Incorporation of Ca$^{2+}$ Ions in Gelatin–Siloxane Hybrids Through a Sol–Gel Process

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Abstract

Several gelatin–siloxane hybrids incorporating Ca$^{2+}$ ions were synthesized through a sol–gel process starting with gelatin, 3-(glycidoxypropyl) trimethoxysilane (GPSM), and Ca(NO$_3$)$_2$ as the starting materials. This paper describes the effect of incorporated Ca$^{2+}$ ions on the structural factors, viscoelastic properties, and in vitro bioactivity of the gelatin–siloxane hybrids.

1. Introduction

In addition to artificial materials like synthetic polymers, ceramics, and metals, natural polymers have been used as tissue substitutes. Some of these are hard and strong, while others are soft and flexible. Silicate-based ceramics like Bioglass$^{4}$ and Glass-Ceramic A-W$^{2}$ are bioactive, being able to bond to bone tissue in vivo thereby spontaneously depositing apatite, however, these silicates are not biodegradable. Polylactic acid, gelatin, chitin, and chitosan$^{3}$,4) are biodegradable as well as biocompatible, but like Bioglass$^{4}$ and Glass-Ceramic A-W$^{2}$ are bioactive, ceramics, and metals, also natural polymers have been used as tissue substitutes. Some of these are hard and strong, while others are soft and flexible.

2. Experimental

Gelatin and Ca(NO$_3$)$_2$ were obtained from Nacalai Tesque, Inc., and GPSM was obtained from Chisso Co. The chemicals used were all commercially available and of reagent grade without further purification. Appropriate amounts of GPSM and Ca(NO$_3$)$_2$ were added to a 12.5 mass % gelatin in 0.1 M HCl solution upon continuous stirring at 40°C in order to obtain compositions, as given in Table 1.

3. Key words: 3-(glycidoxypropyl) trimethoxysilane, Gelatin, Hybrid, Sol–gel, In vitro bioactivity
well reproduces in vivo behavior of implant materials in in vitro experiments. SBF has been prepared as described in the literature, and buffered to pH=7.40 with trishydroxymethylaminomethane (Tris) and HCl aqueous solutions, and kept at 36.5°C throughout the incubation periods. Apatite formed on the hybrid surfaces was detected using thin-film X-ray diffraction (TF-XRD; RAD-II, Rigaku, CuKα, 40kV, 20mA) with an angle of 1° to the direction of the incident X-ray, as well as by SEM observation. The concentrations of the various ions in SBF was measured by inductively coupled plasma emission spectroscopy (ICP; SPS7700, Seiko Electronics).

Table 1. Composition, Homogeneity, Structural Factors, and in vitro Bioactivity of the Hybrids

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>( f_G )</th>
<th>( f_Ca )</th>
<th>Homogeneity</th>
<th>( R_{\text{ GRAT}} )</th>
<th>( U_{\text{SM}} )</th>
<th>NBO</th>
<th>Induction period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
<td></td>
<td>(c)</td>
<td>(d)</td>
<td>(e)</td>
<td>(f)</td>
</tr>
<tr>
<td>A</td>
<td>0.33</td>
<td>0</td>
<td>Homogeneous</td>
<td>0.99</td>
<td>9.7</td>
<td>0.8</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>0.50</td>
<td>0</td>
<td>Homogeneous</td>
<td>0.95</td>
<td>6.3</td>
<td>3.4</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
<td>0.67</td>
<td>0</td>
<td>Heterogeneous</td>
<td>0.99</td>
<td>10.1</td>
<td>1.1</td>
<td>3</td>
</tr>
<tr>
<td>D</td>
<td>0.33</td>
<td>0.11</td>
<td>Homogeneous</td>
<td>0.95</td>
<td>6.1</td>
<td>3.3</td>
<td>3</td>
</tr>
<tr>
<td>E</td>
<td>0.50</td>
<td>0.11</td>
<td>Homogeneous</td>
<td>0.95</td>
<td>6.1</td>
<td>3.3</td>
<td>—</td>
</tr>
<tr>
<td>F</td>
<td>0.67</td>
<td>0.11</td>
<td>Heterogeneous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>0.33</td>
<td>0.56</td>
<td>Homogeneous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>0.50</td>
<td>0.56</td>
<td>Heterogeneous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.67</td>
<td>0.56</td>
<td>Heterogeneous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Mass fraction; 1) \( f_G = \text{GPSM}/(\text{GPSM} + \text{gelatin}) \), 2) \( f_{\text{Ca}} = \text{Ca(NO}_3)_2/(\text{Ca(NO}_3)_2 + \text{GPSM} + \text{gelatin}) \)
b) After SEM photographs
c) The molar fraction of GPSM involved in the grafting
d) The normalized amount of amino acid residues not grafted by GPSM
e) The normalized amount of the non-bridging oxygen atoms derived from the methoxysilane groups of GPSM
f) After TF-XRD patterns
g) No apatite formed after soaking in SBF for 14 days
h) Data not discussed, due to heterogeneity

3. Results

3.1 Effect of Ca\(^{2+}\) ions on the hybrid structure

SEM photographs (not shown) indicated that the morphology of the gelatin-siloxane hybrids was dependent on the composition. Hybrid E, showing a fracture surface without special features, is a typical example of a homogeneous hybrid having \( f_G \approx 0.50 \) and \( f_{\text{Ca}} \approx 0.11 \). With \( f_G < 0.50 \) or \( f_{\text{Ca}} > 0.50 \), hybrids were heterogeneous with bright particles dispersed on their fracture surfaces. An energy-dispersive X-ray analysis indicated that the bright spots were rich in Ca for hybrids G, H, and I with \( f_{\text{Ca}} = 0.67 \) and \( f_{\text{Ca}} \approx 0.11 \). Thus, the heterogeneous hybrids A, B, D, and E may be discussed regarding changes in hybrid structures and in vitro bioactivity due to incorporation of Ca\(^{2+}\) ions.

The GPSM molecules has been grafted on to the gelatin chains, as reported previously. Gelatin may be hydrolyzed in 6N HCl at the peptide bonds in order to distinguish the GPSM grafted and graft-free amino acid of the gelatin chains using an amino acid analyzer. The amount of the graft-free amino acid residues has been normalized to the unit volume of a hybrid (\( U_{\text{SM}} \)). The molar fraction of GPSM (\( R_{\text{ GRAT}} \)) involved in the grafting to the gelatin chains has also been obtained from the results of amino acid analysis. Table 1 shows that \( U_{\text{SM}} \) and \( R_{\text{ GRAT}} \) for hybrid A shows similar values as D, as is the case for B and E, indicating that incorporation of Ca\(^{2+}\) ions has little effect on the formation of gelatin-GPSM graft bonding.

The methoxysilane groups of GPSM were hydrolyzed to give silanol groups, resulting in two or three Si–O–Si bridging bonds. Figure 1 shows the \( ^{29}\text{Si CP-MAS NMR spectra of hybrids A, B, D, and E. Due to the resolution of the NMR spectra, they have been deconvoluted into one T}^2\) siloxane unit and two T\(^3\) siloxane units (T\(^3\), T\(^2\)), where T\(^2\) and T\(^3\) denote R–Si(–OSi)\(^2\)(OCH\(_3\),OH) and R–Si(–OSi)\(^3\)(R is the organic skeleton from GPSM), respectively. The finding that peak T\(^3\) appears at the high field side of peak T\(^2\) suggests that these T\(^3\) units bind to a smaller number of T\(^2\) units than T\(^3\) units. The percentage of each component unit is listed in Table 2. Since one of the oxygen atoms in a T\(^2\) unit is not bridging, the normalized amount of non-bridging oxygen (NBO) atom in the unit volume of hybrids could be derived from the percentage of the T\(^2\) unit. It is indicated from Table 2 that hybrids with a \( f_G \) value of 0.50 or 0.33, were similar in each unit T\(^3\), T\(^2\), and T\(^3\). The same applied to NBO atoms, as shown in Table 1.

The amide I band is found the most intense absorption band in the FT-IR spectra of the gelatin-siloxane hybrids, and is a characteristic for the gelatin secondary structure, representing a number of overlapping \( \alpha \)-helix, \( \beta \)-sheet, and random conformations. The exact vibration frequency of the amide I band depends on the extent of hydrogen bonding involving C=O and N–H bonds. The amide I bands for hybrids A, B, D, and E have been deconvoluted into four or five components with different intensities, and assigned as shown in Fig. 2 for hybrids B and E. The \( \alpha \)-helix conformation shows a peak at 1654 cm\(^{-1}\). A component at around 1644 cm\(^{-1}\) is attributable to a random conformation, and the other three peaks at 1622, 1633, and 1675 cm\(^{-1}\) have been ascribed to the \( \beta \)-sheet conformation. The curve fitting results are summarized in Table 2. It is demonstrated that the percentage of random conformation in the secondary structure of gelatin chains for Ca\(^{2+}\)-free hybrids A (\( f_G =\)
0.33) and B (fG=0.50) exceeded 30%, while Ca2+-containing hybrids D (fG=0.33) and E (fG=0.50) even had larger values of respectively 39.4% and 38.4%.

Figure 3 shows the storage modulus (E') and tanδ for hybrids A, B, D, and E as a function of temperature. The temperature at the tanδ peak has been defined as the glass transition temperature (Tg). The increase in Tg with an increase in fG has been ascribed to the segmental movement of the gelatin chains restrained by bridging or cross-linking bonds with GPSM molecules.5) Figure 3 shows that Ca2+ containing hybrids D (fG=0.33) and E (fG=0.50) gave smaller values of Tg and the storage modulus (E') around Tg than Ca2+-free hybrids A (fG=0.33) and B (fG=0.50), respectively. It was therefore suggested that the presence of Ca2+ ions in gelatin-siloxane hybrids provided flexibility.

Table 2. Analysis of 29Si CP-MAS NMR Spectra and FT-IR Amide I Bands, as well as Viscoelastic Properties of the Homogeneous Hybrids A, B, D, and E

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>fG</th>
<th>fCa</th>
<th>T2^1, T2^3, T2^3</th>
<th>Amide I IR bands (1600 - 1700 cm^-1)</th>
<th>Tg (°C)</th>
<th>E' (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 0.33</td>
<td>0</td>
<td>-57.8, 3.4, -63.5, 15.0, -67.0, 81.6</td>
<td>1654.12, 1622.56, 1644.31, 33</td>
<td>2200</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>B 0.50</td>
<td>0</td>
<td>-57.7, 9.8, -63.6, 15.2, -68.4, 75.0</td>
<td>1654.60, 1622.63, 1644.30, 40</td>
<td>6000</td>
<td>196</td>
<td></td>
</tr>
<tr>
<td>D 0.33</td>
<td>0.11</td>
<td>-57.7, 4.9, -63.6, 17.1, -66.6, 78.0</td>
<td>1654.12, 1622.47, 1644.39, 20</td>
<td>1900</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>E 0.50</td>
<td>0.11</td>
<td>-57.6, 11.2, -63.5, 15.0, -68.4, 73.8</td>
<td>1654.16, 1622.45, 1644.38, 31</td>
<td>5500</td>
<td>114</td>
<td></td>
</tr>
</tbody>
</table>

1) Chemical shift (ppm)
2) The percentage of peak area
3) The position of deconvolution profiles for IR amide I spectra (cm^-1)
4) Not detected

Fig. 1. 29Si CP-MAS NMR spectra of the GPSM monomer and gelatin-siloxane hybrids A, B, D, and E.

Fig. 2. Curve fitting analysis of the FT-IR amide I bands indicated by open dots. The deconvoluted component bands are represented by broken lines, and the sum of the components by the solid line. Symbols α, β, and r represent, respectively, α-helix, β-sheet, and random structure in the gelatin secondary structure.

3.2 In vitro apatite deposition

Figure 4 shows the TF-XRD patterns of Ca2+-containing hybrid E and Ca2+-free hybrid B before and after soaking in SBF up to 14 d. Hybrid E shows XRD peaks assignable to apatite,12) while hybrid B does not. The sharper TF-XRD peak at 26.0° for hybrid E has been ascribed to the (002) diffraction, and a broader one at about 32.0° to an envelope
The induction period for apatite deposition derived from those diffractions is shown in Table 1, indicating that incorporation of Ca\(^{2+}\) ions may be essential to provide hybrids with the in vitro bioactivity, and that hybrids D and E show a similar ability of apatite formation in SBF.

Figures 5(a)–(c) demonstrate the morphology of hybrids B, D, and E after being soaked in SBF for 14 d. Ca\(^{2+}\)-free hybrid B shows only surface erosion, while Ca\(^{2+}\)-containing hybrids D and E have formed hemispherical agglomerates of petal-like apatite having the same morphology as those for common bioactive ceramics.1,2,7,9,10

3.3 Analysis of SBF
Changes in the concentrations of P(V), Ca(II), and Si(IV) in SBF due to soaking the hybrids are shown in Fig. 6. A relative small decrease in P(V) concentration due to adsorption by the Ca\(^{2+}\)-free hybrid B was found. The significant decrease within 2 d in P(V) concentration by the Ca\(^{2+}\)-containing hybrids D and E obviously was attributable to the adsorption of calcium phosphates. Figure 6(b) indicates that the Ca(II) concentration in SBF strongly increased, exceeding 3 mM by the hybrids D and E at the initial soaking stage (<1 d), while it remained constant after soaking for 3 d. However, the Ca(II) concentration in SBF due to hybrid B soaking only changed slightly. The concentration of Si(IV) in SBF, shown in Fig. 6(c), increased rapidly with the soaking time, indicating that Si(IV) ions dissolved from the hybrids, since as-prepared SBF did not contain any Si(IV) ion.

4. Discussion
4.1 Effect of Ca\(^{2+}\) ions on the hybrid structures
It has been demonstrated that under acid catalysis conditions, the epoxy group at one end of a GPSM molecule grafted to the gelatin chain at nucleophilic sites like -NH\(_2\) or -COOH, and that the Si-OH groups, derived from a Si(OCH\(_3\))\(_3\) group at the other end, were polymerized to form <gelatin-R-Si-O-Si-R-gelatin> bridging bonds (R: GPSM organic backbone). It has already been shown that the hydrolysis and polycondensation of GPSM, the ring-opening reaction of GPSM with gelatin, and the viscoelastic properties of the gelatin-siloxane hybrids were strongly dependent on \(f_G\).5) The present study focused on the effect of Ca\(^{2+}\) ions on the hybrid structure. It has been shown that hybrids having the same \(f_G\) values were identical in R\(_{graft}\) and U\(_{aan}\), as well as in the percentage of T\(_4\), T\(_3\), and T\(_2\), independent of \(f_{Ca}\) values. These results indicated that incorporation of Ca\(^{2+}\) ions only minor affect the reactions in the GPSM-H\(_2\)O-gelatin system. This could also be interpreted that hybrids having the same \(f_G\) value had a similar cross-links density, due to GPSM grafting to both gelatin chains and the polymerization of silanol groups. Similar values of the storage modulus at \(-50^\circ C\) in Fig. 3 suggested that incorporation of Ca\(^{2+}\) ions in the hybrids did not affect their
Incorporation of Ca$^{2+}$ Ions in Gelatin-Siloxane Hybrids Through a Sol-Gel Process

primary structure. Nevertheless, the presence of Ca$^{2+}$ ions could provide the gelatin-siloxane hybrids with a certain flexibility: A > D and B > E when comparing $T_g$. Note that the deconvolution of the amide I band indicated that the percentage of the random structure of gelatin increased as Ca$^{2+}$ ions were incorporated. Thus such an ease of molecular movement, i.e. flexibility, can be accounted for by conformational changes of the gelatin chains due to incorporation of Ca$^{2+}$ ions. Even though the ionic bond between Ca$^{2+}$ and NO$_3^-$ is not negligible in the here presented hybrids, it is reasonable to consider that incorporation of Ca$^{2+}$ ions in hybrids lead to stronger electrostatic interactions between Ca$^{2+}$ and negatively charged groups, such as C=O and COO$^-$. This may be an important factor changing ordered conformations of gelatin secondary structures to random ones. That is, the triple helical strands, which are generally accepted to construct gelatin molecules, may be uncoiled into random coils due to Ca$^{2+}$ ions weakening the intermolecular or intramolecular bonding among the gelatin chains that stabilize the helix structure. The random-coil conformation characteristic of a flexible polymer is quite different from the helix conformation where hydrogen bonds fix chains into a rigid rod. Thus the increase in flexibility with incorporation of Ca$^{2+}$ ions may be attributed to the increment of the percentage of the random conformations in the gelatin secondary structures.

4.2 In vitro bioactivity

Pairs of gelatin chains have been bridged by GPSM residues in the way: gelatin-R-Si-O-Si-R-gelatin (R: GPSM organic backbone). It is unlikely that the Si-C bonds in the GPSM residues were susceptible to hydrolysis in SBF. Thus the increase in Si(IV) concentration in SBF, shown in Fig. 6(c), is thought to result from the degradation of the hybrids due to hydrolysis of both the Si-O-Si bonds of the siloxane blocks and the peptide bonds of gelatin chains. The possibility that hydrolysis only took place at the Si-O-Si bonds was considered very high. Such a hydrolysis pathway will keep the dangling fragments from being detached from the gelatin chains and cause the hydrated silanol groups remain in the hybrids. It has been proposed that the Si-OH group is one of the key species for apatite deposition. Once formed, the apatite nuclei are able to grow on the Si-OH groups, thereby incorporating calcium and phosphate ions from the surrounding, with SBF already supersaturated with respect to apatite.

Table I indicates that only Ca$^{2+}$-containing hybrids are able to form a surface apatite layer after soaking in SBF for 14 d. The in vitro bioactivity of the here presented hybrids strongly correlates with incorporation of Ca$^{2+}$ ions. When Ca$^{2+}$-containing hybrids were soaked in SBF, Ca$^{2+}$ ions were released (Fig. 6(b)) to considerably increase the ionic activity product of apatite in SBF, as has been similarly reported for CaO-SiO$_2$ glass. Therefore, the direct correlation of the Ca$^{2+}$ ion release and apatite formation indicated the resemblance in the mechanism of apatite formation on the Ca$^{2+}$-containing hybrids D and E and the common bioactive ceramics. In Fig. 6(b), one should expect a decrease in the Ca(II) concentration due to deposition of apatite. Nevertheless, a constant concentration level beyond the induction period (≥3 d) for hybrids D and E has been found. It is speculated that the dissolution increased the Ca(II) concentration while the apatite deposition decreased it, resulting in a constant net concentration for these two opposing effects.

5. Conclusions

Several Ca$^{2+}$-containing gelatin-siloxane hybrids have been synthesized through sol-gel processing starting with gelatin, 3- (glycidoxypropyl) trimethoxysilane, and Ca(NO$_3$)$_2$. The results of amino acid analysis and deconvolution of $^{29}$Si NMR spectra indicated that the hybrids having the same value of $f_{gs}$ independent of incorporation of Ca$^{2+}$ ions, possessed a similar grafting degree of GPSM to gelatin, polymerizing degree of methoxysilane groups, and cross-link density. However, deconvolution of the IR amide I bands demonstrated that the percentage of the random structure of the gelatin has been increased with incorporation of Ca$^{2+}$ ions. As a consequence, the glass transition...
temperature and storage modulus around $T_g$ also decreased upon incorporation of Ca$^{2+}$ ions. It has been demonstrated that incorporation of Ca$^{2+}$ ions in the hybrids was the most essential factor for apatite deposition on gelatin–siloxane hybrids in vitro.

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References