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Bioactive membranes for bone regeneration applications: Effect of physical and biomolecular signals on mesenchymal stem cell behavior

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ABSTRACT

This study focuses on the in vitro characterization of bioactive elastin-like recombinamer (ELR) membranes for bone regeneration applications. Four bioactive ELRs exhibiting epitopes designed to promote mesenchymal stem cell adhesion (RGDS), endothelial cell adhesion (REDV), mineralization (HAP), and both cell adhesion and mineralization (HAP-RGDS) were synthesized using standard recombinant protein techniques. The materials were then used to fabricate ELR membranes incorporating a variety of topographical micropatterns including channels, holes and posts. Primary rat mesenchymal stem cells (rMSCs) were cultured on the different membranes and the effects of biomolecular and physical signals on cell adhesion, morphology, proliferation, and differentiation were evaluated. All results were analyzed using a custom-made MATLAB program for high throughput image analysis. Effects on cell morphology were mostly dependent on surface topography, while cell proliferation and cell differentiation were largely dependent on the biomolecular signaling from the ELR membranes. In particular, osteogenic differentiation (evaluated by staining for the osteoblastic marker osterix) was significantly enhanced on cells cultured on HAP membranes. Remarkably, cells growing on membranes containing the HAP sequence in non-osteogenic differentiation media exhibited significant up-regulation of the osteogenic marker as early as day 5, while those growing on fibronectin-coated glass in osteogenic differentiation media did not. These results are part of our ongoing effort to develop an optimized molecularly designed periosteal graft.

1. Introduction

Alternative therapies that improve bone regeneration, such as more effective bone grafts, continue to be a major necessity in the medical community [1]. The periosteum plays a critical role during bone fracture healing [2], and its incorporation as an active component of the surgical procedure has been demonstrated to significantly enhance bone regeneration [3]. Therefore, engineering periosteal grafts through membrane-based technologies is an attractive approach to improve bone healing [4]. Membranes based on polyesters [5], periosteal cell sheets [6], or even natural proteins [7] have been used towards this goal. For example, platelet-rich fibrin membranes have been used as scaffolds for periosteal tissue engineering [8] and collagen-based membranes have successfully formed a multiple layer-like tissue in vitro [7]. Furthermore, some studies have reported that platelet-rich plasma-based [9] or chitosan [10] membranes wrapped around an osteoconductive scaffold enhanced the regeneration of bone defects. Nonetheless, limitations, such as a lack of biocompatibility, early degradation, poor mechanical properties, and a lack of control over bioactivity, have limited the success of periosteal grafts [5].

In an effort to develop more bioactive and tuneable periosteal grafts we have recently reported on several approaches using peptide-based materials [11,12]. Elastin-like recombinamers (ELRs) are genetically engineered protein-based polymers inspired by the extracellular matrix protein elastin [13]. ELRs are mainly composed of the repeating pentapeptide domain VPGXG, where X could be any amino acid other than proline. Furthermore, ELRs can easily be enriched with functional epitopes designed to elicit a specific biological response, such as cell adhesion [11,14] or

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biomineralization [15]. Their molecular versatility, biomimetic character, biocompatibility, mechanical properties, and biodegradability [14] are highly attractive elements to incorporate in a periosteal graft strategy.

Biomolecular signaling provided by growth factors, cytokines, and many other proteins and macromolecules plays a key role in the natural process of bone healing. Many short peptide sequences have been identified as potential candidates to enhance therapeutic approaches to bone regeneration [16]. Examples include the well-known cell adhesion sequences RGDS [17] and REDV [14], peptides that nucleate and guide mineralization like DDDEEKFLRRIGRFG [18] and S(P) [19], or peptides that promote osteogenic cell growth like ALKRQGRTLYGFGG [20]. These and many other bioactive epitopes represent a rich toolbox for the fabrication of bioactive peptide-based scaffolds. Similarly, it is well established that surface topographical patterns affect, and in many cases control, cell behavior [21]. For example, microposts have been used to study cell adhesion [22], direct cell migration [23], or stimulate proliferation [24], microchannels have been shown to control the morphology and migration of a large variety of cells [25], and microholes have been utilized to enhance cell differentiation [26] and to study cytoskeletal morphology [27].

The incorporation of both biomolecular and physical signaling within membrane scaffolds could significantly enhance the bioactivity and functionality of periosteal grafts. However, while extensive work has been carried out on stimulating cells through either surface topography or bioactive peptides, little is known about their synergistic or competitive effect. Hence we present the in vitro validation of bioactive ELR membranes that contain a variety of both surface topographies and bioactive epitopes known to play a role in bone regeneration. The membranes were used as substrates for the culture of primary rat mesenchymal stem cells (rMSCs) and their behavior was quantified in terms of cell adhesion, morphology, proliferation, and differentiation.

2. Materials and methods

2.1. ELR molecules

ELRs were supplied by the BIOFORGE group at the University of Valladolid, Spain. Specifically, the materials consisted of repeating pentapeptide domains of VPGIG and VPGKG, with the amino acid lysine (K) enabling ELR cross-linking, the peptides RGDS and REDV for mesenchymal stem cell and endothelial cell adhesion, respectively, the peptide DDDEEKFLRRIGRFG for nucleation of mineralization (HAP), and an ELR that combined the latter and the RGDS sequence (HAP-RGDS) (Table 1).

2.2. Membrane fabrication

Membranes were fabricated according to a recently reported method [11]. Briefly, the fabrication process included: (i) preparation of ELR and cross-linker solutions; (ii) mixing of solutions and the onset of ELR cross-linking; (iii) spin coating of cross-linking ELR; (iv) solvent evaporation, cross-linked ELR assembly, and membrane release. The ELRs were dissolved in anhydrous dimethylformamide (DMF) (Sigma–Aldrich, Germany) at room temperature, and then mixed with hexamethyl diisocyanate (HDI) (Sigma–Aldrich, Germany). The key parameters used to fabricate the ELR membranes included ELR and cross-linker concentrations, the mould, wettability, cross-linking reaction time, temperature, humidity, and solvent evaporation (Fig. 1a). Five ELR membranes were fabricated from the different ELR molecules, containing either one of the bioactive sequences (RGDS, REDV, HAP, and HAP-RGDS) or without bioactivity (IK).

2.2.1. Fabrication of molds for topographical patterned membranes

Direct write laser lithograpy (DWL 66, Heidelberg, Germany) was used to fabricate a silicon chrome photomask (Fig. 1b). A (111)-oriented silicon wafer (Siltronix) was coated with an 8 μm thick layer of SU8–10 photoresist (Microchem Corp., USA), soft baked (65 °C for 2 min and 95 °C for 5 min), and then exposed through the silicon chrome photomask (30 mW cm^{-2} for 3.3 s). It was subsequently post-exposure baked (65 °C for 1 min and 95 °C for 5 min) and developed using SU8 developer (Microchem, USA) for 30 s. Topographies were transferred to polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, USA) using a standard soft lithography process. The PDMS prepolymer was poured on top of the patterned master, degassed under vacuum for 7 min, and then cured at 65 °C for 2 h. The resulting topographically patterned mold was subsequently used to create membranes with the different structural components.

2.2.2. Topographically patterned membranes

A solution of ELR mixed with HDI was prepared and deposited directly on top of the patterned PDMS mold, allowed to air dry for 24 h, released from the mold, and washed thoroughly (Fig. 1c). An array of four 1.5 × 1.5 mm^2 patterned areas was transferred to the ELR membranes. This array consisted of channels that were 7 μm high, 10 μm wide, and separated by 10 μm wide ridges (Channels); holes that were 7 μm deep and either 10 (Holes10) or 40 (Holes40) μm in diameter and separated by 10 μm; posts that were 7 μm high, 10 μm in diameter, and separated by 10 μm (Posts) (Fig. 1d).

2.3. Membrane characterization

Membrane fabrication and pattern reproducibility were analyzed by qualitative observations using scanning electron microscopy (SEM) and profilometry (Fig. 1e–h). Membrane biocompatibility and bioactivity assessment as either the effect of biomolecular or topographical signaling on cell morphology was performed by in vitro culture of rMSCs.

Table 1
Sequence and functionality (bioactivity) of the elastin-like recombinamer (ELR) materials.

ELR Material	ELR sequence (bioactive sequence)	Bioactivity
IK	$(VPGIG\ VPGIG\ VPGKG\ VPGIG\ VPGIG)_{24}$	Control
RGDS	$[[(VPGIG)_2(VPGKG)(VPGIG)_2]_2$AVTG**RGDS**PASS$[(VPGIG)_2(VPGKG)(VPGIG)_2]_2]_6$	Cell Adhesion
HAP	$[[(VPGIG)_2(VPGKG)(VPGIG)_2]_2$ **DDDEEKFLRRIGRFG** $[(VPGIG)_2(VPGKG)(VPGIG)_2]_2]_3$	Mineralization
HAP-RGDS	$[[(VPGIG)_2(VPGKG)(VPGIG)_2]_2$**DDDEEKFLRRIGRFG**$[(VPGIG)_2(VPGKG)(VPGIG)_2]_2]_4[[(VPGIG)_2(VPGKG)(VPGIG)_2]_2$AVTG**RGDS**PASS $[(VPGIG)_2\ (VPGKG)\ (VPGIG)_2]_2]_4$	Mineralization and cell adhesion
REDV	$[(VPGIG)_2\ (VPGKG)\ (VPGIG)_2$EEIQIGHIP**REDV**DYHLYP $(VPGIG)_2\ (VPGKG)\ (VPGIG)_2\ (VGVAPG)_3)_{10}$	Cell Adhesion