surface by simple adsorption of AG73-$\{VPGIG\}_{30}$. PLLA is widely used as a biodegradable scaffold for tissue engineering, but its biological activity has to be supplemented by coating with bio-derived proteins such as collagen. The simple immobilization of AG73-$\{VPGIG\}_{30}$ is expected to replace the coating of bio-derived proteins and shows the potential for future applications, including *in vitro* control of stem cell differentiation and biological functionalization of scaffolds for the regeneration of peripheral nerves.

Acknowledgements

We would like to thank Prof. Ken'ichi Matsumoto and Prof. Seiichi Taguchi of the graduate School of Engineering at Hokkaido University for helpful discussion regarding protein expression. A part of this work was supported by Grant-in Aid from the Comprehensive Research on Disability Health and Welfare, the Ministry of Health, Labour and Welfare of Japan.

Notes and references

1 S. J. Peter, M. J. Miller, A. W. Yasko, M. J. Yaszemski and A. G. Mikos, *J. Biomed. Mater. Res.*, 1998, **43**, 422.
2 F. J. O'Brien, *Mater. Today*, 2011, **14**, 88.
3 X. Liu, J. M. Holzwarth and P. X. Ma, *Macromol. Biosci.*, 2012, **12**, 911–919.
4 L. T. Lim, R. Auras and M. Rubino, *Prog. Polym. Sci.*, 2008, **33**, 820.
5 A. S. Chawla and T. M. S. Chang, *Biomater., Med. Devices, Artif. Organs*, 1985, **13**, 153.
6 G. Perego, G. D. Cella and C. Bastioli, *J. Appl. Polym. Sci.*, 1996, **59**, 37.
7 F. Yang, R. Murugan, S. Wang and S. Ramakrishna, *Biomaterials*, 2005, **26**, 2603.
8 B. Gupta, N. Revagade and J. Hilborn, *Prog. Polym. Sci.*, 2007, **32**, 455.
9 Q. P. Pham, U. Sharma and A. G. Mikos, *Tissue Eng.*, 2006, **12**, 1197.
10 G. Chen, T. Sato, H. Ohgushi, T. Ushida, T. Tateishi and J. Tanaka, *Biomaterials*, 2005, **26**, 2559.
11 D. J. Mooney, D. F. Baldwin, N. P. Suh, J. P. Vacanti and R. Langer, *Biomaterials*, 1996, **17**, 1417.
12 G. Chen, T. Ushida and T. Tateishi, *Mater. Sci. Eng., C*, 2001, **17**, 63.
13 S. G. Kumbar, S. P. Nukavarapu, R. James, L. S. Nair and C. T. Laurencin, *Biomaterials*, 2008, **29**, 4100.
14 M. D. Pierschbacher and E. Ruoslahti, *Nature*, 1984, **309**, 30.
15 K. M. Yamada and D. W. Kennedy, *J. Cell Biol.*, 1984, **99**, 29.
16 D. A. Barrera, E. Zylstra, P. T. Lansbury and R. Langer, *J. Am. Chem. Soc.*, 1993, **115**, 11010.
17 T. Yamaoka, Y. Hotta, K. Kobayashi and Y. Kimura, *Int. J. Biol. Macromol.*, 1999, **25**, 265.
18 Z. Ma, C. Gao, J. Ji and J. Shen, *Eur. Polym. J.*, 2002, **38**, 2279.
19 S. Kakinoki and T. Yamaoka, *Acta Biomater.*, 2010, **5**, 1925.
20 B. S. Week, M. Nomizu, R. S. Ramachandran, Y. Yamada and H. K. Kleinman, *Exp. Cell Res.*, 1998, **243**, 375.
21 T. Yamaoka, S. Uchida, T. Higami and A. Murakami, Immobilization of bioactive molecules onto PLLA porous matrices for tissue regeneration, in *Proceedings of International Chemical Congress of Pacific Basin Societies*, Hawaii, HI, USA, 15–20 December 2005, program no. 482.
22 S. Kakinoki, S. Uchida, T. Ehashi, A. Murakami and T. Yamaoka, *Polymers*, 2011, **3**, 820.
23 J. Rosenbloom, W. R. Abrams and R. Mecham, *FASEB J.*, 1993, **7**, 1208.
24 S. L. Martin, B. Vrhovski and A. S. Weiss, *Gene*, 1995, **154**, 159.
25 B. Vrhovski, S. Jensen and A. S. Weiss, *Eur. J. Biochem.*, 1997, **250**, 92.
26 B. Vrhovski and A. S. Weiss, *Eur. J. Biochem.*, 1998, **258**, 1.
27 D. W. Urry, *J. Protein Chem.*, 1988, **7**, 1.
28 D. W. Urry, *J. Protein Chem.*, 1988, **7**, 81.
29 S. A. Jensen, B. Vrhovski and A. S. Weiss, *J. Biol. Chem.*, 2000, **275**, 28449.
30 L. A. Strzegowski, M. B. Martinez, D. C. Gowda, D. W. Urry and D. A. Tirrell, *J. Am. Chem. Soc.*, 1994, **116**, 813.
31 G. L. Bidwell III, I. Fokt, W. Priebe and D. Raucher, *Biochem. Pharmacol.*, 2007, **73**, 620.
32 D. T. McPherson, J. Xu and D. W. Urry, *Protein Expression Purif.*, 1996, **7**, 51.
33 A. Panitch, T. Yamaoka, M. J. Fournier, T. L. Mason and D. A. Tirrell, *Macromolecules*, 1999, **32**, 1701.
34 E. R. Welsh and D. A. Tirrell, *Biomacromolecules*, 2000, **1**, 23.
35 T. Yamaoka, T. Tamura, Y. Seto, T. Toda, S. Kunugi and D. A. Tirrell, *Biomacromolecules*, 2003, **4**, 1680.
36 E. Kobatake, K. Onoda, Y. Yanagida and M. Aizawa, *Biomacromolecules*, 2000, **1**, 382.
37 M. Mie, Y. Mizushima and E. Kobatake, *J. Biomed. Mater. Res., Part B*, 2008, **86**, 283.
38 A. Minato, H. Ise, M. Goto and T. Akaike, *Biomaterials*, 2012, **33**, 515.
39 R. Ross and P. Bornstein, *J. Cell Biol.*, 1969, **40**, 366.
40 D. W. Urry, B. Haynes, H. Zhang, R. D. Harris and K. U. Prasad, *Proc. Natl. Acad. Sci. U. S. A.*, 1988, **85**, 3407.
41 K. Kaibara, Y. Akinari, K. Okamoto, Y. Uemura, S. Yamamoto, H. Kodama and M. Kondo, *Biopolymers*, 1995, **39**, 189.
42 L. Safinia, N. Datan, M. Höhse, A. Mantalaris and A. Bismarck, *Biomaterials*, 2005, **26**, 7537.
43 K. S. Straley and S. C. Heilshorn, *Front. Neuroeng.*, 2009, **2**, 9.
44 N. Suzuki, N. Ichikawa, S. Kasai, M. Yamada, N. Nishi, H. Morioka, H. Yamashita, Y. Kitagawa, A. Utani, M. P. Hoffman and M. Nomizu, *Biochemistry*, 2003, **42**, 12625.

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Three-layer microfibrous peripheral nerve guide conduit composed of elastin-laminin mimetic artificial protein and poly(L-lactic acid)

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We developed a microfibrous poly(L-lactic acid) (PLLA) nerve conduit with a three-layered structure to simultaneously enhance nerve regeneration and prevent adhesion of surrounding tissue. The inner layer was composed of PLLA microfiber containing 25% elastin-laminin mimetic protein (AG73-$(VPGIG)_{30}$) that promotes neurite outgrowth. The thickest middle layer was constructed of pure PLLA microfibers that impart the large mechanical strength to the conduit. A 10% poly(ethylene glycol) was added to the outer layer to prevent the adhesion with the surrounding tissue. The AG73-$(VPGIG)_{30}$ compositing of an elastin-like repetitive sequence $(VPGIG)_{30}$ and a laminin-derived sequence (RKRLQVQLSIRT: AG73) was biosynthesized using *Escherichia coli*. The PLLA microfibrous conduits were fabricated using an electrospinning procedure. AG73-$(VPGIG)_{30}$ was successfully mixed in the PLLA microfibers, and the PLLA/AG73-$(VPGIG)_{30}$ microfibers were stable under physiological conditions. The PLLA/AG73-$(VPGIG)_{30}$ microfibers enhanced adhesion and neurite outgrowth of PC12 cells. The electrospun microfibrous conduit with a three-layered structure was implanted for bridging a 2.0-cm gap in the tibial nerve of a rabbit. Two months after implantation, no adhesion of surrounding tissue was observed, and the action potential was slightly improved in the nerve conduit with the PLLA/AG73-$(VPGIG)_{30}$ inner layer.

Keywords: poly(L-lactic acid), elastin-laminin mimetic protein, electrospun microfiber, nerve conduit, tissue adhesion prevention

INTRODUCTION

Traumatic nerve injuries in which direct suturing of the proximal and distal stumps is difficult due to the long nerve gap are generally treated with nerve autografts. However, autologous nerve grafting has several disadvantages, such as size mismatch and permanent loss of donor function due to the extraction of normal nerve. As an alternative, artificial nerve conduits have become widely accepted for bridging gaps between nerve stumps (Ichihara et al., 2008; Lohmeyer et al., 2009; Sieminow and Brzezicki, 2009).

Artificial nerve conduits constructed of numerous polymeric materials such as silicone (Lundborg et al., 1982), collagen (Archibald et al., 1995), chitosan (Freier et al., 2005; Ao et al., 2006), hyaluronic acid (Wang et al., 1998), poly(caprolactone) (PCL), poly(glycolic acid) (PGA), and poly(lactic acid) (PLA) (Nakamura et al., 2004; Yoshitani et al., 2007) have been investigated. Recently, an artificial nerve conduit composed of poly(lactic-*co*-glycolic acid) (PLGA) mesh filled with animal-derived collagen has been put into clinical use and has shown good performance (Nakamura et al., 2004). PLGA is hydrolyzed and metabolized *in vivo*, and thus the PLGA conduit is absorbed during nerve regeneration over the course of several months (Mainil-Varlet et al., 1996). Because PLGA does not possess any biological activity, collagen is used for promoting nerve regeneration. Collagen is a major component of the basement membrane of nerve tissue and plays a key role in the reconstruction of the axon network by Schwann cells (Thomas, 1964; Chernousov et al., 2008). Although collagen strongly enhances nerve regeneration, animal-derived materials raise concerns about viral infection and immune responses (Lynn et al., 2004). The mesh structure of this conduit is effective for inhibiting the intrusion of surrounding connective tissues and for allowing the permeation of liquid factors and small molecules. However, the mesh structure promotes adherence with surrounding tissues and can cause painful traction neuropathies (Smit et al., 2004). Hence, the ideal conduits for promoting nerve regeneration and preventing tissue adhesion would not include animal-derived materials.

To avoid the use of animal-derived materials, short peptide sequences isolated from extracellular matrix proteins can be used, because they show biological activity equal to that of full-length proteins. For example, the RGD sequence of fibronectin provides excellent cell adhesive properties (Hersel et al., 2003). Focusing on nerve regeneration, the IKVAV (Tashiro et al., 1989), YIGSR (Iwamoto et al., 1987), and RKRLQVQLSIRT (AG73) (Nomizu et al., 1996) sequences derived from laminin have been well studied in the activation of neural cells. These small peptides are

easily synthesized chemically or biologically and are useful for imparting nerve regenerative activity to polymeric materials (Rao and Winter, 2009; Kakinoki and Yamaoka, 2010). Suzuki et al. prepared IKVAV- or YIGSR-immobilized tendon chitosan tubes and demonstrated the efficacy of peptide immobilization for assisting nerve regeneration in a rat model of nerve injury (Suzuki et al., 2003a). Previously, we reported that poly(L-lactic acid) (PLLA) nerve conduits modified with laminin-derived AG73 peptides and a polyethylene glycol (PEG)-containing outer layer are effective for preventing the adhesion of surrounding tissue (Kakinoki et al., 2011). The AG73 peptide was immobilized onto PLLA microfibers using oligo(D-lactic acid) (ODLA)–AG73 conjugates. The microfibers were fabricated by electrospinning of a mixed solution containing PLLA and the ODLA–AG73 conjugate, resulting in stable immobilization of the AG73 peptide via stereocomplex formation between the PLLA and the ODLA. Regeneration of functional nerve tissue was observed in AG73-immobilized microfibrous nerve conduit in the rat model of nerve injury, but it required a long period of time, approximately 6 months. Thus, the efficiency of AG73-immobilized microfibers is insufficient for nerve regeneration. We speculated that the efficacy of AG73 immobilization was decreased immediately after implantation by release of the low-molecular weight PDLA–AG73 conjugates from the microfibers *in vivo*.

To fabricate a more effective biological material for nerve regeneration, we designed and biosynthesized a high molecular weight elastin-laminin mimetic protein. The basement membrane of nerve tissue is primarily constructed with collagen, laminin, and elastin (Rutka et al., 1988). These proteins cooperatively support the process of nerve regeneration. Specifically, collagen and laminin function in axon extension, and elastin provides elasticity for the tissue structure (Toyota et al., 1990). We previously biosynthesized an elastin-laminin mimetic protein and used it for the functionalization of PLLA scaffolds (Kakinoki and Yamaoka, 2014). This protein is composed of 30 repeats of an elastin-like VPGIG repetitive sequence and a laminin-derived AG73 sequence (AG73-$(VPGIG)_{30}$). AG73-$(VPGIG)_{30}$ showed temperature-dependent coacervation in phosphate buffered saline (PBS) solution at approximately 14°C. Because the AG73-$(VPGIG)_{30}$ was insoluble at body temperature, this protein was expected to serve as a suitable scaffold for nerve regeneration. Our results showed that the adhesion and neurite outgrowth of PC12 cells were enhanced on the AG73-$(VPGIG)_{30}$ adsorbed PLLA films *in vitro*.

In this study, we prepared PLLA microfibrous nerve conduit with a three-layered structure that promoted nerve regeneration and prevented tissue adhesion. The therapeutic efficacy of peripheral nerve regeneration was evaluated using a rabbit model of nerve injury. Microfibrous conduits composed of a PLLA, PLLA/AG73, or PLLA/AG73-$(VPGIG)_{30}$ inner layer, a PLLA middle layer, and a PLLA/PEG outer layer were fabricated using an electrospinning procedure. Adhesion and neurite outgrowth of PC12 cells on PLLA microfibers containing AG73-$(VPGIG)_{30}$ were studied *in vitro*. Nerve autografts and microfibrous conduits were implanted in rabbits to bridge a 2-cm tibial nerve gap. Two months after implantation, nerve regeneration was evaluated by electrophysiological measurements.

MATERIALS AND METHODS

EXPRESSION AND PURIFICATION OF THE ELASTIN-LAMININ MIMETIC PROTEIN

The elastin-laminin mimetic protein (AG73-$(VPGIG)_{30}$) was expressed by *Escherichia coli* BL21(DE3)pLysS (Life Technologies Corporation, Carlsbad, CA, USA) that had been transformed with the pET28(+) vector (Merck KGaA, Darmstadt, Germany) encoding AG73-$(VPGIG)_{30}$, as described previously (Kakinoki and Yamaoka, 2014). AG73-$(VPGIG)_{30}$ expression was automatically induced using an Overnight Express™ Autoinduction System (Merck KGaA). Briefly, *E. coli* cells were incubated in 2 × YT medium supplemented with 34 μg/mL of kanamycin and the reagents of the Overnight Express™ Autoinduction System at 30°C for 24 h. *E. coli* was harvested by centrifugation at 3500 × *g* at 4°C for 15 min. Bacterial pellets were resuspended in lysis solution (8 M urea) and frozen at −80°C. After thawing, bacteria were disrupted by sonication on ice. Insoluble debris was removed by centrifugation at 10,000 × *g* at 4°C for 15 min, and the supernatant was purified on a His-tag affinity column (COSMOGEL His-Accept, Nacalai Tesque, Kyoto, Japan).

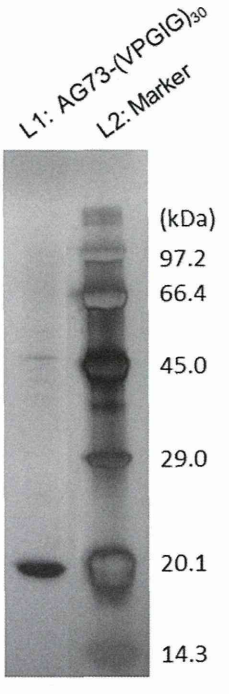

FIGURE 1 | Silver staining of the gel after SDS-PAGE of purified AG73-$(VPGIG)_{30}$.

Following dialysis (MwCo = 10,000 Da) in deionized water at 4°C, purified AG73-$(VPGIG)_{30}$ was obtained by lyophilization. The purity of the AG73-$(VPGIG)_{30}$ was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with silver staining.

FABRICATION OF PLLA/AG73-$(VPGIG)_{30}$ NON-WOVEN MICROFIBERS

A PLLA/AG73-$(VPGIG)_{30}$ solution with a concentration of 20w% was prepared by dissolving PLLA (Mw: 106,000, Mn: 60,000, Mw/Mn: 1.77) (Musashino Chemical Laboratory, Inc., Tokyo, Japan) and AG73-$(VPGIG)_{30}$ in hexafluoroisopropanol (HFIP) at a weight ratio of 4:1. This solution was electrospun using a plastic syringe equipped with a stainless steel needle (length = 15.0 mm, diameter = 20 G) at a constant feed rate of 4 mL/h. An aluminum plate was used as a target and the distance between the target and the needle tip was 100 mm. The solution was electrospun at a high voltage (15 kV) for 5 min. Non-woven PLLA/AG73-$(VPGIG)_{30}$ microfibers were collected and cut for subsequent experiments. Non-woven PLLA and PLLA/AG73 microfibers were prepared using a similar procedure. The concentration of the AG73 peptide (Purity > 82.6%; Sigma-Aldrich, Inc.) in the PLLA/AG73 solution was adjusted to the same molarity as that of the AG73-$(VPGIG)_{30}$ in the non-woven PLLA/AG73-$(VPGIG)_{30}$ microfibers. After immersion in PBS for 24 h at 37°C, the structure of the non-woven microfibers was observed by scanning electron microscopy (SEM; JCM-5700, JEOL, Tokyo, Japan).

NEURITE OUTGROWTH ASSAY

A neurite outgrowth assay was performed using rat adrenal pheochromocytoma (PC12) cells (RIKEN BioResorce Center, Ibaraki, Japan), which are widely used as a model for neural stem cells. PC12 cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin (Life Technologies Corporation, Carlsbad, CA, USA), 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-lysine-coated cell culture dishes (Asahi Glass Co., Ltd., Tokyo, Japan) and maintained at 37°C in a 5% CO_2 atmosphere. Prior to the neurite growth assay, PC12 cells were cultivated in DMEM/F12 medium (Life Technologies Corporation, Carlsbad, CA, USA) with 100 ng/mL nerve growth factor (NGF; Sigma-Aldrich, Inc.) for 24 h on polystyrene cell culture dishes. The medium was refreshed with normal culture medium, and cells were incubated for 30 min at 37°C in a 5% CO_2 atmosphere. Cells were harvested by gentle agitation and resuspended in advanced DMEM/F12 containing 5 mg/mL insulin (Life Technologies Corporation, Carlsbad, CA, USA), 100 ng/mL NGF, 20 nM progesterone, 100 mg/mL transferrin, and 30 nM sodium selenite (Na_2SeO_3) (Nacalai Tesque, Inc., Kyoto, Japan), and seeded on non-woven microfibers fixed with Cell Crown (Scaffdex Ltd., Tampere, Finland) in 24-well cell culture plates at a density of 2.0×10^4 cells/sample. After 48 h incubation at 37°C in a 5% CO_2 atmosphere,

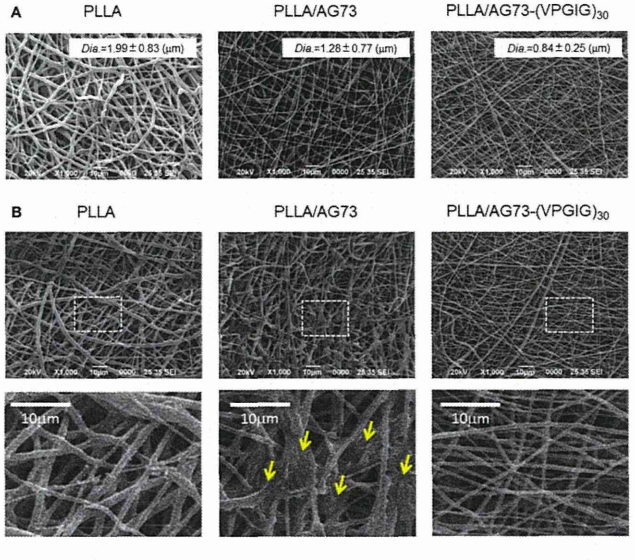

FIGURE 2 | SEM images of electrospun PLLA, PLLA/AG73, and PLLA/ AG73-$(VPGIG)_{30}$ micro-fibers (A) before and (B) after immersion in PBS for 24 h at 37°C. Yellow arrows indicate fusion points.

cells adherent to the non-woven fibers were fixed with 10% formalin and stained with 4% crystal violet/methanol solution. The number of adherent cells observed in the stained images were counted and categorized according to neurite length.

FABRICATION OF PLLA/AG73-$(VPGIG)_{30}$ MICROFIBROUS CONDUIT

We designed the electrospun microfibrous conduits with 3 layer structure, PLLA, PLLA/AG73, or PLLA/AG73-$(VPGIG)_{30}$ inner layer, PLLA middle layer, and PLLA/polyethylene glycol (PEG) outer layer. For fabricating conduits, a rotating stainless steel tube (outer diameter = 2.0 mm, speed = 1500 rpm) was used as a target. Other conditions for electrospinning were completely same to nonwovens. First, inner layer (PLLA, PLLA/AG73, or PLLA/AG73-$(VPGIG)_{30}$) was electrospun as described previously for 5 min. Middle layer was electrospun with 20w% of PLLA solution for 30 min. Then, the mixed solution at 20w% containing PLLA and PEG (9:1) was electrospun for 10 min as an outer layer. The matrices mass for inner, middle, and outer layer were adjusted to 1: 6: 2 for covering inner and outer surface of the mechanically strong middle layer. Microfibrous conduits were cut to length of 22 mm, and immersed in 70%-ethanol for the sterilization. After drying *in vacuo*, conduits were used for animal experiment.

IMPLANTATION OF MICROFIBROUS CONDUITS

Electrospun microfibrous conduits were implanted into 2.0-cm gaps in the left tibial nerve of a total of 12 New Zealand white rabbits (3.0–3.5 kg, male) (Oriental Yeast Co., Ltd., Tokyo, Japan). This study was performed in accordance with the animal experimental guidelines of the National Cerebral and Cardiovascular Center Research Institute. Rabbits were anesthetized by intramuscular injection of 0.1 mL/kg of Selactar (Bayer Yakuhin, Ltd., Osaka, Japan) and anesthesia was maintained by inhalation of 3% Escain isoflurane (Mylan Inc., Canonsburg, PA, USA). Under an operating microscope, the left tibial nerve was exposed and a 2.0-cm segment was removed. A section of microfibrous conduit was filled with physiological saline solution and sutured with 10-0 vicryl (Ethicon, Somerville, NJ, USA) to bridge the gap between the proximal and distal stumps. Both nerve stumps were pulled 1.0 mm inside the conduits. In addition, the removed nerve tissue was inverted on its proximal–distal axis and implanted as an autograft control. Muscle and skin were closed with 3-0 silk sutures (Ethicon), and the rabbits were allowed to recover in a controlled environment.

ELECTROPHYSIOLOGICAL ANALYSIS

Two months after implantation, nerve regeneration was evaluated by electrophysiological analysis. Rabbits were anesthetized in the same manner as for transplantation of the microfibrous conduits, and the implanted site was exposed from the proximal to the distal portions. In order to record electromyograms at the implantation site, a pair of stimulating and recording electrodes was attached to the proximal and distal portions, respectively. Both electrodes were connected to an electric stimulator (SEN-3401, Nihon Kohden, Tokyo, Japan) and a data acquisition system

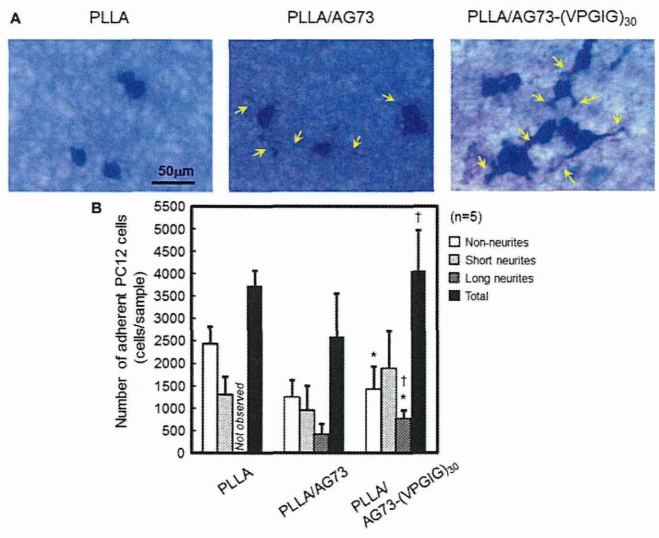

FIGURE 3 | Adhesion and neurite outgrowth of NGF-treated PC12 cells on electrospun nanofibers. (A) Morphology of NGF-treated PC12 cells 48 h after seeding. Yellow arrows indicate neurites. **(B)** The number of adherent PC12 cells categorized as non-neurites, short neurites (< 50 μm), and long neurites (≥ 50 μm). * and † indicate statistically significant differences, $p < 0.05$ vs. PLLA and PLLA/AG73, respectively (Student's *t*-test).

(PowerLab 8/30, ADInstruments, Colorado Springs, CO, USA). The stimulation parameters were as follows: strength = 1 V, current = 1 mA, duration = 10 s, pulse width = 0.1 ms. The active potential was amplified using a PowerLab system (ADInstruments, Burlingame, CA, USA), and recorded as the average of 50 traces.

RESULTS AND DISCUSSION

EXPRESSION AND PURIFICATION OF ELASTIN-LAMININ MIMETIC PROTEIN

SDS-PAGE analysis of purified AG73-$(VPGIG)_{30}$ is shown in **Figure 1**. A single band was observed at 17–18 kDa, which is concordant with the theoretical molecular weight. Sixty milligrams of high-purity AG73-$(VPGIG)_{30}$ was successfully obtained from 1 L of culture medium. As previously reported, PBS solution containing AG73-$(VPGIG)_{30}$ demonstrated temperature-dependent coacervation at 14°C, indicating that the protein is insoluble at 37°C in a physiological environment (Kakinoki and Yamaoka, 2014).

MORPHOLOGY AND STABILITY OF ELECTROSPUN NON-WOVEN MICROFIBERS

SEM images of PLLA, PLLA/AG73, and PLLA/AG73-$(VPGIG)_{30}$ non-woven microfibers before and after PBS immersion are shown in **Figure 2**. The diameter of the PLLA microfibers was approximately 2.0 μm (**Figure 2A**). When AG73 or AG73-$(VPGIG)_{30}$ was mixed with the PLLA, the diameter of the microfibers decreased to approximately 1.3 and 0.9 μm, respectively. The diameter of electrospun microfibers is known to be influenced by solution viscosity and electronic charge (Huang et al., 2003). The PLLA concentration was decreased by the addition of the AG73 and AG73-$(VPGIG)_{30}$, thus decreasing the viscosity of the solution. Furthermore, the electronic charge of the solution should be positive because AG73 is positive due to its Arg and Lys residues. We assumed that the diameter of the microfibers was decreased by the changes in solution viscosity and electronic charge with the addition of the AG73 and AG73-$(VPGIG)_{30}$. The surfaces of the PLLA/AG73 and PLLA/AG73-$(VPGIG)_{30}$ were smooth, without phase separation, suggesting that the AG73 and AG73-$(VPGIG)_{30}$ were homogeneously mixed.

The morphology of the microfibers following immersion in PBS for 24 h is shown in **Figure 2B**. The shape of the PLLA microfibers did not differ before and after immersion in PBS. However, the morphology of PLLA/AG73 microfibers became rough and some fusion appeared (**Figure 2B**; indicated with yellow arrows) after PBS immersion. These morphological changes suggested that the PLLA/AG73 microfibers partially dissolved in PBS. In contrast, the morphology of PLLA/AG73-$(VPGIG)_{30}$ microfibers did not change after PBS immersion, indicating that the PLLA/AG73-$(VPGIG)_{30}$ microfibers were stable in a physiological environment.

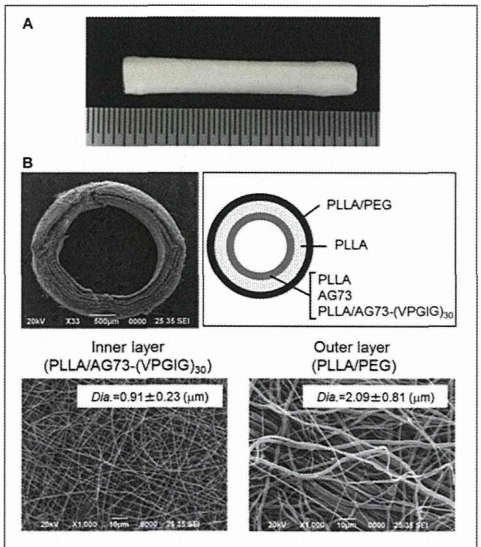

FIGURE 4 | Micro-fibrous nerve conduit with a three-layered structure. **(A)** Whole image, **(B)** SEM images of cross-sections and inner and outer layers.

NEURITE OUTGROWTH OF PC12 CELLS ON NON-WOVEN MICROFIBERS

The morphology and number of PC12 cells adherent on non-woven microfibers are shown in **Figure 3**. On PLLA, the rate of adherence of PC12 cells was approximately 3750/sample. Most of the adherent cells were non-neurites, and cells with long neurites were not observed. On PLLA/AG73, a similar number of adherent cells was observed, but neurite outgrowth was slightly enhanced in comparison to PLLA. PC12 cells have been reported to adhere to AG73-immobilized substrates through syndecans and the NGF pathway has been reported to be activated, resulting in neurite outgrowth (Suzuki et al., 2003b). Thus, AG73 must be stably immobilized on a substrate to express its biological function. The AG73 was removed from the surface of the PLLA/AG73 microfibers in culture medium, resulting in poor enhancement of adhesion and neurite outgrowth of PC12 cells. On PLLA/AG73-$(VPGIG)_{30}$ microfibers, the adhesion and neurite outgrowth of PC12 cells was significantly accelerated compared to PLLA and PLLA/AG73. Because the hydrophobicity of the $(VPGIG)_{30}$ region renders AG73-$(VPGIG)_{30}$ insoluble in PBS, it was stably bound to the PLLA. The hydrophilic AG73 region was available to contact PC12 cells at the outermost surface of the microfibers, resulting in promotion of adhesion and neurite outgrowth of PC12 cells.

NERVE REGENERATION IN THE RABBIT MODEL OF NERVE INJURY

The electrospun microfibrous conduits had a three-layered structure, including a PLLA/PEG outer layer, a PLLA middle layer, and a PLLA, PLLA/AG73, or PLLA/ AG73-$(VPGIG)_{30}$ inner layer, as shown in **Figure 4**. PEG was used in the outer layer to prevent the adhesion of surrounding tissues, because the adhesion of surrounding tissues to nerves causes painful traction

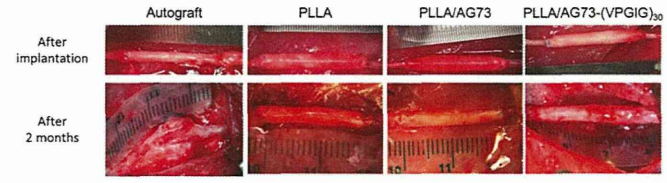

FIGURE 5 | Intraoperative photographs of autograft and micro-fibrous conduits (A) immediately after implantation and (B) 2 months after implantation. The proximal side is shown on the left.

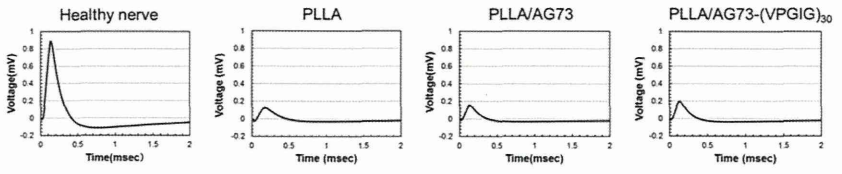

FIGURE 6 | Active potential of healthy nerve and PLLA micro-fibrous conduits 2 months after implantation.

Table 1 | Peak time and intensity of active potentials determined using electrophysiological analysis.

Experimental group	Peak of active potential	*N*			Average	*SD*
		1	2	3		
PLLA	Time (ms)	0.21	0.19	0.17	0.19	0.02
	Intensity (mV)	0.11	0.03	0.12	0.09	0.05
PLUVAG73	Time (ms)	0.16	0.17	0.16	0.16	0.01
	Intensity (mV)	0.28	0.13	0.08	0.16	0.10
PLUVAG73-$(VPGIG)_{30}$	Time (ms)	0.17	0.15	0.14	0.15	0.02
	Intensity (mV)	0.24	0.19	0.19	0.21	0.03

neuropathies (Tashiro et al., 1989). The microfibrous conduits were approximately 500-μm thick, and the diameter of the PLLA/PEG microfiber outer layer was approximately 2.0 μm. Intraoperative photographs of the autografts and the microfibrous conduits in the rabbit tibial nerve gap were taken immediately after the implantation, and are shown in **Figure 5**. The conduits possessed sufficient strength for suturing and maintained their tubular shape after implantation. Two months after implantation, the nerve autografts were strongly adhered to the surrounding tissue and muscle, as shown in **Figure 5**. In contrast, the microfibrous conduits were easily located because the PEG in the PLLA microfibers of the outer layer had suppressed tissue adhesion. All conduits maintained their tubular structure during the 2 months, due to the slow degradation of the high molecular weight PLLA.

Reinnervation by nerve autografts or conduit implantation was analyzed by electrophysiological detection of active potentials (**Figure 6**). The action potential of healthy tibial nerve was 0.9 mV at 0.12 ms. The intensity and peak time of the action potential are summarized in **Table 1**. No action potential was detected in implanted nerve autografts, due to the abnormal adhesion of surrounding tissues, but action potentials were reproducibly detected for PLLA microfibrous conduits. In unmodified PLLA conduit, the time and intensity of the action potential were 0.19 ms and 0.09 mV, respectively. Mixing the AG73 peptide in the inner layers lightly improved the action potential, to 0.16 mV at 0.16 ms. The microfibrous conduit with a PLLA/AG73-$(VPGIG)_{30}$ inner layer carried an action potential of 0.15 ms at 0.21 mV. This recovery is far from functional reinnervation, but nerve regeneration was slightly enhanced by mixing PLLA with AG73-$(VPGIG)_{30}$, compared to PLLA and PLLA/AG73 nerve conduits due to the stable interaction of AG73-$(VPGIG)_{30}$ with PLLA microfibers.

CONCLUSION

PLLA microfibers containing the AG73 peptide or the elastin-laminin mimetic protein AG73-$(VPGIG)_{30}$ were fabricated using an electrospinning procedure. AG73-$(VPGIG)_{30}$ was homogeneously mixed in PLLA microfibers, and the fibers were insoluble in a physiological environment. Neurite outgrowth of PC12 cells was promoted on the PLLA/AG73-$(VPGIG)_{30}$ non-woven microfibers compared to that on the PLLA and PLLA/AG73 non-woven microfibers. In addition, we prepared electrospun microfibrous conduits with a three-layered structure: a PLLA/PEG outer layer, a PLLA middle layer, and a PLLA, PLLA/AG73, or PLLA/AG73-$(VPGIG)_{30}$ inner layer. When microfibrous conduits were implanted into a 2.0-cm gap in an injured rabbit tibial nerve, their tubular structure was maintained after suturing. Although nerve autografts strongly adhered to the

surrounding tissue, PLLA microfibrous conduits did not, owing to PEG mixed in the outer layer. The active potential was slightly improved in PLLA/AG73-$(VPGIG)_{30}$ microfibrous conduit compared to PLLA and PLLA/AG73 microfibrous conduits. However, this recovery was insufficient to achieve functional reinnervation. Microfiber orientation has been reported to influence the differentiation and proliferation of neural stem cell (Bashur et al., 2006; Ghasemi-Mobarakeh et al., 2008). In this study, the structure of the AG73-$(VPGIG)_{30}$ microfibers of the inner layer was random. Alignment of the inner-layer fibers of conduits is expected to enhance reinnervation. The results of this study demonstrate that the electrospun PLLA microfiber conduit with a PEG-mixed outer layer and an AG73-$(VPGIG)_{30}$-mixed inner layershows excellent potential for enhancing nerve regeneration.

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REFERENCES

Ao, Q., Wang, A., Cao, W., Zhang, L., Kong, L., He, Q., et al. (2006). Manufacture of multimicrotubule chitosan nerve conduits with novel molds and characterization *in vitro*. *J. Biomed. Mater. Res. A* 77A, 11–18. doi: 10.1002/jbm.a.30593

Archibald, S. J., Shefner, J., Krarup, C., and Madison, R. D. (1995). Monkey median nerve repaired by nerve graft or collagen nerve guide tube. *J. Neurosci.* 15, 4109–4123.

Bashur, C. A., Dahlgren, L. A., and Goldstein, A. S. (2006). Effect of fiber diameter and orientation on fibroblast morphology and proliferation on electrospun poly(D,L-lactic-co-glycolic acid) meshes. *Biomaterials* 27, 5681–5688. doi: 10.1016/j.biomaterials.2006.07.005

Chernousov, M. A., Yu, W., Chen, Z., Carey, D. J., and Strickland, S. (2008). Regulation of schwann cell function by the extracellular matrix. *GLIA* 56, 1498–1507. doi: 10.1002/glia.20740

Freier, T., Montenegro, R., Koh, H. S., and Shoichet, M. S. (2005). Chitin-based tubes for tissue engineering in the nervous system. *Biomaterials* 26, 4624–4632. doi: 10.1016/j.biomaterials.2004.11.040

Ghasemi-Mobarakeh, L., Prabhakaran, M. P., Morshed, M., Nasr-Esfahani, M.-H., and Ramakrishna, S. (2008). Electrospun poly(ε-caprolactone)/gelatin nanofibrous scaffolds for nerve tissue engineering. *Biomaterials* 29, 4532–4539. doi: 10.1016/j.biomaterials.2008.08.007

Hersel, U., Dahmen, C., and Kessler, H. (2003). RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. *Biomaterials* 24, 4385–4415. doi: 10.1016/S0142-9612(03)00343-0

Huang, Z., Zhang, Y. Z., Kotaki, M., and Ramakrishna, S. (2003). A review on polymer nanofibers by electrospinning and their applications in nanocomposites. *Compos. Sci. Technol.* 63, 2223–2253. doi: 10.1016/S0266-3538(03)00178-7

Ichihara, S., Inada, Y., and Nakamura, T. (2008). Artificial nerve tubes and their application for repair of peripheral nerve injury: an update of current concepts, ainjury. *Injury* 39(Suppl. 4), S29–S39. doi: 10.1016/j.injury.2008.08.029

Iwamoto, Y., Robey, F. A., Graf, J., Sasaki, M., Kleinman, H. K., Yamada, Y., et al. (1987). YIGSR, a synthetic laminin pentapeptide, inhibits experimental metastasis formation. *Science* 238, 1132–1134. doi: 10.1126/science.2961059

Kakinoki, S., Uchida, S., Ehashi, T., Murakami, A., and Yamaoka, T. (2011). Surface modification of poly(L-lactic acid) nanofiber with oligo(D-Lactic acid) bioactive-peptide conjugates for peripheral nerve regeneration. *Polymers* 3, 820–832. doi: 10.3390/polym3020820

Kakinoki, S., and Yamaoka, T. (2010). Stable modification of poly(lactic acid) surface with neurite outgrowth-promoting peptides via hydrophobic collagen-like sequence. *Acta Biomater.* 6, 1925–1930. doi: 10.1016/j.actbio.2009.12.001

Kakinoki, S., and Yamaoka, T. (2014). Thermoresponsive elastin/laminin mimicking artificial protein for modifying PLLA scaffolds in nerve regeneration. *J. Mat. Chem. B*. doi: 10.1039/c4tb00305e

Lohmeyer, J. A., Siemers, F., Machens, H.-G., and Mailänder, P. (2009). The clinical use of artificial nerve conduits for digital nerve repair: a prospective cohort study and literature review. *J. Reconstr. Microsurg.* 25, 55–61. doi: 10.1055/s-0028-1103505

Lundborg, G., Dahlin, L. B., Danielsen, N., Gelberman, R. H., Longo, F. M., Powell, H. C., et al. (1982). Nerve regeneration in silicone chambers: influence of gap length and of distal stump components. *Exp. Neurol.* 76, 361–375. doi: 10.1016/0014-4886(82)90215-1

Lynn, S. K., Yannas, I. V., and Bonfield, W. (2004). Antigenicity and immunogenicity of collagen. *J. Biomed. Mater. Res.* 71B, 343–354. doi: 10.1002/jbm.b.30096

Mainil-Varlet, P., Gogolewski, S., and Nieuwenhuis, P. (1996). Long-term soft tissue reaction to various polylactides and their *in vivo* degradation. *J. Mater. Sci. Mater. Med.* 7, 713–721. doi: 10.1007/BF00121406

Nakamura, T., Inada, Y., Fukuda, S., Yoshitani, M., Nakada, A., Itoi, S., et al. (2004). Experimental study on the regeneration of peripheral nerve gaps through a polyglycolic acid-collagen (PGA-collagen) tube. *Brain Res.* 1027, 18–29. doi: 10.1016/j.brainres.2004.08.040

Nomizu, M., Song, S. Y., Kuratomi, Y., Tanaka, M., Kim, W. H., Kleinman, H. K., et al. (1996). Active peptides from the carboxyl-terminal globular domain of laminin alpha2 and Drosophila alpha chains. *FEBS Lett.* 396, 37–42. doi: 10.1016/0014-5793(96)01060-5

Rao, S. S., and Winter, J. O. (2009). Adhesion molecule-modified biomaterials for neural tissue engineering. *Front. Neuroeng.* 2:6. doi: 10.3389/neuro.16.006.2009

Rutka, J. T., Apodaca, G., Stern, R., and Rosenblum, M. (1988). The extracellular matrix of the central and peripheral nervous systems: structure and function. *J. Neurosurg.* 69, 155–170. doi: 10.3171/jns.1988.69.2.0155

Sieminow, M., and Brzezicki, G. (2009). Current techniques and concepts in peripheral nerve repair. *Int. Rev. Neurobiol.* 87, 141–172. doi: 10.1016/S0074-7742(09)87008-6

Smit, X., van Neck, J. W., Afoke, A., and Hovius, S. E. R. (2004). Reduction of neural adhesions by biodegradable autocrosslinked hyaluronic acid gel after injury of peripheral nerves: an experimental study. *J. Neurosurg.* 101, 648–652. doi: 10.3171/jns.2004.101.4.0648

Suzuki, M., Itoh, S., Yamaguchi, I., Takakuda, K., Kobayashi, H., Shinomiya, K., et al. (2003a). Tendon chitosan tubes covalently coupled with synthesized laminin peptides facilitate nerve regeneration *in vivo*. *J. Neurosci. Res.* 72, 646–659. doi: 10.1002/jnr.10589

Suzuki, N., Ichikawa, N., Kasai, S., Yamada, M., Nishi, N., Morioka, H., et al. (2003b). Syndecan binding sites in the laminin α1 chain G domain. *Biochemistry* 42, 12625–12633. doi: 10.1021/bi030014s

Tashiro, K., Sephel, G. C., Weeks, B., Sasaki, M., Martin, G. R., Kleinman, H. K., et al. (1989). A synthetic peptide containing the IKVAV sequence from the a chain of laminin mediates cell attachment, migration, and neurite outgrowth. *J. Biol. Chem.* 264, 16174–16182.

Thomas, P. K. (1964). The deposition of collagen in relation to schwann cell basement membrane during peripheral nerve regeneration. *J. Cell Biol.* 23, 375–382. doi: 10.1083/jcb.23.2.375

Toyota, B., Carbonetto, S., and David, S. (1990). A dual laminin/collagen receptor acts in peripheral nerve regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 87, 1319–1322. doi: 10.1073/pnas.87.4.1319

Wang, K.-K., Nemeth, I. R., Seckel, B. R., Chakalis-Haley, D. P., Swann, D. A., Kuo, J.-W., et al. (1998). Hyaluronic acid enhances peripheral nerve regeneration *in vivo*. *Microsurgery* 18, 270–275.

Yoshitani, M., Fukuda, S., Itoi, S., Morino, S., Tao, H., Nakada, A., et al. (2007). Experimental repair of phrenic nerve using a polyglycolic acid and collagen tube. *J. Thorac. Cardiovasc. Surg.* 133, 726–732. doi: 10.1016/j.jtcvs.2006.08.089

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解　説

再生医療のための繊維材料修飾とその評価

Surface Modification of Fiber Scaffolds for Regenerative Medicine

山岡哲二・柿木佐知朗

1. はじめに

生分解性ナノファイバーを再生医療に利用しようとする研究発表や論文発表は驚くほど多い。「我々の身体はコラーゲンなどの繊維物質で構成されており……、そのためにナノファイバーはスキャホールドとして最適である。」とは、あまりに楽観的すぎはしないだろうか。繊維径も、微細構造も、力学特性も、表面特性も、何もかもが大きく異なることは明らかである。本当に、ナノファイバーは、スキャホールドとして適しているのだろうか。ちなみに、1990年代に、ポリグリコール酸(PGA)製の生体吸収性縫合糸から作成した不織布をスキャホールドとして用いて軟骨を再生する組織工学(現在の再生医療)が提唱されたが、この時に用いられた繊維径は14μmであった。

2. 再生医療とスキャホールド材料

上述のVacantiとLangerらによる軟骨組織再生の研究では、PGA繊維で作成した不織布をスキャホールドとして使用したが、3次元の組織形状を保持させるために、PGA短繊維をポリ乳酸(PLA)と混合して熱処理することでPGA繊維どうしがPLAで接合されている"bonded fiber structure"を利用した[1-3]。その後、スキャホールド材料は再生医療の中心的役割を担うとして精力的に研究された。しかし、残念ながら、臨床に近い再生医療の中心は、スキャホールドを用いない細胞移植である[4]。その背景には、さまざまな幹細胞の発見がある。1981年にマウス胚性幹細胞が[5,6]、さらに1998年には、ヒト胚性幹細胞の単離が報告され[7]、allogeneicな(他人の)体細胞の入手が可能になると期待された。その後、2006年、2007年には山中らがマウスiPS細胞(induced Pluripotent Stem Cell)[8]、ヒトiPS細胞[9]をあいついで発表し、ご存じの通り、山中らは本年ノーベル医学生理学賞を受賞した。自己由来の体細胞が可能となったことは細胞移植の大きな転換期につながる。自己由来の血小板や赤血球が作成できるとその有用性は計り知れない。つい最近、「文科省を中心に、iPSを利用した立体的な臓器の作成技術を10年以内に」との報道が駆け巡った。立体的の意味にもよるが、我々がイメージする、腎臓、肝臓などの臓器への再生は、これまでと全く異なる新たなイノベーションが無ければ無理ではないかと筆者は考える。立体構造の再現においてこそ、スキャホールドは不可欠である。

3. 臓器・組織再生とナノファイバー

幹細胞研究の発展にともなって、幹細胞の分化増殖を適切にコントロールする細胞周囲の微小環境(ニッチ・Niche)が次々と報告された。生化学的環境のみならず、合成スキャホールド材料の物理化学的特性、すなわち、生体に近い力学的特性も注目され[10,11]、ナノファイバーを中心とするスキャホールド材料には幹細胞ニッチとしての機能も要求されるようになった[12,13]。では、ファイバーとしてのどのような特性が機能性につながる可能性があるのだろうか。表1に、考えられる可能性の一覧を示した。素材に由来するバルク的特性は、もちろん大きな意味を有する

表1　再生医療分野におけるナノファイバー機能の「可能性」

	特　性	機　能（特定の組織）
バルク的特性	分解速度	再生と同調した分解
	高強度	破損回避・3D寸法安定性（血管など）
	柔軟性	拍動との同期（血管） 細胞分化制御
	薬物徐放	組織増殖 組織肥厚化抑制（血管・弁・ステントなど）
構造的特性	形状安定性	再生組織の保持（皮膚・軟骨など）
	繊維径	細胞接着
	繊維間空隙（サイズ・空隙度）	細胞浸潤 物質透過性（神経誘導管・癒着防止材）
	異方性	繊維の配列(細胞分化・神経再生)
表面特性	物理化学的特性	抗血栓性（血管など） 組織非付着性（癒着防止材）
	生理活性	細胞接着・増殖・分化

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