Novel soy protein scaffolds for tissue regeneration: Material characterization and interaction with human mesenchymal stem cells

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1. Introduction

Tissue engineering involves the fabrication of constructs which aid in the repair and regeneration of damaged tissue, providing proper structure, function and integration with the host tissue. One frontier of tissue engineering lies in using a biomaterial scaffold to deliver cell-based therapy. Porous scaffolds provide three-dimensional microenvironments, which can mimic the extracellular matrix and can allow for cell infiltration and space for matrix deposition by cells to form new tissue. An ideal scaffold material should stimulate the formation of tissue which is structurally and functionally robust, while being safe and cost-efficient to obtain, process and manufacture [1,2]. The use of natural proteins to form biomaterials is an attractive therapy because of the ability of the natural material to control stem cell adhesion and growth through inherent binding sites. Human mesenchymal stem cells (hMSC) seeded on collagen and silk protein scaffolds have been shown to proliferate and differentiate into osteoblasts and chondrocytes that were fully functional, biocompatible and able to form tissues resembling native tissue structure and function [3,4].

Soy protein, an isolated component of the soybean, has recently emerged as an attractive alternative to animal-derived protein sources for biomedical applications. The US has led the world production of soybeans for over 50 years, generating 81 million metric tons in 2008 [5]. Soybeans are a natural and abundant resource, which contains 40% pure protein [6]. Two major subunits in the globular structure of soy protein include conglycinin (7S) and glycinin (11S), which contain all amino acids but are rich in glutamate, aspartate and leucine [6]. Soy protein exhibits versatility in processing and is shown to have good biodegradable and biocompatible qualities [7–13].

Different processing strategies of soy protein have been developed to alter its material properties. Thermal and chemical modifications have the capability of tailoring bulk and surface properties during the fabrication of soy structures [10,12]. Heat treatment of soy protein has been shown to induce thermoplasticity, which allows a wide variety of shapes and structures to be formed, including films, granules/pellets and gels [9,13–15]. Glyoxal and tannic acid have been used to extend degradation times of extruded soy protein pellets [12], and soy protein films cross-linked with varying amounts of formaldehyde were capable of controlling model drug release [13]. Transglutaminase, a physiological enzyme used to fabricate novel, porous three-dimensional scaffolds through lyophilization. Physical properties of scaffolds were characterized using scanning electron microscopy, mercury intrusion porosimetry, moisture content analysis and mechanical testing. Human mesenchymal stem cells (hMSC) were seeded and cultured in vitro on the scaffolds for up to 2 weeks, and changes in stem cell growth and morphology were examined. The resulting scaffolds had rough surfaces, irregular pores with size distributions between 10 and 125 μm, <5% moisture content and compressive moduli ranging between 50 and 100 Pa. Enzyme treatment significantly lowered the moisture content. Increasing amounts of applied enzyme units lowered the median pore size. Although enzyme treatment did not affect the mechanical properties of the scaffolds, it did increase the degradation time by at least 1 week. These changes in scaffold degradation altered the growth and morphology of seeded hMSC. Cell proliferation was observed in scaffolds containing 3% soy protein isolate treated with 1 U of transglutaminase. These results demonstrate that controlling scaffold degradation rates is crucial for optimizing hMSC growth on soy protein scaffolds and that soy protein scaffolds have the potential to be used in tissue engineering applications.

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enzyme, has been applied to soy protein in the food industry to modify the textures and mechanical properties of gels [14–16]. The enzyme facilitates the reaction of the γ-carboxamide group of a glutamine side chain with the ε-amino group of lysine side chain to form a γ-(ε-glutamyl) lysine linkage (Fig. 1). Previous research suggests that microbial transglutaminase can increase the mechanical stability of soy protein scaffolds by forming intermolecular or intramolecular covalent bonds [14–16].

Exploration of the in vitro and in vivo biocompatibility of soy, albeit limited, shows promise for the use of soy protein as a biomaterial for drug delivery and tissue engineering purposes [8,10,13,17,18]. Soy curd containing protein and all other soybean components was shown to decrease the level of proinflammatory cytokine production of mononuclear cells from human peripheral blood and to promote osteoblast proliferation [9]. The in vitro biocompatibility of soy protein alone has been explored with l929 mouse fibroblasts in membranes and fiber forms [7,8,10]. For all studies, soy protein substrates were able to sustain cell viability, with no cytotoxic effects. Soy protein blends with cellulose and chitosan were also shown to be biocompatible [7,17,19].

The behavior of cells within three-dimensional soy protein constructs has not yet been explored. This is the first study to investigate the use of three-dimensional porous soy protein scaffolds for tissue engineering applications. The overall aims were: (1) to successfully fabricate soy protein into a porous, three-dimensional scaffold structure; (2) to investigate the effect of transglutaminase and maltodextrin modification on the material properties of soy protein scaffolds; and (3) to determine how scaffold properties affect hMSC growth and proliferation. hMSC are precursors to tissue-forming cells such as osteoblasts, chondrocytes and myocytes and can be obtained from an autologous source [20,21]. Understanding the basic interaction between hMSC and the soy protein scaffold provides insight into developing a potentially safer, lower-cost and more effective biomaterial system which delivers cell therapy for tissue regeneration.

2. Methods and materials

2.1. Scaffold fabrication

Soy protein isolate (SPI) containing ~83% pure soy protein (as verified by bicinchoninic acid analysis from Thermo Fisher Scientific (Rockford, IL, USA)) was obtained from Now Sports (Bloomingdale, IL, USA). Mixtures of 3 and 5 wt.% SPI were dissolved in Millipore water and were homogenized at 5000 rpm for 5 min. The slurries were heated at 90 °C for 1 h. Upon cooling of the solutions to room temperature, glycerol from Sigma–Aldrich (St. Louis, MO, USA) was added in the same weight percentage as SPI. The slurry was homogenized again at 5000 rpm for 5 min. Slurries were cast in 7 cm diameter aluminum weigh boats at volumes of 18 ml and 13 ml for 3 and 5 wt.% SPI solutions, respectively. ACTIVA TI microbial transglutaminase containing maltodextrin from Ajinomoto (Fort Lee, NJ, USA) was added to individual slurries in 1 and 20 U of enzyme activity to 1 g of SPI. The added ACTIVA TI formulation included 1 wt.% transglutaminase and 99 wt.% maltodextrin. Maltodextrin (DE = 4.0–7.0) from Sigma–Aldrich (St. Louis, MO, USA) was added in the same weight to gram protein percentage as 1 U transglutaminase as separate control samples. The slurries were incubated at 37 °C for 1 h.

All slurries were freeze-dried using a VirTis AdVantage BenchTop lyophilizer (Gardiner, NY) via a three-step process: ramping down temperature, solidification and sublimation. The lyophilizer temperature was lowered from 20 to −15 °C at a rate of 0.5 °C min⁻¹ to cool down the slurries (Fig. 2A). The slurries were solidified by holding the lyophilizer temperature constant at −15 °C for 5 h (Fig. 2A). Ice from the water within the slurries was sublimed at 0 °C using a pressure of 100 mtorr for at least 40 h to create a porous scaffold structure. In summary, 3% and 5% SPI heat treatment (HT) and maltodextrin (MD) control groups as well as 1 and 20 U transglutaminase-crosslinked groups (TG 1 U and TG 20 U) were fabricated (Fig. 2B).

2.2. Material characterization

2.2.1. Scaffold microstructure and porosity

Both dry and water-hydrated scaffolds were imaged using scanning electron microscopy (SEM). Dry scaffolds were desiccated for at least 2 h prior to imaging. Hydrated scaffolds were first dehydrated in 95% ethanol for 3 h then rinsed in water for 30 min, and the scaffolds immersed in water were lyophilized prior to imaging. Cross sections of all scaffolds were obtained through liquid nitrogen fracture and coated with 9 nm of osmium. SEM was performed (n = 2 per group) using a LEO Gemini 1525 FEG SEM with an acceleration voltage of 15 kV (Oberkochen, Germany).
to observe the scaffold microstructure. Mercury intrusion porosimetry from Micromeritics (Norcross, GA) was used to determine the volume percentage porosity for all groups (n = 3) using a previously described method [22].

2.2.2. Moisture content

Moisture retention in scaffolds was analyzed immediately upon fabrication (n = 5). Scaffold moisture content was determined for discs 12 mm in diameter using a previously described method [23]. Briefly, the initial weight upon casting (W₀) and weight after drying under vacuum at 40 °C for 24 h (Wₚ) was used to calculate the moisture content (MC) using the formula MC = [(W₀ − Wₚ)/W₀] × 100, immediately after lyophilization.

2.2.3. In vitro degradation

Scaffolds were dehydrated in 95% ethanol for 3 h and rinsed for 30 min in phosphate buffered saline (PBS) containing calcium and magnesium from HyClone (Logan, UT, USA). Scaffolds 7 mm in diameter (n = 4) were punched and subsequently weighed after lyophilization, then incubated at 37 °C in PBS or phenol red free Dulbecco’s Modified Eagle’s Medium (DMEM) media supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution, all from Invitrogen (Carlsbad, CA, USA). Scaffolds were qualitatively checked for robustness with forceps every 3 days.

2.2.4. Compressive mechanical properties

Compression testing was performed using a mechanical tester from JLW Instruments (Chicago, IL, USA). Scaffolds were punched using a 7 mm biopsy punch, and the diameter was measured three times using calipers, and averaged for calculation of stress. Samples were compressed at 0.2 mm min⁻¹ up to 45% strain, with a preload of 0.1 N. The scaffold compressive modulus was determined by calculating the best fit slope of the linear (elastic) regime starting from the lowest strain outside the toe region (0.8% strain) to R² = 0.9. An average compressive modulus was determined for all scaffolds (n = 4–5 per group). Both dry and water-hydrated scaffolds (prepared by 3 h of 95% ethanol immersion and 30 min of rinsing in water) were tested.

2.3. In vitro cell culture

2.3.1. Cell seeding

hMSC (Lonza) were grown in hMSC basal media containing mesenchymal stem cell growth supplement, L-glutamine, and penicillin/streptomycin all from Lonza (Walkersville, MD, USA). Cells were grown to ~90% confluence and trypsinized with 0.05% trypsin-EDTA from Invitrogen (Carlsbad, CA, USA). Passage 5 cells were used for seeding. Scaffolds were sterilized in 95% ethanol for 3–4 h and rinsed three times with PBS. The samples were briefly dried after the third rinse on 11 mm filter paper and immediately incubated at 37 °C for at least 30 min prior to seeding. hMSC were top seeded with 200,000 cells per scaffold and were allowed to adhere to the scaffolds for 10 min at 37 °C before adding media to the wells. Seeded cells were cultured in phenol red free DMEM media supplemented with 10% FBS and 1% antibiotic/antimycotic solution all from Invitrogen (Carlsbad, CA, USA) in a 37 °C, 5% CO₂ humidified environment. Scaffolds were cultured for 1, 7 and 14 days.

2.3.2. Cell morphology and viability

At weekly time points, cell morphology and viability studies were performed using chemicals all acquired from Invitrogen (Carlsbad, CA, USA). Live/Dead analysis was performed (n = 2 per group per time point) using the Live/Dead assay kit, staining live cells with calcein and dead cells with ethidium bromide. Stained cells and the protein scaffold were visualized using an LSM Meta 510 CLSM from Zeiss (Jena, Germany). Cell-seeded scaffolds for SEM imaging (n = 2) were fixed in a 3% sucrose and 2% glutaraldehyde aqueous solution for 3 h, immersed in 70% ethanol overnight, and subsequently dehydrated in graded ethanol and critical point dried prior to imaging using the methods described in Section 2.2.1. DNA quantification was performed (n = 4–5 per group per time point) using a Picogreen assay kit following the manufacturer’s protocol.

2.3.3. Scaffold degradation

Scaffolds with cells were harvested at days 1, 7 and 14 (n = 4–5 per group per time point). At each harvested time point, seeded scaffold weights were determined by measuring dry weight after lyophilization. The relative weight fraction was calculated by normalizing to day 1 values.

2.4. Statistical analysis

The quantitative data were reported as mean ± standard error mean (SEM = standard deviation/n¹/²). One-way analysis of variance (ANOVA) was performed with the treatment group as the fixed factor. P values <0.05 were considered statistically significant. Two-way ANOVA was performed for DNA quantification data, where the two fixed factors were group and time point. Independent t-tests assuming unequal variance were performed between each combination of treatment groups to reduce the likelihood of type I errors.

3. Results

3.1. Scaffold microstructure and porosity

The thickness of all 3% and 5% SPI scaffolds ranged from 1.7 to 1.9 mm and 2.2 to 2.4 mm, respectively. Observations of scaffold microstructure using SEM revealed rough surfaces of scaffold struts (Fig. 3). All scaffolds exhibited irregular pore shapes. Struts of non-hydrated structures appeared thicker compared with hydrated scaffolds. The struts of hydrated scaffolds were thinned along the edges. In the TG 20 U scaffolds, precipitates formed on the surfaces of the protein struts (Fig. 3D). This phenomenon was not observed in the TG 1 U and MD control scaffolds.

All scaffold pore sizes were normally distributed in the range 10–125 μm (Fig. 4). Both 3% and 5% scaffolds followed the same distribution trend with varying treatments. The cumulative volume percentage of pore sizes ranging from 125 to 1000 μm was <5%. All MD control scaffolds yielded a higher median pore diameter. The pore size distribution was wider for MD control scaffolds compared with the HT control scaffolds. The median pore diameter decreased with increased enzymatic crosslinking. Total porosity of scaffolds was consistently >80%, with no significant difference as protein content increased. The 5% SPI TG 20 U group had significantly lower porosities compared with all other groups (P < 0.01) except when compared with the similar 3% group, and 5% SPI TG 1 U scaffolds resulted in significantly different porosity compared with the MD control group (P < 0.05).

3.2. Moisture content

Scaffolds retained 2–5% moisture content (Fig. 5). ANOVA demonstrated that increasing the percentage of soy from 3% to 5% significantly decreased the moisture content for every treatment (P < 0.005). The 3% SPI TG 20 U scaffolds had a significantly lower moisture content compared with all other 3% SPI groups (P < 0.005). Although increasing the amount of enzymatic
crosslinking significantly decreased moisture content for 3% SPI scaffolds, this effect was not significant for 5% SPI scaffolds.

3.3. Compressive mechanical properties

Scaffolds under compression demonstrated viscoelastic behavior. Compressive moduli for dry scaffolds within each treatment group increased as the protein content increased from 3% to 5% (Fig. 6). TG 1 U and TG 20 U groups significantly increased the modulus for 5% SPI scaffolds only \((P < 0.05)\). No difference in modulus was observed when the number of enzyme units applied was increased. Upon hydration, the compressive modulus significantly decreased for all 5% SPI groups \((P < 0.001)\) and for the 3% SPI TG 20 U group \((P < 0.005)\). The 3% MD control scaffolds had higher moduli compared with enzyme-treated scaffolds when hydrated. The 5% SPI TG 1 U scaffolds had the highest moduli compared with all other 5% SPI groups.

3.4. Cell growth and scaffold degradation

Qualitative observations of scaffold robustness using forceps to maneuver the structure revealed that scaffolds soften when hydrated, yet remain intact. Non-seeded scaffolds decomposed over time into many fragments that were not recoverable or able to be lifted out of the well. Comparisons across scaffold groups between the first time points at which scaffolds could not be recovered showed that, in general, scaffolds degraded faster in PBS compared with media, except for the 3% SPI HT control group (Table 1). Cells seeded onto scaffolds allowed the scaffolds to remain intact for a longer time. Enzyme treatment increased time to complete degradation for both 3% and 5% SPI scaffolds, especially for scaffolds in media. Cell-seeded scaffolds remained intact longer with enzyme treatment compared with control groups for 3% SPI scaffolds.

Scaffolds supported cell attachment and cell viability for the 2 week period of this study (Fig. 7). In the HT control group, the amount of DNA significantly increased from day 1 to day 7 and then decreased at day 14 for the 3% SPI scaffolds. At day 14, the amount of DNA for this group was significantly less for 3% SPI scaffolds compared with 5% SPI scaffolds. There was no significant change in DNA content for all MD control scaffolds. In the 3% SPI TG 1 U group, cells proliferated with a threefold increase in DNA content over 2 weeks. The DNA content was constant over 2 weeks for the 5% SPI scaffolds. At day 14, the amount of DNA for the TG 1 U group was significantly higher for the 3% SPI scaffolds compared with 5%SPI scaffolds. In the TG 20 U group, DNA content decreased from day 1 to day 7 and increased from day 7 to day 14 for both 3% and 5% SPI scaffolds.

Cell morphology changed over time as cell growth occurred on and into the surface of the scaffolds, as observed using Live/Dead staining and imaging with confocal fluorescence microscopy (Fig. 8). Blank scaffolds did not yield any Live/Dead fluorescence signal upon staining. However, protein autofluorescence remained visible upon UV excitation (see Supplementary Fig. S1). Observations of 3% SPI scaffolds showed that cells either formed a cell sheet or integrated into the scaffold pores. Cells were spread thinly on the protein scaffold for both control groups (HT and MD control groups) at days 1 and 7, but formed cell layers at day 7 for the TG 1 U group and at all time points for the TG 20 U group. After 2 weeks, the cells had integrated into the pores for the HT control group and the TG 1 U group to a depth of \(~100\) \(\mu m\) (representative images highlighted using red boxes in Fig. 8). A layer of cells covering the outer surface of the scaffold (cell sheet formation) was observed on both the MD control group and the TG 20 U group. The confluent cell sheet appeared denser for the TG 20 U group compared with all other cell sheets observed. For the 5% SPI scaffolds, all groups showed cells that gathered on the same plane at the surface instead of penetrating into the pores of the scaffold (Fig. 9). Cells seeded on HT and MD control...
groups formed surface patches instead of sheets, owing to low cell density (Fig. 9A and B). SEM surface images of 5% SPI TG 1 U scaffolds confirmed the elongated cell morphology and growth pattern observed in the Live/Dead staining, with high cell density all along the scaffold surface and edges (Fig. 9E and F). Other scaffold groups were not robust enough to maintain structure upon fixation for SEM analysis. Comparison of the relative weight fractions of the scaffolds with the Live/Dead images revealed that the image corresponding to the 3% SPI scaffolds which had degraded more than 10% in culture depicted cells integrating into the pores (corresponding images in Figs. 8 and 9). A cell sheet or cell surface patches formed for scaffolds which had minimal degradation, with a relative weight fraction <0.9, representing <10% weight loss (Figs. 8–10). All 5% SPI cell seeded scaffolds maintained integrity, with TG 1 U and TG 20 U groups yielding <10% weight loss over 2 weeks (Fig. 10B). The 5% SPI HT and MD control groups appeared to have gained weight, with relative weight fractions >1 (Fig. 10B).

4. Discussion

Freeze-drying is a fabrication technique used to form three-dimensional porous materials out of collagen and other natural biopolymers [24–26]. Although soy protein is globular, this work demonstrates that heat treatment applied to protein slurries was able to induce interconnected, porous, three-dimensional scaffolds upon freeze-drying. Protein chains relax upon heating, and a degree of denaturation allows proteins to entangle in solution. The denaturation temperature of the 7S and 11S subunits is dependent on the water content of the slurry. Higher water content and lower protein concentration decreases the denaturation temperature of
both subunits; the ideal denaturation temperature to avoid decomposition is 90 °C [27]. Soy protein concentrations <3 wt.% could not retain structure upon hydration, and concentrations >5 wt.% sustained viable cells, but did not yield proliferation (data not shown). Glycerol and water were added as plasticizers to decrease the slurry glass transition temperature, which increased the elasticity of the resulting freeze-dried scaffold. The porosity of the scaffolds between 80% and 95% (Fig. 4) was typical for scaffolds fabricated using freeze-drying [28]. The moisture content was in the same range (5% moisture; Fig. 5) as previously reported soy protein materials [11]. Although the mechanical strength was on the order of Pa, all scaffolds were robust enough to support cell attachment, and strength did not significantly diminish when scaffolds were in the hydrated state (Fig. 6). Differences in hydrated compressive moduli between groups can be attributed to protein struts on the scaffold surface dissolving away from the scaffold upon compression (Fig. 6B). Degradation of protein scaffolds in PBS occurred faster than in media due to the differences in electrostatic interactions (Table 1). It is observed that, in general, the MD control groups degraded faster in PBS compared with other groups (Table 1). This may indicate that electrostatic interactions of maltodextrin with the PBS salts may lead to faster dissolution of the scaffold walls. Addition of transglutaminase increased the degradation time slightly as specific crosslinking occurred, with degradation times longer for TG 20 U groups compared with TG 1 U groups (Table 1). Overall, HT control scaffold groups degraded slower than scaffolds containing maltodextrin (either alone or with transglutaminase) in PBS, which as mentioned previously may be due to the strong interactions between the maltodextrin and salts. Salts in PBS can also interact with the negatively charged soy pro-

<table>
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<th>Bare scaffolds in PBS (days)</th>
<th>Bare scaffolds in media (days)</th>
<th>Cell-seeded scaffolds in media (days)</th>
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<tr>
<td>3% SPI HT control</td>
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<td>MD control</td>
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<td>TG 1 U</td>
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<td>5% SPI HT control</td>
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Fig. 7. DNA quantification per scaffold over various time points, with n = 4–5: (A) HT control scaffolds; (B) MD control scaffolds; (C) TG 1 U scaffolds; (D) TG 20 U scaffolds. *P < 0.05; **P < 0.01.
tein\textsuperscript{[29]} to break up amino acid chains. This may result in faster degradation for the 5\% SPI HT compared with the 3\% SPI HT control group, owing to the greater concentration of scaffold proteins. When scaffolds are immersed in media, serum proteins present in solution may adhere to the scaffold soy proteins and provide stability and resistance to degradation. Therefore, an increase in the amount of soy protein concentration may have increased the stability of the scaffold when immersed in media (Table 1). When cells were present, attached cell processes acted like crosslinks and pulled protein struts together, which increased the degradation time for all scaffold groups, particularly for control groups, and helped maintain scaffold structural integrity over time (Table 1).

Maltodextrin and transglutaminase did not greatly widen the range of the compressive strength, moisture content or crosslink density in the soy protein scaffolds. However, the additives had a significant effect on pore size distributions, degradation properties and biological activity of seeded cells. From the resulting SEM images, maltodextrin appeared to have leached from the protein scaffold walls, since attached particles are seen on the walls of the hydrated scaffolds (Fig. 3C and D). The precipitates were present in large quantities in the TG 20 U scaffolds and in smaller, yet visible amounts in the MD control and TG 1 U groups, but were not observed in the HT control scaffolds (Fig. 3). Although the precipitates were visible upon hydration of the scaffold, the porosity of MD control groups are not significantly different from the HT control groups, owing to the relatively small size of the leached particulates (<1 \textmu m) compared with the bulk scaffold pore sizes (Figs. 3D and 4A). The intensity of protein autofluorescence was diminished for the MD control scaffolds in culture, owing to warping of the

\textbf{Fig. 8.} Representative fluorescence microscopy images at the scaffold surface of representative 3\% SPI scaffolds with Live/Dead staining: blue = protein scaffold; green = live cells; red = dead cells; (A) HT control scaffolds; (B) MD control scaffolds; (C) TG 1 U scaffolds; (D) TG 20 U scaffolds. Left column: day 1 scaffolds. Scale bar = 200 \textmu m. Middle column: day 7 scaffolds. Scale bar = 200 \textmu m. Right column: confocal fluorescence three-dimensional reconstruction of scaffold surface. Grid line equals 90.4 \textmu m. Images in red boxes indicate the scaffolds which have a relative weight fraction of <0.9.
scaffold structure (Figs. 8B and 9B). However, the weight of the scaffolds was maintained throughout culture, showing that scaffolds can soften or warp but maintain mass. Increased amounts of maltodextrin decreased degradation times for scaffolds, owing to leeching of maltodextrin from the protein struts, but the degradation time was slowed by enzyme crosslinking. Enzyme treatment successfully increased the degradation time of the scaffolds; scaffolds containing transglutaminase with maltodextrin degraded more slowly than scaffolds containing the same amount of maltodextrin only (Table 1).

The chemical interactions between the additives with soy protein may have affected cell behavior. The cell layer formation in MD control scaffolds in absence of proliferation (Figs. 7B, 8B and 9B) may be due to cellular digestion of the scaffold and the interaction of cells with the maltodextrin chains. Transglutaminase crosslinking on native type I collagen has been shown previously to promote greater long-term growth, survival and proliferation of human foreskin dermal fibroblast cells compared with non-crosslinked collagen [30]. The bioactivity of hMSC on soy protein scaffolds also improved with the 3% SPI TG 1 U group. Cells seeded on all 3% SPI and 5% SPI TG groups appeared denser, more elongated and aligned compared with cells seeded on HT and MD control groups (Figs. 8 and 9). However, proliferation was not observed on any of the TG 20 U groups or for the 5% SPI TG 1 U group (Fig. 7C and D). Since additives alone did not consistently stimulate cell proliferation on the scaffolds, it is speculated that scaffold degradation was equally important in controlling the growth and morphology of hMSC on soy protein scaffolds.

Cell morphology was varied across the 3% SPI scaffold groups over the 2 week culture period. HT control scaffolds resulted in sparse cell integration (Fig. 8), and the MD control and TG 20 U groups both formed cell sheets (Fig. 8B and D). Cell growth locations for all 5% SPI scaffold groups were similar; cells gathered on the surface. Cell integration within scaffold pores was consistently observed when the relative weight fractions were <0.9, whereas cell sheet or patch formation occurred when relative weight fractions were >0.9 (Figs. 8–10). Lower relative weight fractions indicate greater weight loss of the scaffold (lower resulting scaffold weights). Hence, as scaffold degradation increased, cell integration occurred. High relative weight fractions indicate minimal degradation (relative weight fractions >0.9). Relative weight fractions >1 may indicate minimal scaffold degradation along with matrix synthesis by the cell sheets/patches on the surface of the scaffold. The comparison of relative weight fractions between groups for 3% and 5% SPI scaffolds confirmed that cell morphology and growth behavior into pores or on the surface of the scaffold is influenced by degradation of the soy protein scaffolds (Figs. 8–10).

Porous scaffold microstructure and surface roughness sustained cell viability and spreading for soy protein scaffolds, similar to silk natural protein scaffolds [31]. However, only the cells seeded on 3% SPI TG 1 U group proliferated (Fig. 7). The significant increase in DNA from day 1 to day 14 was similar to proliferation observed on

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**Fig. 9.** (A–D) Confocal fluorescence three-dimensional reconstruction of scaffold surface of representative 5% SPI scaffolds with Live/Dead staining at day 14 and (E, F) representative SEM images of 5% SPI scaffold surface. The cell morphologies observed at this time point for all groups are representative for all time points. For confocal images: blue = protein scaffold; green = live cells; red = dead cells. Grid line equals 90.4 μm. (A) HT control scaffolds; (B) MD control scaffolds; (C) TG 1 U scaffolds; (D) TG 20 U scaffolds; (E) 5% SPI TG 1 U scaffold surface showing cell sheet covering scaffold surface, scale bar = 200 μm; (F) zoomed-in SEM image of area boxed in yellow in (E), scale bar = 50 μm.
silk scaffolds with a similar range of interconnected pore sizes between 20 and 100 μm [32]. Although the cell morphology of the 3% SPI TG 1 U group was similar to HT control, the relative weight fraction uniquely decreased from >0.9 to <0.9 between day 7 and day 14. Optimal strut relaxation and degradation for the 3% SPI TG 1 U group over 2 weeks may have allowed surrounding nutrients to penetrate and flow better through the pores to stimulate cell growth on and within the scaffold. Scaffolds with slower degradation rates limit cell spreading to the exposed surface area when full confluence is reached. Large pore sizes >300 μm were found to be optimal for cell penetration into collagen scaffolds [33], and soy protein scaffold pore sizes were generally <150 μm. The presence of a greater number of cells at earlier time points for all TG 20 U groups (Figs. 7D and 8D) indicated that cells may have become overcrowded. Overcrowded cells remain viable but cannot proliferate; the cells are limited to spreading on the scaffold surface when full confluence is reached, instead of integrating into the depth of the scaffold. These results reiterate the importance of balancing scaffold degradation kinetics with cell proliferation and matrix secretion for tissue deposition [28]. Overall, a combination of degradation rate, scaffold microstructure and presence of additives were shown to affect cell growth and proliferation with the soy protein scaffold.

The ideal tissue engineering scaffold system requires that the material degrades at a rate in which cells can regenerate tissue to fill in pores. Cell integration is preferred over cell sheet or patch formation, since cells can grow into a three-dimensional space rather than being limited to confluence in a flat, two-dimensional plane. This demonstrated that enzyme treatment was capable of modifying the soy protein scaffold to achieve degradation rates optimal for hMSC proliferation. Future work in understanding how degradation rates in vivo differ from in vitro in the presence of other enzymes is crucial for better control of in vivo matrix deposition and tissue formation of hMSC.

5. Conclusion

SPI was assembled into three-dimensional porous structures via freeze-drying. The results from this study show that a range of physical and bioactive properties can be achieved with porous soy protein scaffolds through enzyme crosslinking and additive modifications. All fabricated scaffolds were robust enough to support hMSC viability. Although the addition of the enzyme crosslinker transglutaminase did not increase the mechanical strength of the scaffolds, the stability and degradation time of the scaffolds were increased. Maltodextrin alone hastened scaffold degradation. Cells attached, appeared elongated and grew on the surface and within the pores of the scaffolds near the surface. The 5% SPI scaffolds were stable in media, yet proliferation was not observed. The 3% SPI group with one unit of transglutaminase crosslinking promoted cell proliferation, with cells integrating into the scaffold after 2 weeks. Enzyme treatment of SPI is effective in controlling the degradation time of the scaffolds and optimizing cell growth. These results indicate that SPI in three-dimensional porous scaffold form has the potential for use in tissue engineering applications.

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Appendix A. Supplementary data


Appendix B. Figures with essential colour discrimination

Certain figures in this article, particularly Figure 10, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2011.09.036.

References


