Novel porous soy protein-based blend structures for biomedical applications: Microstructure, mechanical, and physical properties

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Abstract: Use of naturally derived materials for biomedical applications is steadily increasing. Soy protein has advantages over various types of natural proteins employed for biomedical applications due to its low price, nonanimal origin, and relatively long storage time and stability. In the current study, blends of soy protein with other polymers (gelatin, alginate, pectin, polyvinyl alcohol, and polyethylene glycol) were developed and studied. The mechanical tensile properties of dense films were studied in order to select the best secondary polymer for porous three-dimensional structures. The porous soy–gelatin and soy–alginate structures were then studied for physical properties, degradation behavior, and microstructure. The results show that these blends can be assembled into porous three-dimensional structures by combining chemical crosslinking with freeze-drying. The soy–alginate blends are advantageous over soy–gelatin blends, demonstrated better stability, and degradation time along with controlled swelling behavior due to more effective crosslinking and higher water uptake than soy–gelatin blends. Water vapor transmission rate experiments showed that all porous blend structures were in the desired range for burn treatment [2000–2500 g/(m² d)] and can be controlled by the crosslinking process. We conclude that these novel porous three-dimensional structures have a high potential for use as scaffolds for tissue engineering, especially for skin regeneration applications. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 008: 000–000, 2015.

Key Words: soy protein, natural polymers, gelatin, alginate, tissue regeneration

INTRODUCTION
Various natural, synthetic, and semisynthetic materials are currently being utilized in the fabrication of implantable scaffold devices.¹ There is a growing tendency to replace synthetic polymers for biomedical uses with natural, abundant, nonanimal origin, and low-cost biodegradable products such as soy protein.²,³ These plant proteins can be employed to meet the high demand for new materials needed for various tissue engineering applications. They have several advantages over animal proteins (e.g., collagen, fibrinogen, and silk), including lower immunogenicity. These plant proteins, therefore, have a great potential for use as biomaterials for medical applications.

Soy protein
Soy protein presents good water resistance, a low price, and storage stability and is also very versatile. The properties of soy protein can be modified by physical, chemical, or enzymatic treatments.⁴ This enables tailoring soy protein for the diverse requirements of different biomedical applications.

The suitability of soy protein for biomedical applications has recently been investigated, due to its abovementioned advantages and bioactive properties. Proposed applications range from bone cement⁵ to hydrogels,⁶ wound dressing membranes for promoting wound healing,⁷ and, more recently, drug delivery carrier films,⁷,⁸ temporary replacement implants, and tissue engineering scaffoldings.⁹

Soy protein and plant proteins, in general, have lower molecular weights than animal proteins and, as a result, are highly susceptible to enzymatic degradation in the human body. Chemical modifications such as crosslinking are, therefore, essential for achieving the necessary mechanical properties and for ensuring integrity and water stability of plant protein-based biomaterials during the implantation period.¹⁰ Crosslinking agents, including formaldehyde¹¹ and glutaraldehyde,¹² can form bridges between protein chains by reacting with the amino groups of lysine residues and lead to improvement of the mechanical and other properties of crosslinked soy protein isolate (SPI) films.

However, glutaraldehyde has been shown to be toxic even at a relatively low concentration, such as 3.0 ppm, when it is released into the host following biodegradation of biomaterials. Promising solutions for biomedical purposes are glyoxal,⁹ a dialdehyde with lower toxicity compared with similar agents, and water-soluble carbodiimide 1-ethyl-
Another approach to improving the physical properties of SPI materials is to blend the soy protein with different polymers. It is assumed that blending with polymers results in materials with better performance than their separate components. To date, SPI have been blended with various plasticizers and biodegradable polymers such as polysaccharides, proteins, and synthetic polymers in order to achieve the desired properties. Additional natural polymers include sodium alginate, cellulose, chitin, chitosan, gluten, whey protein isolate, and gelatin. Despite numerous studies on composite films based on soy protein, there have been only a few reports on the preparation of porous three-dimensional SPI-based blend structures. Furthermore, there are no published studies on the preparation of chemically crosslinked porous soy-natural polymer blend structures for biomedical applications. Our proposed novel porous soy-based blend structure provides a biostable and cost-effective nonanimal origin solution for biomedical applications and for cell delivery systems in tissue regeneration.

Additional natural polymers used in this study
In the current study, we assumed that combining soy protein with other proteins and polysaccharides and synthetic polymers may result in physical and chemical interactions that may improve the material’s properties and biological performance for better cell affinity. Gelatin and alginate were selected for this study as secondary components due to their suitable range of mechanical properties and bioactive characteristics, as well as their proven history as biomaterials. The polysaccharide pectin was also chosen for examination, because it has recently been reported that it promotes cell adhesion, thus stimulating cell growth and proliferation, and for due to its physical properties, which resemble those of alginate. However, despite their many advantages, these types of biomaterials suffer from the same limitation as other biodegradable polymers, namely uncontrolled rate of degradation and antigenic potential. After crosslinking with EDC or glyoxal, the soy-natural polymer hybrid material will become even more biostable and resistant to degradation, due to the formation of new stable covalent bonds and higher molecular weight, as desired for porous structures. Finally, we chose freeze-drying as our fabrication method for new soy protein blend materials, based on an attempt to create biomimetic three-dimensional structures. It is important to note that the various blends are composed mainly of soy protein, with a relatively small amount of gelatin or polysaccharide (pectin and alginate).

The main goal of this research was to develop and study novel porous soy protein-based blend structures as potential new materials for biomedical applications. These new materials can be used either as an acellular matrix (e.g., wound dressings) or as a carrier system for cells or active biomolecules in the field of tissue engineering, especially for skin and cartilage.

MATERIALS AND METHODS
Materials
Primary component. Soy protein source: Non-GMO SPI (Solpro 910TM; minimum 90% wt/wt protein, on dry basis) was obtained from SolbarTM (Ashdod, Israel) as a donation.

Additional natural and synthetic polymers. Gelatin “type A” from porcine skin (90–100 and 300 bloom) was purchased from Sigma-Aldrich, Rehovot, Israel (G6144 and G2500, respectively). Pectin from apple (76282), alginic acid sodium salt [viscosity ~250 cps, 2% (25°C); A2158], polyethylene glycol (PEG; MW 35,000), and polyvinyl alcohol (PVA; MW 23,000) were purchased from Sigma-Aldrich.

Crosslinkers. N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride, a water-soluble carbodiimide (EDC) E7750, and glyoxal (50649) were purchased from Sigma-Aldrich. Sodium chloride was purchased from Merck (1064041000).

Preparation of dense films for the first step of the study
The effects of a secondary polymer on the mechanical properties of dense blend films were studied. The films were prepared using the solvent-casting method as described elsewhere. The formulation and process are presented in Table I. After completing this step, selected secondary polymers were chosen for the rest of the study (on porous blend structures).

Preparation of porous soy protein-based blend structures
Soy protein-gelatin and soy protein–alginate (or pectin) blends were prepared by hydrating the second component at room temperature for 20 min in double-distilled water at a concentration of 5 wt %. For all blends, a final soy protein concentration of 5 wt % was reached by slowly suspending the soy protein powder under constant stirring in double-distilled water. The soy protein slurry and the second component solution were then mixed together to obtain a series of blends with a final soy protein content of 5 wt % and second biopolymer content of 12, 25, and 50 wt % relative to the SPI (8:1, 4:1, and 2:1 weight compositions, respectively). All blend preparation parameters are presented in Table II.

In order to create porous structures, a crosslinker solution containing EDC or glyoxal in double-distilled water (1 or 4 wt % relative to the soy protein) was cast into a 3-cm diameter Petri dish. The crosslinked samples were then washed three times with saline. Finally, the porous soy protein-based blend samples were obtained by freezing at −40°C for 4 h followed by freeze-drying at −20°C for 18–24 h.

Tensile mechanical properties
The tensile mechanical properties were measured in a dry state, at room temperature, using a 5500 Instron universal testing machine (Instron Engineering, Corp.) with 2-kN load cell, under unidirectional tension at a rate of 50 mm/min according to the standard method ASTM D638-03. The samples were cut into a dumbbell-shape (neck length 2 cm,
The maximal strain was defined as the breaking strain. Five samples were tested for each specimen. Means and standard deviations (SDs) were calculated using Excel.

### Water vapor transmission rate (WVTR)

The moisture permeability of the porous structures was determined by measuring the water vapor transmission rate (WVTR) through the material. A Sheen Payne permeability cup with an exposure area of 10 cm² was filled with 5 mL double-distilled water and was then covered with a circular sponge \((n = 3\) specimens per group). The cup was placed in an upright position inside an oven, which contained 1 kg of freshly dried silica gel at 37°C. The weight of the assembly was measured every hour and a graph of the evaporated water versus time plotted. The WVTR was calculated according to Eq. (1):

\[
\text{WVTR} = \frac{\text{slope} \times 24}{\text{area}} \left( \frac{g}{m^2 \times \text{day}} \right)
\]

### Water uptake

Round specimens (1.5 cm average diameter) of untreated and porous crosslinked soy structures \((n = 3\) specimens per group) were preweighed separately and immersed in a saline solution, pH 7, at 37°C. The weight of the samples was measured after 30 min, 1 h, 2 h, and up to 2 days, by removing the saline and blotting them gently to remove excess fluid with a filter article. The water uptake was calculated using Eq. (2):

\[
\text{Water uptake} (%) = \frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{dry}}} \times 100
\]

### In vitro degradability study

The degradability behavior of the porous soy-based blend structures was studied by both quantitative and qualitative methods.

#### Quantification of noncovalent soluble protein by 10 M urea

Urea in concentrations up to 10 M is a powerful protein denaturant since it disrupts their noncovalent bonds. This property can be exploited to increase proteins’ solubility. A degradation test was performed by immersing the soy-based samples in urea (10 M) for up to 2 days. An aliquot of each sample’s supernatant was taken at different time points. The quantity of the soluble protein was analyzed using the Bradford protein assay. A calibration curve for correlating absorbance at 595 nm to protein content was prepared using soy protein and bovine serum albumin (BSA). The calibration curve was then used to calculate the soluble protein content in the sample. The assay was performed in triplicate.

#### In vitro qualitative degradation

Porous soy protein blend structures of 30 mm diameter \((n = 3\) specimens per group) were placed in a 30-mm diameter Petri dish, and the samples were immersed into 4.0 mL Dulbecco’s Modified Eagle’s Medium (DMEM) at pH 7.4, supplemented with 1% PEN+STREP Nystatin solution, all from Biological Industries, and then incubated at 37°C for up to 21 days. Samples were qualitatively checked for robustness every 3 days, using a tweezers.

### Microstructure of porous blends

The structure of dry porous structures was observed using an environmental scanning electron microscope (ESEM). The cryogenically fractured surfaces were Au-sputtered before observation. Surfaces of selected samples were observed using a Quanta 200 FEI ESEM in a high vacuum mode with an acceleration voltage of 10 kV \((n = 2\) per group). The approximate porosity of the observed

<table>
<thead>
<tr>
<th>TABLE I. The Formulation and Parameters of Preparation of the Dense Blend Films Used in the First Step of the Study</th>
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<tbody>
<tr>
<td>Parameters</td>
</tr>
<tr>
<td>Primary component</td>
</tr>
<tr>
<td>Secondary component</td>
</tr>
<tr>
<td>Concentrations of secondary component</td>
</tr>
<tr>
<td>Temperature of the aqueous solution (°C)</td>
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<td>pH and buffer solutions</td>
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<tr>
<th>TABLE II. The Formulation and Parameters of Preparation of the Soy–Natural Polymer Blend Porous Structures</th>
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<tbody>
<tr>
<td>Parameter</td>
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<tr>
<td>Soy protein concentration</td>
</tr>
<tr>
<td>Natural polymer</td>
</tr>
<tr>
<td>Soy protein:natural polymer ratio</td>
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<tr>
<td>Temperature of soy protein dispersion in double-distilled water</td>
</tr>
<tr>
<td>Temperature of soy protein–natural polymer mixture</td>
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<tr>
<td>Crosslinking agent and concentration</td>
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</table>
morphologies was analyzed using the Scandium software. Porosity was determined as the area occupied by the pores divided by the total area.

Statistical analysis
All data were processed using Excel software. Statistical comparison between more than two groups was performed using the ANOVA (Tukey Kramer) method via SPSS (version 15) software. A value of $p < 0.05$ was considered statistically significant. All errors and error bars indicate the SD from the mean.

RESULTS
Mechanical properties of dense soy protein blend films
The first step in this study was to evaluate the effect of a secondary polymer on the mechanical properties of dense soy protein films. Our assumption was that nonporous soy protein blend film compositions with improved mechanical properties (compared with a reference soy protein film) would be able to maintain mechanical stability also as porous constructs. They could thus serve as the basic selected formulation for the second phase of the study.

Our results showed that the polymer mixture obtained from the 4:1 soy protein:natural polymer composition produced the most suitable blend mixtures, which yielded suitable porous structures. We, therefore, chose this ratio as the preferred composition. In order to choose the most suitable secondary biopolymers, we prepared dense blend films containing soy protein and a secondary biopolymer at a 4:1 weight ratio and measured their tensile properties compared with those of the soy protein reference film. The results are presented in Figure 1.

Reference films prepared from the pH 7 solution resulted in a tensile strength of 5.67 Mpa. The addition of low-molecular weight (LMW) gelatin did not show a significant change in the strength and maximal strain, while addition of high-molecular weight (HMW) gelatin and alginate increased the tensile strength to 7.71 and 7.82 Mpa, respectively. The addition of the synthetic polymers PVA and PEG to the soy protein films decreased the tensile strength of the soy protein films, while addition of alginate drastically decreased the maximal strain of the soy protein films. Addition of PVA and PEG had almost no effect on the maximal strain. Based on the tensile strength results of the dense films, HMW gelatin and alginate were chosen as secondary polymers for the porous structures.

Physical properties
Water vapor transmission rate. WVTR was measured for various types of noncrosslinked and crosslinked porous soy-based blend formulations, and the results are presented in Figure 2. Evaporative water loss through the various porous soy-based blend formulations was linearly dependent on time ($R^2 \geq 0.99$ in all cases, results not shown), resulting in a constant WVTR. Noncrosslinked samples exhibited WVTR values in the range of 2500–2700 g/(m² d), with no significant difference. The WVTR of an exposed aqueous surface was also determined as control. Crosslinking of the soy–alginate substantially increased its WVTR, while crosslinked porous soy–gelatin and pure soy protein structures remained unchanged.

Water uptake. The fluid adsorption capacity was studied on blends containing soy protein and a second component (gelatin or alginate) in relative quantities of 4:1 (wt/wt). Crosslinked samples were used to measure the fluid absorption capacity over time and the results presented in Figure 3(a). The glyoxal crosslinked soy–gelatin and EDC crosslinked...
soy–alginate exhibited a similar water uptake pattern, as follows:

a. A rapid initial water uptake within the first 30 min, reaching the maximum swelling.

b. A decrease in water content during the following 2 h.

c. Constant water uptake between 2 and 25 h.

The water uptake values of the soy–gelatin blends during stages (a) and (b) were higher than those obtained for the soy–alginate blends [Figure 3(a)].

The EDC crosslinked soy–gelatin blends displayed a steady increase in water content over time until equilibrium was achieved after 2 h [Figure 3(a)]. The final water uptake (after 25 h) of this blend was significantly lower than that of the glyoxal crosslinked soy–gelatin or of the EDC crosslinked soy–alginate blends [Figure 3(a) and Table III].

In order to elucidate the effect of crosslinking on the water uptake of the blends, the water uptake of the three types of soy-based blends was compared with that of non-crosslinked blends. The result for the soy–gelatin blend is presented as an example in Figure 3(b). The noncrosslinked blend exhibited rapid water uptake, reaching a value of 435% after 30 min, followed by a decrease in water uptake during the following 2 h, probably due to weight loss of the soluble noncrosslinked fraction. After an additional hour, it was completely dissolved. In contradistinction, the EDC crosslinked soy–gelatin blend exhibited an increase in water uptake with time, reaching an equilibrium after 2 h. It is important to note that the pure crosslinked and noncrosslinked soy samples, as well as noncrosslinked soy–alginate blends, practically dissolved within a very short time of 30 min (results not shown).

The dimensional increase of the crosslinked blends due to water uptake is presented in Table III. It should be noted that the gelatin-containing blends exhibited a greater increase in diameter compared with the alginate-containing blend.

In vitro degradation tests.

a. Quantification of noncovalent soluble protein

Various types of soy blends were immersed in 10 M urea. The in vitro degradation rate was determined by detecting the soluble protein content in water supernatants over time using the Bradford assay. As shown in Figure 4, the crosslinked soy–gelatin [Figure 4(a)] and soy–alginate [Figure 4(b)] samples were significantly less soluble in 10 M urea than their noncrosslinked counterparts. The effect of the EDC concentration on the degree of soy protein solubility of both types of samples are also presented in Figure 4(a,b). Both types of samples showed a tendency toward a decrease in protein solubility, <10% soluble protein, with an increase in the EDC concentration of up to 4 wt %. The in vitro degradation profiles of the soy blend samples crosslinked with 1 wt % EDC are presented in Figure 5. The solubility curve of pure soy protein was higher than that of the soy–gelatin and soy–alginate blends. Minimum solubility was observed for the soy–alginate group.

b. Qualitative in vitro degradation

The degradation time of soy–natural polymer blends at various time points was determined qualitatively by observing robustness using forceps to maneuver the structure. The results are presented in Tables IV and V.

**FIGURE 3.** Water uptake over time (wt %) for: (a) porous crosslinked soy blends using 1 wt % crosslinker: ▲, soy–gelatin crosslinked with EDC; ▼, soy–alginate crosslinked with EDC; ●, soy–gelatin crosslinked with glyoxal. (b) ◡, Noncrosslinked soy–gelatin blend; ●, porous crosslinked soy–gelatin blend, using 1 wt % crosslinker. Data represent the average ± standard deviation of three independent samples. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**TABLE III.** Maximal Water Uptake for Crosslinked (1 wt %) Soy Porous Blends After 24 h and the Dimensional Increase During Swelling After 48 h of Incubation, for Crosslinked Soy Porous Blends

<table>
<thead>
<tr>
<th>Dimensional Increase (Diameter, %)</th>
<th>Maximum Water Uptake (wt %)</th>
<th>Crosslinked Soy Porous Blend</th>
</tr>
</thead>
<tbody>
<tr>
<td>27.2 ± 1.9⁰</td>
<td>308.5 ± 14.2⁰</td>
<td>Soy–gelatin (1 wt % EDC)</td>
</tr>
<tr>
<td>26.5 ± 1.7⁰</td>
<td>609.0 ± 51.4</td>
<td>Soy–gelatin (1 wt % glyoxal)</td>
</tr>
<tr>
<td>18.6 ± 1.0</td>
<td>429.4 ± 51.1⁰</td>
<td>Soy–alginate (1 wt % EDC)</td>
</tr>
</tbody>
</table>

Results marked with the same lowercase letter are not significantly different (p > 0.05).
In order to evaluate the effect of the natural additive concentration on degradation time, a series of cross-linked soy-based blends containing soy protein:natural polymer at ratios of 2:1, 4:1, and 8:1 were prepared. The results for the soy–alginate blends are presented as an example in Table IV. Qualitative robustness observations revealed that a higher alginate concentration in the soy–alginate samples resulted in a decreased degradation rate. Two types of crosslinking agents were used on soy–gelatin samples in order to study the effect of crosslinking agents on degradation time: 1 wt % glyoxal and 1 wt % EDC solutions. Qualitative robustness observations revealed that crosslinked soy–gelatin samples with EDC remained intact and maintained their structure for a longer time (14 days) than samples crosslinked with glyoxal (7 days) (Table V). The noncrosslinked 4:1 soy–natural polymer samples in both groups were completely degraded within 3 days.

Microstructure

Effect of the polymer concentration. Figure 6 shows ESEM fractographs for crosslinked pure soy protein [Figure 6(a,b)] and crosslinked 4:1 and 2:1 soy–gelatin blends [Figure 6(c,d) and 6(e,f), respectively]. A pure soy protein sponge exhibited an irregular porous structure, while the blends exhibited a more organized structure. The 4:1 blend exhibited a smaller and more uniform pore structure than the 2:1 blend.

Effect of the natural polymers. Figure 7 shows three different blends that were used in the current study (soy–gelatin, soy–alginate, and soy–pectin), all crosslinked with 1 wt % EDC and based on the 4:1 soy protein:natural polymer blend formulation. The three blends yielded different resultant matrix microstructures. Analysis of the blends under ESEM revealed a homogenous dispersion of the soy in the gelatin, alginate, and pectin at 4:1 ratios [Figure 7(b,d,f)]. Porous soy–alginate samples [Figure 7(c,d)] yielded a well-interconnected morphology compared with the porous soy–gelatin blend [Figure 7(a,b)]. The pore size and distribution were relatively similar for both soy–gelatin and soy–alginate samples. The total porosity of soy–gelatin and soy–alginate sponges was high (a porosity of approximately >70%). The achieved blend structures combine suitable porosity for skin tissue engineering applications with large pore size (approximately in the range of 100–300 μm). However, the porous structure was not observed for the soy–pectin blend, which presented a different structure than all other soy natural polymer groups, with significantly lower porosities than all other groups [Figure 7(e,f)].

Effect of the crosslinking agent. Figure 8 shows a cross-section of two types of crosslinking agents that were used on soy–gelatin blend samples based on a 4:1 ratio [1 wt % EDC, Figure 8(a), and 1 wt % glyoxal, Figure 8(b)]. Pore orientation structure was found in the soy–gelatin blend samples using 1% glyoxal as the crosslinking agent.
DISCUSSION

Blended soy protein-based materials that are chemically crosslinked with other natural polymers were developed and studied. Gelatin (protein) and alginate or pectin (polysaccharides) were added and chemically crosslinked to soy protein using the crosslinking agents EDC or glyoxal in order to obtain a new porous three-dimensional network through freeze-drying. After completing the first stage of the study, three formulations were selected in order to study their effects on materials characteristics with relation to skin tissue engineering applications.

Mechanical properties of the dense films

The average tensile strength and strain of normal human skin are 7.7 MPa and 100%, respectively. Good mechanical properties are crucial for routine handling and functional stability of biomedical products, especially for tissue engineered- porous construct, as it needs to provide an initial biomechanical profile for the cells before new tissue can be formed. Performance under tensile can affect the ultimate function of the product in vivo. Therefore, in the first step of the study, we compared the mechanical properties (ultimate tensile yield and maximal strain) of a series of dense soy protein blend films to those of the soy protein reference film in order to choose the best secondary components.

The addition of HMW gelatin and alginate to soy protein films at a 4:1 soy:gelatin or soy:alginate ratio showed improvement in tensile strength compared with the reference films. This can be explained by two possible factors: (i) the formation of physical and chemical interactions such as hydrogen bonds or other intermolecular attractions, that is, van der Waals; (ii) chain entanglement between the polymers.

Soy and gelatin have good compatibility, and both are proteins. In the soy–alginate blends, there is an increase in stiffness and brittleness as result of the presence of sugar rings in the polysaccharide chains that limit the flow and sliding movement of polymeric chains over each other. The addition of synthetic polymers such as PVA and PEG at the same ratio decreased the tensile strength. This may be due to a typical plasticizing effect, which causes weakening of the blend film. The plasticizer can interpose itself between the polymer chains and decrease the forces holding the chains together. Furthermore, it can be speculated that the synthetic polymers PEG and PVA might be less soluble, thus causing a decrease in the ability of soy protein to interact with polymer chains, which make them less compatible with soy protein, and they might be easily expelled from the blend mixture. It is also possible that they can crystalize, which could also cause phase separation.

Physical properties

Successful functional biomaterials should have adequate physical properties to be able to perform in a moist environment. For example, an ideal scaffold for wound healing must provide a control of fluid balance over time in order to enable accelerated skin regeneration and should absorb body fluid for transfer of cell nutrients and metabolites through the material. Two parameters must, therefore, be determined: the WVTR through the material and the water uptake ability. This was tested in order to elucidate the affinity of the porous structures to water and the effect of the addition of a second component and crosslinking on the structure’s behavior in an aqueous environment. The degradation behavior of the porous soy blend in aqueous solutions was also studied.

Water vapor transmission rate. An effective wound dressing provides good WVTR management that retains a moist wound bed at the desired levels for the healing course. An excessive WVTR may lead to wound dehydration, whereas a low WVTR might lead to maceration and bacterial contamination. An ideal dressing or graft would control the loss of water from the skin at an optimal rate. It has been claimed that burn wound dressings should ideally possess a WVTR in the range of 2000–2500 g/(m² d).

The WVTR is directly proportional to the degree of crosslinking and is related to the structural properties.
(thickness, porosity) and to the hydrophilicity of the material.\textsuperscript{28–30}

Our results show that all tested porous noncrosslinked soy blends demonstrate a WVTR in the range of 2500–2700 g/(m\textsuperscript{2} d) which is sufficient to prevent exudate accumulation and wound dehydration. These relatively high values, which match the demands for treating burns, are attributed to the hydrophilic nature of the protein polymer and polysaccharide used. Our results show that the WVTR practically does not change due to crosslinking for pure soy protein and soy–gelatin groups. This is probably related to the relatively low crosslinking density in these two cases. When the degree of crosslinking is low, porosity is high, allowing fast passage of water vapor through the material. With a high degree of crosslinking the structure is dense, which may limit the passage of water vapor through the material. Only crosslinking of soy–alginate substantially increased its water vapor resistance. Our WVTR results suggest that soy–alginate blends are more effectively crosslinked with EDC than soy–gelatin and pure soy protein. These results are in agreement with the degradation behavior study, which is presented later. The WVTR of our soy-based scaffolds is similar to the WVTR of commercially available skin graft.\textsuperscript{31}

**Water uptake.** Functional properties such as swelling and water holding capacity are directly related to the manner in which the polymer interacts with water and are usually proportional to the crosslinking density. Water absorption occurs as the hydrophilic polymer gradually absorbs water, followed by chain relaxation. Controlled swelling behavior is of great importance for various applications in the field of biomedical materials because it enables prediction of the materials’ behavior in the hydrated environment, which

\begin{figure}
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\includegraphics[width=\textwidth]{figure6.png}
\caption{ESEM fractographs of: (a,b) pure soy protein, (c,d) 4:1 porous soy–gelatin blends and (e,f) 2:1 porous soy–gelatin blends. All crosslinked using 1 wt % EDC.}
\end{figure}
exists in the human body. A scaffold that absorbs fluid to about 80 times of its initial weight is suitable as skin tissue engineering.\textsuperscript{32} As expected, crosslinked soy–gelatin samples enabled increased stability and decreased water absorption, compared with noncrosslinked soy–gelatin [Figure 3(b)]. Chemical crosslinking of gelatin introduces covalent bonds into the network, thereby decreasing the dissolution of the matrix. Soy–alginate had higher water uptake compared with soy–gelatin although not up to optimum value for skin regeneration scaffolds. These water adsorption results were not completely in accordance with the degradation study. It is usually expected that a higher crosslinking density (as expressed by the degradation study) results in lower water uptake during each of the stages, and enables reaching equilibrium, that is, constant water uptake, within a shorter period of time. An explanation for the deviating water adsorption results might be that soy–alginate scaffolds exhibit larger surface area due to the coarse porosity compared with soy–gelatin scaffolds (Figure 7).

The soy–gelatin crosslinked with glyoxal showed increased water adsorption compared with soy–gelatin crosslinked with EDC, probably due to higher porosity (>80%) and surface area (Figure 8). The changes in the dimensions of the samples as swelling progressed to equilibrium are demonstrated in Table III. The dimensional increases were in accordance with the crosslinking density. A higher crosslinking density prevents expansion and swelling of the network. Soy–alginate samples demonstrated a significant decrease in dimensional changes and in degradation rate compared with both types of soy–gelatin samples.

**In vitro degradation tests.** An ideal tissue engineering porous system requires that the material degrade at a rate at which cells can regenerate tissue to fill in pores. A
degradation time of 25 days is suitable for healing acute wounds (burn and skin excision)\textsuperscript{33} or about 8 weeks for chronic wounds (diabetic ulcer, pressure ulcer). A more rapid or slower biodegradation rate would render it ineffective as a wound closure device and would hinder the wound healing process.\textsuperscript{34} In order to improve our new soy-based material's stability toward biological, chemical, and physical degradations, we hoped to create synergy by combining two approaches: (1) blending soy protein with other natural polymers and (2) creating covalent bonds through chemical crosslinking. Two crosslinking agents suitable for proteins were used in this study. The crosslinking agent EDC creates conjugation between -COOH groups (in polysaccharides and proteins) and amine groups (lysine) in proteins to create stable amide bonds and provides stability and resistance to degradation, while the reactive dialdehyde glyoxal may react with \textepsilon-amino groups of lysine with the involvement of arginine residues, yielding advanced glycation or lipoxidation end products. It should be noted that glyoxal was used only for the soy–gelatin blends in an attempt to improve protein-to-protein crosslinking.\textsuperscript{35,36}

**a. Quantification of noncovalent soluble protein**

The degradability behavior of the porous soy blend was studied by quantifying the noncovalent soluble protein using 10M urea. The soluble protein was analyzed by the Bradford assay. The results indicate that a crosslinking reaction occurred between selected polymeric components that increase stability toward degradation. As the concentration of the crosslinking reagent increased, the material's stability also increased [Figure 4(a,b)]. The results also indicate that soy–alginate blends undergo a more effective crosslinking reaction than soy–gelatin in the presence of EDC (Figure 5). This probably occurs as a result of numerous -COOH groups that are available for reaction in the linear polysaccharide alginate. It is known from the literature that carboxyl groups are much more abundant in gelatin than amino groups, but they appear to be less reactive in crosslinking reactions.\textsuperscript{37} The in vitro degradation behavior of the soy blend samples could also be related to their corresponding crosslinking degree. It can be assumed from the results presented in Figure 5 that soy–alginate blends are more crosslinked than soy–gelatin blends and pure porous soy protein structures. It is important to mention that soy protein is globular, so that these reactive groups may not be as accessible due to the folding of the globular protein. Based on this result, it was worth trying to crosslink soy–gelatin with glyoxal (protein-to-protein crosslinking).

**b. Qualitative in vitro degradation**

The effect of prolonged hydration on the degradability of soy blend samples was evaluated qualitatively. This was done in order to assess the ability of the hydrated soy blend samples to hold structural stability, robustness, and integrity, which provide important information about handling the material in the hydrated state. Qualitative robustness observations revealed that the higher alginate concentration in the soy–alginate samples containing a 2:1 soy:alginate ratio remained intact for longer (21...
days) than samples containing a 4:1 soya-alginate ratio (14 days) and containing an 8:1 ratio (3 days) (Table IV). This is due to the higher molar concentration of the COOH groups in the polysaccharide alginate, which play a key role in protein crosslinking in the presence of EDC. It should be noted, however, that we selected 4:1 as the preferred ratio since it provides satisfactory material stability along with easy to use homogeneous mixtures. The results shown in Table V suggest that crosslinked soya-gelatin with EDC is more effective and resulted in a decreased degradation rate compared with samples crosslinked with glyoxal, due to the fact that EDC reacts via both lysine and COOH groups, whereas glyoxal reacts only via lysine groups. This possibility needs to be examined in order to confirm these results.

In conclusion, the physical properties of our soy protein-based blends demonstrated that crosslinking of soya-alginate is advantageous to the other studied blends due to its higher water vapor resistance and water absorption. The soya-alginate scaffolds also offer the advantage of relatively low dimensional changes. Furthermore, these blends remained intact and stable for longer than the soya-gelatin group and pure soy protein and would thus be more suitable to acute wound healing.

Microstructure

Effect of the polymer concentration. Soy–gelatin blends containing 50 wt % gelatin (2:1 soy:gelatin ratio) showed inconsistency in pore size, with areas of large and areas of small pores [Figure 6(e,f)]. When the gelatin concentration was increased, the soy–gelatin blend exhibited a high viscosity, which may result in nonuniform concentrations. Consequently, it favors the formation of protein aggregates, and even phase separation, leading to a lower efficiency of network formation. Thus, by changing the gelatin concentration, it became possible to tailor both the pore size and the pore distribution. In addition, as demonstrated in Figure 6, more defined pores were obtained with the increase in gelatin concentration compared with the pure porous soya protein structure [Figure 6(a,b)]. This was probably affected by the formation of a more structured and more rigid gel network before freeze-drying (it is known that gelatin begins to set at <15°C).

Effect of the natural polymers. Three natural polymers were used in the current study, and their effect on the microstructure was studied. All three soy blends showed a relatively uniform porous structure at the 4:1 soy:natural polymer ratio. The porosity of the soy–gelatin and soy–alginate blends was high, which is typical for scaffolds fabricated using freeze-drying resulting in pore diameters in the range of 100–300 μm [Figure 7(b,d)]. The pore structure of the soy–alginate samples showed greater connectivity than that of the soy–gelatin samples. High porosity and interconnected porous networks are desirable to permit the attachment and ingrowth of cells and regeneration of new tissue, by providing a component and a three-dimensional extracellular environment similar to that of native tissue, with a preferred pore size of 50–500 μm. In comparison with other studies, our porous soy-based scaffolds exhibited the most suitable pore size range for skin tissue regeneration. As expected, the nature of the interactions between the different secondary polymers and soy protein has a dramatic effect on the structural behavior and had a significant effect on the size and distribution of the pores.

Effect of the crosslinking agent. SEM micrographs [Figure 8(b)] showed that the porous soy-gelatin structures crosslinked with glyoxal possess a well-defined orientated microstructure. Water interactions with polymeric chains have a significant effect on network organization. The template of the arranged ice crystal assembly was imprinted in the soy–gelatin blends during the freeze-drying process. Our results on the effect of glyoxal on the morphology of the soy–gelatin blends are similar to the results obtained by Wu et al., who used glutaraldehyde (also a dialdehyde) as a crosslinking agent.

SUMMARY AND CONCLUSIONS

In the current study, we demonstrated that soy protein blends with gelatin, alginate, and pectin can be assembled into porous three-dimensional structures by combining chemical crosslinking with freeze-drying. The results of this study show that a range of physical properties and porous structures can be achieved through crosslinking and modifications achieved by adding natural polymers. The structure of the soy-based blend sponges was highly dependent on properties of the secondary component and on the soy protein:natural polymer ratios. The achieved blend structures combine suitable porosity with large pore size and adequate interconnectivity.

The preferred weight ratio of soy protein:natural polymer is 4:1, which presented a uniform solution and porous structures. The soy–alginate crosslinked blends are advantageous compared with soy–gelatin blends and showed better stability and degradation time along with controlled swelling behavior and lower water uptake, all obtained due to more effective crosslinking.

The WVTR experiments showed that the porous blend structures were all in the desired range for burn treatment [2000–2500 g/(m² d)] and can be controlled by the crosslinking process.

We conclude that these novel porous three-dimensional structures exhibit physicochemical properties that appear to have a potential for use as scaffolds in tissue engineering applications for skin regeneration. In addition, all crosslinked samples showed promising biocompatibility in the cell cytotoxicity study and remained intact in the presence of human fibroblasts. These results will be presented in a separate publication.

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