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Stem Cell Response to Multiwalled Carbon Nanotube-Incorporated Regenerated Silk Fibroin Films

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Multiwalled carbon nanotubes (MWCNTs) are considered to be the ideal reinforcements for bio-related applications on account of their remarkable structural, mechanical and thermal properties. However, before MWCNTs can be incorporated into new and existing biomedical devices, their toxicity and biocompatibility need to be investigated thoroughly. In this study, regenerated silk fibroin/MWCNT nanocomposite films were prepared using a solvent system with pre-dispersed MWCNTs. Their biocompatibility was examined *in vitro* using human bone marrow stem cells. Scanning electron microscopy and a WST-1 assay demonstrated that the silk fibroin/MWCN film supported BMSC attachment and growth over 7 days in culture similar to the silk fibroin only film.

Keywords: Silk Fibroin, Carbon Nanotube, Nanocomposite, Toxicity.

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1. INTRODUCTION

The silk fibroin produced by the Bombyx mori silkworm is a fibrous biopolymer that has been used for thousands of years as one of the most important materials in the textile industry and as a medical sutures.[1] Recently considerable effort has been directed towards its use as a biotechnological material for such biomedical applications, as tissue-engineering scaffolds, drug-delivery matrices and vascular grafts, on account of its excellent biodegradability and biocompatibility.[2–6] In order for it to be used as a biotechnological material in biomedical applications, it is essential to regenerate silk fibroin into its proper form. However, the crystallinity of silk fibroin decreases during the regeneration process, resulting in a deterioration of the mechanical properties of the regenerated silk compared to natural cocoon fiber.[7,8] Moreover, in a wet state, hydrogen bonds are formed preferentially with water molecules,[9,10] which results in poor mechanical strength. Therefore, the possibility of producing silk-base biomaterials by blending with other polymers or adding reinforcements is of considerable interest.[11,12]

Since their discovery in 1991, carbon nanotubes (CNTs) are considered to be ideal nanomaterials on account of their low density, thermal and chemical stability, and ordered structure with a high aspect ratio.[13–16] The combination of these characteristics makes CNTs a promising material in a wide variety of applications, including biomedical uses.[17] As a result, toxicological studies of CNTs have become a crucial issue. Poland et al. suggested the potential for inflammation and the formation of lesions after exposure to CNTs.[18] However, Schipper et al. reported that the intravenous administration of CNTs to mice does not lead to acute or chronic toxicity.[19] In addition, Lecerda et al. demonstrated that functionalized water-soluble CNTs are compatible with biological fluids, which can lead to their rapid extraction through the renal route, minimizing unwanted tissue accumulation.[20] There is still some controversy regarding the toxicity of CNTs in bio-related applications. To our knowledge, there are few reports on the use of silk-based biomaterials as a substrate for bone marrow stem cells (BMSCs).

In our previous work, we successfully prepared water stable silk films by the incorporation of MWCNTs, which induced their crystallization by acting as a nucleating agent, without any further post annealing processes such as water or methanol treatments.[21] In this study, regenerated silk fibroin/MWCNT nanocomposite films were prepared *in situ* using an all aqueous system with pre-dispersed MWCNTs. The biocompatibility was investigated from the growth of human bone marrow stem cells (hBMSCs) on a silk fibroin/MWCNTs film, and the mechanical properties of MWCNT-incorporated silk films were examined in the wet state.

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2. EXPERIMENTAL DETAILS

2.1. Materials

Cocoons of *Bombyx mori* silkworm silk were kindly supplied by Boeun Sericulture Farm, South Korea. MWCNTs (purity of 97%; Hanhwa Nanotech Co., Korea) that had been synthesized by thermal chemical vapor deposition (CVD) were used. The purity of the pristine, as received MWCNTs was >95%. To eliminate the impurities in the MWCNTs (such as metallic catalysts), they were treated with a mixture of 3 M HNO_3 and 1 M H_2SO_4 at 60 °C for 12 h, followed by refluxing in 5 M HCl at 120 °C for 6 h. The purity of the acid-treated MWCNTs was measured to be 99% using thermo gravimetric analysis (TGA, Q50, TA instruments, UK). These acid treatments are known to introduce carboxylic and hydroxyl functional groups onto the surface of the MWCNTs.[22] The purified MWCNTs were filtered and washed with a large amount of water and then vacuum dried at room temperature overnight.

2.2. Preparation of Silk Fibroin/MWCNTs Films

The cocoons were boiled for 30 min in an aqueous solution of 0.02 M Na_2CO_3 to remove the glue-like sericin proteins. The silk fibroin was obtained by rinsing thoroughly with water. The purified MWCNTs (0.01 wt%) were dispersed in a 9.3 M LiBr aqueous solution. Ultrasound was then applied to the MWCNTs dispersion using an ultrasonic generator (Kodo Technical Research Co., Japan) at a frequency and power of 28 kHz and 600 W, respectively, for 1 h at room temperature. The silk fibroin was dissolved in a LiBr solution containing the purified MWCNTs at 60 °C for 3 h. The solution was dialyzed in water for 48 h using Slide-a-Lyzer dialysis cassettes (Pierce, MWCO 3500). The final concentration of the silk fibroin in the aqueous solution was approximately 8 wt%.[23] The composite films were prepared successfully by solvent casting.

2.3. Characterization

The morphology of the silk/MWCNT films was examined by scanning electron microscopy (SEM, S-4300, Hitachi, Japan) and transmission electron microscopy (TEM, CM200, Philips, Japan). Wide-angle X-ray diffraction (WAXD, Rigaku, DMAX-2500, Japan) was used to characterize the MWCNT-incorporated silk fibroin film structures. The mechanical properties of the composite films were measured at room temperature using a universal test machine. Fourier transform infrared spectroscopy (FT-IR, Bruker, VERTEX 80V, Germany) was used to examine the chemical structure of the films.

2.4. Culture of Human Bone Marrow Stromal Cells

Cryopreserved hBMSCs established from a young woman were kindly donated by the Catholic University of Korea, St. Mary's Hospital. The cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco BRL, USA) and 1%penicillin-streptomycin, and then seeded into tissue culture flasks at 1×10^5 cells/cm^2. The non-adherent hematopoietic cells were removed by replacing the medium on the second day of expansion. The medium was changed every 2 days throughout the study. The adherent cells were rinsed thoroughly with phosphate-buffered saline (PBS) (pH 7.4) and removed with trypsin for use in the experiments.

2.5. *In Vitro* hBMSC Attachment Studies

The cytotoxicity was assayed using a WST-1 kit (Roche, Mannheim, Germany) after culturing the cells on the silk only and composite films for 1, 2, 3 and 7 days. Briefly, 1 mL of the WST-1 reagent was added to each well. The plates were incubated at 37 °C for 4 h, and the samples were then shaken for 1 min. An aliquot from each well (100 μL) was transferred to a 96-well plate (BD Bioscience, Bedford, MA), and the absorbance at 450 nm was measured using a microplate reader (EL808 ultra microplate reader; Bio-Tek Instruments, Vermont, USA). All experiments were repeated 3 times.

At 1, 2, 3 and 7 days, the hBMSCs on the silk only films and MWCNT-incorporated silk film were fixed with 2.5% glutaraldehyde for 24 h, followed by ethanol dehydration. The fixed hBMSCs were coated with a conducting layer of gold by plasma sputtering (Emitech, K575, Japan), and SEM (S-2250N, Hitachi, Japan) images were obtained.

3. RESULTS AND DISCUSSION

The pure silk films were prepared using the solvent casting method from regenerated silk fibroin in an aqueous solution (~8 wt%). The silk fibroin films were crystallized by a water treatment, a well-known method for crystallizing silk films by inducing the formation of the silk I (α-form) structure.[24] The silk fibroin/MWCNT film was also cast from an aqueous silk fibroin solution containing pre-dispersed MWCNTs.

Generally the pristine CNTs form bundles or aggregations due to the van der Waals forces, and thus the preparation of a good CNTs dispersion is important for producing polymer/CNTs composites.[25] To obtain the homogeneous MWCNTs aqueous solution, functional groups, such as carboxyl groups, were introduced to the surfaces of the MWCNTs through the acid treatments. In this way, the MWCNTs were well dispersed in silk fibroin composite films. Figures 1 and 2 show SEM and TEM images of the surface morphology and inner structure of the silk fibroin/MWCNTs film, respectively, which confirmed that the MWCNTs are well dispersed and embedded individually throughout the silk matrix.

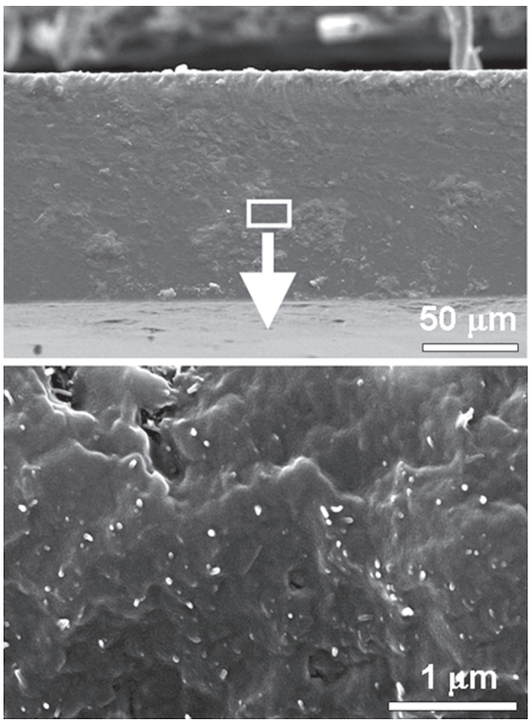

Fig. 1. SEM images of the silk/MWCNT film.

The stability of a silk film in water is one of the important parameters for the practical application of biomaterials. As shown in Figure 3, the as-prepared silk film lost 55.8% of its weight after 48 h. It should be noted that the as-prepared silk films are usually soluble in water without the crystallization processing through their exposure to water or methanol. However, the silk fibroin/MWCNTs film showed no significant decrease in mass because the MWCNTs induced the crystallization of silk fibroin by acting as a nucleating agent.[21]

The crystallinity of the silk fibroin/MWCNTs film was examined by WXRD and FT-IR spectroscopy. The WAXD patterns are shown in Figure 4. The as-prepared silk film

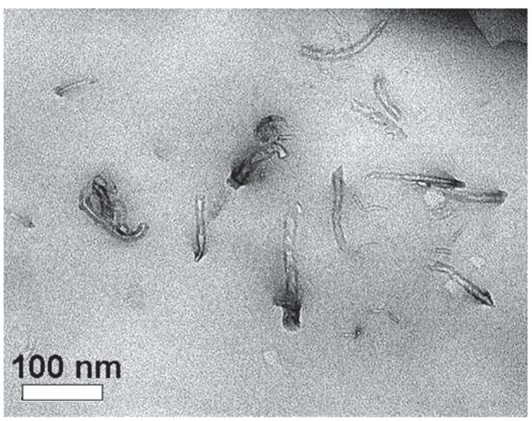

Fig. 2. TEM image of the silk/MWCNT film.

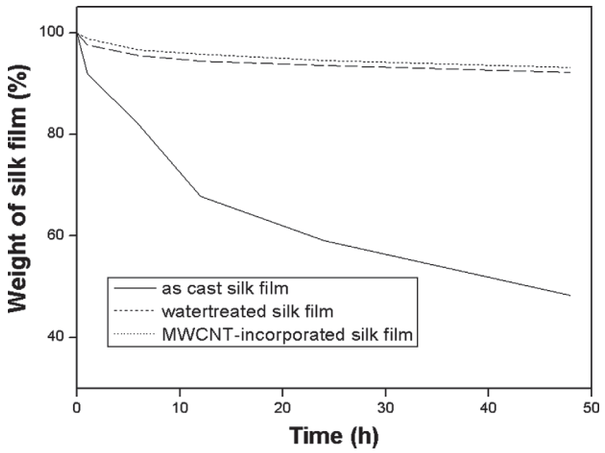

Fig. 3. Solubility of the silk films in water measured at room temperature.

had a typical amorphous pattern, while the water-treated silk film showed broad Bragg reflections at approximately 12.0°, 19.8° and 24.2° corresponding to the silk I (α-form) conformation. The silk fibroin/MWCNT film also showed a silk I structure similar to the water treated silk film with a higher intensity. The FT-IR spectra of the silk films in Figure 4 shows amide I (1,630–1,650 cm^{-1}, C═O stretching) and amide II (1,540–1,520 cm^{-1}, secondary NH bending) vibration bands. The as-prepared silk film initially exhibited an amorphous structure (1538 cm^{-1}) with some silk I (1658 cm^{-1} and 1652 cm^{-1}) (Fig. 5(a)). However, in the water-treated and silk fibroin/MWCNT films, the silk I structure was predominant (Figs. 4(b and c)). The results of both WAXD and FT-IR spectroscopy indicate that the structure of silk fibroin/MWCNTS film had transformed to the silk I structure by incorporating MWCNTs.

The cytotoxicity of the silk film only and the MWCNT-incorporated silk film to hBMSCs was examined over a period of 7 days to determine the ability of these

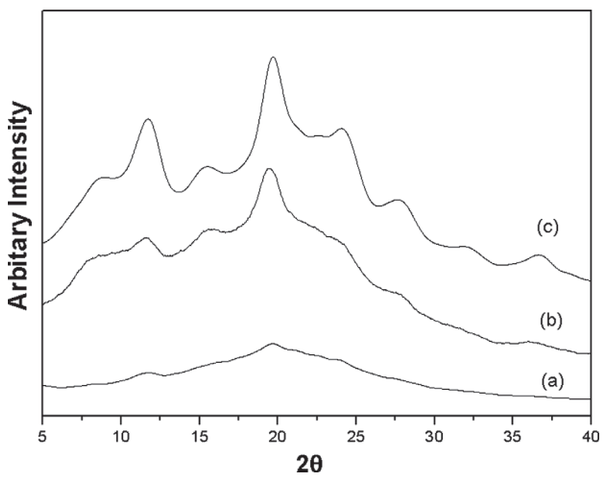

Fig. 4. WAXD spectra of the silk films. (a) as-cast silk film, (b) water treated silk film, (c) silk/MWCNT film.

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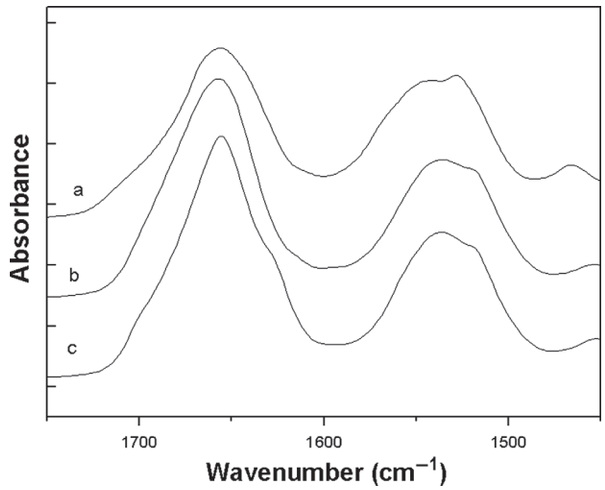

Fig. 5. FTIR spectra of the silk films. (a) as-cast silk film, (b) water treated silk film, (c) silk/MWCNT film.

films to support cell attachment and growth. As shown in Figure 6, at days 1, 2 and 3, a similar number of hBMSCs were counted on the tissue culture plate, silk only film and MWCNT-incorporated silk film. However, at 7 days, there was generally less hBMSCs proliferation on the tissue culture plate (control) than on the silk only film and MWCNT-incorporated silk film. The hBMSCs on each film was monitored by SEM over a 7-day period (Fig. 7). After incubation for 7 days, most of the hBMSCs appeared to have adhered to the silk only and MWCNT-incorporated silk films with a flattened morphology. Most of the attached cells had a flat shape. High-magnification SEM images showed that the hBMSCs were anchored on the silk only and MWCNT-incorporated silk films.

The influence of MWCNTs on the mechanical properties of the regenerated silk film in the wet state (24 h in water at 25 °C) was studied. The water treated silk and

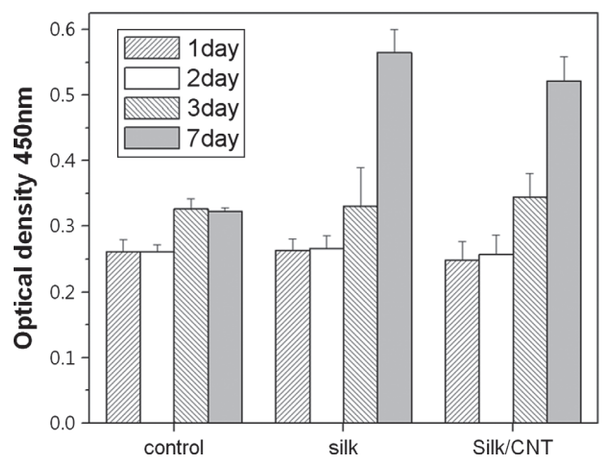

Fig. 6. hBMSC viability for 1, 2, 3, and 7 days on the silk only and MWCNT-incorporated silk films measured using a WST-1 assay. hBMSCs grown on a plain culture plate were used as the control.

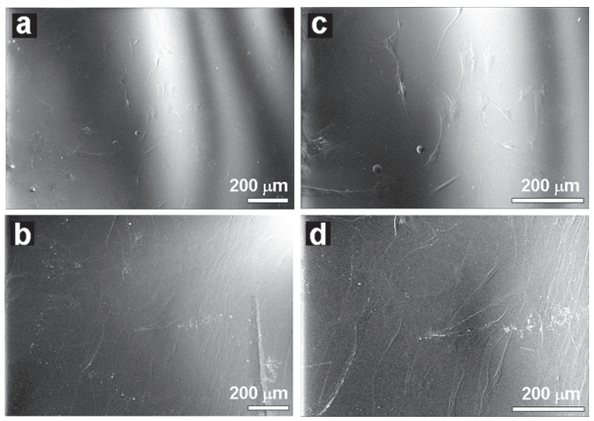

Fig. 7. SEM images of hBMSCs on (a, b) silk film only and MWCNT-incorporated silk film at 7 days. The magnification is X200 (a, c) and X400 (b, d), and the scale bars are 30 μm.

Table I. Mechanical properties of regenerated silk films in the wet state.

	Tensile strength (MPa)	Young's modulus (GPa)	Elongation at break (%)
Silk/MWCNT films	37.20 ±3.93	0.40 ±0.04	73.99 ±2.5
Water treated silk film	27.33 ±8.78	0.28 ±0.08	96.61 ±11.5

silk fibroin/MWCNT films had a thickness of 213±22 and 165±19 μm, respectively. Although the water treated silk and silk fibroin/MWCNT films have a similar structure, the silk fibroin/MWCNT films showed a significantly higher tensile strength and tensile modulus (Table I). As shown in Figures 1 and 2, the MWCNTs were embedded homogeneously in the silk matrix without aggregation. It appears that the carboxyl groups on the surfaces of the MWCNTs introduced by the acid treatment improved the compatibility with the silk matrix. Therefore, the mechanical properties were improved significantly by the incorporation of MWCNTs.

4. CONCLUSIONS

In this study, water insoluble silk films were prepared by incorporating MWCNTs in an all aqueous process. The biocompatibility of the silk fibroin/MWCNT films were examined *in vitro* using hBMSCs. The hBMSCs adhered favorably and proliferated to a similar level on both the silk only and MWCNT-incorporated silk films. This approach should allow MWCNTs to be used in a wide range of biomedical applications.

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References and Notes

1. H. J. Jin, J. Park, R. Valluzzi, P. Cebe, and D. L. Kaplan, *Biomacromolecules* 5, 711 **(2004)**.
2. D. L. Kaplan, C. M. Mello, S. Arcidiacono, S. Fossey, K. Senecal, W. Muller, Protein-Based Materials, edited by K. McGrath and D. L. Kaplan, Birkhauser, Boston **(1997)**, p. 103.
3. J. M. Deitzel, J. Kleinmeyer, D. Harris, and N. C. Beck Tan, *Polymer* 42, 261 **(2001)**.
4. S. Sofia, M. B. McCarthy, G. Gronowicz, and D. L. Kaplan, *J. Biomed. Mater. Res.* 54, 139 **(2001)**.
5. Z. Shao and F. Vollrath, *Nature* 418, 741 **(2002)**.
6. G. H. Altman, R. L. Horan, H. H. Lu, J. Moreau, I. Martin, J. C. Richmond, and D. L. Kaplan, *Biomaterials* 23, 4131 **(2002)**.
7. H. J. Jin, S. V. Fridrikh, G. C. Rutledge, and D. L. Kaplan, *Biomacromolecules* 3, 1233 **(2002)**.
8. Y. Gotoh, M. Tsukada, T. Baba, and N. Minoura, *Polymer* 38, 487 **(1997)**.
9. A. J. Poole, J. S. Church, and M. G. Huson, *Biomacromolecules* 10, 1 **(2009)**.
10. J. Perez-Rigueiro, C. Viney, J. Llorca, and M. Elices, *Polymer* 41, 8433 **(2000)**.
11. E. Marsano, M. Canetti, G.. Conio, P. Corsini, and G. Freddi, *J. Appl. Polym. Sci.* 104, 2187 **(2007)**.
12. E. Marsano, P. Corsini, M. Canetti, and G. Freddi, *Int. J. Biol. Marcromol.* 43, 106 **(2008)**.
13. E. S. Choi, J. S. Brooks, D. L. Eaton, M. S. Al-Haik, M. Y. Hussaini, H. Garmestani, D. Li, and K. Dahmen, *J. Appl. Phys.* 94, 6034 **(2003)**.
14. J. Gao, A. Yu, M. E. Itkis, E. Bekyarova, B. Zhao, S. Niyogi, and R. C. Haddon, *J. Am. Chem. Soc*. 126, 16698 **(2004)**.
15. R. Ramasubramaniam, J. Chen, and H. Liu, *Appl. Phys. Lett.* 83, 2928 **(2003)**.
16. R, Haggenmueller, W. Zhou, J. E. Fischer, and K. I. Winey, *J. Nanosci. Nanotechnol.* 3, 105 **(2003)**.
17. L. Lacerda, A. Bianco, M. Prato, and K. Kostarelos, *Adv. Drug Delivery. Rev*. 58, 1460 **(2006)**.
18. C. A. Poland, R. Duffini, I. Kinloch, A. Maynard, W. A. H. Wallace, A. Seaton, V. Stone, S. Brown, W. Macnee, and K. Donaldson, *Nat. Nanotechnol.* 3, 423 **(2008)**.
19. M. L. Schipper, N. Nakayama-Ratchford, C. R. Davis, N. W. S. Kam, P. Chu, Z. Liu, X. Sun, H. Dai, and S. S. Gambhir, *Nat. Nanotechnol.* 3, 216 **(2008)**.
20. L. Lacerda, A. Bianco, M. Prato, and K. Kostarelos, *Adv. Drug Delivery. Rev.* 58, 1460 **(2006)**.
21. H. S. Kim, W. I. Park, Y. Kim, and H. J. Jin, *Int. J. Mod. Phys. B* 22, 1807 **(2008)**.
22. J. Chen, M. A. Hamon, H. Hu, Y. Chen, A. M. Rao, and P. C. Eklund, *Science* 282, 95 **(1998)**.
23. U. J. Kim, J. Park, C. Li, H. J. Jin, R. Valluzzi, and D. L. Kaplan, *Biomacromolecules* 5, 786 **(2004)**.
24. H. J. Jin, J. Park, V. Karageorgiou, U. J. Kim, R. Valluzzi, P. Cebe, and D. L. Kaplan, *Adv. Funct. Mater.* 15, 1241 **(2005)**.
25. S. M. Kwon, H. S. Kim, and H. J. Jin, *J. Nanosci. Nanotechnol.* 9, 1 **(2009)**.

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