Biocompatibility and Antibacterial Activity of Chitosan and Hyaluronic Acid Immobilized Polyester Fibers

Chi-Hsiung Jou,[1,2] Ling Yuan,[1] Shang-Ming Lin,[1] Mou-Chuan Hwang,[1] Wen-Li Chou,[3] Da-Guang Yu,[3] Ming-Chien Yang[4]

[1]*Department of Materials and Textile, Oriental Institute of Technology, Pan-Chiao City, Taipei County 22064, Taiwan*
[2]*Department of Raw Materials and Yarn Formation, Taiwan Textile Research Institute, Tucheng City, Taipei County 23674, Taiwan*
[3]*Department of Textile Science, Nanya Institute of Technology, Chungli City, Taoyuan County 32091, Taiwan*
[4]*Department of Polymer Engineering, National Taiwan University of Science and Technology, Taipei 10672, Taiwan*

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ABSTRACT: Poly(ethylene terephthalate) (PET) fibers were treated with ^{60}Co-γ-ray and grafted with acrylic acid. The resulting fibers were further grafted with chitosan (CS) via esterification. Afterward, hyaluronic acid (HA) was immobilized onto CS-grafting fibers. The antibacterial activity of CS against *S. aureus*, *E. coli*, and *P. aeruginosa* was preserved after HA-immobilization. After immobilizing HA, the L929 fibroblasts cell proliferation was improved for CS-grafting PET fiber. The results indicate that by grafting with CS and immobilizing with HA, PET fibers not only exhibit antibacterial activity, but also improve the cell proliferation for fibroblast. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 104: 220–225, 2007

Key words: biocompatibility; polyesters; modification; polysaccharides

INTRODUCTION

Poly(ethylene terephthalate) (PET) is frequently used to make prosthetic parts such as artificial vascular, laryngeal, esophageal, and suture.[1] To improve the biomedical applicability, polymeric materials are often immobilized with biologically active components onto the surface. Because PET does not have functional groups available for covalent immobilization, it must be modified to have reactive groups for covalent immobilization of biomacromolecules. Several papers have reported that functional groups such as carboxylic acid, sulfonic acid, amide, amine, acrylate, pyrrolidone, and glycol are introduced into polymeric materials via γ-ray methods.[2–4] Being one of the most effective means to modify polymers, γ-ray can rapidly create uniform active radical sites on the polymer matrix. There are two types of irradiation grafting: simultaneous irradiation and preirradiation. Preirradiation can form less homopolymer.[5] The addition of metal salts can increase the reaction rate and suppress the formation of homopolymer.[6]

Chitosan (CS) is natural biocompatible cationic polysaccharides. When sticking to the bacterial cell wall,[7,8] CS can suppress the metabolism of the bacteria. The antibacterial study of PET grafted with CS and its derivatives has been performed to *S. aureus* and *E. coli*.[9]

Infection often occurs with the implant of biomaterials and can cause death. Methicilin resistant *Staphylococus aureus* (MRSA) of acute abscess infection and *Pseudomonas aeruginosa* are common infectious bacteria found in hospitals. Entrohemorragic *E. coli* O157: H7 is a pathogenic infectious bacterium found in recent years. The symptom ranges from mild to serious hemorrhage, and sometimes may result in hemolytic uremic syndrome and thrombotic thrombocytopenic purpura and other complex.[10,11]

Although CS cannot be considered as a suitable biomaterial for *in vitro* fibroblast cultivation,[12] its cytocompatibility towards fibroblasts allows its use in combination with other materials, such as glycosaminoglycans.[13] CS can promote surface-induced thrombosis and embolization when applied in blood-contacting materials. To reduce the thrombus formation of CS, anionic polysaccharides such as heparin or dextran sulfate was applied to modify the structure or surface of CS.[14]

Hyaluronic acid (HA) is a linear polysaccharide ubiquitous in the organism where it plays important biological roles. Being a component of extracellular matrix, HA can influence several cellular functions such as attachment, migration, and proliferation.[15,16] Recent biomedical applications of HA include scaffolds for wound healing and tissue engineering, as

Correspondence to: M.-C. Yang (myang@mail.ntust.edu.tw).

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well as surgery, arthritis treatment, and as a component of implant materials.

In our previous work, PET fibers were preirradiated with ^{60}Co-γ-ray to graft acrylic acid (AA), followed by the esterification of CS.[17] In this work, HA was covalently bonded to CS-grafted PET fibers with glutaraldehyde (GA). The biocompatibility of these modified fibers was characterized including cytocompatibility and the antibacterial activity against four clinically infectious bacteria.

EXPERIMENTAL

Materials

Poly(ethylene terephthalate) (PET) fibers were obtained from Far Eastern Textile Co. Ltd. (Hsinchu, Taiwan). The fibers were treated by Soxhlet extraction with methanol for 24 h to remove sizing and grease. Chitosan (CS) with molecular mass about 160 kD and a degree of deacetylation of 85.3% was obtained from Taiwan Textile Research Institute (Taipei, Taiwan). Acrylic acid (AA) and 25% glutaraldehyde (GA) were purchased from Ferak Laborat Gmbh (Berlin, Germany). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Acros Organics (New Jersey). Hyaluronic acid, sodium salt (HA) of 757 kD, was purchased from Calbiochem (San Diego, CA).

Grafting of AA

Fiber specimens were evacuated in plastic bags and preirradiated by ^{60}Co γ-ray (Institute of Nuclear Energy Research, Taoyuan, Taiwan) at a total irradiation rate of 10 kGy (dose rate 7.8 kGy/h). Irradiated fibers were placed in 10 wt % AA aqueous solution containing 0.2*M* H_2SO_4 and 0.001*M* $FeSO_4$. The reaction was carried out under nitrogen at 50°C for 4 h. The resulting fibers (PET-AA) were washed with double-distilled water three times in a 250 mL flask, and then soaked in double-distilled water at 75°C for 24 h by frequently replacing water to remove homopolymers from the fiber surface.[2–4]

Grafting of CS

To graft CS onto carboxyl bearing fibers, the fibers were placed in 0.25 mg/mL CS in 5 m*M* acetic acid and 1*M* HCl, and reacted at 60°C for 10 min.[9,18] Afterwards, the fibers (PET-CS) were washed with phosphate-buffered saline (PBS) and double-distilled water three times. The reaction is given as scheme I in Figure 1.

HA immobilization on modified PET fiber

AA grafted fiber were placed in the reacting solution containing 0.2 m*M* EDC at pH 4.8 and 4°C for 24 h, and were washed three times with PBS and double-distilled water, then the fiber was dried in an oven at 65°C. These EDC-treated fibers were then placed in 0.25 mg/mL HA solution and reacted at 4°C for 24 h.[18] Afterwards, these fibers were treated by the Soxhlet extraction with methanol for 24 h to remove residual EDC. The reaction is given as scheme II (1) in Figure 1. The resulting samples were freeze-dried at −40°C in a freeze-dryer for 2 h and were referred as PET-HA.

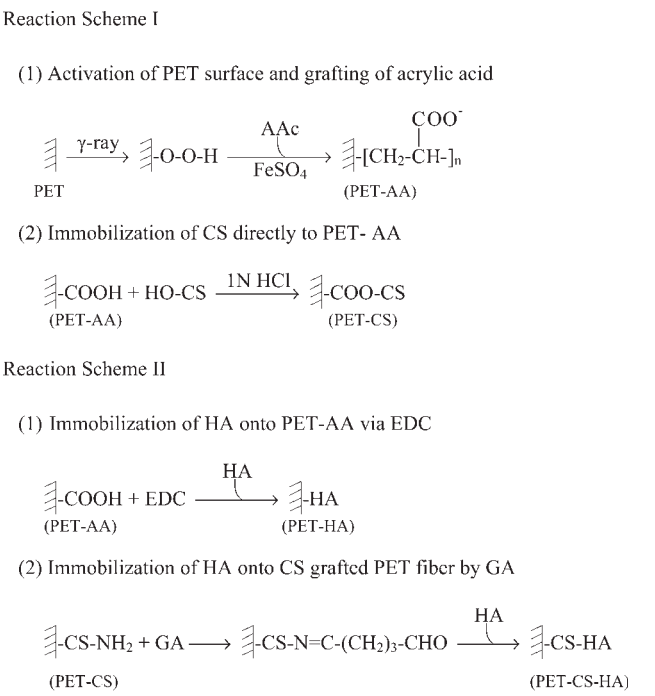

Figure 1 The reaction schemes for grafting of CS onto the fiber surfaces.

To graft HA onto CS-grafted fibers, the fibers were first treated with 0.2 m*M* GA at 25°C for 30 min,[18] washed three times with PBS and double-distilled water, and then were dried in an oven at 65°C. These fibers were then placed in 0.25 mg/mL HA solution in double-distilled water and reacted at 25°C for 30 min. Afterwards, these fibers were treated by the Soxhlet extraction with methanol for 24 h to remove residual GA and HA. The reaction is given as scheme II (2) in Figure 1. The resulting samples were freeze-dried at −40°C using a freeze-dryer for 2 h and were referred as PET-CS-HA.

Determination of surface grafting density

The surface density of carboxyl group for AA or HA was determined by dyeing with 0.01 g/mL of C. I. Basic Blue 17 (Chroma-Gesellschaft GmbH, Müster, Germany) at pH 10 and 30°C for 5 h. After dyeing, the fiber was rinsed with adequate double-distilled water, followed by soaking in 0.1 m*M* NaOH to remove adsorbed dye molecules. Finally, the associated dye molecules were desorbed in 50 vol % acetic

TABLE I
Clinical Source of Bacteria Used in This Study

Bacterium strain	Source
Gram-positive bacteria	
Methicilin resistant *Staphylococus aureus* (MRSA; *S. aureus*-1)	Acute abscess infection culture
Staphylococus aureus strain-2 (*S. aureus*-2)	Wound infection culture due to suture
Gram-negative bacteria	
Escherichia coli O-157:H7	ATCC 43894
Pseudomonas aeruginosa	ATCC10145

acid. The dye concentration was determined at 633 nm using a spectrophotometer (UV 3101 PC, Shimadzu, Tokyo, Japan) and calculated from the calibration curve.

The surface density of amino groups of CS on the fiber surface was determined by dyeing with 0.01 g/mL of C.I. Acid Orange 7 (Tokyo Kaseo Kogyo, Tokyo, Japan) at pH 3 and 30°C for 5 h, and then rinsed with adequate double-distilled water, followed by 1 m*M* HCl to remove adsorbed dye molecules, and finally by 1 m*M* NaOH to desorb the associated dye molecules. The dye concentration was determined at 485 nm and calculated from the calibration curve.[17,18]

Antibacterial activity

Table I lists those four strains of bacteria used in this work. Frozen preserved stock was thawed at room temperature, and then 0.1 mL were pipetted and streaked into quadrant on sheep blood agar plate (Difco Laboratories, USA), and incubated at 37°C overnight. Afterwards, a single colony was scraped with a loop and swabbed to a 15° slant medium (10 mL of nutrient agar) and incubated at 37°C. After incubating for 0–24 h, 20 mL of PBS was added. After mixing, 1 mL of the solution was withdrawn and diluted with 9 mL of nutrient broth (concentration = 8 g/L), and mixed with a vortex mixer. The solution was then diluted with PBS to $1.5 \pm 0.3 \times 10^5$ CFU (colony-forming units), and placed in flasks (six samples of 0.4 g/sample for each group). After incubating at 37°C for 0–24 h, 20 mL of PBS were added and stirred for 30 s. Consecutive dilutions were prepared by taking 1 mL of the previous solution and mixed with 9 mL of PBS. From this solution, 1 mL was transferred to a 50-mL centrifugal tube, mixed with 15 mL of nutrient agar (at 45°C), poured into a 9-cm plate, and then incubated at 37°C for 18–24 h. The number of survival bacteria was then counted.

Activated partial thromboplastin time

The sample was put into 0.5 mL of platelet poor plasma and was incubated at 37°C for 1 h. The activated partial thrombin time (APTT) was then determined using an automated blood coagulation analyzer (CA-50, Sysmex, Kobe, Japan). The control was measured against the glass tube without polymer sample.

Cell culture

Fibroblasts (L929, obtained from Food Industry Research and Development Institute, Hsinchu, Taiwan) were grown in a medium supplemented with a minimum essential medium alpha medium (Gibco, Invitrogen, New Zeeland) with 0.1 m*M* nonessential amino acids, 1 m*M* sodium pyruvate, and 90% Earle's balanced salts (Sigma, USA), 10% horse serum in tissue culture flasks in a CO_2 incubator (5% CO_2 95% air) at 37°C. Confluent monolayers were propagated by trypsinization (0.25% trypsin, 0.02% EDTA) and replating at 1 : 2 dilution. The cells were maintained in a complete medium being replenished every day. After a 3-day culture, the culture was examined using an inverted light microscope (Axiovert 25 CFL, Carl Zeiss, Jena, Germany). At confluency, cells were harvested and subcultivated in the same medium.[19]

Cell proliferation

Modified fibers were taken to the bottom of six 3-cm polystyrene Petri dishes without biomaterial deposit to prevent them from floating in the growth media. Fibroblasts (2500 cells/mL) were plated on the specimens, and the medium was changed every 24 h during incubation in a CO_2 incubator. Cell proliferation on each specimen was determined after 5 days. To remove unattached cells, specimens were gently washed with PBS. The attached cells were separated from the substrate by incubation in 50 μL of 0.25 vol % trypsin solution for 10 min at 37°C, and 100 μL of media was added. After centrifugation, cells were placed in the fresh medium. An aliquot of the resulting cell suspension was stained with trypan blue and counted by using a Neubauer hemacytometer[19,20] on an inverted light microscope.

RESULTS AND DISCUSSION

Surfaces modification of fibers

The surface density of grafted functional groups on each specimen in this work was summarized in Table II. After γ-ray irradiation and grafting with

TABLE II
Surface Density of Modified PET Fibers

	Surface density (nmol/cm^2)	
Sample	Carboxyl group	Amino group
PET-AA	0.64 ± 0.05	NA
PET-CS	NA	10.6 ± 0.24
PET-HA	1.06 ± 0.10	NA
PET-CS-HA	5.82 ± 0.20	1.76 ± 0.12

AA, the surface density of carboxyl groups was 0.64 nmol/cm^2. After esterification for 10 min in 1*N* HCl, the surface density of amino groups from CS was 10.6 nmol/cm^2, whereas that of carboxyl groups was reduced to 0.14 nmol/cm^2. By comparing the surface densities of carboxyl groups on PET-AA and PET-CS, about 78% of the carboxyl groups on PET-AA were esterified with CS. In the third column of Table II, the amino groups after immobilization of HA were from CS, and contributed to the antibacterial activity of the modified PET. Apparently, not all amino groups were reacted during the immobilization of HA.

Antibacterial activity

The cationic amino group of CS can associate with anions on the bacteria wall, suppress its biosynthesis, disrupt the mass transport across the wall, and accelerate the death of the bacteria.[21]

The antibacterial activity was evaluated with those four bacteria listed in Table I. Figure 2 shows the effect of grafting on the growth curves of MRSA. When contacting with untreated PET, the bacteria grew from 1.5×10^5 to 3.2×10^7 CFU after incubating at 37°C for 24 h. Similarly, the bacteria grew to 4.2×10^7 CFU for PET-HA. On the other hand, when contacting PET-CS, the concentration of bacteria reduced rapidly and died out after incubating at 37°C for 4 h. After HA immobilization, the reduction in the number of MRSA began to occur at 6 h, which was less than the 4 h of PET-CS. However, after 24 h of incubation, all the bacteria died out for all four samples for both PET-CS and PET-CS-HA. Similar results were observed for *S. aureus*-2, *P. aeruginosa*, and *E. coli* O-157:H7. This phenomenon is also observed for CS-grafted polyester fiber in our previous work.[17]

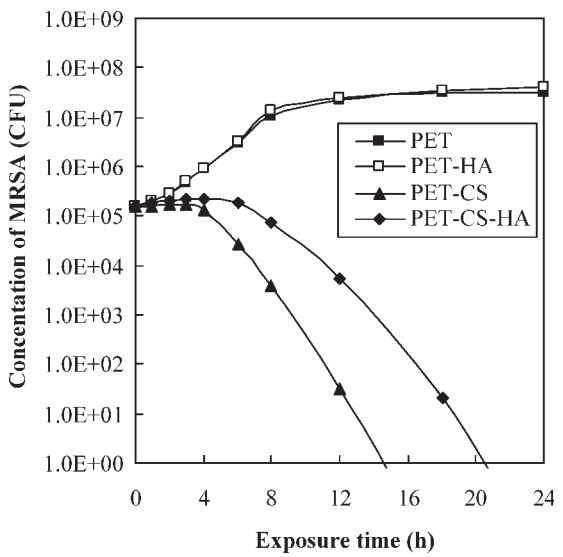

Figure 2 Change in the viable cell number of MRSA with the time exposing to the modified fibers.

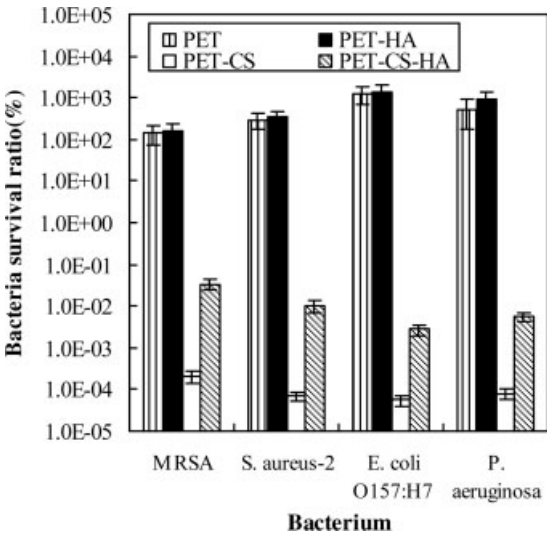

Figure 3 Comparison of antibacterial activity of fibers for four pathogenic bacteria after 12 h of incubation.

Figure 3 compares the antibacterial activity of these modified PET fibers based upon the survival ratio after incubating at 37°C for 12 h. Without grafting CS, bacteria would grow at least 140%. Although the antibacterial activity did drop after grafting with HA, the bacterial survival ratio of PET-CS-HA was still decreased to less than 10% after 12 h. Therefore, we can conclude that fibers grafted with CS can suppress the growth of all three bacteria used in this study.

Figure 3 shows that the order of antibacterial activity of CS-grafting samples is *P. aeruginosa* > *S. aureus*-2 > MRSA. The water contact angle of MRSA (49°) is higher than that of *S. aureus* (42°), as reported in our previous work.[22] Larger contact angle represents higher hydrophobicity, thus MRSA is more hydrophobic than *S. aureus*-2. Hydrophobic MRSA is less prone to be adsorbed on the positive-charged surface or hydrophilic surface of the fiber. This makes MRSA less interactive with CS grafted PET than *S. aureus*-2. The extracellular wall of *E. coli* O157:H7 and *P. aeruginosa* have fimbriae. Fimbriae secrets glycocalyx, polysaccharide, monosaccharides, hetropolysaccharides, polypeptide, and other compounds. These secretions may be absorbed more by a hydrophilic surface.[23] Hydrophilic *E. coli* O157:H7 has more fimbriae and hydrophilic secretions than *P. aeruginosa*. Glycocalyx carries net negative charges,[24] and can easily attach to hydrophilic and positive charged surface, thus increasing bacteria inhibition. Therefore, the grafting of CS is more antibacterial to *P. aeruginosa* than other bacteria tested.[17]

The results of this part of experiment indicate that by grafting CS, PET can acquire antibacterial activity, even after the additional immobilization of HA.

APTT

The blood compatibility of these samples is represented by APTT, and is summarized in Table III. The

TABLE III
Blood Compatibility Evaluated by APTT ($n = 6$)

Fibers	APTT (s)
Negative plasma	39 ± 3
PET	40 ± 3
PET-AA	44 ± 3
PET-CS	32 ± 3
PET-HA	46 ± 4
PET-CS-HA	44 ± 4

APTT of PET-CS was 32 s. It was shorter than that of the control, although not very significant. After HA immobilization, the APTT was extended to 44 s. These results indicate that HA does not improve much the blood compatibility of the substrate material. Magnani et al. studied the anticoagulation of differently sulfated HA and reported that unsulfated HA does not show anticoagulating ability for blood.[22] This agrees with our results.

Cell proliferation

The effect of surface modification on the proliferation of fibroblast is shown in Figure 4. The order of cell proliferation is PET-HA > PET-CS-HA > PET > PET-CS > PET-AA. Chen et al. reported that HA shows growth-promotion effect.[23] This agrees with our finding that HA immobilization did improve the proliferation of fibroblast. On the other hand, CS grafting can reduce the number of cells. Chatelet et al. suggested that CS seems to be cytostatic toward fibroblast: it is not cytotoxic, but inhibits cell proliferation.[12] This is in agreement with our results of CS grafting fibers.

Figure 5 shows that cell proliferation depends on the surface density of amino group. Those amino groups for PET-CS-HA were the remaining amino groups of CS after the immobilization of HA. Both CS and HA are polysaccharides. The difference is that CS has one amino group for each saccharide unit, whereas HA has one carboxyl group and one amide group for each dissacharide unit. Among these samples, PET-HA has the highest cell proliferation, whereas PET-CS has the lowest cell proliferation (in fact, reduction). For comparison, PET-AA, which bears only carboxyl groups on the surface, has even lower cell proliferation. It appears that other functional groups, for example, hydroxyl group in CS and HA, are also important to cell proliferation.

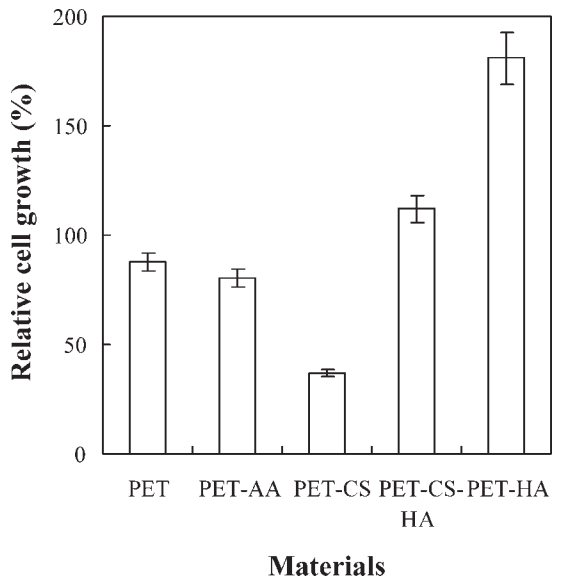

Figure 4 Relative cell growth ratio of fibers. The initial concentration of fibroblast was 2500 cells/mL, and incubated at 37°C for 5 days.

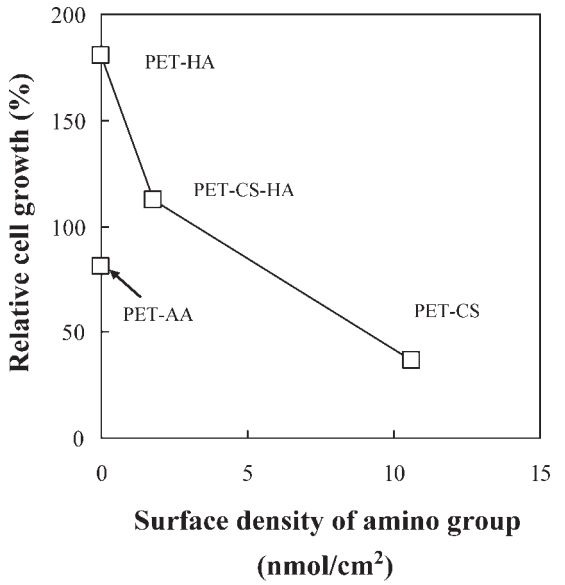

Figure 5 Effect of surface density of amino groups on the cell proliferation of fibroblast.

CONCLUSIONS

The biocompatibility of PET fibers can be affected by grafting CS. Because of the introduction of amino group, the grafting of CS can endow PET with antibacterial activity against four pathogenic bacteria. However, grafting CS onto PET caused the reduction in the number of fibroblasts. Immobilization of HA without CS can promote the proliferation of fibroblast to 180%. On the other hand, by immobilizing both CS and HA onto PET, the fibroblast proliferation can still be promoted 112%. In addition, PET-CS-HA also exhibits the antibacterial activity against four pathogenic bacteria. Therefore, by grafting CS and immobilizing HA, we can improve the applicability of PET fiber as a material for making implant devices.

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