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Apatite mineralization behavior on polyglutamic acid hydrogels in aqueous condition: effects of molecular weight

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Abstract
Apatite-polymer composites mimicking specific structure of natural bone are promised as bone substitutes with moderate flexibility able to be fabricated into desired shapes as well as bone-bonding bioactivity. In order to precipitate the apatite on polymer surfaces, aqueous processing using solution supersaturated to the apatite has been attracting as much attention. Polyglutamic acid (PGA) is a promised candidate of the polymer, since it has high apatite-forming ability owing to abundant carboxyl groups able to trigger the heterogeneous apatite nucleation. Although combination of PGA with different molecular weight is expected to provide design of organic-inorganic composites with moderate bioresorbability, precise relationship between the molecular weight of the PGA and its apatite-forming ability has been remained unclear. In the present study, PGA hydrogels with different molecular weight were prepared by covalent cross-linking using ethylenediamine. Difference in apatite formation in simulated body fluid (SBF) was interpreted in terms of their chemical structure. It was found that hydrogels prepared from PGA with higher molecular weight showed tendency to have higher apatite-forming ability. It was attributed to high content of the carboxyl group remaining on the hydrogel due to low degree of the cross-linking.

Keywords: polyglutamic acid (PGA), apatite, simulated body fluid (SBF), molecular weight, Organic-inorganic composites
1. Introduction

Several kinds of ceramics including sintered hydroxyapatite, Bioglass, glass-ceramics A-W are known to make direct bond to surrounding bone tissues after implanted in bone defects (Hench 1991). Such specific bone-bonding property is called bioactivity. Bioactive ceramics are clinically utilized in the fields of orthopedic surgery, neurosurgery and dentistry. However, hard and brittle characters make application of these ceramics under loaded conditions difficult. In addition, many medical doctors desire development of flexible bioactive materials able to be fabricated into desired shapes according to the shape of the bone defect. Inspired by the fact that natural bone also takes composite structure composed of apatite and collagen, apatite-polymer composites have been attracting much attention as novel bioactive materials with analogous mechanical performance to natural bone and excellent workability. They are also useful for scaffolds supporting bone tissue regeneration, when bioresorbable polymers are selected for an organic component.

As a method to fabricate such composites, aqueous process using simulated body fluid (SBF) has been attracting much attention (Kokubo et al. 2003). In this process, apatite layer is precipitated on organic polymer substrates in SBF under ambient temperature. This is quite attractive because thermal damage of the polymer can be avoided during conventional ceramics processing. If the apatite-forming ability of the materials is not so high, more concentrated solutions with higher ion concentration than SBF such as 1.5SBF and 5.0SBF are also used for acceleration in the apatite deposition (Tanahashi et al. 1994 and Barrere et al. 2002). The heterogeneous apatite nucleation is induced by specific surface functional groups on material surfaces. Several functional groups such as Si-OH (Cho et al. 1995), Ti-OH (Li et al. 1994), Ta-OH (Miyazaki et al. 2001), Nb-OH (Miyazaki et al. 2001a), COOH (Tanahashi et al. 1997) are known to be effective. In addition, it is significantly accelerated by Ca$^{2+}$ release from the materials into SBF because degree of supersaturation of the
surrounding fluid with respect to the apatite is locally increased (Ohtsuki et al. 1992).

Several kinds of natural and synthetic polymers containing carboxyl groups are used for the organic component of the apatite-polymer composites (Kawashita et al. 2003, Miyazaki et al. 2003 and Ichibouji et al. 2009). Among them, we have focused our attention on polyglutamic acid (PGA). This is a kind of biologically compatible polypeptide composed of only glutamic acid containing carboxyl group in side chain. Several researches on biomedical application of PGA for drug delivery system (DDS) carrier and novel biodegradable polymer have been proposed (Matsusaki et al. 2002 and Matsumura 2008). PGA is expected to have apatite-forming ability due to abundant carboxyl groups. We previously revealed that some PGA-based materials form the apatite in a short period after soaking in SBF (Sugino et al. 2008 and Koh et al. 2011). This means that these materials have potential to be highly bioactive in vivo.

Molecular weight is known as an important factor governing degradation rate of the organic polymer in aqueous conditions (Shenwu et al. 2001 and Pekcan et al. 2002). Control in the degradation rate is quite important for design of scaffolds with moderate bioresorbability. It is expected that this is achieved by appropriate combination of polymers by different molecular weight. However, effects of molecular weight of PGA on its ability of the apatite formation are not fully investigated.

In the present study, PGA hydrogels with different molecular weight were prepared by covalent cross-linking using ethylenediamine and their apatite formation was compared in SBF. Difference in the apatite-forming ability was interpreted in terms of their chemical structure.

2. Materials and Methods
2.1 Specimen Preparation

PGA sodium salt with molecular weight range of 1.5-2.5 x 10^6 and 4.0-6.0 x 10^6 and CaCl₂ were dissolved in ultrapure water to form aqueous solutions. PGA
concentration was fixed at 10 mass%, and CaCl$_2$ concentration at 0.125, 0.250, 0.375 M (=kmol·m$^{-3}$). Ethylenediamine-2(N-hydroxysuccinimide) was prepared by adding dropwise 150 cm$^3$ of 2N-hydroxysuccinimide ethyl acetate solution with 1.7 mass% to 10 cm$^3$ of ethylenediamine ethyl acetate solution with 6.3 mass%. Then 0.87 g of the prepared ethylenediamine-2(N-hydroxysuccinimide) and 1.17 g of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl) was added into 20 cm$^3$ of the PGA solution prepared by the above process to progress cross-linking reaction.

The solution was poured into a Teflon dish and dried at 30°C for 2 days. The specimens prepared from PGA with molecular weight of 1.5-2.5 x 10$^6$ and 4.0-6.0 x 10$^6$ are hereafter abbreviated as “LMwPGA” and “HMwPGA”, respectively. All the reagents used for preparation of the hydrogels were purchased from Wako Pure Chemical Industries, Japan, except for EDC·HCl (Tokyo Chemical Industry Co., Ltd. Japan).

2.2 Soaking in SBF

The obtained bulk hydrogels of 0.05 g were cut and then soaked in 30 cm$^3$ of SBF with inorganic ion concentrations (Na$^+$ 142.0, K$^+$ 5.0, Mg$^{2+}$ 2.5, Cl$^-$ 147.8, HCO$_3^-$ 4.2, HPO$_4^{2-}$ 1.0 and SO$_4^{2-}$ 0.5 mM) at 36.5°C for 3 days. The pH of the fluid was buffered at 7.40 by 50 mM tris(hydroxymethyl)aminomethane and an appropriate amount of HCl. SBF was prepared according to the previous literature (Cho et al. 1995). All the reagents used for preparation of SBF were purchased from Nacalai Tesque Inc., Japan. After soaking, the specimens were removed from the SBF, immersed in ultrapure water for 12 hours to remove excess water-soluble salts remaining in the gels and dried at 30°C for 2 days.

2.3 Characterization
The surface structural changes of the specimens were characterized using a scanning electron microscope (SEM; Model S-3500N; Hitachi Co., Japan), an energy dispersive X-ray analyzer (EDX; Model EX-400; Horiba Co., Japan) and a thin-film X-ray diffractometer (TF-XRD; MXP3V; Mac Science Ltd., Japan). The TF-XRD can detect crystalline phase of the surface layer about 1 µm in depth. The apatite formation can occur not only on the surface but also inside the hydrogels due to swelling by SBF. In order to quantify amount of the whole deposited apatite, the specimens after soaking in SBF were immersed in 20 cm$^3$ of 1M-HCl for 1 day at room temperature to completely dissolve the apatite. P concentration of the HCl solution was subsequently analyzed by inductively coupled plasma (ICP) atomic emission spectroscopy (Model Optima 4300DV Cyclon, Perkin-Elmer Co., England).

Release of Ca$^{2+}$ from the specimens was measured by soaking the hydrogels of 0.05 g in 30 cm$^3$ of Tris-NaCl buffer solution. The Tris-NaCl solution contained 142 mol·m$^{-3}$ NaCl and 50 mM tris(hydroxymethyl)aminomethane and was buffered at pH 7.40 by an appropriate amount of HCl. The Ca$^{2+}$ concentrations were measured by ICP. Swelling ratio was measured by immersion of the specimens in 30 cm$^3$ of Tris-NaCl buffer for 3 days at room temperature, and weighing the specimens before and after the immersion.

The amount of carboxyl group in the specimens was measured by acid-base titration as follows (Saito et al. 2006). The specimen was soaked in 30 cm$^3$ of 0.5M-HCl for 10 minutes. After taking from the solution and washing with ultrapure water, they were then soaked in 30 cm$^3$ of 0.5M-NaCl for 10 minutes. The NaCl solution was titrated by 0.1M-NaOH while monitoring pH by a pH meter (Model F-23IIC; Horiba Co., Japan).

2.4 Measurement of in vitro degradation behavior

LMwPGA and HMwPGA hydrogels without containing CaCl$_2$ were prepared
by the procedure indicated in Section 2.1. The obtained hydrogels of 0.05 g were soaked in 30 cm$^3$ of Tris-NaCl buffer solution at pH 7.40 for various periods up to 15 days. After soaking, the specimens were removed from Tris-NaCl, washed with ultrapure water and dried at 30°C for 2 days. Weight ratio of the hydrogels before and after the soaking was calculated.

3. Results

Figure 1 shows SEM photographs of the surfaces of the specimens after soaking in SBF for 3 days, as a function of molecular weight of PGA and CaCl$_2$ concentration of the precursor solution. Deposites of fine particles with size ranging 1 to 3 µm are observed on all the specimens after the soaking.

Figure 2 shows TF-XRD patterns of the surfaces of the specimens after soaking in SBF for 3 days, as a function of molecular weight of PGA and CaCl$_2$ concentration of the precursor solution. Broad peaks corresponding to (002) diffraction and envelope of (211)(112)(300) diffractions of the apatite (JCPDS #09-432) were observed at 26 and 32° in 2θ, respectively. HMwPGA gave slightly higher diffraction peaks than LMwPGA. The diffraction at 26° was not observed for LMwPGA with CaCl$_2$ concentration of 0.125 and 0.250 M.

Figure 3 shows P concentration of HCl solution which dissolved the precipitates formed on the specimens after immersion in SBF for 3 days. HMwPGA showed significantly higher P concentration than LMwPGA. The specimens with CaCl$_2$ concentration of 0.250 and 0.375 M showed significantly higher P concentration than those of 0.125 M for both LMwPGA and HMwPGA.

Figure 4 shows Ca concentration of Tris-NaCl buffer after soaking of the specimens for 3 days. The Ca concentration showed tendency to increase with increase in CaCl$_2$ concentration of the precursor solution. Both LMwPGA and HMwPGA showed similar Ca release with the same CaCl$_2$ concentration.
Figure 5 shows swelling ratio of the specimens, as a function of molecular weight and CaCl2 concentration of the precursor solution. The swelling ratio showed tendency to decrease with increase in CaCl2 concentration. HMwPGA showed slightly higher swelling ratio than LMwPGA.

Figure 6 shows amount of carboxyl groups of the specimens, as a function of molecular weight and CaCl2 concentration of the precursor solution. HMwPGA showed significantly higher amount of the carboxyl groups than LMwPGA.

Figure 7 shows changes in weight ratio of the PGA hydrogels with different molecular weight after soaking in Tris-NaCl for various periods. Mass of both the specimens decreased by about 40% within 4 days, and afterwards became almost constant. HMwPGA showed slightly larger degradation in Tris-NaCl buffer than LMwPGA.

4. Discussion

The obtained PGA hydrogels formed the apatite on their surfaces in SBF irrespective of molecular weight, when they were incorporated with Ca2+ ions in advance. The formed apatite took low-crystalline structure similar to typical bone mineral. It is expected that the bone-like apatite on the PGA-apatite composites would tightly integrate with bone tissues due to structural similarity after implantation in vivo.

The amount of the deposited apatite on HMwPGA was significantly higher than that on LMwPGA as shown in Figs. 2 and 3. The results in Fig. 4 indicate that the hydrogels with the same Ca2+ content show almost similar Ca2+ release into Tris-NaCl irrespective of the molecular weight. This suggests that increase in degree of supersaturation of the surrounding fluid with respect to the apatite is kept at almost similar level also in SBF.

Therefore the difference in the apatite-forming ability of the PGA hydrogels would be mainly attributed to the difference in the amount of carboxyl groups. In
general, swelling ratio of organic polymer gels generally decreases with increase in molecular weight due to molecular interaction such as hydrogen bonding under constant degree of the cross-linking. In spite of that, HMwPGA showed higher swelling ratio and \textit{in vitro} degradation than LMwPGA as shown in Figs. 5 and 7. This means that degree of the cross-linking of the former is lower than that of the latter. During the hydrogel preparation in the present study, carboxyl groups are consumed by the dehydration condensation with ethylenediamine to form the covalent cross-linking as follows:

\[
2R\text{-COOH} + \text{NH}_2\text{(CH}_2\text{)}_2\text{NH}_2 \rightarrow R\text{-CONH(CH}_2\text{)}_2\text{NHCO-R} \tag{1}
\]

where R represents alkyl groups in PGA. In the case of HMwPGA, reduced diffusion of the cross-linking agent into PGA molecular chains would provide hydrogels with low degree of the cross-linking. Similar phenomenon is reported on hyaluronic acid hydrogels cross-linked with glutaraldehyde (Collins \textit{et al.} 2007). Consequently, it is assumed that HMwPGA exhibits higher apatite-forming ability than LMwPGA, because the former remains large amount of carboxyl groups even after the hydrogel formation. This assumption is supported by the results in Fig. 6 showing large amount of the carboxyl groups in HMwPGA.

In comparison with other polymer scaffolds previously reported, degree of the degradation of the present PGA hydrogels after 14 days is similar to that of typical covalently cross-linked collagen sponges (Vrana \textit{et al.} 2007), and higher than porous poly(lactic acid) (Niu \textit{et al.} 2009). However, initial \textit{in vitro} degradation about 40 mass\% was observed within 4 days. This may be too fast for application as scaffolds, because the hydrogels may be completely dissolved before sufficient bone regeneration. In the present study, degradation was evaluated for the hydrogels without apatite formation. As a solution of this drawback described above, the PGA-apatite composites would be effective for suppression of the fast initial degradation. The apatite layer will play a role
as a barrier against penetration of the surrounding water into the interior of the PGA hydrogels, since solubility of the apatite in neutral water is quite low.

5. Conclusions

Apatite formation on PGA hydrogels with different molecular weight was examined in SBF. Apatite-forming ability and degree of in vitro degradation increased with increase in molecular weight. This is because large amount of carboxyl groups are remained on the PGA hydrogels with high molecular weight due to low degree of cross-linking. Selection of PGA with appropriate molecular weight is an important factor to fabricate apatite-PGA composites with moderate bioresorbability.

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**Figure Captions**

**Fig. 1** SEM photographs of the surfaces of the specimens after soaking in SBF for 3 days, as a function of molecular weight of PGA and CaCl$_2$ concentration of the precursor solution.

**Fig. 2** TF-XRD patterns of the surfaces of the specimens after soaking in SBF for 3 days, as a function of molecular weight of PGA and CaCl$_2$ concentration of the precursor solution.

**Fig. 3** P concentration of HCl solution which dissolved the precipitates formed on the specimens after immersion in SBF for 3 days (n=3).

**Fig. 4** Ca concentration of Tris-NaCl buffer after soaking of the specimens for 3 days (n=3).

**Fig. 5** Swelling ratio of the specimens, as a function of molecular weight and CaCl$_2$ concentration of the precursor solution (n=3).

**Fig. 6** Amount of carboxyl groups of the specimens, as a function of molecular weight and CaCl$_2$ concentration of the precursor solution.

**Fig. 7** Changes in weight ratio of the PGA hydrogels with different molecular weight after soaking in Tris-NaCl for various periods (n=3).