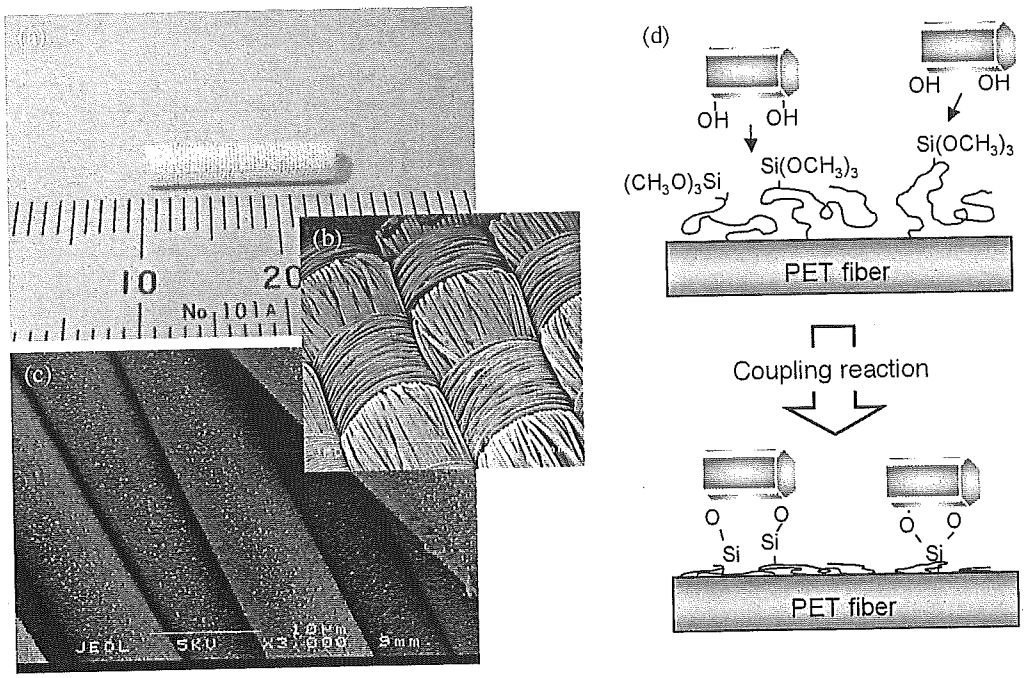

Figure 25 Furuzono et al.

Increase of cell adhesiveness on poly(ethylene terephthalate) fabric by coating of sintered hydroxyapatite nanocrystals for development of an artificial blood vessel

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Running head: HAp/PET composite for artificial vessel (less than 40 characters)

Keywords: poly(ethylene terephthalate); hydroxyapatite; nanocrystal; surface modification; cell adhesiveness

Abstract

Nano-scaled sintered hydroxyapatite (HAp) were covalently linked onto a poly(ethylene terephthalate) (PET) fabric substrate chemically modified by graft polymerization with γ-methacryloxypropyl triethoxysilane (MPTS). The weight gain of graft polymerization with poly(MPTS) on PET in benzyl alcohol containing H_2O_2 as an initiator increased with increasing the reaction time and finally reached a plateau value of about 3.5 wt%. The surface characterization of surface modification with poly(MPTS)-grafting was conducted by X-ray photoelectron spectroscopy. HAp nanocrystals about 50 nm in diameter, mono-dispersed in pure ethanol, were coupled with alkoxysilyl groups of the poly(MPTS)-grafted PET substrate. The HAp nanocrystals were uniform and strongly coated on the surface of the PET fabrics, although HAp particles adsorbed physically on the original PET without poly(MPTS) grafting were almost removed by ultrasonic wave treatment. Human umbilical vein endothelial cells (HUVEC) adhered more plentifully on the HAp/PET composite fabric compared to original PET and collagen-coated PET after only 4 h of initial incubation. The coating of sintered HAp nanocrystals was able to impart bioactivity to the polyester substrate that is a widely used biomedical polymer without a coating of adhesion proteins derived from animals, such as collagen or gelatin.

Introduction

Hydroxyapatite (HAp) has attracted considerable attention as hard-tissue-compatible material for implants and bone augmentation procedures, because it bonds directly to bone when implanted,[1-5] resulting in the formation of a strong bone-implant interface. In addition, HAp ceramics are also known as being compatible with soft tissue such as skin through the development of percutaneous devices.[6] We have developed an inorganic-organic composite consisting of nano-scaled sintered HAp crystals[7-9] and biomedical polymers (such as silk fiber) via covalent bonding at the interface.[10-12] Recently, a novel percutaneous device was developed using that inorganic-organic composite.[13] The actual effectiveness as a percutaneous device has now been evaluated by animal implant experiment. In these continuous studies, one of the reasons for using silk fiber as a polymer substrate is simply for surface-modification due to having so many functional groups on the polymer surface.

If our HAp nanocrystal coating technique can be applied to other medical polymers as well as silk fiber, the uses are expected to spread widely in medical fields. In this regard, polyester has been used as a typical and popular biomedical polymer in medical fields -- as an artificial blood vessel[14] or a ligament,[15] and so on. Medical devices made of polyester, for example, artificial blood vessels, generally, are coated with collagen or gelatin in order to increase interaction with living cells or tissue. The use of animal-derivative proteins is, however, feared due to the possible outbreak of infectious diseases such as bovine spongiform encephalopathy (BSE) but the coating of HAp shows that bioactivity is biologically safe due to having no biological derivative substances.

In this study, we developed a novel composite consisting of nano-scaled HAp

crystals and a poly(ethylene terephthalate) (PET) fabric as a polymer substrate through covalent linkage for the purpose of development of an artificial blood vessel. Donation of covalent boding between HAp and the substrate was carried out by a coupling reaction between hydroxyl groups in a HAp crystal and alkoxysilyl groups of the graft-polymer on the PET. Surface modification of polyester is relatively more difficult compared to a silk substrate because functional groups seldom exist on the polyester surface. Hydrophilic moieties were, therefore, introduced on the surface by alkaline hydrolysis as a pretreatment for graft polymerization on PET.

Materials and methods

Materials

PET fabric (NBC Inc., Tokyo, Japan) selected as a typical polyester was cleaned by soxhlet extraction with methanol for 24h, rinsed with distilled water, and dried at 60°C for 24 h. γ-Methacryloxypropyl triethoxysilane (MPTS) was kindly donated by (Shin-Etsu Chemical Industries Co. Tokyo, Japan). Benzyl alcohol (guaranteed reagent; Nakarai Teque Inc., Kyoto, Japan), methanol (superior quality of reagent; WAKO, Pure Chemicals Co. Ltd., Osaka, Japan), and 30 content H_2O_2 (superior quality of reagent; SIGMA, Tokyo, Japan) were used without further purification. Water was purified with a Milli-Q system (Millipore Corp., Bedford, Mass.). HAp crystals with an average diameter of 50 nm were prepared by an alternating emulsion system using a sintering agent such as calcium hydroxide and potassium dihydrogen phosphate and subsequently calcinated at 800°C for 1 h, as described in our previous reports.[7-9]

Graft-polymerization

Graft polymerization of MPTS onto alkaline-hydrolyzed PET fabric[16] was conducted using H_2O_2 as an initiator.[17] PET fabric was carefully immersed in a 0.2 N aqueous NaOH solution for 30 min at 60°C and then rinsed with Milli-Q water in order to generate carboxyl groups on the surface. Carboxylate-functionalized PET fabric (0.03g) was added into a 100-ml flask equipped with an inlet of N_2, a reflux condenser, and a stirrer, and then purged with N_2 for 30min. 40 ml of benzyl alcohol containing 785 μl of H_2O_2 (100 meq/L) as an initiator was added to the flask. 10 ml (50 v/v%) of MPTS monomer in benzyl alcohol was subsequently added, and the content was stirred occasionally during polymerization. For a defined period after the polymerization, the PET fabric was washed with ethanol several times to stop polymerization in order to remove ungrafted homopolymers formed during polymerization, and then dried under reduced pressure for 30 min at 70°C. The weight gain after polymerization was calculated from the following equation:

$$\text{Weight gain} = (W_2 - W_1) / W_1 \times 100$$

where W_1 and W_2 are the weight of the dried original PET and the PET after the polymerization, respectively.

Coating of HAp nanocrystals on PET

The poly(MPTS)-grafted PET fabric was soaked in the HAp suspension (2.0 wt/v%) in ethanol for 1 h at room temperature to adsorb the crystals on the grafted PET. The fabric adsorbed with HAp particles was strongly washed by stirring in ethanol, and heated at 80°C for 2 h under vacuum (1 mmHg) in order to achieve the reaction between OH groups on the HAp crystals and

ethoxysilyl groups on the poly(MPTS)-grafted PET. The composite was washed in ethanol using an ultra sonic generator for 1 min (output: 20 kHz and 35W) to remove unreacted HAp particles physically adsorbed on other particles. The composite was finally washed in a large amount of ethanol and pure water for 1 day to remove the residual organic solvents used in polymerization.

Cell adhesiveness

Human umbilical vein endothelial cells (HUVEC) were also incubated in Endothelial cell basal medium-2 (EGM-2, supplemented with heat-inactivated 5% FBS, 1mg/ml of gentamicin/amphoterisin B) in air containing 5 CO_2 at 37°C. HUVEC were plated onto the HAp/PET composite fabric in 24-well multiplates at 1 x 10^5 cells/ml in EGM-2, and incubated at 37°C for 4 h. After the fabrics adhered to the cells, they were washed twice in phosphate-buffered saline [PBS (-)], the cells were fixed with 2.5% buffered glutaraldehyde for 20min at 30°C then rinsed with PBS(-) three times. The cells were dehydrated with aqueous ethanol (50-100%) and 100% n-butanol for 5 min at room temperature step by step. The samples were lyophilized and coated with gold. The morphology of the cells on the samples were observed by SEM. In the fluorescence observation, The nuclei of HUVEC on the fabric were stained with 1 μM Hoechst 33342 (ECLIPSE TE300, Calbiochem, Tokyo, Japan), and observed by fluorescence microscopy (Nikon Tokyo, Japan). In the cell adhesion experiment, 5 collagen-coated (Cellmatrix Type I-C, Nitta Gelatin Inc. Osaka, Japan) PET fabric was used as a positive control.

Measurements

The HAp/PET composite and its cell morphology were observed with a 5 kV scanning

electron microscope (SEM; JSM-6301F, JEOL, Tokyo, Japan). To characterize the surface-modified samples, X-ray photoelectron spectroscopy (XPS, 1600S type, PHI Inc., Tokyo, Japan) was used. The power of the non-monochromated MgKα source was 100W with an investigated size of 0.8 x 2.0 mm.

Results and Discussion

Graft-polymerization

HAp nanocrystals were covalently coated on the PET fabric through poly(MPTS)-grafted polymers. Alkoxysilyl groups of MPTS can be coupled with OH groups on HAp surfaces. Before the coating of HAp crystals, it is necessary that the PET surface was modified chemically by radical donation. There are many methods of radical donation on a polymer surface in order to graft polymerize with vinyl monomers on PET, such as using high-energy radiation of γ-rays,[18] benzoyl peroxide,[19] hydrogen peroxide,[20] persulfate,[21] etc. In our case of graft polymerization of MPTS on PET, H_2O_2 in benzyl alcohol was used as an initiator according to the method of Hebeish *et al* [17] because H_2O_2 treatment is easy to handling and a large facility is not necessary. The graft polymerization of MPTS on PET after the radical donation with H_2O_2 could not actually be accomplished, although the procedure of graft polymerization was, at first, faithfully carried out. The process of weak hydrolysis for the 0.2 N aqueous NaOH solution to donate carboxylate functional groups, therefore, was added before the H_2O_2 treatment. Figure 1 shows the weight gain of poly(MPTS) on the PET fabric, which was plotted as a function of the reaction time. The weight gain of poly(MPTS) increased with increase in the reaction time, eventually reaching a plateau value of about 3.5 wt% using PET fabric consisting of a fiber

diameter of 30 μm. On the other hand, Hebeish *et al.* reported the maximum weight gain of graft polymerization of polyacrylic acid (AAc) on 1.2-denier PET consisting of a fiber diameter of 11μm in our calculation showed 4.0 wt% in the same reaction solvent.[17] From comparison of our simple calculation, the existing monomer ratios of poly(MPTS) and poly(AAc) per a unit of PET surface shows 0.9 x 10^{-3} and 1.4 x 10^{-3} mol/m^2, respectively. The difference might depend on monomer reactivity for the solvent.

In the characterization of graft polymerization of poly(MPTS) on PET using attenuated total reflection Fourier transform infrared spectrometry (ATR FT-IR), the sign of the grafting with 3.5 wt% unfortunately was not observed because the band of the Si-O-C stretching vibration of the alkoxysilyl groups overlapped with strong adsorption of an ester (C-O) stretching vibration of the PET substrate at around 1,100 cm^{-1}. Thus, XPS measurement, which enables a more sensitive surface analysis, was carried out in order to characterize the graft surface (3.5 wt%) of PET. Table 1 shows the change in the atomic proportions on the outermost surface of the original and poly(MPTS)-grafted PET. Although the atomic ratio of the carbon of poly(MPTS)-grafted PET was lower than that of the original PET, the silicon of the grafted PET was higher than that of the original one. The original PET initially contained a very small quantity of silicon contamination. The impurity is assumed to get mixed in during the manufacturing process of PET. Figure 2 shows XPS C_{1s} and O_{1s} spectra of the original and grafted PET and Table 2 indicates the quantified data from the area of the curve fitting these XPS signals. In high resolution of XPS C_{1s} spectra, they were resolved at 284.6, 28.62, 288.5 and 291.0 eV due to the C-C/C-H, C-O, -COO- and $\pi \rightarrow \pi^*$ components, respectively. Although the area of the C-C/C-H component of the grafted PET increased compared to that of the original PET, the area of the C-O and –COO- components

decreased. In O_{1s} spectra, on the other hand, they were resolved at 531.5 and 533.0 eV attributing to the O=C(O-Si) and O-C/O-H components, respectively. After graft polymerization of poly(MPTS), the area of O=C(O-Si) and O-C/O-H increased and decreased, respectively. For the control of poly(MPTS)-grafted PET, XPS measurement was conducted against the sample fabric which was adsorbed with MPTS homopolymers and washed strongly with a large amount of ethanol. The value of the silicon component was almost the same as the original PET in the XPS spectra. From the XPS results, it was clear that graft polymerization on PET was well conducted by our process.

Coating of HAp nanocrystals on PET

HAp crystals were coated on poly(MPTS)-grafted PET fabric through covalent bonding. Since the covalent bonding could not be observed directly, the chemical bonding was estimated indirectly using the FT-IR analysis described in previous literature.[10] Figure 3(a, b) show SEM photographs of HAp/PET fabric after treatment with an ultrasonic generator. As for the negative control, an SEM image of the original PET surface after ultrasonic treatment on the non-grafted PET surface adsorbed with HAp nanocrystals is shown in Figure 3 (c). Although the HAp crystals adsorbed on the original fabric were almost removed by the ultrasonic treatment, they strongly remained on the poly(MPTS)-grafted PET surface. The crystals could be uniformly coated with almost every single crystal without severe aggregations, because almost all single-dispersed nanocrystals in a medium can, recently, be developed using anti-sintering agents.[9] In the HAp crystal coating on silk fiber, the mechanical properties were almost the same as the original fiber, except for Young's modulus, which was slightly higher than the original one.[11] The

mechanical properties of HAp crystals coated on PET fiber presumed to maintain those of the original one. Actually, the fabric remained as flexibel as the original of the PET fabric.

Cell adhesiveness

To evaluate cell toxicity simply, at first the interface of cells/fabric of the original PET, poly(MPTS)-grafted PET and HAp/PET composite incubated for 2 days were observed by using an optical microscope (Figure 4). The sample fabrics were cut in half and put into the bottom of 24-well multiplate. The cells around the interface of the fabrics of poly(MPTS)-grafted PET and the HAp/PET composite adhered and proliferated well, the same as those on the original PET. Therefore, it was clear that the composite material did not express cell toxicity. Figures 5 and 6 respectively show SEM images of HUVEC morphologies and fluorescence images of stained nuclei of HUVEC on sample substrates after 4 h of incubation. The initial interaction of HUVEC on substrates was evaluated after 4h of incubation, according to several reports.[22,23] In the SEM images, it seemed that many cells adhered on HAp/PET fabric as well as collagen-coated PET, while only a few cells adhered on the original fabric. The difference in the number of cells which adhered could not be distinguished by SEM observation, since HUVEC were flattened and spread over the substrate. The cells which adhered were then stained by fluorescent stain and observed by a fluorescence microscope. It was found that the number of cells which adhered on HAp/PET was qualitatively the same as that of collagen-coated PET, although the cells seldom adhered on the original PET for such a short period of incubation. This phenomenon can be explained because cell adhesion proteins such as fibronectin or vitronectin in a culture medium may be favorably adsorbed on the HAp surface.[13] In other words, it is clear that calcinated HAp coating

on popular biomedical substrates is effective to obtain the affinity of cells without using biological scaffold proteins such as collagen or gelatin. It can be said that the HAp/PET composite is very meaningful for biological safety in medical fields today when the danger of BSE infection by using proteins derived from a bovine animal is trumpeted loudly. We fabricated a prototype of artificial blood vessel made of the HAp/PET composite (Fig. 7), and are now evaluating the effect of HAp nanocrystals on the artificial grafts through animal implantation experiments *in vivo*.

Conclusion

A novel composite consisting of nano-scaled HAp crystals and PET thorugh covalent linkage was developed. In a cell toxicity test, it was confirmed that the HAp/PET composite was not toxic, and HUVEC adhered more plentifully on the HAp/PET composite compared to the original PET and to the same degree as collagen-coated PET after only 4 h of incubation. From this result, the coating of sintered HAp nanocrystals is a simple method in order to make a polyester substrate bioactive without a coating of animal-derivative adhesion proteins such as collagen or gelatin. It is also a fact that the coating of HAp nanocrystals is superior in the terms of biological safety.

Acknowledgement

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Table 1 Atomic compositions on PET surfaces measured by XPS

Sample		C	O	Si
Original PET	(atom%)	69.5	29.5	0.64
Poly(MPTS)-grafted PET	(atom%)	60.7	29.5	9.76

Table 2 Results of the peak separations for C_{1s} and O_{1s} spectra measured by XPS

		C_{1s}			O_{1s}		
Binding energy	(eV)	284.6	286.2	288.5	291.0	531.5	533.0
Chemical state		C-H C-C	C-O	-COO-	π→π*	O=C O-Si	O-C O-H
Original PET	(atom%)	67.7	16.2	15.1	1.0	55.4	44.6
Poly(MPTS)-grafted PET	(atom%)	81.5	10.6	7.9	0.0	72.0	28.0

Figure captions

Fig. 1. Weight gain of polyMPTS-grafted PET as a function of reaction time.

Fig. 2. XPS spectrum of (a) original PET and (b) polyMPTS-grafted PET

Fig. 3. SEM photographs of HAp particles covalently coated on a PET fabric.

(a) Lower magnification of HAp/PET composite surface after ultrasonic treatment, (b) higher magnification of HAp/PET composite surface of (a), (c) higher magnification of HAp nanocrystals adsorbed on original PET surface after ultrasonic treatment.

Fig. 4. Optical microscopic images of cell toxicity on (a) original PET, (b)poly(MPTS)-grafted PET and (c) HAp/PET composite were incubated after 24hr.

Fig. 5 SEM photographs of HUVEC adhering on (a) original PET, (b) collagen-coated PET and (c) HAp/PET composite.

Fig. 6 Fluorescence photographs of HUVEC nuclei on (a) original PET fabric, (b) collagen-coated PET and (c) HAp/PET composite.

Fig. 7 The images show prototype of an artificial blood vessel made of HAp/PET composite. (a) An external view of the prototype. (b) A SEM image of inside surface of HAp/PET fibers of the prototype.

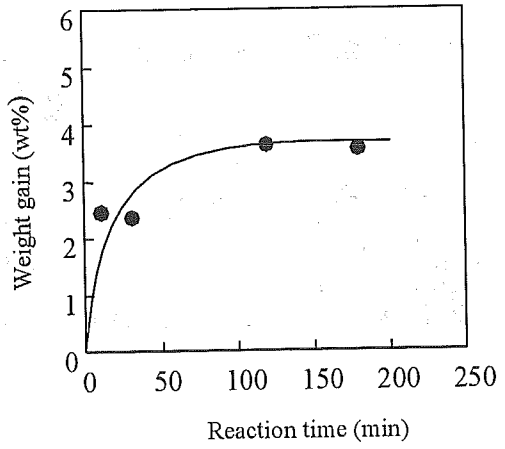

Fig. 1 FURUZONO et al.

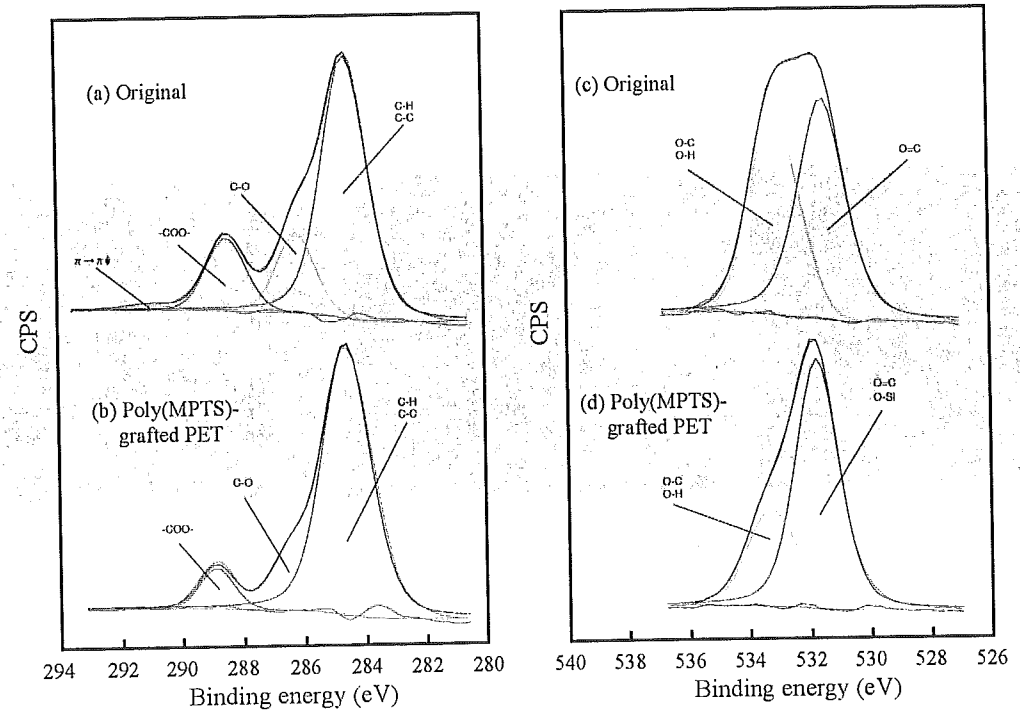

Fig. 2 FURUZONO *et al.*

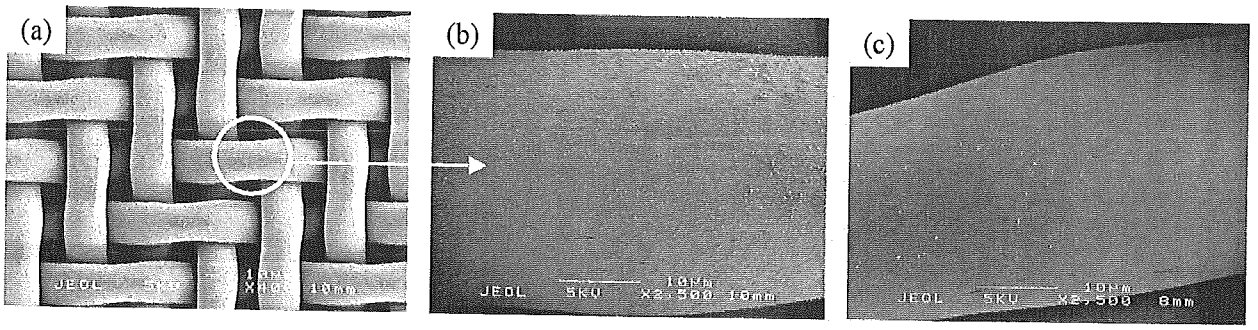

Fig. 3 FURUZONO et al.

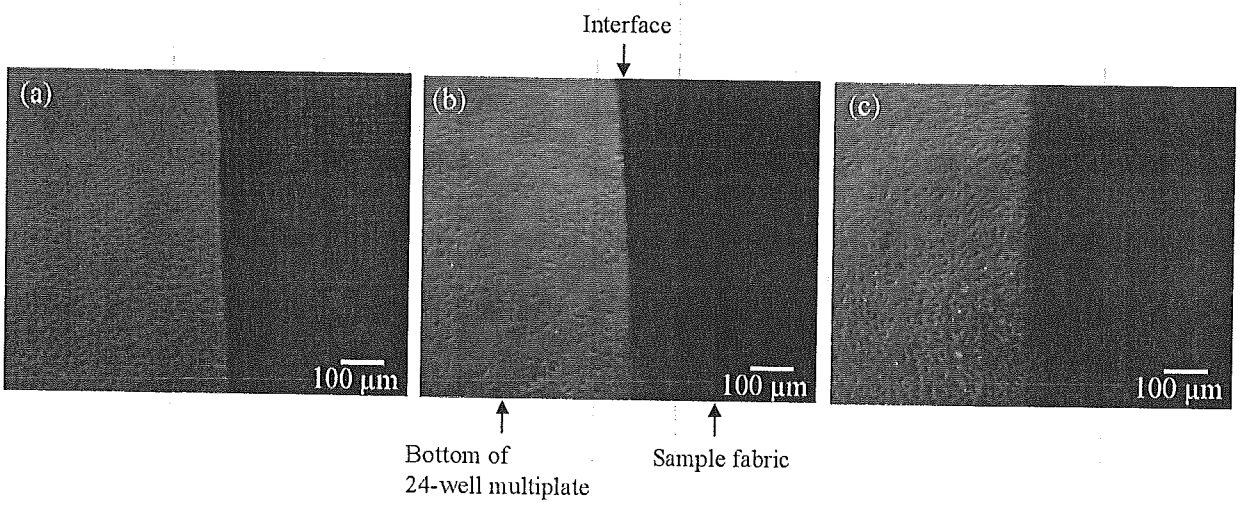

Fig. 4 FURUZONO *et al.*