Casein and soybean protein-based thermoplastics and composites as alternative biodegradable polymers for biomedical applications


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Abstract: This work reports on the development and characterization of novel meltable polymers and composites based on casein and soybean proteins. The effects of inert (Al$_2$O$_3$) and bioactive (tricalcium phosphate) ceramic reinforcements over the mechanical performance, water absorption, and bioactivity behavior of the injection-molded thermoplastics were examined. It was possible to obtain materials and composites with a range of mechanical properties, which might allow for their application in the biomedical field. The incorporation of tricalcium phosphate into the soybean thermoplastic decreased its mechanical properties but lead to the nucleation of a bioactive calcium-phosphate film on their surface when immersed in a simulated body fluid solution. When compounded with 1% of a zirconate coupling agent, the nucleation and growth of the bioactive films on the surface of the referred to composites was accelerated. The materials degradation was studied for ageing periods up to 60 days in an isotonic saline solution. Both water uptake and weight loss were monitored as a function of the immersion time. After 1 month of immersion, the materials showed signal of chemical degradation, presenting weight losses up to 30%. However, further improvement on the mechanical performance and the enhancement of the hydrolytic stability of those materials will be highly necessary for applications in the biomedical field. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 65A: 60 –70, 2003

Key words: soybean; casein; thermoplastic proteins; biomaterials; biodegradable polymers; degradation; bioactivity

INTRODUCTION

The concept of combining the biodegradability and high availability of natural polymers is attracting scientists from diverse areas. A good example is the proposal of blends of several starch based blends as an alternative to the most common biodegradable polymers applied in the biomedical field, such as poly(lactic acid), polyhydroxybutyrate, and polyglycolic acid. However, the number of biopolymers in clinical use is rather small and stills presenting several weaknesses. A potential solution to overcome these difficulties may rely on the development of new materials, such as new protein-based thermoplastics (from vegetable or animal origin). Their high chemical versatility and similarity to tissue constituents may lead to the introduction of novel biomaterials into the clinical area. To our knowledge, until now only collagen and gelatine have been extensively studied and used. However, the introduction of other protein-based biomaterials may be of extreme importance if they could combine 1) the main advantages of collagen and gelatine; 2) reduced susceptibility to thermal degradation (allowing for its easy processing by melt based technologies into complex 3D implants); and 3) convenient degradation behavior. The good processability, both in aqueous media and in the melt, good film-forming properties, good adhesion to various substrates, and surface active properties can differentiate positively proteins from other biopolymers. Furthermore, proteins are very versatile materials, both by source and because of a wide variety scale of possible modifications, so their properties can be tailored towards the diverse requirements of a specific application. As a result, proteins may be regarded as an eventual ideal template suitable for being used as biomaterials. Temporary replacement implants, tissue-engineering scaffolding,
Membranes for promoting wound healing, and drug delivery carriers are the most promising target applications. In this preliminary work, new casein and soybean thermoplastic formulations were developed by extrusion compounding. The effects of inert (alumina, used as a model system) and bioactive (tricalcium phosphate, a bone-like ceramic) ceramic reinforcements over the composites mechanical properties, its degradation behavior, and bioactivity character were examined.

**MATERIALS AND METHODS**

**Materials**

Soybean protein isolate (SI) with a protein content of 90–91% (dry basis) was provided by Protein Technology International. DMV International (The Netherlands) supplied the whole casein protein (CAS). Additionally, a food-grade plasticizer (glycerol) and a coupling agent (Neopentyl(diallyl)oxytri(dioctyl)phosphate zirconate, NZ 12, pH 6) were used as received from the manufacturers: Merck (The Netherlands) and Ken-React, (Kenrich Petrochemicals), respectively. The SI and CAS polymers were reinforced with an inert and a bioactive ceramic filler, namely 1) alumina (Al$_2$O$_3$, Hüber, The Netherlands) and 2) tricalcium phosphate (TCP, Ca$_3$(PO$_4$)$_2$, Merck, The Netherlands), both with an average particle size smaller than 20 μm.

**Materials processing**

SI and CAS formulations with 0, 10, and 30% (wt %) of Al$_2$O$_3$ (respectively, SI, SI + 10Al$_2$O$_3$, and SI + 30Al$_2$O$_3$) were compounded in a corotating twin-screw extruder (Berstorff ZE 25(CL) × 40D) in the presence of 10% of glycerol and 30% of water (both wt % of the protein weight). SI-based formulations also were mixed with 10 and 30% (wt %) of TCP (respectively, SI, SI + 10TCP, and SI + 30TCP). For the preparation of the coupled composites, the above formulations were also mixed, during the extrusion procedure, with 1% (based on the total amount of the filler) of the coupling agent NZ12.

The inert alumina was used as a model to optimise the extrusion compounding setup adequate for the efficient producing of CAS and SI composites. After the adjustment of the referred parameters, the SI polymeric matrix was reinforced with the bioactive TCP.

The extruded compounds (in the form of pellets) were molded into ASTM tensile test bars (2 × 4 mm$^2$ of cross section), after being conditioned (60°C over 24 h) until the respective moisture content reached 10–12%. These specimens were molded using a DEMAG D25 NC 4 under optimized processing conditions. Subsequently, the injection-molded samples were conditioned at 23°C and 60% relative humidity (RH) for at least 1 week.

**Mechanical and morphological characterization**

To assess the mechanical performance of the new SI and CAS thermoplastics, tensile tests were performed on a Zwick Z010 universal mechanical testing machine, in a controlled environment (20°C and 55% relative humidity). The secant modulus at 1% strain ($E_{1%}$), the ultimate tensile strength (UTS), and the strain at break ($\varepsilon_r$) were evaluated.

Tensile fracture surfaces were observed by scanning electron microscopy (SEM) in a Leica Cambridge S360 microscope for morphological characterization of the fracture surfaces, filler distribution, and interfacial interactions between the filler and the polymeric matrix.

**In vitro degradation tests**

Molded specimens of SI and CAS materials and composites were weighed and aged up to 60 days in an isotonic

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**TABLE I**

**Mechanical Properties of the Developed Protein Materials and Their Respective Composites**

<table>
<thead>
<tr>
<th>Material</th>
<th>$E_{1%}$ (MPa)</th>
<th>UTS (MPa)</th>
<th>$\varepsilon_r$ (%)</th>
<th>Moisture Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAS</td>
<td>2534 ± 60</td>
<td>48.5 ± 7.0</td>
<td>2.2 ± 0.5</td>
<td>3.90 ± 0.13</td>
</tr>
<tr>
<td>CAS+10Al$_2$O$_3$</td>
<td>2010 ± 55</td>
<td>39.2 ± 0.5</td>
<td>6.4 ± 2.4</td>
<td>3.74 ± 0.02</td>
</tr>
<tr>
<td>CAS+30Al$_2$O$_3$</td>
<td>3479 ± 59</td>
<td>44.1 ± 1.6</td>
<td>2.4 ± 0.6</td>
<td>3.22 ± 0.31</td>
</tr>
<tr>
<td>SI</td>
<td>1256 ± 101</td>
<td>33.3 ± 4.6</td>
<td>16.5 ± 6.9</td>
<td>5.06 ± 0.50</td>
</tr>
<tr>
<td>SI+10Al$_2$O$_3$</td>
<td>1699 ± 83</td>
<td>33.4 ± 2.3</td>
<td>2.8 ± 0.5</td>
<td>4.40 ± 0.01</td>
</tr>
<tr>
<td>SI+30Al$_2$O$_3$</td>
<td>2481 ± 136</td>
<td>32.3 ± 0.9</td>
<td>3.1 ± 0.2</td>
<td>3.34 ± 0.17</td>
</tr>
<tr>
<td>SI+10TCP</td>
<td>680 ± 20</td>
<td>17.0 ± 0.3</td>
<td>23.2 ± 5.8</td>
<td>8.57 ± 0.08</td>
</tr>
<tr>
<td>SI+30TCP</td>
<td>896 ± 58</td>
<td>17.8 ± 0.2</td>
<td>12.1 ± 1.6</td>
<td>7.65 ± 0.23</td>
</tr>
<tr>
<td>SI+NZ</td>
<td>299 ± 20</td>
<td>6.4 ± 0.2</td>
<td>3.7 ± 0.6</td>
<td>9.74 ± 0.09</td>
</tr>
<tr>
<td>SI+10Al$_2$O$_3$+NZ</td>
<td>425 ± 28</td>
<td>12.1 ± 0.6</td>
<td>23.5 ± 8.0</td>
<td>9.09 ± 0.51</td>
</tr>
<tr>
<td>SI+30Al$_2$O$_3$+NZ</td>
<td>382 ± 25</td>
<td>10.7 ± 0.1</td>
<td>23.1 ± 7.7</td>
<td>7.76 ± 0.05</td>
</tr>
<tr>
<td>SI+10TCP+NZ</td>
<td>586 ± 24</td>
<td>15.0 ± 0.2</td>
<td>26.4 ± 2.7</td>
<td>8.96 ± 0.37</td>
</tr>
<tr>
<td>SI+30TCP+NZ</td>
<td>601 ± 31</td>
<td>13.8 ± 0.3</td>
<td>13.1 ± 4.4</td>
<td>7.87 ± 0.07</td>
</tr>
</tbody>
</table>
saline solution (ISS; NaCl 0.154 M, pH = 7.4 ± 0.02) at 37°C. A volume of 50.0 ± 0.5 mL of ISS was used for three specimens (one batch) of approximately 1.300 ± 0.005 g each. After being removed from the degradation solution, all the bars were immediately weighed to evaluate the respective water uptake as described in Equation (1):

Water uptake (%) = 100 × [(W_w - W_i) / W_i]  (1)

where, W_i and W_w are the initial and wet weight of each sample, respectively. Subsequently, the samples were dried up to exhaustion in a vacuum oven at 40 ± 2°C for 1 day to determine the respective weight loss as described in Equation (2):

Weight loss (%) = 100 × [(W_i - W_d) / W_i]  (2)

where W_i and W_d are the initial and dry weight of each sample, respectively.

The degradation solutions were analyzed by atomic

Figure 1. SEM micrographs of the fracture surfaces of (a) CAS (×50) and (b) CAS + 30Al_2O_3 (×1000).

Figure 2. SEM micrographs of the fracture surfaces of (a) SI (×50); (b) SI + 30Al_2O_3; and (c) SI + 30TCP (×1000).
emission spectrometry (inductive coupled plasma) to quantify the calcium and phosphorous release kinetics as a function of the immersion time ([Ca]_{deg} and [P]_{deg}).

Bioactivity tests

Molded bars of SI and CAS materials and composites were immersed for several prefixed aging periods (0, 5, 7, 14, and 30 days) in a simulated body fluid (SBF) solution (prepared as described by Kim\textsuperscript{21}) at 37°C and pH = 7.4 ± 0.02. After being removed from the solutions, the samples were dried for 1 day in an oven at 40 ± 2°C. The specimen surfaces, subjected to different immersion times, were morphologically examined by SEM and compared with the surface of the correspondent material without immersion (native material). The degree of extension of the apatite layer formed on the materials surface is an indication of their level of bioactivity. The dried materials were further analyzed by thin-film X-ray diffraction (TF-XRD). The referred analysis was performed in a diffractometer Philips X'Pert MPD at 1 s of path time and with a divergent beam at 1 degree. The SBF
solutions, after removal of the samples, were also analyzed by inductive coupled plasma for the quantification of the calcium and phosphorous concentrations ([Ca]bioact and [P]bioact). To subtract the amounts of calcium and phosphorous present in the SBF solution as a result of the materials degradation ([Ca]deg and [P]deg), the final calcium and phosphorous concentrations ([Ca]final and [P]final) were corrected by the following:

\[
\text{[Ca or P]_{final}} = \text{[Ca or P]_{bioact}} - \text{[Ca or P]_{deg}}
\]  

(3)

RESULTS AND DISCUSSION

Mechanical properties

The effects of the alumina and TCP content and of the coupling agent on the mechanical properties of the CAS and SI thermoplastics are compared in Table I. CAS-based polymers and composites exhibit higher
stiffness than the SI materials. For 3.90% of moisture content, the E-modulus of CAS mouldings is 2534 MPa, being increased by the alumina content. In fact, CAS/H11001 30Al2O3 is a rigid and brittle thermoplastic with 3479 MPa of stiffness and 3.22% strain at break (with 3.22% of moisture content). SEM micrographs [Fig.1(a)] show the brittle character of CAS, which exhibited a smooth surface center region where the crack initiated and grew slowly. The CAS composites fracture surfaces revealed a good distribution of the alumina particles and a good polymer/ceramic interfacial interaction [Fig. 1(b)].

SI materials also present a brittle behavior, but with fairer mechanical properties, namely 1256 MPa of E-modulus and 16.5% strain at break (5.06% of moisture content). The introduction of alumina proved to be even more effective in reinforcing SI (97% gain for SI + 30Al2O3). The SEM micrograph in Figure 2(a) illustrates a typical smooth and brittle fracture of SI. The alumina composites reveal a good filler distribution and good polymer/ceramic interfacial interaction [Fig. 2(b)].

When reinforced with TCP, the E-modulus was found to decrease sharply (29% loss for SI + 30TCP; Table I). The observed reduction in properties of these SI composites is consistent with the fracture surface observations [Fig. 2(c)], which present a poor distribution of the filler, a poor polymer/ceramic interaction and a tendency for filler/filler contact, which also is known to reduce the mechanical properties. The introduction of the amino-based coupling agent NZ12 also caused the plasticization of the SI matrix, especially when reinforced with alumina. NZ12 showed some affinity in relation to the TCP composite avoiding an even higher loss in stiffness.

Figure 7. SEM micrographs of the protein materials and composites surfaces after immersion in a SBF solution for 1) 0 and 2) 14 days (×2000): (a) CAS, (b) SI, (c) SI + 30TCP, and (d) SI + 30TCP + NZ12.
In vitro degradation tests

Three degradation stages were noticeable in the in vitro degradation behavior of SI material and composites. The first one, between 0 and 6 immersion days, was essentially related with the leaching of plasticisers (glycerol) and low molecular weight \( M_w \) polymeric chains (resulted from thermo-oxidative degradation occurred during extrusion). A high water uptake (Fig. 3) and a fast degradation occurred during the first week of immersion (weight loss of 20%; Fig. 4). When compatibilized with the coupling agent NZ 12, SI presented an even higher weight loss (45%) because of the plasticizing and destructurizing effect of this agent over the polymeric matrix.

The second degradation stage, typically between 15 and 30 immersion days, was essentially characterized by a decrease in the degradation rate. The stabilization of the weight loss (around 20%; Fig. 4) and decrease in water uptake to around 75% (Fig. 3) were the main relevant aspects.

During the first and the second degradation stages, the SI material and composites released all the calcium \([\text{Ca}]_{\text{deg}} = 25 \text{ mg/L}\) and phosphorous \([\text{P}]_{\text{deg}} = 140 \text{ mg/L}\) to the degradation solutions (Fig. 5 and 6). The only exception was SI + 30TCP + NZ12, which presented a slower release of calcium as a function of immersion time. During the first 2 weeks of immersion, this composite only released half of its total calcium concentration. This fact confirms the existence of a certain affinity of the coupling agent in relation to the TCP ceramic.

The third degradation stage, between 30 and 60 im-

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Figure 7. (Continued from the previous page)
mersion days, was characterized by the apparent begin-
ing of a chemical degradation (hydrolysis and chain breakage). The weight loss doubled (approximately 50%; Fig. 4) and the water uptake (Fig. 3) remained constant. This final stage should correspond to the fragmentation of the proteins internal structure and, consequently, dissolution of the resulting fragments.

The CAS-based compounds also showed three differ-
ent stages in the in vitro degradation tests. The leaching of plasticizers (30% glycerol for CAS) was the main factor responsible for the weight loss of CAS during the first stage (Fig. 4), between 0 and 6 immersion days.

The second degradation stage, typically during the second week of immersion, was characterized mainly by a decrease in the degradation rate. The stabilization of the weight loss and water uptake (Figs. 3 and 4), as well as the complete release of calcium to the degra-
dation solutions (Fig. 5) were the most important phe-
nomena occurring during this stage.

During the third degradation stage, after 2 weeks of

Figure 8. TF-XRD pattern of the protein materials and composites surfaces after immersion in a SBF solution for (a) 0 and (b) 30 days.
immersion, the CAS degradation rate increased. The weight loss was four times higher (approximately 45%; Fig. 4), the water uptake remained constant (Fig. 3), and the CAS released its total phosphorous amount (Fig. 6). This stage should correspond to a beginning of the chemical degradation, with respective breakage of the CAS internal structure, which was probably initiated by the decrease in pH of the degradation solutions.

Bioactivity tests

After 30 days of immersion in a SBF solution, CAS showed no evidence of nucleation of a bioactive film (Ca-P) on its surface, as observed by SEM (Fig. 7) and confirmed by TF-XRD (Fig. 8).

This result indicates a nonbioactive behavior of CAS, which may result from the impossibility of precipitation of a Ca-P film in the surface of the CAS samples resulting from the continuous increase of [P]final and decrease of [Ca]final in the SBF solution, as a function of time (Figs. 9 and 10).

When immersed in a SBF solution, after 1 week of immersion, SI evidenced by SEM (Fig. 7) the formation of only some Ca-P nuclei on its surface. However, even after 1 month of immersion in SBF, no distinct Ca-P layers could be observed. The TF-XRD results (Fig. 8) were in agreement with the SEM observations and peaks correspondent to crystalline Ca-P phases (mainly hydroxylapatite or TCP) could be hardly detected in the surface of the protein.

As evidenced by SEM (Fig. 7), SI + 30TCP showed nucleation and growth of a bioactive Ca-P film on its surface, characteristics of a highly bioactive material. The TF-XRD results (Fig. 8) reinforced the SEM observations, which showed an increment of the crystalline phases (peak intensity) on the composite surface after 14 days of immersion. The XRD pattern resulting from the Ca-P film is similar to that of the bone apatite. The decreased of [Ca]final and [P]final in the SBF solution, as a function of time, resulted in the Ca-P film precipitation, confirming the bioactivity of the referred material (Figs. 9 and 10).

When treated with NZ12, the nucleation and growth of the Ca-P film was faster, as confirmed by SEM (Fig. 7) and TF-XRD (Fig. 8). This fact can be explained by the influence of the coupling agent on the decrease of the kinetics of calcium and phosphorous release to the degradation solutions (Figs. 5 and 6). As a result, a decrease of [Ca]final and [P]final in the SBF solution was observed (Figs. 9 and 10), as a function of time, leading to an in vitro bioactive behavior. Consequently, it is expected that these materials will be able to directly bond to bone, without fibrous encapsulation, when implanted. Nevertheless, further testing, mainly in terms of their immunogenicity, obviously need to be performed.

Concerning this point, it is known that soybean proteins have immunological activity and can act as allergens in humans. However, as pointed out in the literature, there is no evidence that soy proteins possess any special allergenic property when compared with other proteins. As the use of animal origin proteins, such as collagen and gelatin, in biomedical applications is a common practice, it is also expected the possibility of introduce alterna-
CONCLUSIONS

This preliminary study confirms that the developed casein and soybean protein-based thermoplastics present a suitable range of mechanical and degradation properties, as well as a bioactive character (especially when reinforced with bone-like ceramics) which might eventually allow for their use as biomaterials.

For many applications, it will be necessary to create further improvements in their mechanical strength and to enhance their hydrolytic stability. However, as a result of the high versatility of this type of materials, a broad range of physical and chemical modifications can be performed to tailor their properties towards the diverse requirements of different biomedical applications.

References