Effect of crosslinking, thermal treatment and UV irradiation on the mechanical properties and in vitro degradation behavior of several natural proteins aimed to be used in the biomedical field

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Gelatine (GEL), soy (SI), casein (CAS) and sodium-caseinate (NaCAS) solutions were cast to produce protein films. All the proteins were chemically modified by adding glyoxal to the film-forming solutions in amounts varying from 0 to 0.9% (w/w based on the protein content). After casting, the same films were also submitted to a heat treatment performed at 80 °C or UV irradiation. The effect of those chemical/physical modifications on the mechanical properties and on the hydrolytic stability of the protein films was evaluated. As a result, a large variety of protein films with different mechanical properties and degradation profiles were developed. CAS and NaCAS even when chemically/physically modified do not resist to hydrolysis longer than 2 weeks. GEL, only when chemically modified with glyoxal, become water resistant. Due to its hydrolytic stability, SI become a very attractive material for biomedical applications where long term treatments are a requisite.

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1. Introduction
The human body is best represented by a combination of macroscopic structures such as skin, bones, arteries, and biological and biochemical processes, which take place at a molecular or cellular dimension. This concept presents interesting challenges in terms of the proposal and redesign of novel materials and devices to be used in specific biomedical applications, namely in drug delivery applications. The selection of the materials that can be used in these different devices depends on their similarity to the structures to be replaced and on the availability of suitable processing methods. Until now, only few materials have been successfully developed for these purposes. In the case of implantable or injectable systems, where biocompatibility and toxicity are important considerations, biodegradable polyesters such as polyactic acid [1,2] and polyglycolic acid [2] have been the most selected materials until now. A major alternative group involves natural biopolymers, such as polysaccharides (e.g. starch) or proteins. The three proteins that have been most used in the biomedical field so far are collagen, gelatine and albumin [3–5]. However, as potential complications may arise from the risk of diseases like BSE, there is an increasing interest to replace these proteins. In fact, other industrial proteins are largely available, but the respective research effort towards an eventual application as biomaterials has not been performed yet. The main examples are proteins obtained from soy, pea, potato, whey and casein.

The good performance in terms of: (i) processability (in aqueous media and in melt) [6,7]; (ii) film-forming properties [6]; (iii) mechanical behavior [6–8]; (iv) adhesion to various substrates [9]; (v) radiation resistance [10] and; (vi) surface active properties [11], opens a wide range of applications to these proteins in technical applications. Some examples are: (i) adhesives [12]; (ii) coatings [12]; (iii) thermoplastic-processed products (disposables) [12]; (iv) cosmetics [13] and; (v) food compounds [14]. During the years, natural proteins based materials have been extensively developed and studied.
and many processing technologies have been proposed and optimized [12]. The present work reports an evaluation study of several protein materials, in order to assess the respective potential to be used as biomaterials, aimed to be used in: (i) controlled release applications; (ii) wounds dressings or (iii) scaffolds for skin regeneration. It involves the preparation by film casting of different protein films (soy (SI), gelatine (GEL), casein (CAS) and sodium-caseinate (NaCAS)), and their characterization in terms of mechanical performance and degradation behavior.

2. Materials and methods

2.1. Materials

The studied materials were: (i) SI, provided by Loders Crocklaan B.V. (Wormerveer, The Netherlands), (ii) CAS and NaCAS, both supplied by DMV International (Tilburg, The Netherlands); and (iii) GEL, obtained from Delft Gelatine B.V. (Delft, The Netherlands). Further characteristics of the above-mentioned proteins are presented in Table I. Glycerol and glyoxal (40% v/v) were used as a plasticizer and a crosslinking agent, respectively. These agents were employed as received from the manufacturer, Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). The other chemicals used (NaCl, NaOH and HCl) were all of analytical grades.

2.2. Protein films preparation

2.2.1. Non-crosslinked films

Film-forming solutions were prepared by slowly suspending the protein powders (10% w/w), under constant speed (propeller stirrer, Janke & Kunkel, RW20, 300–2000 rpm), in distilled water with glycerol (20% w/w based on the protein content). All the film-forming solutions were prepared at pH 6.8 ± 0.2, except for CAS, which was prepared at pH 4.0 ± 0.1 (adjusted with 1 M HCl). 15 ml of the film-forming solutions were cast into Petri dishes (10 × 10 cm²) and followed by air-drying for 24 h at 25 ± 2°C (room temperature, RT) and 58 ± 3% relative humidity (RH). After drying, the obtained films were removed from the dishes, accurately measured for its thickness (using a digital micrometer) and cut into appropriate shapes (dumb-bell tensile bars, 0.1 × 4 mm²). Subsequently, all the specimens were conditioned in an exsiccator containing a NaBr saturate solution (58% RH) and 25°C until the equilibrium moisture content was reached (7 days).

The preparation conditions and characteristics of the produced films are detailed in Table II.

2.2.2. Glyoxal crosslinking

During the preparation of the SI, NaCAS and GEL (except CAS) film-forming solutions (see Section 2.2.1) the pH was increased to 8.0 ± 0.1 with 1 M NaOH and a glyoxal solution was added to the referred solutions at a level of 0, 0.3, 0.6 and 0.9% (w/w based on the protein content), leading respectively to 0, 0.3X-protein, 0.6X-protein and 0.9X-protein. The resulting solutions were cast and the respective films were dried, measured and conditioned as described in Section 2.2.1.

2.2.3. Heat treatment

Dried films of SI, CAS, NaCAS and GEL, produced as described in Section 2.2.1, were mounted on glass plates by applying tape round the film edges and heated at 80°C in a forced convection oven for 2 and 24 h (2TT-protein and 24TT-protein, respectively). The tape held films flat and prevented curling and rippling during heating. After being removed from the oven, the films were allowed to cool down at 25°C and 58% RH for 1 h and consequently measured and conditioned as described in Section 2.2.1.

2.2.4. Irradiation by UV

2.2.4.1. UV irradiation of protein film-forming solutions.

Film-forming solutions of SI, CAS, NaCAS and GEL were cast into the Petri dishes, as described in Section 2.2.1, and, before drying, were exposed to UV radiation (366 nm) during 2 h, using a 125 W Philips UV lamp, placed 45 cm above the film (UV₝ₒₜ⁻ protein). After treatment, the films were first allowed to dry, then measured and finally conditioned following the methods described in Section 2.2.1.

2.2.4.2. UV irradiation of protein films.

Dried films of SI, CAS, NaCAS and GEL, produced as described in Section 2.2.1, were also exposed for 2 h to UV radiation, using the same conditions referred in Section 2.2.4.1, (UV-protein). After these post-treatments, the films were also measured and conveniently conditioned as mentioned in Section 2.2.1.

2.3. Characterization of the protein films

2.3.1. Total protein content

50 mg of SI, CAS, NaCAS and GEL protein samples, previously ground using liquid N₂ and sieved (mesh size of 1 mm), were dispersed in a mixture of demi-water and concentrated H₂SO₄. After adequate digestion of the protein materials, carried out at 420°C during 50 min, the
TABLE II  Processing parameters of the protein films

<table>
<thead>
<tr>
<th>Material</th>
<th>rpm*</th>
<th>T(°C)</th>
<th>pH</th>
<th>Drying conditions</th>
<th>UV irradiation</th>
<th>Heat treatment</th>
<th>Thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>t (hrs)</td>
<td>T(°C)</td>
<td>RH (%)</td>
<td>t (hrs)</td>
</tr>
<tr>
<td>SI</td>
<td>500</td>
<td>25</td>
<td>6.8</td>
<td>24</td>
<td>25</td>
<td>58</td>
<td>—</td>
</tr>
<tr>
<td>CAS</td>
<td>1000</td>
<td>25</td>
<td>4.0</td>
<td>24</td>
<td>25</td>
<td>58</td>
<td>—</td>
</tr>
<tr>
<td>NaCAS</td>
<td>1500</td>
<td>&gt; 40</td>
<td>6.8</td>
<td>24</td>
<td>25</td>
<td>58</td>
<td>—</td>
</tr>
<tr>
<td>GEL</td>
<td>500</td>
<td>&gt; 40</td>
<td>6.8</td>
<td>24</td>
<td>25</td>
<td>58</td>
<td>—</td>
</tr>
<tr>
<td>X-protein</td>
<td>700</td>
<td>25</td>
<td>8.0</td>
<td>24</td>
<td>25</td>
<td>58</td>
<td>2</td>
</tr>
<tr>
<td>UV-protein</td>
<td>500–1500</td>
<td>25 &gt; 40</td>
<td>6.8</td>
<td>24</td>
<td>25</td>
<td>58</td>
<td>2</td>
</tr>
<tr>
<td>TT-protein</td>
<td>500–1500</td>
<td>25 &gt; 40</td>
<td>6.8</td>
<td>4.0</td>
<td>25</td>
<td>58</td>
<td>2</td>
</tr>
</tbody>
</table>

* screw rotating speed.

**total protein content (N_{prot}) of the resulting solution was determined by Kjeldahl analysis and calculated as:**

\[
N_{prot}(\%) = \left[ \frac{V (\text{HCl}) \times 0.1 \times 14 \times \phi_N}{W_o} \right] \times 100 \tag{1}
\]

where \(V (\text{HCl})\) is the volume of HCl 0.1 M used during the Kjeldahl titration, 14 is the \(M_w\) of nitrogen (N) and \(\phi_N\) the Kjeldahl factor of each protein (6.25 for SI, 6.38 for CAS/NaCAS and 5.55 for GEL). \(W_o\) is the dry weight of the protein powder sample tested.

### 2.3.2. Free amino group measurement

The free amino group content of the protein films was determined using the OPA method [15]. An OPA solution was made by mixing 25 ml of 0.1 M sodium borate (pH 9.2), 2.5 ml of 20% (w/w) sodium-dodecyl sulfate (SDS), 40 μg of OPA (N,N-diphenyl-diehdy) (dissolved in 1 ml methanol) and 100 μl of β-mercaptoethanol. The final volume was adjusted to 50 ml with deionized water. To determine the degree of alkylation, an aliquot (50 μl containing 2 g/l protein in sodium tetraborate buffer 0.0125 M + 2% SDS) was added directly to 1.0 ml of OPA reagent in a cuvette. The solution was mixed rapidly and incubated for 2 min at room temperature before the absorbency was read at 340 nm against water. A calibration curve was previously established by using L-leucine as a standard.

### 2.3.3. Tensile testing and moisture content

The mechanical performance of the films was assessed in tensile mode. The experiments were performed in a Zwick Z010 universal mechanical testing machine, in a controlled chamber (20 °C and 55% RH). A 200N load cell, a pre-load of 0.03N and a loading speed of 1 mm/min were used. E-modulus (\(E\)), yield stress (\(\sigma_y\)) and strain at break (\(\epsilon_b\)) were computed from the respective data.

After testing, specimens were milled using liquid N₂ and weighed into aluminum dishes for subsequent drying for 24 h in a vacuum oven at 40 °C [16]. Moisture content (MC) was determined in triplicate for each type of material as percentage of initial weight (\(W_0\)) lost during drying (\(W_{ad}\)):

\[
MC = \left[ \frac{(W_0 - W_{ad})}{W_0} \right] \times 100 \tag{2}
\]

### 2.3.4. In vitro degradation

Protein films were also submitted to in vitro degradation tests. Pre-weighted dry specimens were immersed up to 15 days at 37 °C in an isotonic saline solution (ISS – NaCl, 9 g/l + 1% sodium azide (NaNO₃)) buffered at pH = 7.40 ± 0.02 with a solution 0.2 M tris(hydroxymethyl)aminomethane/0.2 M hydrochloric acid (HCl). After pre-fixed aging periods (0, 1, 7 and 15 days), the films were removed from the aging solution, washed with distilled water and dried in a vacuum oven (40 °C/24 h) [16]. The percentage weight loss of the protein films were then calculated using the Equation 3

\[
WL_t = \left[ \frac{(W_0 \times N_0 - W_t \times N_t)}{W_0 \times N_0} \right] \times 100 \tag{3}
\]

where WP, is the weight loss of the films after a certain time, \(t\), of immersion. \(W_t\) denotes the weight of the films at aging time \(t\) and \(N_t\) the respective protein content at that aging time \(t\). \(W_0\) is the initial dry weight of the film and \(N_0\) the correspondent protein content. Each experiment was repeated 3 times and the average value was taken as the weight loss.

### 3. Results and discussion

#### 3.1. Glyoxal crosslinking

##### 3.1.1. Tensile properties

E-modulus (\(E\)), yield stress (\(\sigma_y\)) and strain at break (\(\epsilon_b\)) values for the different films are shown in Tables III–VI and graphs of Figs. 1–3.

As expected, glyoxal crosslinking of the protein structure increased the mechanical strength and reduced the ductility for all the proteins studied, except gelatine.

The yield stress (\(\sigma_y\)) increased 20% for SI (Table III and Fig. 2(a)), 30% for CAS (Table IV and Fig. 2(a)) and 30% for NaCAS (Table V and Fig. 2(a)).

The E-modulus (\(E\)) also increased upon glyoxal crosslinking. Increments on the E-modulus were in the range of 50% for SI, CAS and NaCAS (Tables III–V and Fig. 1(a)). As expected, these increments in strength and stiffness were followed by an important embrittlement of the materials, with a strain at break decrease in the range of 50% (Tables III–V and Fig. 3(a)). Furthermore, these data also evidenced some scatter which is usually associated to a brittle mechanical behavior. Enhancements in \(\sigma_y\) have been previously reported for formaldehyde-treated films and coatings such as corn zein [17, 18]. Also, a substantial three-fold increase in \(\sigma_y\).
was obtained with cast soy films when treated with formaldehyde [19].

Glyoxal has been shown to react with arginine and lysine (or hydroxylysine) residues in proteins in aqueous solutions between pH 8 and 9 but, like with other aldehydes, the nature of the derivatives formed is still unknown [20]. However, the increased strength (higher $\sigma_y$) and stiffness (higher $E$) of the glyoxal-treated protein films should be mainly attributed to the crosslinking reaction between the aldehyde groups of the glyoxal and the free $\varepsilon$-amine groups of lysine (or hydroxylysine) residues of the studied proteins [21]. The degree of crosslinking between glyoxal and the proteins was followed by the quantification of the percentage of occupied amino groups (−NH$_2$) within the protein. As presented in Tables III–V, the incorporation of 0.9% glyoxal was responsible for an increase of the crosslinking degree in about 30% for CAS and NaCAS and 20% for SI. As expected these increases in crosslinking degrees cause the increments in the yield strength increments observed for CAS, NaCAS and SI.

The distinct behavior of the GEL films (Table VI and Figs. 1, 2 and 3(a)), with a variation of the mechanical properties within the range of the respective data scatter, is mainly due to: (i) lower amount of lysine available for crosslinking (Table I), when compared with the other

**Table III** Crosslinking degree and tensile properties of soy (SI) protein films

<table>
<thead>
<tr>
<th>Materials</th>
<th>Free−$\text{NH}_2$ groups (%)</th>
<th>$\sigma_y$ (MPa)</th>
<th>$\varepsilon_b$ (%)</th>
<th>$E_{0.05-0.25%}$ (MPa)</th>
<th>Thickness (μm)</th>
<th>MC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI</td>
<td>67.1 ± 1.3</td>
<td>10.5 ± 0.5</td>
<td>41.8 ± 11.9</td>
<td>299 ± 13</td>
<td>0.119 ± 0.010</td>
<td>8.9 ± 0.1</td>
</tr>
<tr>
<td>0.3X-SI</td>
<td>68.9 ± 2.6</td>
<td>11.2 ± 0.4</td>
<td>86.9 ± 44.6</td>
<td>347 ± 7</td>
<td>0.124 ± 0.009</td>
<td>8.7 ± 0.1</td>
</tr>
<tr>
<td>0.6X-SI</td>
<td>51.3 ± 3.6</td>
<td>12.8 ± 1.1</td>
<td>14.6 ± 11.5</td>
<td>413 ± 42</td>
<td>0.130 ± 0.030</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>0.9X-SI</td>
<td>46.3 ± 1.2</td>
<td>12.6 ± 0.6</td>
<td>24.1 ± 14.9</td>
<td>421 ± 47</td>
<td>0.176 ± 0.034</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>2TT-SI</td>
<td>65.6 ± 3.2</td>
<td>13.5 ± 2.1</td>
<td>38.4 ± 16.0</td>
<td>407 ± 27</td>
<td>0.131 ± 0.07</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>24TT-SI</td>
<td>62.7 ± 4.1</td>
<td>12.1 ± 1.1</td>
<td>20.3 ± 11.5</td>
<td>471 ± 42</td>
<td>0.133 ± 0.020</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>UV$_{10}$-SI</td>
<td>83.2 ± 2.0</td>
<td>9.4 ± 0.2</td>
<td>33.5 ± 16.0</td>
<td>309 ± 27</td>
<td>0.121 ± 0.008</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>UV-SI</td>
<td>80.8 ± 7.1</td>
<td>9.3 ± 1.1</td>
<td>50.1 ± 11.5</td>
<td>256 ± 42</td>
<td>0.123 ± 0.009</td>
<td>3.5 ± 0.2</td>
</tr>
</tbody>
</table>

%X-SI = glyoxal crosslinked soy (% glyoxal based on the protein content); (hrs) TT = soy heat treated for 2 and 24 h; UV-SI = soy film irradiated by UV; UV$_{10}$-SI = soy film-forming solution irradiated by UV. $\sigma_y$ = yield stress (MPa); $\varepsilon_b$ = strain at break (%); $E_{0.05-0.25\%}$ = E-modulus between 0.05–0.25% strain (MPa). MC = moisture content of the films.

![Figure 1](image1.png)

**Figure 1** (a) Effect of glyoxal crosslinking on the E-modulus of: SI, CAS, NaCAS and GEL protein membranes. (b) Effect of heat treatment on the E-modulus of: SI, CAS, NaCAS and GEL protein membranes.

![Figure 2](image2.png)

**Figure 2** (a) Effect of glyoxal crosslinking on the tensile strength of: SI, CAS, NaCAS and GEL protein membranes. (b) Effect of heat treatment on the tensile strength of: SI, CAS, NaCAS and GEL protein membranes.
proteins [22]; and (ii) its helical nature [22]. Because gelatine is a scleroprotein built of rigid triple helices, the crosslinking efficiency is lower than in the case of the other more flexible proteins, namely soy and casein [23].

### 3.1.2. Degradation tests

Protein-based materials can be stabilized by chemical crosslinking. For instance, the crosslinking of dermal sheep collagen (DSC) with glutaraldehyde [24], using a water-soluble carbodiimide [25], proved to be very efficient on the decrease of the degradation kinetics of the protein. Crosslinking using glyoxal involves the reaction between the aldehyde groups of the glyoxal and the free ε-amine groups of lysine or hydroxylysine residues of the polypeptide chains. The results obtained revealed that the treatment with glyoxal decreased the weight loss rate for all the proteins, specially during the first 24 h of immersion (Figs. 4–7).

The resistance of the different protein films to degradation is directly proportional to the degree of crosslinking (Tables III–VI). In general, only crosslinking degrees superior to 30% result in protein films resistant to degradation. For this reason, the stability of CAS films is only obtained, and only efficient for short periods, with the use of 0.9% glyoxal (Fig. 5). In the case of NaCAS films, 0.3% glyoxal already resulted in films with a crosslinking degree of 35% and a 60% decrease of the degradation rate (Fig. 6). This degradation rates can eventually be reduced in 90% if NaCAS is crosslinked with 0.9% glyoxal (Fig. 7). In both cases, CAS and NaCAS films are only resistant to degradation up to 7 days of immersion (Figs. 5 and 6) when crosslinked with 0.9% glyoxal. This means that to reach hydrolytic stability for a longer term, CAS and NaCAS need to have at least 50% of their structures crosslinked (Tables IV and V). Although GEL presented a lower crosslinking efficiency with glyoxal (Table VI), as proved by a slight increase in the mechanical properties due to crosslinking (see Section 2.1.1), the established crosslinking seemed enough to stabilize the structure of GEL (Fig. 7). When crosslinked with 0.9% glyoxal, which corresponds to a crosslinking degree of 34% (Table VI), GEL is resistant to degradation.

![Figure 3](image_url) **Figure 3** (a) Effect of glyoxal crosslinking on the strain at break of: SI, CAS, NaCAS and GEL protein membranes. (b) Effect of heat treatment on the strain at break of: SI, CAS, NaCAS and GEL protein membranes.

![Figure 4](image_url) **Figure 4** Weight loss as a function of immersion time for SI membranes without treatment (○), crosslinked with 0.3% (0.3X-SI — ■), 0.6% (0.6X-SI — □) and 0.9% glyoxal (0.9X-SI — ■), submitted to 24 h heat treatment (24TT-SI — △) and irradiated by UV (UV-SI — ●).
to hydrolysis during at least the first 2 weeks of immersion (Fig. 7). SI protein, crosslinked or not, is the most resistant to hydrolysis, presenting a weight loss of only 33% after 2 weeks of immersion (Fig. 4). The crosslinking does not really change their degradation behavior. These differences in behavior should be mainly related with the internal structure of each one of the studied proteins. In general, proteins with a globular structure (like SI) seem to be more resistant to hydrolysis than those with randomly coil (like CAS and NaCAS) or helical structures (like GEL). Randomly coil proteins need high crosslinking degrees (>50%) to achieve resistance to hydrolysis. Proteins with helical structures seem to be stabilized with lower degrees of crosslinking and globular proteins are naturally more water resistant, even when not crosslinked.

3.2. Heat treatment

3.2.1. Tensile properties

Tables III–VI and Figs. 1(b), 2(b) and 3(b) disclose the results of yield stress ($\sigma_y$), E-modulus ($E$) and strain at break ($\varepsilon_b$) for the heat treated protein films. Like glyoxal crosslinking, heat treatment resulted in an increase in stiffness and strength in all the films except for gelatine. Again, a two-fold increment in $E$ was achieved for NaCAS and CAS (Tables IV and V and Fig. 1(b)) and 1.5-fold increment for SI (Table III and Fig. 1(b)).

These effects can be partially attributed to the development of heat-induced crosslinking within the film structure. As reported before [23], thermal treatment of proteins promotes formation of intra- and intermolecular covalent bonds. Such crosslinks result from condensation of lysine and cysteine amino acid residues with a residue of dehydroalanine, the latter being formed by an $\beta$-elimination reaction starting with cysteine or phosphoserine residues.

Presumably, such crosslinks (through the free e-amine groups of lysine or hydroxylysine residues), disulphides linkages (through reaction with the cysteine residues), and also hydrogen bonds [4, 23], contributed to the higher stiffness and strength of the heat-treated protein films. Because heat treatment promoted the formation of intra- (disulphide bonds) and intermolecular (crosslinking through amino groups) bonds, it showed to be a more efficient method to increase stiffness of the protein materials than only crosslinking with glyoxal. In the case of SI protein, the intramolecular bonding is the main responsible for the increase of stiffness of the protein materials. The percentage of amino groups used for crosslinking and contributing for the increase of mechanical properties is of only ~5% (Table III). In the case of CAS and NaCAS, both types of molecular bonding give a high contribution for the observed increment of the tensile properties (Tables IV and V).

In the case of gelatine films, the decrease in stiffness (Table VI) is mainly due to: (i) absence of cysteine residues (Table I), that are the responsible for the establishment of intra-molecular bonding; and (ii) the
low gelation point of GEL (40°C) [22]; so, the thermal treatment resulted in the melting and degradation of this protein. As described in Section 3.1.1, only the establishment of intermolecular bonding through the amino groups of lysine is not an efficient way to increase the mechanical properties of gelatine.

3.2.2. Degradation tests
As described before, the thermal treatment on the films at 80°C proved to be another successful way of cross-linking proteins. As previously reported, SI films are resistant to hydrolysis by nature. Consequently, thermal treatment does not really affect their degradation behavior (Fig. 4), as observed before with the cross-linking with glyoxal (see Section 3.1.2). Heat treated CAS samples present only more 5% of crosslinking degree than the glyoxal-modified CAS (Table IV). However, the degradation stability is increased about 50% which proves the positive influence of the disulphide linkages in the reduction of its degradation rate (Fig. 5). The same trend is observed with the NaCAS heat treated films (Fig. 6). When heat treated, GEL films melt (as explained in Section 2.2.1) and, consequently, become highly soluble in water (Fig. 7).

3.3. UV Irradiation

3.3.1. Tensile properties
The mechanical properties of UV treated films of SI, CAS and NaCAS were slightly improved as compared to the reference ones (Tables III–VI). This slight increase in stiffness should be mainly due to the lower moisture content presented by these films during testing. As discussed before [22], UV irradiation at λ = 366 nm does not result in any significant change in the protein structures. Only in the case of gelatine films, the UV irradiation was responsible for an increase of almost two-fold of the E-modulus (Table VI). E-modulus increased from 658 MPa for the reference to 1130 MPa and 1071 MPa for the UV treated-gelatine films (UV applied on the film forming solution and applied on the film, respectively). This behavior was attributed to the aggregation of the gelatine helices as reported by Kato et al. [22].

3.3.2. Degradation tests
Irradiation by UV did not change the degradation behavior of the studied proteins relatively to the non-treated ones. This should be associated to the fact that the rearrangements of protein helices did not cause cross-linking and, consequently, no positive effect on their degradation behavior.

4. Conclusions
Due to its hydrolytic stability, soy becomes a very attractive protein to be used in biomedical applications, namely as drug delivery carrier or as tissue engineering scaffold, where long term treatment are a requisite. CAS, NaCAS samples are not resistant to hydrolysis in the human body-like conditions. However, the resistance to hydrolysis can be enhanced by chemical crosslinking or heat treatment. Anyway, they do not resist to hydrolysis longer than 2 weeks. GEL, when crosslinked with high amounts of glyoxal, become resistant to hydrolysis and, due to its already proved non-cytotoxic and non-immunogenic behaviors, confirms why it is already a material of election for biomedical applications.

In general, glyoxal crosslinking lead to a decrease of the susceptibility towards hydrolysis and favored the mechanical strength and reduced the ductility for all the proteins. Thermal treatment may eventually be an interesting alternative for glyoxal crosslinking (except for gelatine) when a non-chemical process is needed to improved both mechanical performance and degradation behavior of proteins.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Free–NH₂ groups (%)</th>
<th>σy (MPa)</th>
<th>εb (%)</th>
<th>E0,05–0,25% (MPa)</th>
<th>Thickness (µm)</th>
<th>MC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEL</td>
<td>74.4 ± 0.2</td>
<td>32.6 ± 0.5</td>
<td>52.2 ± 9.3</td>
<td>658 ± 102</td>
<td>0.129 ± 0.017</td>
<td>10.4 ± 0.1</td>
</tr>
<tr>
<td>0.3X-GEL</td>
<td>73.3 ± 1.2</td>
<td>25.6 ± 4.5</td>
<td>41.6 ± 11.2</td>
<td>476 ± 107</td>
<td>0.143 ± 0.013</td>
<td>9.9 ± 0.1</td>
</tr>
<tr>
<td>0.6X-GEL</td>
<td>69.7 ± 1.3</td>
<td>27.7 ± 3.9</td>
<td>41.0 ± 15.0</td>
<td>605 ± 92</td>
<td>0.120 ± 0.011</td>
<td>9.2 ± 0.1</td>
</tr>
<tr>
<td>0.9X-GEL</td>
<td>66.4 ± 0.8</td>
<td>30.6 ± 1.7</td>
<td>37.2 ± 23.5</td>
<td>659 ± 269</td>
<td>0.123 ± 0.051</td>
<td>8.6 ± 0.1</td>
</tr>
<tr>
<td>2TT-GEL</td>
<td>75.0 ± 2.6</td>
<td>11.5 ± 1.6</td>
<td>72.5 ± 10.1</td>
<td>178 ± 62</td>
<td>0.152 ± 0.011</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>24TT-GEL</td>
<td>74.9 ± 1.3</td>
<td>14.4 ± 1.8</td>
<td>60.5 ± 9.0</td>
<td>295 ± 87</td>
<td>0.135 ± 0.010</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>UV&lt;sub&gt;ad&lt;/sub&gt; – GEL</td>
<td>78.9 ± 2.3</td>
<td>36.6 ± 3.2</td>
<td>36.6 ± 11.0</td>
<td>1130 ± 188</td>
<td>0.096 ± 0.005</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>UV-GEL</td>
<td>75.7 ± 0.7</td>
<td>33.8 ± 3.3</td>
<td>28.5 ± 14.0</td>
<td>1071 ± 234</td>
<td>0.099 ± 0.008</td>
<td>4.3 ± 0.1</td>
</tr>
</tbody>
</table>

%X-GEL – glyoxal crosslinked gelatine (% glyoxal based on the protein content); (hrs) TT – gelatine heat treated for 2 and 24 h; UV-GEL – gelatine film irradiated by UV; UV<sub>ad</sub> – gelatine film-forming solution irradiated by UV; σy – yield stress (MPa); εb – strain at break (%); E0,05–0,25% – E-modulus between 0.05–0.25% strain (MPa). MC – moisture content of the films.
List of abbreviations and symbols

SL – soy protein
CAS – casein protein
NaCAS – sodium-caseinate protein
GEL – gelatine protein
%X-protein – protein crosslinked with a certain % of glyoxal (% based on the protein content)
\( \tau \) – protein – heat treated at 80°C during a certain time \( t \)
UV\(_{\text{sol}}\) – protein–protein film (UV–) or protein film-forming solution (UV\(_{\text{sol}}\)–) submitted to UV irradiation
ISS – isotonic saline solution (NaCl 9 g/l)
E – E-modulus
\( \sigma_s \) – yield stress
\( \varepsilon_b \) – strain at break
MC – moisture content
RH – relative humidity
RT – room temperature
UV – ultra-violet
\( W_0 \) – initial weight
\( W_{\text{dry}} \) – dry weight
\( W_L \) – weight loss for aging time \( t \)
\( W_t \) – weight at aging time \( t \)
\( N_{\text{tot}} \) – total protein content
\( N_0 \) – initial protein content
\( N_t \) – protein content at aging time \( t \)
\( \phi_N \) – Kjeldahl factor
OPA – \( \alpha \)-phthalaldehyde
SDS – sodium-dodecyl sulfate

Acknowledgments
Cláudia M. Vaz acknowledges the Portuguese Foundation for Science and Technology (FCT), Ministry of Science and Technology, Portugal, for the attribution of a PRAXIS XXI PhD Grant; and Dr Johan Vereijken, ATO BV, The Netherlands, for its valuable comments on this manuscript.

References
17. R. L. CLARK and R. C. GRALOW, Mod. Packag. 22 (1949) 122.

Received 22 October and accepted 23 December 2002