

# Properties of novel bone hemostat by random co-polymer of ethylene oxide and propylene oxide with sugar-containing hydroxyapatite

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## ABSTRACT

Novel hemostatic agents were prepared using (i) phosphoryl oligosaccharides of calcium (POs-Ca<sup>®</sup>45 and POs-Ca<sup>®</sup>50 with calcium contents being 4.5 and 5.0 mass %, respectively), (ii) sugar-containing hydroxyapatites (*s*-Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub>; *s*-HAp) obtained by the hydrolyses of POs-Ca<sup>®</sup>45 and POs-Ca<sup>®</sup>50, respectively (*s*-HAp(45); Ca/P ratio = 1.56, 61.2 mass % HAp and 38.8 mass % organic materials and *s*-HAp(50); Ca/P ratio = 1.61, 79.3 mass % HAp and 20.7 mass % organic materials), and (iii) thermoplastic resin (random copolymer of ethylene oxide (EO) and propylene oxide (PO)) (EPO). The gels formed by mixing the EPO with water (EPO/water (mass ratio): 0.20) were freeze-dried at -50 °C for 15 h, and then blended with POs-Ca or *s*-HAp ((POs-Ca or *s*-HAp)/EPO-EO (mass ratio): 0.2). The noted findings due to the addition of POs-Ca or *s*-HAp to the composite gels were: (a) the stanching time of more than 80 min due to the addition of POs-Ca<sup>®</sup>50 to the composite gel with EPO : EO : water = 25 : 15 : 60 (mass ratio) (25EPO-15EO) and (b) formation of osteoid at the drilled hole within the tibia and femur of Japanese white rabbits due to the addition of *s*-HAp(45). The combined addition of POs-Ca and *s*-HAp to the EPO-EO were expected to enhance the hemostasis and bone regeneration. Copyright © 2016 VBRI Press.

**Keywords:** Hydroxyapatite; phosphoryl oligosaccharides of calcium; thermoplastic resin; hemostasis.

## Introduction

When the bones are cut at the surgery operation, a large amount of bleeding occurs from the bone's vessel. In order to quickly stop bleeding, the hemostasis treatment may be generally conducted through the following manners: (i) the coherence of platelet on the bleeding parts, (ii) the blocking of wound through von Willebrand factor (platelet plug), (iii) the coating of fibrin film over the whole platelet plug due to the coagulation of proteins in blood, and (iv) the hardening and hemostasis being completed (fibrin thrombus) [1]. Quick stanching techniques can be conducted by the thermal, mechanical, and chemical hemostasis [2]. Most useful technique for the quick stanching from the bones is a mechanical hemostat and, typically, a bone wax whose major component is bee wax is currently used for that purpose. Unfortunately, the bone wax is not bio-absorbable and may have the possibility to inhibit the bonding of bones. Moreover, such animal-derived hemostat has the risks to cause infection [3]. Thus researchers' interests are directed toward the development of novel hemostats with excellent bio-absorbability and bone regeneration, as well as the hemostasis.

Previously, Kabanov *et al.* [4] reported that the block copolymer of "ethylene oxide (EO) and propylene oxide (PO)" (EPO) may be suitable for the drug delivery. On the other hand, the addition of water to a random copolymer of

EPO quickly becomes the pasty and glutinous gel with the excellent flexibility, as well as the excellent biocompatibility [5]. Such EPO, therefore, suits the hemostat but lacks the ability of bone regeneration. Thus additional materials have to be incorporated into the EPO gel in order to encourage the bone regeneration. The phosphoryl oligosaccharides of calcium (POs-Ca) possess not only the molecular structure with Ca<sup>2+</sup> and PO<sub>4</sub><sup>3-</sup> groups, but also excellent dissolution behavior into the water, both of which are regarded as the promising characteristics of re-calcification [6]. By making use of such noted properties, the present authors have successfully prepared a sugar-containing hydroxyapatite (*s*-Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub>; *s*-HAp) from POs-Ca solution through the hydrolysis technique [7]. Thus the combination of POs-Ca or its hydrolyzed materials (HAp and organic components, *s*-HAp), may be expected for the novel hemostat having both excellent bio-absorbability and bone regeneration. Our previous research was conducted by using POs-Ca (Ca content: 4.5 mass % (POs-Ca<sup>®</sup>45)) and its hydrolyzed materials (*s*-HAp(45)) [8]. Recently, POs-Ca with higher Ca content being 5.0 mass % (POs-Ca<sup>®</sup>50) has started to be commercially available and, therefore, it becomes possible to comprehensively investigate the effect of POs-Ca and *s*-HAp addition on the properties of EPO gel for the application of the present materials to the bone hemostat. On the basis of such background, the present

paper describes the investigation on the optimization of the preparation conditions for the application to bone hemostat, using POs-Ca, *s*-HAp and thermoplastic resin.

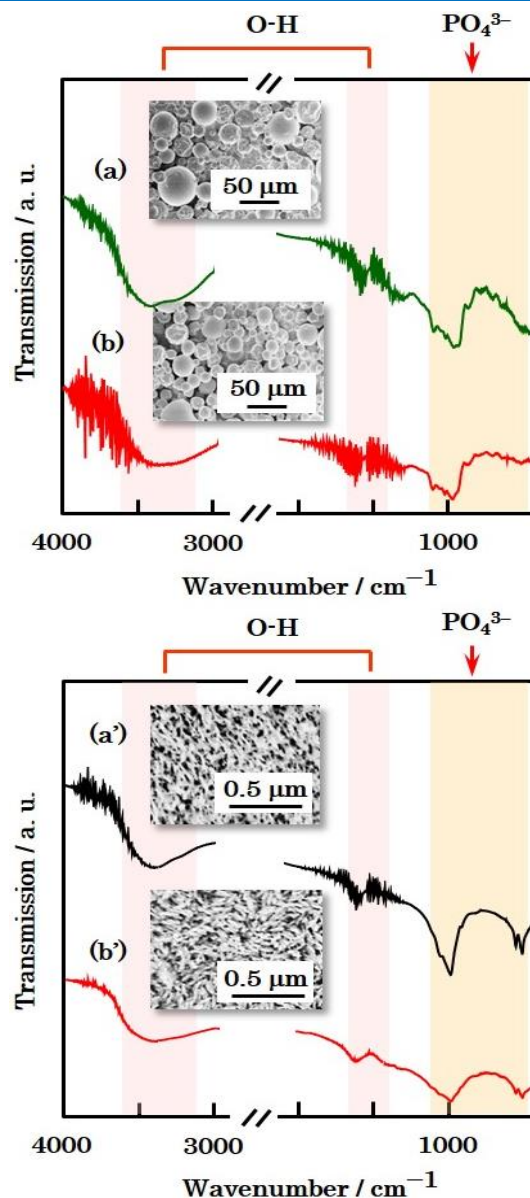
## Experimental

### Starting materials

Among the starting materials, the commercially available POs-Ca<sup>®</sup>45, POs-Ca<sup>®</sup>50 and EPO gel were used with no further purification. On the other hand, 10 mass % POs-Ca<sup>®</sup>45 and POs-Ca<sup>®</sup>50 solutions were hydrolyzed at 100 °C for 5 h in order to form *s*-HAp, *i.e.*, *s*-HAp(45) and *s*-HAp(50), respectively; since no precipitation occurred only by the hydrolysis procedure of POs-Ca solution, ethanol was added to the solution in order to encourage the precipitation of *s*-HAp after cooling the solution down to room temperature. On the other hand, EPO powder was mixed with de-ionized water to form gel (EPO to water (mass ratio): 2.0), flash frozen by liquid nitrogen and then freeze-dried at -50 °C for 1 d. The freeze-dried materials were mechanically ground and sieved in order to obtain the powder with the particle sizes of 1.0 - 2.0 mm. Such granulation was conducted to encourage the gelation of EPO or EPO-EO and tightly adhere to the bleeding sites, after the addition of water to the powder, thereby expecting the provision of effective hemostasis. Further, EPO-EO gels were mixed with POs-Ca or *s*-HAp powder (POs-Ca or *s*-HAp)/EPO-EO (mass ratio): 0.2) in order to prepare the composite gels.

### Measurements and characterizations

Phase identification was conducted using an X-ray diffractometer (XRD; RINT 2000V/P, Rigaku Corp., Tokyo, 40 kV and 40 mA) with monochromatic CuK $\alpha$  irradiation, and a Fourier transform infrared spectrometer (FT-IR; 8600PC, Shimadzu, Kyoto). On the other hand, phase changes during the heating from room temperature up to 1000 °C were examined by differential thermal analysis and thermogravimetry (DTA-TG; Model Thermo plus TG8120, Rigaku, Tokyo). The specific surface area of the starting powder was measured by BET method, using N<sub>2</sub> as adsorption gas. The particle sizes and shapes were observed using a field-emission scanning electron microscope (FE-SEM; Model SU-8000, Hitachi, Tokyo) with an accelerating voltage of 5 kV. The amounts of calcium and phosphorus were determined using an energy-disperse X-ray microanalyzer (EDX; EX-210, Horiba, Kyoto), attached to the FE-SEM. Such amounts were calculated on the basis of the average amounts after repeating this operation five or more times to enhance the accuracy. In order to check the operational ability of composite gels, the consistency was checked by measuring the spread size of gel, after the gel (1 g) had been set between two plates with the mass of 500 g. The stanching time was checked by measuring the time that the blood came out from the composite gels implanted into the drilled holes (diameter: 4 mm) within the femur and tibia of Japanese white rabbits. In order to check the bone regeneration, some of the composite gels were implanted into the drilled holes within the femur and tibia of Japanese white rabbits for four weeks. The degree of bone



**Fig. 1.** FT-IR spectra of (a) POs-Ca<sup>®</sup>45, (b) POs-Ca<sup>®</sup>50, (a') *s*-HAp(45) and (b') *s*-HAp(50).

regeneration was evaluated on the basis of the non-decalcified specimens prepared with a Villanueva bone stain.

## Results and discussion

### Characterization of starting materials

The starting materials used in this research, *i.e.*, POs-Ca<sup>®</sup>45 and POs-Ca<sup>®</sup>50, contained no distinct reflections in the XRD patterns. This fact indicates that POs-Ca<sup>®</sup>45 and POs-Ca<sup>®</sup>50 do not contain crystalline materials (data not shown here). **Fig. 1** shows typical powder properties of POs-Ca and *s*-HAp. Regarding the properties of POs-Ca powders, the grain sizes of both POs-Ca powders were around 0 - 50 μm (Mean particle diameters of POs-Ca<sup>®</sup>45 and POs-Ca<sup>®</sup>50 being 11.7 and 12.4 μm, respectively). FT-IR spectra of POs-Ca powder showed that the absorption peaks appeared at 530, 570, 940, 1030, 1160, 1200 and 1650 cm<sup>-1</sup> and the absorption band of 3200 ~ 3700 cm<sup>-1</sup>.

The absorption peaks at 530, 570, 940, 1030, 1160 and 1200  $\text{cm}^{-1}$  are assigned to the orthophosphate ions ( $\text{PO}_4^{3-}$ ), and those at 1650 and 3200 ~ 3700  $\text{cm}^{-1}$  to physically-adsorbed water. No marked difference in the absorption peak positions and intensities of  $\text{PO}_4^{3-}$  indicates that the molecular structures of  $\text{POs-Ca}^{\textcircled{45}}$  and  $\text{POs-Ca}^{\textcircled{50}}$  are essentially the same but only different from the amount of  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$ .

Regarding the case of *s*-HAp, the particle sizes were always less than 0.1  $\mu\text{m}$ . These submicrometer-scaled particles were stuck together to form agglomerates. FT-IR spectra of *s*-HAp powders contained absorption peaks at 560, 600, 1030, 1080 and 1150  $\text{cm}^{-1}$ , which were assigned to the characteristic bands of  $\text{PO}_4^{3-}$ . The adsorption band in the range of 3200 ~ 3700  $\text{cm}^{-1}$  was a characteristic band of physically-adsorbed water/ $\text{OH}^-$  groups, regardless of the background of XRD pattern being broad (data not shown here). This fact indicates that *s*-HAp is composed of low-crystalline HAp and organic materials. Since the *s*-HAp is found to contain not only inorganic materials (*i.e.*, HAp) but also organic materials (derived from  $\text{POs-Ca}$ ), the amounts of inorganic and organic materials were checked by using DTA-TG. According to the DTA-TG curves, the mass reductions occurred in the range of room temperature up to around 450  $^{\circ}\text{C}$  and that no marked mass changes were found to occur above 500  $^{\circ}\text{C}$ . This mass reduction indicates (i) the release of physically-adsorbed water (room temperature ~ 150  $^{\circ}\text{C}$ ), (ii) thermal decomposition of organic materials derived from  $\text{POs-Ca}$  to form carbon (150 ~ 450 $^{\circ}\text{C}$ ) and (iii) oxidation of residual carbon (around 450  $^{\circ}\text{C}$ ). On the basis of such mass losses, we found that *s*-HAp (45) contains HAp and organic materials being 61.2 and 38.8 mol %, respectively, whereas *s*-HAp (50) contains HAp and organic materials being 79.3 and 20.7 mol %, respectively. On the other hand, Ca/P ratios of *s*-HAp (45) and *s*-HAp (50), which had been measured by EDX, were 1.56 and 1.61, respectively. These data indicate that *s*-HAp (50) contains calcium-deficient HAp and organic materials, and that *s*-HAp (50) possesses the higher Ca/P ratio (=1.61), rather than the case of *s*-HAp (45) (=1.56). The difference in such Ca/P ratio is attributed to the higher calcium content of  $\text{POs-Ca}^{\textcircled{50}}$ , rather than the case of  $\text{POs-Ca}^{\textcircled{45}}$ .

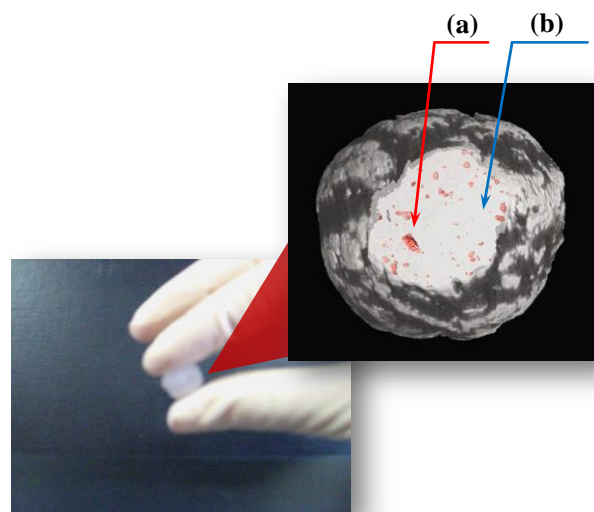
Regarding the broad XRD patterns of *s*-HAp (45) and *s*-HAp (50), the crystallite sizes were further checked on the basis of Scherer's formula by using the characteristic (111) reflection. The crystallite size of *s*-HAp (45) and *s*-HAp (50) were 8.83 and 13.48 nm, respectively. The crystallite size of *s*-HAp (50) was smaller than that of *s*-HAp (45), which indicates that the larger amount of organic materials within *s*-HAp (45), compared to the case of *s*-HAp (50), may inhibit the crystallite growth of HAp.

The specific surface areas of *s*-HAp (45) and *s*-HAp (50) were 22.90 and 63.41  $\text{m}^2\text{g}^{-1}$ , respectively. When the specific surface areas of *s*-HAp (45) and *s*-HAp (50) are calculated on the basis of crystallite sizes, the specific surface areas are estimated to be 215.8 and 140.6  $\text{m}^2\text{g}^{-1}$ , respectively. These values are contrary to what we have anticipated; however, such difference is explained by assuming that *s*-HAp (45) powder may be composed of smaller HAp crystallites but covered with the larger amount of organic compounds, thereby reducing the surface area.

Regarding the thermoplastic resin, EPO was pasty and glutinous gels. Such properties could be controlled by the additional incorporation of EO, on the basis of the hydrophilic nature of EO gel. In this research the gel with EPO: EO: water = 25: 15: 60 (mass ratio) (25EPO-15EO) was used as thermoplastic resin (random co-polymer), together with EPO. When the EPO and 25EPO-15EO were immersed into the water till 40 days, 25EPO-15EO was found to be a little slowly dissolved, compared to the case of EPO. This phenomenon seems to be attributed to the difference in molecular weight between EPO and 25EPO-15EO.

#### Properties of composite gels

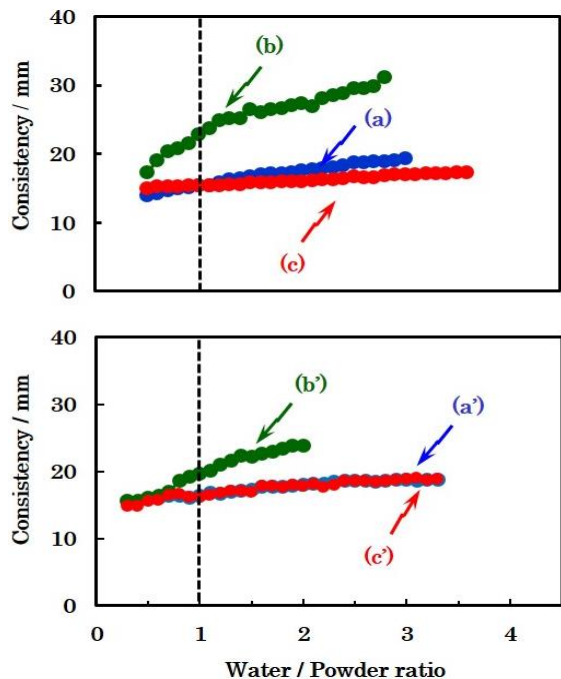
Appearance of composite gel: **Fig. 2** shows the typical appearance and micro-CT images of *s*-HAp-added 25EPO-15EO composite gel. The addition of water to a random copolymer of 25EPO-15EO composite powder was quickly changed into the pasty and glutinous gel (see whole image on the left side). By making use of the difference in X-ray absorbance coefficients between inorganic and organic materials, the micro-CT observation of the cross section within composite gel showed that *s*-HAp was found to be homogeneously distributed within the composite gels.



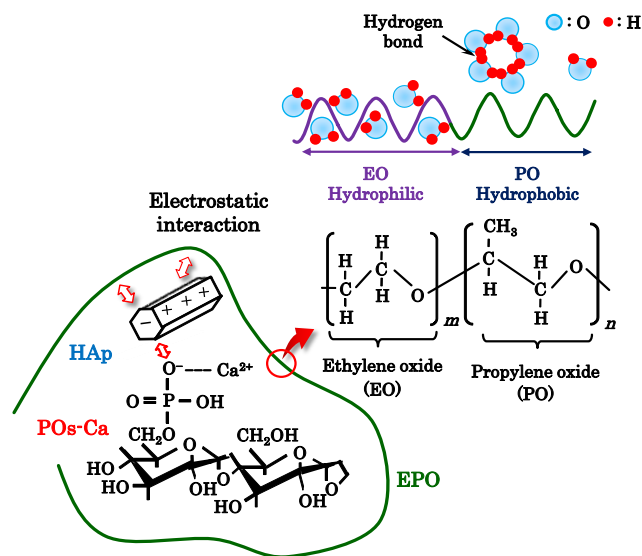
**Fig. 2.** Appearance of *s*-HAp added 25EPO-15EO composite gel and micro-CT showing the presence sites of (a) *s*-HAp and (b) EPO-EO matrix.

#### Consistencies of composite gels

The consistencies were measured in order to investigate the effect of water addition on the composite gel. The results are shown in **Fig. 3**. The consistency of EPO gel increased from 14.90 to 19.52 mm with increasing water/powder ratio from 0.5 to 3.3 (**Fig. 3(a)**). On the other hand,  $\text{POs-Ca}^{\textcircled{50}}$  helped enhancing the consistency of EPO composite gel from 17.26 up to 31.17 mm with increasing water/powder ratio (**Fig. 3(b)**). Further, the consistency of *s*-HAp (50) added EPO composite gel increased from 15.70 to 18.30 mm with increasing water/powder ratio (**Fig. 3(c)**). When the water/powder ratio was fixed at 1.0, consistencies of the composite gels were arranged as follows:  $\text{POs-Ca}^{\textcircled{50}}/\text{EPO}$  (22.80 mm) > *s*-HAp/EPO (15.90 mm) > EPO (15.50 mm).



**Fig. 3.** Consistencies of POs-Ca<sup>50</sup>- and s-HAp(50) added EPO (above) and 25EPO-15EO gels (below) (a) EPO, (b) POs-Ca<sup>50</sup>/EPO, (c) s-HAp(50)/EPO, (a') 25EPO-15EO, (b') POs-Ca<sup>50</sup>/25EPO-15EO, (c') s-HAp(50)/25EPO-15EO



**Fig. 4.** Schematic diagram regarding the chemical bonding of HAp with POs-Ca and water affinity of EO and PO sites within EPO structure. Note the electrostatic bonding of HAp to POs-Ca and EPO, and the affinity of EO and PO sites to the water.

On the other hand, the consistency of 25EPO-15EO gel increased from 16.20 to 19.74 mm with increasing water/powder ratio from 0.3 to 3.4 (**Fig. 3(a')**). The consistency of POs-Ca<sup>50</sup>-added 25EPO-15EO composite gel helped enhancing the consistency from 15.72 to 23.80 mm with increasing water/powder ratio to 2.0 (**Fig. 3(b')**); on further increase in water/powder ratio above 2.0, however, the rapid increase in consistency made the measurement of consistency difficult. Further, the consistency of s-HAp(50)-added 25EPO-15EO composite gel increased from 15.64 to 18.70 mm with increasing

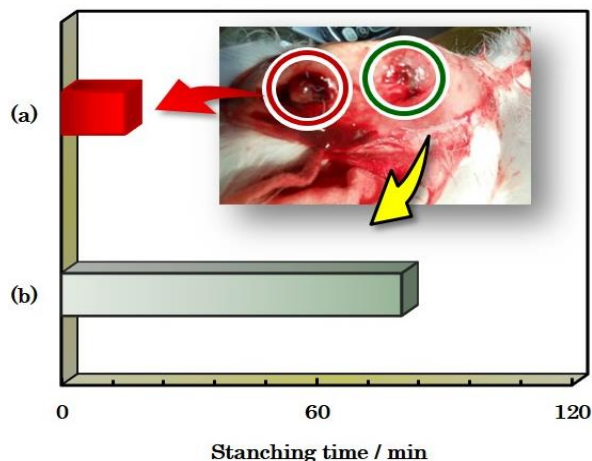
water/powder ratio from 0.3 to 3.4 (**Fig. 3(c')**). When the water/powder ratio was fixed at 1.0, consistencies of the hemostats were arranged as follows: 23.80 mm (POs-Ca<sup>50</sup>/25EPO-15EO) > 18.01 mm (25EPO-15EO) > 17.82 mm (s-HAp (50)/25EPO-15EO).

The consistency of POs-Ca<sup>50</sup>-added EPO gel notably increases with increasing water/powder ratio. In contrast, the consistency of s-HAp (50)-added EPO gel is restricted to increase, regardless of the increase in water/powder ratio, and is almost comparable to the case of EPO gel. Also, the consistency of POs-Ca<sup>50</sup>-added 25EPO-15EO composite notably increases with increasing water/powder ratio. In contrast, the consistency of s-HAp (50)-added 25EPO-15EO gel is restricted to increase, regardless of the increase in water/powder ratio, and is almost comparable to the case of 25EPO-15EO gel.

**Fig. 4** showed the mechanism on the electrostatic bonding of POs-Ca or s-HAp to EPO gel. Regarding the molecular structure of s-HAp, HAp electrostatically bonds to POs-Ca, due to the difference in positive and negative charges between side and top of HAp structure. Such difference in charge, HAp may be electrostatically bonded to EPO. Further, EPO is composed of the hydrophilic molecule (EO) and hydrophobic molecule (PO), EO side seems to be preferentially dissolved into water [9].

*Stanching times of composite gels*

The stanching times of these composite gels were checked using Japanese white rabbits. Some parts of tibia and femur were drilled and the composite gels were put into such parts in order to stop bleeding. Typical stanching times of composite gels are shown in **Fig. 5**, together with the photograph regarding the test of stanching times. Regardless of the stanching times of the most composite gels being less than 15 min (**Fig. 5(a)**), the stanching time of POs-Ca<sup>50</sup>-added 25EPO-15EO gel was more than 80 min (**Fig. 5(b)**).



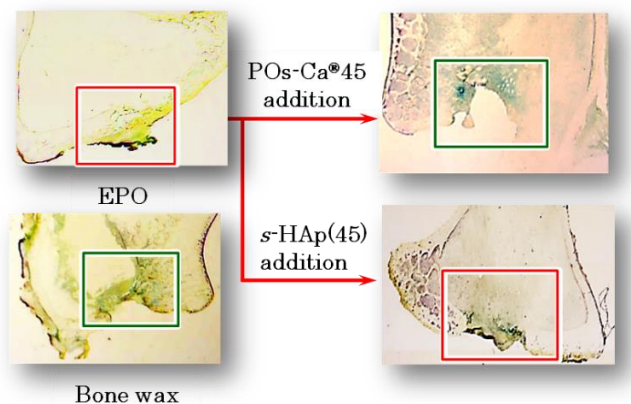
**Fig. 5.** Stanching time of composite gels, together with the photograph showing the test on the measurement of hemostasis times. (a) s-HAp(50)/25EPO-15EO composite gel (b) POs-Ca<sup>50</sup>/25EPO-15EO composite gel

Regardless of the limited stanching times of s-HAp-added composite gels, POs-Ca<sup>50</sup>-added composite gel showed a noted stanching time. On the basis of these results, it is found that the stanching time may be affected

by the consistency of composite gel, *i.e.*, the higher consistency of POs-Ca<sup>®</sup>50-added 25EPO-15EO composite gel being higher stanching time (more than 80 min).

### Bone regeneration

The bone regenerations of composite gels with POs-Ca or *s*-HAp addition were evaluated by implanting them into the drilled holes of tibia and femur using Japanese white rabbits. Typical results are shown in Fig. 6, together with the photographs regarding the case of EPO and bone wax. The squared places in the photographs indicate the original drilled holes. After the implantation of bone wax and EPO into the tibia and femur for nine weeks, the noted bone regeneration was found for the case of EPO, compared to the case of bone wax. Then the further investigation was conducted in order to make clear the effect of POs-Ca<sup>®</sup>45 and *s*-HAp (45) addition on the bone regeneration. In contrast to the case of POs-Ca<sup>®</sup>45-added EPO gel, the noted bone regeneration was found after the implantation of *s*-HAp (45)-added EPO gel for four weeks.



**Fig. 6.** Photographs showing the Villanueva bone sustain after implanting the POs-Ca<sup>®</sup>45/EPO composite gel and *s*-HAp(45)/EPO composite gel (implantation for 4 weeks), together with bone wax and EPO (implanted for 9 weeks).

Since EPO does not have the ability of bone regeneration, the bone regeneration due to its implantation indicates that EPO does not inhibit the bonding of bone regeneration. On the other hand, POs-Ca-derived organic materials within *s*-HAp seem to be dissolved away during the implantation to remain the original drilled holes, whereas HAp within *s*-HAp (45) may help regenerating the bones.

The present materials (*i.e.*, EPO/EPO-EO, POs-Ca and *s*-HAp) are considered to possess excellent biocompatibilities, due to no inflammation being checked by using Japanese white rabbits. On the other hand, the excellent biodegradation (*i.e.*, the degree of dissolution) was found for EPO/EPO-EO and POs-Ca, which plays a role in the stop of bleeding. On the other hand, the bone regeneration capacity was notably found for *s*-HAp, due to the remaining within the implanted place.

### Conclusion

Novel hemostatic agents were prepared using (i) POs-Ca (POs-Ca<sup>®</sup>45 and POs-Ca<sup>®</sup>50 with calcium contents being

4.5 and 5.0 mass %, respectively), (ii) *s*-HAp obtained by the hydrolyses of POs-Ca<sup>®</sup>45 and POs-Ca<sup>®</sup>50, respectively (*s*-HAp(45); Ca/P ratio = 1.56, 61.2 mass % HAp and 38.8 mass % organic materials and *s*-HAp(50); Ca/P ratio = 1.61, 79.3 mass % HAp and 20.7 mass % organic materials), and (iii) EPO and 25EPO-15EO gels. The results obtained were summarized as follows:

- (i) The gels formed by mixing the EPO and 25EPO-15EO with water ((EPO or 25EPO-15EO)/water (mass ratio): 0.20) were freeze-dried at -50°C for 15 h, and then blended with POs-Ca or *s*-HAp ((POs-Ca or *s*-HAp)/EPO-EO (mass ratio): 0.2). The addition of water to 25EPO-15EO composite powder was quickly changed into the pasty and glutinous gel.
- (ii) The noted findings due to the addition of POs-Ca or *s*-HAp to the composite gels were: (a) the stanching time of more than 80 min due to the addition of POs-Ca<sup>®</sup>50 to the composite gel with 25EPO-15EO gel and (b) regeneration of osteoid at the defect parts due to the addition of *s*-HAp(45). The combined addition of POs-Ca and *s*-HAp to the EPO-EO was expected to enhance the hemostasis and bone regeneration.

### Acknowledgements

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