APATITE FORMATION BEHAVIOR ON PECTIN HYDROGELS IN SIMULATED BODY ENVIRONMENT

（生体模倣環境下におけるペクチン水和ゲル上でのアパタイト形成挙動）

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Contents

General Introduction

1. Bioactive materials

Recently bone damage caused by diseases, accidents and aging has increased. It severely reduces our quality of life (QOL). In most cases, bone defects formed by the damage are currently repaired with bone grafts such as allografts and autografts. Allograft is extracted from another patient, whereas autograft is extracted from another portion of the same patient. However allografts have the problems such as confection by virus and prion. On the other hand, autograft has the problems such as limited supply of the grafts. In addition, it forces the patient much physical damage because healthy tissues must be injured in order to extract the autograft. Therefore, developments of artificial materials which can repair the bone defects are desired. However artificial materials implanted into bone defects are generally encapsulated by a fibrous tissue of collagen and become isolated from the surrounding bone [1]. This is normal reaction to protect human's body from foreign substances. As a result, the material implanted into the bone defect is isolated from the surrounding bone and does not bond to the living bone directly.

To overcome this problem, so-called "Bioactive ceramics" have been developed as bone substitutes that make direct bonding to living bone after implantation in bone defects. In the early 1970's, Hench *et al.* discovered that some glasses in the Na_2-CaO-SiO_2-P_2O_5 system, later called Bioglass® [2-4]. Since the discovery of these glasses, several ceramics such as sintered hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) [5,6], glass-ceramic A-W containing crystalline oxyfluorapatite ($Ca_{10}(PO_4)_6(O,F_2)$) and wollastonite ($CaSiO_3$) in a MgO-CaO-SiO_2 glassy matrix [7,8] were developed. These are currently used for bone repair due to their attractive features including their direct bone-bonding activities, i.e. bioactivities. When these ceramics are implanted into bone defects, they form bone-like apatite layer on their surfaces in the body environment and consequently bond to living bone through this apatite layer [9]. This apatite is quite similar to

1

hydroxyapatite in living bone in terms of composition and crystallinity. Figure 1 shows schematic representation of the bonding mechanism of bioactive materials to living bone. Therefore, these materials have been used as periodontal fillers, bone fillers, iliac crests, artificial vertebrae, and intervertebral discs in the orthopedic field.

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 [10]. Furthermore, bioactive metallic materials such as titanium, titanium alloy and tantalum have higher fracture toughness than foregoing bioactive ceramics, were recently developed. These metallic materials are provided with bioactivity by chemical treatment with sodium hydroxide solution and heat treatment [11-19] or treatment with hydrogen peroxide containing various kinds of metal chlorides [20]. The metals also have a problem that they have higher Young's modulus than cortical bone, which may cause resorption of the surrounding bone due to stress shielding effects. Therefore, novel materials exhibiting both flexibility similar to natural bone and bioactivity are required for bone repair in medical fields.

2. Biomimetic process

Natural bone is known to take organic-inorganic hybrid structure in which inorganic nanocrystals of the calcium-deficient carbonate apatite with low crystallinity [6] are deposited on organic collagen fibers woven into a three-dimensional structure. This apatite takes a form of needle-like crystals with 20-40 nm in length and 1.5-3.0 nm in thickness in collagen fiber matrix by biomineralization. These hydroxyapatite contained collagen fibers are arranged into a higher ordered organization and result in the anisotropic structure of bone shown in Figure 2 [21-23]. 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) is protein-free and acellular aqueous solution with inorganic ion concentrations that are nearly equal to those found in human blood plasma. Table 1 shows ion concentrations of SBF and human blood plasma. SBF or more concentrated solutions are popularly used to deposit the apatite [24, 25]. Several functional groups, such as Si-OH [26], Ti-OH [26], Zr-OH [27], Ta-OH [28], Nb-OH [29], COOH [30], PO_4H_2 [30] and SO_3H [31], have been reported to induce apatite nucleation in SBF. Figure 3 shows the mechanism of hydroxyapatite formation on substrates with those functional groups. The nucleation of hydroxyapatite is initiated by incorporation of Ca^{2+} ions into those functional groups on the substrate surfaces, and then HPO_4^{2-} and OH^- ions subsequently bind to the positive surface [32]. Once hydroxyapatite nuclei are formed, they spontaneously grow into the hydroxyapatite layer. Additionally, 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.

3. **Biomedical application of polysaccharide**

Generally, biomaterials can be classified into bioresorbable and nonbioresorbable. Bioresorbable materials become extinct to be absorbed into human body after having achieved a purpose as the materials when it had been implanted in the body. Figure 4 shows the degradation style of bioresorbable materials [33]. Although most of traditionally-developed materials are nonbioresorbable, bioresorbable materials have been recently attracted much attention. As

representative polysaccharides, cellulose, chitin, starch, alginic acid, hyaluronic acid and pectin have been known.

Various studies on medical application of bioresorbable polysaccharides, e.g. scaffold for tissue regeneration (Figure 5 [33]) and drug delivery system (DDS) (Figure 6 [34]) have been making much progress. Hyaluronan is highly non-antigenic and non-immunogenic, owing to its high structural homology across species, and low interaction with blood components [35-38]. For these reasons, hyaluronan hydrogel crosslinked with divinylsulfone (DVS) are widely used to relieve joint pain and as a tissue spacer without using surgical adhesions.

In addition, polysaccharides and these derivatives are also used as wound dressers for reconstruction of skin tissues, because it has highly moisture-retaining property. Main polysaccharides used for these purposes are nonwoven chitin [39], nonwoven calcium alginate [40] and the hydro-colloid type wound dressing composed of fine particles of hydrophilic pectin or carboxymethylcellulose dispersed in the matrix of hydrophobic synthetic polymer [41]. In the future, it is expected that novel biomaterials such as sponges of the alginic acid or the hyaluronic acid impregnated with biologically active agent are developed [42].

Furthermore, polysaccharides have applied as the DDS carrier with bioresorbability. Drug delivery system (DDS) is originally aimed to deliver drugs to the target tissue to improve the therapeutic effect as well as minimize the side effects [43]. Water-soluble cellulose derivatives, chitin, chitosan and starch and so on have been used for these purposes [34].

As described above, the polysaccharides had been widely used as medical materials. However mechanical strength of these hydrogels is lower compared with human bone to substitute hard tissue. Recently, it has been reported that several organic polymers containing carboxyl (-COOH) groups form apatite on their surfaces in the body environment [44-46]. Consequently, it is thought that information on material design to improve mechanical property of the polysaccharide hydrogels is necessary for development of polysaccharide-based bone substitutes.

4. Pectin as biomaterials

Pectin is a kind of plant-derived polysaccharide enriched with galacturonic acid and galacturonic acid methyl ester units as a component of extracellular matrix. It has high biological compatibility [47], and contains carboxyl groups as the functional groups with apatite-forming ability in body environment. Pectins form skeletal tissues of plants by combined with proteins and other polysaccharides, which are chemically stable and physically strong. Pectins can take various forms ranging from fluid sol to dense gels with high molecular weight and polyanionic character depending on degree of the chemical reacttion. These properties enable pectin polymers to carry signal molecules and support various biologically active substances [48]. The structure of pectin is primarily composed of repeating units of galacturonic acid joined by a 1→4 glycosidic linkages, which create a linear polymer as shown in Figure 7 [49]. The linear structure is cleaved by the presence of neutral sugar side chains such as rhamnose. Furthermore, the carboxyl groups of galacturonic acid may either remain as free acids, which are able to be esterified with methanol or neutralized with cations. For details, Pectins are primarily a polymer of D-galacturonic acid (homopolymer of [1→4]α-D-galactopyranosyluronic acid units with varying degrees of carboxyl groups methylesterified) and rhamnogalacturonan (heteropolymer of repeating [1→2]α-L-rhamnosyl-[1→L] α-D-galactopyranosyluronic acid disaccharide units), making it an α-D-galacturonan [50]. According to Thibault, this rhamnogalacturonan main-chain combined with galactose, arabinose, mannose and glucose as side-chains [51]. Pectin backbone is composed of smooth region (linear galacturonan) with poor side-chains and hairy region (rhamnogalacturonan and side chains) with rich side-chains as shown Figure 8 [52]. The chemical structure of pectin varies by species and states of the plants [53].

Pectin is classified into low methoxyl pectin and high methoxyl pectin depending on amount of methoxyl groups. Namely, amount of carboxyl group is decreased with increase in

amount of methoxyl groups. It is known that low methoxyl pectin make a hydrogel by forming so-called egg-box structure in which polymer chain of pectin is layered via cross-linkage by Ca^{2+} ions, independent of pH and temperature [54]. It is reported that pectin and modified pectin have been found to exhibit anti-inflammatory, antimutagenic activity and inhibit cancer metastasis and proliferation, with no evidence of toxicity or other serious side effects [49,55-61]. Moreover, pectins are relatively inexpensive and easy to obtain in comparison to, for example, recombinant proteins. Additionally, the human body cannot degrade pectins in tissues. It are also reported pectin-containing poly(lactide-co-glycolide) matrices improved cell adhesion and proliferation when compared to plain p(LGA) matrices, as determined in vitro by osteoblast culture [62] and the growth and differentiation of mammalian bone cells such as osteoblast and osteoclast was induced on the substrate that was coated with modified pectin by specific enzymes which have rich rhamnogalacturonans with shorter side chains [63]. Therefore, pectin has been recently attracting much attention as novel biomaterials such as scaffolds for tissue engineering and careers for drug delivery. Thus, their properties and general availability make pectins viable to consider when engineering new biomedical materials.

5. Purpose of this study

The general purpose of this study in this thesis is to investigate the apatite deposition behavior on pectin hydrogels in simulated body environment to establish the novel bone-repairing materials.

In Chapter 1, pectin hydrogels were prepared by calcium salt treatment, and apatite-forming ability in SBF and mechanical property of it were investigated. Pectin is a natural polysaccharide containing carboxyl groups and forms a hydrogel with characteristic structure. The properties of this are different by degree of esterification of the side chain. Therefore, three kinds of pectins were examined. The results are discussed in terms of the relationship between

apatite-forming ability and mechanical properties of the pectin hydrogels.

In Chapter 2, apatite-forming ability in SBF and mechanical property of pectin hydrogels that were prepared through covalent cross-linking with divinyl sulfone (DVS), were investigated and compared with those of the pectin gels cross-linked with Ca^{2+}. In addition, zeta potential of the surfaces of pectin gels cross-linking with DVS or Ca^{2+} was measured and apatite deposition mechanism on the pectin gel is discussed.

In Chapter 3, pectin hydrogels were prepared by mixing various water-soluble calcium salts powder (calcium acetate monohydrate ($Ca(CH_3COO)_2 \cdot H_2O$), calcium chloride ($CaCl_2$) and calcium hydroxide ($Ca(OH)_2$)), and the apatite-forming ability of it was investigated in simulated body fluid (SBF). Pectic acid with the lowest apatite-forming ability and citrus-derived pectin with the highest one as shown Chapter 1 were examined. The results are discussed in effects of the solubility and degree of the pH of calcium salts on gelation and apatite-forming ability of pectin gals.

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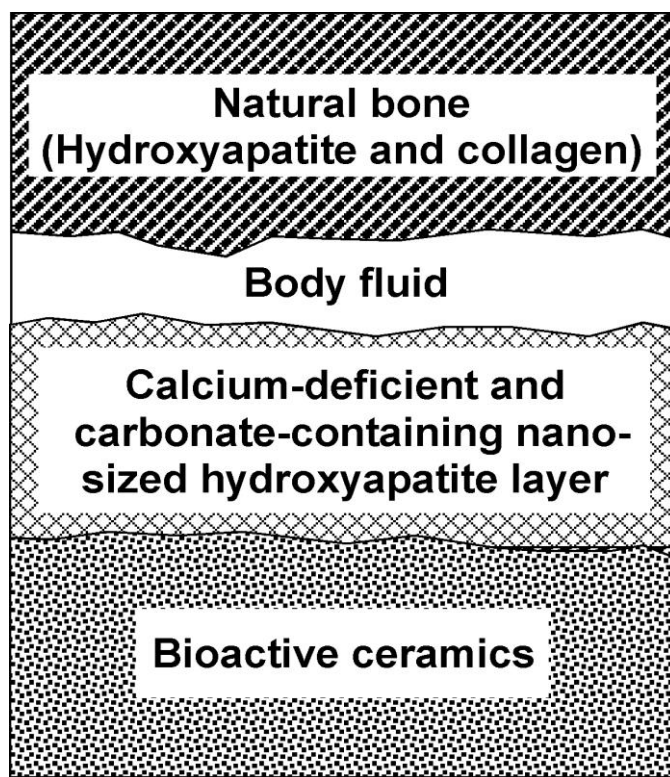
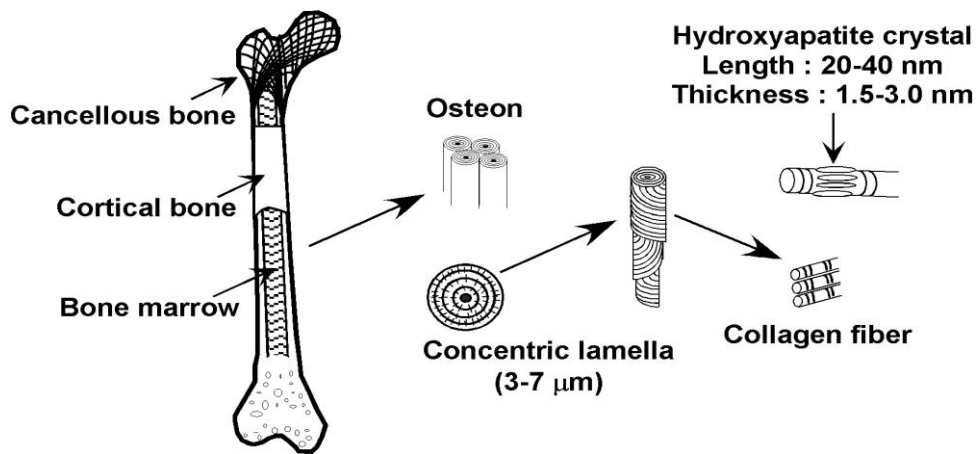Figure 1 Schematic representation of the bonding mechanism of bioactive materials to living bone.
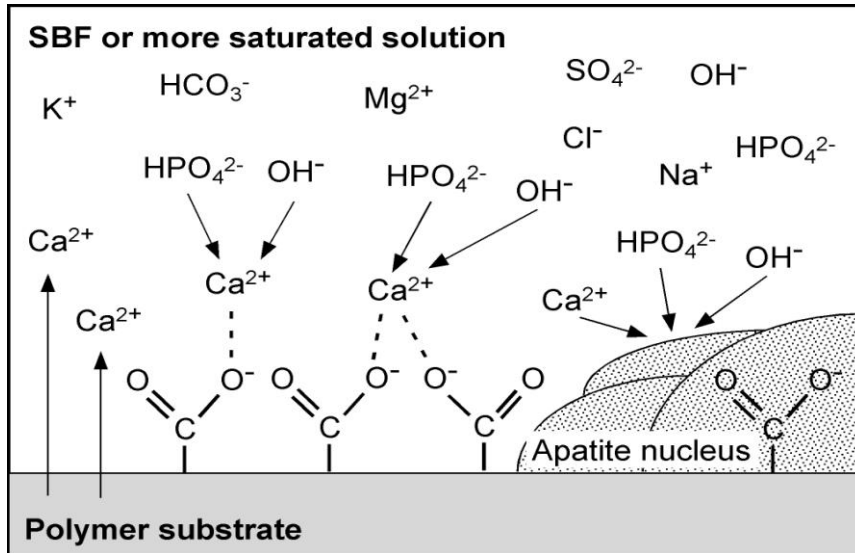
Figure 2 Structure of natural bone.

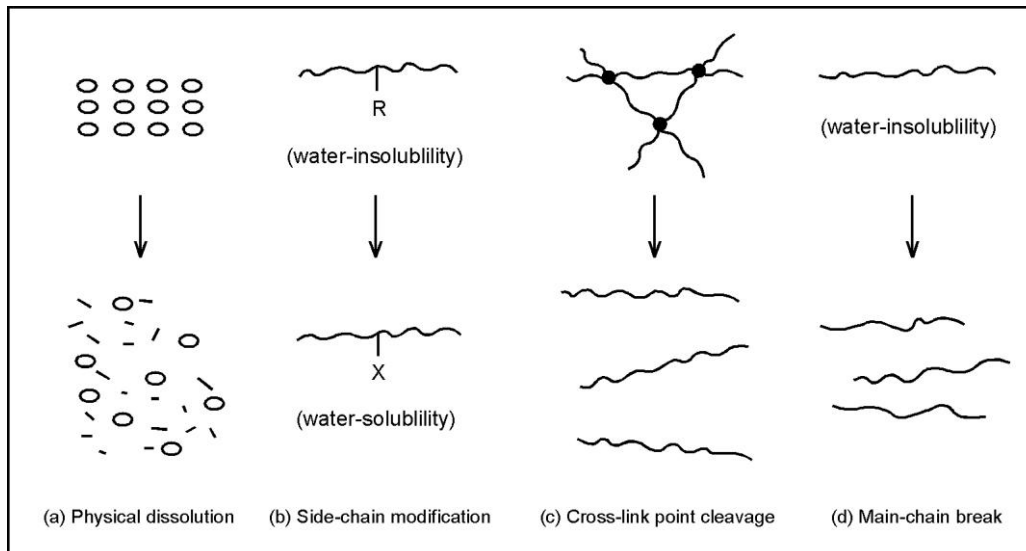Figure 3 Mechanism of hydroxyapatite formation in body environment.

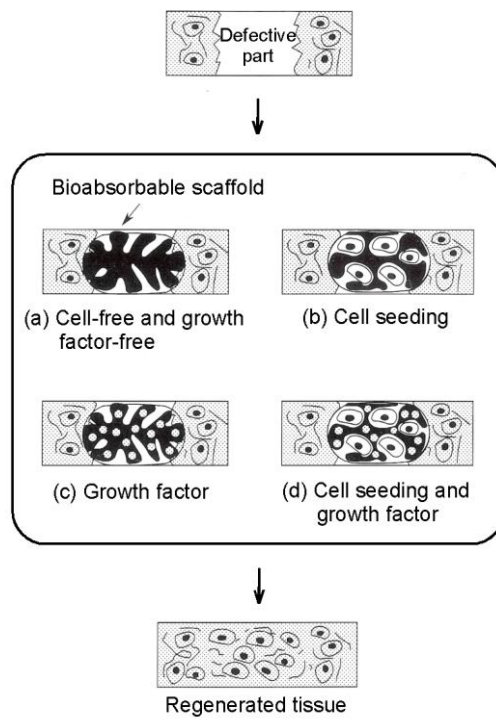Figure 4 Type of degradation of biodegradable materials. [33]

Figure 5 Tissue regeneration supported by scaffolds [33].

a. Diffusion control system
b. Biodegradation control system
c. Interaction control system
d. Osmotic pressure control system
e. Gel swelling-shrinking control system
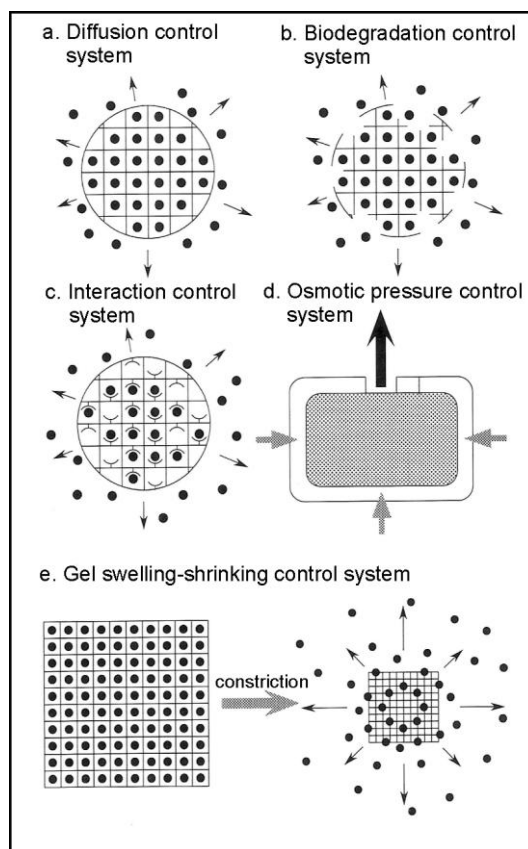
constriction

Figure 6 Mechanism of drug release from organic polymers. [34]
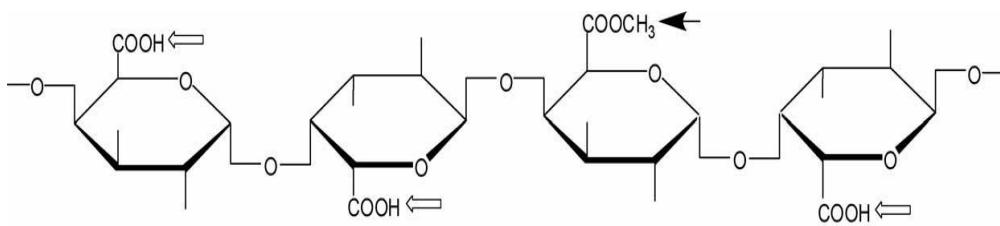
Figure 7 Chemical structure of pectin. The close arrow indicates the esterified carboxyl group, and the open arrow indicates carboxyl sites able to be esterified [49].
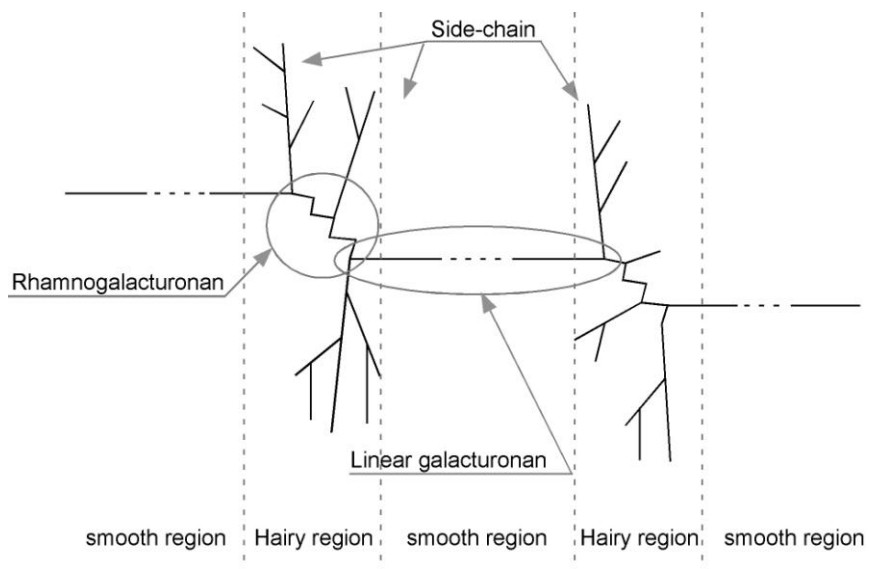
Figure 8. Schematic representation of pectin backbone showing the "hairy" regions (rhamnogalacturonan and side chains) and "smooth" regions (linear galacturonan) [52].

Table 1 Ion concentrations of human blood plasma and SBF.

Ion	Concentration / mM	
	Human blood plasma	SBF
Na^+	142.0	142.0
K^+	5.0	5.0
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^-	103.0	147.8
HCO_3^{3-}	27.0	4.2
HPO_4^{2-}	1.0	1.0
SO_4^{2-}	0.5	0.5
pH	7.20-7.40	7.40

Chapter 1

Evaluation of apatite-forming ability and mechanical property of pectin hydrogels

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

23

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].

Pectin is a natural polysaccharide which contains carboxyl groups as the functional groups, and it is included in most plants as a component of extracellular matrix and has high biological compatibility [20]. Its chemical structure is shown in Figure 1-1. In pectic acid, all of R takes a form of OH. In fact, neutral sugar side chains such as rhamnosyl residue accompanies into some parts of this structure [21,22]. Pectin is classified into low methoxyl pectin and high methoxyl pectin depending on amount of methoxyl groups. Namely, amount of carboxyl group is decreased with increase in amount of methoxyl groups. It is known that low methoxyl pectin make a hydrogel by forming so-called egg-box structure in which polymer chain of pectin is layered via cross-linkage by Ca^{2+} ions, independent of pH [23]. The author prepared pectin hydrogels from different low methoxyl pectins such as pectic acid, apple-derived pectin and citrus-derived pectin, by treatment with $CaCl_2$ solution. The apatite-forming ability of the prepared gels was examined in SBF. In addition, mechanical properties of the pectin gels were also quantitatively evaluated by tensile test.

2. Experimental procedure

2.1. Measurement of degree of esterification and weight-average molecular weight

We measured degree of esterification (DE) to determine amount of COOH group in accordance to the literature by Doesburg *et al* [24]. Chemical reagents of apple-derived pectin

((C$_6$H$_8$O$_6$, C$_7$H$_{10}$O$_6$)$_n$, Wako Pure Chemical Industries, Ltd. Inc., Japan) and citrus-derived pectin ((C$_6$H$_8$O$_6$, C$_7$H$_{10}$O$_6$)$_n$, Wako Pure Chemical Industries, Ltd. Inc., Japan) were dissolved in 20 cm^3 of ultrapure water to form aqueous solutions of 5 mass% at 80 °C. The obtained solutions were titrated with 0.03 M (=kmol·m^{-3})-NaOH aqueous solutions using phenolphthalein indicator and then 5 cm^3 of 0.1 M-NaOH aqueous solutions were added to the solutions. After keeping the solutions overnight, an excess of 0.2 M-H$_2$SO$_4$ aqueous solutions of were added and the solutions were back-titrated with 0.03 M-NaOH aqueous solutions. DE was calculated by the following equation:

$$DE\ (\%) = 100\ (b + L - A) / (a + b + L - A)$$

where a is milliequivalent of NaOH in 0.03 M-NaOH for the first titration, L milliequivalent of NaOH in 0.1 M-NaOH, A milliequivalent of H$_2$SO$_4$ in 0.2 M-H$_2$SO$_4$ and b milliequivalent of NaOH in 0.03 M-NaOH for the back titration. At least 5 solutions were titrated for each composition, and average DE and standard deviation were calculated.

Viscosity-average molecular weight (M$_v$) of pectins was measured by the viscosity technique in accordance to the literature by Owens *et al* [25]. The foregoing two kinds of pectin and polygalacturonic acid (Pectic acid, (C$_6$H$_8$O$_6$)$_n$, Nacalai Tesque Inc., Kyoto, Japan) were dissolved in 0.155 M sodium chloride solutions at 80 °C. Relative viscosity (η_r) of pectin solution (0.1-0.4 g/100ml) was measured by B-type viscometer (BL-type, TOKYO KEIKI Co., Ltd., Japan) at 25°C and specific viscosity ($\eta_{sp} = \eta_r - 1$) was calculated. Pectin concentration (C) and reduced viscosity (η_{sp} / C) were plotted on the horizontal axis and the vertical axis, respectively, and reduced viscosity in 0 g/100ml, namely intrinsic viscosity ([η]) was determined by extrapolation. M$_v$ determined from intrinsic viscosity data according to the equation [η] = 1.4 x 10^{-6} M$_v^{1.34}$. Results presented are the average of at least three separate determinations

2.2. Preparation of pectin gels

The foregoing two kinds of pectin and Pectic acid were dissolved in ultrapure water and 0.2 M-NaOH at 80 °C, respectively. The obtained pectin solutions of 10 mass% were cast in polystylene mold 95 x 60 mm^2 in size, and equivolume $CaCl_2$ (Nacalai Tesque Inc., Kyoto, Japan) aqueous solutions of 0.5 or 1 M were poured gently onto the pectin solutions. After keeping 24 hours at 4 °C, remained $CaCl_2$ solutions were removed from the gels. The obtained hydrogels were rinsed with ultrapure water and dried at 40 °C for 3 days. The examined compositions of the pectin gels were summarized in Table 1-1.

2.3. Evaluation of apatite-forming ability

After a bulk gels were obtained, rectangular specimens 10 x 10 mm^2 in size were cut from the gels and were 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) nearly equal to those of human blood plasma at pH 7.40 at 36.5 °C for various periods within 7 days [11]. After the soaking, the specimens were taken out from SBF, immersed in ultrapure water for 24 hours to remove excess water-soluble salts in the gels, and dried at 40 °C for 24 hours. The surface structural changes of the specimens were characterized by using scanning electron microscope (SEM; Model S-3500N, Hitachi Co., Japan), energy dispersive X-ray analyzer (EDX; Model EX-400, HORIBA Co., Japan) and thin-film X-ray diffractometer (TF-XRD; MXP3V, Mac Science Ltd., Japan).

2.4. Measurement of changes in Ca concentration pH in buffer solutions

The changes in Ca^{2+} concentrations and pH were measured by soaking of the pectin hydrogels in 30 cm^3 of Tris-NaCl buffer solutions. Tris-NaCl contained 142 mM of NaCl and 50 mM of tris(hydroxymethyl)aminomethane and was buffered at pH=7.40 by appropriate amount of HCl. Ca^{2+} concentrations and pH were measured by using calcium ion electrode (Model #6583-10C,

HORIBA Co., Japan) and pH electrode (Model #9621-10D, HORIBA Co., Japan), respectively.

2.5. Evaluation of mechanical properties of pectin gels

Tensile strength of pectin gels was measured according to Japanese Industrial Standards (JIS) K 7127. Each pectin solution of 3 cm^3 was filled into a Teflon$^®$ mold with dumbbell shape as shown in Figure 1-2 and gelled by CaCl$_2$ solutions. After aging at 4 °C for 24 hours, the formed gels were dried at 40 °C for 3 days. They were then subjected to tensile test by using Instron-type testing machine (Model AG-I, Shimadzu Co., Japan) at a cross-head speed of 5.0 mm/min. The tensile stress was calculated from the load at fracture and geometrical area of cross section of the specimens. At least 5 specimens were tested for each composition, and average stress and standard deviation were calculated.

3. Results

3.1. Apatite-forming ability

The degree of esterification of apple-derived pectin and citrus-derived pectin was calculated to be 41.7 ± 0.3 % and 32.0 ± 0.5 %, respectively. This means that amount of carboxyl group increases in the order (apple-derived pectin) < (citrus-derived pectin) < (pectic acid). The viscosity-average molecular weight of pectic acid, apple-derived pectin and citrus-derived pectin was calculated to be 1.51 x 10^4, 3.53 x 10^4 and 5.48 x 10^4, respectively. Namely, molecular weight increases in the order (pectic acid) < (apple-derived pectin) < (citrus-derived pectin). Figure 1-3 shows appearance of the pectin gels after dried at 40°C for 3 days. Crack-free bulk gels were obtained for all the compositions after the gelation.

Figure 1-4 shows SEM photographs of the surfaces of the pectin gels after soaking in SBF for various periods. Deposition of spherical fine particles was observed to cover whole the surfaces of Citrus-Ca1 after 3 days and Apple-Ca1 after 7 days, but not pectic acid. According to the EDX

analysis as shown Figure 1-5, peaks assigned to calcium and phosphorous were detected on the deposits observed under SEM. Amount of the deposits increased with increase in $CaCl_2$ concentration.

Figure 1-6 shows TF-XRD patterns of the surfaces of pectin gels after soaking in SBF for various periods. Broad peaks assigned to hydroxyapatite with low crystallinity were detected on the samples where the deposits covered whole the surfaces at 26° and 32° in 2θ.

Table 1-2 summarizes apatite-forming ability of the pectin gels. We can see that the apatite-forming ability increases in the order (pectic acid) < (apple-derived pectin) < (citrus-derived pectin). In addition, the apatite-forming ability enhances with increase in $CaCl_2$ concentration used for gelation of pectin.

3.2. Changes in Ca concentration and pH of in buffer solutions

Figure 1-7 shows changes in Ca concentration of Tris-NaCl buffer due to soaking of different pectin gels. The concentration increased within 3 days and then decreased for all the compositions. The degree of the initial increase in Ca concentration increased in the order (pectic acid) < (citrus-derived pectin) < (apple-derived pectin). Figure 1-8 shows changes in pH of Tris-NaCl buffer due to soaking of different pectin gels. Pectic acid and citrus-derived pectin showed initial increase in pH within 1 day and subsequent decrease, whereas apple-derived pectin showed monotonous decrease in pH.

3.3. Mechanical properties of pectin gels

The apple-derived and citrus-derived pectin gels showed so high flexibility as to be easily bended by hand. Figure 1-9 shows representative stress-strain curves of the gels in a dry condition. The tensile stress, strain and Young's modulus of the specimens in comparison with those of human cancellous bone are summarized in Table 1-3 [1]. The results showed that Young's modulus

increases in the order (apple-derived pectin) < (citrus-derived pectin) < (pectic acid). The gels prepared by treatment with 0.5 M-CaCl$_2$ showed Young's modulus nearly equal to that of human cancellous bone. Tensile stress showed tendency to decrease with increase in CaCl$_2$ concentration. In addition, mechanical strength of gels with wet condition was deteriorated so as not to be able to measure it.

4. Discussion

Homogeneous bulk gels were obtained from pectin solutions by CaCl$_2$ treatment. This might be attributed to cross-linking of carboxyl groups in pectin with Ca^{2+}. Because, it is known that low methoxyl pectin make a hydrogel by forming so-called egg-box structure in which polymer chain of pectin is layered via cross-linkage by Ca^{2+} ions as shown Figure 1-10 [23].

The results of SEM observation and TF-XRD show that the apple- and citrus-derived pectin gels form the apatite on their surfaces after soaking in SBF within 7 days and the formed apatite is low-crystalline hydroxyapatite similar to bone apatite. Although Citrus-Ca05 formed deposits on their surface in SBF but gave no clear XRD patterns characteristic of the apatite. It is assumed that the formed fine particles are not the apatite but the precursor of the apatite such as amorphous calcium phosphate, or that the amount of the deposit is too low to be detected by TF-XRD. It is apparent from the results seen in Table 1-2, apatite-forming ability increases in the order (apple-derived pectin) < (citrus-derived pectin) and enhances with increase in CaCl$_2$ concentration used for gelation of pectin. This results show apatite formation on the pectin gel is increase by amount of carboxyl groups in the gel. In addition, amount of carboxyl groups in the gel is more dominant than the amount of Ca^{2+} elution from the gel, due to apple-derived pectin gel has lower apatite-forming ability than citrus-derived pectin gel despite the former elutes much more calcium ions than the later as shown Figure 1-6. Takadama *et al.* reported that silanol groups provide a nucleation site for hydroxyapatite formed from amorphous calcium silicate compounds

comprising $\equiv SiOCa^+$ and $(\equiv SiO)_2Ca$, by bond of calcium ions before deposition of the hydroxyapatite [26]. Therefore, carboxyl groups might well be able to form $-COOCa^+$ and $(-COO)_2Ca$ complexes on the surface of pectin hydrogel and elution of Ca^{2+} ions from the gel may increase the number of these complexes as shown Figure 1-11.

However, Pectic acid did not form the apatite in SBF, although it contained the largest amount of carboxyl group among the pectins examined in this study. The pectic acid showed the smallest Ca^{2+} release and the largest pH reduction in Tris-NaCl solutions (See Figures 1-6 and 1-7). Ionic activity product (IP) of a solution for hydroxyapatite can be described as the following equation:

$$IP = \gamma^{10}{}_{Ca^{2+}} \gamma^6{}_{PO_4^{3-}} \gamma^2{}_{OH^-} \left[Ca^{2+}\right]^{10} \left[PO_4^{3-}\right]^6 \left[OH^-\right]^2 \tag{1}$$

Here, γ_i and [i] are the activity coefficient and actual ion concentration of i, respectively. Large amount of carboxyl group in pectic acid makes tight cross-linking with Ca^{2+} via formation of egg-box structure, leading to suppress Ca^{2+} release from the gels. In addition, high acidity of pectic acid significantly lowers pH of the surrounding solution. These effects would suppress increase in IP of the surrounding solutions for hydroxyapatite. These results suggest that too much carboxyl groups in pectin gels rather reduce the apatite-forming ability in SBF. Therefore, in the polysaccharides form egg box structure with Ca^{2+} as well as pectin (for example, alginic acid), excessive carboxyl groups might control the apatite formation on those gels.

The obtained pectin hydrogels possess Young's modulus similar to natural bone, although tensile strength is a little lower. The gel of pectic acid ($M_v = 1.51 \times 10^4$) with the smallest molecular weight in the three kinds of pectin showed the highest Young's modulus, although citrus-derived pectin ($M_v = 5.48 \times 10^4$) with the highest molecular weight showed higher Young's modulus than apple-derived pectin ($M_v = 3.53 \times 10^4$) with middle molecular weight. Therefore, it is revealed that

Young's modulus of the pectin gel depends on not only the molecular weight but also the amount of the carboxyl groups. This cause is that the polymer chain of pectin was regularly arranged in laminas so that there is much quantity of the carboxyl group in it due to the pectin makes a hydrogel by forming so-called egg-box structure in which polymer chain of pectin is layered via cross-linkage by Ca^{2+} ions as shown Figure 1-10.

In addition, although molecular weight of pectic acid was the lowest in three kinds of pectin, the pectic acid gel showed tensile strength equal to apple-derived pectin after 0.5 M-$CaCl_2$ treatment and citrus-derived pectin after 1 M-$CaCl_2$ treatment, respectively. It is thought that cross-linking of the pectic acid gel was so strong since it has the most carboxyl groups in three kinds of pectin. Generally, mechanical strength and Young's modulus of the polymeric material increase so that molecular weight increases. Therefore, it is assumed that pectin with highly molecular weight and lowly degree of esterification should be selected to improve mechanical property of gels. However, it is necessary to be careful so that degree of esterification of the pectin is not too low in the preparation, since the gels with low degree of esterification showed low apatite-forming ability. It is expect that the increase in $CaCl_2$ concentration for the gelation would enhance the cross-linking of the pectin to reinforce the hydrogel. In spite of that, the results in this study indicated that the tensile strength of the gels showed tendency to decrease with increase in $CaCl_2$ concentration. $CaCl_2$ is known as a highly hygroscopic substance. It is therefore considered that the specimens containing a lot of $CaCl_2$ easily adsorb water in air, leading to the decrease in tensile strength. On the other hand, the apatite-forming ability of the gels showed a tendency to reduce so that the $CaCl_2$ concentrations were low. Consequently, it was suggested that pectin gel with highly molecular weight and moderate degree of esterification should be cross-linked with lowly hygroscopic calcium salt to improve mechanical strength of the gel without losing apatite-forming ability. In addition, the mechanical strength of gels was too poor to measure under wet condition. Therefore, it is necessary to improve mechanical strength of pectin hydrogels.

5. Conclusions

The author examined apatite-forming ability in SBF and mechanical properties of pectin hydrogels with different compositions prepared by cross-linking with Ca^{2+}. Apple- and citrus-derived pectin gels formed the apatite in SBF, but not pectic acid gel. It was suggested that the apatite-forming ability of the pectin gels is governed by not only the amount of carboxyl groups but also changes in Ca^{2+} concentration and pH in surrounding solutions. Young's modulus of the pectin gels was similar to that of natural bone, although tensile strength was a little lower. Therefore, it is necessary that these mechanical properties of pectin hydrogels were improved to use it as bone substitute.

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R = OCH$_3$, OH
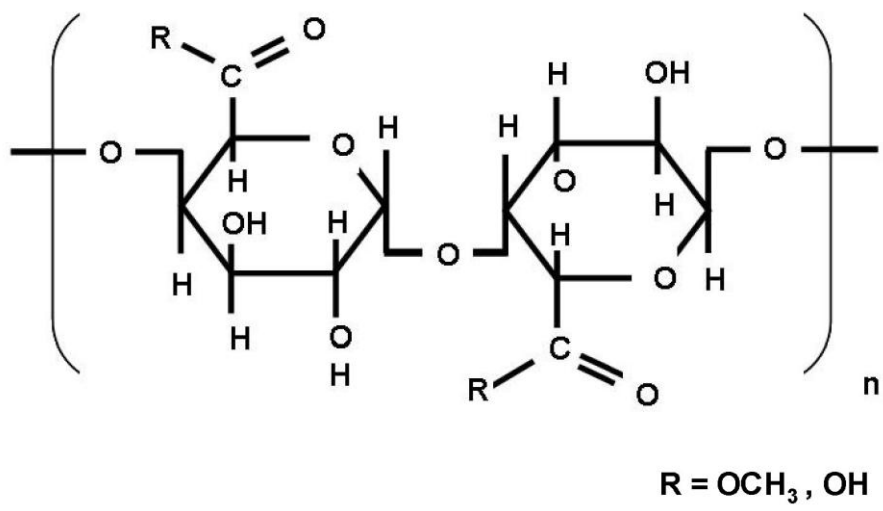
Figure 1-1. Chemical structure of pectin.
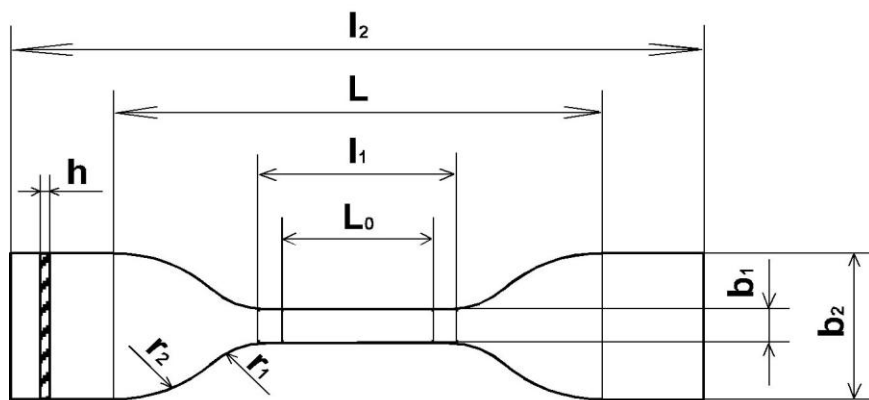
b₁ Width of the narrow parallel part : 6 mm ± 0.4 mm
b₂ width of both ends : 25 mm ± 1 mm
h Thickness : ≦ 1 mm
L₀ Length between the gauge lines : 25 mm ± 0.25 mm
l₁ Length of the narrow parallel part : 33 mm ± 2 mm
L Initial length between the chuck parts : 80 mm ± 5 mm
l₂ Total length : ≧ 115 mm
r₁ Minimum radius : 14 mm ± 1 mm
r₁ Maximum radius : 25 mm ± 2 mm

Figure 1-2. The configuration of the specimens for tensile test.

37
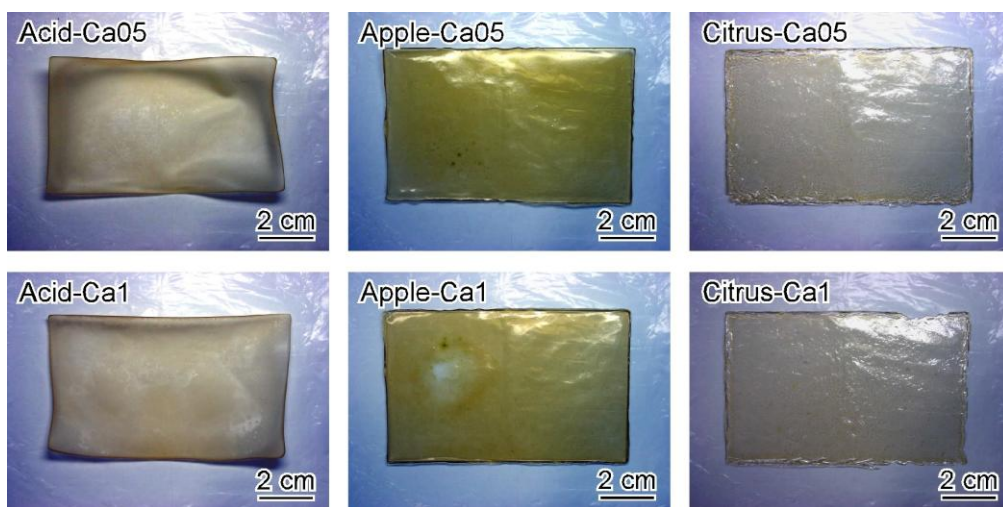
Figure 1-3. Appearance of pectin gels after dried at 40°C for 3 days.

Acid-Ca05 **Apple-Ca05** **Citrus-Ca05**

Acid-Ca1 **Apple-Ca1** **Citrus-Ca1**
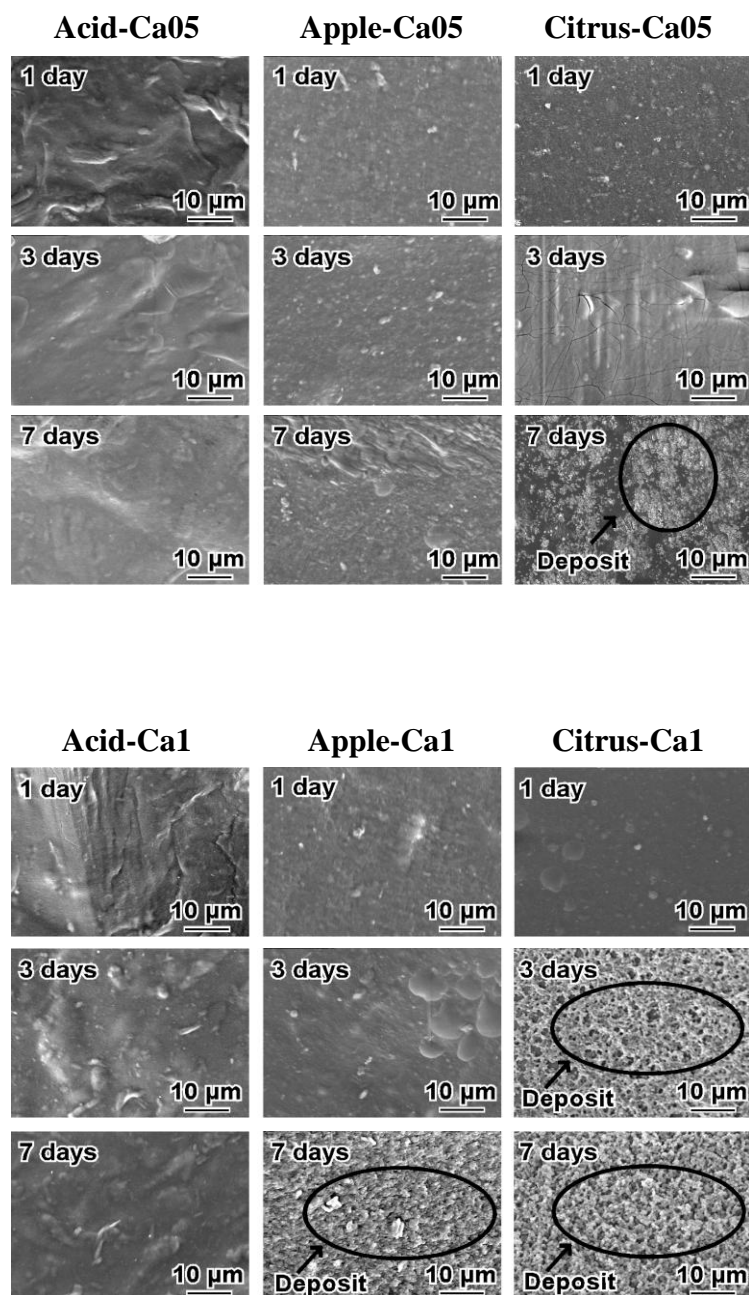
Figure 1-4. SEM photographs of the surfaces of pectin gels prepared by $CaCl_2$ treatment after soaking in SBF for various periods.
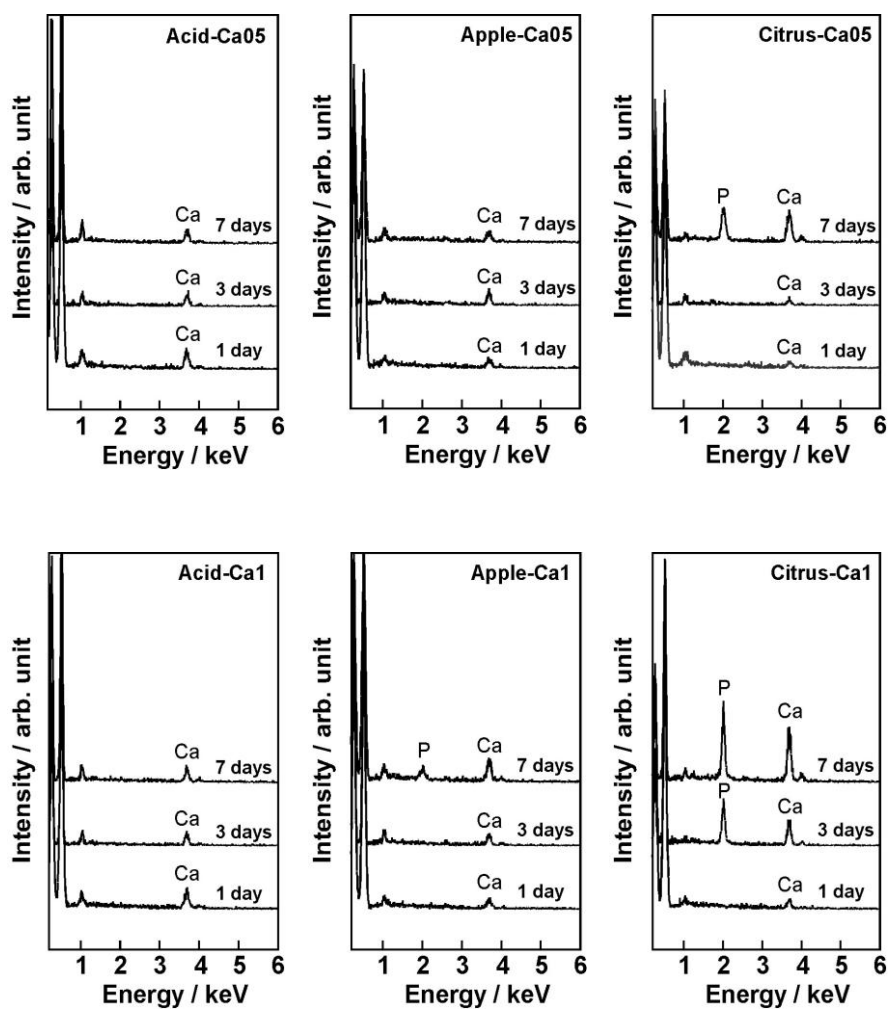
39

Figure 1-5. EDX spectra of the surfaces of pectin gels prepared by $CaCl_2$ treatment after soaking in SBF for various periods.
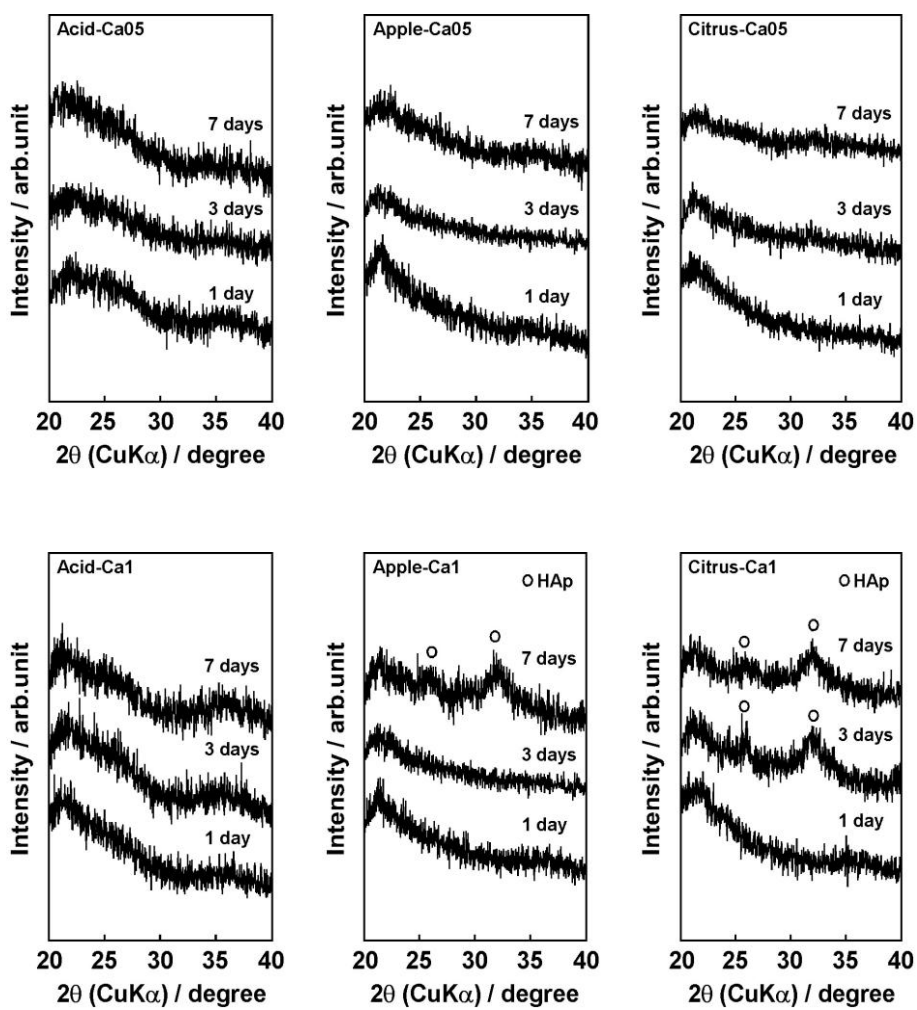
Figure 1-6. TF-XRD patterns of the surfaces of pectin gels prepared by CaCl$_2$ treatment after soaking in SBF for various periods (HAp: hydroxyapatite).
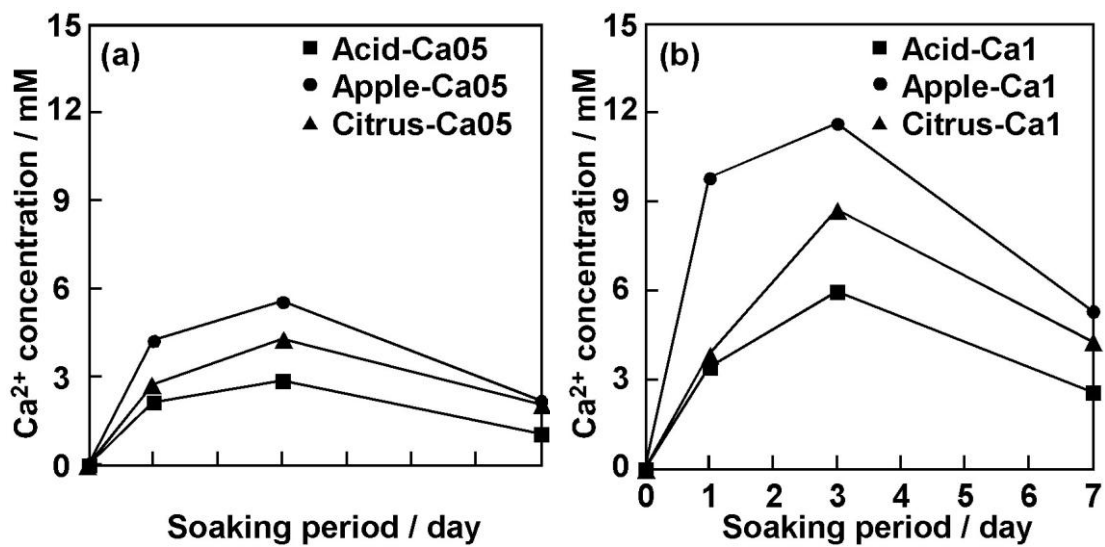
Figure 1-7. Changes of Ca^{2+} concentration in Tris-NaCl buffer due to soaking of various pectin gels treated with $CaCl_2$ solutions of (a) 0.5 M or (b) 1 M.
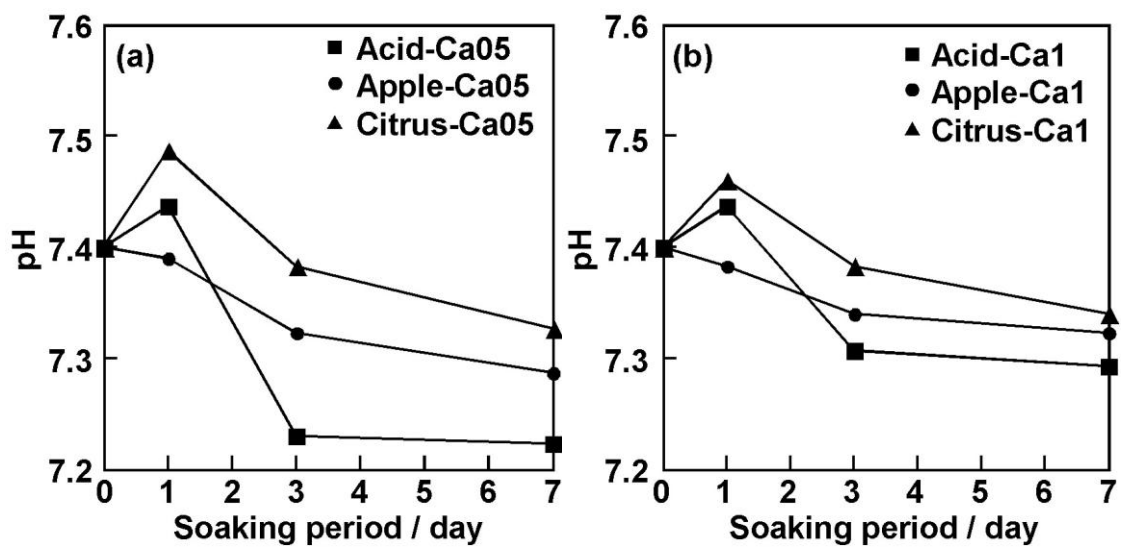
Figure 1-8. Changes of pH in Tris-NaCl buffer due to soaking of various pectin gels treated with CaCl$_2$ solutions of (a) 0.5 M or (b) 1 M.
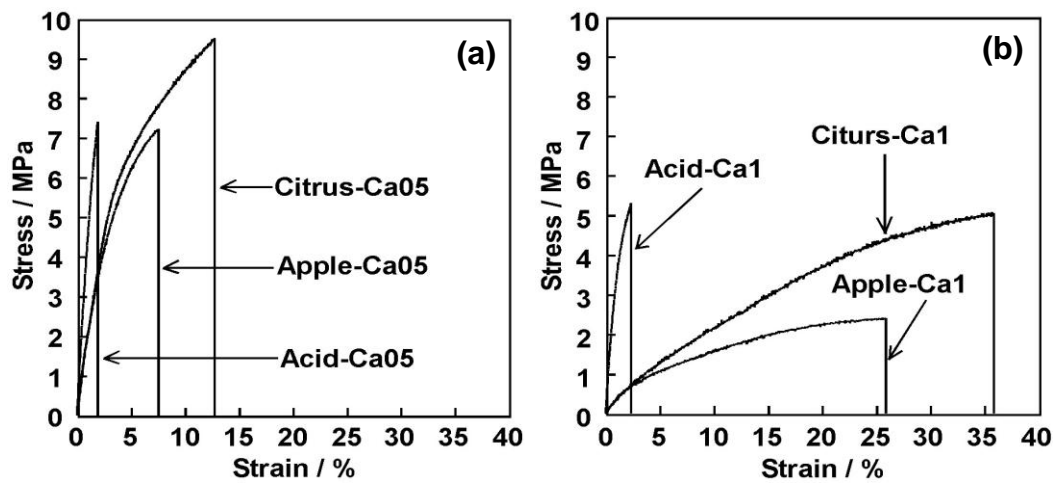
Figure 1-9. Stress-strain curves of pectin gels treated with

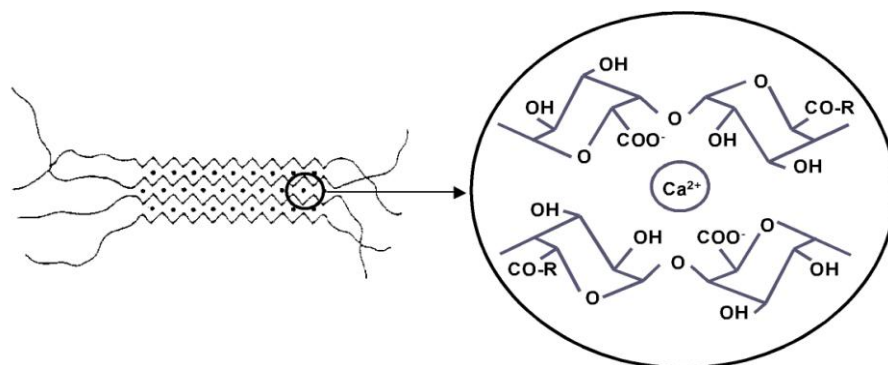CaCl$_2$ solutions of (a) 0.5 M or (b) 1 M.

Figure 1-10. Structure of Cross-linkage with Ca^{2+}.
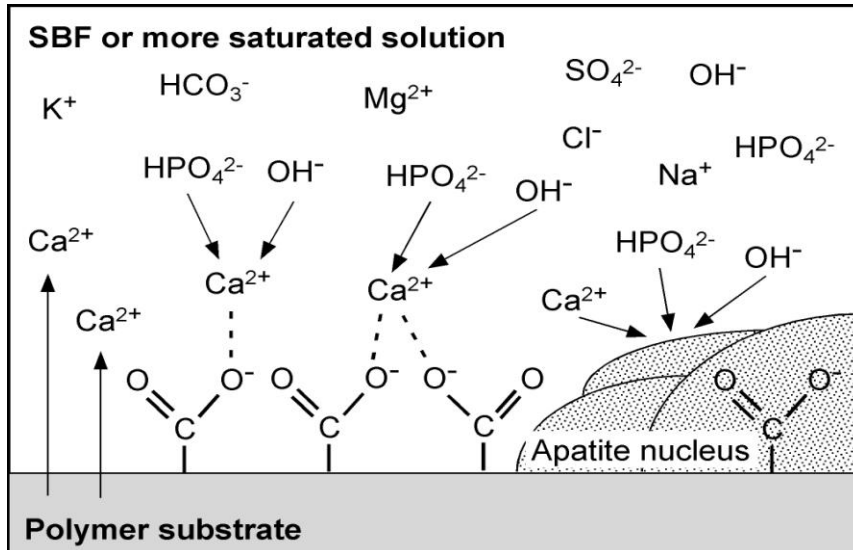
Figure 1-11. Mechanism of hydroxyapatite formation on surface of pectin gels in body environment.

Table 1-1. Concentrations of pectin gels.

Sample	Kind of pectin	Pectin concentration / mass%	CaCl$_2$ concentration / M
Acid-Ca05	Pectic acid	10	0.5
Acid-Ca1			1
Apple-Ca05	Apple-derived	10	0.5
Apple-Ca1			1
Citrus-Ca05	Citrus-derived	10	0.5
Citrus-Ca1			1

Table 1-2. Apatite-forming ability of pectin gels after soaking in SBF for various periods.

Sample	Apatite-formation		
	1 day	3 days	7 days
Acid-Ca05	x	x	x
Acid-Ca1	x	x	x
Apple-Ca05	x	x	x
Apple-Ca1	x	x	o
Citrus-Ca05	x	x	△
Citrus-Ca1	x	o	o

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

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

x : No deposit was formed.

Table 1-3. Mechanical properties of pectin gels in comparison with those of human cancellous bone

Sample	Stress / MPa	Strain / %	Young's modulus / MPa
Acid-Ca05	7.4 ± 1.7	1.9 ± 0.3	417.8 ± 73.7
Acid-Ca1	5.3 ± 0.8	2.4 ± 0.2	402.5 ± 53.7
Apple-Ca05	7.2 ± 2.7	7.5 ± 1.9	202.2 ± 61.9
Apple-Ca1	2.4 ± 0.4	25.9 ± 5.5	26.3 ± 10.9
Citrus-Ca05	9.5 ± 1.4	12.7 ± 3.6	202.1 ± 21.6
Citrus-Ca1	5.1 ± 0.3	35.9 ± 14.7	32.3 ± 8.9
Cancellous bone	10-20	5-7	50-500

Chapter 2

Apatite-forming abilities and mechanical properties of covalently cross-linked pectin hydrogels

1. Introduction

Natural bone has features such as high fracture toughness and bone-bonding bioactivity, is organic-inorganic hybrid composed of collagen and apatite crystals [1]. Therefore, apatite-polymer hybrids designed to mimic the structure of bone represent candidates for high-performance bone substitutes. Biomimetic process has been paid much attention where bone-like apatite layer is deposited on organic polymers in simulated body fluid (SBF, Kokubo solution) with ion concentrations nearly equal to those of human blood plasma or more concentrated solutions [2,3]. Nucleation of the apatite is induced both by dissolution of calcium ions from the materials, and by specific functional groups such as carboxyl group (COOH) [4,5].

In Chapter 1, pectin-apatite hybrid gels were prepared by ionic cross-linking with Ca^{2+}. However, mechanical properties of those differed from human cancellous bone. Therefore, it was expected that these properties might be improved by covalent cross-linking. Covalent cross-linking with divinylsulfone (DVS; $CH_2=CHSO_2CH=CH_2$) is popularly used for preparation of polysaccharide hydrogels. Hyaluronan hydrogel cross-linked with divinylsulfone (DVS) are widely used to relieve joint pain and as a tissue spacer without using surgical adhesions [6-9]. Matsumoto *et al.* reported that DVS cross-linked hyaluronan hydrogel treated with $CaCl_2$ are formed hydroxyapatite on there surface in SBF and this apatite mineralized hyaluronan hydrogel has non cytotoxicity.

It was shown 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, in

Chapter 1. It is expected that process of the cross-linking would affect properties of the pectin hydrogels. The author 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. Analysis of cross-linking mechanism using model compounds

Propylene glycol (PG) was dispersed in 30 cm^3 of 0.2 M (=kmol·m^{-3}) NaOH to form 10 mass% solution. DVS was then added to PG solution at 5 mass% to the PG. After keeping 24 hours at 4°C, the obtained solutions were cast in polystyrene molds of 95×60 mm^2 in size, and dried at 60°C for 3 days. The specimens were pulverized and mixed with KBr powder in a mass ratio of 1 : 100. A thin film was prepared by uniaxially pressing the mixed powder, which was then subjected to the measurement by using Fourier-transform infrared (FT-IR) spectroscopy (Janssen FT-IR Microscope, JASCO, Japan).

2.2. 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. Powdered pectin preparations were dissolved in 0.2 M NaOH (pectic acid) or ultrapure water (apple- and citrus-derived pectins) to form 10 mass% solutions at 80°C. In Chapter 1, the degrees of esterification and viscosity-average molecular weight of pectic acid, apple-derived pectin and citrus-derived pectin were shown to be 0%, 41.7±0.3% and 32.0±0.5% and 1.51 x 10^4, 3.53 x 10^4 and 5.48 x 10^4, respectively. DVS was added to 15 cm^3 of pectin solutions at 5 mass% relative to the pectin. After keeping 24 hours at 4°C, the obtained

solutions were cast in polystyrene molds of 95×60 mm^2 in size, and dried at 40°C for 3 days. The compositions of the pectin gels are summarized in Table 2-1.

2.3. 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 M-CaCl$_2$ (Nacalai Tesque Inc., Kyoto, Japan) 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 mM) at pH 7.40 at 36.5°C for various periods up to 7 days [10]. 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.

2.4. Analysis of pectin gels

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 surface potential of the pectin gels with DVS or Ca^{2+} cross-linking after soaking in SBF was analyzed in terms of zeta potential, which was measured using a laser electrophoresis spectroscopy (Model ELSZ-2, Otsuka Electronics Co., Osaka, Japan). After they were removed from the SBF, the pectin gels (33 x 14 mm^2) 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 surface of

pectin gels. 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. At each condition of immersion time, the zeta potential was measured at least three times to present an average value with standard deviation.

Changes in the Ca^{2+} concentrations and pH were measured by soaking of $CaCl_2$ treated hydrogels of 10×10 mm^2 in size in 30 cm^3 of Tris-NaCl buffer solution. The Tris-NaCl solution contained 142 mM NaCl and 50 mM 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.

2.5. 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. Analysis of cross-linking mechanism using model compounds

Figure 2-1 shows FT-IR spectra of the PG before and after cross-linking with DVS. In PG, several peaks assigned to hydroxyl group (-OH; around 1050 and 3400 cm^{-1}), the methyl group (around 800-1200, 1380 and 2900 cm^{-1}) and the methylene group (-CH$_2$-; around 1450 and 2900 cm^{-1}). In the PG cross-linked with DVS (PG-DVS), peaks assigned to ether bond (-C-O-C-) and sulfonyl group (-C-SO$_2$-C-) were observed at 1080-1150 and 1300-1340 cm^{-1}, respectively. In addition, peaks of the hydroxyl group around 1050 and 3400 cm^{-1} were decreased, whereas those of the methylene group around 1450 cm^{-1} were increased in comparison with PG. This means that hydroxyl groups of PG were cross-linked by DVS. On the basis of the results using model compounds, it is suggested that the hydroxyl groups of the pectin were also cross-linked by DVS.

3.2. Apatite-forming abilities

Figure 2-2 shows appearance of the pectin gels after dried at 40°C for 3 days. For all the compositions other than Acid-DVS, crack-free and flexible gels as to be easily bended by hand were obtained. Figure 2-3 shows SEM photographs of the surfaces of pectin gels after soaking in SBF for various periods. Deposition of fine spherical particles was observed on the predominantly surfaces of Apple-DVS and Citrus-DVS gels after 7 days, but not on pectic acid gels. Figure 2-4 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 predominantly surfaces at 26° and 32° in 2θ.

Figure 2-5 shows EDX spectra and changes in zeta potential of surface of pectin gels cross-linked with DVS and Ca^{2+} after soaking in SBF for various periods. According to the EDX analysis, the peaks assigned to calcium and chlorine were detected before soaking in SBF, and the calcium's peaks decreased and chlorine's peaks were disappeared after the soaking. Then the peaks assigned to calcium and phosphorous were detected on the deposits observed under the SEM. On the results of surface charge analysis, in DVS cross-linked gels, for all gels shown negative charge

before soaking, and decrease within 1 day, increase until 3 days and then to become constant (Acid-DVS) or increase (Apple-DVS and Citrus-DVS) after soaking. In Ca^{2+} cross-linked gels, for all gels shown positive charge before soaking, and decrease within 1 day, increase until 3 days and then to become constant for Acid-DVS and decrease for Apple-DVS and Citrus-DVS after the soaking.

Table 2-2 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+}.

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

Figure 2-6 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. Figure 2-7 shows the changes in pH of Tris-NaCl buffer due to soaking of different pectin gels. The pH of the gels decreased continuously.

3.4. Mechanical properties of pectin gels

Acid-DVS gels could not be subjected to tensile tests because they formed many cracks during drying. Figure 2-8 shows representative stress-strain curves of the remaining gels. Table 2-3 summarizes the tensile stress, strain and Young's modulus of the specimens in comparison with those of human cancellous bone [11]. DVS cross-linked gels showed higher tensile strength as molecular weight is high, although there is little difference in Young's modulus of gels. 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) as shown Chapter 1.

4. Discussion

After drying, Acid-DVS formed many cracks, though Apple-DVS and Citrus-DVS had become the homogeneous gel. It is known that DVS cross-linking is enhanced alkaline condition. Therefore, pectic acid gel would be cracked to inhibit cross-linking due to pectic acid sol had high acidity in comparison with other pectins.

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. In common with Chapter 1, pectic acid gel 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. 2-6 reduces the degree of supersaturation of the surrounding solution with respect to apatite.

The pectin gels cross-linked with DVS formed apatite with a comparable induction period to gels cross-linked with Ca^{2+}, although release of Ca^{2+} into the buffer solution was lower for the gels cross-linked with DVS (0.6-4 mM) than those cross-linked with Ca^{2+} (6-12 mM). According to changes in zeta potential of surfaces of pectin hydrogels, the surface charges of almost gels significantly decreased after soaking in SBF within 1 day and then increased. After the soaking for 1 day, the pectin hydrogels would reveal a negative surface charge by dissociation of carboxyl units in its molecular structure due to dissolution of the calcium salts that bound to the carboxyl groups.

Subsequently, the negatively charged carboxyl groups bind to the positively charged calcium ions in the fluid, thereby surface charge was increased by formation of -COOCa$^+$ and (-COO)$_2$Ca complexes after the soaking for 3 days. The specific functional group with induction ability of nucleation of the apatite has negative charge in body fluid environment, and it is supposed that this negative charge promotes uptake of Ca^{2+} and then uptake of PO$_4^{3-}$ and OH$^-$ and form a nucleus of the apatite [12,13]. Figure 2-9 shows schematic representation of apatite formation on the pectin gels. Therefore, this situation would arise because heterogeneous nucleation of apatite occurred on the gels cross-linked with DVS by enhancement of calcium ion incorporation into the surface, due to the surface negative charge of the gels cross-linked with DVS was higher than the gels cross-linked with Ca^{2+} as shown Figure 2-5. The difference of the surface charge of these two kinds of gel was thought to be due to the negative charge of the sulfonyl groups in DVS. These sulfonyl groups might play a role as a nucleation site of the apatite, too.

The gels cross-linked with DVS showed higher tensile strengths than those cross-linked with Ca^{2+}. The covalent cross-links formed would be stronger than the ionic cross-links formed by Ca^{2+}. A similar tendency was reported for alginate microcapsules prepared through ionic and covalent cross-linking procedures [15]. DVS creates cross-links with hydroxyl groups in pectin according to the following chemical equation [14]:

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

where R represents alkyl groups in the polysaccharides. This is supported by the results in Fig. 2-1 using model compounds that show formation of ether groups. Figure 2-10 shows schematic representation of cross-linking form with DVS or Ca^{2+}. Amount of the hydroxyl group as cross-linking site of DVS is larger than amount of the carboxyl group as cross-linking site of Ca^{2+} in the pectin molecular. It is thought that this is one of the main factors of improvement of mechanical

property of gels cross-linked with DVS. Moreover, 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 [16].

The tensile strength of pectin gels cross-linked with DVS depends on molecular weight as well as gels cross-linked with Ca^{2+}. It would be because degree of cross-linking of gels cross-linked with DVS is constant irrespective kind of pectin, since the amount of hydroxyl group which acts as cross-linking site of DVS have no influence on amount of the carboxyl group. Meanwhile, the Young's modulus did not depend on molecular weight unlike gels cross-linked with Ca^{2+}. This reason would be because cross-linking of gel of pectin with low amount of carboxyl group by DVS became weak due to it have low pH. In addition, 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 Young's moduli of pectin gels cross-linked with DVS were too higher than those of human cancellous bone. Therefore, the mechanical performances of the pectin gels have to be matched with those of human cancellous bone by the degrees of cross-linking are appropriately controlled with changing the kind and amount of the cross-linking agents. However, the mechanical strength of gels cross-linked with DVS was too poor to measure because the gels were swelled and dissolved in the solution as well as gels cross-linked with Ca^{2+}. It is thought that swelling and dissolution of the gel are inhibited if the apatite is immediately deposited on surface or inside of the gel after soaked in SBF, and it is desirable to select the pectin with high molecular weight to improve mechanical strength of the gel, because the tensile strength of DVS cross-linked

gel was higher as molecular weight of pectin is high. In addition, apatite deposition inside the pectin hydrogels will be necessary for application as scaffolds for tissue regeneration. Therefore, it will need to be promoted apatite–forming ability of pectin hydrogels by changing of cross-linking agent, introduction method of the calcium ion into gel or shape of the gel, and so on.

5. Conclusions

The author 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. Therefore, it was found that the mechanical strength of the pectin hydrogel could be improved without losing apatite-forming ability of it by using DVS as cross-linking agent.

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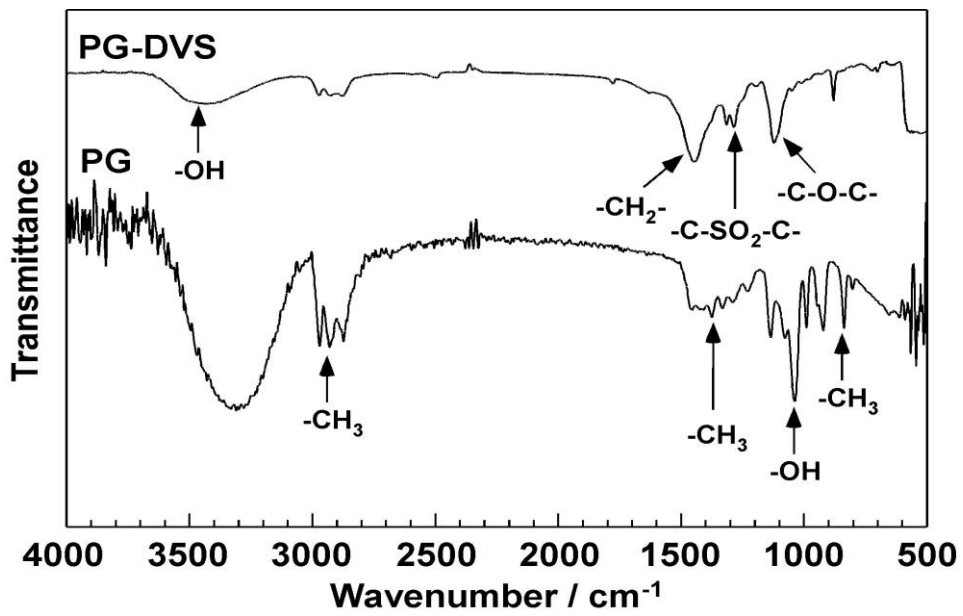
Figure 2-1. FT-IR spectra of the PG before and after cross-linked with DVS.
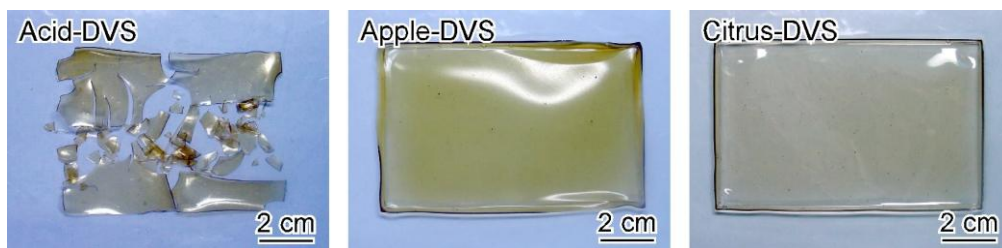
Figure 2-2. Appearance of pectin gels after dried at 40°C for 3 days.
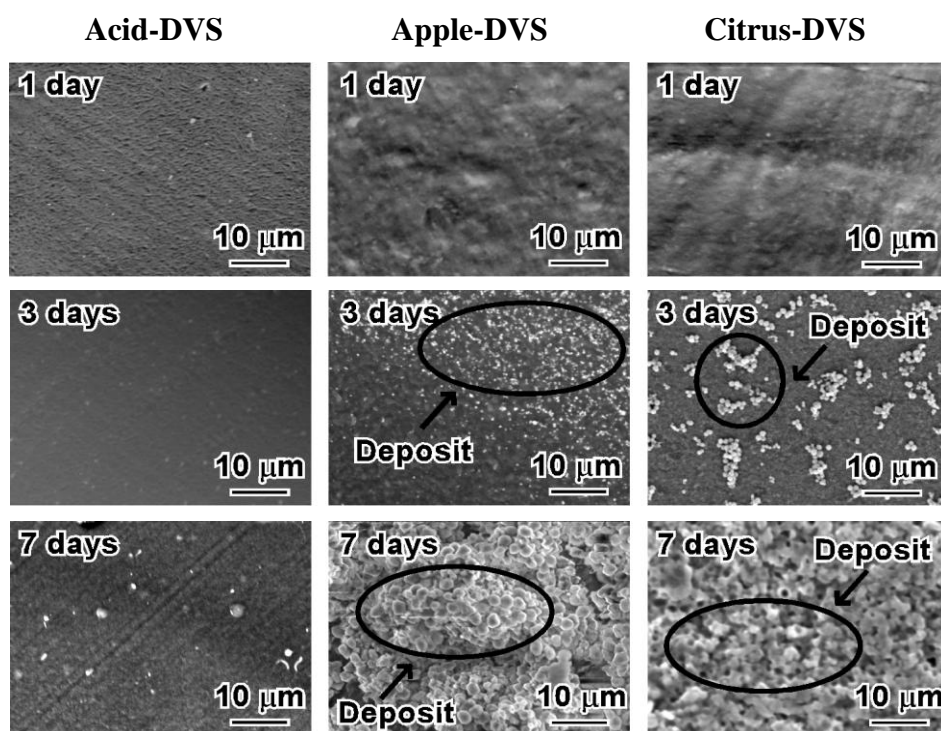
Figure 2-3. SEM photographs of the surfaces of pectin gels cross-linked with DVS after soaking in SBF for various periods.
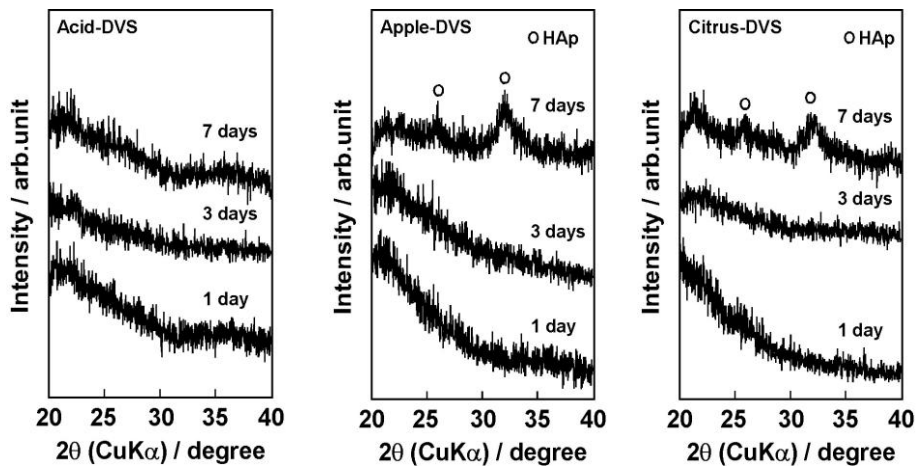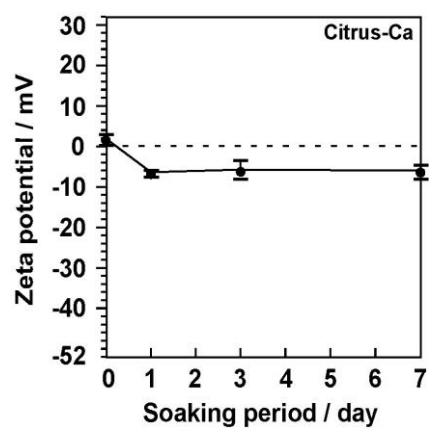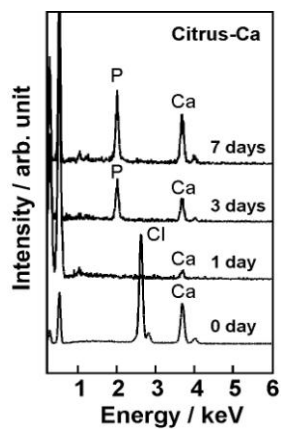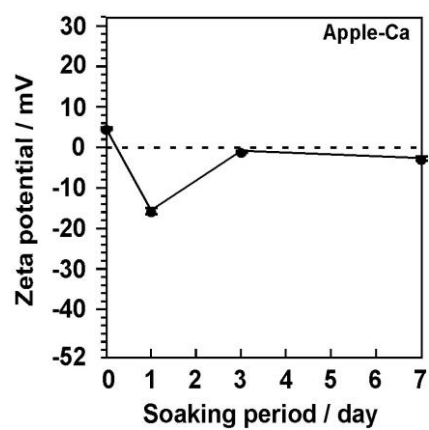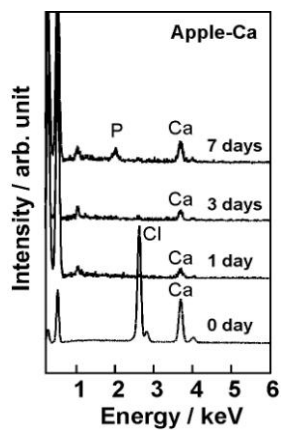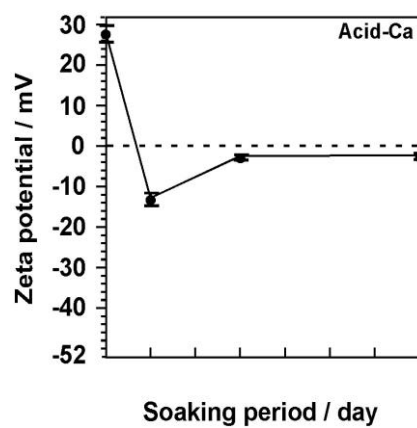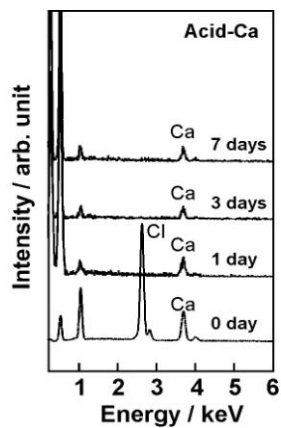
Figure 2-4. TF-XRD patterns of the surfaces of pectin gels cross-linked with DVS after soaking in SBF for various periods (HAp: hydroxyapatite).
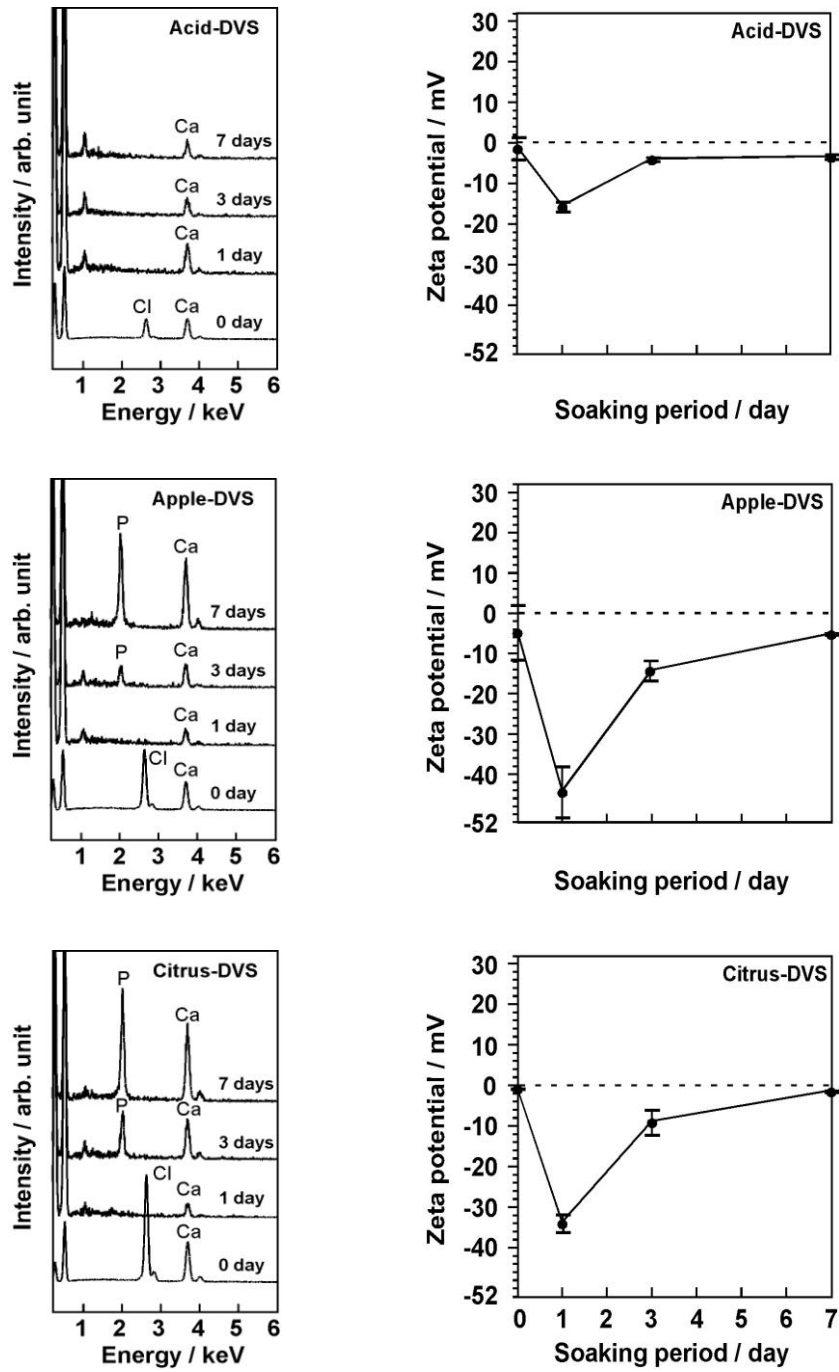
(a) Cross-linked with DVS

Figure 2-5. EDX spectra and changes in zeta potential of pectin gels cross-linked with (a) DVS and (b) Ca^{2+} after soaking in SBF for various periods.
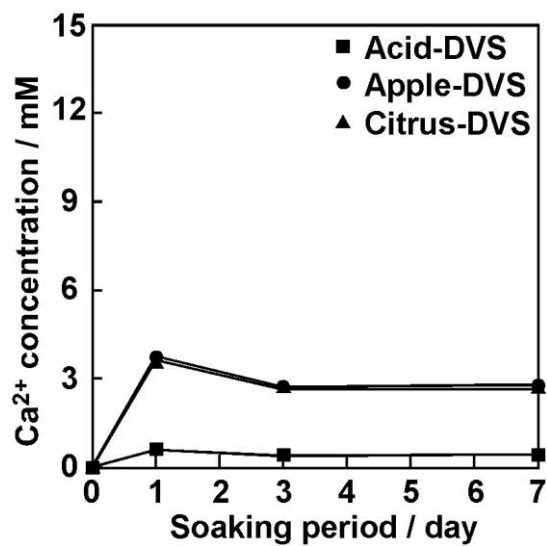
(b) Cross-linked with Ca^{2+}

Figure 2-5. (Continued)

Figure 2-6. Changes of Ca^{2+} concentration in Tris-NaCl due to soaking of pectin gels cross-linked with DVS.
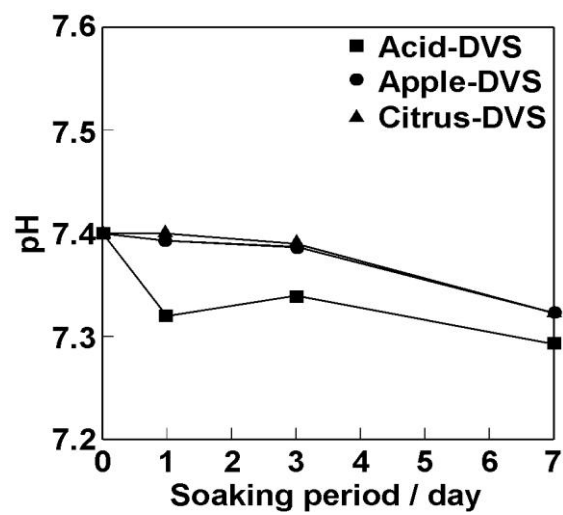
Figure 2-7. Changes of pH in Tris-NaCl due to soaking of pectin gels cross-linked with DVS.
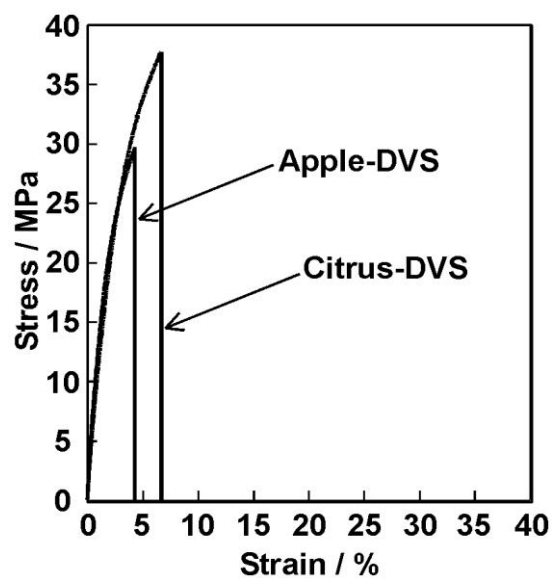
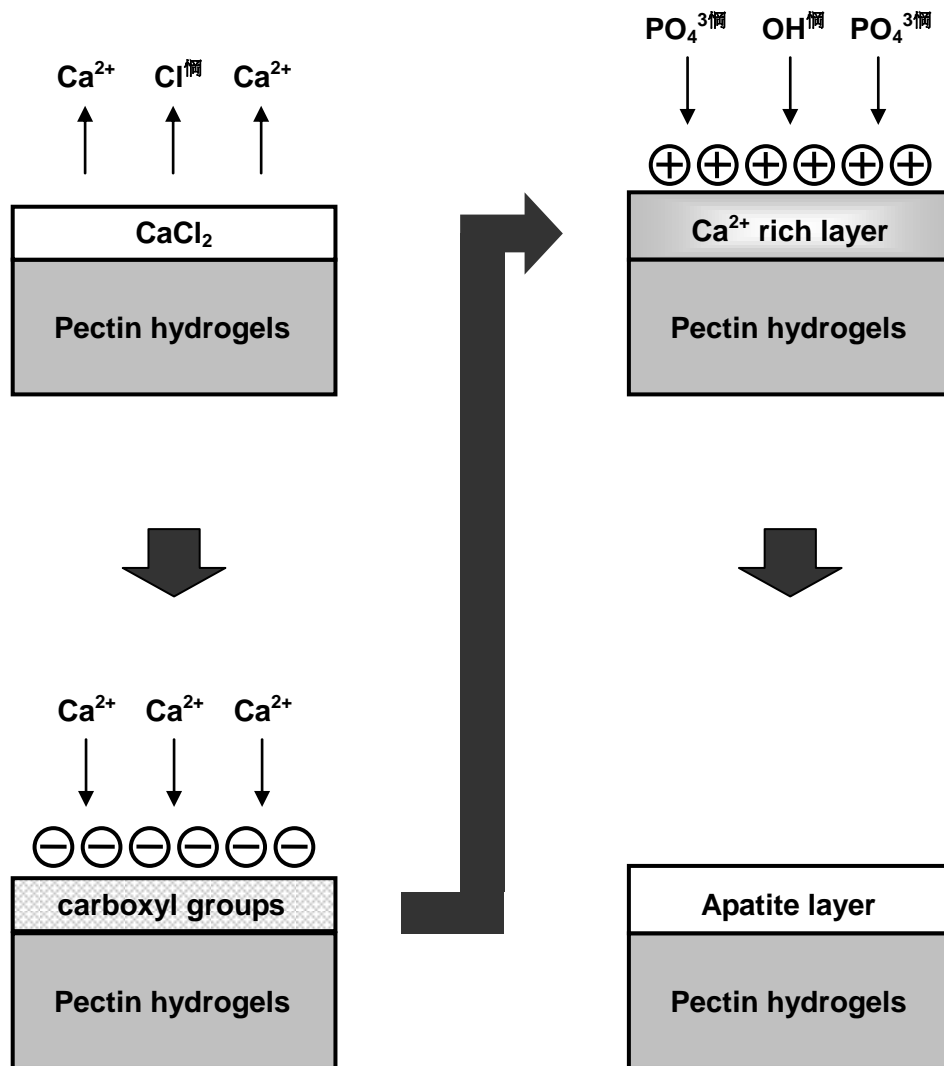**Figure 2-8.** Stress-strain curves of pectin gels cross-linked with DVS.

Ca²⁺ Cl⁻ Ca²⁺

| CaCl₂ |
| Pectin hydrogels |

PO₄³⁻ OH⁻ PO₄³⁻

⊕⊕⊕⊕⊕⊕

| Ca²⁺ rich layer |
| Pectin hydrogels |

Ca²⁺ Ca²⁺ Ca²⁺

⊖⊖⊖⊖⊖⊖

| carboxyl groups |
| Pectin hydrogels |

| Apatite layer |
| Pectin hydrogels |

Figure 2-9. Schematic representation of apatite formation
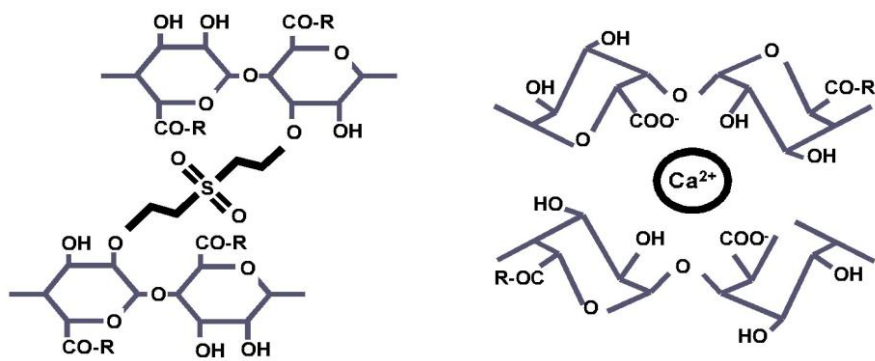
on the pectin gels.

Figure 2-10. Schematic representation of cross-linking form with DVS (left) or Ca^{2+} (right).

Table 2-1. Concentrations of pectin gels.

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

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

Sample	Apatite-formation		
	1 day	3 days	7 days
Acid-DVS	x	x	x
Apple-DVS	x	△	o
Citrus-DVS	x	△	o

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

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

x : No deposit was formed.

Table 2-3. 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

Chapter 3

Apatite-forming ability of pectin hydrogels obtained by different calcium salt treatments

1. Introduction

Apatite-polymer hybrids are expected as novel bone substitutes exhibiting bone-bonding ability and mechanical performances analogous to those of natural bone. As techniques to be deposited apatite on organic polymers, biomimetic processes have been developed by Kokubo and colleagues [1,2]. In this processes, heterogeneous nucleation of apatite is achieved on materials with specific functional groups on their surfaces in protein-free and acellular simulated body fluid (SBF; Kokubo solution) with inorganic ion concentrations nearly equal to human blood plasma. Recently, it has been reported that several organic polymers containing carboxyl (-COOH) groups form apatite on their surfaces in the body environment [3-6].

Pectin is a kind of plant-derived polysaccharide enriched with galacturonic acid and galacturonic acid methyl ester units, and disperses in water and forms the negatively-charged hydrocolloid [7]. It is known that low methoxyl pectin make a hydrogels by forming so-called egg-box structure in which polymer chain of pectin is layered via cross-linkage by Ca^{2+} ions, independent of pH and temperature [8].

In Chapter 1 and Chapter 2, it is showed that pectin hydrogels have potential to apatite formation on their surfaces in SBF by treatment of calcium chloride as water-soluble calcium salt. However, pectic acid gels did not show apatite formation though it have most carboxyl groups. Kind of calcium salts with different solubility and the introduction process of calcium ion into the gels were modified, due to apatite-forming ability of the pectin gel is assumed to be affected calcium dissolution behavior from the gel.

In this study, we prepared pectin hydrogels by mixing pectin solution and various water-soluble calcium salts including calcium acetate monohydrate ($Ca(CH_3COO)_2 \cdot H_2O$), calcium chloride ($CaCl_2$) and calcium hydroxide ($Ca(OH)_2$). Effects of the setting behavior and the apatite-forming ability of the pectin gels was examined in simulated body fluid (SBF). Pectic acid with the lowest apatite-forming ability and citrus-derived pectin with the highest one as shown Chapter 1 were used.

2. Experimental procedures

2.1. Preparation of pectin gels

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. Powdered pectin preparations were dissolved in 20 cm^3 of 0.2 M (=$kmol \cdot m^{-3}$) NaOH (pectic acid) or ultrapure water (citrus-derived pectin) to form 5 mass% solutions at 80°C. The degree of esterification of citrus-derived pectin and pectic acid were reported to be 32.0±0.5% and 0%, respectively, as shown in Chapter 1. The obtained solutions were mixed with three kinds of 0.2 mol calcium salts (calcium acetate monohydrate ($Ca(CH_3COO)_2 \cdot H_2O$, Wako Pure Chemical Industries Ltd. Inc., Japan), calcium chloride ($CaCl_2$, Nacalai Tesque Inc., Kyoto, Japan) and calcium hydroxide ($Ca(OH)_2$, Wako Pure Chemical Industries Ltd. Inc., Japan)) each on the PTFE dish by using PTFE spatula and crushed in a mortar. The obtained gels were cast in PTFE cylindrical molds of $\phi 8 \times 12$ mm^2 in size by using injector or directly filling with hands. The compositions of the pectin gels are summarized in Table 3-1.

2.2. Evaluation of apatite-forming abilities

After filling of the gels in the molds, molds filled with 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 mM) at pH 7.40 at 36.5°C for various periods up to 7 days. 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 3 days.

2.3. Analysis of pectin gels

The structural changes of contact surface of the specimens with SBF 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).

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

Changes in the Ca^{2+} concentrations and pH were measured by soaking molds filling with hydrogels in 30 cm^3 of Tris-NaCl buffer solution. The Tris-NaCl solution contained 142 mM NaCl and 50 mM 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. Degree of saturation for CaCO₃ in SBF after soaking of pectin gels

Changes in the Ca^{2+} concentrations and pH in 30 cm^3 of SBF after soaking molds filling with hydrogels were measured by using a calcium ion electrode and a pH electrode, respectively. Additionally, CO_3^{2-} concentrations were calculated from equilibrium constants for the following equations [9]:

$$HCO_3^- \Leftrightarrow H^+ + CO_3^{2-} \qquad\qquad K = 10^{-10.25} \qquad (1)$$

The degree of saturation (SI) were calculated from the ionic activity products (IP) of SBF for CaCO$_3$ (calcite) given by the following equations.

$$SI = \log(IP / K_{CaCO_3}) \tag{2}$$

$$IP\,(\text{calcite}) = \gamma_{Ca^{2+}} \cdot \gamma_{CO_3^{2-}} \cdot \left[Ca^{2+}\right] \cdot \left[CO_3^{2-}\right] \tag{3}$$

Here, γ_i is the activity coefficient and [i] is the concentration of ion species i, and the solubility product of calcite in an aqueous solution (K_{CaCO_3}) is $10^{-8.6}$ [9]. The activity coefficient of Ca^{2+} is reported 0.36 under normal physiological conditions (ionic strength: $\mu = 0.16$) [10]. The activity coefficient of CO_3^{2-} is calculated from the following Debye-Hückel limiting law [11,12]:

$$\log \gamma_i = -0.5\, Z_i^2\; \mu^{1/2} / [1 + \mu^{1/2}] \tag{4}$$

where Z_i nd μ are valence of ion i and total ionic strength of the fluid, respectively.

3. Results

3.1. Appearance of gels

Figure 3-1 shows appearance of the pectin gels after mixing with various calcium salts. The citrus-derived pectin gels prepared by mixing with calcium chloride and calcium acetate maintained fluidity. However, in the other compositions, pectin sol immediately lost fluidity and hardened after mixing with the calcium salts. Therefore, the former was able to inject into a mold using a syringe, but the latter was not. Therefore the latter gel was pulverized and packed into the mold. After exposure to SBF, the gels were hardened into cylindrical shape to fit on the mold except the gels mixed with calcium hydrate, as shown in Figure 3-2.

3.2. Apatite-forming abilities

Figure 3-3 shows SEM photographs of the surfaces of pectin gels that were mixed with various calcium salts after soaking in SBF for various periods. In pectic acid gels, deposition of aggregate of fine spherical particles was observed in the part of surfaces of Acid-ace and Acid-chl gels after 7 days, and many particles were observed over the whole surface of Acid-hyd gel after 1 day. On the other hand, in the citrus-derived pectin gels, deposition of aggregate of fine spherical particles was observed in the part of surfaces of Citrus-ace and Citrus-chl gels after 1 day and deposition areas were increased until the 7 days, and many particles were observed over the whole surface of Citrus-hyd gel after 1 day. According to the EDX spectra as shown Figure 3-4, peaks assigned to calcium and phosphorous were detected on the deposits of aggregate of fine spherical particles observed under the SEM, but only peak assigned to calcium was detected on the other deposits.

Figure 3-5 shows the TF-XRD patterns of the surfaces of pectin gels that were cross-linked by various calcium salts after soaking in SBF for various periods. Broad peaks assigned to hydroxyapatite with low crystallinity were detected for the samples where the aggregate of fine spherical particles deposited the surfaces at $26°$ and $32°$ in 2θ. The peaks assigned to calcium carbonate were detected for the gels were mixed with calcium hydroxide.

Table 3-2 summarizes the apatite-forming abilities of the pectin gels. We can see that there was little difference in the apatite-forming ability of the gels that were cross-linked with calcium acetate or calcium chloride. Meanwhile, the gels that were cross-linked with calcium hydroxide had not shown apatite-forming ability. In addition, the pectic acid gels were showed apatite-forming ability, although it did not show the ability in Chapter 1 and Chapter 2.

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

Figure 3-6 shows the changes in the Ca^{2+} concentration of the Tris-NaCl buffer due to soaking of different pectin gels. In the gels were cross-linked with calcium acetate or calcium chloride, the concentrations increased within 1 day and then became constant. In the gels were cross-linked with calcium hydroxide, it increased within 3 days. Figure 3-7 shows the changes in pH of Tris-NaCl buffer due to soaking of different pectin gels. The pH of the gels was cross-linked with calcium acetate or calcium chloride decreased continuously and the gels were cross-linked with calcium hydroxide increased continuously.

3.4. Degree of saturation for $CaCO_3$ in SBF after soaking of pectin gels

The degree of saturation for $CaCO_3$ (SI) in SBF after soaking of pectin gels are calculated and summarized in Table 3-3. The SBF did not saturate for $CaCO_3$ before soaking of the gels due to SI was -2.4. After the soaking of the gels that were cross-linked with calcium acetate or calcium chloride, SI was increased up to 0.45-0.63 after the soaking for 1 day, and then decreased until 0.07-0.37 due to CO_3^{2-} concentration was decreased by decrease of the pH with the dissolution of the gel. In contrast, the soaking of the gels that were cross-linked with calcium hydroxide, SI was increased up to 0.89-1.07 after the soaking for 1 day, and then tremendous increased up to 2.42-4.37 due to CO_3^{2-} concentration was increased by increase of the pH with the dissolution of calcium hydroxide.
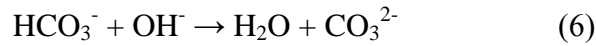
4. Discussion

In the pectic acid gels, the gels rapidly hardened and lost fluidity after exposure to the calcium salts irrespective of the calcium salt. It is assumed that large amount of carboxyl group in pectic acid makes tight cross-linking with Ca^{2+} via formation of egg-box structure. On the other hand, in the citrus-derived pectin gels, gels were prepared by mixing powder of calcium chloride and calcium acetate maintained enough fluidity to be molded into desired shapes. Degree of the cross-linking in the citrus-derived pectin is lower than the pectic acid as shown in Chapter 1, and

81

that the calcium acetate and the calcium chloride with high solubility (34.7 g/100 cm^3 H$_2$O and 74.5 g/100 cm^3 H$_2$O at 20°C, respectively) immediately dissolved in pectin solution to form a uniform solution after the mixing [13]. However, only the gels prepared by mixing with calcium hydroxide immediately lost fluidity, hardened and showed phenomenon of syneresis. Fluidity of the gel would be suppressed by undissolved residue of the calcium hydroxide powder with low solubility (0.17 g/100 cm^3 H$_2$O at 20°C). In addition, gelation would be significantly promoted by rapid increase in pH due to dissolving of calcium hydroxide in the pectin solution. It is reported that alkaline conditions enhance de-methoxylation of pectin leading to full dissociation of carboxyl groups, and that the cations in surrounding solution induce gelation to neutralize the negative charge of the pectin [14]. After exposure to SBF, Citrus-ace and Citrus-chl which maintained fluidity hardened into a cylindrical shape in the mold. It is known that low methoxyl pectin shows the greatest viscosity around pH 6. The pH of the citrus-derived pectin gels is 4-5. Therefore, it is thought that gels lost fluidity due to viscosity of the gel increased by it were soaked in SBF of pH 7.4.

SEM observations and TF-XRD data shows that citrus-derived pectin gels form low crystalline bone-like apatite on their surfaces after soaking in SBF within 1 day and that pectic acid gels formed it after 7 days, when they were mixed with calcium acetate or calcium chloride. Pectin gels released Ca^{2+} of 15-20 mM within 1 day (See Figure 3-6). The amount of Ca^{2+} release was 1.5 - 2 times larger the pectin gels with apatite formation shown in Chapter 1. It is thought that the Ca^{2+} release improved apatite formation on the gels prepared by directly mixing calcium salt powder than those of gels prepared by treatment of calcium salt solution.

Meanwhile, the gels prepared by mixing with calcium hydroxide formed not the apatite but the calcium carbonate on the gel surfaces within 1 day in SBF. It is known that the saturated calcium hydroxide solution easily converts into calcium carbonate by reaction with dissolved carbon dioxide or carbonate ions in the solution. According to Figure 3-7, only the gels mixed with calcium hydroxide increased pH of the buffer solutions. It is assumed that HCO$_3^-$ is dissociated into

H$^+$ and CO$_3$$^{2-}$ with high pH in SBF, and CaCO$_3$ is formed by combining CO$_3$$^{2-}$ and Ca(OH)$_2$ by the following chemical equation:

$$Ca(OH)_2 \rightarrow Ca^{2+} + 2OH^- \qquad (5)$$

$$HCO_3^- + OH^- \rightarrow H_2O + CO_3^{2-} \qquad (6)$$

$$Ca(OH)_2 + CO_3^{2-} \rightarrow CaCO_3 + 2OH^- \qquad (7)$$

In addition, according to results of calculation of the degree of saturation of SBF for CaCO$_3$, it is found that SBF after soaking of all compositions of pectin gels have become supersaturated, though SBF is normally unsaturated with respect to the CaCO$_3$. In the gels that were cross-linked with calcium acetate or calcium chloride, it is thought that apatite was deposited not calcium carbonate due to degree of saturation of SBF for CaCO$_3$ is relatively small (0.07-0.65). On the other hand, in the gels that were cross-linked with calcium hydroxide, calcium carbonate would be subject to be deposited because degree of saturation of SBF is high (0.89-4.37) in comparison with the other gels. The degree of saturation for calcite increases according to the increase in pH and concentration of calcium ion. Therefore, calcite was deposited on the surfaces of the gels mixed with calcium hydroxide, since the degree of saturation of the surrounding solution is rapidly increased by complete dissolution of calcium hydroxide.

Mori *et al.* reported that the rate of the apatite deposition was increased by increase in solubility of the calcium salts added to the PMMA-based bone cement. [15]. In the pectin hydrogels, the apatite-forming ability was increased in the order: Ca(OH)$_2$ (no deposit) << Ca(CH$_3$COO)$_2$ ≈ CaCl$_2$. The solubility of calcium salts was increased in the order: Ca(OH)$_2$ << Ca(CH$_3$COO)$_2$ < CaCl$_2$. Therefore, it is revealed that the apatite-forming ability was affected by the solubility of the calcium salts even in the case of pectin gel. Namely, the calcium salts with low solubility have no effect on the apatite-forming ability.

5. Conclusions

The author examined the apatite-forming abilities of pectin hydrogels prepared by mixing pectin solution and calcium salts powder, in SBF. Homogeneous gel was obtained by mixing with calcium chloride and calcium acetate, but not calcium hydroxide. The former precipitated the apatite after exposure to SBF, whereas the latter precipitated the calcium carbonate. The difference was attributed to degree of increase in pH of the surrounding solution. It was found that the apatite-forming ability of the pectin hydrogels can be enhanced by appropriate selection of the calcium salt with high solubility and a little pH changes after dissolving. The results in this chapter will provide material design novel self-setting bone substitutes based on pectin.

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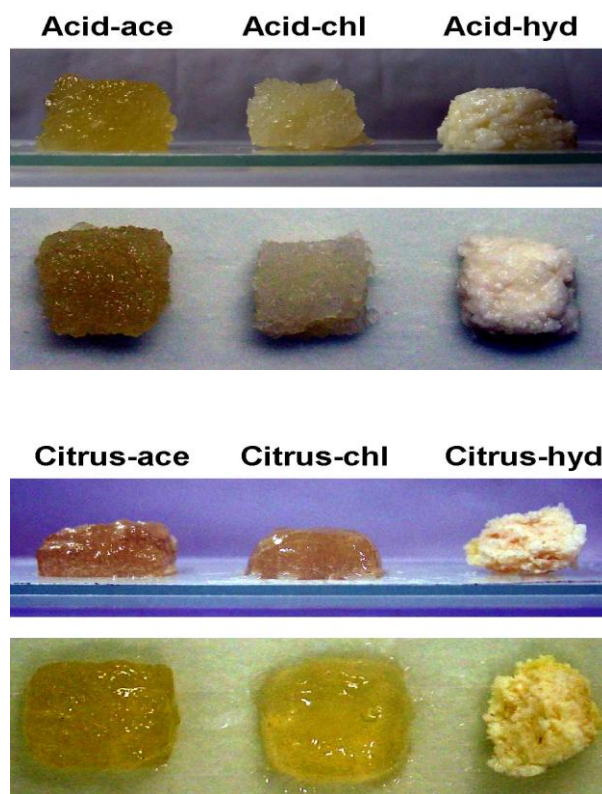
Figure 3-1. Appearance of the pectin gels after mixing with calcium salts.
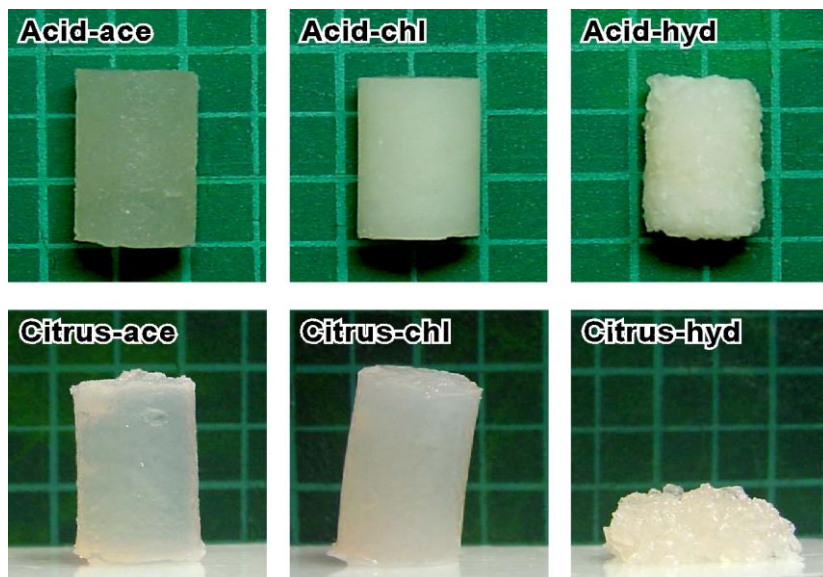
Figure 3-2. Appearance of the pectin gels after soaking in SBF for 7 days.
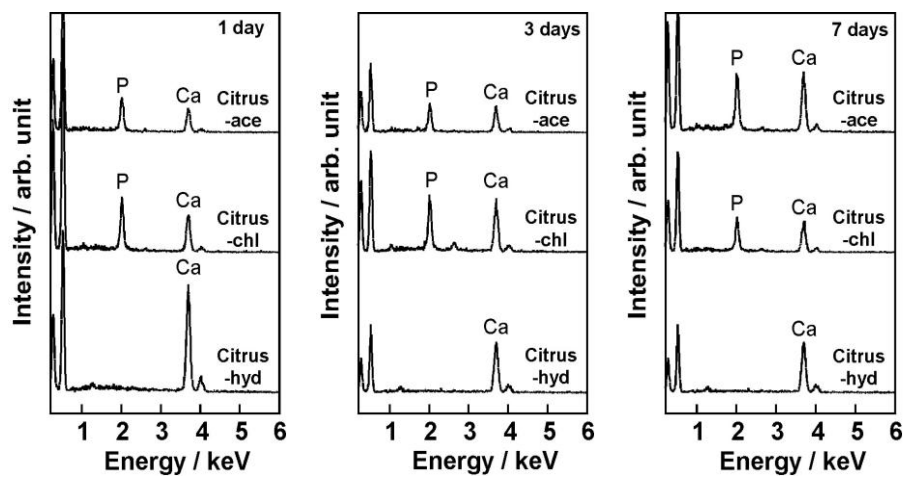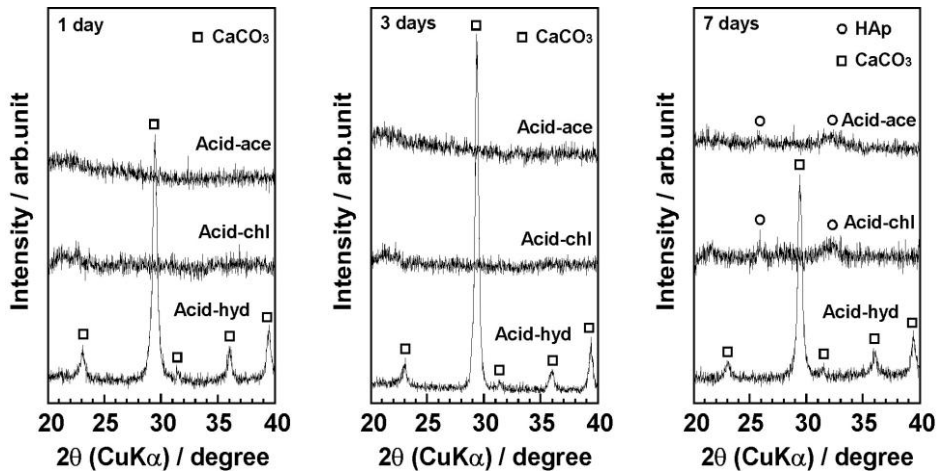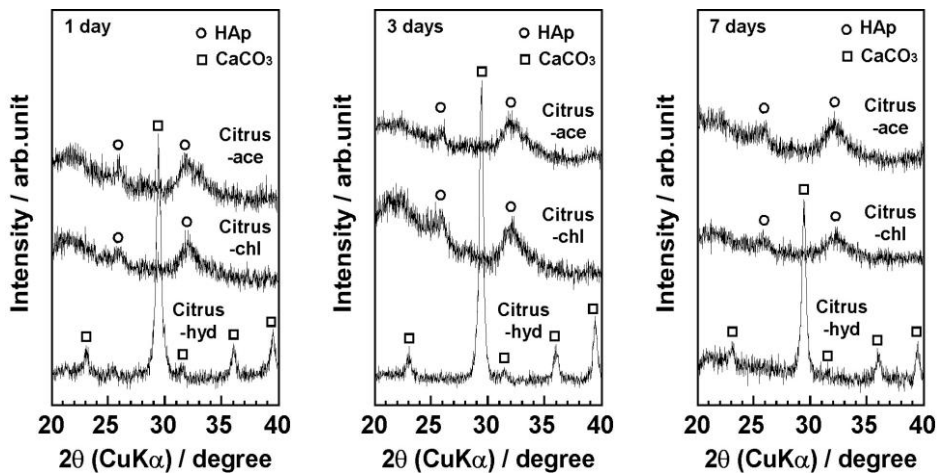
1 day
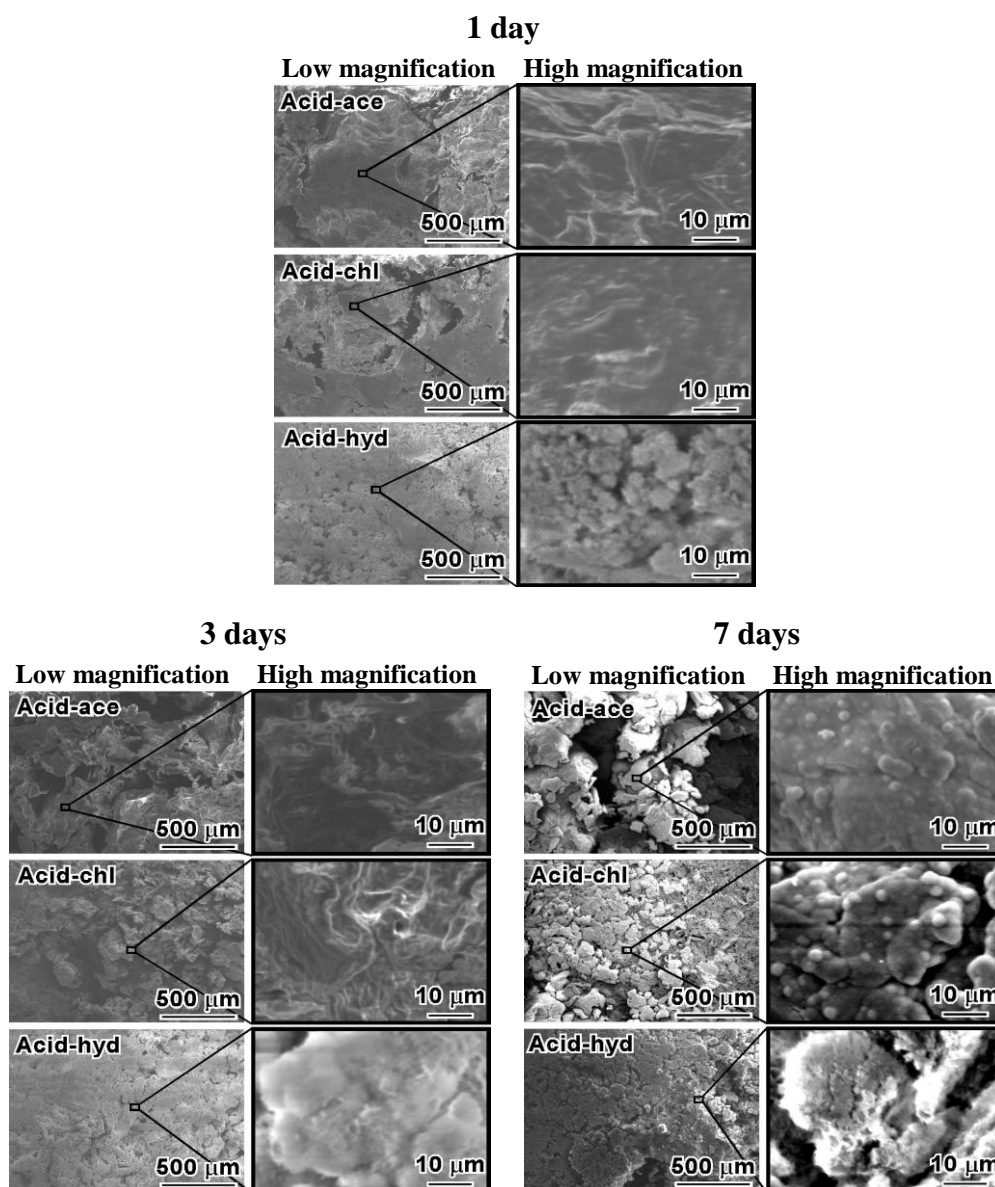
(a) Pectic acid

Figure 3-3. SEM photographs of the surfaces of pectin gels after soaking in SBF

for various periods. (a) is pectic acid gels and (b) is citrus-derived pectin gels.

1 day

Low magnification   High magnification

Citrus-ace

500 μm   10 μm

Citrus-chl

500 μm   10 μm

Citrus-hyd

500 μm   10 μm

3 days

Low magnification   High magnification

Citrus-ace

500 μm   10 μm

Citrus-chl

500 μm   10 μm

Citrus-hyd

500 μm   10 μm

7 days

Low magnification   High magnification

Citrus-ace

500 μm   10 μm

Citrus-chl

500 μm   10 μm

Citrus-hyd

500 μm   10 μm
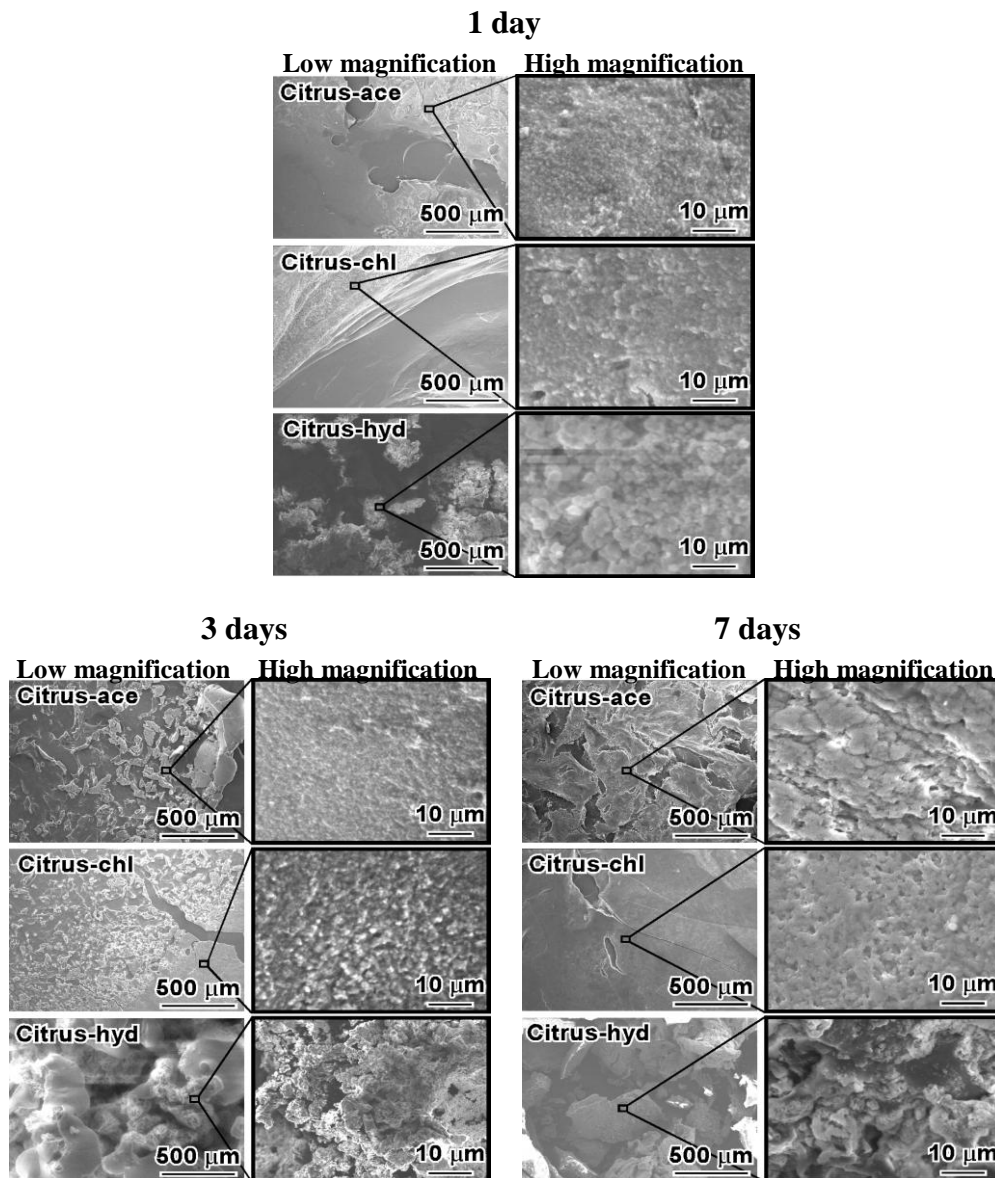
(b) Citrus-derived

Figure 3-3. (Continued)

Pectic acid
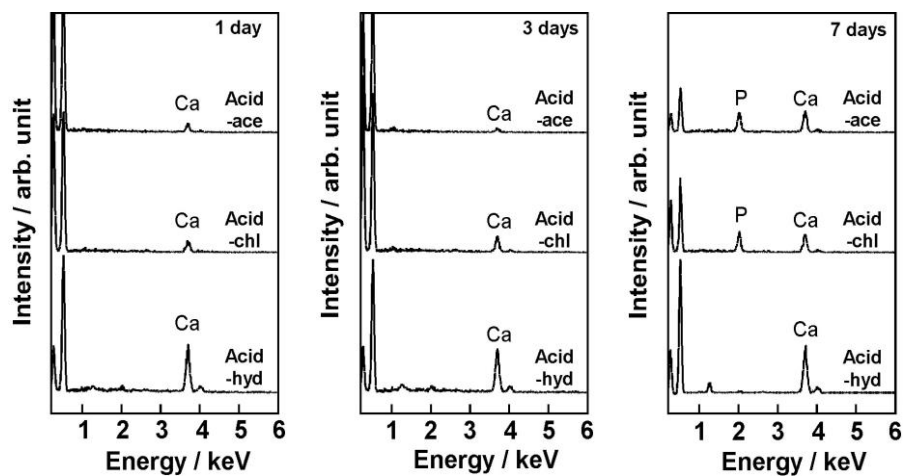
Citrus-derived

Figure 3-4. EDX spectra of the surfaces of pectin gels after soaking in SBF for various periods. Left is pectic acid gels and light is citrus-derived pectin gels.

Pectic acid

Citrus-derived

Figure 3-5. TF-XRD patterns of the surfaces of pectin gels after soaking in SBF

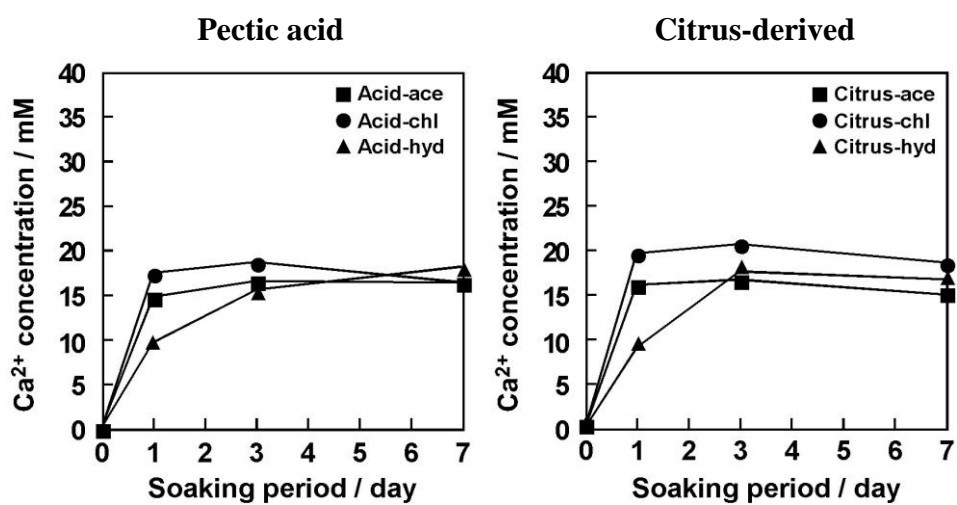for various periods. Left is pectic acid gels and right is citrus-derived pectin gels.

Pectic acid　　　　　**Citrus-derived**

Figure 3-6. Changes of Ca concentration in Tris-NaCl buffer solution due to soaking of various pectin gels. Left is pectic acid gels and right is citrus-derived pectin gels.
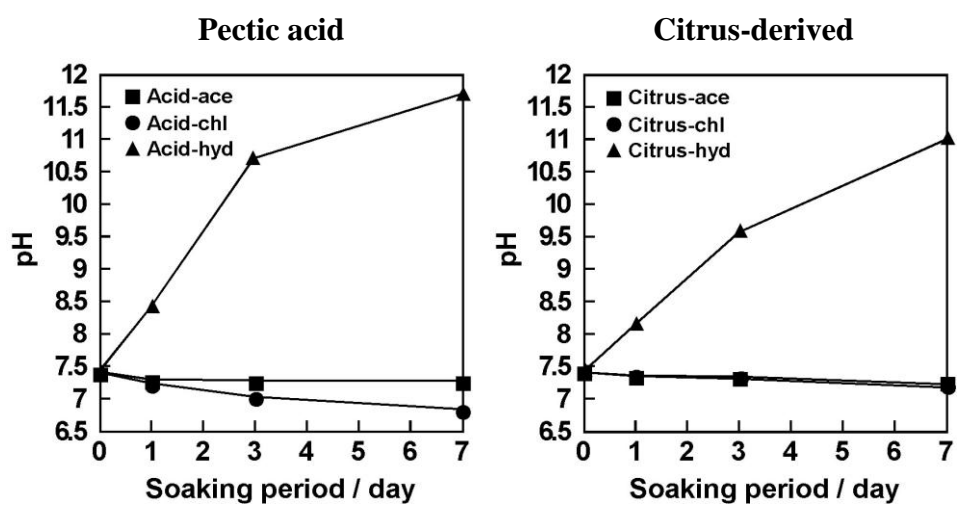
Pectic acid   **Citrus-derived**

Figure 3-7. Changes of pH in Tris-NaCl buffer solution due to soaking of various pectin gels. Left is pectic acid gels and right is citrus-derived pectin gels.

Table 3-1. Concentrations of pectin gels.

Sample	Kind of pectin	Pectin concentration / mass%	Cross-linking agent	Cross-linking agent concentration / $kmol \cdot m^{-3}$
Acid-ace			$Ca(CH_3COO)_2 \cdot H_2O$	
Acid-chl	Pectic acid	5	$CaCl_2$	1
Acid-hyd			$Ca(OH)_2$	
Citrus-ace			$Ca(CH_3COO)_2 \cdot H_2O$	
Citrus-chl	Citrus-derived pectin	5	$CaCl_2$	1
Citrus-hyd			$Ca(OH)_2$	

Table 3-2. Apatite-forming ability of pectin gels after soaking in SBF for various periods.

Sample	Apatite-formation		
	1 day	3 days	7 days
Acid-ace	x	x	o
Acid-chl	x	x	o
Acid-hyd	x	x	x
Citrus-ace	o	o	o
Citrus-chl	o	o	o
Citrus-hyd	x	x	x

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

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

Table 3-3. Degree of saturation for $CaCO_3$ in SBF after soaking of pectin gels.

Sample	Degree of saturation ($\log(IP/K_{CaCO_3})$)			
	0 day	1 day	3 day	7 day
Acid-ace	-0.24	0.45	0.38	0.37
Acid-chl	-0.24	0.44	0.18	0.07
Acid-hyd	-0.24	1.07	2.22	4.37
Citrus-ace	-0.24	0.54	0.38	0.23
Citrus-chl	-0.24	0.63	0.41	0.22
Citrus-hyd	-0.24	0.89	2.06	2.42

General Conclusions

This thesis has described the investigation into the apatite deposition behavior on pectin hydrogels in simulated body environment to establish the novel bone-repairing materials. The results of this study were summarized as follows.

In Chapter 1, hydrogels were prepared from three kinds of pectin with different degree of esterification of the side chain by $CaCl_2$ treatment and these apatite-forming ability in SBF and mechanical property were investigated. Apple- and citrus-derived pectin gels formed the apatite in SBF, but not pectic acid gel. It was suggested that the apatite-forming ability of the pectin gels is governed by not only the amount of carboxyl groups but also changes in Ca^{2+} concentration and pH in surrounding solutions. Young's modulus of the pectin gels with dry condition was similar to that of natural bone, although tensile strength was a little lower. It is necessary that these mechanical properties of pectin hydrogels were improved, due to those were too poor to measure under wet condition.

In Chapter 2, apatite-forming ability in SBF and mechanical property of pectin hydrogels that were prepared through covalent cross-linking with divinyl sulfone (DVS), were investigated and compared with those of the pectin gels cross-linked with Ca^{2+}. 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. Consequently, it was found that the mechanical strength of the pectin hydrogel could be improved without losing apatite-forming ability of it by using DVS as cross-linking agent. However, the mechanical strength of gels cross-linked with DVS was too poor to measure under wet condition as well as gels cross-linked with Ca^{2+}. It was thought that this is because apatite was deposited only on the contact surface with SBF. Apatite deposition inside the pectin hydrogels will be necessary for application as scaffolds for tissue regeneration by changing cross-linking agent, method of incorporation of the calcium ion into gel or

morphology of the gels.

In Chapter 3, pectin hydrogels were prepared by mixing pectin solution and various water-soluble calcium salts powder including calcium acetate monohydrate, calcium chloride and calcium hydroxide. Their apatite-forming ability was investigated in SBF. Homogeneous gel was obtained by mixing with calcium chloride and calcium acetate, but not calcium hydroxide. The former precipitated the apatite after exposure to SBF, whereas the latter precipitated the calcium carbonate. The difference was attributed to degree of increase in pH of the surrounding solution. It was found that the apatite-forming ability of the pectin hydrogels can be enhanced by appropriate selection of the calcium salt with high solubility and a little pH changes after dissolving.

On the basis of these results, fundamental material design for development of pectin-apatite composites has been revealed. Chemical treatment of pectin with appropriate calcium salt allows to make the composites. On the contrary to initial expectation, large amount of carboxyl group in the pectin rather inhibits the apatite formation. In addition, the mechanical strength of the pectin hydrogel can be improved by covalent cross-linking with divinyl sulfone.

In the future, the mechanical strength of the pectin hydrogels in body environment is required to be further improved. The results in this thesis will also provide material design of novel self-setting bone substitutes based on polysaccharides.

List of Publications

Journal Papers

1. <u>Ichibouji T</u>, Miyazaki T, Ishida E, Ashizuka M, Sugino A, Ohtsuki C and Kuramoto K. Evaluation of apatite-forming ability and mechanical property of pectin hydrogels. *J. Ceram. Soc. Japan* 2008;116:74.

2. <u>Ichibouji T</u>, Miyazaki T, Ishida E, Sugino A and Ohtsuki C. Apatite mineralization abilities and mechanical properties of covalently cross-linked pectin hydrogels. *Mater. Sci. Eng. C,* in press.

Conference Proceedings

1. <u>Ichibouji T</u>, Miyazaki T, Ishida E, Ashizuka M, Sugino A, Ohtsuki C and Kuramoto K. Preparation of organic-inorganic hybrids consisting of apatite and pectin. In:, Watari F, Akazawa T, Uo M and Akasaka T, editors. *Archives of Bioceramics Research Vol. 5*: Asian Bioceramics Symposium; 2005. p. 95.

2. <u>Ichibouji T</u>, Miyazaki T, Ishida E, Ashizuka M, Sugino A, Ohtsuki C and Kuramoto K. Bioactivity and mechanical property of organic-inorganic hybrids based on pectin. In: Organizing Committee of 6th Asian Bioceramics Symposium, editor. *Archives of Bioceramics Research*; 2006. p. 75.

3. <u>Ichibouji T</u>, Miyazaki T, Ishida E, Ashizuka M, Sugino A, Ohtsuki C and Kuramoto K. Apatite-forming ability of pectin gels in simulated body environment. In: Zhang X, Li X, Fan H and Liu X, editors. *Bioceramics Vol. 19*: Trans Tech Publications; 2007. p. 675.

4. <u>Ichibouji T</u>, Miyazaki T, Ishida E, Ashizuka M, Sugino A, Ohtsuki C and Kuramoto K. Apatite-forming ability and mechanical property of pectin gels with different cross-linking. In: Yokogawa Y, Kawashita M, Miyazaki T, Nakahira A, Miyaji F, Takigawa T, Furuzono T, Hirose M, Takadama H, Nakamura A and Kishida I, editors. *Archives of BioCeramics Research*, Vol. 7:

Tadasu Printing Co., Ltd.; 2007. p. 79.

5. <u>Ichibouji T</u>, Miyazaki T, Ishida E, Ashizuka M, Sugino A, Ohtsuki C and Kuramoto K. Influence of cross-linking agents on apatite-forming ability of pectin gels. In: Daclusi G and Layrolle P, editors. *Bioceramics Vol. 20*: Trans Tech Publications; 2008. p. 559.

Related Publications

1. Miyazaki T, Sugino A, <u>Ichibouji T</u>, Ashizuka M, Doi K and Kuramoto K. Paste for artificial bone. *Japanese patent*, No. JP2007054278.

Acknowledgments