

NOTE

Solid-state NMR studies for the development of non-woven biomaterials based on silk fibroin and polyurethane

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Silk fibroin (SF) possesses several characteristics that are favorable for tissue engineering. However, the mechanical properties must be modified in some cases. For instance, soft tissues such as those of the circulatory system are more elastic than tissue-engineered materials. The development of polymer blends is a simple method with the potential to provide materials with extended useful properties. In this study, we developed a non-woven SF and thermoplastic polyurethane-blend sheet from a polymer solution. The structure and miscibility of the blend sheet was evaluated using solid-state nuclear magnetic resonance (NMR) methods. In the ¹³C cross-polarization and magic angle spinning NMR spectra, no peak shift was observed between the pure and blended samples. The miscibility of the sample was investigated using proton spin-lattice relaxation times in the laboratory frame (T_1^H). The T_1^H measurement clearly revealed that the molecular chains of SF and Pellethane exist in close proximity of several tens of nanometers.

Polymer Journal (2017) 49, 583–586; doi:10.1038/pj.2017.16; published online 29 March 2017

INTRODUCTION

Silk fibroin (SF) from *Bombyx mori* is a good candidate for regenerative medical materials because of its high biocompatibility, slow biodegradation and superior workability.^{1–3} The fibers of SF consist of highly organized β -sheet structures in crystalline regions that are responsible for the water insolubility and the high strength of the material.^{4,5} SF also contains several semi-crystalline and amorphous regions that are responsible for the water solubility and elasticity. However, because of the high ratio of crystalline regions in SF fibers,⁶ the elasticity of SF is insufficient for developing medical scaffolds that are suitable for use in soft tissue. The low elasticity creates several problems, including a highly fragile material and a mismatch in mechanical properties with soft materials. It is very important to match the mechanical properties of the medical scaffolds with the target tissue because a mismatch causes serious problems such as aneurysm or occlusion, particularly in circulatory system tissues.⁷ Therefore, adjustment of the mechanical properties is one of the most important topics to investigate in the development of a new medical scaffold for soft tissues such as circulatory system tissue engineering.

Development of polymer blends is a simple method with the potential to provide materials with extended useful properties. Understanding the structure and miscibility in the blended materials is of interest because they influence the mechanical properties.

In this study, Pellethane (2363-80 AE, Lubrizol Co., USA) was selected for use as an elastic material because it is the only medical grade thermoplastic polyurethane that is available, and it has been used for decades in the fabrication of implantable devices for soft tissues. In addition to the unique characteristics of polyurethanes, including biocompatibility, toughness and functionality, significant improvement has been to the biostable mechanical properties. However, the mechanical properties may suffer from excessive plastic deformation or limited biostability, especially in porous implant applications. By blending SF and Pellethane, we aim to combine the elastic character of Pellethane and the high strength of SF in a non-woven blend sheet to prevent such deformations. An electrospinning method was applied to an SF/Pellethane blend polymer solution to fabricate non-woven sheets that mimic bio-tissues. Non-woven sheets that have a similar fiber diameter and pore size to bio-tissues are anticipated to promote cell compatibility and invasion into the sheet when used as a medical scaffold. SF consists of heavy (H) and light (L) chain proteins that are linked by a single disulfide bond.² A previous analysis of the *B. mori* gene determined that the fibroin amino acid sequence is 5263 residues long and that the molecular weight is 391 367 Da.⁸

Solid-state nuclear magnetic resonance (NMR) is a powerful tool for elucidating the morphology and miscibility of blended materials.

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Received 28 September 2016; revised 21 December 2016; accepted 20 February 2017; published online 29 March 2017

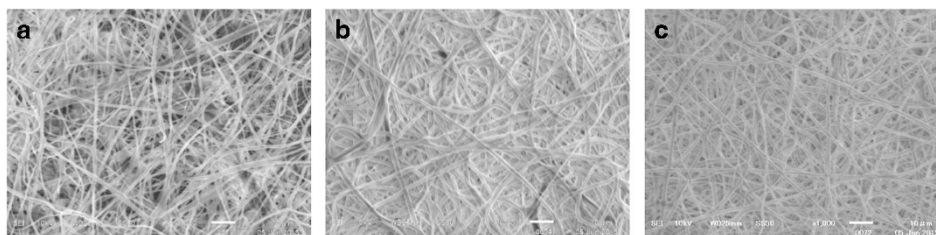


Figure 1 SEM images of SF (a), blend (b) and Pellethane (c) non-woven sheets. Scale bar, 10 μm . SEM, scanning electron microscopy; SF, silk fibroin.

In this paper, the molecular structures of pure SF, Pellethane and a SF/Pellethane-blended non-woven sheet were investigated using ^{13}C spectra obtained by the combined use of cross-polarization and magic angle spinning methods. The miscibility of the SF/Pellethane-blended non-woven sheet was evaluated using the proton spin-lattice relaxation times that were estimated from the well-resolved ^{13}C signals.

EXPERIMENTAL PROCEDURE

Production of SF/Pellethane composite non-woven sheets

Aqueous solutions of SF from *B. mori* were obtained by dialyzing degummed fibers dissolved in 9.0 M LiBr (Wako Pure Chemical Industries, Ltd, Osaka, Japan) against ultra-pure water according to a previous study.⁹ In the current study, the final concentration of the SF solution was 10 (w/v)% at 37 °C. Cellulosic dialysis membranes with a molecular weight cut-off of 14 000 were used for this process. SF sponges were obtained by lyophilization of diluted SF aqueous solutions (1 (w/v)%). The obtained SF sponge and Pellethane were blended in a weight ratio of 5/5 and dissolved in 1,1,3,3,3-hexafluoro-2-propanol (HFIP, Wako Pure Chemical Industries, Ltd) at 6 (w/v)%. Next, the SF/Pellethane HFIP solution was transferred to a syringe fitted with a needle and electrospun for 3 h at 12 μl per minute of the injection speed with a high-voltage power supply under laboratory conditions (Esprayer ES-2000S, Fuenue, Tokyo, Japan). The distance between electrodes was fixed at 10 cm. The applied voltage was regulated at 16 kV. An aluminum plate was used as a collector. The obtained non-woven sheets were dried in air overnight, then exposed to 100% relative humidity at 37 °C for 24 h to convert the structure of SF into its insoluble form. Pure SF and Pellethane sheets were also prepared using the same method except the applied voltages in electrospinning process were changed to 17 and 20 kV, respectively.

Characterization of SF/Pellethane complex non-woven sheets

Scanning electron microscopy measurements. After sputter gold coating, the surfaces of the non-woven sheets were observed using a scanning electron microscope (JEOL Ltd. JSM-6510, Tokyo, Japan) at an applied voltage of 10 kV. The diameter of the fibers was calculated using ImageJ software. The measured fibers were randomly selected from 50 fibers in each image. All results are presented as the mean standard deviation. Statistical analysis was carried out based on one-way analysis of variance test and the differences were considered significant at $*P < 0.05$.

Solid-state NMR measurements. The structure of the non-woven sheets was evaluated following the humidity treatment. All solid-state NMR measurements were performed using a Varian NMR system 400 WB spectrometer (Agilent Technologies, Santana Clara, CA, USA) with an operating frequency of 100.57 MHz for ^{13}C using a 6.0-mm ϕ rotor. The magic angle spinning rate was set to 7 kHz and the TPPM method was used for ^1H decoupling. The ^{13}C chemical shifts were measured relative to tetramethylsilane using the methine carbon signal at 29.47 p.p.m. for solid adamantane as an external standard. ^{13}C spectra were obtained from the combined use of cross-polarization and magic angle spinning. The contact time of cross-polarization was 1 ms. Proton spin-lattice relaxation times in the

laboratory frame (T_1^{H}) were indirectly measured from the well-resolved ^{13}C signals enhanced by cross-polarization of 1 ms applied after the inversion-recovery pulse sequence for ^1H .

RESULTS AND DISCUSSION

Morphological observation

Figure 1 depicts the scanning electron microscopy images of the fabricated non-woven sheet samples of pure SF (a), the SF/Pellethane blend (b) and pure Pellethane (c). The scanning electron microscopy images clearly indicate that all of the sheet samples consisted of long electrospun fibers. The fiber diameter was determined to be approximately 1 μm in each sample. No significant difference was observed between the fiber diameters of the three samples. The measured diameters were as follows: SF sheet: $1.1 \pm 0.3 \mu\text{m}$ (Figure 1a), SF/Pellethane sheet: $1.2 \pm 0.4 \mu\text{m}$ (Figure 1b) and Pellethane sheet: $1.2 \pm 0.3 \mu\text{m}$ (Figure 1c).

The blended sheet exhibited more continuous fibers compared with the pure SF sample. The electrospinning process was successful for the SF/Pellethane-blended sheet, producing a material with a large surface area and porous structure. Such a structure is desirable for cell and tissue incorporation and the growth of tissue engineering materials because it is similar to the structure of the extracellular matrix of native tissue.¹⁰

Structural evaluation

The ^{13}C cross-polarization/magic angle spinning NMR spectra of pure SF, pure Pellethane and the SF/Pellethane-blended non-woven sheets are displayed in Figure 2 along with their peak assignments. The spectrum of SF is in good agreement with a previously measured spectrum.¹¹ The detected chemical shifts of the pure SF non-woven sheet (a) indicate that the sheet mainly consisted of a β -sheet structure (19.6 p.p.m. from Ala C β , 42.4 p.p.m. from Gly C α , 48.7 p.p.m. from Ala C α , 169.1 and 171.8 p.p.m. from the carbonyl carbons of Gly and Ala, respectively).¹¹ Shoulder peaks at approximately 51 and 177 p.p.m. that are assigned to Ala C α and the carbonyl carbon, respectively, and a peak at 16.9 p.p.m. that is assigned to Ala C β suggest that the pure SF non-woven sheet partially includes β -turn and/or α -helical conformations, although these morphologies cannot be confirmed only from the chemical shifts. Several types of conformations are known to be possible in SF depending on the species of silkworm and the conditions of sample preparation. Previous studies have shown that SF from *B. mori* adopts two dimorphic structures in the solid state: silk I and silk II.^{12,13} Silk I is less stable compared with silk II and rapidly undergoes a transition to the silk II conformation after spinning. Thus, the current spectrum reflects the structure of silk II. A recent study of SF/Polyurethane-blend films demonstrated that the mechanical properties of the films were strongly correlated to the silk content.¹⁴ However, the structural properties of SF, which are influenced by treatment methods and manufacturing methods, have a significant impact on the mechanical

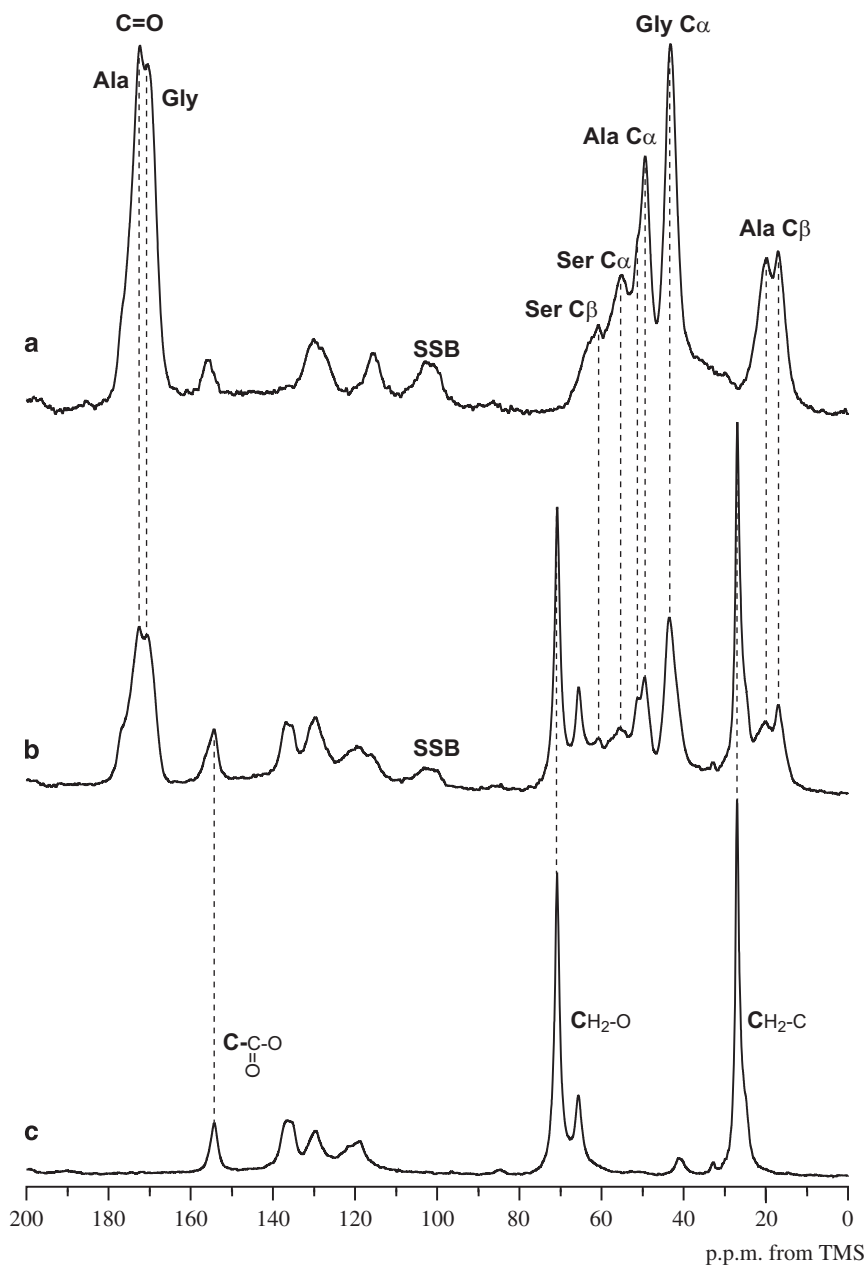


Figure 2 ^{13}C CP/MAS NMR spectra of SF (a), blend (b) and Pellethane (c) non-woven sheets. CP, cross-polarization; MAS, magic angle spinning; NMR, nuclear magnetic resonance; SF, silk fibroin.

properties. Here, we evaluated the effect of the treatment and manufacturing methods on the SF structure.

The spectrum of the pure Pellethane sheet (Figure 2c) contains two sharp methylene (CH_2) peaks, indicating high mobility of the CH_2 chains. The spectrum of the SF/Pellethane blend (Figure 2b) shows complete superposition of the spectra of pure SF and pure Pellethane. This measurement clearly revealed that Pellethane does not break the microstructure and morphology of SF. Furthermore, the high chain mobility of Pellethane was also not affected by the presence of SF because the linewidth of the CH_2 peak remained unchanged.

Miscibility evaluation

Figure 3 depicts the proton spin-lattice relaxation (T_1^{H}) curves obtained from the Ala C β peak at 16.9 p.p.m. of pure SF (filled square

symbols) and the CH_2 peak at 27.0 p.p.m. of Pellethane (filled circle symbols). Solid lines represent the results of the least-squares fitted T_1^{H} decay times of pure SF and Pellethane, and the slopes indicate the T_1^{H} relaxation times. The estimated T_1^{H} value of SF was 1.01 s and that of Pellethane was 0.55 s. Open symbols represent the T_1^{H} decay times obtained from the SF/Pellethane blend sheet. Open circles represent the Pellethane CH_2 carbon of the blend sheet and the squares represent the SF Ala C β . The curves rapidly approach each other, indicating efficient ^1H spin diffusion between SF and Pellethane on the T_1^{H} timescale. When fast ^1H spin diffusion occurs between SF and Pellethane domains, their different spin energies are averaged and become identical. Thus, the distinct T_1^{H} values converge to the same value. Moreover, such a phenomenon occurs when the polymers are in close proximity within several tens of nanometers.¹⁵ The T_1^{H} value of

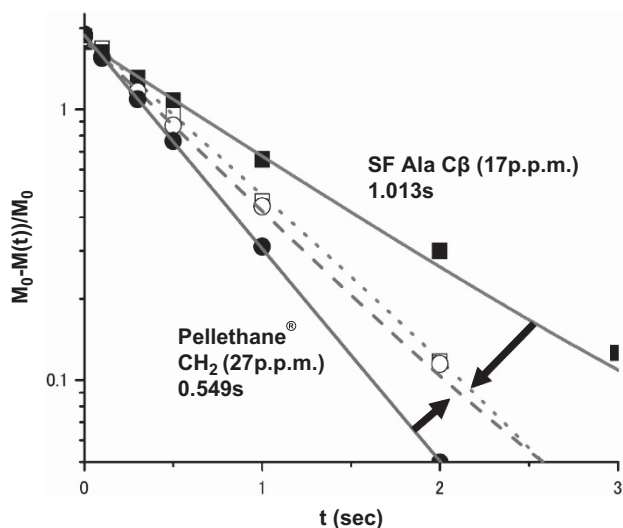


Figure 3 T_1^H relaxation curves of the methyl peak from Ala C β in SF at 17 p.p.m. (square) and the methylene peak from Pellethane at 27 p.p.m. (circle). Filled symbols and solid lines are from pure SF and Pellethane non-woven sheets. Open symbols and dotted lines are from the blend sample. SF, silk fibroin. A full colour version of this figure is available at the *Polymer Journal* journal online.

SF changed from 1.01 to 0.77 s and that of Pellethane changed from 0.55 to 0.69 s. The final T_1^H values are in good agreement with each other. This measurement clearly revealed that the molecular chains of SF and Pellethane are in close proximity within several tens of nanometers.

From the above results, we concluded that the ordered structures of SF and Pellethane are preserved in the blend sample. For SF, the inter- and intramolecular hydrogen bonds maintained the β -sheet structure, and the interaction was not severely affected by the presence of Pellethane. Therefore, SF molecular chains might aggregate with one another in the blend. However, Pellethane was in close contact with the SF chains. As discussed, SF and Pellethane were highly miscible with each other even though SF maintained the strong β -sheet crystal structure. This result suggests that SF/Pellethane-blended sheets possess strength that is derived from SF and elasticity that is derived from Pellethane. Therefore, this blended sheet should be a good medical material for soft tissues, such as in circulatory system tissue engineering.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This project was entrusted to us by MAFF as part of a scientific technique research promotion program from the agriculture, forestry, fishers and food industry (26051A). Several achievements were supported by grant KAKENHI from the Grant-in-Aid for Scientific Research (15H03020) by the Ministry of Education, Culture, Sports, Science and Technology, Japan.

- Asakura, T., Suzuki, Y., Nakazawa, Y., Yazawa, K., Holland, G. P. & Yarger, J. L. Silk structure studied with nuclear magnetic resonance. *Prog. Nucl. Magn. Reson. Spectrosc.* **69**, 23–68 (2013).
- Charu, V. & Kaplan, D. L. Silk as a biomaterial. *Prog. Polym. Sci.* **32**, 991–1007 (2007).
- Leal-Egana, A. & Scheibel, T. Silk-based materials for biomedical applications. *Biochemistry* **55**, 155–167 (2010).
- Suzuki, Y., Aoki, A., Nakazawa, Y., Knight, D. P. & Asakura, T. Structural analysis of the synthetic peptide (Ala-Gly-Ser-Gly-Ala-Gly)₅, a model for the crystalline domain of Bombyx mori silk fibroin, studied with ¹³C CP/MAS NMR, REDOR, and statistical mechanical calculations. *Macromolecules* **43**, 9434–9440 (2010).
- Suzuki, Y., Takahashi, R., Shimizu, T., Tansho, M., Yamauchi, K., Williamson, M. P. & Asakura, T. Intra- and intermolecular effects on ¹H chemical shifts in a silk model peptide determined by high-field solid state ¹H NMR and empirical calculations. *J. Phys. Chem. B* **113**, 9756–9761 (2009).
- Kameda, T., Nakazawa, Y., Kazuhara, J., Yamane, T. & Asakura, T. Determination of intermolecular distance for a model peptide of Bombyx mori silk fibroin, GAGAG, with rotational echo double resonance. *Biopolymers* **64**, 80–85 (2002).
- Ravi, S. & Chaikof, E. Biomaterials for vascular tissue engineering. *Regen. Med.* **5**, 1–21 (2010).
- Zhao, C. & Asakura, T. Structure of Silk studied with NMR. *Prog. Nucl. Magn. Reson. Spectrosc.* **39**, 301–352 (2001).
- Aytemiz, D., Sakiyama, W., Suzuki, Y., Nakaizumi, N., Tanaka, R., Ogawa, Y., Takagi, Y., Nakazawa, Y. & Asakura, T. Small-diameter silk vascular grafts (3 mm diameter) with a double-raschel knitted silk tube coated with silk fibroin sponge. *Adv. Healthc. Mater.* **2**, 361–368 (2013).
- Miyazu, K., Kawahara, D., Ohtake, H., Watanabe, G. & Matsuda, T. Luminal surface design of electrospun small-diameter graft aiming at *in situ* capture of endothelial progenitor cell. *J. Biomed. Mater. Res. B Appl. Biomater.* **94B**, 53–63 (2010).
- Asakura, T., Hamada, M., Nakazawa, Y. & Ha, S. Conformational study of silk-like peptides containing the calcium-binding sequence from calbindin D 9k using ¹³C CP/MAS NMR spectroscopy. *Biomacromolecules* **7**, 627–634 (2006).
- Saito, H., Iwanaga, Y., Tabet, R., Narita, M. & Asakura, T. A high resolution ¹³C NMR study of silk fibroin in solid state by the cross polarization-magic angle spinning method: conformational characterization utilizing conformation-dependent ¹³C chemical shifts. *Chem. Lett.* **4**, 427–430 (1983)
- Asakura, T., Kuzuhara, A., Tabet, R. & Saito, H. Conformation characterization of Bombyx mori silk fibroin in the solid state by high-frequency ¹³C cross polarization-magic angle spinning NMR, X-ray diffraction, and infrared spectroscopy. *Macromolecules* **18**, 1841–1845 (1985).
- Park, H. S., Gong, M.-S. S., Park, J.-H. H., Moon, S., II, Wall, I. B., Kim, H.-W. W., Lee, J. H. & Knowles, J. C. Silk fibroin-polyurethane blends: physical properties and effect of silk fibroin content on viscoelasticity, biocompatibility and myoblast differentiation. *Acta Biomater.* **9**, 8962–8971 (2013).
- Asano, A. Polymer blends. in *Modern Magnetic Resonance* (ed. Webb, G. A.), 627–631 (Springer, 2006).