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Chapter 3

SILOXANE-CONTAINING VATERITE / POLY (LACTIC ACID) FIBREMATS WITH IMPROVED DUCTILITY

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ABSTRACT

Siloxane-containing vaterite (SiV)/poly (L-lactic acid) (PLLA) hybrid fibremats (SiPVHs) have outstanding potential for bone regeneration applications. SiPVHs were prepared by using melt-blending and electrospinning protocols and modified by increasing PLLA content from 40 to 70 wt% (denoted by SiPVH60 and SiPVH30, respectively). The tensile strength of SiPVH30 was 10 times higher than that of SiPVH60. The elongation before the failure of SiPVH30 was twice as large as that for SiPVH60. The necking of fibers occurred at the failure points in SiPVH30 but not in SiPVH60. Ionic silicon-species in Tris-buffer solution from SiPVH30 was the relatively constant release. SiPVH30 could also be coated with hydroxyapatite by being soaked in simulated body fluid. A new type of membrane was prepared by bonding SiPVH60 fibremat with PLLA. The bilayered membrane showed

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flexibility, relatively high mechanical strength to allow easy handling, and not only functions as the barrier to control the intrusion of soft tissue but also the function of a 3-D scaffold which enhances the cell activation and the bone formation.

Keywords: Siloxane, Calcium, Poly(lactic acid), Fibremat, Electrospinning

INTRODUCTION

There are many materials for bone reconstruction on the market. Calcium phosphate ceramics such as hydroxyapatite and tricalcium phosphate and silica-based glasses such as Novabone® (bioactive glasses) are very popular[1]. They have been used clinically as bone graft substitute materials as they can bond to natural bone directly. Composite materials out of such ceramic or glass and a biodegradable polymer have been widely investigated to create materials with bioactivity, high toughness and flexibility[2-4]. Biodegradable polymers used clinically are poly (L-lactic acid) (PLLA) and poly(glycolic acid) (PGA), etc. Calcium phosphate ceramics or bioactive glasses can release calcium and phosphate ions, which contribute to the formation of hydroxycarbonate apatite (HCA) and new bone, through the biodegradation of the polymer in living body.

Scaffolds out of the biodegradable polymer have been developed for use in bone reconstruction, tissue engineering and drug delivery systems[5,6]. Three-dimensional and porous scaffolds are common and regarded to be useful in biomaterials. The pores in the scaffolds are regarded to interconnect with each other and have pore diameters of at least 100 μm to allow cell migration, bone ingrowth and eventually vascularization [7,8]. Various types of porous scaffolds out of calcium phosphate ceramics, bioactive glasses and their composites with biodegradable polymers have been developed.

Electrospinnig is one of the processes that can generate polymer fibremats with high porosity and flexibility, both essential components of the scaffolds[9-11]. The process of electrospinning is affected by both system parameters and process parameters. System parameters include polymer molecular weight and polymer solution properties, such as viscosity and conductivity. Process parameters, on the other hand, involve the flow rate of the polymer solution, electric potential and the distance between the capillary and collector, among others [9]. Fibre diameter and thickness of fibre mesh scaffolds can be controlled by optimizing these parameters.

It is necessary to choose the appropriate materials for optimizing the mechanical and biomimetic properties of a fibre mesh, since the orientation and diameter of the fibres in electrospun scaffolds can influence cellular compatibility [12-14]. The fibre diameters in electrospun scaffolds have, for example, been reported to influence osteoblast proliferation and mineralization.

Guided bone regeneration (GBR) is one of the techniques for regenerating bones in particular to achieve the dental implant. When a dental implant is fixed in jaw bone, if thickness of alveolar bone is not enough for achieving the implantation, bone regeneration is needed. A defect formed in alveolar bone for the implantation is covered with a membrane, so called "GBR membrane", is prone to prevent the intrusion of soft tissue such as gingival epithelial and connective tissues into the defect and to keep enough space for the proliferation and mineralization of osteogenic cells. The bone regeneration in the defect should be accelerated by the selective growth of the osteogenic cells there. However, it usually takes for about six months to completely reconstruct the alveolar bone, even if the GBR is applied. The GBR membranes require flexibility to adapt to the bone shape with defect and strength to maintain the space for bone formation and to connect with the soft tissues. They require an additional property, enhancement of the bone formation.

Ionic dissolution products from inorganic biomaterials have been attracting attention and regarded as a key to understand bioactivities of the materials in body. Many elements, calcium, phosphorus, strontium, copper, zinc, boron, vanadium, magnesium and cobalt, have been reported to have stimulatory effects on osteogenic cells[15]. Silicon species is one of the essential, trace elements for healthy bone and have been recently reported to stimulate functions of osteogenic cells[7, 16-20]. This has been supported by the reports about bioactive glasses, such as Bioglass® (45 % SiO_2, 24.5 % CaO, 24.5 % Na_2O, 6 % P_2O_5). Ionic dissolution products released from Bioglass® stimulated osteogenic cells at the genetic level. Xynos *et al*. reported that some gene expressions in human osteoblasts were stimulated by silicon species in a culture medium, derived from Bioglass®[17,18]. They also reported that the expression of insulin-like growth factor II in human osteoblasts was enhanced by silicon species in the culture medium, which stimulated the cells to proliferate. Reffitt*et al.* reported that orthosilicic acid in a culture medium was effective to stimulate collagen type I synthesis and osteoblastic differentiation in human osteoblast-like cells [20].

Our previous studies reported that a composite material of PLLA and siloxane-containing calcium carbonate (vaterite) (SiV) exhibits outstanding

potentials for bone regeneration applications as it enhances the proliferation and mineralization of osteoblast-like cells *in vitro* and the formation of mineralized tissue *in vivo*[21, 22]. This composite material releases a trace amount of silicon in a cell culture medium and shows an HCA-forming ability in simulated body fluid in one day. Osteoblast-like cells (MC3T3-E1 cells) proliferated and mineralized on the composite membrane.

Fibremats out of the composite material were successfully prepared by an electrospinning method [22-25]. SiV should play an important role in the enhancement of their osteoconductivity, since it releases both calcium and silicon ionic species. We have found that it is possible to prepare the composite material containing ~60 wt% of SiV in the system without the aggregation of SiV particles in a polymer matrix. Too much SiV, however, should cause a brittleness of the composite material. In spite of the success of the fibremat, mechanical properties such as tensile strength and ductility (in the present work, ductility refers to the elongation of the fibremats before failure) are still required to be improved for the applications. The present communication shows the fibremat out of the SiV/PLLA composite material improved in mechanical properties by manipulating its composition or incorporating with a PLLA membrane.

SILOXANE-CONTAINING VATERITE

Silicon-containing vaterite (SiV) powders were prepared by a carbonation process with methanol. A sample of 150 g of $Ca(OH)_2$ was mixed with 60 mL of aminopropyltriethoxysilane (APTES) and 2000 mL of methanol with CO_2 atmosphere for 75 min at 2000 mL min^{-1}. The resulting slurry was dried at 110 ˚C, resulting in the SiV powders.The amount of silicon in SiV was estimated to be approximately 2 wt% by X-ray fluorescence analysis. Results of X-ray diffractometry indicated that SiV consisted of only vaterite crystalline phase. The specific surface area of SiV was estimated to be 67.2 m^2g^{-1} according to BET theory using nitrogen gas adsorption.

The morphology of SiV particles was observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SiV particles were aggregates consisting of several primary particles with the size of several hundreds nm (Figure 1). The TEM images in Figure 2 show the morphologies of the SiV particles. The low-magnification TEM image (Figure 2(a)) showed the particle size of the SiV, which is approximately 1 μm and these results were consistent with the literature[21]. The high-magnification

TEM image (Figure 2(b)) revealed the nanostructure of the SiV particles, consisting of smaller primary nanoparticles with diameters approximately 20 nm.

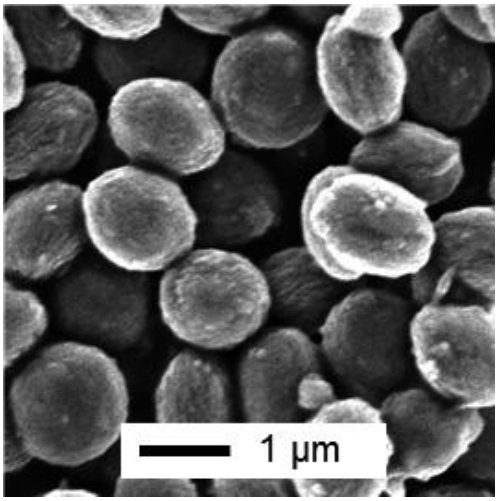

Figure 1. SEM image of SiV particles.

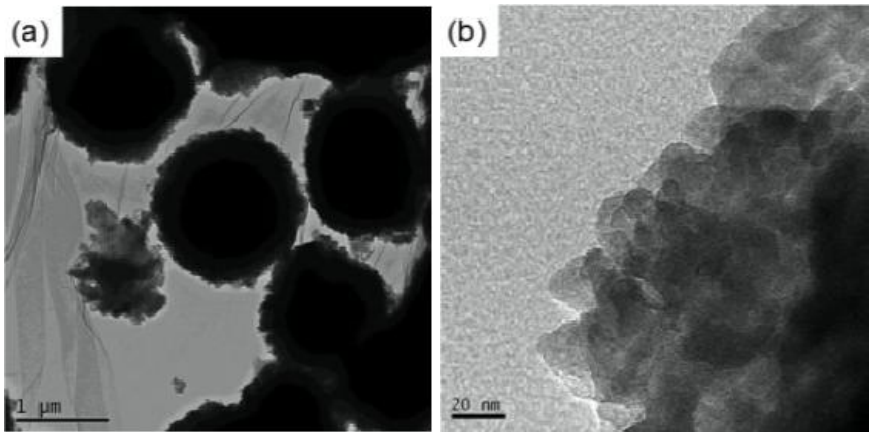

Figure 2. TEM images of SiV particles. (a) lower magnification, (b) higher magnification.

SiV/PLLA Composite Fibremats

Electrospinning of Composite Materials

The fibremats were prepared by melt-blending 40 wt% PLLA (molecular weight (M_W): 160 kDa) with 60 wt% SiV particles at 200 ^oC for 10 min. The products after the melt-blending were dissolved in chloroform to prepare 10 wt% solution, which was used to conduct electrospinning at 20 kV to obtain siloxane/poly(lactic acid)/vaterite hybrid fibremats (denoted by SiPVHs). In order to modify SiPVHs, the SiV particles content was changed from 60 wt% to 30 wt% (denoted by SiPVH60 and SiPVH30, respectively). The morphology of samples was observed by SEM. Comparison between the two materials was conducted in terms of mechanical properties and ion releasability.

SEM images in Figure 3 show the morphologies of SiPVH30 and SiPVH60. Microscopically, there were many pores (< 1 μm) in SiPVH30. Since chloroform is a relatively poor solvent for PLLA, the interaction between PLLA and chloroform is not sufficient to prevent phase separation in the SiPVH30 solution. The quick evaporation of pure chloroform phases could leave the pores shown in Figure 3(a). There were few pores in SiPVH60, which is due to the relatively lower polymer content in SiPVH60 and less evaporated chloroform during the electrospinning process. The primary fibre diameters of all the samples were found to be approximately 10 μm.

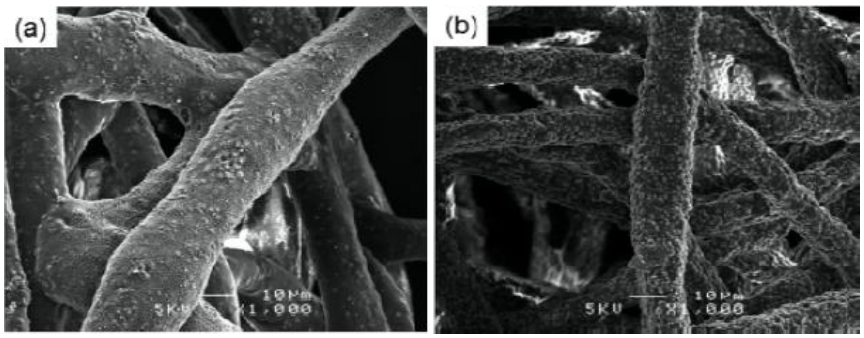

Figure 3. SEM images of (a) SiPVH30 and (b) SiPVH60.

The viscosity of chloroform solutions before the electrospinning process was measured with a viscosimeter. The differences in viscosity could also be explained by the different affinities between the chloroform and the polymer. The viscosity of SiPVH30 chloroform solution was 4582 Pa·s. The viscosity of the solution could affect the stabilisation of the Taylor cone during the electrospinning process. The PLLA chloroform solution was relatively less well-detangled and the entangled PLLA polymer chains increased the solution viscosity and enhanced the single-fibre electrospinning. This indicates that the affinity between the organic solvent and the polymer during the electrospinning process is critical to control the solution viscosity for electrospinning and in turn control the final fibre diameter.

Ion Release from Fibremats

The amount of silicon released from SiPVH30 or SiPVH60 fibremat in a buffer solution was measured. The fibremats of 15 mm in diameter with 100 ~ 120 μm in thickness were soaked in 4 mL of Tris buffer solution (TBS) at 37 ^{o}C for 5 days. The TBS media after 1, 3, and 5 days of soaking were refreshed and analysed by inductively coupled plasma atomic emission spectroscopy (ICP-AES). The silicon ion concentrations released from the fibremat in TBS are shown in Figure 4. Almost no ions released from SiPVH60 during the 4th and 5th days of the soaking test. SiPVH30 constantly released the ions. The ion release behaviour of the fibremat may be influenced by the composite structure in the fibres, that is, the condition of SiV particles in the polymer matrices. In SiPVH60, much SiV particles were exposed to TBS and immediately released the ions in 3 days after soaking. The particles might be in contact with each other in the fibre and there should be a lot of path for TBS to permeate into the inside of fibre. The amount of SiV particles exposed to TBS and such path in the fibre should be little in SiPVH30. The ions were expected to be released slowly from each sample with the polymer degradation when they were soaked in TBS for longer, since the amount of the ions released from each sample over 5 days of soaking was just a little bit of the total amount of the ions contained in the sample.

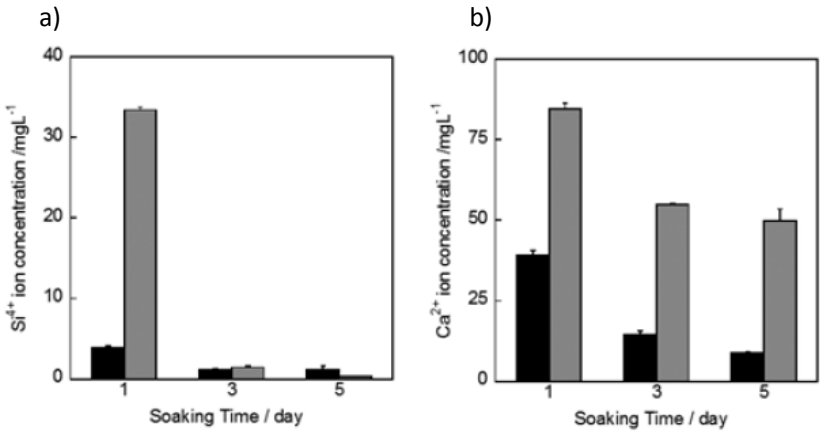

Figure 4. Concentrations of (a) Si4+ and (b) Ca2+ ions released from SiPVH30 and SiPVH60 after soaking in TBS for 5 days.

Mechanical Properties of Fibremats

In order to obtain fundamental data on mechanical properties, Japanese industrial standard tensile tests JIS L1015 were conducted in air at room temperature to measure their tensile strengths and elongation before failure (test details: span length, 20 mm; sample dimension, 40×5×0.1 mm^3; crosshead speed, 20 mm min^{-1}). The failure points of the samples after the tensile tests were observed by using SEM.

The tensile strength and elongation before failure of fibremats SiPVH30 and SiPVH60 are shown in Figure 5. The mechanical test results showed that the tensile strength and elongation before failure of SiPVH30 were 3.3 MPa and 3.0%, respectively, which were significantly higher than those of SiPVH60 (0.3 MPa and 1.6%).The SEM images in Figure 6 show the failure points of fibremats SiPVH30 and SiPVH60 after the tensile tests. Necking occurred at the failure points of SiPVH30, whereas no necking occurred at the failure points of SiPVH60 fibres.

Since PLLA is much more ductile than SiV particles, the PLLA matrix in the SiPVH30 fibres is suggested to induce the necking, which resulted in strain hardening and elongation of the fibres. The strain hardening and elongation of the fibres in SiPVH30 can in turn contribute to the enhanced tensile strength and enlarge elongation before failure, respectively. This indicates the high

polymer content in the fibremats could enhance the mechanical properties of bone filler materials.

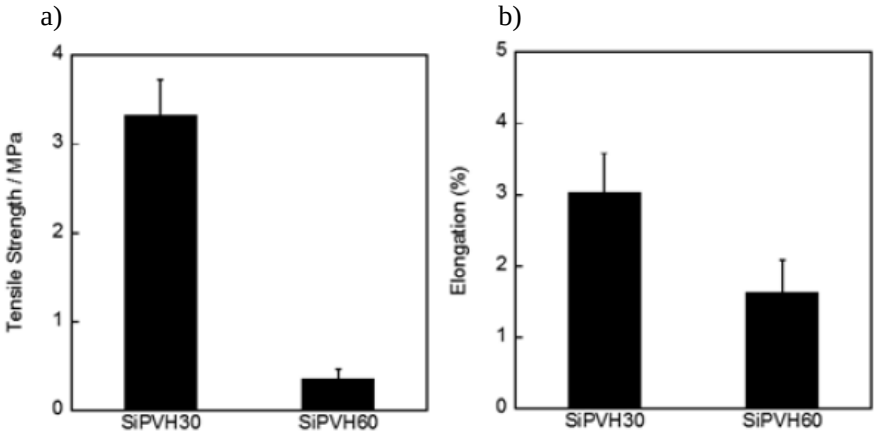

Figure 5.Mechanical properties of SiPVH30 and SiPVH60.(a) Tensile strength and (b) elongation before failure.

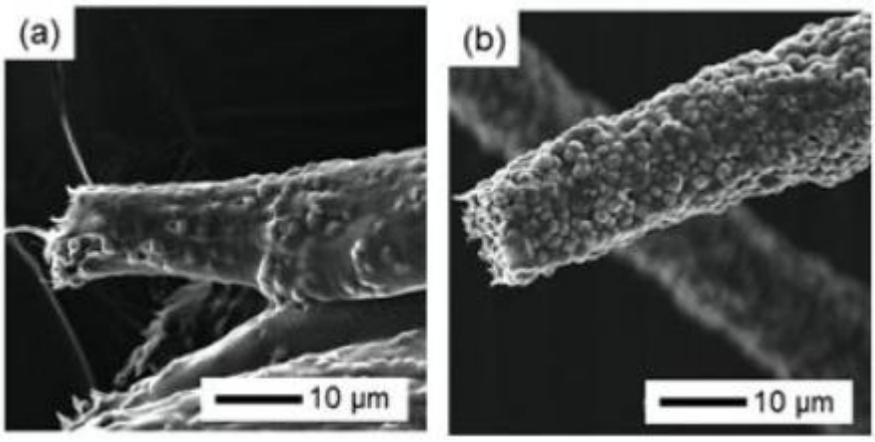

Figure 6. SEM images of the failure points of (a) SiPVH30 and (b) SiPVH60 after the tensile tests.

HCA-Coating on Fibre Surfaces

The fibremats coated with HCA can enhance cellular activities such as attachment and proliferation. HCA-forming on SiPVH30 and SiPVH60 fibremat was done by soaking in concentrated simulated body fluid (1.5 SBF) at 37 °C for 1 and 3 days. The 1.5 SBF consists of 3.75 mM of Ca^{2+}, 213.0 mM of Na^{+}, 2.25 mM of Mg^{2+}, 7.5 mM of K^{+}, 223.2 mM of Cl^{-}, 6.3 mM of HCO_3^{-}, 1.5 mM of HPO_4^{2+} and 0.75 mM of SO_4^{2-}, adjusted at pH 7.4 by including $(CH_2OH)_3CNH_2$ and HCl[26]. The surfaces of the fibremats after soaking were observed by SEM (Figure 7) to be almost completely covered with HCA, which was confirmed by XRD. Cauliflower-like deposits were partially observed on the fibre surfaces in SiPVH30 after 1day-soaking although the fibre surfaces in SiPVH60 were covered with the deposits. The fibre surfaces in SiPVH30 were completely covered with the deposits after 3days soaking. The HCA deposition obviously related to the content of SiV particles in the fibremat. In our preliminary experiment, no HCA deposited on the fibre surfaces in SiPVHfibremats with ~20 wt% of SiV particles, when they were soaked in 1.5SBF for 1day. We could conclude the SiPVH containing more than 30 wt% of SiV particles should be one of attractive candidates for biomaterials with enhancement of bone formation in body.

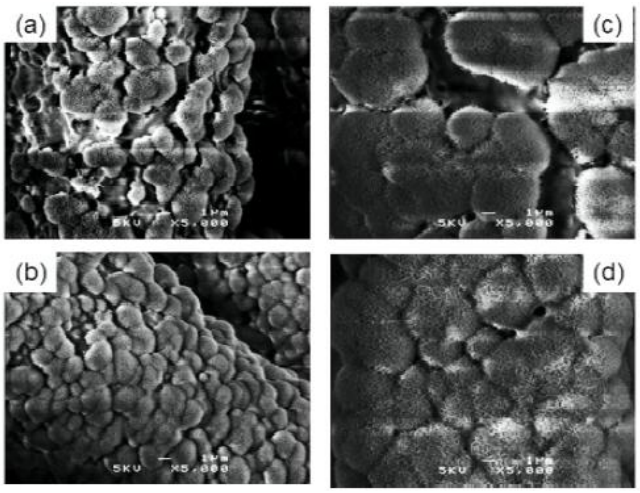

Figure 7. SEM images of the surfaces of (a,c) SiPVH30 and (b,d) SiPVH60 after (a,b) 1 days and (c,d) 3 days of soaking in 1.5SBF.

BILAYERED FIBREMATS

Two Different Fibremats

PLLA fibremats were prepared by an electrospinning method and pressed at 15 MPa at room temperature to reduce the sizes of the spaces between the fibres for the purpose of preventing the intrusion of soft tissue. The pressed PLLA fibremat was incorporated with as-prepared SiPVH60 fibremat by hot-pressing. The pressed PLLA fibremat with ~100 μm in thickness was placed on top of the SiPVH60 one, and then pressed at ~ 0.2 MPa with a stainless steel mesh with an opening size of ~ 400 μm which was heated at 150 ^{o}C for 10 s, resulting in a bilayeredfibremat. The bilayeredfibremat was soaked in 1.5SBF.

Figure 8 shows the cross-section and the surface of the bilayeredfibremat. The fibres were fused at the hot-pressed regions. There were no significant changes in the shapes of fibres and the spaces between the fibres in the area that was not hot-pressed. No coating was observed on the PLLA fibres even after soaking in 1.5SBF, although SiPVH60 fibres were coated with cauliflower-like HCA. The resulting bilayeredfibremat was flexible and also strong enough to avoid detachment and damage when pulled by hand.

Cell Attachment

Mouse osteoblast-like cells (MC3T3-E1 cells) were seeded onto the bilayeredfibremat (SiPVH60 surface) placed in a 24-well plate at a density of 30,000 cells/well and incubated for 5 days. Alpha minimum essential medium (αMEM) containing 10 % fetal bovine serum (FBS) was used as a culture medium. After culturing, the cells were incubated for Alexa Fluor 488 Phalloidin and viewed with a fluorescence microscope.

Figure 9 shows the cell morphology on the bilayeredfibremat. An image of the bilayeredfibremat after culturing which was taken with a light microscope was superimposed on the cell image taken with a fluorescence microscope to find where in the fibremat the cells attached. The cells were observed around the bonded portion, which was formed by the hot-pressing. The fibre structure with small-sized spaces, that is, the bonded portion might not be favoured for the cell attachment in this sample.

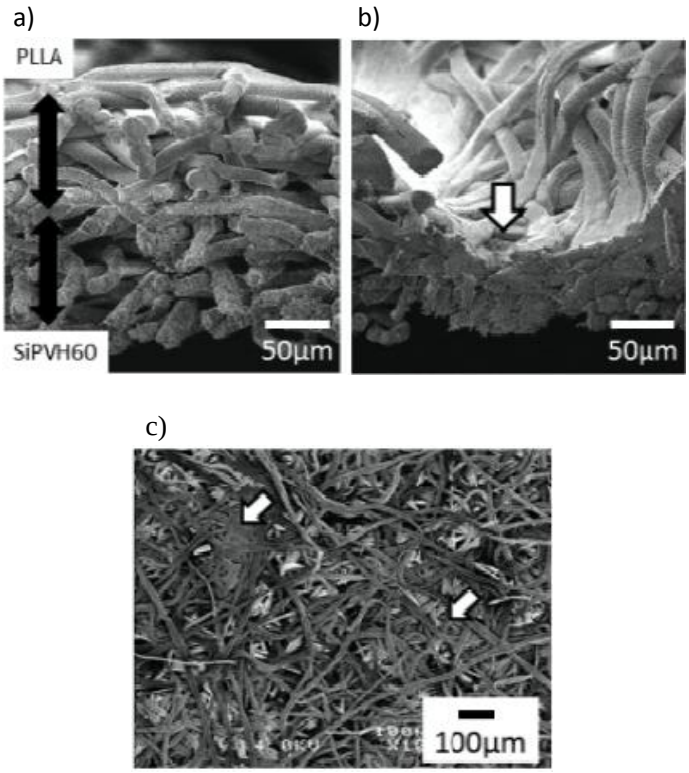

Figure 8. SEM images of the cross-sections (a) in an unbonded portion and (b) around the bonded portion and (c) the surfaces of SiPVH60 after hot-pressing. Black arrows indicate the area of PLLA or SiPVH60.White arrows indicate the bonded portion.

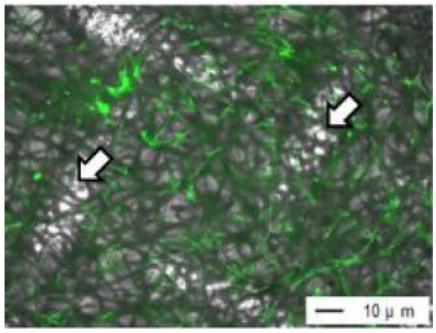

Figure 9. Morphology of osteoblast-like cells (MC3T3-E1 cells) on the bilayeredfibremat after 5 days-culturing. Green indicates the cells. Arrows indicate the bonded portion.

Tissue Compatibility

The tissue compatibility and bone-forming ability of the bilayeredfibremat was evaluated by *in vivo* tests using rabbits. An 8-mm diameter hole was drilled into the front midline of the animal's calvaria using a bone cutter and then covered with the bilayeredfibremat. An 8-mm diameter hole in bone is generally not regenerated if remained to be done. The bilayeredfibremat was fixed by putting between skin and bone. The bilayeredfibremat was implanted with SiPVH60 fibremat on the side in contact with the hole and dense PLLA one on the side in contact with the skin.

Figure 10 shows the histology of *in vivo* response to the bilayeredfibremat and the new bone formation at the center of the holes. Villanueva Goldner stain showed that mineralized tissue formation started in the bonded portion formed by hot-pressing. Mineralized tissue was formed over almost the entire area of implanted SiPVH60 fibremat after 12 weeks. There was no tissue inflammation observed by histology. The bone formation started from the inside of SiPVH60 fibremat and not from the edge of the hole. In our preliminary experiment, a poly(lactide-*co*-glycolide) fibremat was implanted in the same position for 12 weeks and new bone was not to be formed in/on the mesh at the center of an 8-mm hole. The bilayeredfibremat is expected to show not only the function of the barrier membrane to control the intrusion of the soft tissue but also importantly the function of a scaffold with three dimensional structure, cell activation and enhanced bone-forming ability.

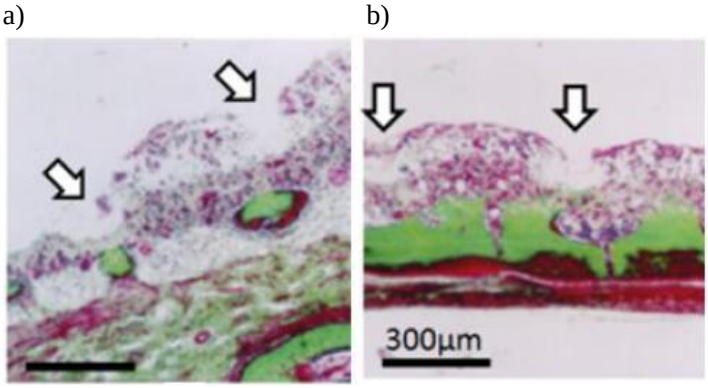

Figure 10. Histology of in vivo response to the bilayeredfibremats after (a) 4 weeks and (b) 12 weeks. Villanueva-Goldner staining shows mineralized tissue formed in the fibremat at the center of the holes. Green indicates mineralized tissues. Arrows indicate the bonded portion.

CONCLUSION

The PLLA-based composite fibremat releasing silicon and calcium species was prepared by an electrospinning method. The results showed here suggested the fibremat as an attractive candidate for GBR membrane with an ability enhancing bone formation in body. SiV particles, which are source of the species, were successfully incorporated with a PLLA matrix. The mechanical properties such as tensile strength and elongation of the fibremat strongly related to the content of SiV in the composite. It is necessary to find an appropriate weight ratio of polymer matrix: SiV particle to get a fibremat showing flexibility, strong, and releasing ability of silicon and calcium species enough for enhancement of bone regeneration. We suggest a new technique for fabricating such fibremat; incorporating two different fibremat, PLLA fibremat and SiPVH60 one, by hot-pressing with a stainless mesh. The bonded portion formed by hot-pressing played an important role both in the cell attachment *in vitro* and bone formation *in vivo*, particularly it was a very interesting to find the mineralized tissue formation started around the bonded portion.

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