4 h at 130 °C, the reaction product was poured into ethanol (EtOH) for precipitation. The precipitate was dissolved in chloroform. The EtOH was evaporated to obtain the white solid of lactic acid oligomer. The molecular weight of LAo prepared was determined by 1H NMR spectroscopy (JNM-EX, JEOL, Ltd., Tokyo, Japan).

2.3. Synthesis of LAo-grafted gelatin

Gelatin (1.0 g: 1.0 × 10−5 mol) was dissolved in anhydrous dimethyl sulfoxide (DMSO, 30 ml) at room temperature. LAo with molecular weights of 950 was dissolved in 15 ml DMSO, and then DSC and DMAP (9.0, 23, and 45 × 10−5 mol) were dissolved in 2.5 ml of DMSO, respectively. The solution was mixed to allow to react for 3 h under stirring at room temperature to activate the hydroxyl groups of LAo. The solution of activated LAo was slowly added to the gelatin solution, while the mixture was stirred overnight at room temperature for LAo grafting to gelatin. The product solution was dialyzed against DDW with a dialysis tube with the cut off molecular weight of 12,000–14,000 for 72 h at room temperature, followed by freeze-drying to obtain the LAo-grafted gelatin (Fig. 1).

2.4. Water-solubilization of 20(S)OHC by LAo-g-gelatin micelles

LAo-grafted gelatin solution (1.0 mg/ml, 900 μl) in DDW and a 20(S)OHC solution (1.0 mg/ml, 100 μl) in EtOH were mixed at 37 °C. The mixed solution was centrifuged (8000 rpm, 10 min, 4 °C) to separate water-insoluble 20(S)OHC, and the supernatant was freeze-dried to obtain the 20(S)OHC water-solubilized by LAo-grafted gelatin micelles [20(S)OHC–micelle]. To measure the amount of 20(S)OHC incorporated into the 20(S)OHC–micelle, the freeze-dried 20(S)OHC–micelle was dissolved in EtOH and sonicated in 1 min. The amount of 20(S)OHC was measured by high performance liquid chromatography (HPLC) while the 20(S)OHC concentration was determined from the standard curve prepared with the EtOH containing various amounts of 20(S)OHC. 20(S)OHC was separated on a TSK-GEL ODS-100V column (25 cm × 4.6 cm i.d., particle size: 5 μm) purchased from Tosoh Corporation (Tokyo, Japan). Ten μl of sample solution were injected into the Prominence LC-20AT HPLC analysis system purchased from Shimadzu Corporation (Kyoto, Japan) after it was dissolved in ethanol. The mobile phase for separation was a mixture of methanol and acetonitrile (60:40, vol/vol %). The solvent was maintained at a flow rate of 1.0 ml/min. UV detection was performed simultaneously at wavelengths of 205 nm.

2.5. Evaluation of 20(S)OHC–micelle bioactivity

An *in vitro* bioassay was performed to evaluate the biological activity of 20(S)OHC–micelle by alkaline phostaphase (ALP) activity. C3H10T1/2 murine embryonic mesenchymal cell line obtained from ATCC (Manassas, VA, USA) were cultured in growth medium consisting of DMEM (Mediatech, Inc., Manassas, VA, USA) with 10% of FBS (Omega Scientific Inc., Tarzana, CA, USA) and 1% of penicillin/streptomycin (Invitrogen Corp., Carlsbad, CA, USA) at 37 °C in a 5% CO_2 medium was changed every 3 days. Cells were seeded into each well of 24-well multi-well culture plate (Corning Inc., Corning, NY) at a density of 2 × 10^4 cells/cm^2. After 24 h culture, the medium was changed to the osteogenic medium (OM) containing 10 mM β-glycerophosphate, and 2 mM L-ascorbic acid 2-phosphate for osteogenic differentiation. The 20(S)OHC–micelle containing 0.4 μg of 20(S)OHC was added into the medium and the 20(S)OHC dissolved in EtOH; the same amount empty micelle was added to the control mixtures. Following 4 days of cell culture under the various experimental conditions described above, colorimetric ALP activity assays on whole cell extracts were

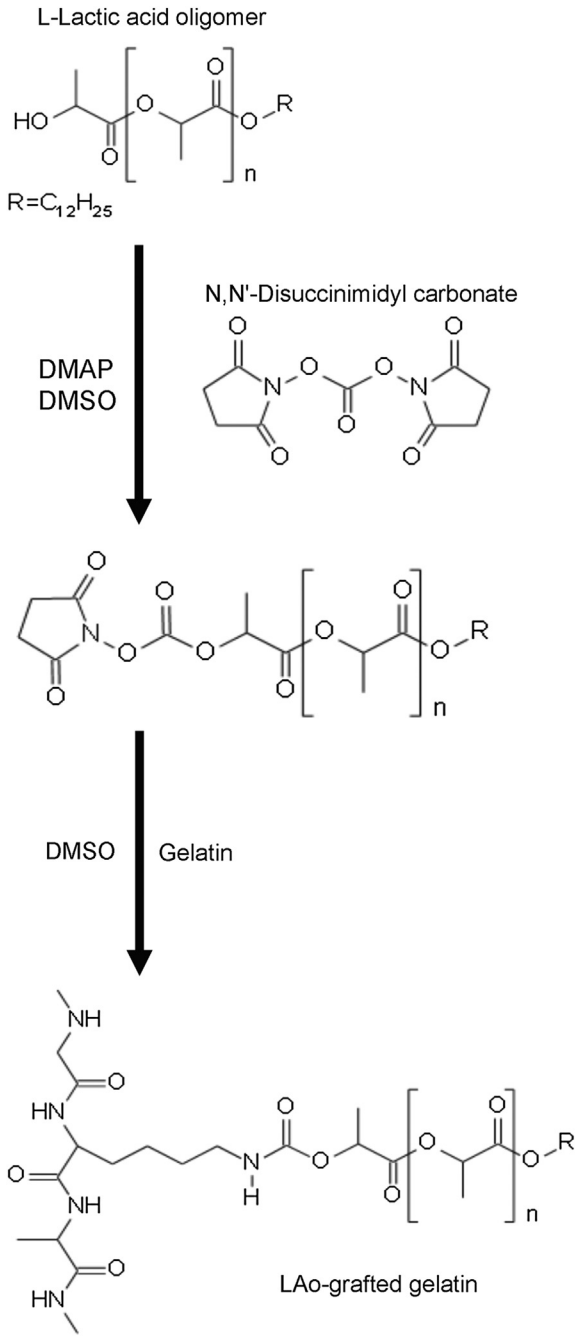

Fig. 1. Schema of LAo-grafted gelatin synthesis. L-lactide acid oligomer was dissolved in DMSO, and then disuccimidyl carbonate (DSC) and 4-dimethylaminopyridine (DMAP) were dissolved in DMSO. These were mixed to activate the hydroxyl groups of LAo. The solution of activated LAo was added to the gelatin dissolved in DMSO to prepare the LAo-grafted gelatin.