Apatite Mineralization Abilities and Mechanical Properties of Covalently Cross-linked Pectin Hydrogels

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Abstract

Natural bone has features such as high fracture toughness and bone-bonding bioactivity, is organic-inorganic hybrid composed of collagen and apatite crystals. Therefore, apatite-polymer hybrids designed to mimic the structure of bone represent candidates for high-performance bone substitutes. In this study, we prepared pectin hydrogels through covalent cross-linking using divinylsulfone (DVS) and investigated their apatite-forming abilities of in SBF and mechanical properties. The obtained results were also compared with pectin hydrogels prepared by ionic cross-linking with Ca^{2+}. The gels cross-linked with DVS showed similar apatite-forming abilities to those cross-linked with Ca^{2+}. The former gels showed higher tensile strengths than the latter ones.

Keywords: pectin, apatite, cross-linking, simulated body fluid, mechanical properties

1. Introduction

Generally, artificial materials implanted into bone defects become encapsulated by fibrous tissue and isolated from the surrounding bone [1]. However, several ceramics, such as Bioglass® [2-4], sintered hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) [5,6] and glass-ceramic A-W [7,8] are currently used for bone repair due to their attractive features including their direct bone-bonding activities, i.e. bioactivities. These ceramics form bone-like apatite on their surfaces in the body environment and consequently bond to living bone through this apatite layer. However, their clinical applications are limited to areas under low load due to their inappropriate mechanical performance, such as lower fracture toughness and higher Young's modulus than natural bone. Therefore, novel materials exhibiting both high flexibility and bioactivity are required for bone repair in medical fields. Natural bone is a kind of organic-inorganic hybrid composed of apatite nanocrystals and collagen fibers [9]. This structure provides specific mechanical properties such as high fracture toughness and high flexibility. Therefore, if organic polymers are provided with apatite-forming ability, they are expected to act as novel artificial bone substitutes exhibiting not only bioactivity but also mechanical performance analogous to natural bone.

As techniques for depositing apatite on organic polymers, biomimetic processes have recently been proposed. In these processes, heterogeneous nucleation of apatite is achieved on materials with specific functional groups on their surfaces in aqueous solutions supersaturated with respect to the apatite. Simulated body fluid (SBF; Kokubo solution) with inorganic ion concentrations that are nearly equal to those found in human blood plasma or more concentrated solutions are popularly used to deposit the apatite [10,11]. Several functional groups, such as Si-OH [12], Ti-OH [12], Zr-OH [13], Ta-OH [14), Nb-OH [15], COOH [16], PO_4H_2 [16] and SO_3H [17], are known to induce apatite nucleation. The apatite formation is accelerated by the release of calcium ions

(Ca^{2+}) from the material surfaces into the surrounding solution, since the release of Ca^{2+} increases the degree of supersaturation of the surrounding fluid with respect to the apatite. Recently, it has been revealed that several organic polymers containing carboxyl (-COOH) groups form apatite on their surfaces in the body environment [18,19].

We focused our attention on pectin, a kind of plant-derived polysaccharide, which contains carboxyl groups [20,21]. Pectin has been recently attracting much attention as novel biomaterials such as scaffolds for tissue engineering and careers for drug delivery [22]. We previously reported that pectin hydrogels prepared by treatment with $CaCl_2$ solution deposit apatite in SBF, although their tensile strengths are lower than that of human cancellous bone [23]. It is expected that process of the cross-linking would affect properties of the pectin hydrogels. Covalent cross-linking with divinylsulfone (DVS; $CH_2=CHSO_2CH=CH_2$) is popularly used for preparation of polysaccharide hydrogels. We prepared the pectin hydrogels through covalent cross-linking with DVS and examined the apatite-forming abilities and mechanical properties. In addition, the measured properties were compared with those of the pectin gels cross-linked with Ca^{2+}.

2. Experimental procedures

2.1. Preparation of pectin gels

Three kinds of pectin, namely apple-derived pectin ($(C_6H_8O_6, C_7H_{10}O_6)_n$, Wako Pure Chemical Industries Ltd. Inc., Japan), citrus-derived pectin ($(C_6H_8O_6, C_7H_{10}O_6)_n$, Wako Pure Chemical Industries Ltd. Inc., Japan) and polygalacturonic acid (Pectic acid, $(C_6H_8O_6)_n$, Nacalai Tesque Inc., Kyoto, Japan), were used in this study. All the pectins were manufactured by Wako Pure Chemical Industries Ltd. Inc., Japan. Powdered pectin preparations were dissolved in 0.2 $kmol \cdot m^{-3}$ NaOH (pectic acid) or ultrapure water (apple- and citrus-derived pectins) to form 10 mass% solutions at 80°C. The

degrees of esterification of apple-derived pectin, citrus-derived pectin and pectic acid were previously reported to be 41.7±0.3%, 32.0±0.5% and 0%, respectively [23]. DVS was added to 15 cm^3 of pectin solutions at 5 mass% relative to the pectin. The solutions were cast in polystylene molds of 95×60 mm^2 in size, and dried at 40°C for 3 days. Although scaffolds generally take a form of porous body, dense specimens were prepared to facilitate fundamental analysis of chemical reaction on their surfaces in SBF. The compositions of the pectin gels are summarized in Table 1.

2.2. Evaluation of apatite-forming abilities

After bulk gels were obtained, rectangular specimens of 10×10 mm^2 in size were cut from the gels. The gels cross-linked with DVS were subsequently soaked in 1 $kmol \cdot m^{-3}$ $CaCl_2$ aqueous solution at 36.5°C for 24 hours. All gels were 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 $mol \cdot m^{-3}$) at pH 7.40 at 36.5°C for various periods up to 7 days [11]. After soaking, the specimens were removed from the SBF, immersed in ultrapure water for 24 hours to remove excess water-soluble salts remaining in the gels and dried at 40°C for 24 hours.

2.3. Analysis of pectin gels

The surface potential of the pectin gels with DVS or Ca^{2+} cross-linking was analyzed in terms of zeta potential, which was measured using a laser electrophoresis spectroscopy (Model ELSZ-2, Otsuka Electronics Co., Japan). The pectin gels 33 x 14 mm^2 in size were put on a polyacrylamide-coated high-purity silica glass cell and Tris-NaCl buffer solution dispersed with Latex monitor particles was filled in the cell, which was immediately equipped into the electrophoresis system to measure the zeta potential of surface of the pectin gels. This system adopts laser Doppler electrophoresis

to measure the electrophoretic mobility of monitor particles and the electroosmotic flow mobility of surfaces of the specimens. The zeta potential (ζ) is given by the Smoluchowski equation,

$$\zeta = 4\pi\eta U/\varepsilon \qquad (1)$$

where U is the electroosmotic flow mobility of surface of pectin gels, η is the viscosity of the solution and ε is the dielectric constant of the solution.

The surface structural changes of the specimens were also 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).

Changes in the Ca^{2+} concentrations and pH were measured by soaking hydrogels of 10×10 mm^2 in size in 30 cm^3 of Tris-NaCl buffer solution. The Tris-NaCl solution contained 142 $mol \cdot m^{-3}$ NaCl and 50 $mol \cdot m^{-3}$ tris(hydroxymethyl)aminomethane and was buffered at pH 7.40 by an appropriate amount of HCl. The Ca^{2+} concentrations and pH were measured using a calcium ion electrode (Model #6583-10C; HORIBA Co.) and a pH electrode (Model #9621-10D; HORIBA Co.), respectively.

2.4. Evaluation of mechanical properties of pectin gels

The tensile strengths of pectin gels were measured according to the Japanese Industrial Standards (JIS) K 7127. Each pectin solution (3 cm^3) was placed into a Teflon® mold with a dumbbell shape. After aging at 4°C for 24 hours, the formed gels were dried at 40°C for 3 days, and then subjected to tensile tests using an Instron-type testing machine (Model AG-I; Shimadzu Co., Japan) at a cross-head speed of 5.0 $mm \cdot min^{-1}$. The tensile stress was calculated from the load at fracture and the

geometrical area of cross-sections of the specimens. At least 5 specimens were tested for each composition, and the mean stress and standard deviation were calculated.

3. Results

3.1. Apatite-forming abilities

For all the compositions other than Acid-DVS, crack-free specimens were obtained. Fig. 1 shows zeta potential of the pectin gels with and without cross-linking with DVS, which were all treated with 1 $kmol \cdot m^{-3}$ $CaCl_2$. Hydrogels of pectic acid, apple-derived pectin and citrus-derived pectin treated only with 1 $kmol \cdot m^{-3}$ $CaCl_2$ were denoted as Acid-Ca, Apple-Ca and Citrus-Ca, respectively. The pectin gels cross-linked with DVS were negatively charged, whereas those cross-linked with Ca^{2+} were positively charged.

Fig. 2 shows SEM photographs of the surfaces of pectin gels after soaking in SBF for various periods. Deposition of fine spherical particles was observed over the whole surfaces of Apple-DVS and Citrus-DVS gels after 7 days, but not on pectic acid gels. According to the EDX analysis, peaks assigned to calcium and phosphorous were detected on the deposits observed under the SEM (data not shown).

Fig. 3 shows the TF-XRD patterns of the pectin gels after soaking in SBF for various periods. Broad peaks assigned to hydroxyapatite with low crystallinity were detected for the samples where the deposits covered the whole surfaces at 26° and 32° in 2θ.

Table 2 summarizes the apatite-forming abilities of the pectin gels. According to the previous results, apatite formation in SBF was observed for citrus- and apple-derived gels cross-linked with Ca^{2+} after 3 and 7 days, respectively, but not pectic acid. We can see that the apatite-forming abilities of the pectin gels cross-linked with DVS were comparable with those of the pectin gels cross-linked with Ca^{2+}.

3.2. Changes in Ca^{2+} concentrations and pH of the buffer solutions

Fig. 4 shows the changes in the Ca^{2+} concentration of the Tris-NaCl buffer due to soaking of different pectin gels. The concentrations increased within 1 day and then slightly decreased. Fig. 5 shows the changes in pH of Tris-NaCl buffer due to soaking of different pectin gels. The pH of the gels decreased continuously.

3.3. Mechanical properties of pectin gels

Acid-DVS gels could not be subjected to tensile tests because they formed many cracks during drying. Fig. 6 shows representative stress-strain curves of the remaining gels. Table 3 summarizes the tensile stress, strain and Young's modulus of the specimens in comparison with those of human cancellous bone [1]. The DVS cross-linked gels showed tensile stress about 30 MPa. These values are higher than those of the pectin gels cross-linked with Ca^{2+} (2.4-5.3 MPa) [23].

4. Discussion

Our SEM observations and TF-XRD data revealed that apple- and citrus-derived pectins formed apatite on their surfaces after soaking in SBF for up to 7 days. The apatite formed had the characteristic of low crystallinity, similar to the case for bone apatite. However the deposits initially formed on the gels surfaces within 3 days in SBF gave no clear TF-XRD patterns characteristic of apatite. It is assumed that the fine particles were comprised of precursors of apatite, such as amorphous calcium phosphate, rather than apatite itself or that the amount of the deposit was too low to be detected by TF-XRD.

The pectin gels cross-linked with DVS formed apatite with a comparable induction period to gels cross-linked with Ca^{2+}. Release of Ca^{2+} into the buffer solution

was higher for the gels cross-linked with Ca^{2+} (6-12 $mol \cdot m^{-3}$ [23]) than those cross-linked with DVS. This situation would arise because not only heterogeneous nucleation of apatite but also homogeneous nucleation occurred on the gels cross-linked with Ca^{2+}, judging from the observation that the solutions became cloudy after soaking of the gels. Pectic acid did not form the apatite in SBF, despite the fact that it has the largest amount of carboxyl groups among the three kinds of pectin. Release of Ca^{2+} from the gels would be suppressed because Ca^{2+} ions incorporated into the gels bind tightly to the abundant carboxyl groups via the formation of the egg-box structure. In addition, the high acidity of pectic acid shown in Fig. 5 reduces the degree of supersaturation of the surrounding solution with respect to apatite.

The gels cross-linked with DVS showed higher tensile strengths than those cross-linked with Ca^{2+}. DVS creates cross-links with hydroxyl groups in pectin according to the following chemical equation [24]:

$$2R\text{-}OH + CH_2{=}CHSO_2CH{=}CH_2 \quad \text{----}> \quad R\text{-}OCH_2CH_2SO_2CH_2CH_2O\text{-}R \qquad (2)$$

where R represents alkyl groups in the polysaccharides. The gels cross-linked with DVS were more negatively charged than those cross-linked with Ca^{2+} as shown in Fig. 1. These results also support the assumption that DVS is successfully incorporated into the gels, since DVS contains negatively charged sulfonyl groups. The covalent cross-links formed would be stronger than the ionic cross-links formed by Ca^{2+}. Similar tendency was reported for alginate microcapsules prepared through ionic and covalent cross-linking procedures [25]. In addition, the former gels would have larger amounts of cross-linking sites than the latter gels. In other words, the former gels have not only covalent cross-linking with DVS but also ionic cross-linking with Ca^{2+}, since they are subjected to $CaCl_2$ treatment.

In addition, the gels cross-linked with DVS have another advantage. Namely they can suppress rapid degradation in body environment, since covalent cross-linking in them is maintained. If only ionic cross-linking with Ca^{2+} exists in the gels, reduction in mechanical strength can be occurred by the cleavage of the cross-linking due to release of Ca^{2+}. Similar phenomenon has been reported by Kamitakahara et al [26].

The tensile strength and Young's modulus of human cancellous bone are intermediate between the pectin gels cross-linked with Ca^{2+} and those cross-linked with DVS. The mechanical performances of the pectin gels can be matched with those of human cancellous bone if the degrees of cross-linking are appropriately controlled by changing the kind and amount of the cross-linking agents.

5. Conclusions

We examined the apatite-forming abilities in SBF and mechanical properties of pectin hydrogels with different cross-links. The gels cross-linked with DVS showed comparable apatite-forming abilities to those cross-linked with Ca^{2+}, whereas the former gels showed higher tensile strengths than the latter gels. Overall, it was found that the bioactivities and mechanical properties of pectin gels can be easily controlled by changing the kind or degree of cross-links.

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Table captions

Table 1. Compositions of the specimens

Table 2. Apatite-forming ability of pectin gels after soaking in SBF for various periods

Table 3. Mechanical properties of pectin gels in comparison with those of human cancellous bone

Figure captions

Fig. 1. Zeta potential of the pectin gels with and without cross-linking with DVS, which were all treated with 1 $kmol \cdot m^{-3}$ $CaCl_2$.

Fig. 2. SEM photographs of the surfaces of pectin gels cross-linked with DVS, after soaking in SBF for various periods.

Fig. 3. TF-XRD patterns of the surfaces of pectin gels cross-linked with DVS, after soaking in SBF for various periods (HAp: hydroxyapatite).

Fig. 4. Changes in Ca concentration of Tris-NaCl due to soaking of pectin gels cross-linked with DVS.

Fig. 5. Changes in pH of Tris-NaCl due to soaking of pectin gels cross-linked with DVS.

Fig. 6. Stress-strain curves of pectin gels cross-linked with DVS.

Table 1. Compositions of the specimens

Sample	Kind of pectin	Pectin concentration / mass%	Cross-linking agent
Acid-DVS	Pectic acid	10	DVS
Apple-DVS	Apple-derived	10	
Citrus-DVS	Citrus-derived	10	

Table 2. Apatite-forming ability of pectin gels after soaking in SBF for various periods

Sample	Apatite-formation		
	1 day	3 days	7 days
Acid-DVS	×	×	×
Apple-DVS	×	Δ	○
Citrus-DVS	×	Δ	○

○: Deposit was formed and identified with the apatite by TF-XRD.

Δ: Deposit was formed but not identified with the apatite by TF-XRD.

× : No deposit was formed.

Table 3. Mechanical properties of pectin gels in comparison with those of human cancellous bone

Sample	Stress / MPa	Strain / %	Young's modulus / MPa
Acid-DVS	-	-	-
Apple-DVS	29.7 ± 4.0	4.3 ± 1.4	1312.6 ± 174.1
Citrus-DVS	37.7 ± 5.9	6.7 ± 1.2	1279.6 ± 186.3
Cancellous bone	10 -20	5 - 7	50 - 500

- : not measured

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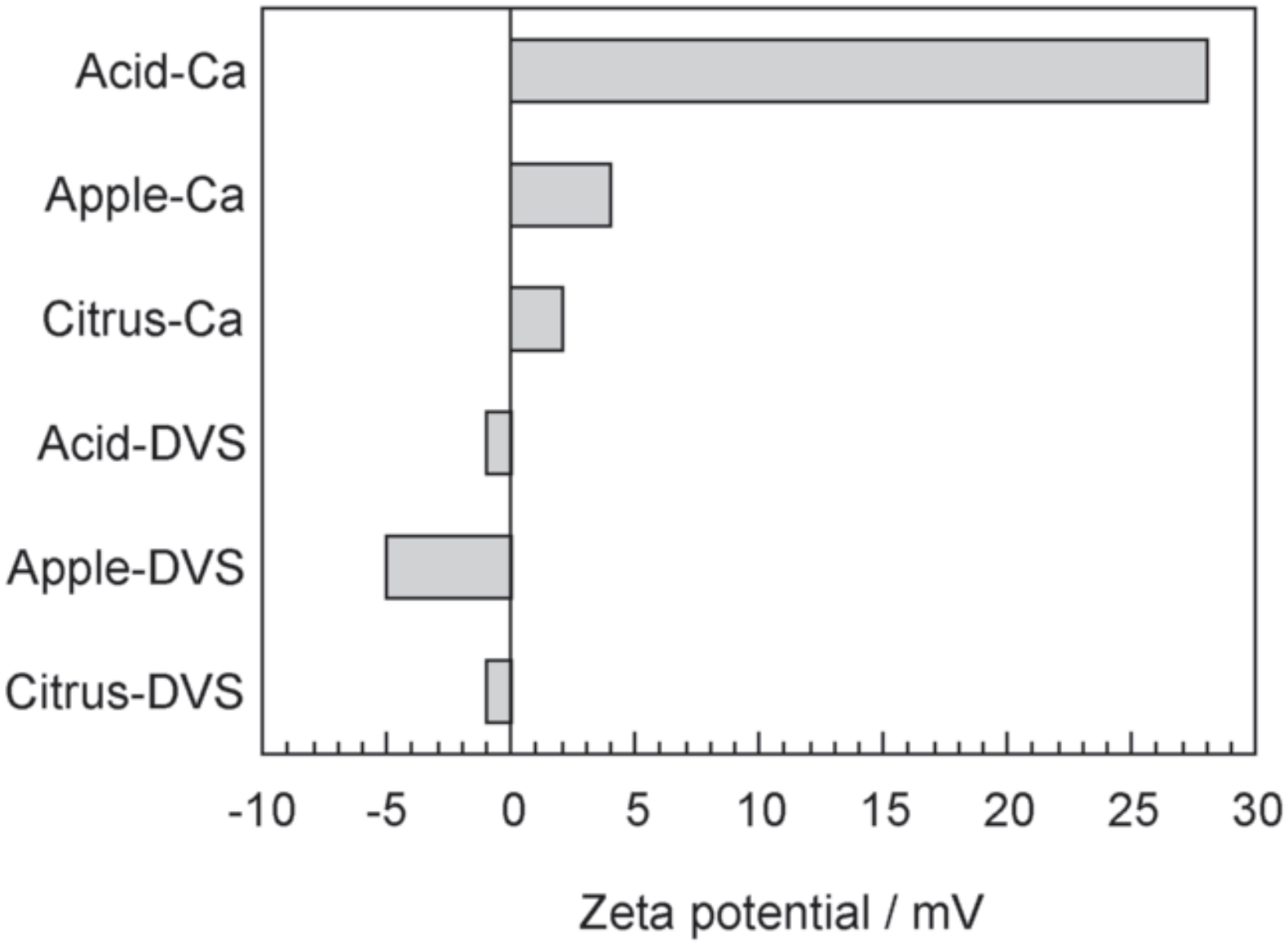

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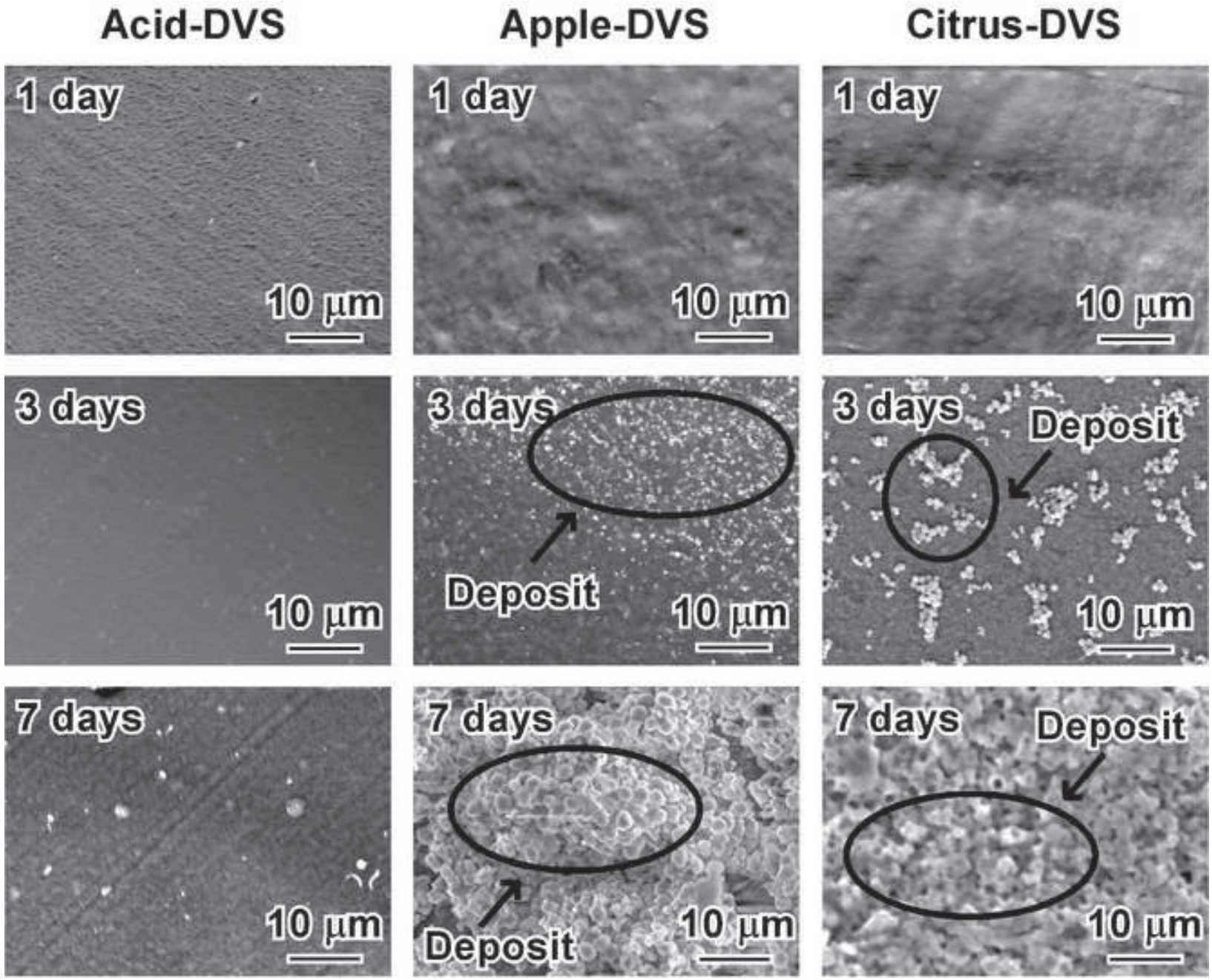

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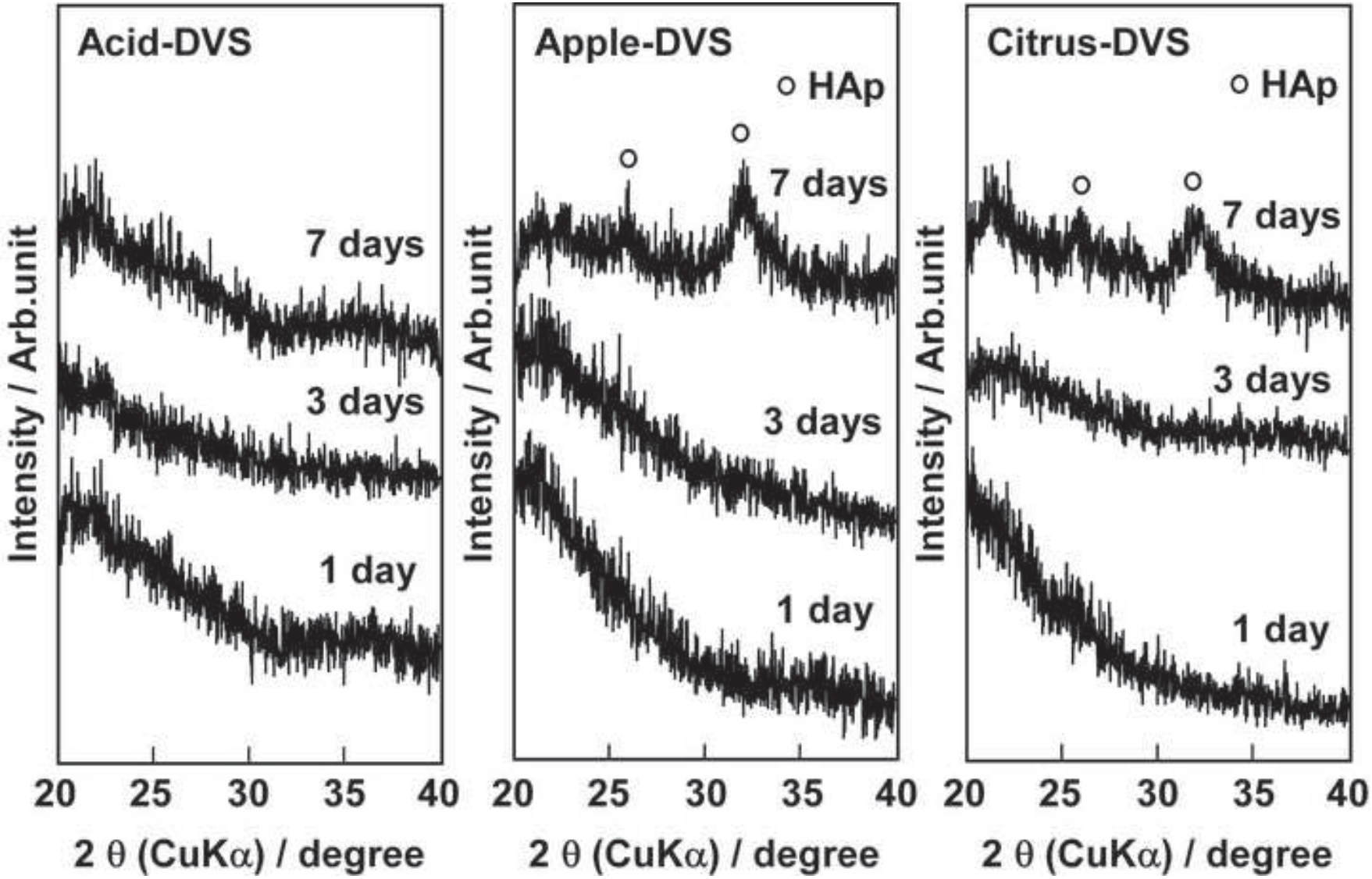

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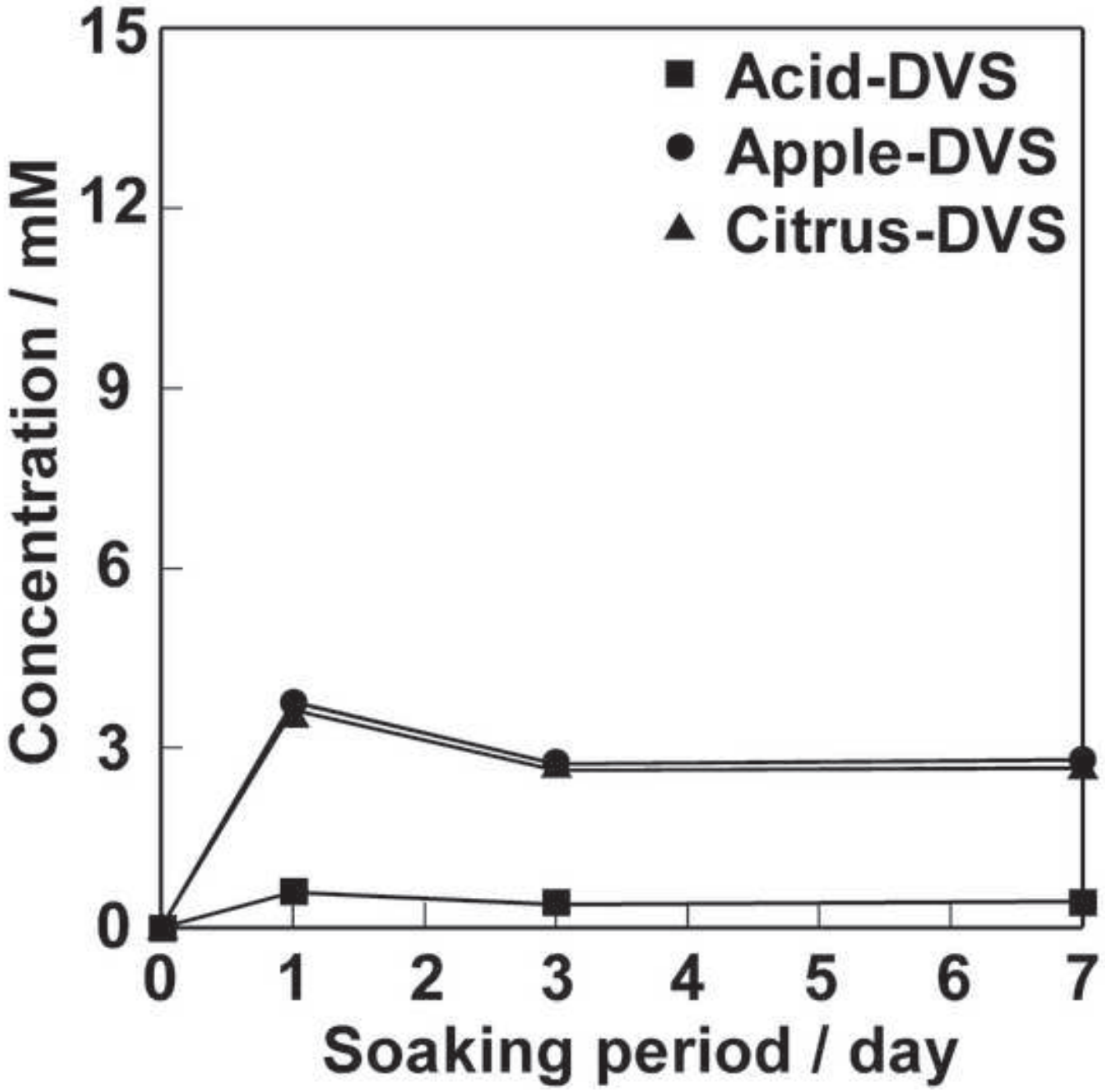

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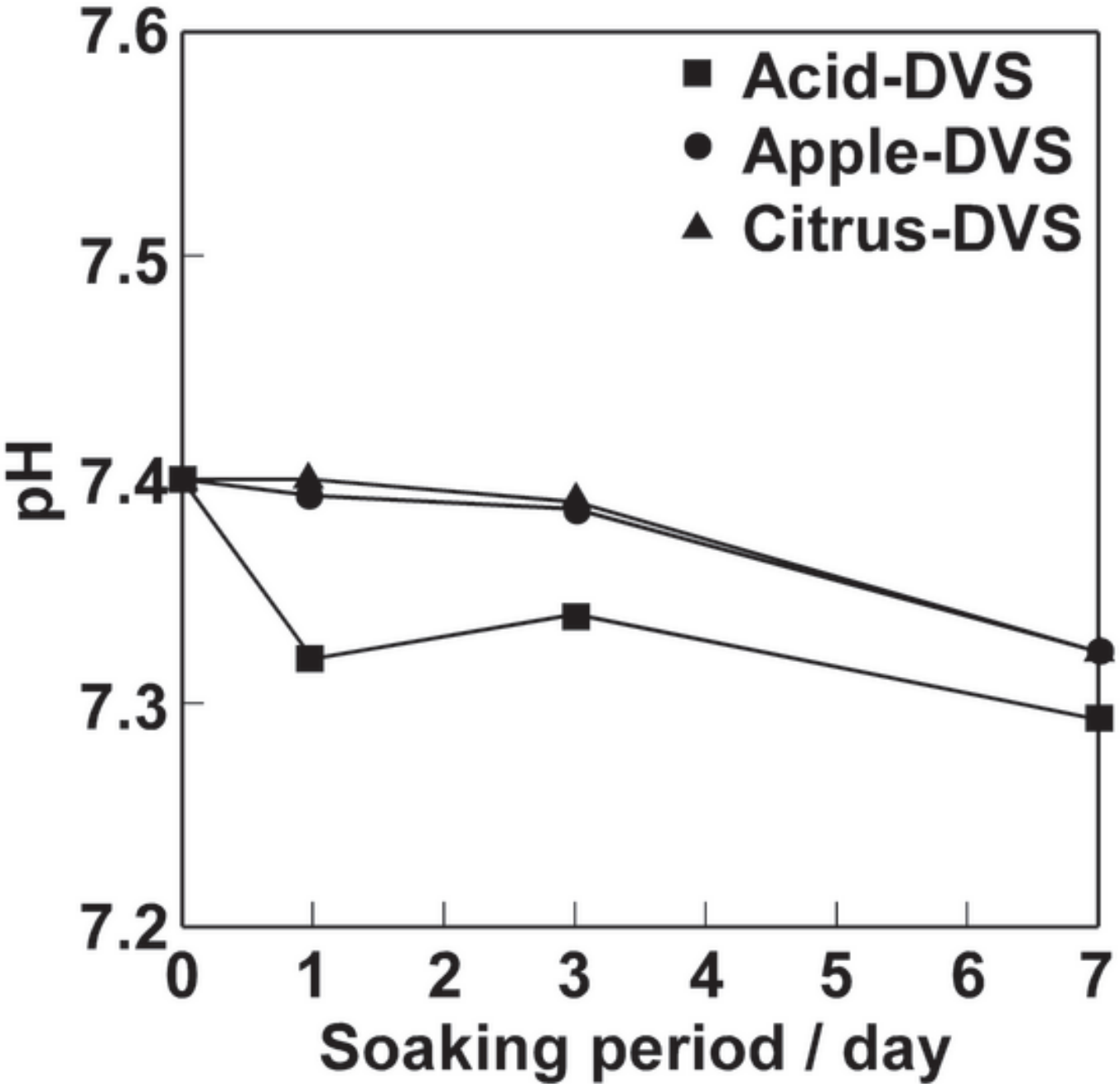

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