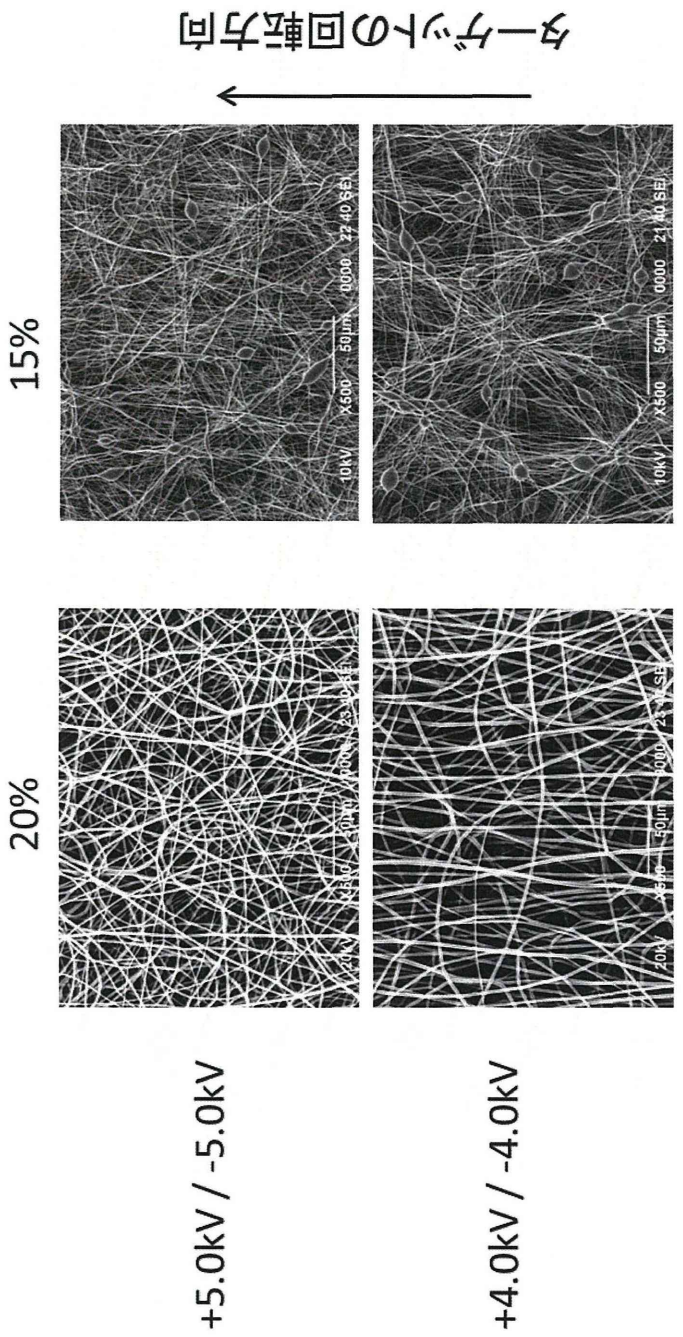

図３２．円板型ターゲットへ電界紡糸したポリ L-乳酸ファイバーの形態
[走査型電子顕微鏡による観察]

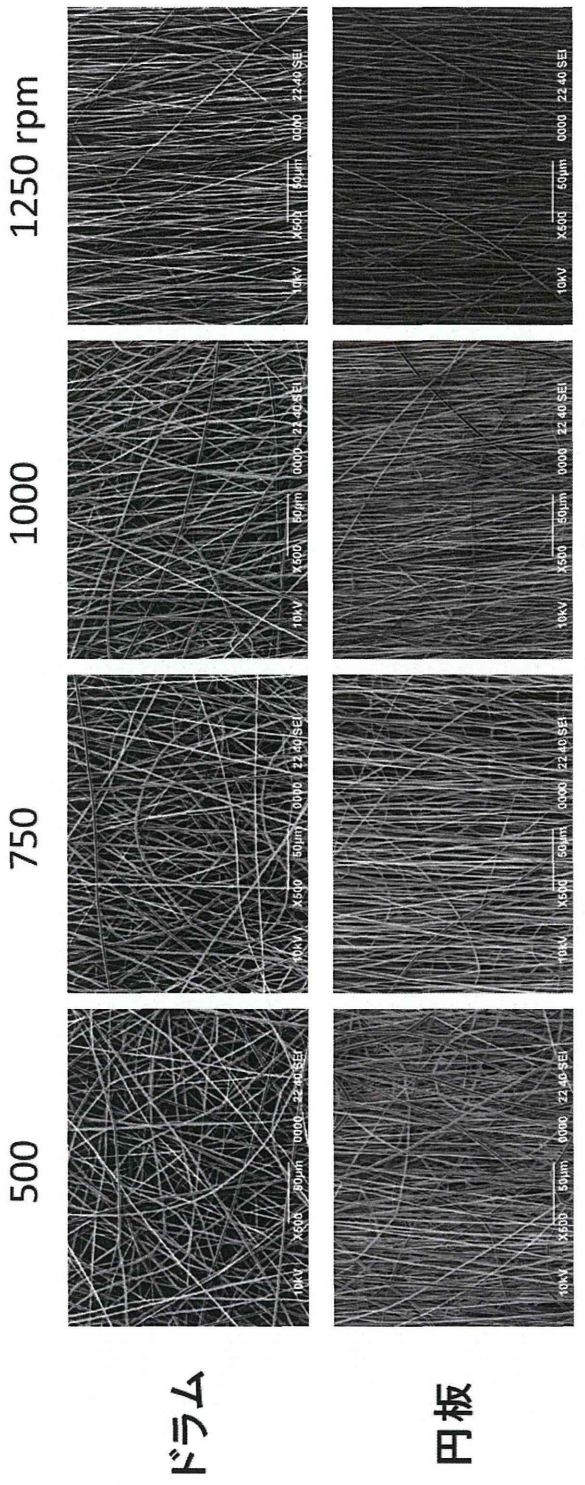

図３３．ポリ L-乳酸マイクロファイバーの配向化条件の検討
ドラム型および円板型ターゲットの比較

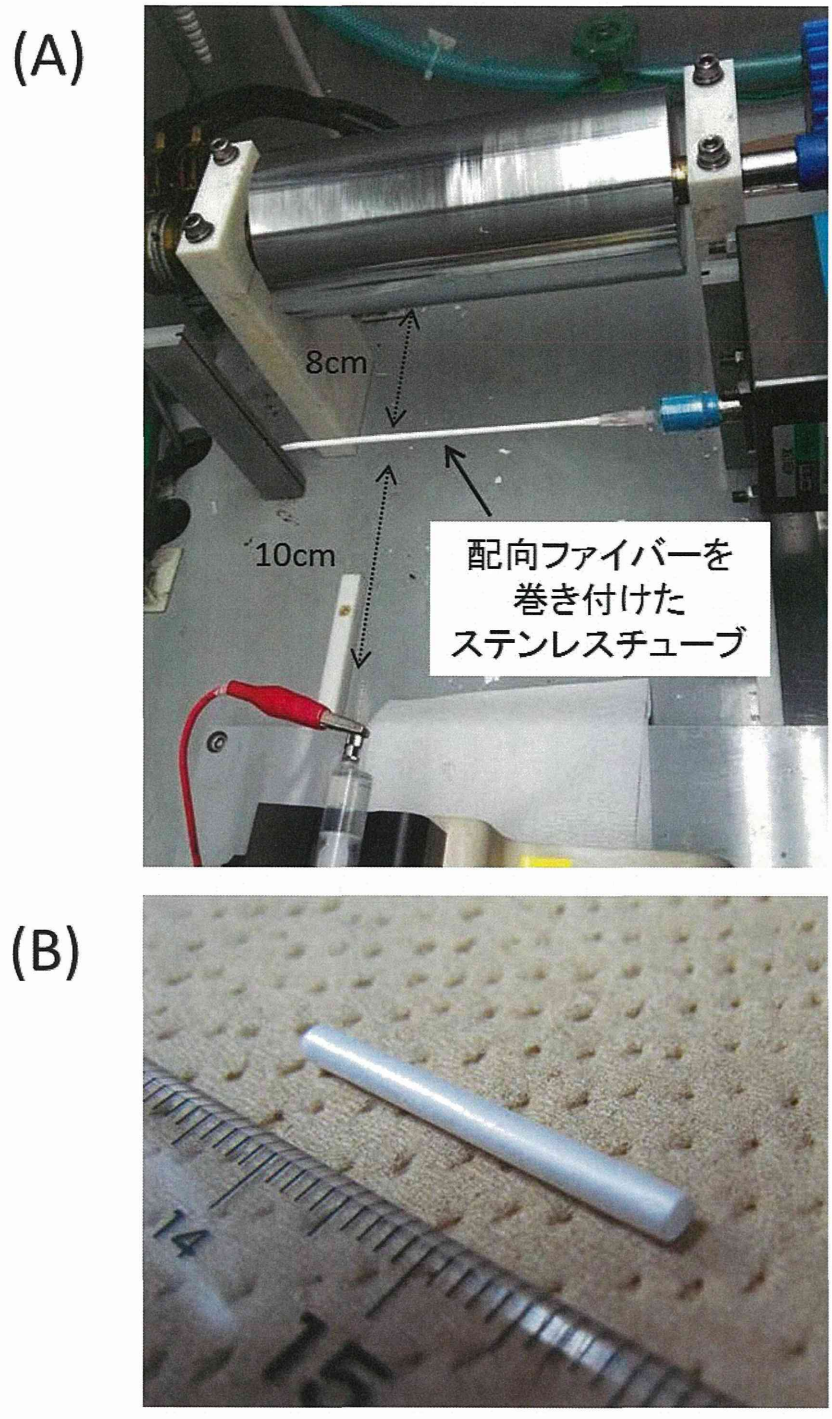

図３４．配向性ポリ L-乳酸マイクロファイバーを内層にもつチューブの試作
（A）チューブ作製に用いた装置
（B）試作したチューブ

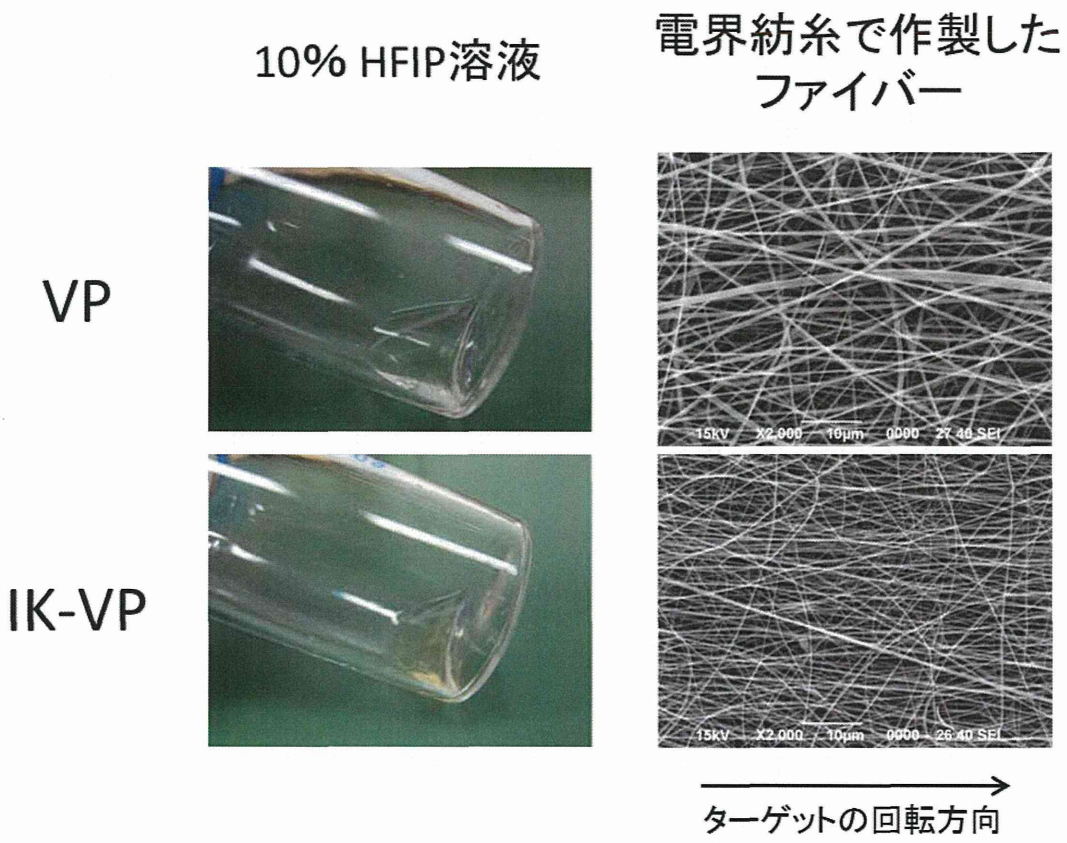

図３５．ＶＰおよびＩＫ－ＶＰのみでなるマイクロファイバーの電解紡糸

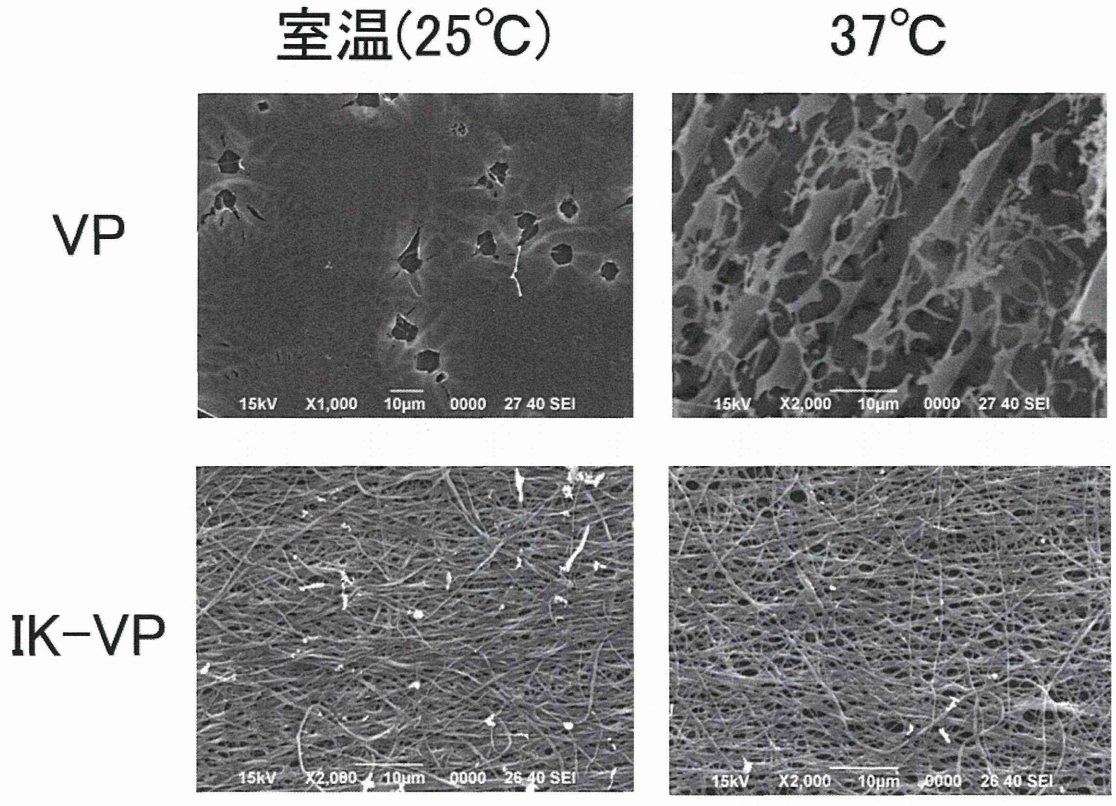

図３６．ＶＰおよびＩＫ－ＶＰマイクロファイバーの
ＰＢＳ中３時間浸漬時の形態変化

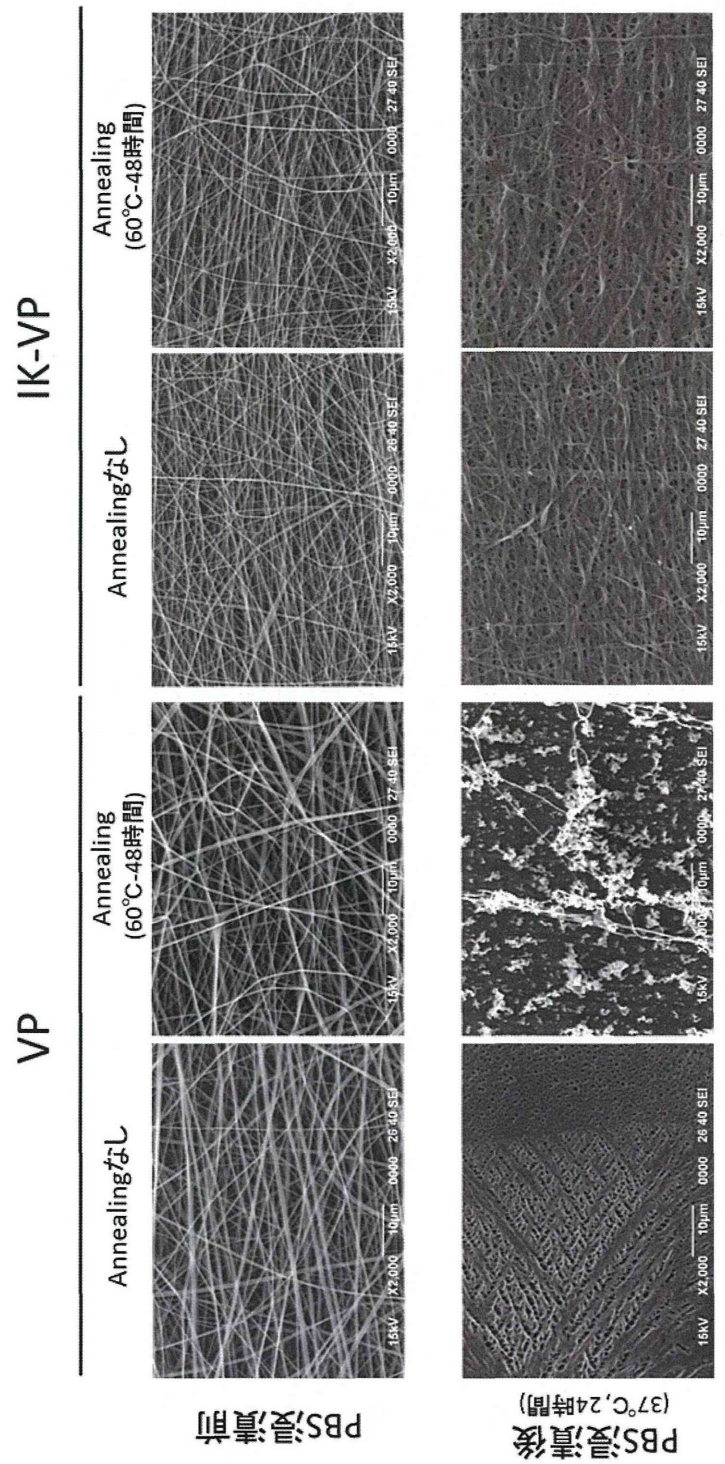

図３７．ＶＰおよびＩＫ－ＶＰマイクロファイバーの安定性に対する
アニーリングの効果の検証

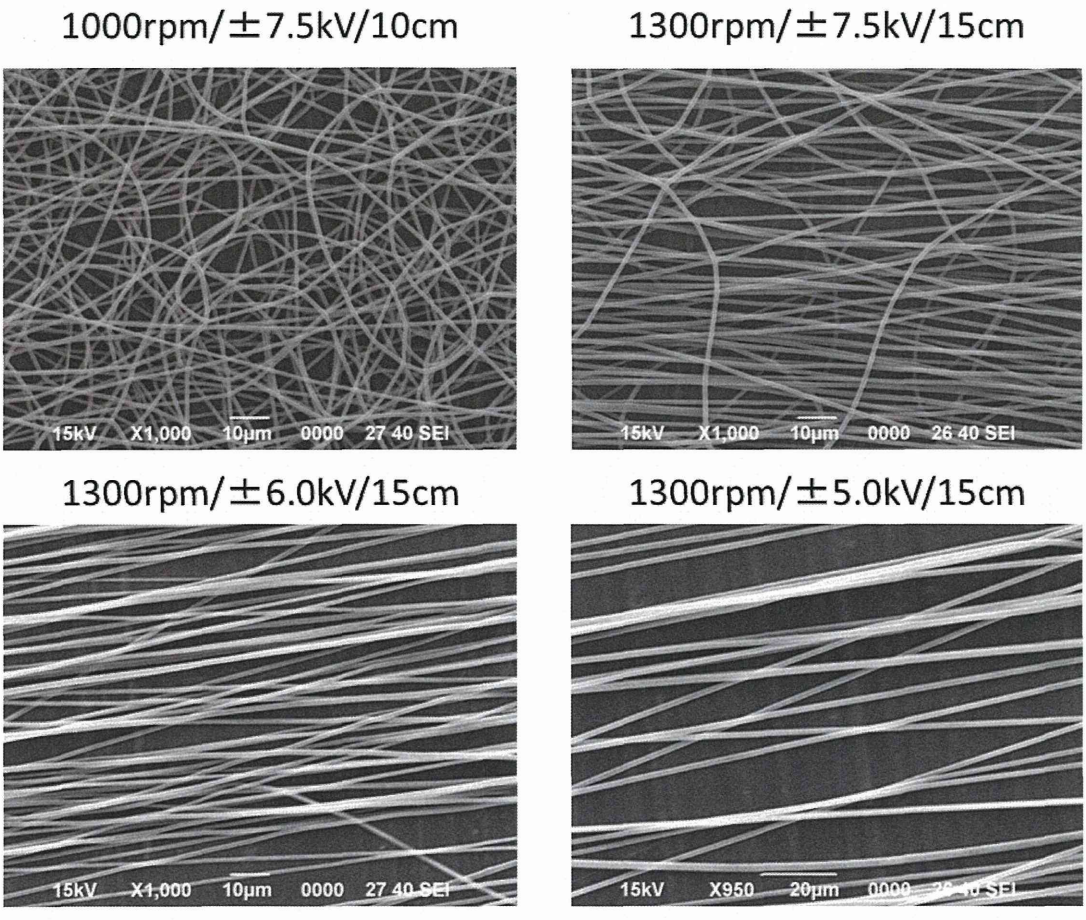

図３８．ＶＰとポリＬ－乳酸混合マイクロファイバーの作製と
配向化条件の検討

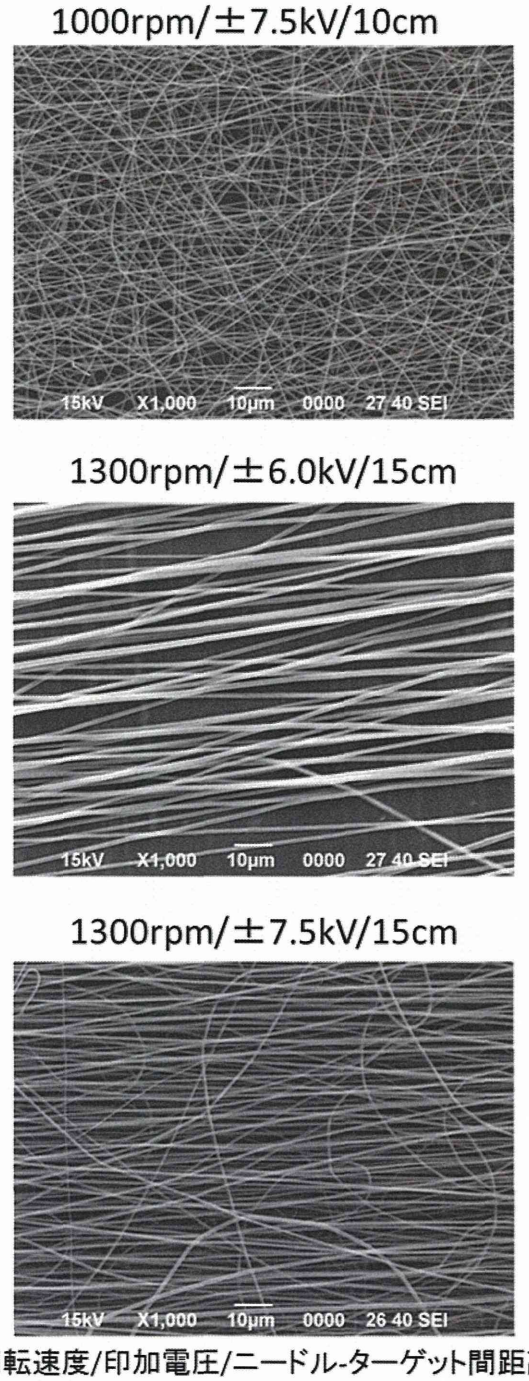

図３９．ＩＫ－ＶＰとポリＬ－乳酸混合マイクロファイバーの作製と
配向化条件の検討

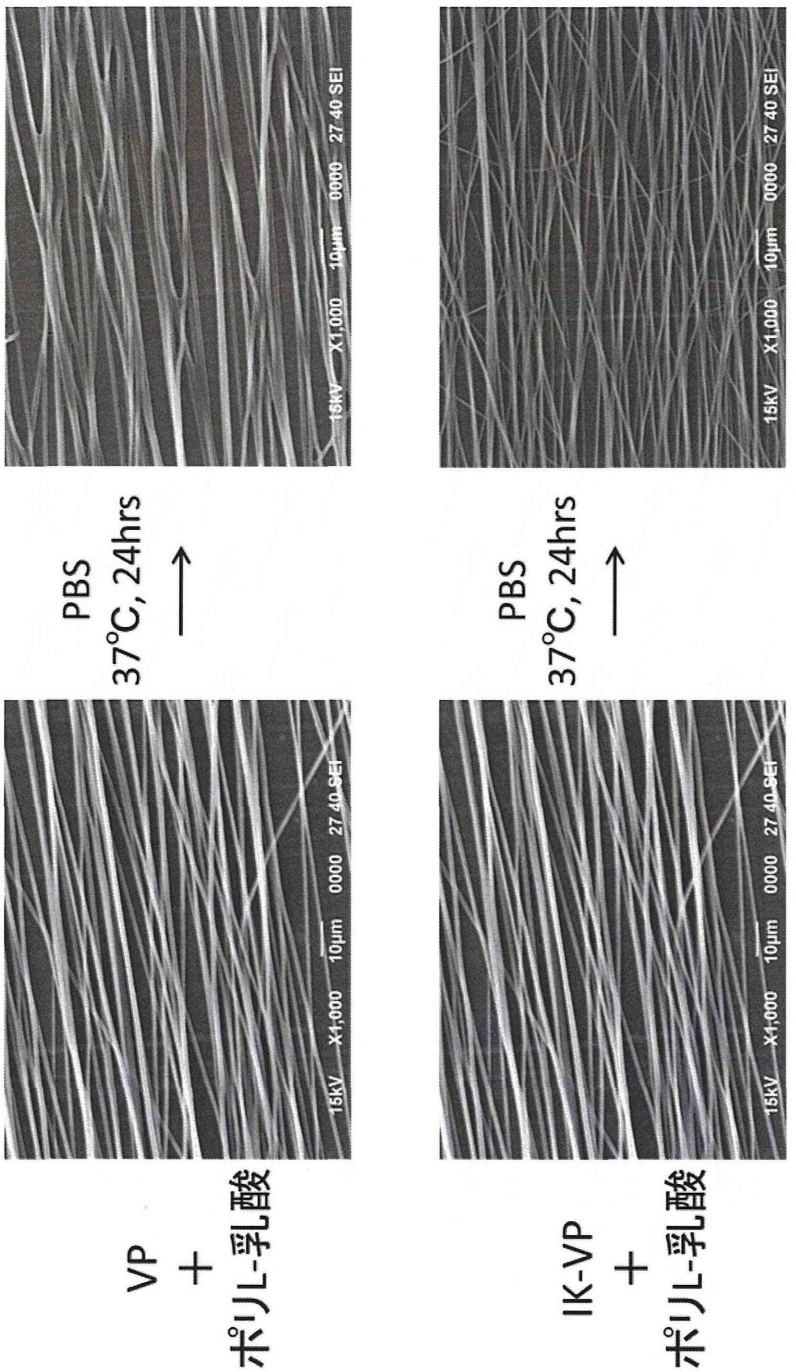

図４０．ＶＰもしくはＩＫ－ＶＰとポリＬ－乳酸との混合マイクロファイバーのＰＢＳ中での安定性評価

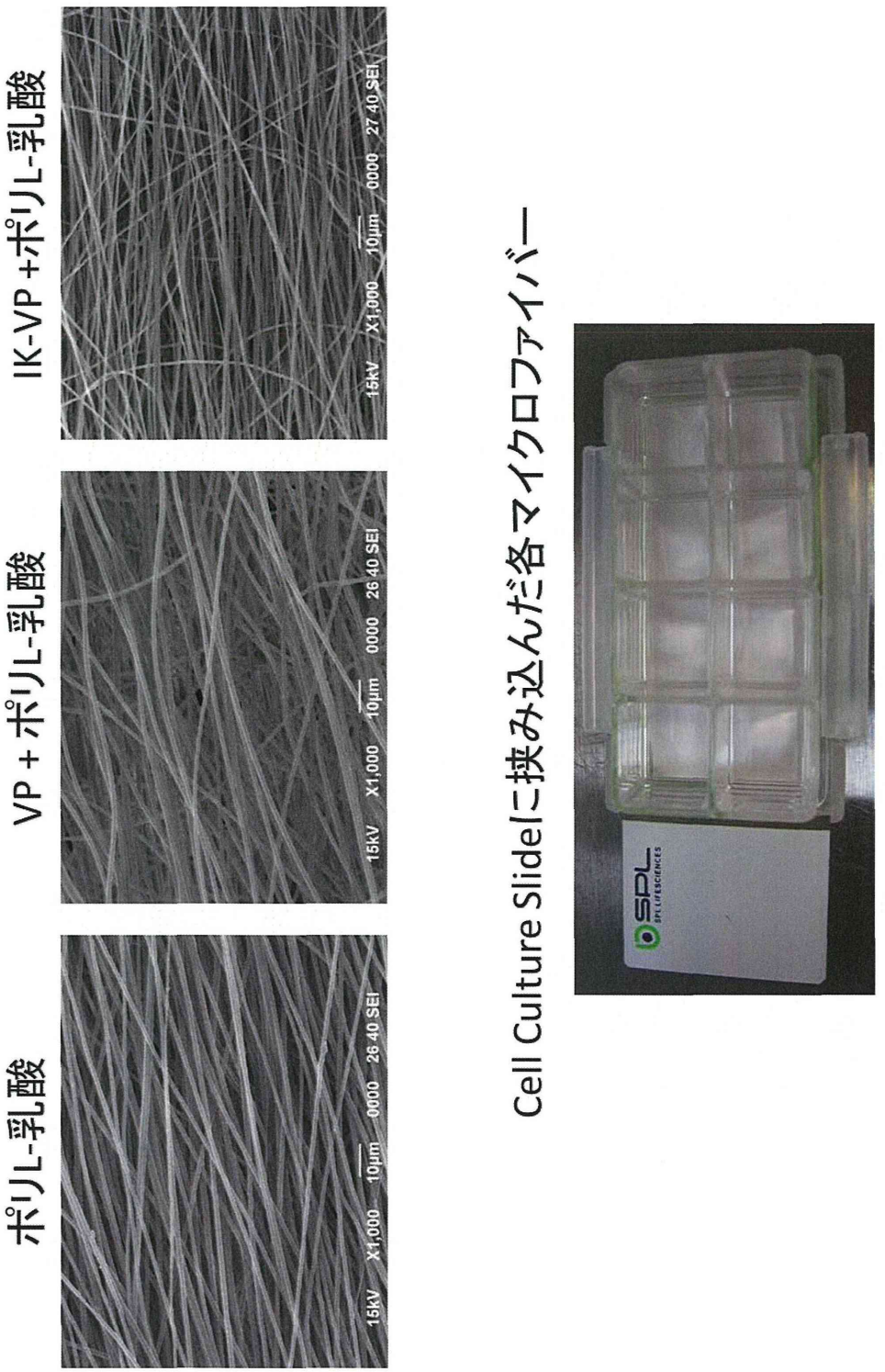

図４１．ポリＬ－乳酸および、ＶＰもしくはＩＫ－ＶＰとポリＬ－乳酸との混合マイクロファイバーとそのＣｅｌｌ Ｃｕｌｔｕｒｅ Ｓｌｉｄｅへの固定（*Ｉｎ ｖｉｔｒｏ*実験用）

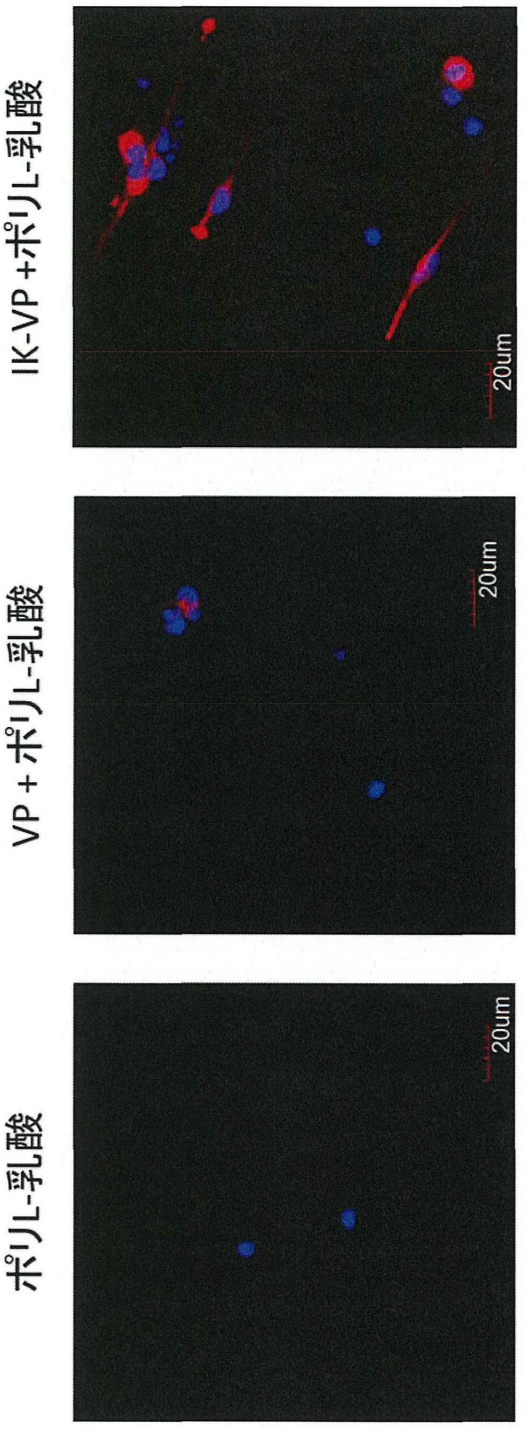

図４２．ポリＬ－乳酸および、ＶＰもしくはＩＫ－ＶＰとポリＬ－乳酸との混合マイクロファイバー上でのラットＤＲＧニューロンの軸索伸長性挙動
（赤; Ｎｅｕｒｏｆｉｌａｍｅｎｔ、青；ＤＡＰＩ）

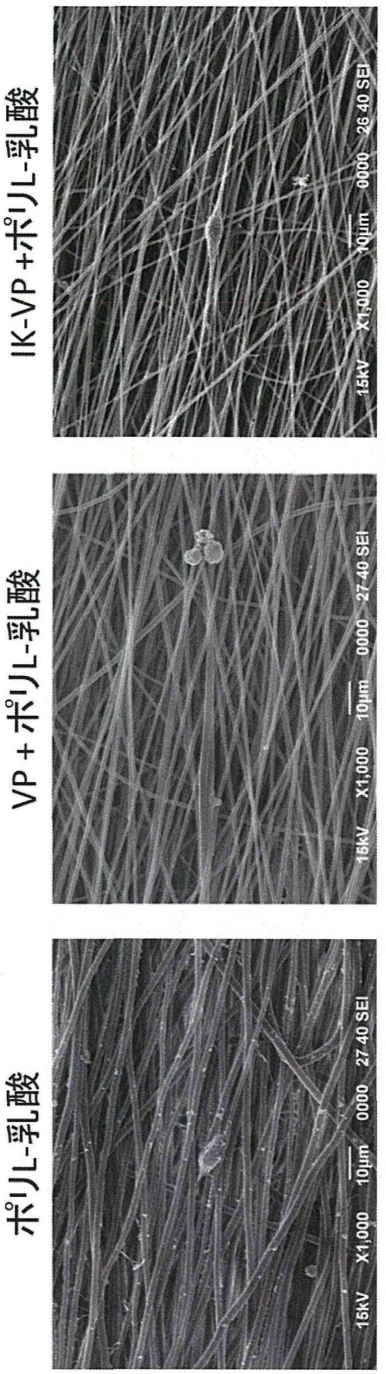

図４３．ポリＬ－乳酸および、ＶＰもしくはＩＫ－ＶＰとポリＬ－乳酸との混合マイクロファイバー上でのラットＤＲＧニューロンの軸索伸長性挙動
（走査型電子顕微鏡による観察）

別添４

雑誌

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Sachiro Kakinoki, Tetsuji Yamaoka	Thermoresponsive elastin/laminin mimicking artificial protein for modifying PLLA scaffolds in nerve regeneration	*J. Mat. Chem. B*	2	5061 - 5067	2014
Sachiro Kakinoki, Midori Nakayama, Toshiyuki Moritan, Tetsuji Yamaoka	Three-layer microfibrous peripheral nerve guide conduit composed of elastin-laminin mimetic artificial protein and poly(L-lactic acid)	*Frontiers in Chemistry*	2	Article 52	2014

総説・著書等

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柿木佐知朗 山岡哲二	神経	技術情報協会	再生医療における臨床研究と製品開発	技術情報協会	東京	2013	35 －
柿木佐知朗	ペプチド複合型バイオアクティブバイオマテリアルーポリ乳酸スキャホールドへのペプチド修飾法の開発と末梢神経再生への展開－	日本ペプチド学会	PEPTIDE NEWS LETTER JAPAN	日本ペプチド学会	大阪	2013	9 － 13

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Thermoresponsive elastin/laminin mimicking artificial protein for modifying PLLA scaffolds in nerve regeneration†

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Poly(L-lactic acid) (PLLA) is widely used as a scaffold but does not possess biological functions. Here, we described the biosynthesis of the elastin-like repetitive polypeptide $(VPGIG)_{30}$ containing a laminin-derived neurite outgrowth-promoting sequence (RKRLQVQLSIRT: AG73) (AG73-$(VPGIG)_{30}$). The expression vector for AG73-$(VPGIG)_{30}$ was constructed using the self-ligation technique to elongate the VPGIG repetitive sequence. The coacervation temperature of the purified AG73-$(VPGIG)_{30}$ protein was 20 and 14 °C in water and phosphate-buffered saline (PBS), respectively. AG73-$(VPGIG)_{30}$ was quickly adsorbed on PLLA films *via* a hydrophobic interaction by raising the temperature from 4 °C to 37 °C. On the AG73-$(VPGIG)_{30}$-modified PLLA surface, the neurite outgrowth of PC12 cells was strongly promoted. We successfully induced the neurite outgrowth activity on PLLA films by treating the novel surface modifier AG73-$(VPGIG)_{30}$, which could be applicable in developing PLLA scaffolds for nerve regeneration.

Introduction

One of the key steps of successful tissue engineering is to develop functional biodegradable scaffolds.[1–3] Since scaffolds act as a temporary extracellular matrix (ECM) and support cell adhesion, migration, or growth during the repair and regeneration of damaged tissues, they mimic well the three-dimensional network structure and biological functions of the native ECM.

Poly(L-lactic acid) (PLLA) is utilized to fabricate scaffolds because of many desirable features.[4] PLLA is non-enzymatically hydrolyzed to low-toxic lactic acid and metabolized *in vivo*, and its degradation rate can be controlled by the molecular weight.[5] The mechanical properties of PLLA are very high and also controllable by tuning the molecular weight and crystallinity.[6] In addition, PLLA is easily processed in nano/micro-fiber,[7–9] mesh,[10] and porous structures[11,12] that have been used for skin,[13] nerve,[7] cartilage, and bone[12] regeneration. However, since these chemical features are not satisfactory for tissue regeneration, many efforts have been invested for improving the biological properties on their surface.

ECM proteins such as collagen, fibronectin, and laminin and ECM-derived cell adhesive peptides such as Arg-Gly-Asp-Ser (RGDS) peptide[14,15] have been used for surface modification of PLLA. Barrera *et al.* synthesized poly(lactic acid-*co*-lysine) and introduced a cell adhesive RGD peptide through the condensation reaction with amino groups.[16] Yamaoka *et al.* also successfully synthesized PLLA with malate units and fabricated a thin film, and then immobilized the RGD peptide through the condensation reaction with carboxyl groups.[17] They showed that fibroblast adhesion was drastically improved on the RGD-immobilized PLLA films. Many researchers also reported the immobilization of the ECM proteins onto PLLA surfaces. For example, Ma introduced hydroxyl or carboxylic groups on PLLA by grafting of poly(hydroxyethyl methacrylate) or poly(methacrylic acid) and immobilized gelatin or collagen type-I.[18] These strategies are useful but require complicated processes to immobilize peptides or proteins. Furthermore, they may lead to the loss of the mechanical strength of PLLA or to the acceleration of the degradation rate. Thus, simpler and milder methods without any chemical reactions are preferable to immobilize peptides or proteins on PLLA scaffolds.

We previously reported on two simple methods of peptide immobilization on PLLA scaffolds for nerve regeneration. One exploits the hydrophobic adsorption of a collagen-like repetitive peptide (CLP).[19] A peptide composed of CLPs [$(PPG)_5$] with a laminin-derived neurite outgrowth-promoting sequence, AG73 (RKRLQVQLSIRT),[20] was synthesized with the typical Fmoc solid phase procedure, and successfully immobilized onto PLLA films through hydrophobic adsorption. Neurite outgrowth of rat adrenal pheochromocytoma cells (PC12) was slightly enhanced on peptide-immobilized PLLA surfaces *in vitro*. We reported on the other peptide-immobilization techniques on PLLA films or nano-fibers using a stereo-complex formation between poly(L-lactic acid) and oligo(D-lactic acid).[21,22] The conjugates of

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oligo(D-lactic acid) with the AG73 peptide were blended into a PLLA solution, and the films and nano-fibers were fabricated by employing the casting or electrospinning methods, respectively. By adding 1–3 wt% of conjugates in PLLA, neurite outgrowth of PC12 cells was progressed on the peptide-immobilized films and nano-fibers. Furthermore, we successfully fabricated nerve conduits with the inner layer of the PLLA/oligo(D-lactic acid)-AG73 conjugate nano-fiber, and these nerve conduits promoted the regeneration of the gap (1.0 cm) of rat peripheral nerve. These results indicated that the adsorption and conjugation of biological peptides without chemical reactions could be utilized for the surface modification of PLLA scaffolds.

We have been recently focusing on the elastin-like peptide (ELP) as a novel surface modifier of PLLA scaffolds. Elastin is an important ECM protein providing elastic properties to tissues such as blood vessels, skin, and lung.[25] Interestingly, the precursor of elastin, tropoelastin, is soluble in water and its solution shows temperature-dependent coacervation.[24–26] Tropoelastin contains repetitive segments composed of the VPGXG sequence, where X is mainly V or I, and synthetic small peptides mimicking this sequence have been studied as ELPs.[27–29] The chemically synthesized ELP shows temperature-dependent coacervation as well as tropoelastin, and ELPs have been widely investigated as thermoresponsible materials such as hydrogels[30] and carriers of gene delivery.[31] To design and synthesize an ELP having a predetermined amino acid sequence, expression systems in *Escherichia coli* have been reported. Urry *et al.* have succeeded in the biosynthesis of 251 repeats of the VPGVG sequence.[32] Tirrell and coworkers have designed and biosynthesized an artificial ECM protein composed of the CS5 region of fibronectin with the REDV sequence and the VPGIG repetitive sequence.[33–35] ELP-bearing cell recognition sites also showed the coacervation properties in aqueous solution depending on the temperature. Kobatake and coworkers have attempted to produce thermoresponsible surfaces using a genetically engineered ELP containing RGD ligands for the harvest of cell sheets.[36,37] Recently, the ELP was used for the immobilization of insulin-like growth factor binding protein 4 (IGFBP4) on polystyrene cell culture dishes for the cardiomyocyte differentiation of embryonic stem cells.[38] A solution of the fusion protein of IGFBP4 with the ELP region was incubated on polystyrene cell culture dishes at temperature higher than the coacervation temperature, and IGFBP4 was stably immobilized due to the hydrophobization by coacervation. Since PLLA is also hydrophobic like polystyrene, the ELP should be a good surface modifier to induce the biological peptides onto PLLA scaffolds.

In the present study, the laminin-derived neurite outgrowth peptide, AG73, was immobilized on PLLA scaffolds by simple treatment utilizing the temperature-dependent coacervation of ELPs. The VPGIG repetitive ELP bearing an AG73 sequence was designed and constructed by genetically engineering *E. coli* expression systems. The constructed ELP consists of His-tag, AG73 sequence, and 30 repeats of the VPGIG sequence [AG73-$(VPGIG)_{30}$] (Fig. 1(A)). PLLA films were immersed in AG73-$(VPGIG)_{30}$ solution below the coacervation temperature and heated up to a temperature higher than the coacervation temperature. The ELP aggregated and adsorbed onto PLLA films by the hydrophobic interaction, resulting in the functionalization of the PLLA surfaces by AG73. Since AG73-$(VPGIG)_{30}$ becomes insoluble at high temperatures due to coacervation, AG73-$(VPGIG)_{30}$ was expected to be effectively adsorbed onto the PLLA surface compared to the low hydrophilic peptide like the previously described CLP. Furthermore, the immobilization of the AG73 peptide using AG73-$(VPGIG)_{30}$ did not require any chemical reaction and blending before the fabrication of the scaffolds, that is, it was available to the introduction of the neurite outgrowth activity onto PLLA scaffolds with various shapes such as porous, tubular, and fibrous scaffolds. The immobilization of AG73 onto the PLLA surface would lead to excellent neurite outgrowth activity and become a powerful strategy for the development of artificial nerves.

Results and discussion

Gene construction and AG73-$(VPGIG)_{30}$ expression

Synthetic DNA cassette encoding $(VPGIG)_5$ flanked at the *Ban*I sticky-end was designed to avoid the insertion of unnecessary amino acids between the VPGIG repetitive units. The VPGIG repetitive sequence was elongated by self-ligation of $(VPGIG)_5$ DNA cassettes and, at the same time, it was inserted into pUC18 encoding AG73 at the *Bbs*I digested site. The restriction enzyme *Bbs*I recognizes the GAAGACTG′NNNN sequence and exposes the NNNN sticky-end after digestion. In this study, the NNNN fragment was designed so that the $(VPGIG)_n$ sequence could be inserted at the sticky-end of *Ban*I (GTGC). The sticky-end of *Ban*I is not a palindromic sequence; therefore, the DNA cassette of $(VPGIG)_5$ can stitch only in a head-to-tail manner by self-ligation. The pUC18 encoding AG73-$(VPGIG)_{5n}$, where $n = 1, 2, 3, 5$, and 6, was obtained (Fig. S1†). The longest DNA encoding AG73-$(VPGIG)_{30}$ was chosen and introduced into the expression vector. The DNA encoding AG73-$(VPGIG)_{30}$ was digested by *Sal*I/*Xho*I, and ligated into pET28a(+) having His-tag and stop codon sequences at the *Xho*I digestion site (Fig. 1(B)). The expression plasmid pET28a(+) encoding AG73-$(VPGIG)_{30}$ with the His-tag was expressed in *E. coli* BL21 (DE3) pLysS by IPTG induction at 30 °C. The expressed AG73-$(VPGIG)_{30}$ was purified by using a His-tag affinity column into high purity (approximately 95%) (Fig. 1(C)). Approximately 20 mg L^{-1} purified AG73-$(VPGIG)_{30}$ was successfully obtained.

Characterization of AG73-$(VPGIG)_{30}$

The expressed AG73-$(VPGIG)_{30}$ was dissolved at a concentration of 10 mM in PBS at 4 °C and allowed to aggregate at 37 °C as shown in Fig. 2(A). This temperature-dependent coacervation was reversible as well as the previously reported behavior of tropoelastin.[27,28] The coacervation of AG73-$(VPGIG)_{30}$ was evaluated in detail by measuring the particle size change upon heating (Fig. 2(B)). AG73-$(VPGIG)_{30}$ gradually aggregated from 20 °C in water with coacervation. The coacervation temperature of elastin-A is also around 23 °C in water. In PBS, the coacervation temperature of AG73-$(VPGIG)_{30}$ decreased to 14 °C and its behavior was more sharply compared with that in water. On

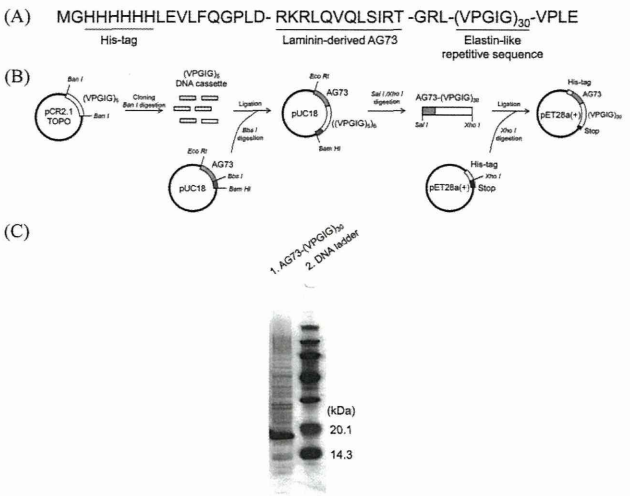

Fig. 1 (A) Amino acid sequence of AG73-$(VPGIG)_{30}$, (B) strategy for cloning and expression of the artificial gene encoding AG73-$(VPGIG)_{30}$ and (C) SDS–PAGE of the purified AG73-$(VPGIG)_{30}$ with silver staining.

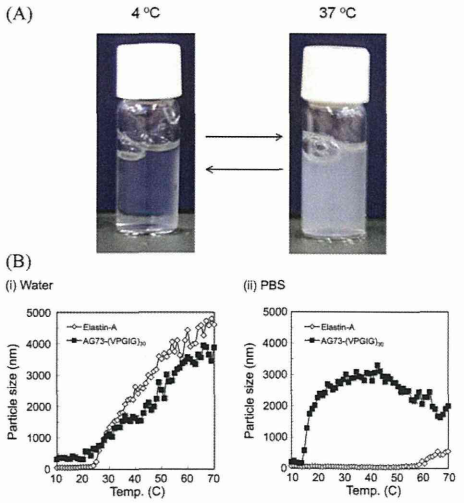

Fig. 2 Temperature dependence coacervation of AG73-$(VPGIG)_{30}$. (A) Coacervation of 10 μM AG73-$(VPGIG)_{30}$ in PBS. (B) Temperature dependence of the particle size of elastin-A and AG73-$(VPGIG)_{30}$ in (i) water and (ii) PBS measured by dynamic light scattering.

the other hand, the aggregation temperature of elastin-A rose up to approximately 60 °C in PBS. Elastin-A was prepared by the hydrolysis of elastin extracted from porcine aorta and contained both acidic and basic amino acids in addition to the VPGXG repetitive sequence. It is reported that the composition ratio of acidic amino acids (Asp and Glu) in hydrolyzed elastin is higher than that of the basic amino acids (Lys and Arg).[39] Urry reported that the aggregation temperature of chemically synthetic poly-(VPGVG) containing Glu became higher depending on the increase of the pH value.[40] In addition, Kaibara *et al.* showed that the coacervation temperature of water soluble α-elastin depended on the solution pH, namely, it was 20 °C at pH 5.5 and >60 °C at pH 7.2.[41] Since the pH value of water and PBS is 5.5 and 7.4, respectively, it is thought that elastin-A became more hydrophilic in PBS than in water because of the dissociation of the acidic amino acids, resulting in a higher coacervation temperature. On the other hand, AG73-$(VPGIG)_{30}$ does not possess acidic and basic amino acids in the ELP domain. The coacervation temperature of poly(VPGVG) is decreased by the addition of metal cations such as Na^+, Mg^{2+}, and Ca^{2+}, but it is not affected by the pH value.[41] Therefore, the coacervation temperature of AG73-$(VPGIG)_{30}$ might have decreased in PBS due to the effects of sodium salts, and it successfully formed coacervates under physiological conditions (37 °C).

Surface analysis of AG73-$(VPGIG)_{30}$-adsorbed PLLA films

The water contact angle of non-coated and protein-coated PLLA films is shown in Fig. 3. The water contact angle of approximately 72.0° of the non-coated PLLA film indicated its hydrophobic properties. After incubation in AG73 peptide solution at 4 or 37 °C, the water contact angle of PLLA films decreased to 34.0°. The mechanism of AG73 adsorption onto the PLLA surface could be the electrostatic interaction because AG73 is a

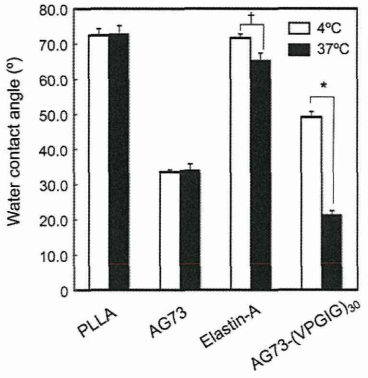

Fig. 3 Water contact angle of PLLA films untreated or treated with AG73, elastin-A, and AG73-(VPGIG)$_{30}$ at 4 or 37 °C (n = 3) (*p < 0.01 and †p < 0.02, Student's t-test).

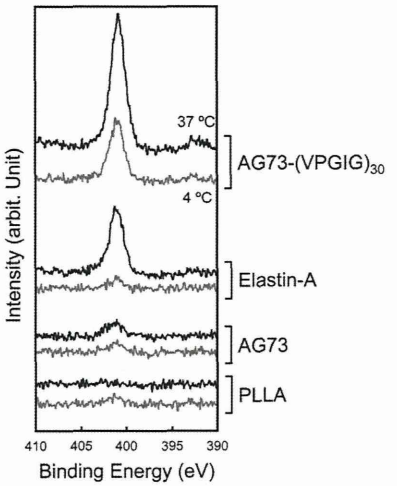

Fig. 4 XPS spectra in the N1s region of PLLA films untreated or treated with AG73, elastin-A, and AG73-(VPGIG)$_{30}$ at 4 or 37 °C.

positively charged peptide and the PLLA surface shows a negative ζ-potential at physiological pH.[42] The water contact angle of PLLA films treated with elastin-A solution at 4 and 37 °C was 71.6° and 65.4°, respectively. Although elastin-A did not aggregate at 37 °C in PBS, it slightly adsorbed onto PLLA films probably due to the hydrophobic interaction. Interestingly, AG73-(VPGIG)$_{30}$ treatment at 37 °C drastically decreased the contact angle to 21.4°, suggesting that its adsorption was greatly accelerated due to its temperature-dependent aggregation at 37 °C. Furthermore, the nearly identical contact angle after AG73 and AG73-(VPGIG)$_{30}$ treatment indicated that the AG73 segment of the adsorbed AG73-(VPGIG)$_{30}$ exposed toward the outmost surface.

The XPS spectrum in the N1s region is shown in Fig. 4. The N1s peak was not observed on non-treated PLLA films because PLLA does not possess nitrogen atoms. After incubation with AG73 solution, a weak N1s peak derived from the peptide was detected and its strength was almost similar between 4 and 37 °C, suggesting that the AG73 adsorption was not affected by the temperature. In the case of elastin-A, the N1s peak slightly appeared at 4 °C and its intensity increased at 37 °C. After incubation in the AG73-(VPGIG)$_{30}$ solution, a very strong N1s peak was detected at 4 °C and its intensity largely increased at 37 °C. The elemental ratios calculated from the XPS spectra are summarized in Table 1. After AG73 treatment at 4 and 37 °C, N1s/C1s and N1s/O1s ratios were low (~0.04). These values increased more than 10-fold upon elastin-A and AG73-(VPGIG)$_{30}$ treatment at 37 °C, indicating that the adsorption of elastin-A and especially that of AG73-(VPGIG)$_{30}$ were accelerated at 37 °C.

Table 1 Elemental ratios of the surface of PLLA films untreated or treated with AG73, elastin-A, and AG73-(VPGIG)$_{30}$ at 4 or 37 °C

	N1s/C1s		N1s/O1s	
Temp. (°C)	4	37	4	37
PLLA	0.02	N.D.	0.01	N.D.
AG73	0.04	0.05	0.03	0.03
Elastin-A	0.24	0.41	0.22	0.50
AG73-(VPGIG)$_{30}$	0.24	0.39	0.22	0.51

Neurite outgrowth of PC12 cells on of AG73-(VPGIG)$_{30}$-immobilized PLLA films

The morphology and number of adherent PC12 cells on PLLA films treated with proteins are shown in Fig. 5. On non-treated PLLA films, PC12 cells adhered roundly without neurites and their number was about 100 cells per mm^2. PC12 cells adhered and spread with neurites on the AG73-treated PLLA surface and no differences were observed between the treatment temperatures. The morphology and number of adhered PC12 cells on PLLA treated with elastin-A at 4 °C were similar to those on non-treated PLLA but were largely inhibited by the treatment with elastin-A at 37 °C. The surface of PLLA films became hydrophilic by the adsorption of elastin-A at 37 °C as shown in Fig. 3, and the PC12 cell adhesion was suppressed. In addition, Heilshorn *et al.* reported that the ELP containing the VPGIG repetitive sequence was bio-inert for PC12 cells.[43] The backbone of elastin-A also contains a VPGXG repetitive sequence and does not possess the cell recognition site; thus, it is considered that the PLLA surface treated with elastin-A at 37 °C showed bio-inert properties for PC12 cells. After AG73-(VPGIG)$_{30}$ treatment at 4 °C, the number of adherent PC12 cells was approximately 150 cells per mm^2, and more than half of them had neurites. Moreover, adhesion and neurite outgrowth of PC12 cells on the

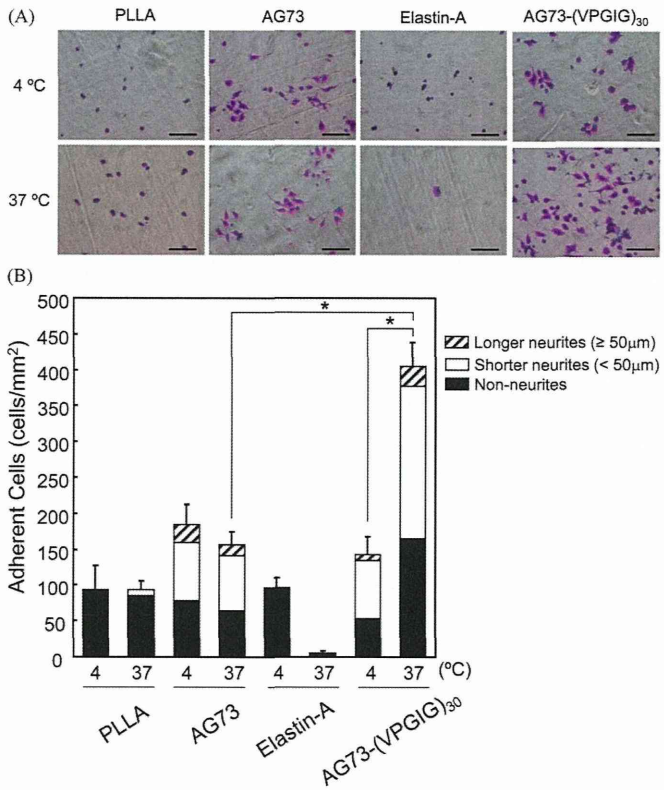

Fig. 5 (A) Morphology (scale bar = 100 μm) and (B) the number of adherent PC12 cells with non-, shorter (less than 50 μm), and longer (greater than or equal to 50 μm) neurites ($n = 3$) (*$p < 0.01$, one-way ANOVA and turkey post-hoc test).

PLLA surface were drastically enhanced by AG73-$(VPGIG)_{30}$ treatment at 37 °C, *i.e.*, the number of adherent PC12 cells was about 400 cells per mm^2, and >60% of the cells showed short or long neurites. This result indicates that AG73-$(VPGIG)_{30}$ adsorption was enhanced at 37 °C by temperature-dependent aggregation, the hydrophilic AG73 regions exposed toward the outmost surface, and then PC12 cells easily recognized AG73 through syndecan,[44] resulting in the activation of their neurite outgrowth.

Experimental

Gene construction for AG73-$(VPGIG)_{30}$ expression

The scheme of AG73-$(VPGIG)_{30}$ expression is shown in Fig. 1(B). Synthetic oligonucleotide encoding $(VPGIG)_5$, which has a non-palindrome *Ban*I sticky-end at both termini, was purchased from Life Technologies Corporation (Carlsbad, CA, USA). This oligonucleotide was ligated into various shapes pCR®2.1-TOPO® vector (Life Technologies Corporation) by using the TA cloning method. After cloning in the *E. coli* DH5α strain (Takara, Kyoto, Japan), this vector was extracted by using the QIAGEN-plasmid mini kit (QIAGEN, Valencia, CA, USA) and digested with *Ban*I. The $(VPGIG)_5$ DNA cassette was obtained by agarose gel electrophoresis of the digested solution. Moreover, the synthetic oligonucleotide encoding AG73 flanked with the *Bbs*I recognition site was inserted into pUC18 (Takara) by using the Ligation high kit (TOYOBO, Osaka, Japan). The *Bbs*I recognition site was designed to produce a *Ban*I sticky-end after digestion. The DNA cassette of $(VPGIG)_5$ was elongated by self-ligation and introduced into pUC18 carrying the AG73 sequence after *Bbs*I digestion. Then, pUC18 encoding AG73-$(VPGIG)_{30}$ was purified by agarose gel electrophoresis. After *Sal*I–*Xho*I ligation, the DNA fragment encoding AG73-$(VPGIG)_{30}$ was ligated into pET28a(+) (Merck KGaA, Darmstadt, Germany), which was completed by inserting a His-tag fragment and a stop codon sequence having a *Xho*I recognition site beforehand.

Expression and purification of AG73-$(VPGIG)_{30}$

E. coli BL21 (DE3) pLysS (Life Technologies Corporation) was transformed with the expression vector pET28(+) encoding AG73-$(VPGIG)_{30}$, and fermented in 2xYT medium supplemented with 34 μg mL^{-1} of kanamycin at 30 °C. After incubation (OD_{600} = 0.5–0.6), protein expression was induced by the addition of 0.1 M β-isopropyl thiogalactoside (IPTG) and *E. coli* was harvested by centrifugation at 3500 × *g* at 4 °C for 15 min following 3 h of continued growth. The bacterial pellet was resuspended with a lysis solution (8 M urea) and frozen at −80 °C. After thawing, bacteria were disrupted by sonication. Insoluble debris was removed by centrifugation at 10 000 × *g* at 4 °C for 15 min, and then the supernatant was purified by using a His-tag affinity column (COSMOGEL His-Accept, Nacalai Tesque, Kyoto, Japan). After dialysis (MwCo = 10 000 Da) in deionized water at 4 °C, purified AG73-$(VPGIG)_{30}$ was obtained by lyophilization.

Characterization of AG73-VPGIG

Purified AG73-$(VPGIG)_{30}$ was dissolved in Milli-Q water (18 MΩ cm^{-1}; Millipore, Billerica, MA, USA) or phosphate-buffered saline (PBS; pH 7.2, ionic strength 0.167) (Life Technologies Corporation) at 4 °C at a final concentration of 10 μM. The solution of water-soluble elastin derived from porcine aorta [elastin-A (25.2 kDa); Wako Chemical Co., Osaka, Japan] was also prepared at the same concentration to compare the properties with those of AG73-$(VPGIG)_{30}$. The temperature-dependent coacervation of AG73-$(VPGIG)_{30}$ was determined by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Southborough, UK). The temperature was gradually increased at a rate of 1 °C per hour from 10 °C to 70 °C, and the particle size was detected.

AG73-VPGIG adsorption on PLLA films

PLLA (M_w = 106 000 Da) (Musashino Chemical Laboratory, Inc., Tokyo, Japan) was used to fabricate the films. PLLA films (diameter = 13.0 mm, thickness = 0.5 mm) were prepared by using a hot-shrinking machine at 180 °C and sterilized by UV irradiation. In addition to AG73-$(VPGIG)_{30}$ and elastin-A, the AG73 peptide synthesized by the Fmoc solid phase procedure was dissolved in PBS at a concentration of 10 μM. PLLA films were placed into a 24-well cell culture plate, and 1 mL of PBS (abbreviated as PLLA in all figures) or each sample solution was poured onto PLLA films at 4 °C. PLLA films were incubated for 24 h at 4 °C or 37 °C. Samples were washed three times with 1 mL of PBS at 37 °C. Samples were dried for surface analysis or immediately used for the cell adhesion test.

Surface analysis of PLLA films

The water contact angle was measured by using a contact-angle meter (CA-X; Kyowa Interface Science Co., Ltd., Saitama, Japan). Images of the water spreading on the sample were recorded by using a camera and then analyzed. Three samples were measured for each group.

The surface composition of the protein-adsorbed PLLA films was determined by means of X-ray photoelectron spectroscopy (XPS; ESCA-3400, Shimadzu Co., Kyoto, Japan). The X-ray source was a monochromatic Mg K_α X-ray emitted from a rotating anode. Survey scans were measured from 0 to 1200 eV. Peak positions and areas were analyzed and ratios for C1s, N1s, and O1s were calculated using the software provided by the manufacturer.

Neurite outgrowth assay

The neurite outgrowth assay was performed using rat adrenal pheochromocytoma (PC12) cells (RIKEN BioResource Center, Ibaraki, Japan) as the model for neural stem cells. PC12 cells were maintained in DMEM supplemented with 100 units per mL penicillin, 100 μg mL^{-1} streptomycin (Life Technologies Corporation), 10% fetal bovine serum (FBS; MP Biomedicals, Inc., Solon, OH, USA), and 7.5% horse serum (HS; Sigma-Aldrich, Inc., St. Louis, MO, USA). PC12 cells were cultured in poly-D-Lys-coated cell-culture dishes (Asahi glass Co., Ltd., Tokyo, Japan) and maintained at 37 °C in an atmosphere composed of 5% CO_2 and 95% air. For the neurite outgrowth assay, PC12 cells were cultivated in the DMEM/F12 medium (Life Technologies Corporation) containing 100 ng mL^{-1} nerve growth factor (NGF; Sigma-Aldrich, Inc.) for 24 h on polystyrene cell-culture dishes. Then, the medium was gently changed to the normal culture medium and cells were incubated for 30 min at 37 °C in an atmosphere composed of 5% CO_2 and 95% air. The cells were collected by gentle agitation and resuspended with advanced DMEM/F12 containing 5 mg mL^{-1} insulin (Life Technologies Corporation), 100 ng mL^{-1} NGF, 20 nM progesterone, 100 mg mL^{-1} transferrin, and 30 nM sodium selenite (Na_2SeO_3) (Nacalai Tesque, Inc., Kyoto, Japan). Collected cells were seeded on PLLA films on which proteins were previously adsorbed at 4 or 37 °C in 24-well cell-culture plates at a density of 2.0 × 10^4 cells per film, and incubated at 37 °C for 24 h. Three wells were evaluated for each experimental condition. Adherent cells on PLLA films were fixed with 10% formalin and stained by using 4% crystal violet/methanol solution. The number of PC12 cells was counted and they were categorized based on the neurite length according to the photographs taken at five arbitrary positions in each well. The statistical significance of total adhering PC12 cells among each surface was determined by using one-way ANOVA and the Turkey post-hoc test.

Conclusions

An elastin-like peptide containing the laminin-derived neurite outgrowth-promoting sequence AG73-$(VPGIG)_{30}$ was designed and genetically synthesized. AG73-$(VPGIG)_{30}$ showed temperature-dependent coacervation at 14 °C in PBS solution. AG73-$(VPGIG)_{30}$ more efficiently adsorbed and immobilized onto PLLA films *via* a hydrophobic interaction when the temperature was changed from 4 °C to 37 °C. Adhesion and neurite outgrowth of PC12 cells were significantly enhanced on AG73-$(VPGIG)_{30}$-immobilized PLLA films. This result showed that the bioactive AG73 domain was easily introduced on the PLLA