Acta Biomaterialia 10 (2014) 5021–5033

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Novel osteoinductive photo-cross-linkable chitosan-lactide-fibrinogen hydrogels enhance bone regeneration in critical size segmental bone defects

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ARTICLE INFO

Article history:
Received 23 April 2014
Received in revised form 20 August 2014
Accepted 22 August 2014
Available online 28 August 2014

Keywords:
Chitosan
Lactide
Fibrinogen
Photo-cross-linkable hydrogel
BMP-2

ABSTRACT

The purpose of this study was to develop and characterize a novel photo-cross-linkable chitosan-lactide-fibrinogen (CLF) hydrogel and evaluate the efficacy of bone morphogenetic protein-2 (BMP-2) containing a CLF hydrogel for osteogenesis in vitro and in vivo. We synthesized the CLF hydrogels and characterized their chemical structure, degradation rate, compressive modulus and in vitro BMP-2 release kinetics. We evaluated bioactivities of the BMP-2 containing CLF hydrogels (0, 50, 100 and 500 ng ml^{-1}) in vitro using W-20-17 preosteoblast mouse bone marrow stromal cells and C2C12 mouse myoblast cells. The effect of BMP-2 containing CLF gels (0, 0.5, 1, 2 and 5 μg) on bone formation was evaluated using rat critical size segmental bone defects for 4 weeks. Fourier transform infrared spectroscopy spectra and scanning electron microscopy images showed chemical and structural changes by the addition of fibrinogen into the chitosan-lactide copolymer. The incorporation of fibrinogen molecules significantly increased the compressive modulus of the hydrogels. The in vitro BMP-2 release study showed initial burst releases from the CLF hydrogels followed by sustained releases, regardless of the concentration of the BMP-2 over 4 weeks. Cells in all groups were viable in the presence of the hydrogels regardless of BMP-2 doses, indicating non-cytotoxicity of hydrogels. Alkaline phosphate activity and mineralization of cells exhibited dose dependence on BMP-2 containing CLF hydrogels. Radiography, microcomputed tomography and histology confirmed that the BMP-2 containing CLF hydrogels prompted neo-osteogenesis and accelerated healing of the defects in a dose-dependent manner. Thus the CLF hydrogel is a promising delivery system of growth factors for bone regeneration.

1. Introduction

Bone morphogenetic protein-2 (BMP-2) is a potent osteoinductive factor and plays an important role in bone regeneration by stimulating endochondral ossification via chemotaxis, migration, proliferation and osteogenic differentiation of mesenchymal stem cells [1–4]. However, BMP-2 administered in the form of a buffer solution has a short biological half-life, rapid clearance, potentially harmful side-effects and is very costly to deliver systematically [2,5,6]. Improved efficacy of BMP-2 induced bone formation can be achieved by using biodegradable delivery systems to overcome the aforementioned limitations [2,7]. BMP-2 in combination with an absorbable collagen sponge (ACS) is a device approved by the US Food and Drug Administration for clinical applications, including lumbar spine fusion, open tibial fractures and sinus augmentation [4,7–9]. However, the concerns about side-effects associated with a rapid burst release and the risk of immunogenic responses have necessitated new delivery strategies that enable a controlled and sustained release of BMP-2 at the physiologically relevant dose to enhance therapeutic outcome [10–14]. The ideal delivery system should maintain the structural integrity of the defects, spatially and temporally present bioactivity to guide surrounding cells and gradually degrade corresponding to the rate of tissue regeneration [15,16].

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http://dx.doi.org/10.1016/j.actbio.2014.08.028
1742-7061/

In this regard, hydrogels have been broadly studied for the delivery of growth factors to facilitate tissue integration and regeneration, as well as to restore the function of a damaged tissue [13–16]. Recent studies have demonstrated that the introduction of growth factor binding ligands to polymer-based delivery systems can maintain bioactivities of the growth factors over extended periods of time and allow cell-mediated proteolysis to transiently release the bound growth factors [12,16,17]. The growth factor binding ligands enhance the strength of interactions between growth factors and the materials, leading to improved tissue regeneration [15–17]. The choice of growth factor binding protein depends on its affinity to specific growth factor in the specific target of interest. For example, BMP-2 binds to the components of the extracellular matrix (ECM) by interaction with heparan-sulfate proteoglycans [12,18]. The basic N-terminal domains of BMP-2, including arginine, lysine and histidines, are key components of the heparin-binding sites [18–20]. Therefore, the heparin-binding affinity can be used to enhance controlled release of BMP-2 in response to cell-mediated enzymatic activity during bone healing [17–23].

Herein, we present a photo-cross-linkable chitosan-lactide-fibrinogen (CLF) hydrogel as a carrier for delivery of BMP-2 and evaluated the delivery efficacy of BMP-2 containing CLF hydrogels on osteogenesis in vitro and in vivo. This CLF hydrogel was developed based on our previous work on a chitosan-lactide (CL) hydrogel by the addition of fibrinogen [24]. Fibrinogen contains a heparin-binding domain, which was used to improve BMP-2 binding affinity [21,25]. Fibrinogen has been extensively studied for human use in a number of clinical applications, such as a tissue sealant, growth factor delivery, and a tissue engineered scaffold [21,22,26]. Fibrinogen has proteolytically degradable sites and adhesive ligands for cell surface integrins [10,17,26]. Our CLF hydrogel was designated to contain tunable mechanical properties, hydrolytically degradable amide and ester linkages and excellent protein binding affinities. The cross-linked hydrogel networks are formed by a radical polymerization upon application of ultraviolet (UV) light. The properties of the CLF hydrogels, such as swellability, stiffness and degradability, can be readily adjusted by changing ratios of chitosan to lactide and cross-linking density via UV exposure time. In this regard, we have optimized the CLF hydrogels for sustained delivery of BMP-2 over several weeks to promote new bone formation.

In this study, we first synthesized the photo-cross-linkable CLF hydrogels and characterized their chemical structure, compressive modulus, degradation rate and in vitro BMP-2 release kinetics. Then, we examined bioactivities of the CLF hydrogel mediated BMP-2 using W-20-17 preosteoblast mouse bone marrow stromal cells and C2C12 mouse myoblast cells in vitro. The delivery efficacy of BMP-2 was evaluated by measuring cytotoxicity, alkaline phosphatase (ALP) activity and mineralization according to different doses (0, 50, 100 or 500 ng ml^{-1}). In addition, we investigated the new bone formation in a critically sized rat femoral defect by implanting the CLF hydrogels loaded with BMP-2 (0, 0.5, 1, 2 and 5 μg) for 4 weeks. This study has demonstrated that the photo-cross-linkable CLF hydrogel can effectively deliver BMP-2 to regulate cell response and enhance osteogenesis, both in vitro and in vivo.

2. Materials and methods

2.1. Materials

Chitosan (⩾310 kDa, 75% or greater degree of deacetylation) and methacrylic anhydride were purchased from Sigma-Aldrich (St Louis, MO). D,L-Lactide was purchased from Ortec (Piedmont, SC). Human fibrinogen was obtained from Enzyme Research Labs (South Bend, IN). Human bone morphogenetic protein-2 (BMP-2) was obtained from Medtronic (Minneapolis, MN). All other chemicals were reagent grade and were used as received. A UV light source (Omnicure S2000) was purchased from Lumen Dynamics Group Inc (Ontario, Canada).

2.2. Synthesis of CLF hydrogels

A 1% (w/v) chitosan solution was prepared by stirring powdered chitosan in 0.75% (v/v) aqueous acetic acid at room temperature overnight. The insoluble particles in the chitosan solution were removed by filtration. An aqueous solution of lactic acid was prepared by dissolving powdered D,L-Lactide in DMSO (DMSO) at 80 °C. The mass ratio of chitosan to lactide was 8:1. The mixture of chitosan and lactide was stirred using a magnet stirrer for 1 h at 80 °C. Tin (II) 2-ethylhexanoate and triethylamine (TEA) were added dropwise. The mixture was reacted at 80 °C with magnetic stirring for 20 h in a nitrogen atmosphere. The mixture was dialyzed in distilled water using dialysis tubing (molecular weight cut off (MWCO): 14,000) for 1 day. 2.5% (w/v) methacrylic anhydride was added into the dialyzed mixture dropwise, and the reaction was continued for 8 h at 60 °C. The mixture was dialyzed in distilled water using dialysis tubing (MWCO: 14,000) for 7 days. The obtained solution was then freeze-dried for 2–3 days and stored at −20 °C until use. For the CLF hydrogel formulation, the freeze-dried samples were reconstituted as a 2.5% (w/v) in distilled water. The prepolymer solution was mixed with fibrinogen (3.6 mg ml^{-1}) at 4 °C overnight. The photoinitiator (Irgacure 2959, CIBA Chemicals) was dissolved completely into distilled water at 70 °C. The photoinitiator solution was sterile-filtered through a 0.22 μm filter and then added to the prepolymer solutions to make a final concentration of 0.5% (w/v). The prepolymer solutions were then exposed to 6.9 mW cm^{-2} UV light to allow for free radical polymerization by photo-cross-linking.

2.3. Characterization of CLF hydrogels

2.3.1. Fourier transform infrared spectroscopy (FTIR) spectra

In order to investigate chemical structure of prepolymer solutions, including CL and CLF, FTIR spectra were obtained using a Bruker Vertex 70 FTIR spectrometer coupled to a PC with analysis software. Samples were placed in the holder directly in the IR laser beam. All spectra were recorded by transmittance mode (40 times scanning, 800–4000 cm^{-1}).

2.3.2. Scanning electron microscopy (SEM)

The internal microstructures of the CL and CLF hydrogels were investigated by SEM. The effect of fibrinogen on the morphological change of the CL hydrogels was observed. The hydrogel samples were incubated into phosphate buffered saline (PBS; pH 7.4) at 37 °C for 1 day and lyophilized overnight (Freezone, LABCONCO). The samples were sputter-coated with gold and examined under a scanning electron microscope (Hitachi S-3400N VP SEM) operated at 10 kV.

2.3.3. Mechanical testing

Unconfined compression tests were performed to determine the mechanical properties of the CL and CLF hydrogels using an Instron 5944 materials testing system (Instron Corporation, Norwood, MA) fitted with a 10 N load cell (Interface Inc., Scottsdale, Az). The prepolymer solution was pipetted into a cylindrical Teflon mold and exposed to 6.9 mW cm^{-2} UV light for 200 s. The diameter (∼6 mm) and thickness (∼3 mm) of the samples were measured using digital calipers and the material testing system's position read-out, respectively. Before each test, a preload of ∼2 mN was