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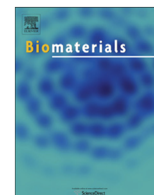

A tunable silk–alginate hydrogel scaffold for stem cell culture and transplantation

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ABSTRACT

One of the major challenges in regenerative medicine is the ability to recreate the stem cell niche, which is defined by its signaling molecules, the creation of cytokine gradients, and the modulation of matrix stiffness. A wide range of scaffolds has been developed in order to recapitulate the stem cell niche, among them hydrogels. This paper reports the development of a new silk–alginate based hydrogel with a focus on stem cell culture. This biocomposite allows to fine tune its elasticity during cell culture, addressing the importance of mechanotransduction during stem cell differentiation. The silk–alginate scaffold promotes adherence of mouse embryonic stem cells and cell survival upon transplantation. In addition, it has tunable stiffness as function of the silk–alginate ratio and the concentration of crosslinker – a characteristic that is very hard to accomplish in current hydrogels.

The hydrogel and the presented results represents key steps on the way of creating artificial stem cell niche, opening up new paths in regenerative medicine.

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1. Introduction

Stem cell therapy is a powerful therapeutic intervention that has the potential to combat several autoimmune, cancer and metabolic diseases [1–3]. Unfortunately, due to the rarity and fragility of progenitor cell populations, limited ex-vivo growth potential, few successful stem cell culture systems, and poor integration into host tissues upon transplantation, the widespread use of stem cell therapies in the clinic is limited [4,5].

The primary obstacle in developing stem cell based therapies subsequently, lies within the ability to recreate the microenvironment in which stem cells naturally reside in. *In vivo*, the stem cells live within a niche, which is described as a highly specialized microenvironment. This milieu integrates both established supportive cells, as well as a complex extracellular matrix (ECM) consisting of a network of proteins, such as collagens, or elastin arranged in a three-dimensional network. The orientation, elasticity and fluid handling properties of these network fibers help to dictate the biomechanical properties of the niche. In addition, these properties of the microenvironment determine the stem cell fate (i.e., self-renewal vs. differentiation) through a number of different, complementary mechanisms, including the well-defined presentation of various signaling molecules, the creation of cytokine gradients, and the modulation of matrix stiffness [6–8].

The microenvironment plays a pivotal role in determining cell identity and behavior by providing a suitable niche to sustain self-renewal and differentiation capacity [6,7,9]. Therefore, mimicking the stem cell niche, (i.e., preparing an artificial niche), is key to facilitating in vitro expansion of freshly isolated stem cells pre- or post-transplantation [8,10].

Current approaches to prepare a suitable environment that supports stem cell survival and differentiation, are based upon mimicking the host environment to the stem cell niche as much as possible [11]. For this purpose, scaffolds have been prepared using biopolymers and other molecules found in the ECM, such as

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collagen, elastin [12], fibrinogen, fibrin, hyaluronic acid, glycosaminoglycans (GAGs), hydroxyapatite, Matrigel, silk, alginate or chitosan to accomplish this goal [12–19]. These polymers have the advantage of being bioactive.

Besides natural polymers, synthetic polymers are widely used to form scaffolds for stem cell cultivation [20]. The most prominent examples include poly(ethylene glycol) (PEG) and acrylated hydrogels [14]. Among the synthetic class of polymers, are biodegradable ones such as, polyglycolic acid (PGA), polylactic acid (PLA), poly(ε-caprolactone) (PCL) and the copolymer poly(lactic-co-glycolic acid) (PLGA). These synthetic polymers have been used extensively as synthetic 3D scaffold materials for evaluating cell behavior, however, fail in recreating the same biomechanical properties and structural complexity found naturally in the ECM.

Different studies show that stem cell survival and phenotype can be controlled by attenuating the mechanical properties of biosynthetic matrices [8,9,15,21]. Gaining the ability to generate and control the mechanical properties of stem cell scaffolds is therefore very important and practical in developing stem cell based therapies. In this proof of concept study we address the need for simulating the mechanical and structural properties of the niche. We report on the preparation and characterization of a newly designed silk–alginate based hydrogel, of defined molecular composition and topology that addresses the unmet need for a mechanically adjustable scaffold to support and with the potential to guide stem cell survival and differentiation, respectively. The combination of these two very different types of biomaterials, silk and alginate, results in a hybrid class of rapidly gelling, physically stable hydrogels overcoming the biomechanical limitations of current hydrogels for several applications in regenerative and pharmaceutical applications, such as 3D printed organs, or organs-on-a-chip as e.g. drug screening platform [22].

2. Materials and methods

2.1. Scaffold formation

Hydrogel precursor mixtures were prepared from alginate 4w/v% in distilled water (Protanal® LFR 5/60 Sodium Alginate with high alpha-L-guluronate (G) residues kindly provided by FMC Biopolymers, Ewing, NJ) and 7.4 − 7.8 w/v% silk solution in distilled water (freshly prepared *Bombyx mori* silkworm silk solutions kindly provided by Prof. David Kaplan, Tufts University, Boston, MA). Precursor mixtures were supplemented by one of the following: 0.5 mg/mL mouse laminin (Roche, Indianapolis, IN), 0.5 mg/mL fibronectin (Roche, Indianapolis), 1 mg/mL in pH 6.5 in BIS–TRIS (10 mM) Bovine collagen I (MP Biomedicals, Santa Ana, CA), or 0.5 mg/mL cyclic RGD (Peptides International, Louisville, KY). Concentrations of the final precursor solutions were adjusted using distilled water to reach a final concentration of 1.5% w/w silk and 1% w/w alginate. Precursor mixtures were mixed until they appeared homogeneous. For all experiments the bubble-free precursor solution was injected into a custom mold, enabling the simultaneous preparation of 6 disc-shaped hydrogel samples (8 mm diameter, 1.56 mm thickness). The mold was covered on both sides by a dialysis membrane (50,000 MWCO, Spectrum Laboratories, Houston, Tx), allowing calcium ions to enter and induce gelation. Following injection of the precursor solution into the mold, gelation was induced by immersing the mold in a buffered 25 mM $CaCl_2$ solution (Sigma Aldrich) (10 mM BIS–TRIS, 100 mM NaCl, pH 6.5, Sigma–Aldrich, USA, and Anachemia, Reno, NV, respectively). Gelation time ranged from 20 to 60 min. Scaffolds for cell adherence experiments were cast in 12-well transwell plates (Corning, Lowell, MA) using the same precursor solutions and gelation conditions described above.

2.2. Cell culture and bioluminescence imaging

Following casting of the different hydrogel precursor mixtures in 12-well transwell plates, D3 mouse embryonic stem cells (mESC) expressing firefly luciferase (Fluc) were plated (200,000 cells/well) and cultured with the appropriate medium (D3 mESCs were cultured on gelatin-coated 10 cm dishes with knock-out Dulbecco's modified Eagle's medium (D-MEM) (GIBCO/BRL, Grand Island, NY, USA), containing 15% knock-out fetal bovine serum (FBS) (Invitrogen, Grand Island, NY, USA), 1% nonessential amino acids (GIBCO/BRL), 0.1 mmol/L 2-mercaptoethanol (GIBCO/BRL), 1000 U/mL mouse recombinant leukemia inhibitory factor (LIF) (GIBCO/BRL), 100 IU/mL penicillin, and 0.1 mg/mL streptomycin). 72 h after plating, the cells were tested for Fluc expression as an indicator for cell presence and viability using bioluminescence imaging (BLI). The luciferase substrate, D-Luciferin (BIOSYNTH, Itasca, IL), was added to each well (2 μg/mL). Cells were imaged immediately after the addition of substrate using an IVIS-200 imaging system equipped with a cooled charge-coupled device camera (Caliper). Imaging was performed using open filters. Regions of interest were drawn over each well, and the average radiance was determined using Living Image software (V4.1, Caliper Life Sciences).

2.3. Window chamber implantation

All animal handling was performed in accordance with Stanford University's Animal Research Committee guidelines.

A dorsal skinfold window chamber was surgically implanted in female Balb/C mice (Charles River, 10 weeks of age). Animals were anesthetized by intraperitoneal (IP) injection of a mixture of 1 mg/mL of xylazine and 10 mg/mL ketamine in 300 μL final volume. Hair was removed from the mice's backs using hair clippers and depilatory cream (Nair, naircare.com). Next, medium-sized titanium dorsal skinfold window chambers (APJ Trading, Cat.# MD100) were surgically implanted on the back of the animals, as previously described [23]. Briefly, following the midline, a titanium frame was sutured to the dorsal side using surgical sutures (Blue Polypropylene, 5-0, FS-2) (Med Rep Express, Patricia Brafford, MA). Both layers of the skin flap were punctured in two instances to secure two stainless steel screws. A round-shaped epidermal layer was removed from the upward-facing skin flap and covered by a sterile 12 mm diameter glass coverslip. Following this, both frames were screwed together and sutured to the skin flap. The animals were allowed to recover over a period of 3–4 days, after which the scaffold was implanted. For the implantation, the coverslip was removed using pliers, followed by placement of the scaffold onto the dermal layer inside the window chamber and covering with a fresh coverslip.

2.4. Fluorescent alginate synthesis

For the purpose of window chamber intravital microscopy (IVM) experiments, we transplanted fluorescent scaffolds, in which the alginate (Sigma–Aldrich, USA) was labeled with rhodamine B isothiocyanate (RBITC) (Sigma Aldrich, Milwaukee, WI). Alginate was labeled with RBITC according to a method previously reported by Mladenovska et al. [24]. Briefly, an aqueous 2w/w% alginate solution was prepared and adjusted to a pH = 8 by adding 1 M sodium hydroxide (Anachemia, Reno, NV). An RBITC solution was prepared by mixing 1 mg of RBITC in 1 mL DMSO (Fisher, Fairlawn, NJ) followed by slow addition into the alginate solution. The alginate–RBITC mixture was stirred for 1 h at 40 °C. After stirring, 0.5349 g of NH_4Cl (Sigma Aldrich) was added and mixed until fully dissolved. The alginate–RBITC solution was dialyzed in darkness overnight. Water baths were frequently changed with distilled deionized water. Subsequently, the alginate–RBITC solution was poured into 50 mL polypropylene conical vials until approximately three-quarters full, flash frozen at −80 °C and lyophilized until fully dry. Lyophilized alginate–RBITC was stored at −20 °C until use.

2.5. Intravital microscopy (IVM)

Fluorescent scaffolds were transplanted in mice with a dorsal skinfold window chamber ($n = 4$). Intravital Microscopy (IVM) was used to track the scaffold's degradation for 10 days following transplantation.

An intravital laser-scanning microscope optimized for *in vivo* imaging (Olympus IV 100, Olympus, Center Valley, PA) was used with Olympus UplanFL objectives and Olympus FluoView IV10-ASW 1.2 software. Regions within the scaffold and the tissue were excited with a laser at 488 nm to outline the scaffold area. Regions of interest were analyzed using the FluoView FV300 software (V4.3, Olympus).

2.6. Scanning electron microscope (SEM) imaging

Hydrogel samples were placed on clean 1 cm aluminum SEM post (Ted Pella, Redding, CA). The samples were then air dried under a glass petri dish for a minimum of 6 h. After 6 h the samples were transferred to a SEM sample storage box, and placed in a vacuum desiccator overnight. After the samples were fully dehydrated they were permanently adhered to the SEM posts using high performance silver paste (Ted Pella, Redding, CA) to prevent the samples from coming loose in the microscope. The samples were then coated with a thin layer of AuPd using a Cressington 108 sputter coater (Ted Pella, Redding, CA) to improve conductivity. After preparation the samples were imaged in the Magellan 400 XHR SEM (FEI, Portland, OR) at 3 kV with a beam current of 25 pA. These operating conditions were chosen to minimize beam damage, while providing excellent topographical information about the hydrogel. Images were taken at several positions across the hydrogel samples at multiple magnifications to obtain information about the pore size distribution. As the hydrogel dehydrated it collapsed on itself. Areas of the hydrogel with pores collapsed further than the surrounding areas because there was less material present. This created topographical features at the surface of the dehydrated hydrogel that correspond to the pore size of the hydrated hydrogel allowing for the measurement of the pore size from the dehydrated samples.

Hydrogel samples were also imaged in EVO LS15 variable pressure SEM to obtain images of the hydrogel in its hydrated state. Samples were placed on peltier cooled stage and maintain just above the freezing point as the pressure was decreased to

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