Modification of Sericin-free Silk Fibers for Ligament Tissue Engineering Application

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Received 23 March 2006; revised 14 July 2006; accepted 8 August 2006
Published online 22 February 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.b.30714

Abstract: Biomedical application of silk requires the removal of sericin that is the gumming material of native silk fibers. This is because sericin can elicit an adverse immune response after implantation in the human body. However, the removal of sericin causes the silk fiber to fray and weakens its structural property, making it very difficult to knit or braid them into a scaffold for ligament tissue engineering applications. The aim of this study was to replace sericin with gelatin using NDGA as a cross-linking agent to biomimic the natural structure of native silk fibers. The physical properties and biocompatibility of the modified and native silk fibers were compared by *in vitro* and *in vivo* models. The mechanical and swelling properties of sericin-free silk fibers were greatly increased after modification with gelatin. Both modified and native silk fibers were shown to be nontoxic by *in vitro* cytotoxicity tests. The *in vivo* study demonstrated that the modified silk fibers, after 4 weeks' subcutaneous implantation in rats, caused little or no inflammatory reaction as compared with native silk fibers. The superior mechanical properties and lower inflammatory potential of modified silk fibers make them a promising candidate for ligament tissue engineering applications. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 82B: 129–138, 2007

Keywords: silk fiber; gelatin; NDGA; surface modification; biocompatibility

INTRODUCTION

Ligaments are bands of dense connective tissue that mediate normal joint movement and stability. Injury to these structures may result in significant joint dysfunction because they either heal by production of inferior matrix or do not heal at all. Reconstruction of anterior cruciate ligament (ACL) is one of the major concerns and tissue engineering strategies for its repair face the challenge of fabricating ideal biomaterial scaffolds. Scaffolds provide a structural and logistic template for cell attachment and tissue development, and should biodegrade in parallel with the accumulation of new tissue components.

Collagen is one of the best candidates for adhesion, proliferation, and differentiation of reparative cells (fibroblasts) and progenitors cells and so most of the material surface modification techniques are based on coating with collagen (or its derivatives) or attaching Arg-Gly-Asp (RGD) sequences that are abundant in collagen.[1,2] However, high cost, batch-to-batch variability, hydrophilicity, complex handling properties, potential for immune response and disease transmission are existing disadvantages.[3] PLLA and PLGA have been extensively investigated as possible materials for ligament tissue engineering applications in the authors' institution.[4] However, tissues engineered using these biomaterials demonstrated rapid degradation rates and inferior mechanical properties compared with the normal tissues. Superior mechanical properties and environmental stability of silk make silk fibroin (core protein of silk) a more suitable biomaterial for ligament tissue engineering applications. Silk has already been investigated as a possible scaffold material for ligament tissue engineering, particularly by Kaplan's group at Tufts University.[5,6] The fundamental knowledge and capabilities of silk are well known. The core silk fibers are encased in a sericin coat, a family of glue-like proteins that hold the fibroins together to form the composite fibers. Sericin is a globular protein and water-soluble glue, unlike silk fibroin which is an oriented fiber protein. When subjected to physical, chemical, or enzymatic processes, sericin

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Contract grant sponsor: Biomedical Research Council, Singapore.

TABLE I. Mechanical Properties of Sericin-free, Gelatin-modified and Native SilkFibers[a]

Samples	Tensile Stress (MPa)	Modulus (GPa)	Failure Strain
sericin-free	338.13 ± 7.7	5.99 ± 0.2	0.27 ± 0.017
0.25%	360.76 ± 8.7	5.97 ± 0.7	0.25 ± 0.022
0.5%	367.84 ± 15.8	6.57 ± 0.4	0.21 ± 0.009
1%	386.23 ± 9.5	7.44 ± 0.1	0.23 ± 0.018
3%	391.89 ± 14.7	8.56 ± 0.2	0.19 ± 0.023
5%	483.85 ± 3.9	10.50 ± 0.6	0.21 ± 0.006
native	421.60 ± 10.7	8.50 ± 0.2	0.25 ± 0.015

[a] Values shown are means ± SD for five specimens.

can be degraded into sericin peptides or partially hydrolyzed. Sericin proteins are known to be the major cause of adverse biocompatibility and hypersensitivity reactions to native silk.[7,8] Therefore, sericin must be removed if silk fibers are to be used as a biomaterial. In the current study, following the removal of sericin, the silk fibers frayed out into many individual silk fibroins [(Figure 2(A)] and their maximum tensile strength and stiffness decreased significantly (Table I). This would make it extremely difficult to knit or braid the silk scaffolds for tissue engineering application. Moreover, the surface of silk fibers also changed from hydrophilic sericin to hydrophobic fibroin.

The aim of the present study was to investigate the possibility of replacing sericin with gelatin, a biocompatible polysaccharide, with an aim to biomimic the natural structure of native silk fibers. A broader objective was to improve the physical property and biocompatibility of sericin-free silk fibers for ligament tissue engineering application. To accomplish this goal, sericin was removed from native silk fibers, which were subsequently modified with different concentrations of gelatin solution. The modified silk fibers were then cross-linked with nordihydroguaiaretic acid (NDGA). The physical properties and biocompatibility of modified silk fibers and native silk fibers were compared using both *in vitro* and *in vivo* models.

MATERIALS AND METHODS

Raw *Bombyx mori* silk fibers (local name: Nang Lai silk fibers) were provided by the Silk Innovation Center at Mahasarakham University in Thailand. Gelatin and the cross-linking agent, NDGA, were purchased from Sigma Chemical Co. (St. Louis, MO).

Degumming and Modification With Gelatin

Raw silk fibers were immersed in an aqueous solution of 0.1% (w/v) Na_2CO_3 at 98–100°C for 0.5 h, and the process was repeated thrice to remove sericin.

The sericin-free silk fibers were immersed in different concentrations (0.25, 0.5, 1, 3, and 5%) of aqueous gelatin solution for 1 h at room temperature, clamped at one end, and then hung vertically in air to dry at room temperature. The dried silk fibers were hydrated in PBS solution (pH 7.0) for 30 min. NDGA was suspended in 0.1*N* NaOH solution. Complete solubilization of NDGA required addition of 10 μL of 10*N* of NaOH. One milliliter of the NDGA solution was added directly to PBS solution in which the fibers were suspended to make a final concentration of 3 mg/mL. The fibers were agitated in the NDGA solution for 24 h at room temperature, after which they were washed thoroughly with 70% ethanol followed by PBS and then hung vertically to dry. The cross-linking treatment with NDGA was then repeated.

SEM Observation

Scanning electron microscopy (JEOL JSM-5600LV) was used to observe and compare the surface and cross-section morphologies of native silk fibers and modified silk fibers.

Swelling Properties

To assess the hydrophilicity of native, sericin-free and modified silk fibers, the following procedure was used. The different silk fibers were washed with distilled water and dried under reduced vacuum over P_2O_5. The dried silk fibers were weighed and then hydrated in PBS or culture medium till there was no increase in their weight. The "weight degree of swelling" (E) was calculated as the ratio of weight of PBS or culture medium taken up by the fibers normalized to their initial dry weight:

$$E = [(W_e - W_0)/W_0] \times 100$$

where W_e is the weight of the fibers fully swollen and W_0 is initial weight of the dried fibers.[9]

Mechanical Tests

The native silk fibers and the silk fibers modified with different concentrations of gelatin solution were tested under tension using an Instron testing system (Model 5543, Instron Inc., MA) to determine their mechanical properties ($n = 5$). The gauge length was set at 30 mm and the specimens tested at a speed of 10 mm/min. Maximum tensile stress was taken as the highest load attained before failure normalized to cross-sectional area. The linear portion of the stress/strain curve was used to calculate the elastic modulus.

In Vitro and *In Vivo* Models

The protocols for the use of animals for both *in vitro* and *in vivo* studies were approved by the Institutional Animal Care and Use Committee of the National University of Singapore.

Bone Marrow Stem Cell Isolation and Culture

Bone marrow stem cells (BMSCs) were isolated and cultured according to previously published techniques using density gradient centrifugation and short-term adherence to plastic culture plates.[10] In brief, bone marrow was aspirated from the

iliac crest of an anesthetized adult New Zealand white rabbit (weight 2.5 kg) and mononuclear cells concentrated by Ficoll gradient centrifugation and resuspended in culture medium containing Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, CA), 15% fetal bovine serum (HyClone, Logan, UT), penicillin, and streptomycin. The nucleated cells were plated at a density of 5 million cells per 100-mm dish and incubated at 37°C with 5% humidified CO_2. After 24 h, nonadherent cells were discarded and adherent cells were cultured, changing the medium every 3 days. When culture dishes became nearly confluent after about 14 days, the cells were detached and serially subcultured. The second-passage cells were used for *in vitro* cytotoxicity test.

In Vitro Cytotoxicity

It is not possible to seed cells directly onto single silk fibers to evaluate cytotoxicity. Therefore, according to ISO 10993-5, 50 m of modified and native silk fibers were incubated with 10 mL of culture medium for 72 h at 37°C, after which the medium was extracted and filtered through 0.22 μm filters (Millipore). Confluent rabbit BMSC monolayers in a 96-well plate were incubated in 100 μL of the extracted medium or normal culture medium (control) and cell viability was assessed by MTT assay after 24, 48, and 72h of incubation.[11]

In Vivo Biocompatibility

Surgical procedure was performed according to ISO 10993-6 (Biological evaluation of medical devices, Part 6: Tests for local effects after implantation). Native, sericin-free, 0.5% and 5% gelatin-modified silk fibers were used for this study and sterile 1-0 chromic catgut surgical suture (Johnson & Johnson, Mumbai, India) was used as control. All silk samples were used after immersing in 70% ethanol for 20 min and rinsing thrice with PBS, while catgut sutures were directly used after rinsing with PBS.

Three syngeneic Wistar rats weighing 280–350 g were anesthetized with 7% chloralhydrate (400 mg/kg, intraperitoneal). Under aseptic conditions, a 4-cm long dorsal midline skin incision was made on the back of each rat and five subcutaneous pockets were created by dissection. Implants were inserted into each pocket individually, taking care to prevent any contact between them. The incisions were closed with surgical sutures after operation and the rats were kept for 4 weeks before being sacrificed. Implantation sites were harvested immediately after sacrifice and fixed in 10% neutral buffered formalin for 3 days. Five micrometer-thick paraffin sections were stained with haematoxylin and eosin for histological evaluation.

Statistical Analysis

All data are expressed as mean ± standard deviation. Single factor analysis of variance (ANOVA) technique was used to assess the statistical significance of results between groups. The statistical analysis was performed with the software OriginPro (version 6.1) using a confidence level of 95%.

RESULTS

Morphological Characterization

The surface and cross-section morphologies were observed by SEM. Boiling at 98–100deg;C for 1.5 h in Na_2CO_3 solution completely removed the sericin, revealing the smooth surface of underlying core silk fibroins having an average diameter of about 10 μm [Figure 1(A)]. Increasing the concentration of gelatin solution resulted in increasingly thicker fibers with rougher surfaces [Figure 1(B–F)] and the surface of 5% gelatin modified silk fibers closely resembled that of native silk fibers [Figure 1(G)]. Additionally, sericin-free fibers frayed out into several individual silk fibroins that were separated from each other [Figure 2(A)]. Cross-sectional SEM images reveal that in the modified silk fibers [Figure 2(B–G)], with increasing gelatin concentration, individual silk fibroins become more closely adhered to again form an integrated silk fiber resembling the native silk fiber, with the interstices filled with gelatin.

Swelling Properties

Figure 3 illustrates the swelling properties of native and gelatin-modified silk fibers in culture medium and in PBS. Similar trends were observed in both liquids, with native silk fibers demonstrating very low swelling compared with sericin-free and modified silk fibers. The least swelling was observed with 1% gelatin-modified silk fibers; the highest swelling occurred with 5% gelatin-modified silk fibers, and was about 16-fold and 45-fold higher than that of the native silk fibers in culture medium and PBS respectively.

Mechanical Properties

After removing sericin, the maximum tensile stress and modulus of native silk fibers decreased significantly (Table I). The tensile stress decreased by 20% and the modulus decreased by 29%. Interestingly, gelatin modification and cross-linking with NDGA increased the tensile stress and modulus of silk fibers greatly, showing a direct relationship with the concentration of gelatin solution used for modification. The maximum tensile stress of 5% gelatin-modified silk fibers was 15 and 43% higher than that of native and sericin-free silk fibers respectively. Similarly, the modulus of 5% gelatin-modified fibers was 75 and 24% higher than that of the native and sericin-free fibers.

In Vitro Cytotoxicity

According to the ISO 10993-5 (Tests for cytotoxicity: *In vitro* methods), the silk fibers were incubated at 37°C over an extended period of 72 h to ensure the extraction of significant amounts of soluble products from the silk fibers.

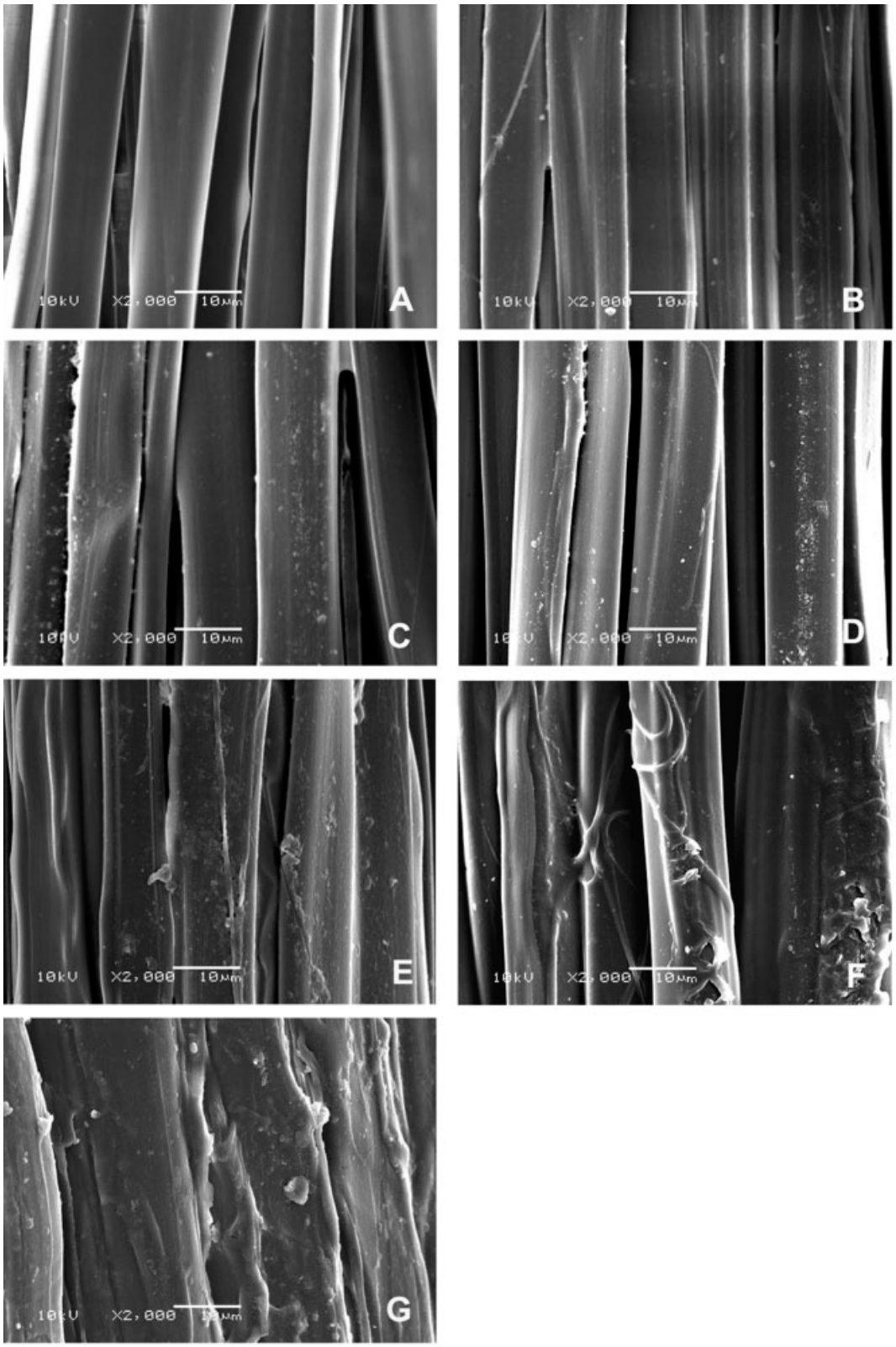

Figure 1. SEM images illustrating the changes in surface texture of (A) sericin-free silk fibers, silk fibers modified with different concentrations of gelatin solution: (B) 0.25%, (C) 0.5%, (D) 1%, (E) 3%, (F) 5%, and (G) native silk fibers (original magnification ×2000).

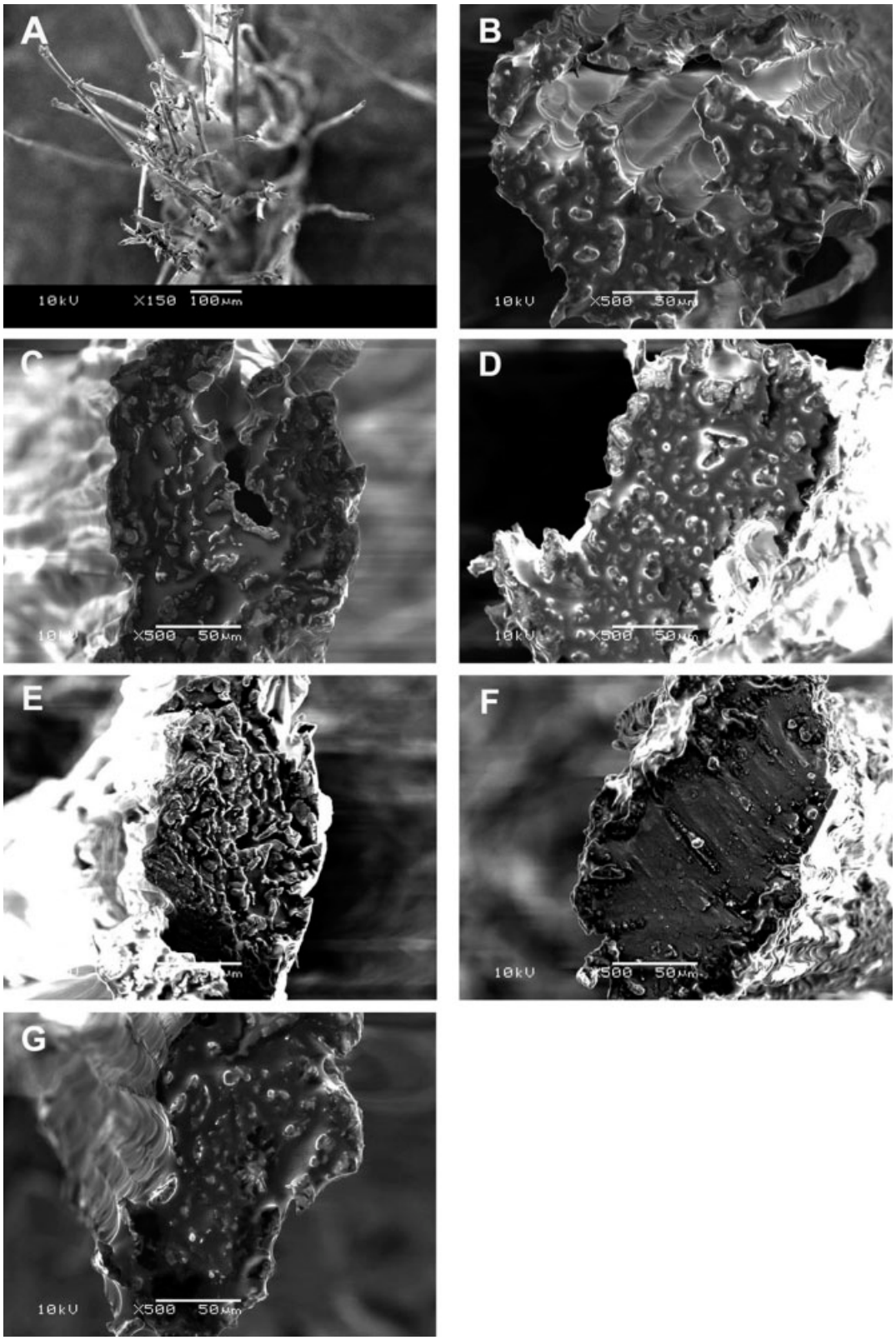

Figure 2. SEM images illustrating the changes in cross-sectional texture of (A) sericin-free silk fibers (original magnification ×150), silk fibers modified with different concentrations of gelatin solution: (B) 0.25%, (C) 0.5%, (D) 1%, (E) 3%, (F) 5%, and (G) native silk fibers (original magnification ×500).

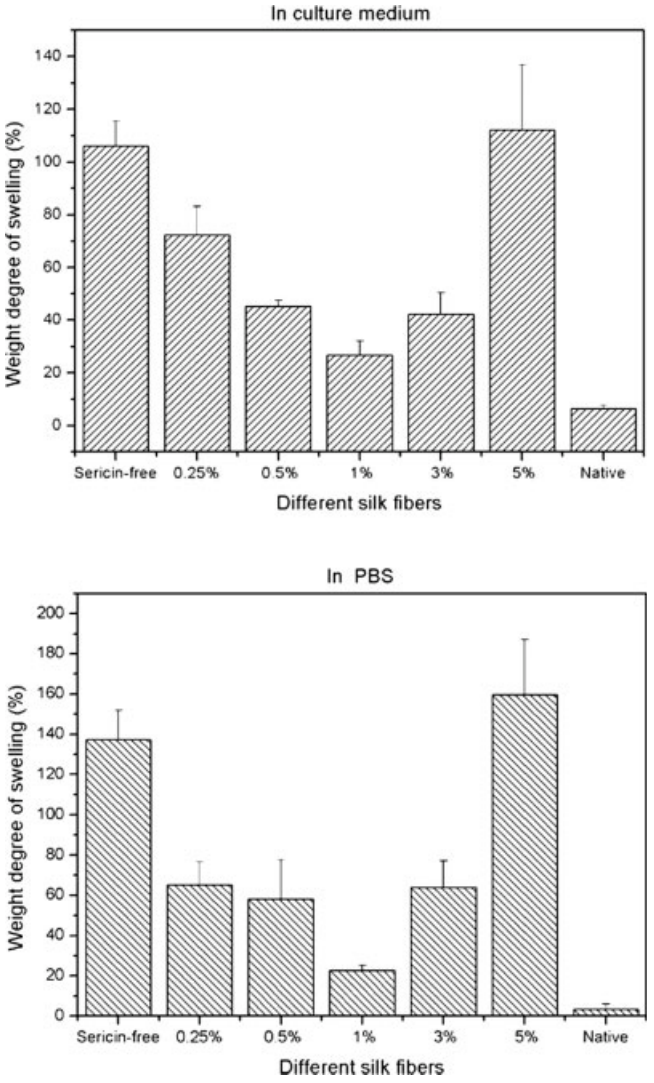

Figure 3. Swelling properties in culture medium and in PBS of sericin-free, gelatin-modified and native silk fibers. Values shown are means ± SD for five specimens.

Cytotoxicity was inversely correlated to cell viability, which was quantitatively measured using established MTT assays. The MTT assay (Figure 4) indicated a relatively low cell viability of 85–90% in all the medium-extracts as compared with normal culture medium (control), during the first 2 days. A significant increase was observed on day 3 of incubation, when the cell viability was 89–94% of control values, with the maximum viability occurring with the culture medium extracted from 5% gelatin-modified silk fibers. It was noticed that cell viability in the extract from native silk fibers was not the lowest, but was in fact better than in most of the extracted media, with the exception of that extracted from 5% gelatin-modified fibers and the control medium.

In Vivo Biocompatibility

Histology results (Figure 5) showed that after 4 weeks' subcutaneous implantation, all the specimens, except the native silk fibers, were covered with a soft granular tissue without any vascularization. No morphological changes were observed in the silk fibers and tissue ingrowth with fibroblasts could be seen between the fibers in all the groups. Large numbers of inflammatory cells, including polymophonuclear leucocytes, eosinophils, macrophages, and multinucleated cells, were found around native silk fibers while very few inflammatory cells were seen in other groups. Thick fibrous capsules were found around native silk fibers while relatively thin fibrous capsules surrounded sericin-free and 0.5% gelatin-modified silk fibers. Notably, there was no sign of inflammation and fibrosis around the 5% gelatin-modified silk fibers as in the control group using catgut sutures.

DISCUSSION

In order to improve the physical property and biocompatibility of sericin-free silk fibers for ligament tissue engineering application, gelatin was chosen to modify the sericin-free silk fibers with an aim to biomimic and re-create the natural structure of native silk fibers. Gelatin is a partially-denatured soluble derivative of the fibrous insoluble protein collagen, obtained by breaking the triple-helical structure of collagen into single-stranded molecules. It contains repeating amino acid sequences of -(Glycine-X-Y)n-, where X and Y are frequently proline and hydroxy-proline, respectively. Being biodegradable, hydrophilic, biocompatible and non-immunogenic, gelatin is a suitable biomaterial.[12–14] After modification with 5% gelatin, the surface of the silk fibers became very similar to that of native silk fibers, with individual silk fibroins becoming closely packed to yield an integrated silk fiber again.

Removal of sericin from the silk fibers resulted in a change in the surface from hydrophilic sericin to hydrophobic fibroin, which is expected to reduce water-binding and aqueous swelling of the silk fibers. However, the sericin-free fibers exhibited a higher swelling; this might have resulted from the fact that sericin removal caused individual silk fibers to fray out into several silk fibroins forming a loose and porous structure that could physically trap significant amounts of water. Subsequent modification with gelatin is expected to make the surface hydrophilic again, and aqueous swelling is expected to increase proportionately with gelatin concentration. However, the silk fibers modified with 1% gelatin exhibited the least swelling among the sericin-free and modified silk fibers. This might have been caused by decreasing porosity of the modified silk fibers with increasing gelatin concentration from 0 to 1%, so that the 1% gelatin-modified silk fibers were the least porous. From this gelatin concentration, the surface hydrophilicity of the modified silk fibers would be a major determinant of the swelling property. At concentrations above 1%, increasing amounts of gelatin were deposited onto the surface of the silk fibroins causing a great increase in their swelling property.

The material used to fabricate scaffolds greatly influences the adhesion, proliferation, and differentiation of seeded cells, with its surface properties playing a major role in determining the biocompatibility. [15] Cell adhesion on a biomaterial is

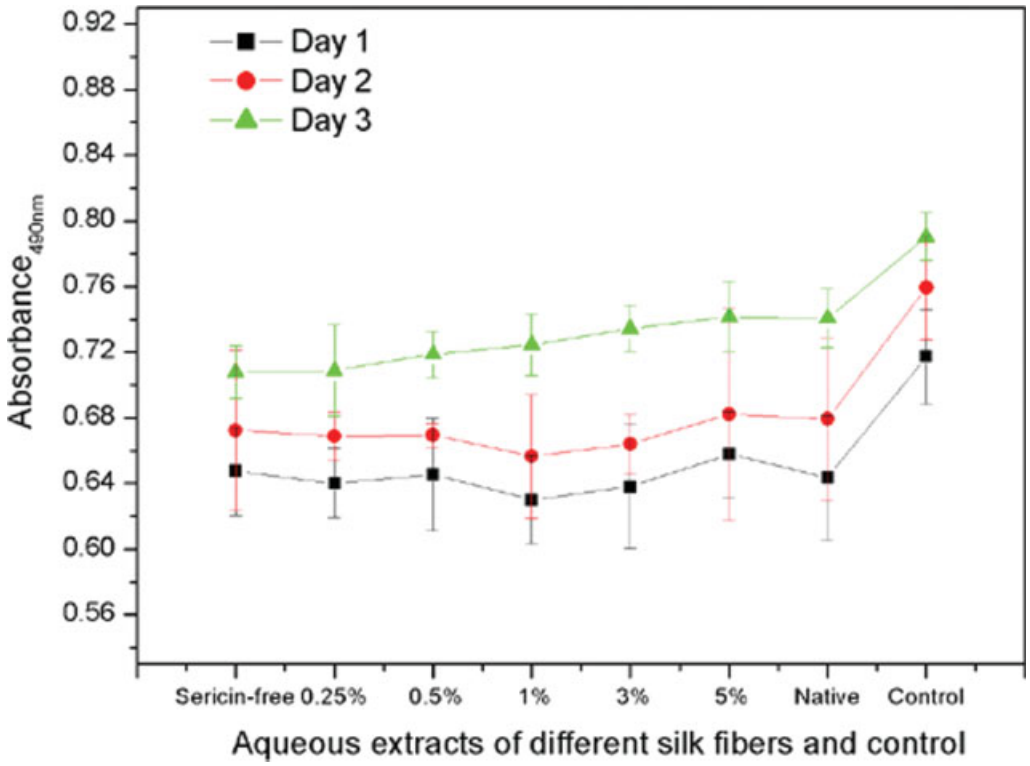

Figure 4. MTT assay formazan absorbance at 490 nm was expressed as a measure of cell viability of BMSCs cultured in aqueous extracts of different silk fibers and control (normal culture medium). Values shown are means ± SD for five specimens. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

influenced not only by biological factors like metabolic status of the cells, its membrane properties and the period of contact, but also by material factors like surface hydrophilicity or hydrophobicity, free energy, charge type and density, chemical structure and topography.[16] Hydrophilic surfaces promote cell attachment, spreading, and cytoskeleton reconstruction during the process of cell adhesion.[17] Rough surfaces are more suitable for cell adhesion and rapid regeneration of a biologic membrane.[18] Surface modification by introduction of amido, amide, hydroxyl, carboxy, or sulfanilamide groups also enhances cell adhesion and proliferation.[19] In this study, silk fibroins were modified with hydrophilic gelatin and the surface of modified silk fibers became as rough as native silk fibers. As a consequence, the silk fibers modified with 5% gelatin were more suitable for cell adhesion, migration, and proliferation.

Gelatin-modified silk fibers need to be cross-linked in order to withstand normal *in vivo* conditions, to prevent any significant attenuation of their mechanical, thermal, or chemical properties. Many chemical cross-linking methods have been applied to gelatin in order to produce a stable biomaterial scaffold for tissue engineering. One such method uses glutaraldehyde, which forms adducts with free primary amines in proteins, particularly on lysine side chains; such adducts react to form cross-links between neighboring macromolecules. Though glutaraldehyde cross-linking greatly improves the mechanical and thermal properties of gelatin-based materials,[20] a major disadvantage of using glutaraldehyde is its cytotoxicity and the risk of *in vivo* release of cytotoxic agents after biodegradation of glutaraldehyde cross-linked materials.[21] Another cross-linking strategy uses carbodiimide or hydroxysuccinimide, which form intermolecular isopeptide bonds, and can yield thermally stable biomaterials[22] with better biocompatibility as compared with glutaraldehyde-treated materials.[23] However, carbodiimide-treated materials have poorer mechanical properties than glutaraldehyde-treated materials and are more easily biodegraded in enzyme solutions. We chose NDGA, an antioxidant composed of two ortho-catechols separated by a short, 6-carbon alkane chain, as the cross-linking agent. The reactive end catechol groups of NDGA are slowly auto-oxidized to ortho-quinones at neutral pH and are responsible for polymerization (Figure 6).[9] NDGA has been shown to be an optimal cross-linking agent for synthetic collagen fibers. Thomas et al. have reported that NDGA cross-linked-collagen fibers were not only mechanically stronger, but also noncytotoxic, permitting better cell attachment, migration, and proliferation.[24,25] Our study also confirmed that the maximum tensile stress and modulus of sericin-free silk fibers were greatly increased after modification with gelatin and cross-linking using NDGA, with the highest value obtained with 5% gelatin solution.

Because of practical difficulties associated with seeding cells directly onto single silk fibers to evaluate their cytotoxicity, an alternative method provided by ISO 10993-5 was used. The MTT assay primarily confirmed that both the modified

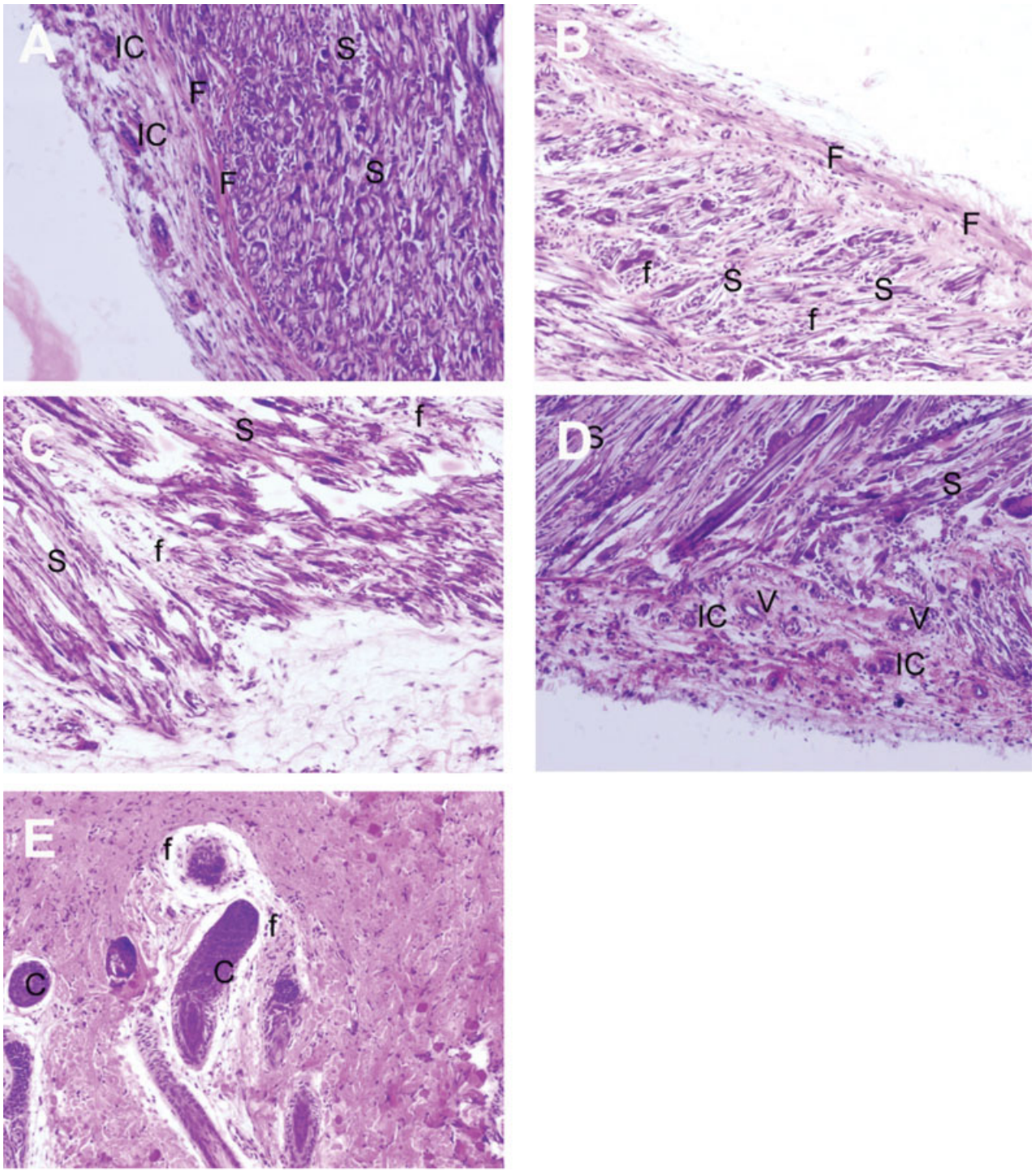

Figure 5. Cellular response to subcutaneously implanted (A) sericin-free silk fibers, silk fibers modified with (B) 0.5% and (C) 5% gelatin solution, (D) native silk fibers and (E) catgut sutures (control) in rats for 4 weeks post surgery. Haematoxylin and Eosin staining. (original magnification ×100). S indicates silk scaffold in all forms; C indicated catgut suture used as control; F indicates fibroblasts with capsulation; f indicates fibroblasts; IC indicates inflammation cells; V indicates vascular formation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

and native silk fibers were noncytotoxic *in vitro*. The *in vivo* biocompatibility of a biomaterial is very important for its application in tissue engineering. All implants derived from nonautologous sources elicit some level of foreign body response, which is influenced by biomaterial characteristics, including implantation site, size, geometry, and surface topography. Our *in vivo* study showed that, after 4 weeks of subcutaneous implantation, native silk fibers elicited strong inflammatory response while modified silk fibers caused very little immune reaction, with the 5% gelatin-modified silk fibers proving as

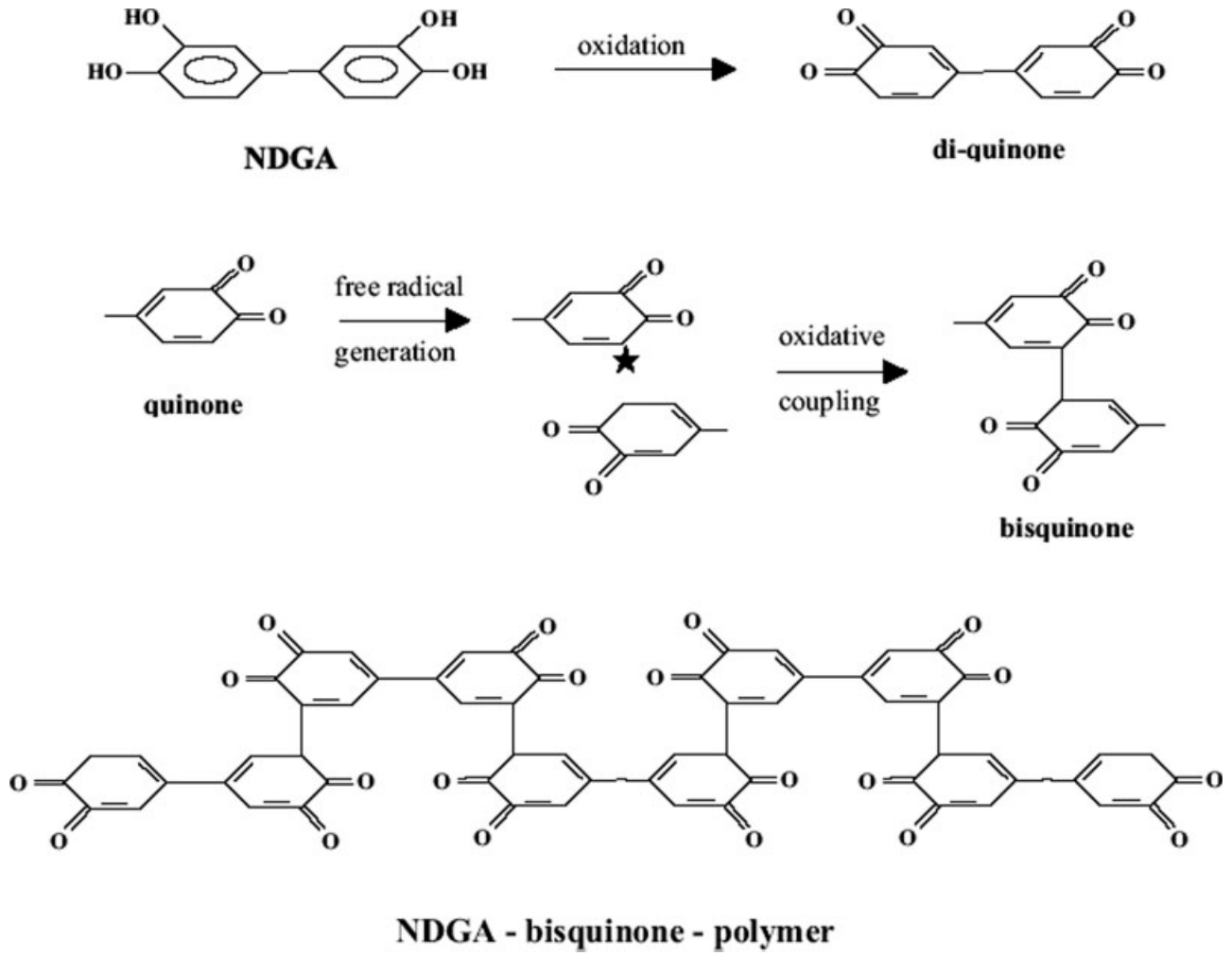

Figure 6. The putative polymerization scheme of NDGA (redrawn with permission from Koob's article[9]).

good as the catgut sutures. The present study thus showed that 5% gelatin-modified and NDGA-cross-linked sericin-free silk fibers possessed not only the best mechanical properties but also higher *in vitro* and *in vivo* biocompatibility.

CONCLUSIONS

The physical properties and biocompatibility of modified silk fibers and native silk fibers were compared using *in vitro* and *in vivo* models. After modification with gelatin, the mechanical and swelling properties of silk fibers were greatly increased. More importantly, the low inflammatory potential and higher biocompatibility of modified silk fibers make them promising candidates for ligament tissue engineering applications. Moreover, the modification technique can also be adapted to coat sericin-free silk fibers with other biocompatible polysaccharides and growth factors in according with various tissue engineering requirements.

We thank Dr. Sambit Sahoo for his careful editing of this article. In addition, we thank Mr. Yong Soon Chiong and Mr. Wu Yingnan for their technical assistance in SEM observation.

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