Simulation of novel soy protein-based systems for tissue regeneration applications

Dafna Knani⁎, Hilla Barkay-Olami, David Alperstein and Meital Zilberman

In the present research, molecular modeling methods were used to study novel porous soy protein conjugates with gelatin or alginate, which were recently developed as potential scaffolds for tissue engineering applications. Gelatin (protein) and alginate (polysaccharides) were chemically crosslinked to soy protein isolates (SPI) in order to obtain a porous 3D network. Computational tools were applied to estimate the crosslinking degree and compare the degradation rate of soy–gelatin or soy–alginate conjugates. Soy protein 3D structure was obtained from the Protein Data Bank (PDB). Alginate and gelatin structures were built and subjected to dynamic simulation using the molecular modeling package Material Studio 7.0. The crosslinking degree was estimated by the miscibility of the two reactants and the interaction with the crosslinking agents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) or glyoxal. The calculations revealed that soy protein mixes well with gelatin but not with alginate. Radial distribution function (RDF) calculations showed that the interaction distance between alginate and EDC is significantly shorter than between gelatin and EDC, probably because of ionic attraction between the ammonium groups of EDC and the carboxylate groups in alginate, which facilitates the crosslinking reaction. The degradation rate of soy protein conjugates was related to their interaction with water. It was found that the solubility of soy–gelatin in water is higher than soy–alginate and that water molecules form more hydrogen bonds with soy–gelatin than with soy–alginate. These findings might be the reason for the observed difference in degradation rate of the two conjugates; the soy–gelatin degrades faster than soy–alginate. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: MD simulation; soy protein isolates; alginate; gelatin; scaffold for tissue engineering

INTRODUCTION

Recently, there is an increasing interest in using bio-based polymers of non-animal origin to fabricate biodegradable materials. Plant polymers can be employed to meet the high demand for new materials needed for various biomaterials applications in the food industry, agriculture and medical applications, especially tissue engineering. One biopolymer that has drawn attention lately is soy protein that has advantages over various types of natural proteins used as biomaterials because of its low price and relatively long storage time and stability. Soy is a protein with reproducible resource, good biocompatibility, biodegradability, and processability. Various soy protein isolate (SPI)-based materials were prepared mostly for applications in the food industry, especially as films used for packing. For instance, Barbut et al. found that adding small amounts of SPI, gelatin or whey protein isolate (WPI) to calcium cross-linked “wet” alginate can produce films with a similar puncture strength as some commercially used manufactured collagen. On the other hand, Denavi and coworkers studied the structure–function relationship of composite films obtained from SPI and cod gelatin and found that although some physical properties have been improved as compared to pure gelatin or pure SPI films, these composite films are nevertheless highly water sensitive, making them still far from being a real alternative to the synthetic polymers.

In recent work, Barkay-Olami and Zilberman explored novel porous soy protein-based systems for use as scaffolds in tissue engineering, especially for skin regeneration applications. Blends of soy protein with other polymers (gelatin, alginate, pectin, polyvinyl alcohol, and polyethylene glycol) were prepared. Porous soy–gelatin and soy–alginate conjugates obtained by combining chemical crosslinking with freeze-drying were studied for physical properties, degradation behavior, and microstructure. The results show that soy–alginate blends are advantageous over soy–gelatin blends, demonstrating better stability and degradation time along with controlled swelling behavior because of a more effective crosslinking and higher water uptake than soy–gelatin blends. Water vapor transmission rate experiments showed that all porous blend structures were in the desired range for burn treatment and can be controlled by the crosslinking process. However, the interactions between the two molecules cannot be directly observed by current laboratory methods.

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studies, and therefore molecular dynamics (MD) simulation was applied to complement laboratory studies.

Only few works were published concerning simulation of soy protein mixtures. Pang and coworkers[5] used MD simulation to study the interactions between the polysaccharide konjac glucomannan (KGM) and SPI. They found that the sum potential energy of the mixed KGM/SPi gel dropped, while that of single KGM gel increased. The surface area of both KGM and SPI in the mixed system decreased. The results indicate that the stability of KGM/SPi was improved and that hydrogen bond is formed between the —OH groups in KGM and the amide linkage group on Histidine, Asparagine, and Leucine in SPI. The hydrogen bond was formed directly or indirectly by the bridge of waters.[6] Withana-Gamage and Wanasundara[6] used soybean globulin as a model protein to demonstrate that food protein scientists can utilize bioinformatics to screen or investigate suitability of a protein for specific functionalities needed in food. This approach resembles designing of drugs in pharmaceutical and medicinal chemistry.[6]

Alginate and gelatin systems were simulated as well. Alginate is a polysaccharide extracted from seaweeds. Isolated alginates and wastewaters. The model organic compounds chosen were sodium alginate, bovine serum albumin (BSA), and humic acid.

The restraint properties of alginate by calcium rather than sodium ions. Gray et al.[10] examined the binding modes that provide a rationale for the observed gelling activity occurs when alginate out a number of industries because of their gelling and water retaining properties.[2] This gelling activity occurs when alginate chains aggregate in the presence of divalent cations (most commonly Ca2+) that facilitate interchain interactions. The chains of alginate consist of arrangements of β-D-mannuronic acid (M) and, α-L-guluronic acid (G) monomers, bound via (1→4) glycosidic linkages arranged in three distinct sequences of block copolymers: polygulurionate (poly-G), poly-mannuronate (poly-M), or alternating GM sequences (poly-GM). Stewart and coworkers[6,9] conducted a series of MD simulations to account for the observed aggregation of various alginate chains (poly-G, poly-M poly-GM) in the presence of calcium ions. A number of junction zones involving calcium ions have been identified that result in chain aggregation. The results have revealed binding modes that provide a rationale for the observed gelling of alginate by calcium rather than sodium ions. Gray et al.[10] conducted a study to identify possible interactions between organic compounds that are commonly found in natural waters and wastewaters. The model organic compounds chosen were sodium alginate, bovine serum albumin (BSA), and humic acid.

MD simulations were designed in order to provide insights into the interaction between BSA and humic acid, as well as alginate–humic acid system. The alginate models used were decamer chains of the three sequences found naturally in algal-sourced alginates. It was found that the dominant interaction in the alginate–humic acid system was water-mediated Ca2+ bridging between the deprotonated carboxylate moiety of the humic acid molecule and a binding pocket on the alginate.[10] Leng et al.[11] used MD simulations to elucidate the fouling mechanism of polyamide membrane used in water purification. They investigated the properties of hydrated amorphous polyamide membrane and its binding with alginate, which is believed to have a significant contribution to membrane fouling. Simulation results showed that the carboxylate groups in both the polyamide surface and alginate exhibit strong binding with metal ions (Ca2+ and Na+). This binding mechanism plays a major role in the polyamide–alginate fouling through the formation of an ionic binding bridge.[11] Similar results were obtained for crosslinked polyamide with alginate. Alginate is also produced in an infection process by opportunistic human pathogens, for instance Pseudomonas aeruginosa.[13] The infection process involves production of a biofilm mucoid containing several hydrolytic enzymes and the extracellular polysaccharide alginate. Tielen and coworkers[14] studied the interaction between the extracellular lipase LipA and alginate by molecular modeling. They demonstrated that lipase LipA interacts with the alginate via electrostatic interactions suggesting a role of this interaction for enzyme immobilization and accumulation within biofilms. The enzyme is retained near the cell surface, with the catalytic center exposed toward the substrate and is protected from denaturation and proteolytic degradation.[14]

Gelatin is a mixture of polypeptides obtained by partial hydrolysis of collagen extracted from connective tissues of animals, primarily from bovine and porcine skin and bones.[15] The amino acid composition of several types of gelatin is presented in Table 1.[16] Gelatin “type A” from porcine skin was used in our experimental study.[4]

Examination of the amino acids sequence shows that Gly is present as every third residue, producing a (Gly-X-Y)n repeating pattern.[17] The most common tripeptide sequence found in gelatin and collagen is Gly-Pro-Hyp, but in contrast to the restrictive nature of every third position being Gly, the other two positions in each repeating unit, X and Y, can accommodate any of the 20 amino acids, including imino acids, without distortion.[17] Gelatin is a well-known bioadhesive that could be utilized as the active layer of composite membrane because of its high affinity toward water and good film forming property. The COOH and NH2 groups in gelatin molecules can provide “active-sites” for the formation of adhesive bonds. Jiang et al.[18] prepared a composite gelatin – polyacrylonitrile (GE/PAN) membrane by dip-coating method and crosslinked the gelatin by glutaraldehyde to manipulate the swelling of the composite membranes. MD simulation was utilized to probe the interfacial compatibility and the interfacial interaction of the composite membrane by calculation of the interfacial energy, the solubility parameters of membrane.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Type A</th>
<th>Type B</th>
<th>Type B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Pork skin)</td>
<td>(calf skin)</td>
<td>(bone)</td>
</tr>
<tr>
<td>Alanine</td>
<td>8.6–10.7</td>
<td>9.3–11.0</td>
<td>10.1–14.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.3–9.1</td>
<td>8.5–8.8</td>
<td>5.0–9.0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>6.2–6.7</td>
<td>6.6–6.9</td>
<td>4.6–6.7</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.1</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>11.3–11.7</td>
<td>11.1–11.4</td>
<td>8.5–11.6</td>
</tr>
<tr>
<td>Glycine</td>
<td>26.4–30.5</td>
<td>26.9–27.5</td>
<td>24.5–28.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.9–1.0</td>
<td>0.74–0.8</td>
<td>0.4–0.7</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>1.0</td>
<td>0.91–1.2</td>
<td>0.7–0.9</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>13.5</td>
<td>14.0–14.5</td>
<td>11.9–13.4</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.4</td>
<td>1.7–1.8</td>
<td>1.3–1.5</td>
</tr>
<tr>
<td>Leucine</td>
<td>3.1–3.3</td>
<td>3.1–3.4</td>
<td>2.8–3.5</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.1–5.2</td>
<td>4.5–4.6</td>
<td>2.1–4.4</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.8–0.9</td>
<td>0.8–0.9</td>
<td>0.0–0.6</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.1–2.6</td>
<td>2.2–2.5</td>
<td>1.3–2.5</td>
</tr>
<tr>
<td>Proline</td>
<td>16.2–18.0</td>
<td>14.8–16.4</td>
<td>13.5–15.5</td>
</tr>
<tr>
<td>Serine</td>
<td>2.9–4.1</td>
<td>3.2–4.2</td>
<td>3.4–3.8</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.2</td>
<td>2.2</td>
<td>2.0–2.4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.4–0.9</td>
<td>0.2–1.0</td>
<td>0.0–0.2</td>
</tr>
<tr>
<td>Valine</td>
<td>2.5–2.8</td>
<td>2.6–3.4</td>
<td>2.4–3.0</td>
</tr>
</tbody>
</table>
materials, and the mean-square displacement of GE polymer chains. They concluded that the intermolecular interaction between GE and PAN was stronger than the intramolecular force of GE polymer.\(^{[18]}\) Lendlein et al.\(^{[19]}\) explored the potential of side chain functionalization of gelatin with the aim to develop defined polymer systems with tailorable properties by enabling specific noncovalent interactions. They employed molecular modeling methods to explore the influence of the functionalized gelatins as amorphous bulk materials. The models predicted an increasing number of specific π–π interactions and hydrogen bonds of gelatins functionalized with increasing numbers of tyrosine-derived phenol moieties.\(^{[19]}\)

The aim of this work is to study soy–protein blends with gelatin or alginate using MD simulation, which may lead to a better understanding of the interaction nature of the mixed systems.

**EXPERIMENTAL**

**Computational tools**

The simulation tools used were as follows:

1. Material Studio 7.0 (by Biovia, previously Accelrys)\(^{[20]}\) molecular modeling package. Three simulation modules were used:
   - Forcite—A forcefield simulation tool performing molecular mechanics and molecular dynamics tasks. The forcefield used was COMPASS II (condensed-phase optimized molecular potentials for atomistic simulation studies).
   - Amorphous Cell—A simulation tool capable of building 3D periodic boundary cells.
   - DMOL3—A quantum mechanics module, modeling the electronic structure and energetics of molecules using density functional theory (DFT).
2. GOLD (Genetic Optimization for Ligand Docking) Suite, developed by CCDC (The Cambridge Crystallographic Data Centre).\(^{[21]}\) GOLD Suite includes software tools for protein structure visualization and manipulation (Hermes), for protein-ligand docking (GOLD) and for post-processing (GoldMine) and visualization of docking results.

**Molecular models**

**Soy protein**

Soy is a globular protein composed of four fractions, which are classified according to their sedimentation properties. These fractions include the 2S, 7S, 11S, and 15S fractions and they comprise 8%, 35%, 52%, and 5% of the total protein content, respectively.\(^{[1]}\) Two major subunits in the globular structure include β-conglycinin (7S, 35%) and glycinin (11S, 52%). The molecular weights of the 7S and 11S fractions are about 180 and 350 kDa, respectively. Both β-conglycinin and glycinin consist of several chains.

The solved X-ray diffraction crystal structures of β-conglycinin and glycinin as archived in the Protein Data Bank (PDB) database\(^{[22]}\) are shown in Fig. 1. β-Conglycinin (PDB code 1UIK)\(^{[23]}\) is a trimer in the shape of a triangle with three chains: two chains are identical and one that is almost identical with a minor difference. Each chain is built of three sub-units. Glycinin (PDB code 1OD5)\(^{[24]}\) consists of two identical chains, each of which consists of four sub-units connected by two SS bonds.

The size and sequence of each chain were viewed using Hermes, GOLD software viewer. The sub-chain chosen for the simulation was one of glycinin’s containing 52 amino acids and has molecular mass of 5698.5 Da, with amino acid composition quite similar to the experimental data.\(^{[4]}\) The sub-chain was named Glycinin A chain f3, and its sequence is given in Table 2.

The structure of Glycinin A chain f3 was downloaded from the PDB and processed by adjusting bonds to double bonds where it was suitable and addition of hydrogen atoms (Fig. 2). Out of eight carboxylic acid groups it contains, seven were provided an ionized form, and one was left free. Seven positively charged amine groups balance the negative charges.

**Alginate**

Alginate was built as a block copolymer containing 12 sugar units: 6 units of α-L-guluronate (G) and 6 units of β-D-mannurionate (M) with a molecular mass of 2107 Da (Fig. 3). According to the literature,\(^{[7]}\) the pKa of alginic acid is 1.5–3.5. Therefore, at pH 5.5–6.0 in which the crosslinking reaction is performed, the concentration of the ionized carboxylate groups is at least 100 times larger than the concentration of free carboxylic acid groups. Because the chain of the alginate includes only 12 sugar units, this ratio cannot be created. Therefore, out of 12 carboxylic groups, one was free, and eleven were ionized. Eleven sodium ions were added to balance the charges.

**Gelatin**

The molecular model of gelatin was built as a decapeptide chain with a composition that resembles that in Table 1 (Type A pork

<table>
<thead>
<tr>
<th>Glycinin A chain f3 sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLU200 GLY201 HIS211 LYS210</td>
</tr>
<tr>
<td>GLY202 PHE212 ASP222 THR223</td>
</tr>
<tr>
<td>GLY203 LEU213 THR223 GLU233</td>
</tr>
<tr>
<td>GLY204 ALA214 ALA224 ARG234</td>
</tr>
<tr>
<td>GLU205 GLN215 GLU221 ASP231</td>
</tr>
<tr>
<td>VAL204 LEU213 THR223 GLU233</td>
</tr>
<tr>
<td>GLY206 SER216 LYS226 GLN236</td>
</tr>
<tr>
<td>GLY207 PHE217 LEU227 ILE237</td>
</tr>
<tr>
<td>PHE208 ASN218 ARG228 VAL238</td>
</tr>
<tr>
<td>SER209 THR219 SER229 THR239</td>
</tr>
</tbody>
</table>

The sequence of the 10 amino acids was as follows, with a molecular mass of 965 Da:

\[
\text{H}_2\text{N-}\text{Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro-Lys-COOH}
\]

The optimized structure of the gelatin molecule appears in Fig. 4.

In this sequence, the amino acids that are chargeable are Arg, Glu, and Lys. The guanidinium group of Arg was set to be positively charged and the carboxylic acid group of Glu as negatively charged. The amino side group of Lys was left free, and it was used to link to soy protein. The terminal amino group was set to be positive and the terminal carboxylic group to be negatively charged. Therefore, the net charge of the gelatin molecule is zero (2 positively charged amino groups and 2 negatively charged carboxylic groups).

**Soy protein–gelatin conjugate**

Soy protein was conjugated to gelatine by creation of an amide bond between gelatin’s side amino group of Lys and soy protein’s side carboxylic group of Glu. The molecular mass obtained was 6645.5 Da (14.5% w/w gelatin).

**Soy protein–alginate conjugate**

Soy protein was conjugated to alginate by creation of an amide bond between alginate’s free carboxylic group in the middle of the molecule and the terminal amino group Glu of soy protein. The molecular mass was 8040.5 (29.4% w/w of sodium alginate).

**Crosslinking agents EDC and glyoxal**

The crosslinking procedure was performed using the crosslinking agents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) or glyoxal (Fig. 5). EDC is a water-soluble carbodiimide usually obtained as the hydrochloride and is used to couple carboxyl groups to amine groups forming an amide linkage.[25] Glyoxal is the smallest dialdehyde and it reacts initially to form a Schiff base adduct between the carbonyl and the amine moiety; then, the aldimine rearranges to a more stable ketoamine or Amadori product.[26] The molecular models of EDC and glyoxal were built and minimized.

![Figure 2](image1.png) **Figure 2.** Structure of glycinin A chain f3. Color code: red, O; white, H; gray, C; blue, N.

![Figure 3](image2.png) **Figure 3.** Structure of sodium alginate after geometry optimization. Color code: red, O; white, H; gray, C; purple, Na.
Computation details

Preparation of the soy protein

The structure of Glycinin A chain f3 was downloaded from the PDB. The 3D structure was read into HEMERS visualizer of GOLD Suite. The protein was processed by the following stages:

1. Adjustment of bonds to single or double bonds according to their proper situation.
2. Addition of hydrogen atoms—To be consistent with the experimental conditions, it was examined which and how many amino acids should be protonated.

Dynamic simulation

Alginate and gelatin structures were built using the builder tool of the molecular modeling package Material Studio 7.0. The geometry of the molecules was optimized using Forcite module. Soy protein–gelatin and soy protein–alginate were also built and optimized. The dynamic simulation was conducted according to the following steps:

Step 1 Building cubic cells

A simulation cubic box (about 20 Å edge) was constructed using Amorphous cell module at a temperature of 298°K for each of the following:

a. Soy protein; gelatin; alginate
b. Soy protein + gelatin; soy protein + alginate
c. Soy protein; gelatin; alginate; + edc or glyoxal
d. Soy protein; gelatin; alginate; + water molecules
e. Soy–gelatin; soy–alginate
f. Soy–gelatin + water molecules (Fig. 6); soy–alginate + water molecules (Fig. 7).

Step 2 Molecular dynamics simulation
Dynamic simulation was performed at 298°K. The cells were subjected to 100,000 dynamic steps of 1 fs each at constant moles number, pressure, and temperature (NPT ensemble) to determine their density. This stage was followed by a constant moles number, volume, and temperature (NVT ensemble) refinement stage of 100,000 dynamic steps and a data collection stage of additional 400,000 NVT steps.

All MD simulations were conducted using Forcite module with COMPASSII force field. Electrostatic term was considered using Ewald and van der Waals term using Atom based summation methods with an accuracy of $10^{-3}$ kcal/mol. The repulsive cut-off for Van der Waals term was chosen as 12.5 Å. For NPT molecular dynamic simulations, Nose thermostat and Berendsen barostat were chosen.

Step 3 Analysis

The resulted dynamic trajectories were analyzed using Forcite module analysis tools.\[^{[20]}\] The following properties were calculated:

- **Cohesive energy density (CED) and solubility parameter.** Cohesive energy is the energy required to break the interactions between molecules. Generally, it is measured as the heat of vaporization of a liquid. The cohesive energy density (CED) corresponds to the cohesive energy per unit volume. Solubility parameter $\delta$ is the square root of the CED. The solubility parameter is a measure of the ability of materials to dissolve each other.

- **Enthalpy of mixing.** CED values can be used to calculate the enthalpy of mixing (per unit volume) using the following equation:
  \[
  \Delta H_{\text{mix}} = (\phi_a E_{\text{coha}} + \phi_b E_{\text{cohb}}) - E_{\text{cohab}}
  \]
  where: $E_{\text{coh}}$ is CED of constituent $a$, $b$, or the blend $(ab)$; $\phi_a$ and $\phi_b$ are the volume fractions of the two components in the blended system.

- **Radial distribution function (RDF).** RDF (also referred to as pair correlation function) gives a measure of the probability that, given the presence of an atom at the origin of an arbitrary reference frame, there will be an atom with its center located in a spherical shell of infinitesimal thickness at a distance $r$ from the reference atom. RDF may serve as a tool to estimate intermolecular interactions like hydrogen bonding.

### RESULTS

**Interactions of soy protein with gelatin and alginate**

Density, CED, and solubility parameter ($\delta$) were calculated for soy protein, gelatin, and sodium alginate (see Table 3). The solubility parameter of alginate is very high (106.42 (J/cm$^{3}$)$^{0.5}$) because it is charged, and those of gelatin and soy protein are considerably lower (33.59 and 28.15(J/cm$^{3}$)$^{0.5}$, respectively). To estimate the energetic benefit gained from mixing them, enthalpy of mixing was calculated according to eqn 1. Whereas the enthalpy of mixing of soy protein with gelatin is negative ($-82.94$ J/cm$^{3}$), the value obtained for soy and alginate is positive ($140.52$ J/cm$^{3}$). This result indicates that soy protein mixes well with gelatin but not with alginate, which is reasonable because the similarity between soy and gelatin is higher than between soy and alginate. RDF was calculated between carboxylate and ammonium groups of alginate or gelatin and soy protein. The main interaction distance is similar for both mixtures ($r = 1.58$ Å), but the intensity for soy protein with gelatin is higher ($g(r) = 22.68$) than for soy protein with alginate ($g(r) = 9.84$) (Fig. 8). This result might be attributed to stronger interaction between soy protein and gelatin as indicated by the $\Delta H_{\text{mix}}$ values.

**Interactions of soy protein, gelatin, and alginate with EDC and glyoxal**

Two crosslinking agents were used in this study. The crosslinking agent EDC conjugates COOH groups (in polysaccharides and proteins) to amine groups in proteins, forming amide bonds. The reactive dialdehyde glyoxal reacts with amine groups and...
is used to crosslink proteins. According to the experimental study, soy–alginate undergoes a more effective crosslinking reaction than soy–gelatin in the presence of EDC, as indicated by water vapor resistance measurements. Soy–alginate had a higher water vapor resistance, and it degraded slower than soy–gelatin. It was also found that crosslinking of soy–gelatin with EDC is more effective than with glyoxal.

Simulation was performed to account for the difference in the crosslinking behavior of EDC and glyoxal. Solubility parameter was calculated for the systems of sodium alginate, gelatin, and soy protein with EDC or gelatin and soy protein with glyoxal (Table 4). The solubility parameter of EDC is higher than glyoxal because it is charged and glyoxal is a neutral molecule. Enthalpy of mixing of the mixtures of gelatin with EDC, soy protein with glyoxal, and alginate with EDC is positive, indicating that they do not mix well. The last result is surprising because EDC is charged, and therefore it was expected that it would mix well with sodium alginate. The $H_{mix}$ of the mixtures of soy protein with EDC and gelatin with glyoxal are negative. According to these results, gelatin mixes better than alginate with the crosslinking agents, and this may lead to a higher degree of crosslinking. However, the experimental results indicate that the crosslinking of soy protein with alginate is more effective than with gelatin, which might suggest that solubility of the crosslinking agents does not govern the crosslinking reaction.

To gain a better understanding of the interaction between gelatin or alginate and the crosslinking agents, a quantum geometry optimization was conducted using DMOL3 module.

![RDF between soy protein and gelatin or alginate](image)

**Figure 8.** RDF of soy protein with gelatin or alginate.

| Table 3. Density, solubility parameter, and enthalpy of mixing of soy protein, gelatin, alginate, soy–gelatin, and soy–alginate with water |
|---|---|---|---|---|
| Substance | w/w of polymer (%) | Density (g/cm³) | Solubility parameter (J/cm³) 0.5 | Volume fraction of polymer | Enthalpy of mixing (J/cm³) |
| Soy protein | 100 | 1.208 | 28.15 | — | — |
| Gelatin | 100 | 1.271 | 33.59 | — | — |
| Alginate | 100 | 1.732 | 106.42 | — | — |
| Soy protein + 2 gelatin | Soy 74.7 | 1.191 | 30.90 | soy 0.756 | —82.94 |
| | Gelatin 25.3 | — | — | — | — |
| Soy protein + alginate | Soy 70.6 | 1.284 | 54.87 | soy 0.776 | 140.52 |
| | Alginate 29.4 | — | — | — | — |
| Soy protein + 100H₂O | 76.0 | 1.200 | 37.12 | 0.719 | —206.54 |
| 5 Gelatin + 100H₂O | 72.8 | 1.231 | 42.18 | 0.677 | —529.42 |
| 3 Alginate + 100H₂O | 79.8 | 1.622 | 95.97 | 0.682 | —793.02 |
| Soy–gelatin | 100 | 1.201 | 27.63 | — | — |
| Soy–alginate | 100 | 1.275 | 56.44 | — | — |
| Soy–gelatin + 100H₂O | 78.7 | 1.224 | 38.43 | 0.754 | —353.54 |
| Soy–alginate + 100H₂O | 82.2 | 1.315 | 56.04 | 0.774 | —156.40 |
| H₂O | 0 | 0.976 | 47.21 | — | — |
| Soy–gelatin | 100 | 1.201 | 27.63 | — | — |
| Soy–alginate | 100 | 1.275 | 56.44 | — | — |
| Soy–gelatin + 100H₂O | 78.7 | 1.224 | 38.43 | 0.754 | —353.54 |
| Soy–alginate + 100H₂O | 82.2 | 1.315 | 56.04 | 0.774 | —156.40 |
| H₂O | 0 | 0.976 | 47.21 | — | — |

*Calculated according to eqn 1.
Because quantum calculations are highly demanding in computation resources, only the fragment that is directly involved in the crosslinking reaction was simulated. That is, glutamic acid (in gelatin) with EDC, di-β-D-mannuronate (in alginate) with EDC, and lysine (in gelatin) with glyoxal (Fig. 9a, b, and c, respectively). The interaction distances measured were as follows:

- The distance between the carboxyl group of glutamic acid in gelatin and C of EDC is 5.222 Å.
- The distance between the carboxyl group of di-β-D-mannuronate and C of EDC is 4.385 Å and 4.427 Å.
- The distance between the amino group of lysine in gelatin and C of glyoxal is 7.336 Å.

These results show that the interaction distance is significantly shorter between alginate and EDC than between gelatin and EDC. The shorter interaction distance between alginate and EDC might be because of ionic attraction between the ammonium group of EDC and the carboxylate group in alginate, and it facilitates the crosslinking reaction. Indeed a distance of 2.649 Å was measured between the ammonium group of EDC and the second carboxyl group of di-β-D-mannuronate (see Fig. 9b). The interaction distance between gelatin’s lysine side chain and glyoxal is considerably longer which might account for the lower efficiency of the crosslinking reaction with glyoxal. Another factor to be considered is the fact that EDC reacts via both lysine and COOH groups, whereas glyoxal reacts only via lysine groups.

### Table 4. Density, solubility parameter, and enthalpy of mixing of soy protein, gelatin, and alginate with EDC and glyoxal

<table>
<thead>
<tr>
<th>Substance</th>
<th>w/w of polymer (%)</th>
<th>Density (g/cm³)</th>
<th>Solubility parameter (J/cm³) 0.5</th>
<th>Volume fraction of polymer</th>
<th>Enthalpy of mixing (J/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDC</td>
<td>0</td>
<td>0.907</td>
<td>51.015</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Soy protein + EDC</td>
<td>96.7</td>
<td>1.191</td>
<td>30.68</td>
<td>0.957</td>
<td>−73.40</td>
</tr>
<tr>
<td>Gelatin + EDC</td>
<td>96.2</td>
<td>1.222</td>
<td>34.68</td>
<td>0.948</td>
<td>2.89</td>
</tr>
<tr>
<td>Alginate + EDC</td>
<td>97.6</td>
<td>1.684</td>
<td>101.69</td>
<td>0.949</td>
<td>567.69</td>
</tr>
<tr>
<td>Glyoxal</td>
<td>0</td>
<td>1.087</td>
<td>26.79</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Soy protein + 3 glyoxal</td>
<td>97.0</td>
<td>1.241</td>
<td>26.92</td>
<td>0.957</td>
<td>66.70</td>
</tr>
<tr>
<td>Gelatin + 3 glyoxal</td>
<td>96.5</td>
<td>1.226</td>
<td>36.52</td>
<td>0.960</td>
<td>−277.70</td>
</tr>
</tbody>
</table>

*Calculated according to eqn 1.

**Interactions of soy, gelatin, and alginate with water molecules**

The tendency of soy, gelatin, and sodium alginate to mix with water was approximated by calculating enthalpy of mixing (Table 3). Negative values of Δ_Hmix were obtained for all three biopolymers, suggesting that they dissolve in water. The value found for the mixture of alginate with water is the most negative (−793.02 J/cm³), then gelatin (−529.42 J/cm³) and soy protein (−206.54 J/cm³). These results indicate that the order of solubility in water is alginate > gelatin > soy protein. RDF analysis gave an opposite order when calculated between the oxygen atoms of the carboxylate group in soy protein, gelatin, or alginate and the hydrogen atoms of water molecules (Fig. 10). The main interaction distance is similar for all three biopolymers (r = 1.51 Å), but the intensity varies. For the soy protein the intensity is g(r) = 7.33, for gelatin g(r) = 6.19 and for alginate g(r) = 2.30. The contradiction between the comparatively small Δ_Hmix of soy protein with water and the high RDF value may be attributed to the globular structure of soy protein in which the inner part is hydrophobic. This structure limits the solubility of soy protein in water, but the hydrophilic carboxylate groups in the outer part form many hydrogen bonds with the water molecules. The comparatively low RDF intensity value of alginate might be because of the participation of the carboxylate groups in ionic binding with the Na⁺ ions, as shown by Leng et al. [11]

RDF was also calculated between the hydroxyl groups in soy protein, gelatin or alginate, and water. No interaction was found.

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**Figure 9.** Optimized structures of: a) Glutamic acid (in gelatin) with EDC (right); b) Di-β-D-mannuronate (in alginate) with EDC (right); c) Lysine (in gelatin) with glyoxal.
indicating that hydrogen bonds are mostly formed between carboxylate groups and water. The hydroxyl groups do not contribute to the intermolecular interactions.

**Interactions of soy–gelatin and soy–alginate with water molecules**

The degradation rate of soy protein conjugates can be related to their interaction with water. Results of solubility parameters and enthalpy of mixing calculations of soy–gelatin, soy–alginate, and their mixture with water are shown in Table 3. The negative values of $\Delta H_{\text{mix}}$ obtained for both biopolymers suggest that they dissolve in water. The value observed for the mixture of soy–gelatin with water ($-353.54 \text{ J/cm}^3$) is more negative than for soy–alginate ($-156.40 \text{ J/cm}^3$). These results suggest that the solubility of soy–gelatin in water is higher than soy–alginate.

RDF was calculated between the oxygen atoms of the carboxylate group in soy–gelatin or soy–alginate and the hydrogen atoms of the water molecules (see Fig. 11). The main interaction distance is similar for the two conjugates ($r = 1.51 \text{ Å}$), but the intensity for soy–gelatin is $g(r) = 7.79$ and for soy–alginate is $g(r) = 4.75$. These results indicate that hydrogen bonds are formed between water molecules and each of the conjugates but to a greater extent for soy–gelatin than for soy–alginate, similar to the results obtained for gelatin and alginate. RDF was also calculated between the oxygen atom of the amide group and the hydrogen atoms of the water molecules. No significant interaction was found between those groups, indicating that the amide groups do not contribute to the interaction with water.

The experimental study revealed that the solubility of soy–gelatin was higher than that of soy–alginate conjugate (see Fig. 12), as predicted by the simulation.
SUMMARY AND CONCLUSIONS

In the present research, molecular modeling methods were used to study soy protein conjugates with gelatin or alginate as potential scaffolds for tissue engineering, especially for skin regeneration applications. Computational tools were applied to estimate the crosslinking degree of soy–gelatin and soy–alginate and compare their degradation rates.

The crosslinking degree depends on several factors including the miscibility of the two reactants and the interaction with the crosslinking agents. The calculations revealed that soy protein mixes well with gelatin but not with alginate. However, the crosslinking agents EDC and glyoxal mix better with gelatin than with alginate. RDF calculations showed that the interaction distance between alginate and EDC is significantly shorter than between gelatin and EDC, probably because of ionic attraction between the ammonium groups of EDC and the carboxylate groups in alginate, which facilitates the crosslinking reaction. No significant interaction was noticed between gelatin’s lysine side chain and glyoxal, which might account for the lower efficiency of the crosslinking reaction with glyoxal. Another factor to be considered is the fact that EDC reacts via both lysine and COOH groups, whereas glyoxal reacts only via lysine groups. These results are in accordance with the experimental observation that the crosslinking of soy protein with alginate is more effective than with gelatin.[4]

The degradation rate of soy protein conjugates was related to their interaction with water. First, the solubility of soy protein, alginate, and gelatin in water was calculated separately. The calculations indicate that the order of solubility in water is alginate > gelatin > soy protein. Opposite results were obtained for the conjugates; the solubility of soy–gelatin in water is higher than soy–alginate. RDF calculations support these results and indicate that water molecules form more hydrogen bonds with soy–gelatin than with soy–alginate. These observations might be the reason for the difference in degradation rate of the two conjugates; the soy–gelatin degrades faster than soy–alginate.

It was found experimentally that the water uptake of soy–alginate crosslinked by EDC is higher than that of soy–gelatin. According to the calculation, the miscibility of soy–gelatin with water is higher. That contradiction might be related to different crosslinking degree of the two systems. This hypothesis is supported by the fact that in the other crosslinking system (with glyoxal) the water uptake of soy–gelatin is much higher.

REFERENCES