Novel soy protein wound dressings with controlled antibiotic release: Mechanical and physical properties

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ARTICLE INFO

Article history:
Received 31 March 2011
Received in revised form 26 July 2011
Accepted 24 August 2011
Available online 30 August 2011

Keywords:
Soy protein films
Water vapor transmission rate
Controlled drug delivery
Gentamicin

ABSTRACT

Naturally derived materials are becoming widely used in the biomedical field. Soy protein has advantages over various types of natural proteins employed for biomedical applications due to its low price, non-animal origin and relatively long storage time and stability. In the current study soy protein isolate (SPI) was investigated as a matrix for wound dressing applications. The antibiotic drug gentamicin was incorporated into the matrix for local controlled release and, thus, protection against bacterial infection. Homogeneous yellowish films were cast from aqueous solutions. After cross-linking they combined high tensile strength and Young's modulus with the desired ductility. The plasticizer type, cross-linking agent and method of cross-linking were found to strongly affect the tensile properties of the SPI films. Selected SPI films were tested for relevant physical properties and the gentamicin release profile. The cross-linking method affected the degree of water uptake and the weight loss profile. The water vapor transmission rate of the films was in the desired range for wound dressings (~2300 g m⁻² day⁻¹) and was not affected by the cross-linking method. The gentamicin release profile exhibited a moderate burst effect followed by a decreasing release rate which was maintained for at least 4 weeks. Diffusion was the dominant release mechanism of gentamicin from cross-linked SPI films. Appropriate selection of the process parameters yielded SPI wound dressings with the desired mechanical and physical properties and drug release behavior to protect against bacterial infection. These unique structures are thus potentially useful as burn and ulcer dressings.

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1. Introduction

Soybean is one of the most important and widely consumed legume crops in the world. It is composed of approximately 38% proteins, 30% carbohydrates, 18% oil and 14% minerals, other materials and moisture [1]. Soybeans are widely consumed in the food industry in the form of inexpensive high protein soy meals, soy oil, soy milk and tofu. Soybean protein has been explored mainly in the polymer, food and agriculture fields. The use of soybean protein as a food source is still increasing due to its functional and nutritional value, availability and low price [2]. The carbohydrate and oil components of the soybean can be removed to obtain soy protein (at least 90%) that can be used for various applications. Such soy protein was used in the current study. In the materials industry soy protein has been studied as an adhesive and as a “green” plastic [3].

The soy protein matrix possesses several advantages over various types of biodegradable polymers and natural proteins used for biomedical applications, in particular low price makes it economically competitive. Its non-animal origin eliminates the risk of transmissible diseases which poses a danger in human- and animal-derived products. In addition, soy protein has good water resistance, a relatively long storage time and stability, and it degrades into natural components [4].

It has also been reported that soybean-based products promote tissue regeneration, such as new bone growth. Furthermore, these materials integrate into blood clots and stimulate collagen deposition and, therefore, have significant potential for wound healing applications. They stimulate cells to produce new tissue, with no need for expensive growth factors [1].

Protein films and adhesives are usually processed in water or extruded under low moisture conditions. Several methods have been used to prepare films from soy protein, including solvent casting [5], extrusion [6], spinning in coagulating buffer [7] and thermal compaction [8]. Solvent casting of polymers is performed by a three step process: preparation of aqueous protein solutions, casting, and drying by solvent evaporation. The structure and properties of the formed film can be modified by changing various parameters during preparation, such as the pH and temperature of the solution, amount of protein, drying conditions (temperature, humidity, and duration) and heat treatment after film formation. The film properties can also be modified by additives such as plasticizers and cross-linkers. During the solvent evaporation step cross-linking occurs through intermolecular covalent disulfide bonds.

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doi:10.1016/j.actbio.2011.08.022
Soy protein can be cross-linked by a cross-linker, heat treatment, enzymatic treatment or irradiation. It has many reactive groups (e.g. –NH₂, –OH and –SH) which enable cross-linking reactions. Low molecular aldehydes (e.g. formaldehyde, glutaraldehyde and glyoxal) react primarily with the free ε-amine groups of arginine, lysine and hydroxylsine residues of the protein, thereby forming intra- and intermolecular cross-links [14]. Formaldehyde and glutaraldehyde are the most widely used agents, although concerns regarding post-implantation cytotoxic effects following use of these two agents have been raised, due to monomer release from the cross-linked matrices [15,16]. Glyoxal is a potentially less toxic alternative for use in biomedical devices, as shown with collagen-based matrices [17]. Cross-linking of soy protein can also be carried out by adding cysteine or Ca²⁺ ions.

Despite its functionality and good qualities, few groups have investigated the potential of soy, particularly soy protein, in the biomedical field as a natural biomaterial for various applications. Soy protein-based thermoplastics reinforced with tricalcium phosphate have been investigated for orthopedic biomedical applications [18]. These thermoplastics were found to be non-cytotoxic and even encouraged cell proliferation during in vitro tests [19]. Soybean-based materials have been investigated as bioactive bone fillers and wound dressings [20]. Two types of blends, poly(ethylene glycol)–soy protein hydrogel blends [21] and chitosan–soy blends [12], were studied for wound dressing applications. Their investigation focused mainly on the microstructure and the physical and mechanical properties.

The main goal in wound management is to achieve rapid healing with functional and esthetic results. An ideal wound dressing can restore the milieu required for the healing process, while simultaneously protecting the wound bed against bacteria and environmental threats. The dressing should also be easy to apply and remove. Most modern dressings are designed to maintain a moist healing environment and to accelerate healing by preventing cellular dehydration and promoting collagen synthesis and angiogenesis [22]. However, over-restriction of water evaporation from the wound should be avoided, since accumulation of fluid under the dressing may cause maceration and facilitate infection. The water vapor transmission rate (WVTR) from the skin has been found to vary considerably depending on the wound type and healing stage, increasing from 204 g m⁻² day⁻¹ for normal skin to 278 and as much as 5138 g m⁻² day⁻¹ for first degree burns and granulating wounds, respectively [23]. The physical and chemical properties of the dressing should therefore be adapted to the type of wound as well as to the degree of wound exudation.

2. Materials and methods

2.1. Materials

Donated non-genetically modified organism soy protein isolate (SPI, Solpro 910™, minimum 90 wt.% protein, on a dry basis, Solbar™, Ashdod, Israel) was used as the soy protein source. Glycerol (G-7893) and sorbitol (S-1876) and were used as plasticizers, glyoxal (50650) and l-cysteine (C-7352) were used as cross-linking agents and gentamicin sulfate (G-1264), an antibiotic drug (450–477 g mol⁻¹, Tₘ = 218–237 °C), was used as the incorporated active agent (all purchased from Sigma-Aldrich, Rehovot, Israel).

Trypsin/EDTA solution A (03-050-1), composed of 0.02% EDTA and 0.25% trypsin with Phenol red, was purchased from Biological Industries (Beit Haemek, Israel).

2.2. Preparation of soy protein films

Films of soy protein isolate (SPI) were prepared using the solvent casting method. SPI solutions were prepared by slowly dissolving SPI in constantly stirred distilled water. When needed, the pH was adjusted with 1 M sodium hydroxide or 1 M HCl (using a Mettler Toledo MP220 pH meter). Then the plasticizer, cross-linker and gentamicin were added to the mixture with constant stirring. All film preparation parameters are presented in Table 1. While stirred, the solution was heated at a constant temperature for 30 min and cooled at room temperature for another 30 min to remove bubbles. Finally, the solution was cast in low density polyethylene plates and dried at the ambient temperature and humidity for 72 h. The thickness was controlled by casting the same amount of solution (50 ml) per plate and was determined to be approximately 0.5 mm (using a micrometer). Dried films were removed from the plates and specimens were cut for each test. For certain samples a thermal treatment was conducted in an oven. The heated samples were placed in glass Petri dishes and were held down with covering plates to prevent curling and rippling during heating. The samples were stored in desiccators (room temperature, 30% relative humidity) until use.

2.3. Tensile mechanical properties

The SPI film tensile mechanical properties were measured at room temperature, under unidirectional tension at a rate of 50 mm min⁻¹ (according to the standard test method ASTM D6338-03), using a 5500 Instron machine. Each wound dressing sample was cut into a dumb-bell-shape (neck length 2 cm, width 5 mm). The tensile strength was defined as the maximum strength in the stress–strain curve. The maximal strain was defined as the breaking strain. The Young’s modulus was defined as the slope of the stress–strain curve in the elastic (linear) region. At least three samples were tested for each type of specimen. The means and standard deviations were calculated using Student’s t-test (Excel).

2.4. Water uptake ability

The fluid absorbing capacity of a wound dressing is an important criterion in maintaining a moist environment over the wound bed. A swelling test was performed in order to determine the water sorption capacities of the various samples. Prior to testing all specimens were conditioned for 10 days in a desiccator (room temperature, 30% relative humidity), for moisture content equilibrium. Round specimens (2 mm diameter) were pre-weighed and immersed in phosphate-buffered saline (PBS), pH 7.0 at 37 °C in an incubator. The weight of the samples was measured at several...
time points up to 24 h by removing the PBS and blotting them gently to remove excess fluid. The water uptake was calculated as:

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

(1)

2.5. Water vapor transmission rate

The moisture permeability of the wound dressings was determined by measuring the water vapor transmission rate (WVTR) across the film. Prior to testing all specimens were conditioned for a period of 10 days in a desiccator (room temperature, 30% relative humidity) to achieve moisture content equilibrium. A Sheen Payne permeability cup with an exposure area of 10 cm² was filled with 5 ml of PBS and covered with a circular wound dressing. The cup was placed in an oven containing 1 kg of freshly dried silica gel in order to maintain a relatively low humidity, at 37 °C. The weight of the assembly was measured every hour and a graph of water evaporation versus time was plotted. Measurements were taken until at least seven points gave a straight line (\( R^2 \geq 0.99 \)). The slopes of the curves (water loss rates) were calculated and the WVTR values were evaluated using the equation:

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

(2)

2.6. In vitro weight loss profile

The in vitro weight loss profile of the SPI films was studied in aqueous medium. Round dry specimens (2 mm diameter) were pre-weighed (after 10 days in a desiccator, 30% humidity, room temperature) and immersed in PBS solution at 37 °C in an incubator for 28 days. Sodium azide (0.05% w/v) was added as a preservative. The films were removed at certain time points (1, 7, 14, 21, 28, 35, 49 and 56 days) and fresh medium introduced.

2.7.1. Gentamicin assay

Determination of the medium gentamicin content was carried out using a TDX Therapeutic Drug Monitoring System (Abbott Laboratories) according to the manufacturer’s instructions. This machine enables determination of the gentamicin concentration based on a polarization fluorimunoassay using fluorescein as the tracer. Briefly, the latter is excited by polarized light. Polarization of the emitted light is dependent on molecule size. Free and labeled drug compete for binding sites. The drug concentration in the sample is proportional to the scatter of polarized light caused by free labeled drug. The measurable concentration range without dilution is 0.0–10.0 μg ml⁻¹. Higher drug concentrations were measured after dilution.

2.7.2. Residual drug recovery

Residual drug recovery from the SPI films was measured as follows. Drug remaining in the films were extracted by cleaving the film in trypsin A solution at 40 °C for 24 h. Trypsin completely cleaved the protein chains and the gentamicin concentration was estimated using the above described assay. The experiments were performed in triplicate.

3. Results

Yellowish, transparent, homogeneous, soy protein films were created using the solution casting technique under various processing conditions and with addition of various chemical agents. The films were approximately 0.5 mm thick and easily peeled off the plates. Low density polyethylene plates prevented film adherence, in contrast to polystyrene or glass Petri dishes. The potential of SPI films to serve as drug eluting wound dressings was assessed by studying the effects of the process parameters and additives on the mechanical and physical properties of the films and on the drug release profile.

3.1. Mechanical properties

Certain desirable mechanical properties, i.e. a combination of strength with ductility and flexibility, are crucial for routine handling and mechanical stability during application. It was therefore important to understand which parameters significantly affect the mechanical properties and choose reference samples for the following studies. The effects of the plasticizer type, cross-linking method, and temperature and pH of the solution on the tensile properties of SPI films were studied.

3.1.1. Plasticizer effect

Preliminary films prepared from soy protein alone without additives were highly brittle and could not be handled. A plasticizer must be added in order to soften the films. The samples were prepared from 5% w/v SPI solution at pH 7.2 and 55 °C. Our preliminary assumption was that cross-linking is necessary in order to create durable films. Therefore, 1 wt.% glyoxal was also added to the solution, and the effect of the plasticizers glycerol and sorbitol on the properties of SPI films were studied. The effect of further cross-linking by means of thermal treatment after the film preparation step was also studied. All results are presented in Table 2.

The results for non-treated films show a slight advantage of glycerol over sorbitol. However, when films were thermally treated (80 °C, 24 h) 50 wt.% sorbitol or less yielded films that were highly brittle and cracked during handling, and only 80 wt.% sorbitol

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values used in the study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy protein isolate</td>
<td>5% w/v</td>
</tr>
<tr>
<td>pH values of the aqueous solution</td>
<td>6, 7.2 and 10</td>
</tr>
<tr>
<td>Temperature of the aqueous solution</td>
<td>25 °C, 40 °C, 55 °C and 70 °C</td>
</tr>
<tr>
<td>Plasticizer</td>
<td>30, 50 and 80 wt.% sorbitol relative to SPI</td>
</tr>
<tr>
<td>Cross-linker</td>
<td>1 wt.% cysteine relative to SPI</td>
</tr>
<tr>
<td>Drug</td>
<td>1 and 3 wt.% gentamicin sulfate relative to SPI</td>
</tr>
<tr>
<td>Heat treatment temperature</td>
<td>80 °C for 24 h</td>
</tr>
</tbody>
</table>

The medium was removed (completely) at each sampling time (2, 6 and 12 h and 1, 2, 3, 5, 7, 14, 21, 28, 35, 49 and 56 days) and fresh medium introduced.
enabled mechanical testing. Therefore, thermally treated 50 wt.% glycerol films were compared with thermally treated 80 wt.% sorbitol and 80 wt.% glycerol/sorbitol (50/50) films. The results (Table 2) show that only thermally treated samples that were plasticized with glycerol exhibited increased tensile strength and Young's modulus with a very small non-significant decrease in maximal strain. Sorbitol plasticized films exhibited very low maximal strain and 50/50 glycerol/sorbitol films exhibited a relatively low Young's modulus. Glycerol was therefore chosen as the preferred plasticizer in our study.

3.1.2. Cross-linking effect

Cross-linking is essential for film integrity and stabilization during swelling, degradation and drug release. Therefore, the next step was to evaluate and choose an appropriate cross-linking method for the SPI films. Glyoxal (dialdehyde) cross-linked films were compared with L-cysteine (amino acid) cross-linked films. Glyoxal is considered a very efficient cross-linker, while the other potential cross-linker, L-cysteine, is considered less toxic than cross-linkers from the aldehyde family. Although the results are not significantly different (Table 3), they show a tendency of glyoxal enabling better and less variable mechanical properties than L-cysteine. It was therefore decided that glyoxal is indeed more favorable for our SPI system.

The effect of glyoxal and thermal treatment cross-linking was studied separately and the combined effect was also investigated and compared with non-cross-linked films. The results are presented in Fig. 1 for two types of films: cast from high temperature and high pH solutions (70 °C, pH 10) and cast from low temperature and neutral pH solutions (55 °C, pH 7.2). Both cross-linking methods increased the tensile strength and Young's modulus and decreased the maximal strain. However, solution conditions of 55 °C and pH 7.2 gave better results in terms of tensile strength and Young's modulus than 70 °C and pH 10.

3.1.3. Effect of pH and solution temperature

A series of experiments at various pH values (6, 7.2 and 10) and temperatures (25, 40, 55 and 70 °C) was carried out in order to evaluate the effect of pH and temperature of the solution on the tensile properties of the cross-linked films. All films were prepared from 5% w/v SPI solutions with glycerol (50 wt.%) as plasticizer and glyoxal (1 wt.%) as cross-linking agent, and underwent thermal treatment (80 °C, 24 h). The results are presented in Fig. 2. When the SPI films were cast from solutions with pH values of 6 and 7.2 the tensile strength and Young's modulus showed a gradual increase with temperature, while the maximal strain decreased with increasing solution temperature. At pH 10, however, the maximal values of strength and elastic modulus were acquired at 55 °C. The effect of the solution pH in the selected range was mostly minimal except for films prepared at 70 °C, at which temperature the tensile modulus decreased and the maximal strain increased with increasing pH.

It is important to mention that films prepared from pH 6 solutions exhibited a denser and more crumpled texture, while films prepared from pH 10 solutions were less homogeneous. Based on these experiments the optimal solution parameters are pH 7.2 and 55 °C. These conditions produced high quality homogeneous films which combined a relatively high tensile strength and

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Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Maximal strain (%)</th>
<th>Young's modulus (MPa)</th>
<th>Ultimate tensile strength (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol 50 wt.%, non-treated</td>
<td>158.8 ± 20.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>128.5 ± 12.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.3 ± 0.2</td>
</tr>
<tr>
<td>Sorbitol 50 wt.%</td>
<td>151.5 ± 17.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>104.3 ± 18.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.3 ± 1</td>
</tr>
<tr>
<td>Glycerol 50 wt.%, thermally treated (80 °C, 24 h)</td>
<td>144.5 ± 24.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>190.6 ± 35.6</td>
<td>16 ± 1.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sorbitol/glycerol 50/50 (total 80 wt.% plasticizer)</td>
<td>146.9 ± 14.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>127.8 ± 16.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.9 ± 0.9</td>
</tr>
<tr>
<td>Sorbitol 80 wt.%</td>
<td>9.0 ± 3.7</td>
<td>273.2 ± 28.2</td>
<td>18.9 ± 1.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results marked with the same lower-case letter are not significantly different (<i>P</i> > 0.05).

Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Maximal strain (%)</th>
<th>Young's modulus (MPa)</th>
<th>Ultimate tensile strength (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyoxal 1 wt.%</td>
<td>128.2 ± 12.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>244.8 ± 8.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.5 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>L-cysteine 1 wt.%</td>
<td>110.5 ± 14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>142.8 ± 57.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.5 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results marked with the same lower-case letter are not significantly different (<i>P</i> > 0.05).
24 h) was used. The combined cross-linking method (1% glyoxal and thermal treatment at 80 °C for 24 h; films cross-linked using 1 wt.% glyoxal; films cross-linked using 1% glyoxal and thermal treatment at 80 °C for 24 h) was used.

Young's modulus with good ductility, and were therefore chosen for the physical property experiments.

3.2. Physical properties

The physical properties of the SPI films determine their function as a wound dressing and were therefore examined. All films were cast from 5% w/v SPI films plasticized with 50 wt.% glycerol. The water uptake, WVTR and weight loss profile of the following four samples were determined: non-cross-linked films; films cross-linked with 1 wt.% glyoxal; films cross-linked using 1% glyoxal and thermal treatment at 80 °C for 24 h; films cross-linked using 1 wt.% glyoxal and thermal treatment at 80 °C for 24 h.

3.2.1. Water uptake

All samples were immersed in PBS (37 °C) to simulate the water absorption behavior in the presence of wound fluids. The water uptake values of the SPI films studied are presented in Fig. 3. Non-treated films exhibited rapid water uptake, reaching a value of 257% after 6 h. A slight decrease in water uptake was obtained after 24 h, probably due to some degradation.

Cross-linked films exhibited reduced water uptake with similar water absorption patterns, consisting of the following stages: a rapid initial water uptake within the first 30 min; a slight decrease in water content during the following 30 min; a slight increase in water content during the following 23 h.

Films which underwent both cross-linking processes displayed a water uptake peak of 67% after 15 min and a constant water uptake of approximately 54% after 1 h.

3.2.2. Water vapor transmission rate (WVTR)

The WVTR values of the four selected samples were measured as described in Section 2.5. The evaporative water loss through the various dressings was linearly dependent on time (R² > 0.99 in all cases), resulting in constant WVTR values. All samples exhibited WVTR values in the range 2300–2700 g m⁻² day⁻¹ (Fig. 4), with no statistical differences (P > 0.05). The WVTR of an exposed aqueous surface was also determined experimentally in order to simulate the condition in which no dressing was applied to the wound surface. In this case a WVTR of 6329 g m⁻² day⁻¹ was measured.

3.2.3. In vitro weight loss profile

The weight loss profiles of the four samples studied are presented in Fig. 5. All samples lost 30–40% of their initial weight after 1 day immersion in aqueous medium. This was followed by slow weight loss for a period of 28 days. The films maintained their structural integrity during the entire experiment and did not disintegrate upon handling. Non-cross-linked films showed the highest weight loss rate and lost 50.5% of their initial weight after 1 month. During the same period glyoxal cross-linked and thermally treated films lost 42.8% and 41.2%, respectively, and films that were cross-linked by the two methods lost only 37.4% of their initial weight. The weight loss rates (slope of the curve) of the non-cross-linked films were higher than those of the three types of cross-linked films.

3.3. Gentamicin release from the SPI films

SPI films which exhibited the desired mechanical and physical properties were chosen for use in the release studies. These were cast from 5% w/v solutions (55 °C, pH 7.2), plasticized with 50 wt.% glycerol and cross-linked using 1 wt.% glyoxal. Two such films were studied, non-treated and thermally treated at 80 °C for 24 h, so as to be able to evaluate the effect of crosslinking on the drug release profile. Each film was loaded with either 1 or 3 wt.% gentamicin and the drug release kinetics were studied for 2 months in triplicate. The cumulative release profiles from the four types of samples are presented in Fig. 6 and the burst release values and calculated release rates are presented in Table 4.

The release profiles can be divided into three main stages: burst release during the first 6 h; exponentially decreasing release rate during the first week; approximately constant release rate during weeks 2–8. Heat treatment showed a significant effect on the gentamicin release profile of both samples, loaded with 1 and 3 wt.% gentamicin. The burst effect (stage 1) and total drug quantity released during the first week (stage 2) by the non-treated films were approximately 40% higher than those of the thermally treated films (Fig. 6). In contrast, the rate of release from the thermally treated films during the third phase of release was higher than from the non-treated films. After approximately 2 months both the non-treated 1 and 3 wt.% gentamicin loaded films released their total encapsulated drug, while the thermally treated films released only 73% and 83% of the total encapsulated drug, respectively. The effect of drug content on the release profile was clearly evident only during the third stage. Increasing the drug content from 1 to 3 wt.% had no significant effect on the first two stages of release, but resulted in a higher release rate during the third stage, especially in the thermally treated films.
4. Discussion

4.1. Mechanical properties

The mechanical properties of a wound dressing constitute an important factor in its wound protection performance. The dressing should withstand different stresses and not tear, whether it is used topically to protect dermal wounds or as an internal wound support, e.g. for surgical tissue defects. Furthermore, in clinical settings appropriate mechanical properties of dressing materials are needed to ensure routine handling. The initial mechanical properties of natural polymers such as collagen and gelatin can be satisfactory. However, a considerable decrease in these properties is expected to occur quickly due to hydration and enzymatic activity. It is thus important that naturally occurring dressings are cross-linked and retain relatively high strength. The effect of various processing parameters on the mechanical properties of SPI films was studied and the results are discussed below.

Addition of a plasticizer to the SPI films was found to be essential. Without it the films are highly brittle and cannot be handled. Cross-linked films (5% w/v SPI, 1 wt.% glyoxal, pH 7.2, 55 °C) with sorbitol as the plasticizer exhibited mechanical properties similar to glycerol plasticized films. However, when the films were also thermally treated the sorbitol plasticized films showed poor maximal strain compared with glycerol plasticized films (Table 1). As is known from the literature, polyol-based plasticizers (compounds containing multiple hydroxyl groups) reduce stiffness and induce flexibility by penetrating between protein chains, forming hydrogen bonds and lowering the $T_g$ [25]. Sorbitol is known to crystallize when films are stored under conditions of low humidity [26]. Therefore, heat treatment, which reduces the film water content, probably led to sorbitol crystallization and thus to the high brittleness of these films.

Glycerol as the plasticizer in our SPI films resulted in very good mechanical properties. However, it is important to note that a high glycerol content can lead to high water absorption and water vapor permeability, which may be undesirable [27]. Our results also show that if one wishes to reduce the hydrophilicity of the thermally treated films by mixing glycerol and sorbitol a 50/50 ratio and total quantity of 80 wt.% plasticizer could be a good alternative to glycerol only films.

It is known that cross-linking of hydrophilic biopolymers improves their mechanical strength and susceptibility to hydrolytic and enzymatic degradation [11,13]. Among the various methods for cross-linking proteins it was decided to examine the effects of two methods on the tensile properties, i.e. cross-linking by a chemical agent and by thermal treatment, and a combination of the two methods. Glyoxal as cross-linker was found to be more effective than i-cysteine in imparting good mechanical properties to the

![Fig. 3. Water uptake of SPI films (5% w/v) plasticized with glycerol (50 wt.%).](image-url)
Our study shows that a combination of both cross-linking methods yielded a film much stronger than the films obtained by applying each method separately, with preservation of good ductility. Glyoxal creates cross-links mostly via free ε-amine groups, while heat treatment is believed to promote cross-linking mostly by encouraging S–S bonds and the formation of new hydrogen and hydrophobic interactions [29]. The combined effect of using both methods is presumably achieved because each method acts on different types of protein–protein interaction. Furthermore, the cross-linker reaction with the protein chains is probably enhanced during heat treatment and more cross-links are created via amine groups.

The pH and temperature of the SPI solution had only a slight effect on the tensile properties of the films (Fig. 2). High temperatures, usually above 50 °C, are known to cause protein chains to unfold and expose hydrophobic zones and active groups, such as amines, for cross-linking. Therefore, when pH values of 6 and 7.2 were used increasing the solution temperature probably resulted in some increased cross-linking which led to a higher tensile strength and Young’s modulus. However, at relatively high pH values, such as 10, at which more charged COO⁻ groups are present, dispersion of the chains probably causes higher rejection and less effective cross-linking, i.e. more intra- than inter-chain linkages that reduce the film strength. This explains the unexpected decrease in strength and Young’s modulus obtained for films processed at 70 °C and pH 10. It should be mentioned that the number of carboxylic (COOH) groups in soy protein is approximately twice that of amine groups (NH₂) and they usually dictate the tone.

4.2. Physical properties

Successful wound healing requires a moist environment. Two parameters must therefore be determined: the water uptake ability of the dressing and the WVTR through the dressing. The weight loss profile in aqueous solutions affects the drug release rate and the mechanical strength, and was also studied.

Some degree of water uptake is desirable in order to provide adequate gaseous exchange and absorption of wound exudates. However, rapid water penetration should be avoided in order to prevent rapid release of the active agent from the wound dressing. Water uptake by the SPI membranes occurs as their hydrophilic polymer gradually absorbs water, chain relaxation occurs and the matrix swells.

As expected, swelling of our cross-linked films by water uptake is significantly lower than that of non cross-linked films (Fig. 3). The cross-linked samples demonstrated a three-stage water uptake pattern during the first 24 h of immersion in aqueous medium. In the first stage there is a rapid influx of water into the film matrix. During the second stage there is a small decrease in water content, probably due to a “spring-like” contraction of the cross-linked network and the secretion of water and glycerol molecules. In the third stage water uptake increases gradually as the chains undergo relaxation and the matrix swells again. While the cross-linked films undergo the second stage after 15–30 min of immersion, in the non cross-linked films the second stage only begins after 6 h of immersion. Finally, it is important to note that a higher cross-linking

<table>
<thead>
<tr>
<th>Film type</th>
<th>Burst release (6 h) (%)</th>
<th>Total second phase release (%)</th>
<th>Third phase constant release rate (µg day⁻¹)</th>
<th>(% day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated (1% drug)</td>
<td>66.7 ± 6.1</td>
<td>94.3 ± 7.4</td>
<td>0.21 (R² = 0.79)</td>
<td>0.094 (R² = 0.79)</td>
</tr>
<tr>
<td>Thermally treated (1% drug)</td>
<td>46.2 ± 4.1</td>
<td>65.7 ± 6.7</td>
<td>0.34 (R² = 0.91)</td>
<td>0.150 (R² = 0.91)</td>
</tr>
<tr>
<td>Non-treated (3% drug)</td>
<td>63.2 ± 6.5</td>
<td>94.7 ± 8.2</td>
<td>0.80 (R² = 0.97)</td>
<td>0.119 (R² = 0.97)</td>
</tr>
<tr>
<td>Thermally treated (3% drug)</td>
<td>47.8 ± 7</td>
<td>70.6 ± 3.6</td>
<td>2.12 (R² = 0.98)</td>
<td>0.314 (R² = 0.98)</td>
</tr>
</tbody>
</table>
density (as expressed by the mechanical properties) results in lower water uptake during each of the stages and enables equilibrium to be reached, i.e. constant water uptake, within a shorter period of time.

The WVTR of normal human skin is around 204 g m⁻² day⁻¹ [30] and may reach 5138 g m⁻² day⁻¹ in severe burn wounds. An effective wound dressing provides good WVTR management that maintains a moist wound bed at the desired level for the course of healing. An excessive WVTR may lead to wound dehydration, whereas a low WVTR might lead to maceration and bacterial contamination [31,32]. It has been claimed that wound dressings should ideally possess a WVTR in the range 2000–2500 g m⁻² day⁻¹ [32].

However, commercial dressings do not always correspond to this range, and have been shown to cover a larger spectrum of WVTR, ranging from 90 (Dermiflex®, J J [33]) to 3350 g m⁻² day⁻¹ (Beschitín®, Unitika [34]). Indeed, the WVTR value is related to the structural properties (thickness and porosity) of the dressing as well as to the hydrophilicity of the material. The results for all SPI films tested clearly demonstrate a WVTR in the desired range for a wound dressing, i.e. ~2300 g m⁻² day⁻¹. This relatively high value could be explained by the intrinsic hydrophilicity of the soy and glycerol matrix. Our results show that WVTR practically does not change due to cross-linking. This is probably related to the relatively low density films that were prepared using the solution casting technique. In the case of denser melt processed (thermoplastic) SPI films it was reported that cross-linking resulted in lower WVTR values [19].

The primary weight loss occurred during the first day, as SPI films lost 30–40% of their initial weight due to leaching out of the plasticizer and small non-cross-linked protein chains. This was a result of rapid water uptake and was probably enhanced by the hydrophilic nature of glycerol. Then a slow rate of weight loss was observed for 28 days. It is assumed that protein chains are cleaved and diffuse out of the matrix during that period. Indeed, a similar degradation profile was previously observed for chitosan/soy blended membranes [12]. As expected, the cross-linked SPI films were found to have slower weight loss rates than the non cross-linked films. Cross-linking is a known method to decrease the degradation rate of polymers. It should be noted that a relatively high initial plasticizer content was used here in order to obtain the desired ductility when handling the wound dressing. During usage the wound exudate will cause diffusion of the plasticizer, but this will occur in parallel with water uptake by the wound dressing. Water is known to act as a plasticizer of polymers, especially hydrophilic ones.

4.3. Gentamicin release profile

The gentamicin release profile obtained for the structures studied showed a moderate burst effect (46–66%) during the first 6 h, followed by a continuous decrease in release rate during the first week. This was followed by a third stage of zero order release (constant release rate) that lasted until the end of the experiment. It should be mentioned that the specimens maintained their integrity throughout the entire test period. The burst effect and release profile during the first week were typical of diffusion controlled systems. The third phase of constant release rate presumably involved degradation of the soy protein matrix combined with diffusion of the remaining drug that was more firmly attached to the protein chains. The release profile obtained could be beneficial for application as antibiotic eluting wound dressings. During the first hours after wounding a relatively high drug release is essential to eliminate infections that may not have been eliminated during wound cleansing and might create a resistant biofilm. Later, a continuous low release rate can keep the wound “infection free” for more than 2 weeks, which is the time usually required for wound healing.

The burst release and amount of drug released during the first week from the thermally treated films were significantly lower than from the non-treated films. This phenomenon is attributed to the limited swelling capacity of the former. The relatively low water uptake of the highly cross-linked cured films limited drug diffusion during the first days. After degradation of the SPI host polymer during the first week more water could penetrate the film and leach out the remaining drug. Thus, after the second release phase, when higher drug amounts remained in the heat-treated film, it exhibited a higher diffusion gradient and faster release rate during the third phase of release compared with the untreated films. These differences in the release profile could be applicable for different wound dressing uses. Thus the thermally treated films can be used for burns, which are not infected immediately after trauma but require a relatively long period of infection prevention, whereas the non-treated films can be used for unclean infected wounds.

It should be noted that most of the gentamicin was released within the first week of the study (thermally treated 66%, non-treated 95%), due to the hydrophilic nature of the antibiotic and the SPI matrix. The hydrophilic nature of soy protein enables relatively rapid water intake, leading to full swelling of the matrix within a few hours of immersion (Fig. 3). Unfavorable more rapid drug release rates have been reported in the literature for other antibiotic eluting systems [35]. Controlling the release of antibiotics from these systems is challenging due to the hydrophilic nature of both the drug and the host polymer. In most cases the drug reservoir is depleted in less than 2 days, resulting in a very short antibacterial effect. Thus our new antibiotic eluting SPI film has advantages over other systems. This advantage can be explained by the fact that many antibiotic drugs bind proteins via van der Waals or ionic interactions. The bonded portion may act as a reservoir and be released more slowly than the unbound form. 0–30% of adsorbed gentamicin binds to albumin [36]. Furthermore, gentamicin is a highly charged polycation (+3.5, pH 7.4 [37]), whereas soy protein has many negatively charged carboxyl groups. It is thus probable that some ionic bonding took place. Such a binding mechanism has been shown before between deproteinized carboxyl groups of succinylated collagen and positive anion groups of the gentamicin molecule [38].

5. Conclusions

Novel biodegradable wound dressings based on soy protein films were developed and studied. These dressings were prepared from aqueous solutions and their investigation focused on the effects of the formulation and process parameters on the mechanical and physical properties and on the release profile of the antibiotic drug gentamicin from the dressings.

The initial mechanical properties of our wound dressing structures are mainly affected by the plasticizer and cross-linking agent and can also be controlled by the cross-linking method. Glycerol was chosen as the preferred plasticizer for our SPI films and glyoxal was chosen as the preferred cross-linking agent. The pH and temperature of the starting solution have only a slight effect on the tensile properties of the SPI films. Films that were cross-linked by a combination of cross-linking agent and thermal treatment were found to be superior, combining a relatively high resistance to tearing (tensile strength of 17 MPa) and ductility (maximal strain of 160%).

The physical properties of the wound dressing (water absorbance and weight loss profile) can be controlled by the cross-linking process. Films that were cross-linked by thermal treatment or
addition of cross-linking agent exhibited lower water uptake and weight loss rate than non-cross-linked films. A combination of both cross-linking methods resulted improvements in these properties. The WVTR of the films was in the desired range for wound dressings (~2300 g m⁻² day⁻¹) and was not affected by the cross-linking method.

The gentamicin release profile exhibited a moderate burst effect followed by a decreasing release rate which lasted for at least 4 weeks. The dominant release mechanism of gentamicin from cross-linked SPI films is diffusion. Cross-linking by a combination of glyoxal and thermal treatment resulted in a lower burst release and lower total released drug, compared with cross-linking by glyoxal only.

Our novel SPI structures combine good mechanical properties with the desired physical properties and controlled release of the antibiotic drug gentamicin and could, therefore, be potentially very useful as burn and ulcer dressings. Changing the process parameters allows the desired properties to be adapted to the wound characteristics, and could thus enhance wound healing.

Acknowledgement

The authors would like to thank Solbar™ (Ashdod, Israel) for kindly providing the soy protein isolate used in this study.

References