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Silk ionomers for encapsulation and differentiation of human MSCs

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ABSTRACT

The response of human bone marrow derived human mesenchymal stem cells (hMSCs) encapsulated in silk ionomer hydrogels was studied. Silk aqueous solutions with silk-poly-L-lysine or silk-poly-L-glutamate were formed into hydrogels via ultrasonication in situ with different net charges. hMSCs were encapsulated within the hydrogels and the impact of matrix charge was assessed over weeks in osteogenic, adipogenic and maintenance growth media. These modified silk charged polymers supported cell viability and proliferative potential, and the hMSCs were able to differentiate toward osteogenic or adipogenic lineages in the corresponding differentiation media. The silk/silk-poly-L-lysine hydrogels exhibited a positive effect on selective osteogenesis of hMSCs, inducing differentiation toward an osteogenic lineage even in the absence of osteogenic supplements, while also inhibiting adipogenesis. In contrast, silk/silk fibroin-poly-L-glutamate hydrogels supported both osteogenic and adipogenic differentiation of hMSCs when cultured under induction conditions. The results demonstrate the potential utility of silk-based ionomers in gel formats for hMSCs encapsulation and for directing hMSCs long term functional differentiation toward specific lineages.

Mesenchymal stem cells from human bone marrow (hMSCs), due to their intrinsic self-renewing ability and multilineage potential, can differentiate to various cell types of connective tissues including bone, cartilage, fat, muscle, tendon and stroma, thus representing one of the most significant cell sources for regenerative medicine [1,2]. To fully exploit the potential of stem cells in tissue engineering and regeneration, well-controlled techniques for *in vitro* cell expansion, maintenance and specific differentiation are needed. The most common methods to induce and direct stem cell differentiation *in vitro* involve the use of soluble chemical and biochemical factors. In contrast, numerous studies indicate that besides growth factors such as cytokines, chemokines and serum proteins, many other factors have a profound influence on cell differentiation [3]. In particular, hMSCs fate *in vitro* is dependent on physical and chemical clues from the environment as well as from the complex interactions between cells [4,5].

Cell function can be controlled using tailored biomaterials that mimic the native stem cell niche [6]. The engineering of functional tissues *in vitro* relies on the accurate combination of biomaterial, cells, biological signals and biophysical stimulation [7,8]. Therefore, there is a need to develop suitable biomaterial platforms in which hMSCs can be expanded and stimulated to lineage-specific differentiation, or maintained in an undifferentiated state [9,10]. In this context, cell encapsulation within hydrogels has many advantages for regenerative medicine, providing a hydrated three-dimensional environment with excellent permeability to nutrients and oxygen necessary for tissue healing or regeneration [11].

Silks are versatile protein biomaterials with useful physicochemical and mechanical properties that undergo biodegradation *in vivo*, exhibit long-term stability and biocompatibility and have been successfully used in a range of tissue engineering applications [12–17]. In particular, native silk fibroin and its derivatives have been extensively studied as starting materials for hydrogels due to their self-assembly via physical beta sheet crosslinks, avoidance of chemical crosslinkers, and the mechanical properties and slow biodegradability of the hydrogels [12,18]. However, the gelation of silk aqueous solutions obtained by protein self-assembly occurs slowly under physiological conditions, often limiting cell encapsulation [19]. Therefore, we have developed more rapid modes of silk gelation useful for cell encapsulation, including vortexing [20] and ultrasonication [21].

As previously reported, we have developed two silk ionomer derivatives, silk fibroin-poly-L-lysine (SF-PL) and silk fibroin-poly-L-glutamic acid (SF-PG) [22], which physically interact via charge complexation to form reversible silk-based hydrogels suitable for cell encapsulation [22]. Furthermore, using layer by layer techniques, these silk ionomers can be used for the formation of pH-sensitive shell microcapsules for the encapsulation and delivery of macromolecules or cells [23].

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In previous studies, we have also investigated the impact of biophysical signaling in control of hMSC function and differentiation [24,25]. These results indicate that the bioelectrical properties of stem cells, such as the transmembrane potential (V_{mem}), can strongly influence the differentiation state in hMSCs, and that the modulation of cell V_{mem} could be a useful tool to control stem cell differentiation [24,25]. In particular, the differentiating cells exhibited a hyperpolarized V_{mem} compared with undifferentiated hMSCs and improved osteogenic differentiation upon hyperpolarization [24].

The aim of the present study was to develop a new method for the encapsulation of hMSCs within charged silk-based hydrogels in order to investigate how stem cells fate is affected by the net charge in the three-dimensional environment. Part of the motivation for such studies was based on our prior studies of the role of stem cell membrane potential on hMSCs fate and function as mentioned above [24,25]. For the present study, ultrasonication was used to induce fast polymer gelation and in situ hMSCs encapsulation. The cells were cultured within the hydrogels in adipogenic, osteogenic or maintenance growth media for up to 56 days to assess differentiation.

1. Materials and methods

1.1. Reagents

All reagents were purchased from Sigma–Aldrich (St. Louis, MO, United States), unless otherwise stated.

1.2. Silk fibroin extraction

Silk fibroin (SF) was extracted from *Bombyx mori* cocoons as we have previously reported [26]. In brief, the cocoons (purchased from Tajima Shoji Co., Ltd., Yokohama, Japan) were cleaned, cut in small pieces, and boiled for 30 min or 1 h in 0.2 M Na_2CO_3 aqueous solution. The degummed silk was washed several times in distilled water and dried at room temperature. After drying, the extracted SF was dissolved in 9.3 M LiBr at 60 °C for 3–4 h (depending on the boiling time), yielding a 20% w/v solution, and then dialyzed against water for 3 days (MWCO 3500).

1.3. Synthesis of SF ionomers

SF ionomers derivatives were obtained as previously reported [22]. In brief, SF, obtained after 1 h degumming, was enriched in pendant carboxyl groups by two reactions involving tyrosine and serine residues, respectively. In the first step, tyrosine residues were reacted with 4-aminobenzoic diazonium salt [26], leading to the SF-(Y)COOH derivative; in the following step the serine residues were reacted with chloroacetic acid, leading to the SF-(Y,S)COOH derivative. Then, the SF-(Y,S)COOH product was covalently conjugated with poly-L-lysine or poly-L-glutamic acid via carbodiimide-mediated chemistry, to obtain silk fibroin-poly-L-lysine (SF-PL) and silk fibroin-poly-L-glutamic acid (SF-PG) ionomers, respectively. To confirm the structures of the intermediates and final products, each solution was lyophilized, dissolved in deuterium oxide and subjected to ^{1}H NMR analysis with a Bruker AVIII-500 MHz spectrometer (Billerica, MA, USA).

1.4. Silk ionomers gelation

In order to develop optimal sonication parameters and conditions for gelation, solutions of SF and SF blended with SF-PL or SF-PG ionomers were sonicated with a Branson 450 Sonifier (Branson Ultrasonics Co., Danbury, CT), equipped with an externally threaded disruptor horn and a 3.175 mm diameter-tapered microtip. Solutions of 30 min boiled SF, pure or blended with SF-PL or SF-PG ionomers (SF concentrations ranging from 0.4 to 2% w/v, and ionomer concentrations ranging from 0.1 to 2% w/v), in water or in DMEM at pH 7.4, were sonicated at 15 and 20% amplitude for different times. After sonication, each solution was incubated at 37 °C and the gel–sol transition was screened as we have previously reported [27].

1.5. Cell culture and encapsulation in SF and SF-ionomers hydrogels

hMSCs were isolated from human bone marrow aspirates (Lonza, Gaithersburg, MD, USA) as we have previously reported [28]. The cells were expanded, until P2, in tissue culture polystyrene flasks in maintenance growth medium, consisting of high glucose DMEM supplemented with 10% fetal bovine serum (FBS), 1 ng/mL basic fibroblast growth factor (bFGF), 1% Gibco® Antibiotic–Antimycotic, 0.01 M non-essential amino acids, all purchased from Gibco (Life Technologies, Grand Island, NY, USA). When the cells were 80% confluent they were trypsinized, resuspended in fresh growth medium, and encapsulated in SF or SF ionomers hydrogels, using different densities and polymer concentrations. Preliminary cell encapsulation experiments were performed by sonicating solutions of 2% SF or 2% SF blended with each SF-ionomer with final concentrations ranging from 0.1 to 1% w/v, and adding hMSCs to the sonicated polymer solutions to a final cell density of 5×10^4 per gel.

The following samples were extensively investigated: SF 2% w/v, SF 2% w/v + SF-PL 0.5% w/v, and SF 2% w/v + SF-PG 0.5% w/v hMSCs-hydrogels (cell density = 4×10^5/mL; gel volume = 50 μL). In brief, SF 2.5% w/v stock solutions (30 min degummed SF), and SF-PL or SF-PG 3.5% w/v stock solutions in DMEM were sterilized by filtration (0.22 μm filter) under sterile conditions, immediately before use. Then, proper volumes of each stock solution were mixed in order to obtain the targeted final polymer concentration, and aliquots of 3.3 mL of each polymer blend sonicated for 20 s at 20% amplitude. After 5 min, 200 μL of hMSCs suspension (7×10^6 cells/mL in growth medium) were added to the sonicated polymer solutions or to the same amount of growth medium (in order to obtain a 2D control), and each final mixture was pipetted in 96-well plates (50 μL per well). A similar procedure was used to make control gels, with no cells encapsulated. The plates were incubated at 37 °C in a humidified, 5% CO_2 atmosphere, until gelation was complete. Then, 150 μL of fresh differentiation media (osteogenic or adipogenic induction), or maintenance growth medium were added on the top of each gel, and the plates were incubated at 37 °C in a humidified, 5% CO_2 atmosphere, replacing the media every 3 days. The osteogenic differentiation medium was comprised of high-glucose DMEM supplemented with 10% FBS, 1% Gibco® Antibiotic–Antimycotic, 100 nM dexamethasone, 50 μM ascorbic acid, and 10 mM β-glycerophosphate. The adipogenic induction medium was comprised of high-glucose DMEM supplemented with 3% FBS, 1% Gibco® Antibiotic-Antimycotic, 1 μM dexamethasone, 1 μM insulin, 33 μM biotin, 17 μM pantotenate, 500 μM 3-isobutyl-1-methylxanthine (IBMX), 5 μm 2,4-thiazolidinedione (TZD). After one week, the adipogenic induction medium was replaced with the adipogenic maintenance medium, having the same composition of the induction medium, but without IBMX and TZD.

1.6. Cell proliferation

Cell proliferation was assessed by Pico Green and MTS assay. MTS assay was performed by following the manufacturer's instructions (Promega Corporation, Madison, WI, USA). In brief, after replacing the media with 100 μL of fresh media per well, 20 μL of the combined MTS/PMS solution was added into each well and the plate was incubated for 1.5 h at 37 °C in a humidified, 5% CO_2 atmosphere. After incubation, the absorbance at 490 nm was measured using an ELISA plate reader. dDNA was quantified by performing the Pico Green assay. After predefined times, the media was removed, the samples were rinsed with Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco-Life Technologies, Grand Island, NY, USA), and DNA was extracted with 0.2% (v/v) Triton X100, 5 mM $MgCl_2$ and by manual homogenization of the hydrogels. The lysates were collected and centrifuged for 5 min at 12,000 rpm and 4 °C, to eliminate cellular debris. The supernatants were collected and dDNA was assayed with Quant-iT™ Pico Green® kit (Invitrogen-Life Technologies, Grand Island, NY, USA), following the manufacturer's protocol.

For both of the assays, blank hydrogels from the same time points were used as controls to subtract the absorbance or the fluorescence of the material from the sample readings.

1.7. Live/dead staining and confocal fluorescence microscopy

After predefined times, the hMSCs encapsulated in SF/SF-ionomer hydrogels were stained with live/dead staining according to the manufacturer's instructions (Molecular Probes – Life Technologies, Grand Island, NY, USA). Briefly, after removing the media from each well, the samples were gently washed with DPBS and incubated with a 2 μM calcein AM and 4 μM ethidium homodimer-1 (EthD-1) solution for 45 min at room temperature. After this time, the samples were analyzed with a Leica DMIRE2 confocal microscope with a TCS SP2 scanner (Wetzlar, Germany).

1.8. Adipogenic differentiation

Adipogenic differentiation of hMSCs while encapsulated within SF and SF-ionomers hydrogels was investigated by Oil red O staining for lipid droplet accumulation and by the leptin release assay. The cells encapsulated within the hydrogels or cultured in 2D in adipogenic differentiation or maintenance growth media, were fixed in a 10% formalin solution in PBS at each time point, and stained with a 60% Oil red O solution in isopropyl alcohol. After staining, the samples were observed under a light microscope. The cell culture supernatants, collected from the samples at each time point, were analyzed for human leptin content using the Quantikine Human Leptin Immunoassay (R&D Systems, Inc., Minneapolis, MN, USA) according with the manufacturer's instructions.

1.9. Osteogenic differentiation

The osteogenic differentiation of hMSCs while encapsulated within SF and SF-ionomers hydrogels was assessed by measuring the alkaline phosphatase (ALP)