

Biomimetic modification of the TiO₂/glass composite Ecopore with heparinized collagen and the osteoinductive factor BMP-2

M. von Walter^{a,*}, C. Herren^a, T.J. Gensior^a, G.C.M. Steffens^b,
B. Hermanns-Sachweh^c, W. Jahnen-Dechent^d, M. Ruger^a, H.J. Erli^a

^a Clinic of Trauma Surgery, RWTH Aachen University, Germany

^b Institute of Biochemistry, RWTH Aachen University, Germany

^c Institute of Pathology, RWTH Aachen University, Germany

^d Department of Biomedical Engineering, Biointerface Laboratory, RWTH Aachen University, Germany

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Abstract

The porous TiO₂/glass composite Ecopore has potential applications in hard tissue replacement. We describe the modification of Ecopore with the growth factor bone morphogenetic protein-2 (BMP-2) to add osteoinductive properties. Ecopore covalently coated with BMP-2 caused a weak induction of alkaline phosphatase in murine embryonal fibroblasts. In a rabbit bone defect model, BMP-2-coated Ecopore had moderately higher bone apposition rates and ingrown bone quantities at 6 weeks after implantation. To overcome loss of function due to chemical surface coupling, we filled the pore system of Ecopore with heparinized collagen sponge and loaded this secondary matrix with BMP-2. Heparinization of collagen filling increased the BMP-2 loading capacity of the matrix approximately 1.28-fold. Within 96 h, 17.0 ± 0.1 and $10.1 \pm 0.2\%$ of the used BMP-2 was released from non-modified and heparinized Ecopore/collagen, respectively, indicating that the heparin modification retarded BMP-2 release. Revealed by energy-dispersive X-ray spectroscopy analysis of implant cross-sectional areas, BMP-2-loaded Ecopore/collagen had significantly higher bony ingrowth quantities in rabbits, with the heparinized modification yielding the highest value ($16.09 \pm 3.51\%$, $p < 0.005$) compared with the non-heparinized matrix ($10.72 \pm 4.07\%$, $p < 0.05$) and the BMP-2-free controls ($5.60 \pm 1.47\%$). This suggested a beneficial effect of the biomimetic modification of Ecopore with heparinized collagen for bone healing and integration.

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1. Introduction

The TiO₂/glass composite Ecopore is a porous, non-toxic, load-bearing and easy-to-manufacture biomaterial [1]. In our previous work, we studied material properties and surface modification of Ecopore in view of a possible application as a mechanically stable and modifiable carrier in hard tissue replacement. Modification of Ecopore with the cell adhesion mediator fibronectin led to enhanced spreading and growth of human osteoblasts [2]. In a rabbit

bone defect model, an enhanced osteoconductivity was observed, resulting in improved ingrowth of bone tissue in fibronectin-coated Ecopore [3]. In the present study, we employed Ecopore modifications with the osteoinductive factor bone morphogenetic protein-2 (BMP-2) in order to find out whether bone ingrowth could be further enhanced by this modification.

BMP-2 is a morphogen and growth factor with osteoinductive properties, amongst other inductive effects in embryology [4,5]. Since its discovery, BMP-2 has been thoroughly studied to enhance the integration of bone replacement materials and thus to accelerate bone healing. Different strategies have been applied to combine bulk materials with BMP-2. In several studies, BMP-2 was

* Corresponding author. Tel.: +49 241 8085581; fax: +49 241 8082415.
E-mail address: UC-labor@ukaachen.de (M. von Walter).

incorporated into degradable carriers, e.g., calcium phosphate [6,7], while in another approach the protein was covalently coupled onto the surface of passivated metal implants [8]. In analogy to the latter approach, we first employed covalent surface coating of Ecopore with BMP-2 to find out whether this simple configuration could be biologically effective. Here, we show an overall limited efficiency of this modification. The next part of our study thus comprises a secondary matrix of heparin-modified collagen to enable reversible storing of BMP-2 in the pore system of Ecopore and to take advantage of the natural function of BMP-2 as soluble factor.

In release matrix design, collagen is often employed due to its biological compatibility and resorbability [9,10]. Collagen sponges biochemically resemble the collagenous part of the extracellular matrix (ECM) and thus can act as an initial support for new ECM generation. The use of BMP-2-loaded collagen has been described in bone regeneration studies [11]. As an example of a commercial product, the combination of an interbody fusion cage and a BMP-2-loaded collagen sponge has been developed and approved (InFuse[®], Medtronic Sofamor Danek).

The natural activity and distribution of several growth factors is modulated by glycosaminoglycans (GAGs) as part of the ECM [12,13]. Against this background, artificial collagen matrices can be chemically supplemented with GAGs to modify binding properties of GAG-binding cytokines. Covalent coupling of heparin to a collagen matrix has been employed to modify binding and release of the angiogenic factor VEGF [10,14,15]. In this case, heparinization led to a noticeable retardation of VEGF release. Like VEGF, BMP-2 contains a binding site for heparin [16]. Thus we reasoned that heparinized collagen matrix should bind BMP-2 better than collagen alone, resulting in a sustained release of the factor. We studied BMP-2 binding and release of non-heparinized and heparinized Ecopore/collagen, as well as the biological activity of released BMP-2. We show that BMP-2-loaded implants outperformed non-loaded material in a bone defect healing model.

2. Materials and methods

2.1. Production of Ecopore

Ecopore was produced as described previously [2]. Cylindrical specimens with a height of 10.0 mm and diameters of 10.0 and 5.6 mm were manufactured for the cell culture and animal experiments, respectively. The specimens were cleaned by sonication in acetone for 10 min and air dried.

2.2. Surface modification of Ecopore

Etching, aminosilanization of Ecopore and the choice of linker were performed as described previously [2]. Briefly, recombinant human BMP-2 (Osteogenetics GmbH, Würzburg, Germany) was coupled onto amino-functionalized

Ecopore using a homobifunctional linker with a polyethylene glycol (PEG) spacer (SPA-PEG-SPA; Shearwater Polymers Inc., Huntsville, USA). Bound BMP-2 was detected using a commercial enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, USA). To test the biological activity of bound BMP-2, we employed murine embryonal fibroblasts (MC3T3) cultured on sterile BMP-2-coated Ecopore specimens in α -minimum essential medium (α -MEM) with 10% fetal calf serum (FCS; Bio-Whittaker). After 3 days of incubation, the cells were scored for BMP-2-induced alkaline phosphatase (AP). To this end, the cells were first assayed with an XTT kit (Roche Diagnostics GmbH, Mannheim, Germany) for estimation of cell numbers. They were then rinsed with Ringer solution twice and incubated in a solution of 18 mM *para*-nitrophenyl phosphate in 200 mM NaHCO₃ buffer, pH 10.3, and 20 mM MgCl₂ for 1 h. Then 2 × 100 μ l of each supernatant was transferred into a 96-well plate and measured at 402/498 nm in an ELISA reader. Finally, the AP values were normalized by dividing them by the corresponding values gained from the XTT assay.

2.3. Biomimetic modification of Ecopore with heparinized collagen and BMP-2

The cylinders were incubated in a solution of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h prior to further modification in order to block unspecific surface binding. After thorough rinsing in water, the specimens were blotted dry on paper tissues and used immediately for the further modification.

A suspension of collagen (insoluble, from bovine achilles tendon, Sigma/Aldrich, Taufkirchen, Germany) in 10% acetic acid (Sigma/Aldrich) was prepared using a vortex blender. Ecopore specimens were submerged in the collagen suspension and evacuated to remove air bubbles. The filled specimens were frozen at -80°C and subsequently freeze-dried overnight. Loosely adhering collagen was removed. The amount of collagen filling was quantified by total hydrolysis in HCl (110°C , 1 h) and subsequent amino acid analysis.

A solution of 4 mg ml⁻¹ heparin, 20 mg ml⁻¹ 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 20 mg ml⁻¹ *N*-hydroxysuccinimide in 50 mM 2-(*N*-morpholino)-ethanesulfonic acid buffer, pH 5.8, was prepared for the covalent coupling of heparin to the collagen matrix (all Sigma/Aldrich). After 10 min of activation time, the Ecopore/collagen specimens were submerged in the solution, evacuated and incubated for 4 h at room temperature. Subsequently, the specimens were repeatedly rinsed with water, frozen at -80°C and freeze-dried overnight. Modified specimens were sterilized in 70% ethanol for 1 h and subsequently air dried in a flow bench.

BMP-2 was loaded into the heparin–collagen-filled Ecopore specimens by soaking with 300 μ l of BMP-2 solutions in PBS + 0.1% BSA per specimen. To estimate release of BMP-2, the specimens were placed into inert plastic vessels

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