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Collagen I derived recombinant protein microspheres as novel delivery vehicles for bone morphogenetic protein-2

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ABSTRACT

Bone morphogenetic protein-2 (BMP-2) is a powerful osteoinductive protein; however, there is a need for the development of a safe and efficient BMP-2 release system for bone regeneration therapies. Recombinant extracellular matrix proteins are promising next generation biomaterials since the proteins are well-defined, reproducible and can be tailored for specific applications. In this study, we have developed a novel and versatile BMP-2 delivery system using microspheres from a recombinant protein based on human collagen I (RCP). In general, a two-phase release pattern was observed while the majority of BMP-2 was retained in the microspheres for at least two weeks. Among different parameters studied, the crosslinking and the size of the RCP microspheres changed the *in vitro* BMP-2 release kinetics significantly. Increasing the chemical crosslinking (hexamethylene diisocyanide) degree decreased the amount of initial burst release (24 h) from 23% to 17%. Crosslinking by dehydrothermal treatment further decreased the burst release to 11%. Interestingly, the 50 and 72 μm-sized spheres showed a significant decrease in the burst release compared to 207-μm sized spheres. Very importantly, using a reporter cell line, the released BMP-2 was shown to be bioactive. SPR data showed that N-terminal sequence of BMP-2 was important for the binding and retention of BMP-2 and suggested the presence of a specific binding epitope on RCP (K_D: 1.2 nM). This study demonstrated that the presented RCP microspheres are promising versatile BMP-2 delivery vehicles.

1. Introduction

Bone has a high self-regeneration capacity and most of the fractures heal without any scar formation [1]. However, in critical-size bone defects, a surgical procedure using bone grafts is often required [2,3]. To replace the use of natural bone graft in clinics, there has been a tremendous effort to develop biomaterial based synthetic bone substitutes [4]. The biomaterials have been used in combination with growth factors to create an osteoinductive environment and to induce substantial amount of bone formation. These osteoinductive growth factors are known to recruit osteoprogenitor cells and guide their differentiation during the regeneration of the bone [5]. Bone morphogenetic protein-2 (BMP-2) and bone morphogenetic protein-7 (BMP-7) are growth factors that are already clinically used in bone regeneration [6]. BMP-2 adsorbed onto a collagen sponge matrix has been approved by the FDA for spinal applications [7]. However, off-label use [8] of BMP-2 especially in the cervical spine fusion resulted in adverse events such as hematoma, swallowing/breathing difficulties or swelling without hematoma [9]. Therefore, to obtain better clinical outcomes utilizing BMP-2, it is crucial to reduce the applied dose and/or to regulate its spatio-temporal delivery using appropriate delivery systems [10].

Several materials, especially natural biopolymers, have been investigated as controlled release systems for BMP-2 [11–14]. Natural

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biopolymers are often chosen as a delivery system in tissue engineering not only due to their biocompatibility and biodegradability, but also due to their intrinsic features mimicking the extracellular matrix. As such, collagen was widely studied since it is a major component of the organic bone matrix [15]. However, in case of collagen sponge, one of the first natural scaffolds used for BMP-2 delivery, an early burst release of BMP-2 is typically observed [16]. More sophisticated BMP-2 release systems based on collagen's degradation product, gelatin have been developed [17]. For example, genipin-crosslinked gelatin microspheres have been shown to provide a slower release compared to poly(lactic-*co*-glycolic acid) (PLGA) microspheres [18]. However, animal-derived collagen or gelatin-based materials bear the risk of antigenic response, and batch-to-batch variability which complicates their clinical translation [19].

We have previously developed a human collagen type I-derived recombinant protein (RCP) as designer biomaterial, which is produced by a fermentation process using genetically modified yeast *Pichia pastoris* [20]. The major advantage of RCP over animal-derived proteins is that genetically engineered RCP offers a versatile and powerful platform to create functional (collagen-based) proteins with a low immunogenic response. The specific RCP variant used in this study is enriched with several RGD units. RGD enriched material was selected in this study because it improved cell attachment [21]. Furthermore, RCP is advantageous over animal-derived collagen/gelatin not only for enhanced cell binding, but also for its well-defined protein sequence, the reproducibility of the production process, and low immunogenicity [22]. Previous *in vivo* studies have shown that RCP-based microspheres do not elicit a strong immune response or foreign body reaction when injected subcutaneously [23].

In this study, we have developed novel RCP microspheres for a defined release of recombinant BMP-2. The aim of this research was to identify the parameters that control BMP-2 release kinetics. Detailed understanding of different parameters affecting these release kinetics is required for the final design of a controlled release system. Based on literature, we selected crosslinking [17], particle size [24] and pore size [25] as potential parameters to modulate release. Hence, we used different crosslinking techniques and different preparation methods to create a small library of microspheres. In brief, the effect of crosslinking was studied by using high and low amounts of chemical crosslinker (hexamethylene diisocyanide); and long and short dehydrothermal treatment. The effect of size was studied creating three different size of spheres: 50 and 72 and 207 μm; and pore size studied by creating pore sizes of 1 μm, 10 μm and macropores (> 10 μm). These microspheres were subsequently loaded with BMP-2 by adsorption and their release profiles were investigated by ELISA. Furthermore, the bioactivity of the released protein was confirmed by cellular assays using a reporter C2C12 cell line. To gain better understanding of the interaction between BMP-2 and RCP on a molecular level a surface plasmon resonance (SPR) study was performed using multiple BMP-2 variants.

2. Materials and methods

2.1. Materials

Recombinant protein (RCP) based on human collagen I, commercially available from Fujifilm as Cellnest™, was produced by a fermentation process using genetically modified yeast *Pichia pastoris* as described previously [20,23]. RCP comprises 571 amino acids, has an isoelectric point (pI) of 10.02 and a molecular weight of 51.2 kDa. The mature part of rhBMP-2 (amino acids 283 to 396 plus an N-terminal Met-Ala) was expressed in *E. coli*, isolated from inclusion bodies, renatured and purified as previously described [26]. In the variant EHBMP-2 the N-terminal segment of the mature part of BMP-2, which contains two triplets of basic amino acid residues (QA KHK Q RKR …), was replaced by the sequence (MAPTSSSTKKTQL) [27]. In T4BMP-2 the N-terminal sequence motif harboring two basic triplets (shown above) was doubled (QA KHK Q RKR A KHK Q RKR…) [28]. Both protein variants were expressed and purified identical to wildtype BMP-2 [26]. For use in SPR, the extracellular domain of the BMP type I receptor BMPR-IA ($BMPRIA_{ec}$) was expressed in *E. coli* and purified as described [26].

Hexamethylene diisocyanide (HMDIC), corn oil, calcium carbonate ($CaCO_3$), picrylsulfonic acid (TNBS, 5% w/v solution in H_2O), bovine serum albumin (BSA) and collagenase from *Clostridium histolyticum* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, acetone and hydrochloric acid were purchased from Millipore (Billerica, MA, USA). ELISA development kit and reagents were ordered from Peprotech (Rocky Hill, NJ, USA). Gibco products: Dulbecco's Modified Eagle's Medium (DMEM) and penicillin-streptomycin were ordered from Thermofisher Scientific (Waltham, MA, USA). SteadyLite Plus was ordered from Perkin Elmer (Waltham, MA, USA). MilliQ water was used in the experiments.

2.2. Preparation of RCP microspheres

RCP microspheres were produced by emulsification using calcium carbonate ($CaCO_3$) crystals as pore-forming agent. Briefly, a 20% (w/v) aqueous RCP solution was mixed with $CaCO_3$ powder (fine powder with particle size of < 1 μm or 10 μm) in a 1:1 weight ratio of RCP to $CaCO_3$. The suspension was added dropwise to preheated corn oil at 50 °C while stirring the emulsion at 800 rpm for 20 min. Then, the emulsion was cooled using an ice bath and the emulsified microspheres were washed three times with acetone. After overnight drying at 60 °C, microspheres were sieved to the desired size (Sieves Retsch GmbH, Haan, Germany). In the experiments 32–50 μm, 50–72 μm or 200–300 μm sieve fractions of microspheres were used. Subsequently, the microspheres were crosslinked. DHT (dehydrothermal) crosslinking was conducted at 160 °C in vacuum ($\sim 5 \times 10^{-3}$ mbar) for 1 day or 4 days. Hexamethylene diisocyanide (HMDIC) crosslinking was conducted by suspending 1 g of spheres, and 1 mL of HMDIC (high) or 30 μL of HMDIC (low) in 100 mL ethanol for 1 day. (High HMDIC corresponds to an excess amount of chemical crosslinker, while low HMDIC corresponds to less than the amount required to crosslink all amino groups present in the RCP). After crosslinking, the $CaCO_3$ porogen was removed by suspending the microspheres in 0.23 M HCl for 30 min until the formation of carbon dioxide stopped. The microspheres were washed repeatedly with water until a neutral pH was achieved. Complete removal of $CaCO_3$ was confirmed by Energy-dispersive X-ray spectroscopy (EDX) mapping of calcium on spheres (Jeol JSM-6335F Field Emission Scanning Electron Microscope). In order to produce 200–300 μm microspheres, the viscosity of corn oil was decreased during emulsification by adding n-heptane. Macroporous microspheres with pores larger than 10 μm were produced by a double emulsification method as described elsewhere [29]. No $CaCO_3$ porogen was used in the preparation of these macroporous microspheres.

2.3. Characterization of microspheres

The microsphere size was measured by a particle size analyzer (Mastersizer 2000, Malvern instruments, Malvern, United Kingdom). Dry microspheres were resuspended and measured in ethanol. The swollen sphere size was determined after overnight incubation in pure water. All measurements were performed in triplicate. The average microsphere size was calculated based on volume weighted mean. The size distribution of spheres is indicated as 10% (D0.1), which means 10% of spheres are below this size; and 90% (D0.9), which means 90% of spheres are below this size. The swelling ratio for each type of microsphere was calculated using the ratio of swollen diameter in water (dt) to dry diameter (do) and subsequently converted to volume $(dt/do)^3$.

Carboxyl and amine groups of RCP are crosslinked by DHT; whereas, only amines are crosslinked by HMDIC. Therefore, the degree