JOURNAL OF THE ROYAL SOCIETY Interface

J. R. Soc. Interface (2005) **2**, 373–378
doi:10.1098/rsif.2005.0052
Published online 13 July 2005

Heterogeneous nucleation of hydroxyapatite on protein: structural effect of silk sericin

Akari Takeuchi[1], Chikara Ohtsuki[1,†], Toshiki Miyazaki[2], Masanobu Kamitakahara[1], Shin-ichi Ogata[1], Masao Yamazaki[3], Yoshiaki Furutani[4], Hisao Kinoshita[4] and Masao Tanihara[1]

[1]*Graduate School of Materials Science, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan*
[2]*Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu, Kitakyushu, Fukuoka 808-0196, Japan*
[3]*Kyoto Prefectural Institute for Northern Industry, 139-1 Tamba, Mineyama, Kyotango, Kyoto 627-0011, Japan*
[4]*Centmed Inc., 2-185-2-302, Issha, Meitoh, Nagoya, Aichi 465-0093, Japan*

Acidic proteins play an important role during mineral formation in biological systems, but the mechanism of mineral formation is far from understood. In this paper, we report on the relationship between the structure of a protein and hydroxyapatite deposition under biomimetic conditions. Sericin, a type of silk protein, was adopted as a suitable protein for studying structural effect on hydroxyapatite deposition, since it forms a hydroxyapatite layer on its surface in a metastable calcium phosphate solution, and its structure has been reported. Sericin effectively induced hydroxyapatite nucleation when it has high molecular weight and a β sheet structure. This indicates that the specific structure of a protein can effectively induce heterogeneous nucleation of hydroxyapatite in a biomimetic solution, i.e. a metastable calcium phosphate solution. This finding is useful in understanding biomineralization, as well as for the design of organic polymers that can effectively induce hydroxyapatite nucleation.

Keywords: biomineralization; biomimetic process; hydroxyapatite; metastable calcium phosphate solution; silk protein

1. INTRODUCTION

Bone and teeth are biocomposites with a unique structure: nano-crystalline hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), a form of calcium phosphate, is packed and aligned cooperatively between organic matrices, such as collagen and some acidic proteins. This structure is constructed as a result of mineralization in a living body, a process known as biomineralization. During mineral formation, the organic matrix plays an important role, as, for example, it controls the location and organization of nucleation sites, and structure and orientation of hydroxyapatite. However, the mechanism of protein-mediated mineralization is not well understood (Mann 2001).

The mineralization of hydroxyapatite has also been documented to occur on the surface of materials that can bond to living bone directly in bony defects (Kokubo 1991; Hench 1991; Neo *et al.* 1992). This formation of hydroxyapatite can be reproduced in a solution called a simulated body fluid (SBF), which is a metastable calcium phosphate solution with inorganic ion concentrations almost equal to those in human blood plasma (Kokubo *et al.* 1990*a*,*b*; Filgueiras *et al.* 1993). Recently, it has been shown that the nucleation of hydroxyapatite in a SBF can be induced by certain types of surface functional groups on a material, such as silanol (–SiOH), carboxyl (–COOH), phosphate ($-OPO_3H_2$) groups (Li *et al.* 1992, 1994; Ohtsuki *et al.* 1992; Tanahashi & Matsuda 1997). Nevertheless, the relationship between the arrangement of a functional group and hydroxyapatite formation is poorly understood.

An investigation into hydroxyapatite nucleation on proteins under biomimetic conditions utilising a SBF may provide significant information to further the understanding of the mechanism of biomineralization. Our study focused on examining the structural effect of a protein on hydroxyapatite formation. Sericin, a type of silk protein, was adopted as a suitable protein to study structural effect on hydroxyapatite deposition, since it forms a hydroxyapatite layer on its surface in a

†Author for correspondence (ohtsuki@ms.naist.jp).

Received 5 March 2005
Accepted 25 May 2005

solution mimicking body fluids (Takeuchi *et al.* 2003), and its structure has been reported (Komatsu 2000*a*; Lee *et al.* 2003). We investigated the relationship between the structure of sericin and its ability to deposit hydroxyapatite in the solution. Four types of sericin film were prepared under different conditions, and the ability to form hydroxyapatite examined using a metastable calcium phosphate solution that had 1.5 times the ion concentrations of a normal SBF (1.5SBF). Our findings will be useful to further the understanding of the mechanism of mineralization in biological systems, as well as in the design of novel organic polymers for the preparation of hybrid materials with structures similar to bone.

2. EXPERIMENTAL

Four types of films were prepared from sericin solutions, formed under the different conditions listed in table 1. The solutions were prepared by the degumming raw silk fibre of *Bombyx mori* in ultra-pure water without any agents such as sodium carbonate to avoid contaminations. The degumming process was performed using an autoclave (SV-302 II, Advantec Toyo, Ltd, Japan) at either 105 or 120 °C for a period of 1 h. Some of the samples were stored at 4 °C for a period of two weeks. The molecular weight of the sericin in the solutions was determined using gel permeation chromatography (GPC) employing an ÄKTA purifier system (Amersham Biosciences Corp., NJ, USA), using the following experimental parameters: column = Superdex 200 HR 10/30, elution buffer = 10 mol m^{-3} phosphate buffer containing 150 mol m^{-3} NaCl (PBS, pH = 7.4), flow rate = 0.5 ml min^{-1}, and detection wavelength = 215 nm. The peak molecular weight was calculated from a standard curve obtained using an Amersham Biosciences gel filtration calibration kit. The concentration of sericin in the extracted solution was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). The solution was diluted with ultra-pure water to a concentration of approximately 0.3 mg ml^{-1}. Then, 0.3 mg ml^{-1} of the sericin solution was analysed using circular dichroism (CD) spectroscopy (J-820, JASCO, Japan).

The sericin films were fabricated by drying solutions containing 6 mg ml^{-1} of sericin. A volume of 150 µl of the solution was cast onto a polyethylene film, and then dried under ambient conditions at room temperature. The sericin layer on the polyethylene film was analysed using Fourier transform infrared (FT-IR) spectroscopy (Spectrum One, Perkin Elmer Ltd, UK). Films were also prepared by casting the solution in a Petri dish (diameter = 60 mm, height = 15 mm) to form a thin sericin film on the bottom of the dish.

The cast films were exposed to the 1.5SBF, whose ion concentrations are listed in table 2. The solution was buffered at pH = 7.25 using 75 mol m^{-3} of tris(hydroxymethyl) aminomethane, along with an appropriate volume of hydrochloric acid, following the method reported by Kokubo *et al.* (Tanahashi *et al.* 1994; Cho *et al.* 1995). The temperature of the solution was maintained at 36.5 °C. A volume of 15 ml of the 1.5SBF was poured into a Petri dish coated with a sericin film, and kept for a period of 7 days at 36.5 °C. The films were observed both before, and after soaking in the 1.5SBF using a scanning electron microscope (SEM; S-3500N, Hitachi Ltd, Japan). In the SEM observations, the surfaces of some of the samples were coated with a sputtered gold film. The surfaces of the films were characterized using thin-film X-ray diffraction (TF-XRD; MXP3V, MAC Science Ltd, Japan) and X-ray fluorescence element analysis (XRF; MESA-500, Horiba, Ltd., Japan) employing a rhodium target. In the TF-XRD apparatus, the incident beam was set at an incident angle of 1° against the sample surface.

Table 1. The experimental samples and their preparation conditions.

samples	extraction	storage
S105	105 °C, 1 h	none
S120	120 °C, 1 h	none
S105-2w	105 °C, 1 h	4 °C, 2 weeks
S120-2w	120 °C, 1 h	4 °C, 2 weeks

Table 2. Ion concentrations of an SBF and a 1.5SBF, and human plasma.

	concentration (mM)		
ion	human plasma	SBF	1.5SBF
Na^+	142.0	142.0	213.0
K^+	5.0	5.0	7.5
Mg^{2+}	1.5	1.5	2.3
Ca^{2+}	2.5	2.5	3.8
Cl^-	103.0	147.8	221.7
HCO_3^+	27.0	4.2	6.3
HPO_4^{2-}	1.0	1.0	1.5
SO_4^{2-}	0.5	0.5	0.8

3. RESULTS

Figure 1 shows the GPC profiles of sericin extracted under various conditions. The most frequent distribution in the molecular weight (Mw) appeared at approximately 159 and 43 kDa for samples S105 and S120, respectively. Both these sericin samples showed molecular weights almost equal to those of the as-prepared solutions stored at 4 °C for a period of two weeks (Mw = 141 and 37 kDa, respectively). Figure 2 shows the CD spectra of these sericin samples. The CD spectrum of sample S105 was assigned as a random coil structure from the typical negative peak occurring near to 198 nm (Townend *et al.* 1966; Greenfield & Fasman 1969; Madison & Schellman 1972; Brahms & Brahms 1980). After storage for two weeks storage at 4 °C, the intensity of the negative peak at 198 nm decreased. This shows that the content of β sheet structure of

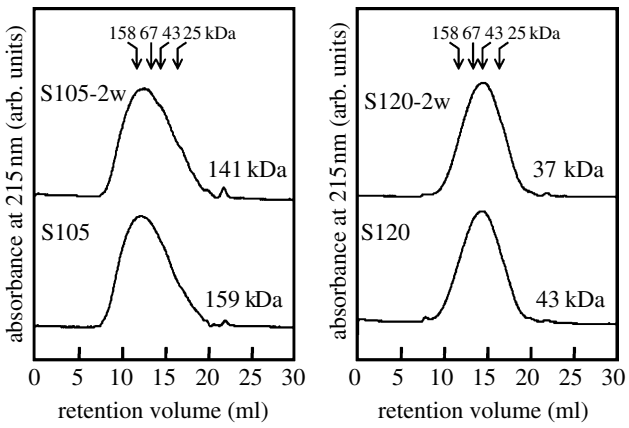

Figure 1. GPC profiles of sericin samples prepared under various conditions.

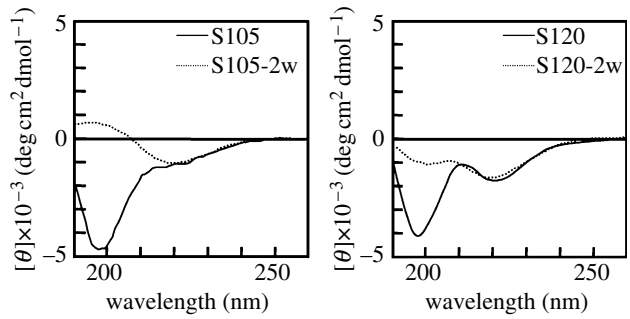

Figure 2. CD spectra of the extracted sericin prepared under various conditions.

sericin increased during storage of the solution. The behaviour of the sericin extracted at 120 °C was similar to that of the sericin extracted at 105 °C. However, the content of the β sheet structure in sample S105-2w was higher than that in sample S120-2w.

Figure 3 shows the FT-IR spectra of the above sericin films. The two absorption bands located at 1655 and 1550 cm^{-1} were assigned to the amide I (νC=O) and amide II (δN-H) stretches, respectively. The absorption peak located at 1655 cm^{-1} was attributed to a random coil structure (Miyazawa & Blout 1961). A shoulder peak located at 1626 cm^{-1} was attributed to the β sheet structure. The intensity of this latter peak was significantly higher for the film prepared using sample S105-2w than for the film prepared using sample S105. This same tendency was also observed for films prepared from sericin extracted at 120 °C. However in this case, the increase in intensity was less distinct than that detected in the spectrum of films prepared from sericin extracted at 105 °C. These results indicate that the random coil structure is dominant in sericin films prepared immediately after extraction. The proportion of the β sheet structure increased during ageing at 4 °C, and the proportion of the β sheet structure in sericin extracted at 105 °C was higher than that in sericin extracted at 120 °C.

Figure 4 shows SEM images of the surfaces of sericin films soaked in a 1.5SBF for a period of 7 days. Among the films prepared from the extracted sericin, only the

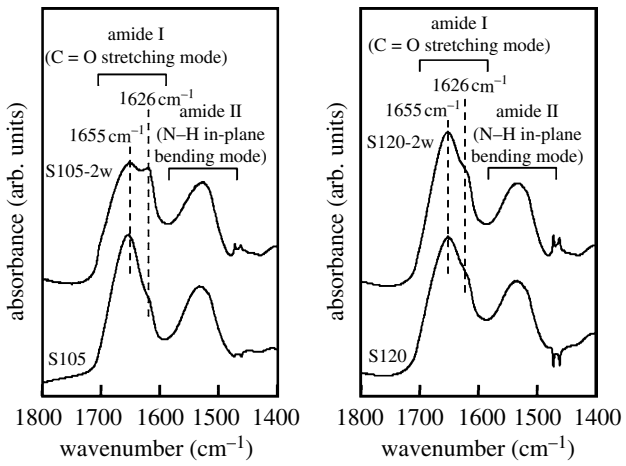

Figure 3. FT-IR spectra of sericin films prepared from various solutions.

film prepared from sample S105-2w had particles deposited on its surface. On the surfaces of the films prepared from the other samples, any changes in their morphology were not observed.

Figure 5 shows the XRF spectrum of the surface of the sericin film of sample S105-2w after soaking in the 1.5SBF for a period of 7 days, showing that the particles were mainly composed of calcium and phosphorus. The TF-XRD patterns of the sericin films soaked in the 1.5SBF for a period of 7 days are shown in figure 6. A broad peak assigned to hydroxyapatite was detected in the film prepared from sample S105-2w after soaking in the 1.5SBF. These results confirm that the particles formed on the film after soaking in the 1.5SBF were bone-like apatite.

4. DISCUSSION

From the results described above, hydroxyapatite deposition was not observed in all the sericin film samples, but was observed only for a certain type of sericin film. Deposition of hydroxyapatite on a substrate in a metastable calcium phosphate solution is initiated by the existence of a substance that can induce heterogeneous nucleation of hydroxyapatite. Therefore, only the sericin film prepared from sample S105-2w had the potential to induce heterogeneous nucleation of hydroxyapatite. The CD and FT-IR spectra data show that the film made from sample S105-2w had the highest content of β sheet structure among the prepared sericin solutions, while the GPC data showed that sericin solutions extracted at 105 °C had a higher molecular weight than those extracted at 120 °C. These findings indicate that a sericin film can induce hydroxyapatite nucleation when it has both a high molecular weight and a high β sheet structure content.

Previous studies have reported that hydroxyapatite deposition can be initiated by functional groups existing on the surface of a material (Li *et al.* 1994; Kokubo *et al.* 2000). Carboxyl groups are effective in the nucleation of hydroxyapatite from a solution that mimics body fluid, i.e. a metastable calcium phosphate solution. (Tanahashi & Matsuda 1997). We therefore

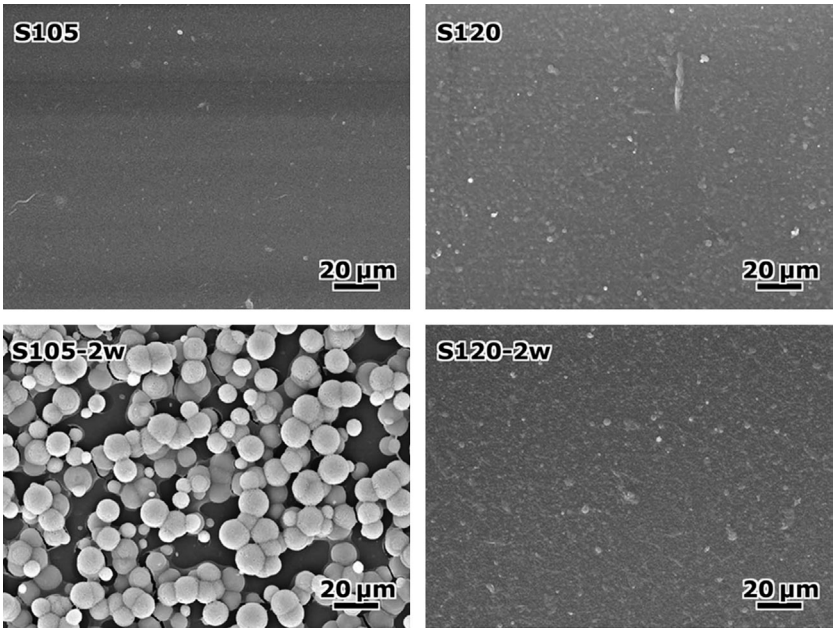

Figure 4. SEM images of the surfaces of sericin films prepared from various solutions after soaking in the 1.5SBF for 7 days.

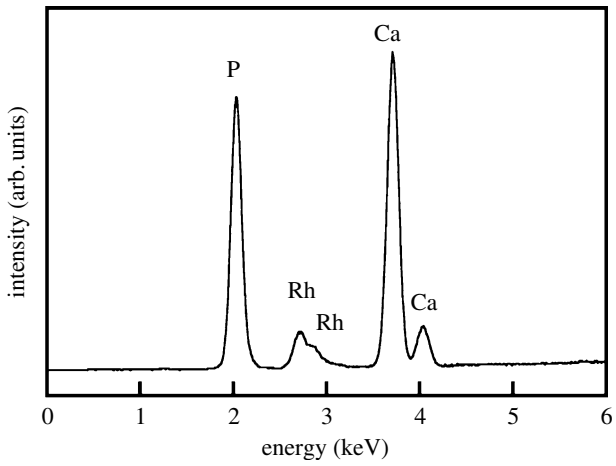

Figure 5. XRF spectrum of the surface of sample S105-2w after soaking in the 1.5SBF for 7 days.

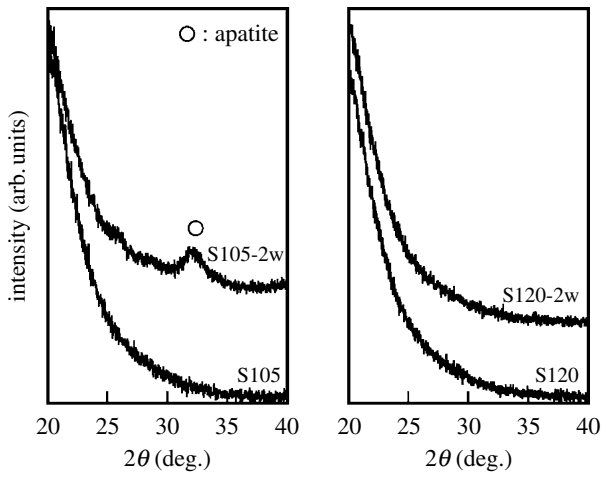

Figure 6. TF-XRD patterns of sericin films prepared from various solutions after soaking in the 1.5SBF for 7 days.

hypothesized that the induction of heterogeneous nucleation on sericin arises from a high number of carboxyl groups on the sericin (Takeuchi *et al.* 2003) from a comparison of the amino acid content between sericin and fibroin (Komatsu 2000*b*; Shimura & Katagata 2000). Interestingly, the present work indicates an important finding, in that the heterogeneous nucleation of hydroxyapatite on sericin is governed by its molecular weight and secondary structure. This means that induction of heterogeneous nucleation on hydroxyapatite depends on the carboxyl group content and also on their arrangement. When a protein has a β sheet structure, functional groups of side chains in amino acids generally point above and below the β sheet alternately. The β sheet structure in sericin molecules allows for an orientation of carboxyl groups, as shown schematically in figure 7, whereas random coil does not. Namely, 10% of the carboxyl groups can be arranged perpendicular to the sheet when the sericin has an ideal β sheet structure. A higher molecular weight may lead to a specific relationship between each β sheet in the sericin molecular structure. Such a specific arrangement of the functional groups would result in sites that were suitable for hydroxyapatite nucleation. The importance of the arrangement of the functional groups for the deposition of hydroxyapatite in SBF has also been reported for Ti–OH groups (Uchida *et al.* 2003) and for Zr–OH groups (Uchida *et al.* 2002) groups. We have shown that there is a suitable arrangement of functional groups on organic substrates for hydroxyapatite nucleation for biomimetic mineralization. Consequently, hydroxyapatite nucleation on a substrate in a solution mimicking a body fluid is very sensitive to such structural functional group arrangements. This finding is useful in understanding biomineralization, as well as for the design of organic polymers that can effectively induce hydroxyapatite nucleation.

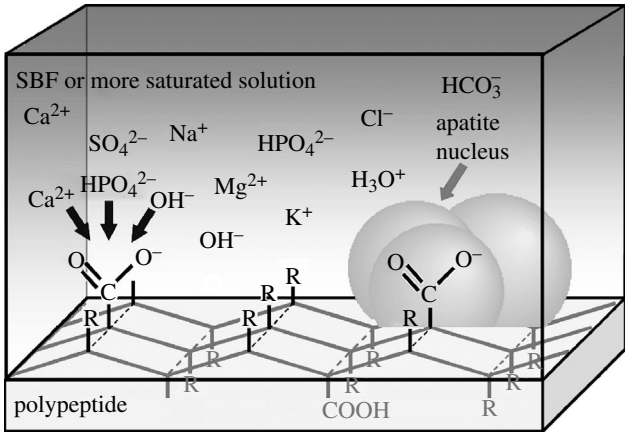

Figure 7. Mechanism of hydroxyapatite nucleation on a polypeptide substrate with a β sheet structure in a highly saturated SBF.

5. CONCLUSIONS

Sericin, a natural silk protein, can induce hydroxyapatite deposition on its surface in a metastable calcium phosphate solution, such as a 1.5SBF, when it has a high content of β sheet structure and a high molecular weight. This indicates that the induction of hydroxyapatite nucleation is governed by the arrangement of carboxyl groups on the protein. Our findings are valuable to further the understanding of the mechanism of mineralization in biological systems, as well as in the design of novel organic polymers for the preparation of hybrid materials with structures similar to bone.

This work was conducted under the auspices of the research project, 'Technology Development for Medical Materials Merging Genome Information and Materials Science', in the Kansai Science City Innovative Cluster Creation Project, supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

One of the authors (T.M.) acknowledges the support of a grant-in-aid for young scientists ((B)16700365) from the Japan Society for the promotion of science.

REFERENCES

Brahms, S. & Brahms, J. 1980 Determination of protein secondary structure in solution by vacuum ultraviolet circular dichroism. *J. Mol. Biol.* **138**, 148–178.

Cho, S. B., Nakanishi, K., Kokubo, T., Soga, N., Ohtsuki, C., Nakamura, T., Kitsugi, T. & Yamamuro, T. 1995 Dependence of apatite formation on silica gel on its structure: effect of heat treatment. *J. Am. Ceram. Soc.* **78**, 1769–1774.

Filgueiras, M. R., La Torre, G. & Hench, L. L. 1993 Solution effects on the surface reactions of a bioactive glass. *J. Biomed. Mater. Res.* **27**, 445–453.

Greenfield, N. & Fasman, G. D. 1969 Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* **8**, 4108–4116.

Hench, L. L. 1991 Bioceramics: from concept to clinic. *J. Am. Ceram. Soc.* **74**, 1478–1510.

Kokubo, T. 1991 Bioactive glass ceramics: properties and applications. *Biomaterials* **12**, 155–163.

Kokubo, T., Ito, S., Huang, Z. T., Hayashi, T., Sakka, S., Kitsugi, T. & Yamamuro, T. 1990*a* Ca,P-rich layer formed on high-strength bioactive glass-ceramic A-W. *J. Biomed. Mater. Res.* **24**, 331–343.

Kokubo, T., Kushitani, H., Sakka, S., Kitsugi, T. & Yamamuro, T. 1990*b* Solutions able to reproduce in vivo surface-structure changes in bioactive glass-ceramic A-W. *J. Biomed. Mater. Res.* **24**, 721–734.

Kokubo, T., Kim, H. M., Kawashita, M., Takadama, H., Miyazaki, T., Uchida, M. & Nakamura, T. 2000 Nucleation and growth of apatite on amorphous phases in simulated body fluid. *Glastech. Ber. Sci. Technol.* **73C1**, 247–254.

Komatsu, K. 2000*a* Chemical and structural characteristics of silk sericin. In *Structure of silk yarn, part B: chemical structure and processing of silk yarn* (ed. N. Hojo), pp. 47–85. Enfield, USA: Science Publishers, Inc.

Komatsu, K. 2000*b* Chemical and structural characteristics of wild cocoon and silk. In *Structure of silk yarn, part B: chemical structure and processing of silk yarn* (ed. N. Hojo), pp. 21–46. Enfield, USA: Science Publishers, Inc.

Lee, K. G., Kweon, H. Y., Yeo, J. H., Woo, S. O., Lee, Y. W., Cho, C. S., Kim, K. H. & Park, Y. H. 2003 Effect of methyl alcohol on the morphology and conformational characteristics of silk sericin. *Int. J. Biol. Macromol.* **33**, 75–80.

Li, P., Ohtsuki, C., Kokubo, T., Nakanishi, K., Soga, N., Nakamura, T. & Yamamuro, T. 1992 Apatite formation induced by silica gel in a simulated body fluid. *J. Am. Ceram. Soc.* **75**, 2094–2097.

Li, P., Ohtsuki, C., Kokubo, T., Nakanishi, K., Soga, N. & de Groot, K. 1994 The role of hydrated silica, titania and alumina in inducing apatite on implants. *J. Biomed. Mater. Res.* **28**, 7–15.

Madison, V. & Schellman, J. 1972 Optical activity of polypeptides and proteins. *Biopolymers* **11**, 1041–1076.

Mann, S. 2001 Organic matrix-mediated biomineralization. In *Biomineralization* (ed. R. G. Compton, S. G. Davies & J. Evans), pp. 89–124. New York: Oxford University Press.

Miyazawa, T. & Blout, E. R. 1961 The infrared spectra of polypeptides in various conformations: amide I and II bands. *J. Am. Chem. Soc.* **83**, 712–719.

Neo, M., Kotani, S., Nakamura, T., Yamamuro, T., Ohtsuki, C., Kokubo, T. & Bando, Y. 1992 A comparative study of ultrastructures of the interfaces between four kinds of surface-active ceramic and bone. *J. Biomed. Mater. Res.* **26**, 1419–1432.

Ohtsuki, C., Kokubo, T. & Yamamuro, T. 1992 Mechanism of apatite formation on $CaO–SiO_2–P_2O_5$ glasses in a simulated body fluid. *J. Non-Cryst. Solids* **143**, 84–92.

Shimura, K. & Katagata, Y. 2000 Chemical structure of silk fibroin. In *Structure of silk yarn, part B: chemical structure and processing of silk yarn* (ed. N. Hojo), pp. 3–20. Enfield, USA: Science Publishers, Inc.

Takeuchi, A., Ohtsuki, C., Miyazaki, T., Tanaka, H., Yamazaki, M. & Tanihara, M. 2003 Deposition of bone-like apatite on silk fiber in a solution that mimics extracellular fluid. *J. Biomed. Mater. Res.* **65A**, 283–289.

Tanahashi, M. & Matsuda, T. 1997 Surface functional group dependence on apatite formation on self-assembled monolayers in a simulated body fluid. *J. Biomed. Mater. Res.* **34**, 305–315.

Tanahashi, M., Yao, T., Kokubo, T., Minoda, M., Miyamato, T., Nakamura, T. & Yamamuro, T. 1994 Apatite coating on organic polymers by a biomimetic process. *J. Am. Ceram. Soc.* **77**, 2805–2808.

Townend, R., Kumosinski, T. F., Timasheff, S. N., Fasman, G. D. & Davidson, B. 1966 The circular dichroism of the β-structure of poly-L-lysine. *Biochem. Biophys. Res. Commun.* **23**, 163–169.

Uchida, M., Kim, H. M., Kokubo, T., Tanaka, K. & Nakamura, T. 2002 Structural dependence of apatite formation on zirconia gels in a simulated body fluid. *J Ceram Soc Jpn.* **110**, 710–715.

Uchida, M., Kim, H. M., Kokubo, T., Fujibayashi, S. & Nakamura, T. 2003 Structural dependence of apatite formation on titania gels in a simulated body fluid. *J. Biomed. Mater. Res.* **64**, 164–170.