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Synthetic design of growth factor sequestering extracellular matrix mimetic hydrogel for promoting in vivo bone formation

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ABSTRACT

Synthetic scaffolds that possess an intrinsic capability to protect and sequester sensitive growth factors is a primary requisite for developing successful tissue engineering strategies. Growth factors such as recombinant human bone morphogenetic protein-2 (rhBMP-2) is highly susceptible to premature degradation and to provide a meaningful clinical outcome require high doses that can cause serious side effects. We discovered a unique strategy to stabilize and sequester rhBMP-2 by enhancing its molecular interactions with hyaluronic acid (HA), an extracellular matrix (ECM) component. We found that by tuning the initial protonation state of carboxylic acid residues of HA in a covalently crosslinked hydrogel modulate BMP-2 release at physiological pH by minimizing the electrostatic repulsion and maximizing the Van der Waals interactions. At neutral pH, BMP-2 release is primarily governed by Fickian diffusion, whereas at acidic pH both diffusion and electrostatic interactions between HA and BMP-2 become important as confirmed by molecular dynamics simulations. Our results were also validated in an in vivo rat ectopic model with rhBMP-2 loaded hydrogels, which demonstrated superior bone formation with acidic hydrogel as compared to the neutral counterpart. We believe this study provides new insight on growth factor stabilization and highlights the therapeutic potential of engineered matrices for rhBMP-2 delivery and may help to curtail the adverse side effects associated with the high dose of the growth factor.

1. Introduction

Strategies involving delivery of growth factors using scaffolds are widely explored for regenerative medicine applications [1]. They are particularly important for treating large bone defects (critical size) that fail to heal by themselves. Large bone defects are commonly treated using autologous bone grafting where healthy bone tissue from patient is harvested and implanted at the defect site. However, lack of transplantable bone, donor site morbidity and lack of good quality bone tissue in elderly patients results in sub-optimal clinical outcome [2]. Thus, regenerative medicine-based approaches are developed in order to accelerate bone healing. To differentiate bone-forming mesenchymal stem (progenitor) cells towards osteoblasts, specific growth factors are needed, where the most potent are the morphogenetic proteins (BMPs) namely BMP-2 and BMP-7 [3]. Recombinant human (rh) version of both these

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proteins are approved by FDA (US Food and Drug Administration) as well as EMA (European Medicines Agency), to be used in treatments of spinal fusion [4] however, due to severe risks of side-effects, rhBMP-7 also called OP-1 (Osteogenic Protein-1) is withdrawn from the market whereas rhBMP-2 (InductOs®) is currently having limited sale in Europe. Recently, an extensive debate has taken place on the clinical use of rhBMP-2 since several complications are observed in patients, which include acute respiratory, neurological and inflammatory complications [5]. This led the FDA to issue a public health notification of life-threatening complications [6]. The primary cause of such severe complications is due to the supraphysiological dose of rhBMP-2 (1.5 mg/mL) that is used clinically with a collagen sponge-based carrier. Such high doses are needed because collagen-based scaffolds are not effective in stabilizing rhBMP-2 in vivo [7]. It is interesting to note that at high doses, BMP-2 induces adipogenic differentiation of MSCs by activating *PPAR*-γ (peroxisome proliferator-activated receptors) pathway [8] and at the same time activates bone resorbing osteoclasts [9], resulting in poor quality bone.

BMP-2 is an unstable protein that undergoes rapid clearance under physiological conditions with $t_{1/2} = 6.7$ min in non-human primates after IV administration [10]. We have recently confirmed that rhBMP-2 undergoes aggregation at physiological pH and adheres to hydrophobic surfaces such as cell culture plastics and Eppendorf tubes [11]. Recently, this association of BMP-2 to hydrophobic surfaces has been utilized to develop nanocarriers for efficient delivery of the protein in vivo [12]. In living systems, soluble forms of growth factors are stabilized by the biopolymers present in the ECM (extracellular matrix) that regulate dynamic stem cell niche [13]. Thus several researchers, including our group, have attempted to develop ECM mimetic scaffolds to deliver functional BMP-2 in vivo [14,15]. Several strategies have been employed to increase the bioavailability and stability of this protein. These strategies include engineering recombinant protein with a specific ECM binding motif to control sequestration [16], immobilizing protein by covalent conjugation [17], electrostatic binding to modified surfaces [18], and incorporating specific BMP-2 binding peptides or glycosaminoglycans (GAGs) such as heparan sulfate into the scaffold [19]. Although these strategies are promising, they are cumbersome entailing complex bioengineering steps that often result in the loss of function due to covalent/electrostatic immobilization. It is noteworthy that rhBMP-2 is stable and remains biologically active when constituted at acidic pH (~4.5) resulting in efficient ectopic bone formation [20]. Scaffolds also play a crucial role in dictating the bioactivity of this growth factor. The BMP-2 complexed to different scaffolds may result in either stimulatory or inhibitory effects in bone formation. The stimulatory effect is attributed to the increase in BMP-2 half-life by minimizing interaction with BMP antagonist noggin [19], whereas the inhibitory effect is due to inefficient binding of BMP-2 to its cognate receptor [21] or inefficient release of the protein in vivo [22]. Interestingly, incorporation of BMP-2 in an 'acidic' form of gelatin based scaffold did not show much affinity for BMP-2, though it promoted binding with other proteins such as bFGF and TGF-β1 [23]. This is presumably due to the fact that neutralization of the scaffold results in desorption of the protein from the matrix similar to what is observed with collagen-based scaffolds. BMP-2 complexing biomolecules such as HA, chondroitin sulfate and heparin also have a unique biological function related to the coagulation and complement system of the blood that also affect the biological outcome [24]. These results prompted us to design a BMP-2 sequestering 3D scaffold without using any special complexing agents that could enhance the stability and bioavailability of BMP-2.

In this article, we describe a novel strategy to engineer HA hydrogels having different initial carboxylic acid protonation states that regulate the release kinetics of bioactive BMP-2 over 28 days at physiological pH. In order to gain insight on the influence of backbone protonation of HA to bind BMP-2 within the HA-gels, we performed a multiscale modeling approach combining fundamental mass balances with simulations at the molecular level. The results of the computational analysis as well as the in vitro release experiments suggest that the incorporation of BMP-2 in an acidic gel controlled its release over 28 days as compared to that at neutral pH. This is mainly attributed to the fact that upon neutralization of HA, the polymer rearranges to maximize the Van der Waals interactions between the HA chains and the protein, resulting is reduced ionic repulsion. Such a controlled release system demonstrated a significant improvement in ectopic bone formation in a rat subcutaneous model with nearly twice the bone volume in acidic gel as compared to the neutral gel. In summary, we present a new insight in material design that is simple and translatable, which could pave the way to develop safe and cost-effective strategies for treating large bone defects and non-unions and overcome the current limitations in the clinical use of rhBMP-2.

2. Results

2.1. Hydrogel preparation and characterization

To prepare HA hydrogels having different pH, we fabricated two HA hydrogels having different pH, by modifying the synthetic procedure from our previously optimized hydrazone crosslinked hydrogel that was obtained by mixing carbohydrazide modified HA derivative with aldehyde modified HA derivative [15]. The hydrazone crosslink exhibited excellent stability at physiological pH, which is attributed to the extensive delocalization of charges over the urea-type linkage [15]. To prepare hydrogel components having acidic pH, we synthesized the HA derivatives as previously reported and performed dialysis under acidic pH (~3). However, to develop neutral gels, we neutralized the purified hydrogel components to pH 7.4 by using 0.1 M NaOH and subsequently dialysed them against water (see experimental section for details). Both HA hydrogels were prepared by mixing 1:1 ratio (*v:v*) of the HA-aldehyde and HA-hydrazide components in phosphate buffer saline (PBS, pH 7.4). The final pH of the hydrogel was determined by measuring the pH of the gel components before mixing and was found to be ~4.5 for acidic hydrogel and ~7 for neutral gel (Fig. 1A). These HA hydrogels were subjected to rheological assessment before and after swelling in phosphate buffer. Time-sweep rheological evaluation was performed to determine the gel point of the two-hydrogel systems, *i.e.*, pH 4.5 (acidic) and pH 7 (neutral) HA-hydrogels. These experiments indicated that the acidic hydrogel had a shorter gelation time (90 s) whereas the neutral hydrogel took 13 min to reach the gel point (Fig. 1B and C). Gel characteristics after complete crosslinking (i.e., after 24 h of curing) reveals that the neutral hydrogel was more rigid (~300 Pa after swelling) than the acidic hydrogel (Fig. 1D and E and Table 1). This was also reflected in the calculated average molecular weight between crosslinks (Mc) and the pore size (ξ), which shows that neutral hydrogel had lower Mc and smaller ξ (Equations (8) and (9) respectively) [15]. However, the acidic and neutral gels had similar swelling and hyaluronidase (HAse) mediated enzymatic degradation rate (Table 1).

2.2. In vitro release of bioactive rhBMP-2 from acidic and neutral HA hydrogels

Next, we evaluated the release of functional BMP-2 from our HA-gels. Even though it is generally accepted that association of