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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 cues 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

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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 physico-chemical 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].

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.

2. Materials and Methods

2.1. Reagents

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

2.2. 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 minutes or 1 hour 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 hours (depending on the boiling time), yielding a 20% w/v solution, and then dialyzed against water for 3 days (MWCO 3,500).

2.3. Synthesis of SF ionomers

SF ionomers derivatives were obtained as previously reported [22]. In brief, SF, obtained after 1 hour 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 ^1H -NMR analysis with a Bruker AVIII-500 MHz spectrometer (Billerica, MA, USA).

2.4. 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 minutes 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].

2.5. 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 minutes 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 seconds at 20% amplitude. After 5 minutes, 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% CO₂ 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 pantothenate, 500 µM 3-isobutyl-1-methylxanthine (IBMX), 5 mM 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.

2.6. 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 hours at 37°C in a humidified, 5% CO₂ 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 MgCl₂ and by manual homogenization of the hydrogels. The lysates were collected and centrifuged for 5 minutes at 12,000 rpm and 4°C, to eliminate cellular debris. The supernatants were collected and dDNA was assayed with Quant-iT™ PicoGreen® 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.

2.7. 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) solution for 45 minutes at room temperature. After this time, the samples were analyzed with a Leica DMIRE2 confocal microscope with a TCS SP2 scanner (Wetzlar, Germany).

2.8. 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.

2.9. Osteogenic differentiation

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

mineralization (calcium deposition) over time. ALP activity was measured using p-nitrophenyl phosphate, in accordance to manufacturer's instructions (Alkaline Phosphatase Liquicolor, Stanbio Laboratory, Boerne, TX, USA). Briefly, after removing the media, the samples were rinsed twice with DPBS, and the cells were lysed with 0.2% v/v Triton X-100, 5 mM MgCl₂ and by manual homogenization. After cell lysis, the lysate was collected and centrifuged at 4°C for 5 minutes at 12,000 rpm, to eliminate cellular debris. ALP activity was assayed by reacting 80 µL of the supernatant with 120 µL of the substrate solution and measuring the absorbance at 405 nm, related to the hydrolysis by the ALP of the 4-nitrophenyl phosphate to 4-nitrophenol. The ALP activity, the amount of enzyme that produces 1 mmol/L of 4-nitrophenol per minute, was obtained by the slope of the absorbance curve at 405 nm, read over a 60 minutes period, and data were calculated using a standard curve of 4-nitrophenol. Calcium deposition was quantified by using ortho-cresolphthalein complexone in a spectrophotometric assay, in accordance to manufacturer's instructions (Total Calcium LiquiColor, Stanbio Laboratory, Boerne, TX, USA). In brief, after removing the media, the samples were rinsed with DPBS, and calcium was extracted in solution by treating with 5% trichloroacetic acid. After manual homogenization of the cell-hydrogel constructs, the samples were collected and centrifuged for 5 minutes at 12,000 rpm. The supernatants were collected and mixed with the reagent solutions in a ratio 1:10. After 5 minutes of incubation at room temperature, the absorbance at 570 nm was recorded, and the calcium concentration was calculated by using a calibration curve made with calcium standard solutions.

2.10. Statistical analysis

All quantitative measurements were performed at least in 3 replicates. All values are expressed as mean $\pm$ standard deviation. Statistical significance was determined using paired two-tailed t-tests to compare data sets, assuming a significance level of $p < 0.05$.

3. Results and Discussion

The goal was to investigate the behavior of hMSCs while encapsulated within silk-based charged (ionomer) hydrogels. In a previous work, we reported the synthesis of two silk ionomer derivatives, silk fibroin-poly-L-lysine (SF-PL) and silk fibroin-poly-L-glutamic acid (SF-PG), where branched charged-polyamino acids provided net charges and ionomer features [22]. These new properties were successfully exploited to form reversible hydrogels suitable for cell encapsulation [22]. The goal of this project was to further explore the potential for these silk-ionomers for stem cell encapsulation with control of differentiation.

The two ionomers were synthesized, purified and analyzed as previously reported [22]. The ¹H-NMR analysis of the ionomers was in agreement with our previous results (data not shown) [22].

In order to obtain charged hydrogels, we could not use simple mixing to form the hydrogels, as they are based on electrostatic interactions between opposite charged ionomers [22–23]. In previous work we demonstrated the ultrasonication technique as a useful way to obtain silk-based hydrogels [21]. Since gelation time can be customized, this was a useful approach for in situ cell encapsulation. By changing the sonication parameters and the SF concentration, it was possible to obtain different gelation times and hydrogel properties [21,29]. Ultrasonication was therefore used to obtain SF-ionomer-based hydrogels. In order to tailor the gelation rate to the requirements for cell encapsulation, we performed preliminary gelation studies where the polymer concentration, SF/SF-ionomer ratio, solution volumes and sonication parameters were assessed. Representative results from these preliminary studies are summarized in Table 1, included in Supplementary Data.

The sonication of each ionomer alone, at concentrations ranging from 0.1 to 2% w/v, did not result in gelation. We hypothesize that the higher hydrophilicity, compared to that of unmodified SF, and electrostatic repulsion between the charged branched chains, would impede the formation of physical interactions and hydrophobic crosslinks which are responsible for sonication-induced gelation of SF [21,29]. The addition of SF allowed the gelation of SF-ionomer solutions at different concentrations, ranging from 0.1 to 2% w/v. According with our previous results, the ultrasonication of SF solutions allowed gelation in a time-controllable manner [21], showing an overall increase in the gelation rate, by increasing the SF concentration, or the sonication amplitude/time (Table 1, Supplementary Data). Moreover, the presence of SF-ionomers affected the gelation properties of SF solutions: SF-PL ionomers decreased, while SF-PG slightly increased, the gelation rate. For example, by sonication for 15 seconds (20% amplitude), 2% SF and 2% SF + 0.5% SF-PG solutions (volume = 2 mL) gelled within 9–10 minutes, while 2% SF + 0.5% SF-PL solutions gelled in more than 1 hour (Table 1, Supplementary Data).

In order to investigate the cytocompatibility of the encapsulation method and the hydrogels, preliminary screening experiments were also conducted by encapsulating hMSCs within SF 2%, SF 2% + SF-PL 0.1, 0.5 or 1% w/v, and SF 2% + SF-PG 0.1, 0.5 or 1% w/v hydrogels. The dDNA of each culture was extracted and analyzed by Pico Green assay after 2, 3 and 6 weeks. The data indicated that the encapsulation method and the hydrogels did not have any significant adverse effects on the encapsulated cells (Fig. 1). There were no statistically significant changes in DNA concentration for the first 3 weeks. Interestingly, a significant ($p < 0.005$) increase in DNA for most of the samples was measured after 6 weeks, suggesting a positive effect of the encapsulation within the SF-ionomers hydrogels on long term cell proliferation.

To investigate the performance of hMSCs encapsulated within the charged silk hydrogels, one concentration set was selected for detailed study with the following gel forming solutions: SF 2% w/v, or SF 2% w/v blended with SF-PL 0.5% w/v or SF-PG 0.5% w/v in plain DMEM at pH 7.4. After 20 seconds of sonication (amplitude = 20%), the solutions gelled within 15–30 minutes, allowing efficient cell encapsulation based on microscopic examinations. The viability and proliferation of hMSCs while encapsulated within the SF/SF-ionomer hydrogels was investigated over two months by MTS assay, DNA quantification, and live/dead staining imaging.

The MTS assay results (Fig. 2) indicated that the cells retained viability over the entire period of culture in almost all of the hydrogels. After 1 day of encapsulation/differentiation, no significant difference in cell viability was observed between all the samples, confirming that encapsulation did not affect cell viability. In adipogenic media (Fig. 2a), after 1 week, the number of viable cells was significantly higher than the corresponding number in the 2D control ($p < 0.005$); while after 8 weeks a decrease in cell viability was observed for cells encapsulated within ionomer hydrogels, with a value near zero for the SF/SF-PL hydrogels (Fig. 2a). In osteogenic media (Fig. 2b), the cells proliferated over time, while for the 2D control cell numbers decreased after 4 weeks. Cell viability at 4 and 8 weeks was significantly higher for the cells encapsulated within hydrogels than for the 2D controls ($p < 0.05$). Similarly, in maintenance growth media (Fig. 2c) cells proliferated within all hydrogels, showing a peak after 4 weeks and then a plateau until the end of the analysis at 8 weeks. These results suggest that the SF and SF-ionomer hydrogels are suitable encapsulation systems for long term *in vitro* hMSCs maintenance and proliferation.

Due to potential complications with charge-charge interactions of DNA with some of the polymers, and therefore incomplete extraction from the gels, the Pico Green results may not accurately reflect the total number of live cells (data not shown). Therefore, the

differentiation data reported are normalized to cell number instead of to DNA extracted from each cell-hydrogel sample.

The presence of live cells was confirmed by Live/Dead staining and confocal microscopy analysis. Representative confocal images of hMSCs stained live (Calcein AM/green) or dead (EthD/red), are shown after 8 weeks of culture in adipogenic, osteogenic and maintenance growth media, while encapsulated in the SF and SF/SF-ionomer hydrogels, or cultured in 2D (Fig. 3). Although there are no quantitative data for the Live/Dead assay, the images were consistent with the MTS results, which support the conclusion that SF and SF ionomer hydrogels are suitable biomaterials for hMSCs encapsulation and long term *in vitro* maintenance. After 8 weeks, the cells were still viable in the SF 2% hydrogels in all the investigated media, showing a rounded shape in adipogenic media (Fig. 3a), an osteocytes-typical stellate shape in osteogenic media (Fig. 3e), and a spindle-shape characteristic for hMSCs when cultured with no differentiation factors (Fig. 3i). These results indicate that the encapsulation within SF hydrogels does not restrict cell morphology, as the cells tend to assume the characteristic shape of the cell-lineage toward which they are differentiating. These results confirmed that hMSCs encapsulated within SF hydrogels have long term viability (Fig. 2) and, if cultured in the presence of the proper osteogenic or adipogenic induction soluble factors, undergo differentiation into the respective lineage. In contrast, in the absence of differentiation media they retain their proliferative mesenchymal potential for the 8 week study (Fig. 3i). The cells encapsulated within SF/SF-PL hydrogels appeared viable in osteogenic and in maintenance growth media, until the end point of the analysis (Figs. 3f and j), while in adipogenic medium a drop in cell viability was observed after 8 weeks (Fig. 3b). Cells encapsulated within SF/SF-PG hydrogels appeared viable in all conditions (Figs. 3c, g and k). The few rounded-shaped live cells, observed in the 2D samples in osteogenic media (Fig. 3h), was due to the lack of the attachment on glass surfaces, which easily occurs for osteogenic differentiated hMSCs cultured onto 2D glass surfaces for longer times.

The osteogenic differentiation of hMSCs encapsulated in SF and SF-ionomer hydrogels, was investigated by the analyses of alkaline phosphatase (ALP) activity and calcium accumulation. ALP activity is a common marker for osteogenic differentiation [30]. *In vitro*, during late osteoblast differentiation, ALP dephosphorylates substrates supplied in the media, releasing inorganic phosphate available for the mineralization of the matrix, and its activity rapidly increases during mineralization until a peak level and then subsequently decreases. In Fig. 4 we report representative data for ALP activity: different amounts and patterns of ALP activity were observed for the encapsulated hMSCs when cultured in the different media conditions. In particular, in osteogenic medium (Fig. 4b) the cells exhibited the typical osteogenic ALP activity pattern, with a rapid increase in marker until a peak level (day 21), followed by a plateau for SF-PG, or a rapid decrease over time for SF and SF-PL. A similar pattern was observed for the 2D controls, but the maximum level was observed on day 42 with a subsequent decrease over time. ALP is an enzyme ubiquitous in the body with highest activity in bone, but usually poor activity in undifferentiated hMSCs. However, in the present study hMSCs encapsulated within SF/SF-PL hydrogels and cultured in maintenance growth media (Fig. 4c) exhibited a peak in ALP activity on day 21, significantly higher than that of the other samples ($p < 0.05$). This result suggests that hMSCs encapsulated within SF-PL based hydrogels were able to differentiate toward an osteogenic lineage, even without the selective induction factors.

Moreover, it has been demonstrated that there is a correlation between ALP activity and lipid accumulation during human pre-adipocyte maturation and adipogenic differentiation [31–32]. Accordingly, we observed a significative increase in ALP activity for the 2D control cells cultured in adipogenic conditions, with a peak at day 42 and a subsequent

decrease at day 56 (Fig. 4a). In contrast, the pattern of ALP activity for the corresponding encapsulated cells showed a late increase for the SF and SF/SF-PG samples, with values similar to that in 2D at day 56. In contrast, no relevant ALP activity was revealed for SF/SF-PL samples, suggesting that in adipogenic conditions SF/SF-PL could inhibit, while both SF and SF-PG could sustain, adipogenic differentiation.

Mineralization, as calcium accumulation, was also investigated as a specific endpoint marker of the metabolic activity of osteoblast differentiated cells (Fig. 5). These data were consistent with the pattern of ALP activity. No calcium accumulation occurred in samples cultured in adipogenic media (Fig. 5a), while when cultured in osteogenic media hMSCs encapsulated in SF and SF-ionomer based hydrogels mineralized the matrices (Fig. 5b), based on the significant increase in calcium concentration over time. There was a peak at 6 weeks ($p < 0.005$) for all the samples, as well as for the 2D controls. In particular, SF samples showed the lowest peak in calcium, whereas SF-PG samples had higher calcium concentrations than the 2D controls ($p < 0.05$) at 6 and 8 weeks. SF-PL samples showed a progressive increase in mineralization until 6 weeks, and then a plateau until the end of the analysis. Interestingly, cells encapsulated in the presence of SF-PL ionomer, even if cultured in the absence of osteogenic induction media (Fig. 5c), exhibited high mineralization, with a significative calcium accumulation starting after 4 weeks, reaching at 6 weeks a value significantly higher than the corresponding peak obtained in osteogenic media for the 2D control ($p < 0.01$). On the other hand, no calcium accumulation was detected for the cells encapsulated in the presence of SF-PL ionomer when cultured in adipogenic conditions (Fig. 5a).

All of these results suggest that hMSCs encapsulated within SF and SF-ionomers hydrogels, cultured in the presence of osteogenic differentiation media, are able to differentiate into osteoblasts inside the gels and mineralize the matrices, as indicated by the increase in ALP activity (Fig. 4b) and the calcium accumulation over time (Fig. 5b). Moreover, an interesting and unexpected finding is the strong effect of the SF-PL ionomer on the encapsulated hMSCs, which stimulated osteogenic differentiation even in the absence of osteogenic-induction cocktail (Figs. 4c and 5c), avoiding at the same time, non-specific osteogenic differentiation in adipogenic conditions, as suggested by the poor ALP activity and calcium accumulation (Figs. 4a and 5a).

The highest mineralization observed at day 42 for the SF-PG samples, cultured under osteogenic conditions (Fig. 5b), could be related to the role of the poly-L-glutamate in the biomineralization process of bone [33]. The effect of poly-L-glutamic acid on the nucleation and growth of hydroxyapatite crystals has been shown [34]. Moreover, it has been reported that poly-L-glutamic acid is one of the primary residues of the acidic non-collagenous proteins associated with bone formation [35] and may have a key role in the biomineralization process of bone tissue [33,36]. The mechanism by which the SF/SF-PL induces hMSCs toward specific osteogenic differentiation may be related to the amino groups of the poly-L-lysine. A positive role of amino groups immobilized onto biomaterial surfaces in stimulating osteogenic differentiation of hMSCs has been reported previously [37–39]; furthermore, poly-L-lysine promotes osteoblast adhesion, spreading, and proliferation, when incorporated into poly(ethylene glycol)-poly(L-lactide) copolymers [40], and induces osteoblastic differentiation of hMSCs cultured in poly-L-Lysine-treated 3D titanium-6-aluminium-4-vanadium materials [41].

In order to investigate the adipogenic differentiation of the hMSCs encapsulated in the SF/SF-ionomer hydrogels, lipid accumulation was assessed by Oil red O staining and microscopic examination and by human leptin release over two months. Representative images are provided of encapsulated hMSCs stained with Oil red O after 3/4 and 6/8 weeks

of culture, respectively, in adipogenic or maintenance media (Figs. 6 and 7). The cells encapsulated within the SF/SF-PG hydrogels cultured in adipogenic medium show lipid droplets accumulation after 3 weeks (Fig. 6b) and then the droplets became larger and more prevalent during the remaining period until the last point of the analysis (Figs. 6h, 7b, and 7h). In contrast, for the first 4 weeks no lipid droplets were observable in the cells encapsulated in the other hydrogels, even if cultured in adipogenic differentiation medium (Fig. 6). Lipid accumulation in cells encapsulated in SF hydrogels started after 6 weeks (Fig. 7a), showing an increase of the size and the extent of the droplets during the residual time of the analysis (Fig. 7g). In cells encapsulated in SF/SF-PL hydrogels, only a few and small lipid droplets were observed at week 6 and 8 (Figs. 7c and i). For the cells cultured in the absence of the adipogenic cocktail, no lipid accumulation was observed over time for the all cell-gel constructs investigated (Figs. 6 and 7). These results suggest that SF-PG ionomer induces lipid accumulation earlier and to a greater extent compared to that observed for SF hydrogels, which apparently delay adipogenic differentiation. On the other hand, SF-PL ionomer inhibited lipid accumulation, with a delay in formation and smaller lipid droplets (Figs. 7c and i). These results are in agreement with the ALP activity pattern observed in adipogenic media (Fig. 4a).

Leptin, a cytokine released by preadipocytes and mature adipocytes, is a specific marker of adipogenic differentiation [42]. In this study, leptin release from hMSCs encapsulated in SF and SF/SF-ionomer hydrogels was monitored for the entire period of analysis (Fig. 8). Leptin levels for the cells cultured under adipogenic conditions were significantly higher (Fig. 8a) than the corresponding values revealed in uninduced cultures (Fig. 8c): in particular, for the cells encapsulated in SF/SF-PG hydrogel, leptin concentration significantly increased at day 42, reaching the maximum value at the last time point of the analysis, thus suggesting that the adipogenic-differentiating cells were continuing to mature over time. A similar pattern was observed for the cells encapsulated in SF hydrogels, with a peak at day 42, and a subsequent decrease until the end of the analysis. Conversely, cells encapsulated in SF/SF-PL hydrogels showed a similar leptin release trend until day 28, followed by a significant drop in concentration over time (Fig. 8a). The 2D control hMSCs differentiated in adipogenic media showed a typical pattern for leptin release, with levels significantly higher (Fig. 8b) than that observed for the corresponding encapsulated cells (Fig. 8a). This large difference in leptin release observed between the investigated samples and the 2D controls may be due to the interactions of the hormone with the hydrogels. This hypothesis was supported by the fact that the encapsulation of hMSCs within SF/SF-ionomer with lower polymer concentrations, such as 0.4% SF and 0.4% SF blended with 0.125% SF-PL or SF-PG hydrogels (data not shown), exhibited leptin release which was almost an order of magnitude higher than the corresponding 2D controls after 8 weeks (Fig. 9). The crosslink density of the hydrogels obtained by sonication of SF solutions depends on the starting polymer concentration [19,21]. It is probable that the smaller pores of the hydrogels obtained from 2% SF-based solutions impeded the diffusion of leptin through the networks, so release from the cells cannot be accurately quantified. Even if the leptin release data does not accurately reflect the adipogenic differentiation pattern, the lipid accumulation results indicate a different effect of the hydrogels on hMSCs adipogenic differentiation. The encapsulation in SF/SF-PG hydrogel improved hMSCs adipogenic differentiation, while SF hydrogel allowed delayed adipogenic differentiation, as demonstrated by the delay in lipid accumulation observed for the latter. Moreover, all the adipogenic markers were low for SF-PL sample, thus suggesting an inhibiting role for this ionomer on adipogenesis.

All of the results indicate a different performance of stem cells if encapsulated within SF or SF-PL or SF-PG based hydrogels. In particular, hMSCs encapsulated in SF 2% hydrogels exhibited long term viability and proliferation (Fig. 2); moreover, if cultured in the presence of osteogenic or adipogenic induction media were able to differentiate into the respective

lineage, while in the absence of differentiation media they retained their proliferative mesenchymal potential up to 8 weeks, as suggested from the spindle shape (Fig. 3i) and from the poor level of the differentiation markers exhibited until 8 weeks (Figs. 4c, 5c and 7j). The encapsulation of hMSCs within SF/SF-PG hydrogels sustained long term cell proliferation and, in the presence of the proper soluble factors, osteogenic or adipogenic differentiation. It seems that SF-PG has a positive effect on adipogenic differentiation, showing a more rapid formation and larger lipid droplets than the other samples (Fig. 6b). The hMSCs encapsulated in SF/SF-PL showed a different performance: undergoing osteogenic differentiation under induction conditions, the cells also exhibited significant osteoblast differentiation behavior even if cultured without induction medium; in contrast, the cells exhibited poor adipogenic differentiation in the three media conditions. This is an important result, considering the mutual correlation between the adipogenic and osteogenic differentiation of hMSCs and the need for regenerative materials, which would inhibit adipogenic, while stimulating the osteogenic differentiation of the cells, or vice-versa [10].

The observed differential effects of the ionomers on hMSC fate may be due to the grafted charged polymeric chains, which can interfere with the interactions between the cells and the extracellular matrix components [43]. Although the use of proper soluble factors is the most established method to regulate stem cell differentiation in vitro, recent findings support a central role for the 3D environment on cell function [3–7,9]. In particular, the promotion or activation of hMSCs differentiation toward a specific lineage induced from biomaterial functional groups is an active area of research, with numerous recent studies highlighting the importance of complex interaction between the biomaterial, the cells and the matrix [3,9,38,43–44]. A potential mechanism by which different functional groups belonging to the biomaterial affect stem cell differentiation may be related to a differential activation of intracellular signaling, followed by the promotion of the differentiation into specific lineages [9]. The biomaterial-related events triggering different signals, that ultimately affect cell differentiation, can be due to selective sequestration of cell-secreted soluble factors by the material functional groups [43], or the biomaterial can specifically adsorb some serum components that interact with cell receptors for the activation of specific differentiation pathways [39,43]. Another mechanism could be related to the different ability of the various biomaterials to form an optimal hydrophobic/hydrophilic balance in the three-dimensional biomaterial-matrix system, which is important for promoting proper cell-matrix interactions, ultimately affecting cell differentiation [44].

In previous studies we investigated the role of hMSCs biophysical properties on function [24–25]. Our findings suggested that the osteogenic and adipogenic differentiating hMSCs have a hyperpolarized transmembrane potential (V_{mem}), compared with undifferentiated hMSCs; moreover, we demonstrated that the control of the V_{mem} can be used to control differentiation [24]. The mechanism by which the variations of the V_{mem} are transduced into specific intracellular signaling, thus regulating the cell behavior, is still unclear but likely involves calcium influx [8,25]. Considering the important role of the V_{mem} of the stem cells on their fate [8,24–25], the effect of the silk ionomers on hMSCs differentiation could be also related to mechanisms associated to biophysical properties and V_{mem} . The effect of poly-lysine in modifying the V_{mem} of different types of cells, via various mechanisms, has been reported [45–48]. For example, by the interaction with the ryanodine receptors, poly-lysine can regulate calcium permeation of cardiac sarcoplasmic reticulum [45]. Moreover, poly-lysine and other polycations altered the V_{mem} of tracheal smooth-muscle cells, via interaction with specific K-channels [46]. Therefore it is reasonable hypothesize that one of the mechanisms involved in terms of the effect of SF-PL on hMSCs differentiation could be related to changes in V_{mem} .

4. Conclusions

A cytocompatible method for in situ hMSC encapsulation within charged or neutral silk fibroin-based hydrogels was studied. All the hydrogels provided suitable three-dimensional platforms for hMSCs long-term maintenance and osteogenic or adipogenic differentiation, when cultured in the proper differentiation media. Moreover, the silk ionomers exhibited different effects on the stem cells differentiation: silk fibroin-poly-L-lysine ionomer hydrogels selectively induced osteogenic differentiation in the presence, and even in the absence, of osteogenic stimuli, but not under adipogenic induction. In contrast, in adipogenic media, silk fibroin-poly-L-glutamic ionomer provided a suitable environment for specific adipogenic differentiation. These results can provide new input to the understanding of the relationships between biomaterial physico-chemical properties and stem cell fate, and enlarge the potential for the use of silk fibroin based-materials in more tailored and specific tissue engineering strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999; 284:143–7. [PubMed: 10102814]
2. Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *J Cell Mol Med*. 2004; 8:301–16. [PubMed: 15491506]
3. Discher DE, Mooney DJ, Zandstra PW. Growth factors, matrices, and forces combine and control stem cells. *Science*. 2009; 324:1673–7. [PubMed: 19556500]
4. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell*. 2006; 126:677–89. [PubMed: 16923388]
5. McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell*. 2004; 6:483–95. [PubMed: 15068789]
6. Lutolf MP, Gilbert PM, Blau HM. Designing materials to direct stem-cell fate. *Nature*. 2009; 462:433–41. [PubMed: 19940913]
7. Jha AK, Xu X, Duncan RL, Jia X. Controlling the adhesion and differentiation of mesenchymal stem cells using hyaluronic acid-based, doubly crosslinked networks. *Biomaterials*. 2011; 32:2466–78. [PubMed: 21216457]
8. Levin M. Large-scale biophysics: ion flows and regeneration. *Trends Cell Biol*. 2007; 17:261–70. [PubMed: 17498955]
9. Phadke, A.; Chang, CW.; Varghese, S. Functional biomaterials for controlling stem cell differentiation. In: Roy, K., editor. *Studies in mechanobiology, tissue engineering and biomaterials. Biomaterials as stem cell niche* Springer; Berlin Heidelberg: 2010. p. 19-44.
10. Liu D, Yi C, Zhang D, Zhang J, Yang M. Inhibition of proliferation and differentiation of mesenchymal stem cells by carboxylated carbon nanotubes. *ACS Nano*. 2010; 4:2185–95. [PubMed: 20218664]
11. Nicodemus GD, Bryant SJ. Cell encapsulation in biodegradable hydrogels for tissue engineering applications. *Tissue Eng Part B*. 2008; 14:149–65.

12. Altman GH, Diaz F, Jakuba C, Calabro T, Horan RL, Chen J, et al. Silk-based biomaterials. *Biomaterials*. 2003; 24:401–16. [PubMed: 12423595]
13. Hofmann S, Knecht S, Langer R, Kaplan DL, Vunjak-Novakovic G, Merkle HP, et al. Cartilage-like tissue engineering using silk scaffolds and mesenchymal stem cells. *Tissue Eng*. 2006; 12:2729–38. [PubMed: 17518642]
14. Wang Y, Rudym DD, Walsh A, Abrahamsen L, Kim HJ, Kim HS, et al. In vivo degradation of three-dimensional silk fibroin scaffolds. *Biomaterials*. 2008; 29:3415–28. [PubMed: 18502501]
15. Zhang Y, Wu C, Friis T, Xiao Y. The osteogenic properties of CaP/silk composite scaffolds. *Biomaterials*. 2010; 31:2848–56. [PubMed: 20071025]
16. Etienne O, Schneider A, Kluge JA, Bellemin-Lapponnaz C, Polidori C, Leisk GG, et al. Soft tissue augmentation using silk gels: an in vitro and in vivo study. *J Periodontol*. 2009; 80:1852–8. [PubMed: 19905955]
17. Sundelacruz S, Kaplan DL. Stem cell- and scaffold-based tissue engineering approaches to osteochondral regenerative medicine. *Semin Cell Dev Biol*. 2009; 20:646–55. [PubMed: 19508851]
18. Ishida M, Asakura T, Yokoi M, Saito H. Solvent- and mechanical-treatment-induced conformational transition of silk fibroins studies by high-resolution solid-state carbon-13 NMR spectroscopy. *Macromolecules*. 1990; 23:88–94.
19. Kim UJ, Park J, Li C, Jin HJ, Valluzzi R, Kaplan DL. Structure and properties of silk hydrogels. *Biomacromolecules*. 2004; 5:786–92. [PubMed: 15132662]
20. Yucel T, Cebe P, Kaplan DL. Vortex-induced injectable silk fibroin hydrogels. *Biophys J*. 2009; 97:2044–50. [PubMed: 19804736]
21. Wang X, Kluge JA, Leisk GG, Kaplan DL. Sonication-induced gelation of silk fibroin for cell encapsulation. *Biomaterials*. 2008; 29:1054–64. [PubMed: 18031805]
22. Serban MA, Kaplan DL. pH-Sensitive ionomeric particles obtained via chemical conjugation of silk with poly(amino acid)s. *Biomacromolecules*. 2010; 11:3406–12. [PubMed: 21028849]
23. Ye C, Shchepelina O, Calabrese R, Drachuk I, Kaplan DL, Tsukruk VV. Robust and responsive silk ionomer microcapsules. *Biomacromolecules*. 2011; 12:4319–25. [PubMed: 22050007]
24. Sundelacruz S, Levin M, Kaplan DL. Membrane potential controls adipogenic and osteogenic differentiation of mesenchymal stem cells. *PLoS One*. 2008; 3:e3737. [PubMed: 19011685]
25. Sundelacruz S, Levin M, Kaplan DL. Role of membrane potential in the regulation of cell proliferation and differentiation. *Stem Cell Rev*. 2009; 5:231–46. [PubMed: 19562527]
26. Murphy AR, St John P, Kaplan DL. Modification of silk fibroin using diazonium coupling chemistry and the effects on hMSC proliferation and differentiation. *Biomaterials*. 2008; 29:2829–38. [PubMed: 18417206]
27. Matsumoto A, Chen J, Collette AL, Kim UJ, Altman GH, Cebe P, et al. Mechanisms of silk fibroin sol-gel transitions. *J Phys Chem B*. 2006; 110:21630–8. [PubMed: 17064118]
28. Moreau J, Anderson K, Mauney JR, Nguyen T, Kaplan DL, Rosenblatt M. Tissue-engineered bone serves as a target for metastasis of human breast cancer in a mouse model. *Cancer Res*. 2007; 67:10304–8. [PubMed: 17974972]
29. Zhang W, Wang X, Wang S, Zhao J, Xu L, Zhu C, et al. The use of injectable sonication-induced silk hydrogel for VEGF₁₆₅ and BMP-2 delivery for elevation of the maxillary sinus floor. *Biomaterials*. 2011; 32:9415–24. [PubMed: 21889205]
30. Bruedigam C, van Driel M, Koedam M, van de Peppel J, van der Eerden BCJ, Eijken M, et al. Basic techniques in human mesenchymal stem cell cultures: differentiation into osteogenic and adipogenic lineages, genetic perturbations, and phenotypic analyses. *Curr Protoc Stem Cell Biol*. 2011; 17:1H.3.1–1H.3.20.
31. Bianco P, Costantini M, Dearden LC, Bonucci E. Alkaline phosphatase positive precursors of adipocytes in the human bone marrow. *Brit J Haematol*. 1988; 68:401–3. [PubMed: 3377984]
32. Ali AT, Penny CB, Paiker JE, Psaras G, Ikram F, Crowther NJ. The relationship between alkaline phosphatase activity and intracellular lipid accumulation in murine 3T3-L1 cells and human preadipocytes. *Anal Biochem*. 2006; 354:247–54. [PubMed: 16750158]

33. Thula TT, Svedlund F, Rodriguez DE, Podschun J, Pendi L, Gower LB. Mimicking the nanostructure of bone: comparison of polymeric process-directing agents. *Polymers*. 2011; 3:10–35. [PubMed: 22328971]
34. García-Ramos JV, Carmona P. The effect of some homopolymers on the crystallization of calcium phosphates. *J Crys Growth*. 1982; 57:336–42.
35. Fisher LW, Torchia DA, Fohr B, Young MF, Fedarko NS. Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. *Biochem Biophys Res Commun*. 2001; 280:460–5.
36. Chi C, Shi Y, Zheng H, Zhang Y, Chen W, Yang W, et al. Biomineralization process of calcium phosphate: modulation of the poly-amino acid with different hydroxyl/carboxyl ratios. *Mater Chem Phys*. 2009; 115:808–14.
37. Curran JM, Chen R, Hunt JA. Controlling the phenotype and function of mesenchymal stem cells in vitro by adhesion to silane-modified clean glass surfaces. *Biomaterials*. 2005; 26:7057–67. [PubMed: 16023712]
38. Curran JM, Chen R, Hunt JA. The guidance of human mesenchymal stem cell differentiation in vitro by controlled modifications to the cell substrate. *Biomaterials*. 2006; 27:4783–93. [PubMed: 16735063]
39. Phillips JE, Petrie TA, Creighton FP, García AJ. Human mesenchymal stem cell differentiation on self-assembled monolayers presenting different surface chemistries. *Acta Biomater*. 2010; 6:12–20. [PubMed: 19632360]
40. Peng H, Xiao Y, Mao X, Chen L, Crawford R, Whittaker AK. Amphiphilic triblock copolymers of methoxy-poly(ethylene glycol)-*b*-poly(L-lactide)-*b*-poly(L-lysine) for enhancement of osteoblast attachment and growth. *Biomacromolecules*. 2009; 10:95–104. [PubMed: 19063715]
41. Galli D, Benedetti L, Bongio M, Maliardi V, Silvani G, Ceccarelli G, et al. In vitro osteoblastic differentiation of human mesenchymal stem cells and human dental pulp stem cells on poly-L-lysine-treated titanium-6-aluminum-4-vanadium. *J Biomed Mater Res A*. 2011; 97:118–26. [PubMed: 21370441]
42. Gregoire FM. Adipocyte differentiation: from fibroblast to endocrine cell. *Exp Biol Med* (Maywood). 2001; 226:997–1002. [PubMed: 11743135]
43. Benoit DSW, Schwartz MP, Durney AR, Anseth KS. Small functional groups for controlled differentiation of hydrogel-encapsulated human mesenchymal stem cells. *Nature Mater*. 2008; 7:816–23. [PubMed: 18724374]
44. Ayala R, Zhang C, Yang D, Hwang Y, Aung A, Shroff SS, et al. Engineering the cell-material interface for controlling stem cell adhesion, migration, and differentiation. *Biomaterials*. 2011; 32:3700–11. [PubMed: 21396708]
45. Yano M, Yamamoto T, Kohno M, Hisaoka T, Ono K, Tanigawa T, et al. Polylysine-induced rapid Ca^{2+} release from cardiac sarcoplasmic reticulum. *J Cardiovasc Pharm*. 1998; 32:96–100.
46. Oshiro T, Sasaki T, Nara M, Tamada T, Shimura S, Maruyama Y, et al. Suppression of maxi-K channel and membrane depolarization by synthetic polycations in single tracheal myocytes. *Am J Respir Cell Mol Biol*. 2000; 22:528–34. [PubMed: 10783123]
47. Katsu T, Tsuchiya T, Fujita Y. Dissipation of membrane potential of *Escherichia coli* cells induced by macromolecular polylysine. *Biochem Biophys Res Commun*. 1984; 122:401–6. [PubMed: 6378203]
48. Reuveni M, Lerner HR, Poljakoff-Mayber A. Changes in membrane potential as a demonstration of selective pore formation in the plasmalemma by poly-L-lysine treatment. *Plant Physiol*. 1985; 79:406–10. [PubMed: 16664422]

Fig. 1.

Representative results of Pico Green assay performed on hMSCs encapsulated within SF, or SF/SF-PL or SF/SF-PG hydrogels, cultured in: a) adipogenic, b) osteogenic and c) maintenance growth media. The asterisks ** indicate significant differences between 6 weeks and 2/3 weeks data ($p<0.005$).

Fig. 2.

Representative results of MTS assay performed on hMSCs while encapsulated within SF 2%, SF 2% + SF-PL 0.5% and SF 2% + SF-PG 0.5% hydrogels, or cultured in 2D (controls), in a) adipogenic, b) osteogenic and c) maintenance growth media. Significant differences are indicated by one * ($p < 0.05$) or two asterisks ** ($p < 0.005$).

Fig. 3. Representative images of hMSCs stained as live (Calcein AM/green) or dead (EthD/red), after 8 weeks of culture in adipogenic (a, b, c, d), osteogenic (e, f, g, h) and maintenance growth media (i, j, k, l), while encapsulated in SF 2% (a, e, i), SF 2% + SF-PL 0.5% (b, f, j) and SF 2% + SF-PG 0.5% (c, g, k) hydrogels, or cultured in 2D (d, h, l).

Fig. 4. ALP activity (U/L, normalized to 10^3 cells per sample) for hMSCs encapsulated in SF 2%, SF 2% + SF-PL 0.5% and SF 2% + SF-PG 0.5% hydrogels, or cultured in 2D in: a) adipogenic, b) osteogenic and b) regular maintenance media. The asterisk * indicates significant statistical difference ($p < 0.05$).

Fig. 5.

Calcium concentration (mM Ca^{++} , normalized to 10^3 cells per sample) for hMSCs encapsulated in SF 2%, SF 2% + SF-PL 0.5% and SF 2% + SF-PG 0.5% hydrogels, or cultured in 2D in: a) adipogenic, b) osteogenic and c) maintenance growth media. One asterisk * indicates the significant statistical differences between the samples at day 42 ($p < 0.05$), and two asterisks ** indicate the significant statistical differences between the value of each sample at different times in b and c, or between SF/SF-PL and the other samples at day 28, 42 and 56 in c ($p < 0.005$).

Fig. 6. Representative images of hMSCs stained with Oil red O after 3 (a–f) or 4 weeks (g–l) of culture in adipogenic (a–c and g–i) or maintenance growth media (d–f and j–l), while encapsulated in SF 2% (a, d, g, j), SF 2% + SF-PL 0.5% (b, e, h, k) and SF 2% + SF-PG 0.5% (c, f, i, l) hydrogels.

Fig. 7.

Representative images of hMSCs stained with Oil red O after 6 (a–f) or 8 weeks (g–l) of culture in adipogenic (a–c and g–i) or maintenance growth media (d–f and j–l), while encapsulated in SF 2% (a, d, g, j), SF 2% + SF-PL 0.5% (b, e, h, k) and SF 2% + SF-PG 0.5% (c, f, i, l) hydrogels.

Fig. 8.

Human leptin release (pg/mL, normalized to 10^5 cells) from hMSCs while encapsulated in SF 2%, SF 2% + SF-PL 0.5% and SF 2% + SF-PG 0.5% hydrogels, or cultured in 2D in adipogenic (a, b) or maintenance growth media (c). The asterisks indicate significant statistical difference * ($p < 0.05$) and ** ($p < 0.005$).

Fig. 9.

Human leptin release (pg/mL, normalized to 10^5 cells) from hMSCs while encapsulated in SF 0.4%, SF 0.4% + SF-PL 0.125% and SF 0.4% + SF-PG 0.125% hydrogels, or cultured in 2D in adipogenic medium. The asterisks indicate significant statistical difference ** ($p < 0.005$).