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Improvement of Histocompatibility of Silk Fibroin/Polyurethane Membrane with Controlled Release of Aspirin

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ABSTRACT: The histocompatibility of polyurethane (PU) modified by superfine silk-fibroin (SF) powder (1 : 1) with different dosage of aspirin to determine the best dosage for histocompatibility was investigated. Aspirin powder was incorporated into SF–PU (1 : 1) blend mixture at doses of 0%, 5%, or 10% (w/w). The influence of different doses of aspirin on histocompatibility of the hybrid material was studied by using morphology examination, infrared spectroscopy, *in vitro* drug release study, and *in vivo* tests including an acute toxicity test, an assessment of local reaction in the muscle, hematological examination, and histological examination. SF–PU (50 : 50) blend film had superior performance over the polytetrafluoroethylene (e-PTFE) film in terms of histocompatibility, and the anti-inflammatory/anti-platelet effects were improved after integration with limited doses of aspirin to yield a valuable drug release vascular graft. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40580.

KEYWORDS: biocompatibility; composites; polyurethanes

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INTRODUCTION

Man-made small diameter vascular graft is an alternative for replacing the great saphenous vein as the bypass graft for CABG. Nevertheless, thrombosis and restenosis remain the biggest problem that affects its long-term efficacy. In our previous studies, we employed the anti-thrombotic and anti-inflammatory properties of aspirin to improve the histocompatibility of the synthetic polymeric membrane of polyurethane (PU) modified by superfine silk-fibroin (SF) powder, and achieved better histocompatibility as compared with polytetrafluoroethylene (e-PTFE).

The material used in this research was a blend film composed of SF, PU, and aspirin and was made by using the phase separation technique.[1] Our previous research exhibited that the SF–PU (1 : 1) possessed good physical properties.[2] Moreover, the hybrid film in combination with controlled released aspirin not only had good mechanical properties,[3] but also could reduce inflammation.[4] However, an animal experiment showed that the material may induce platelet aggregation at certain concentrations of incorporated aspirin.[4] In this study, we examined the histocompatibility of the SF–PU (1 : 1) material with a different aspirin/SF–PU ratio to determine the optimal dosage of aspirin.

MATERIALS AND METHODS

Materials

Silk cocoons were purchased from SiYuan Textile Co. Ltd., Zhejiang, China. PU (Pellethane 2363-80AE) was supplied by Dow Chemical Corporation (Midland, MI). N,N-dimethylformamide (DMF) of analytical grade was obtained from Sinopharm Chemical Reagent Co, Ltd. (SCRC), China. Aspirin was originally made by SCRC and procured from Shenwei Pharmaceutical Co, Ltd., Hebei, China.

Preparation of Superfine SF Powder

Silk cocoons were first removed of sericin and then were dissolved in an aqueous solution of 0.25% (w/v) sodium lauryl sulfate and 0.25% (w/v) sodium carbonate at 80°C for 100 min. The extract was then ground into a fine powder with a specially made grinding machine,[5,6] made by Wuhan University of Science and Engineering, to obtain particles of 3.58 μm (on average) in diameter.

Preparation of Composite SF–PU Material with Different Aspirin/SF–PU Ratio

SF–PU–aspirin blend film was fabricated by using immersion precipitation phase inversion. In detail, PU (50 g) and DMF (400 mL) were stirred together in a rockered flask. After PU was fully dissolved in DMF, superfine SF powder (50 g) was put into the

solution and was mingled with PU by stirring for 10 min to obtain the SF–PU (1 : 1) blend mixture. Aspirin was grounded into fine powder in a mortar. Subsequently, aspirin powder was incorporated into the SF–PU (1 : 1) blend mixture at different doses (0, 5, or 10 g aspirin). Each SF–PU–aspirin blend mixture was 100 g in total weight. PU, DMF, SF, and aspirin powder was mixed, by stirring, for 3 h, until a homogeneous blend mixture was obtained. Then the flask was vacuumed to remove any bubbles in the mixture, which was then poured onto a clean glass plate at room temperature. Then the glass plate was submerged in a bath containing distilled water for 10 min to allow for the exchange between the solvent and non-solvent. After desiccation for 72 h at room temperature, the SF–PU (1 : 1) blend films (thickness = 120–150 μm) with different doses of aspirin were harvested from the glass plate.

Five experimental groups were set up: control, or sham-operated group, e-PTFE group, 0%-SF–PU (pure SF–PU 1 : 1) group, 5%-SF–PU group, and 10%-SF–PU group (0%, 5%, 10% refers to the content of aspirin, w/w).

Measurement of Particle Size of SF Powder

Laser analyzer (JL-1166 model, Chengdu Jingxin Powder Analysis Instrument Co. Ltd., China) was used to measure the average particle size and size distribution of the SF powder.

Morphological Observation of the Composite Blend Films

SF–PU blend films modified with different doses of aspirin, after gold coating, were morphologically observed under a scanning electron microscope (SEM) (Hitachi X-650 SEM, Japan), at 20 kV acceleration voltage. For morphological characterization, the blend films were frozen in liquid nitrogen and cut into slices to observe their cross sections.

Infrared Spectroscopy of the Composite Polymeric Materials

Attenuated total reflection Fourier transform infrared (ATR-FTIR) was used for the analysis of chemical composition of SF–PU–aspirin. In brief, a 1-mg sample film was taken from 0%-SF–PU, 5%-SF–PU, and 10%-SF–PU, respectively, placed on an FTIR spectrometer (TENSOR-27, Bruker Optics, Germany) and examined with the reflection method. The scan range was 500–4000 nm. The acquisition parameters were: 32 scan and 4/cm (spectra resolution).

In Vitro Release of Aspirin from the Blend Films

The modified SF–PU blend films integrated with 5% and 10% aspirin (for each group, $n = 3$, weight = 1 mg) were immersed in a 20 mL PBS (pH 7.4) at 37°C for 24 h. The same amount of fresh PBS solution was replaced at a predetermined time point in order to ensure a full immersion. Subsequently, samples were taken out and rinsed with pure water and vacuum-dried at 37°C. The release of aspirin was detected on a high performance liquid chromatographer (Hitachi L-2000, Japan) by using a C18 column (Knauer Eurospher-100, Berlin, Germany) with a pore size of 10 μm. The samples were dissolved in 2-mL DCM for 1 h, and 18 mL methanol was added to precipitate the polymer from DCM and methanol. Then they were centrifuged at 5000 rpm for 10 min and the supernatant was filtered (pore size of the filter: $\Phi = 0.2$ μm) before being injected into the HPLC.[7] The mobile phase consisted of 50% methanol and 50% glacial acetic acid, with a flow rate of 1 mL/min. About 10 mL of the sample was injected into HPLC system and detected at 280 nm.

Animal Care

All animal experiments were performed in strict accordance with the Regulations for Administration of Laboratory Animals, Hubei, China. About 30 Sprague-Dawley (SD) rats (aged 6–8 weeks, including 15 males and 15 females, mean weight being 189.6 g), were obtained from the Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

Implantation Process

The 30 rats were randomly divided into five groups, with each group having three male and three female animals. Each rat was weighed and numbered prior to operation. The rats were anesthetized by Su-Mian-Xin II [a mixture of haloperidol, 2,4-xylidine thiazole, ethylenediaminetetraacetic acid (EDTA), and dihydrotestosterone etorphine] (Huamu Shouyao Co. Ltd., China). The materials for each group were cut into thin slices, rinsed in triple-distilled water, and irradiated by Co^{60} for sterilization. The hair on the back of the rats was shaved off and the operation region was disinfected by Iodophor. An incision was made in the skin, and the muscles adjacent to the spine were fully exposed and separated from the subcutaneous tissue. The materials (e-PTFE or SF–PU with different doses of aspirin) were implanted into corresponding groups. The materials were embedded in the muscles along the long axis of the muscle fiber and the skin was disinfected after closure of skin. The rats in the control group underwent the same operation, without receiving implantation of material. The sutures were removed 1 week after the operation.

Acute Toxicity Test

After operation, the animals were observed for tissue responses and the survival rate 72 h after the operation.

Hematological Examination

The rats were euthanized in two batches: the first group were sacrificed 1 week post-implantation and the second group 4 week post-implantation. The blood from the heart ventricle of the rats was examined for white blood cell (WBC) and platelet (PLT) counts 1 week after the operation with the first batch and 4 weeks after operation with the second batch.

Methylene Blue Staining 1 Week After Operation

Back muscle specimens were harvested from the first batch, fixed in formalin for 24 h and embedded in paraffin. The specimens were cut into 1 μm semi-thin slices, immersed in methylene blue solution, rinsed, dried, embedded in resin, and observed for inflammatory responses under a light microscope.

SEM and Transmitting Electron Microscopy (TEM) 4 Weeks After Operation

In the fourth week, the implantation sites of the second batch were taken *en bloc* (including the embedded materials and the surrounding muscles). The specimens were fixed in 2.5% glutaraldehyde solution for SEM (Hitachi X-650 SEM, Japan) and TEM (Hitachi H-600 TEM, Japan).

Statistical Analysis

The data were expressed as mean ± standard deviation (SD). Differences between the experimental groups and the control group were evaluated by using ANOVA. Difference was considered to be statistically significant when a $P < 0.05$.

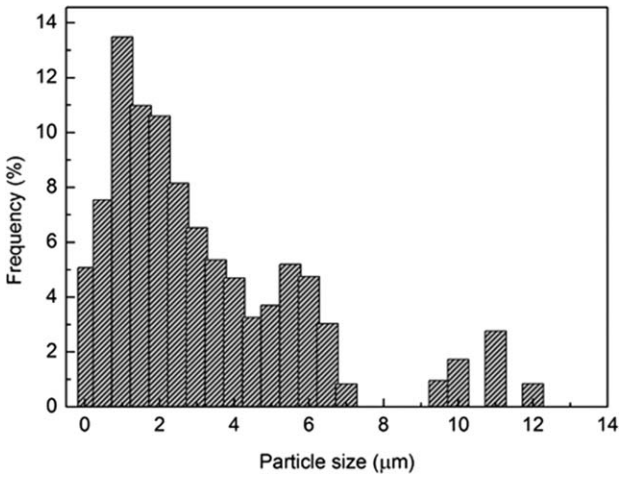

Figure 1. Particle diameter of SF powder.

RESULTS

Structural Characterization of Blend Films

Superfine powder is defined as all powder smaller than 30 μm in diameter. Figure 1 shows the particle size and frequency distribution of the superfine SF powder. The particles measured from 0.50 to 12 μm, and 70% of the SF power was approximately 4 μm, while 13.5% and 11.0% of the powder was about 1 and 2 μm in diameter, respectively. The average particle size of the powder was 3.58 μm.

Figure 2 shows that pure PU had a relatively smooth and dense surface, while the surface of SF–PU blend films was relatively looser and had more micro-pores. The SF powders were evenly distributed in the blend films, and no apparent agglomerations were observed.

Densities of the blend films integrated with different doses of aspirin were essentially similar. The pore diameter of 0% and 5%-SF–PU blend films were virtually identical, suggesting that small doses of aspirin did not exert significant impact on the structure of the blend films. On the cross-section of the films, the evenly distributed pores served as a perfect medium for the growth of cells.[8]

ATR-FTIR Spectra

The results were obtained by using OriginPro Version 8.0 (Figure 3). The absorption peak is appearing as a characteristic frequency region of the molecular group.[9] Figure 3 shows the absorption curves of 0%-SF–PU, 5%-SF–PU, and 10%-SF–PU were practically same. No new characteristic peak was found, indicating that no new functional groups formed. Moreover,

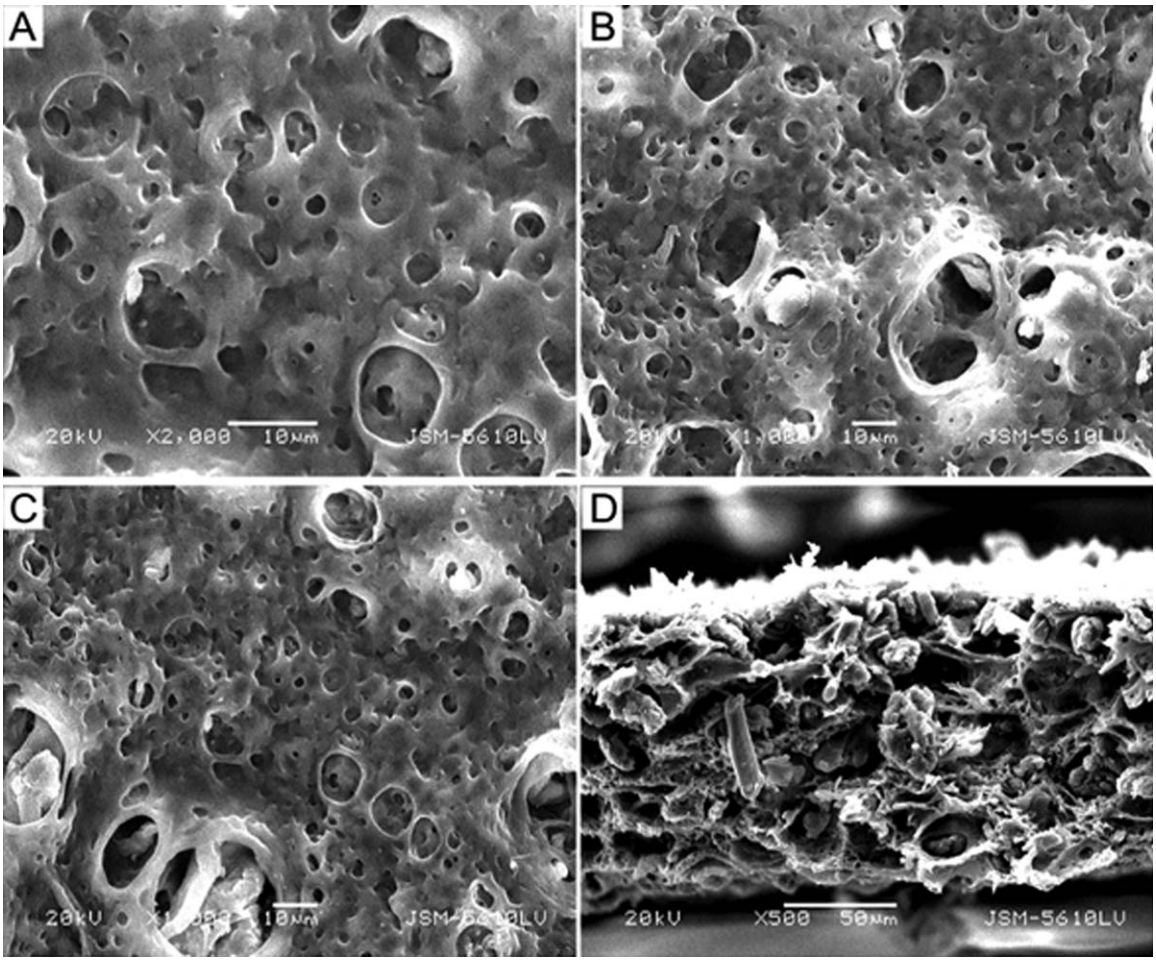

Figure 2. SEM of composite blend films: (**A**) surface of pure PU; (**B**) surface of 0%-SF–PU; (**C**) surface of 5%-SF–PU; (**D**) cross-section of 10%-SF–PU.

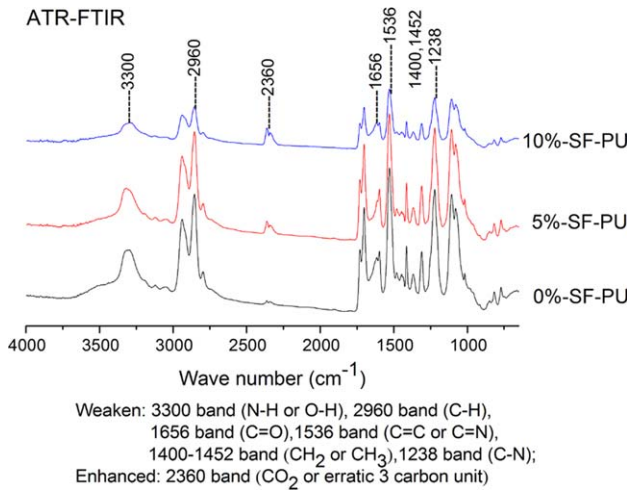

Figure 3. ATR-FTIR spectra of SF–PU materials with different ratios of aspirin. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

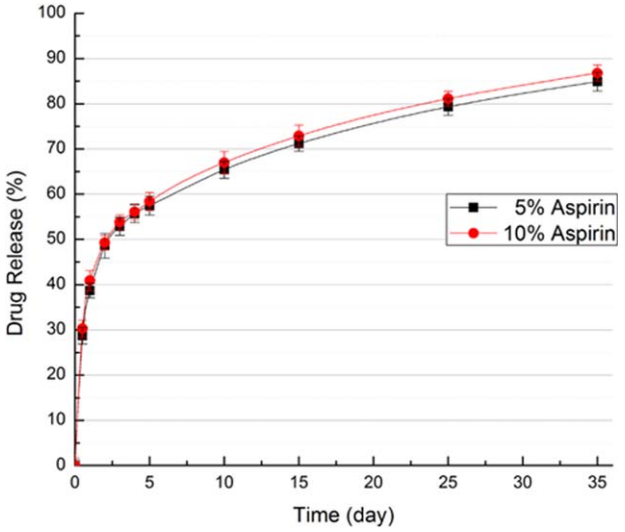

Figure 4. Release of aspirin at 37°C (mean ± s.d., $n = 3$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with the increase of the aspirin content, the absorption intensity was decreased.

Drug Release

We examined the release behavior of aspirin from SF–PU blend films at 37°C (pH = 7.4). The *in vitro* release of aspirin from the composite blend films with different doses are shown in Figure 4. SF–PU with different aspirin doses had a similar short-term rapid release in the first 24 h. Approximately 50% of the aspirin was released in a burst and about 70% was released over a period of 15 days. Almost all the aspirin was released after 35 days. The high burst release was due to the enhanced hydrophilicity of aspirin prepared by the phase separation technique.[10,11] The sustained release of aspirin was probably due to the porous architecture.[12–14] This release profile was very close to the desired profile for aspirin, as it is meant to address both acute thrombosis and late-stage thrombosis.

Acute Toxicity After Operation

During the 72-h observation period, no rat died after operation and no adverse responses were observed. One rat in the e-PTFE group became lethargic, ate less, and lost weight, but the other rats did not show any significant change in body weight. The wounds in three rats in e-PTFE group and one rat in pure SF–PU were infected. However, 1 week after operation, the suture sites healed completely and no obvious back injury was observed in most rats. Clear suture marks could still be seen in one rat in the e-PTFE group and one in the pure SF-PU group. No rat suffered stomach irritation or diarrhea.

WBC Counts at Different Time Points

Figure 5 illustrated that the WBC count in the control group remained normal, while the WBC count was higher in e-PTFE group and the pure SF-PU group. This finding was consistent with the aggravated inflammation in the wounds. The WBC count was a slightly less in 5%-SF–PU group than in the control group, while the WBC count was decreased in 10%-SF–PU group, indicating that aspirin had an anti-inflammatory effect and that a high dosage of aspirin might be a possible cause of bone marrow suppression.[15,16]

Four weeks after surgery, the WBC count of the rats implanted with e-PTFE remained higher than that in the control group whereas the WBC counts in the SF–PU–aspirin groups were decreased (Figure 5). The WBC counts in the pure SF–PU and 5%-SF–PU groups were comparable to those of the control group. The WBC count of 10%-SF–PU was still lower than that in the control group 4 weeks after operation, indicating that SF–PU has better histocompatibility than e-PTFE and that inflammation can be better controlled if a certain amount of aspirin is incorporated into SF–PU.

PLT Counts at Different Time Points

Figure 6 illustrates that 4 weeks after operation, the number of PLT of all the groups was decreased obviously, suggesting that the PLT may be raised as a response to surgical stress. Four weeks after operation, the PLT counts returned to normal. However, no significant differences in the PLT count between the 0%-SF–PU and 5%-SF–PU groups and the control group

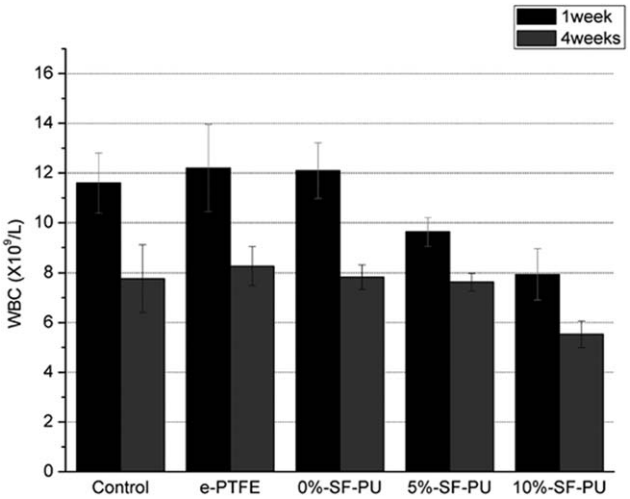

Figure 5. WBC counts of rats receiving different biomaterial implants 1 and 4 week(s) (mean ± s.d., $n = 3$).

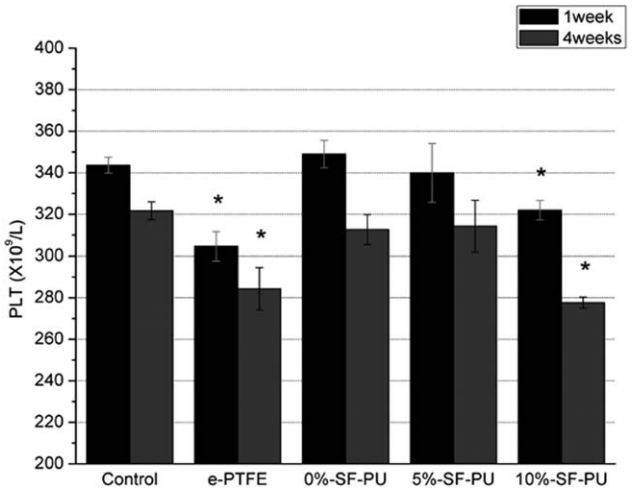

Figure 6. PLT counts of rats receiving different biomaterial implants 1 and 4 week(s) (mean ± s.d., $^*P < 0.05$ vs. control, $n = 3$).

($P > 0.05$). On the other, 1 and 4 week(s) after operation, the PLT counts in the e-PTFE and 10%-SF–PU groups were all significantly decreased as compared with the control group ($P < 0.05$). This finding suggested that e-PTFE might have an inhibitory effect on PLT, while the higher dose (10%) of aspirin might induce bone marrow suppression.

Local Reactions and Histological Changes 1 Week After Operation

The implanted biomaterials were visually observed (Figure 7-1). The biomaterial implants were clearly visible in implanted rats but those receiving e-PTFE. The e-PTFE material was distinctively curly. Although there were still a number of blood clots in the 0%-SF–PU group, no edema or necrosis was noted.

Histological examination showed that myocytes were of a normal size. One week after operation, the e-PTFE material was of golden color and structurally condensed, without cellular infiltration. A pseudomembranous structure composed of mostly necrotic inflammatory cells, encased the material. These findings suggested that the histocompatibility of e-PTFE materials was poor [Figure 7-2(a)]. 0%-SF–PU was sieve-like and of blue colored, was in close contact with surrounding tissues, and no pseudomembranous structure developed, indicating that the histocompatibility of 0%-SF–PU was better than e-PTFE. However, a large number of inflammatory cells invaded into the implant pores [Figure 7-2(b)]. 5%-SF–PU also showed a very close contact with the host tissue and no pseudomembranous structure was observed. Less invading inflammatory cells were seen and the inflammatory cells in the surrounding connective tissue were also significantly less as compared with e-PTFE. Crystal particles of aspirin were seen scattered inside the material [Figure 7-2(c)]. With the 10%-SF–PU material, the contact between the implant and the host tissue was loose as compared with the 5%-SF–PU group. Although no pseudomembranous structure was observed, more inflammatory cells invaded into the implant and the surrounding tissue [Figure 7-2(d)].

SEM and TEM Observations 4 Weeks After Operation

Figure 8 shows that e-PTFE material was microstructurally sieve-like, no invasive cells were observed. A large number of apoptotic inflammatory cells with nuclear uptake and cell degeneration seen in the surrounding connective tissues and the cells formed a pseudomembrane.

The surrounding cells (especially myocytes and inflammatory cells) in the 0%-SF–PU material, were swollen or degenerated, but had no apoptosis, and showed no nuclear uptake.

The 5%-SF–PU material showed mild invasion by few inflammatory cells, which experienced no significant structural change.

The 10%-SF–PU material was invaded by more inflammatory cells. Compared with the *in vitro* SEM photo [Figure 2(D)] before operation, the skeleton of the 10%-SF–PU material was intact, without degradation even after drug was released.[17,18] Some cells were structurally destroyed and some only had only moderate swelling, but none of them developed apoptosis.

The morphological examination showed that e-PTFE material could damage cells, resulting in apoptosis of a large number of cells around the material. As a consequence, the e-PTFE material was detached. Pure SF–PU maintained the structural integrity of the tissue but some cells invaded the pure SF–PU material and suffered from swelling and degeneration. In 5%-SF–PU, the cell damage was conspicuously mild and the inflammatory cells were clearly less. With 10%-SF–PU, a small number of cells swelled and had other damages. The findings revealed that the e-PTFE had the worst histocompatibility, while the SF–PU integrated with 5% aspirin had enhanced histocompatibility.

DISCUSSION

Among the medical composite polymeric materials, PU has been widely used as a component for blood contact materials for its relatively good biocompatibility and excellent mechanical property. PU has perfect mechanical properties as it is formed by flexible segments glassing below room temperature and the rigid segments glassing above room temperature.[19,20] However, its biocompatibility needs to be further improved to meet the requirements of biomedical engineering. Researchers have tried a wide range of technique, physical or chemical, to enhance the biocompatibility of PU, such as surface modification endgroup (SME), surface graft polymerization, interpenetrating network (IPN) method, etc.[21–23] So far, all modified PU materials available fail to satisfy the requirements for small-caliber artificial blood vessels.[24,25] In 2001, Petrini et al.[26] suggested that SF-coatings PU was a suitable scaffold for tissue engineering due to its surface and structural properties, good bio- and hemocompatibilities, the ability to promote cell adhesion and cell growth, and stability under near-physiological conditions.

Our preliminary studies indicated that the SF–PU blend film reinforced with elastic tubular fabric had good physical properties and could achieve the mechanical performance for medical materials.[3,27] However, the histocompatibility of modified PU material varies when mixed with various SF powders at different ratio. A composite of superfine SF powder and PU material at a

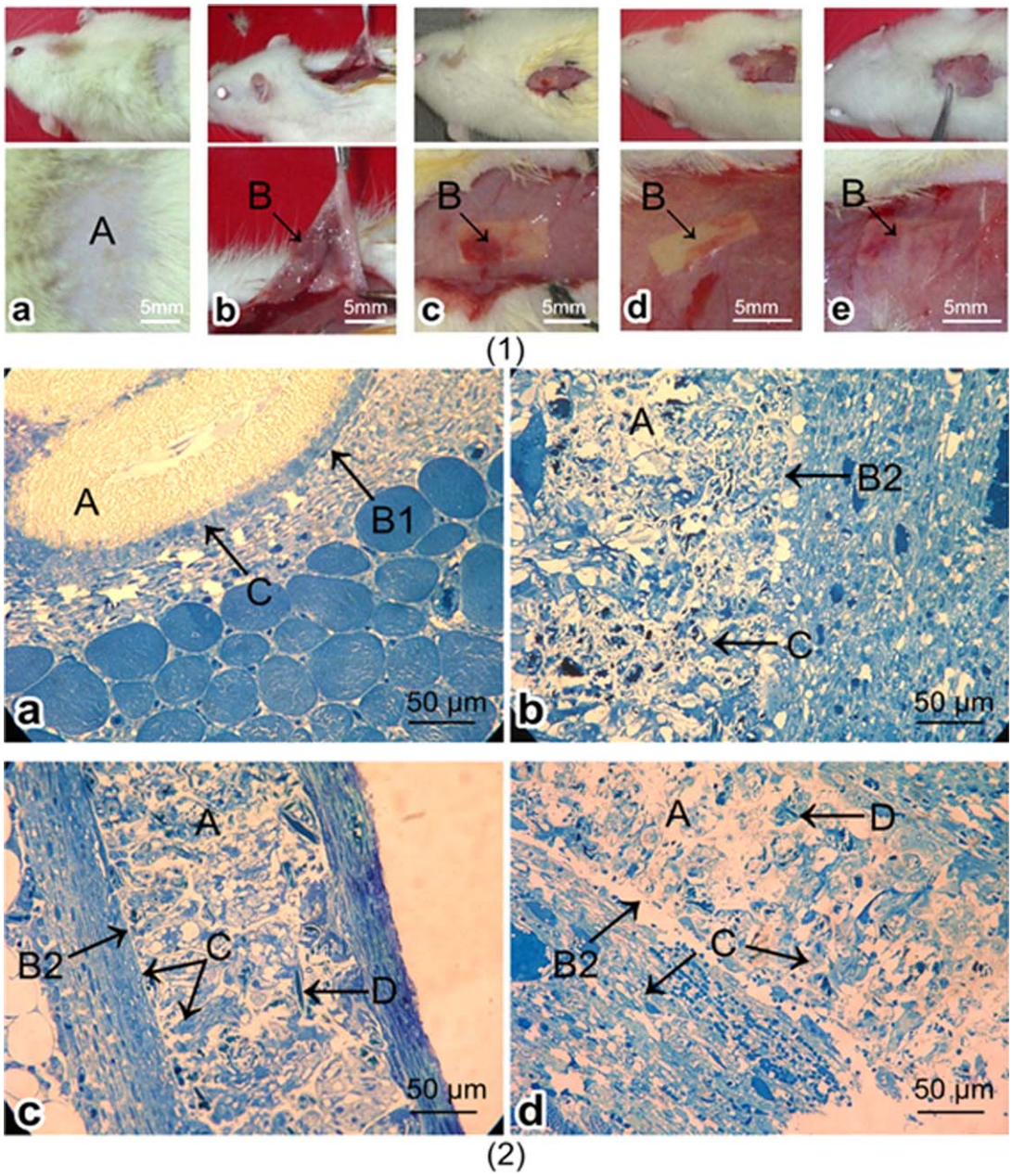

Figure 7. (**1**) Visual observations of different biomaterial implants into rats 1 week after operation. **a**: Control group (sham operated); **b**: e-PTFE group; **c**: 0%-SF–PU group; **d**: 5%-SF–PU group; **e**: 10%-SF–PU group (A: The healing of the incision wound; B: The implanted biomaterials). (**2**) Tissue slices of different biomaterials implanted into rat muscles 1 week after the implantation. Methylene blue staining (40 × 10). **a**: e-PTFE group; **b**: 0%-SF–PU group; **c**: 5%-SF–PU group; **d**. 10%-SF–PU group (A: The implanted biomaterials; B1: The structure of pseudomembrane; B2: The biomaterials contacted with surrounding tissue; C: The inflammatory cells; D: Crystal particles of aspirin). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ratio of 1 : 1 had mechanical properties close to those of human arteries, and at this ratio the histocompatibility of the material was optimal.[2] SF–PU (1 : 1) also showed a better histocompatibility in the acute phase than some established macromolecule biomaterials, such as Dacron, pure PU, and e-PTFE.[28] To further improve the histocompatibility of SF–PU (1 : 1), the heparin and aspirin was studied.[29–31]

In vitro SEM observations showed that the SF–PU blend films had evenly distributed pores and aspirin exerted no significant influence on the structure of the films. ATR-FTIR indicated that the chemical compositions of all aspirin–SF–PU materials were identical. No toxic groups developed after SF–PU was modified with different doses of aspirin. It can be theorized that absorption intensity differences might be due to a difference of Aspirin content. Moreover, drug release study revealed that aspirin could be sustainably released from the composite blend films. A composite material consisting of SF, PU, and aspirin could form an aspirin-releasing system and the release of aspirin could be

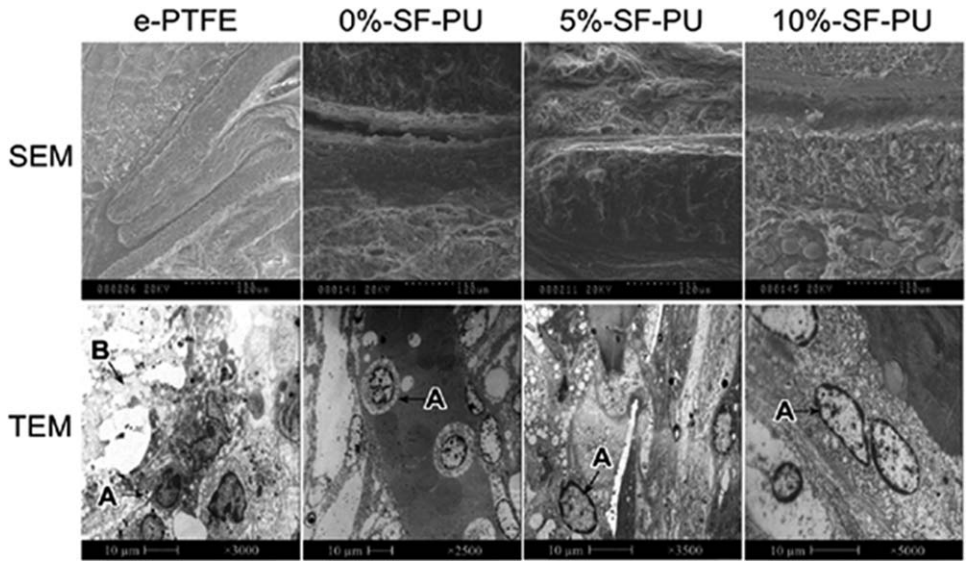

Figure 8. SEM and TEM observation of different biomaterials implanted in rat muscle after 4 weeks. A: The inflammatory cells; B: loose structure of e-PTFE material, without invasive cells.

controlled to achieve slow and sustained release of aspirin. The properties of the system can be used for addressing the acute-phase and long-term thrombosis. *In vivo* experiments illustrated that all the materials tested had good histocompatibility and the acute toxicity tests revealed no significant toxic responses in any animal group. Intra-group comparisons exhibited that the toxicity of the blend films containing aspirin was less as compared with their counterparts without integrated aspirin and e-PTFE. One week after the operation, methylene blue staining and WBC and PLT counting indicated that 0%-SF–PU (1 : 1) had a better anti-inflammatory effect than e-PTFE did, while e-PTFE caused damage to the surrounding cells and PLT. The blend films containing 5% aspirin had not only enhanced anti-inflammatory effect, but also better anti-platelet aggregation property, which could prevent the formation of a thrombus and cause few damage to the surrounding tissue cells. However, the inflammatory response was not so obvious with the blend film with 10% dose of aspirin and some cells in surrounding regions were damaged. This aspirin-integrated material tended to be associated with pancytopenia and bone marrow suppression. Four weeks after operation, the results of EM and blood tests (WBC and PLT counts) were similar to the results from those 1 week after operation.

CONCLUSIONS

Preliminary results suggested that the small diameter SF–PU graft reinforced with elastic tubular fabric had appropriate flexibility and enhanced physical properties close to human blood vessels.[2,27]

This study showed that the SF-PU (1 : 1) blend film had superior performance over the e-PTEF film in terms of histocompatibility, and the anti-inflammatory/anti-platelet effects were improved after integrated with limited doses of aspirin.

Thus, the SF–PU–aspirin hybrid material is a valuable biomaterial of small diameter vascular graft for further study.

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