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Antibacterial and Anticoagulation Properties of Polyethylene/GeneO-MPC Nanocomposites

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ABSTRACT: Nanocomposite technology has been utilized to improve biocompatibility and mechanical property of biomedical catheter materials. In this study, graphene oxide (GeneO), graphene oxide complexes with 2-(methacryloyloxy)ethyl phosphorylcholine (GeneO-MPC), and modified polyethylene (PE/GeneO-MPC) nanocomposites applied in biomedical catheter materials field were synthesized and their bacteriostasis to *E. coli* and *S. aureus* was studied. According to the results of bacterial adhesion, PE/GeneO-MPC nanocomposites inhibited the growth of the mass of predominant bacteria. PE/GeneO-MPC could reduce platelet adhesion greatly as compared to PE, which displayed the improved anticoagulant property. The tensile strength and elongation of PE/GeneO-MPC nanocomposites were enhanced by 15.5 and 97.3%, respectively. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 129: 884–891, 2013

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INTRODUCTION

Polymeric biomaterials are widely used in biomedical fields for the fabrication of medical implants and devices.[1–3] When exposed to the living tissue, they will result in some important reactions like forming of clots or undesired immunoreactions.[4] One of the important issues in developing medical devices that come into contact with blood is to improve their blood compatibility and antifouling properties.[5–8] Polyethylene (PE) is one of the most widely used polymers and the surface modification of PE has been widely investigated for electrical, coating, bonding, and biomedical applications.[9,10] Biologically nonfouling surface properties are of great interest since PE has been widely used to produce biomedical implants such as catheters or sutures. Wu et al. synthesized various kinds of antibacterial function plastics containing quaternary ammonium salt of nano-SiO_2 powder.[11,12] However, up to date, a few reports are closely associated with biosafety of PE in cells or live biosystems.

2-(Methacryloyloxy) ethyl phosphorylcholine (MPC) is a zwitterionic monomer bearing a biomimetic phosphorylcholine group. MPC-based copolymer coatings have excellent biocompatibility, exhibiting antibiofouling properties and antithrombogenicity.[13] It has been used to produce a wide range of copolymer coatings that confer excellent blood compatibility as well as resistance to protein adsorption and cell adhesion.[14] Biomedical applications for MPC-based copolymers include extended-wear soft contact lenses,[15] biocompatible coatings for guidewires and coronary stents,[16] extracorporeal circuits,[17] low biofouling coatings on urological devices, and tympanostomy tube implants.[18] Previously, MPC brush was grafted onto surface of PU through RATRP method in our group, expecting to obtain a new kind of PU with improved resistance to nonspecific protein adsorption and platelet adhesion as well as reduced bacterial adhesion.[4] In addition, several kinds of functionalized graphene oxides were successfully synthesized simultaneously.[19]

Since graphene was first isolated in 2004 with the help of scotch tape, researchers have excitedly turned to the material to discover its potential applications. Graphene oxide (GeneO), having multiple oxygen containing functional groups, such as hydroxyls and epoxies in the basal plane and carboxyl groups at plane edges,[20] can be easily prepared by chemical modification.[17] Surface functionalization leads to good dispersion in water and has become a hotspot so far and has been actively investigated to build new composite materials.[21] Nowadays, much attention has been paid to the preparation of polymer nanocomposites using GeneO as nanofiller whereas few reports deal with GeneO for anticoagulation and antibacterial

applications. Wang et al.[22] blended graphene oxide with epoxy, the obtained epoxy nanocomposites showed excellent thermal conductivities. The nanocomposite with 5 wt % of graphene oxide showed fourfold increment on thermal conductivity as compared to epoxy matrix. These have motivated us to explore the possibility of GO as a reinforcement in PE matrix for a new kind of artificial joints. However, to our knowledge, PE/GeneO composite materials are rarely reported.

In this study, a novel exfoliated polyethylene/modified graphene oxide nanocomposite (PE/GeneO-MPC) was synthesized via melting intercalation. Many modern analytical instrumental methods have been used in characterizing the features of PE functionalized with GeneO-MPC, and this new material was expected to be eventually used in biomedical field.

MATERIALS AND METHODS

Materials

PE was provided by Dongjue Fine Chemicals Co. (Nanjing, China). Graphite powder was purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). MPC was obtained from NanJing Le-Tianran Science and Technology Institute. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was obtained from Amosco. Dulbecco's minimum essential medium (DMEM) was purchased from HyClone. All other chemicals were of analytical grade and used without further purification. *E. coli* and *S. aureus* were provided by Jiangsu Center for Disease Prevention and Control. Fresh anticoagulant whole blood and platelet-rich plasma (PRP) was provided by Blood Center of Jiangsu Red Cross.

Preparation of PE/GeneO-MPC

Graphite oxide was prepared by modified Brodie method using graphite as initial material and graphene oxide was subsequently obtained by exfoliation with mild sonication.[23] GeneO (1 g) and MPC (1 g) were dispersed in 100 mL of distilled water under sonication for 30 min. The dispersion was vigorously stirred at 80°C for 2 h and then centrifuged at 3000 rpm. Finally, the precipitate (GeneO-MPC) was washed with deionized water and was dried in vacuum at 80°C for 24 h.

Premixed PE and GeneO-MPC were blended with Haake torque rheometer. The mass fraction of the modified graphite oxide was varied from 0 to 0.5 wt %. The ternary mixture was mixed at 160°C for 20 min with a rotor speed of 60 rpm. Then the composites were taken out and sheeted through a two-roll mill at 130°C for 5 min with the thickness of 1 mm. The as-prepared PE/GeneO-MPC nanocomposites were subsequently cut into standard dumb bell-shaped specimens for the following investigation.

Characterization

The surface topologies of GeneO-MPC was observed by atomic force microscopy (AFM, Veeco Instruments) under dry conditions, using a tapping mode at a scan rate of 0.5 Hz over an area of 5 μm × 5 μm. The internal organized structure of the sample was examined by transmission electron microscopy (TEM, JEM-200CX). Mechanical properties of the films were evaluated by tensile test using an Instron 4466 all-purpose tester at a speed of 500 mm/min. The morphology of the pristine material, materials adhered with platelets and materials adhered with bacteria were observed on a scanning electron microscope (SEM, JSM-5610).

Hemolysis Test

PE/GeneO-MPC films were cut into 5 mm × 5 mm pieces.[24] Two milliliters of fresh anticoagulated blood from human volunteers was diluted with 2.5 mL of normal saline solution. The 0.2 mL of diluted blood was added to PE/GeneO-MPC samples. The mixture was kept at 37°C for 60 min and then was centrifuged at 1500 rpm for 10 min. The supernatant was transferred to a 96-well plate where the absorbance was measured at 545 nm using a Microplate Reader. Positive controls consisted of 0.2 mL diluted blood in 10-mL deionized water while negative controls consisted of 0.2-mL diluted blood in10-mL normal saline solution. Hemolytic degree was calculated as:

$$\text{Hemolysis } \% = \frac{D_t - D_{nc}}{D_{pc} - D_{nc}} \times 100\%$$

where D_t is the absorbance of sample, D_{nc} is the absorbance of the negative control, and D_{pc} is the absorbance of the positive control.

Plasma Recalcification Profile Tests

Fresh anticoagulated blood from human volunteers (10 mL) was centrifuged at 3000 rpm for 10 min.[25] The supernatant platelet poor plasma (PPP) was collected. The sample solution (0.1 mL), plus 0.025 mol/mL $CaCl_2$ solution (0.1 mL), and PPP (0.1 mL) were added to 96-well plate for recalcification reaction. The plate was immediately placed in a BioTek synergy 2 Multi-Mode Microplate Reader. The absorbance was recorded every 20 s for 50 min at 405 nm wavelength at 37°C. At each time, absorbance of six samples was measured and the average value was used.

Platelet Adhesion Inhibition Method

Antithrombogenic ability was evaluated using platelet adhesion inhibition method as previously described.[26] PE/GeneO-MPC samples were immersed in PBS for 24 h and washed before immersing into PRP at 37°C for 2 h. After that, they were washed three times with PBS solutions to remove free platelet. The samples were then immersed in 2.5% glutaraldehyde of PBS for 30 min. Finally, the samples were washed with PBS, followed by step dehydration with 25, 50, 70, 80, 95, and 100% ethanol for 10 min each. The dried samples were sputter-coated with a thin film of gold for SEM imaging.

Bacteria Adhesion Inhibition Method

Antibacterial activity of PE and PE/GeneO-MPC against *E. coli* and *S. aureus* were assessed by bacteria adhesion inhibition method using SEM images.[27] After the samples were immersed in 10-mL bacteria solutions with bacterial counts of 10^7 CFU/mL at 37°C for 24 h, they were washed three times with PBS solutions to remove nonabsorbed bacterial. The samples were then immersed in 2.5% glutaraldehyde of PBS for 4 h at 37°C to fix bacteria. Finally, the samples were washed with PBS, followed by step dehydration with 25, 50, 70, 95, and 100% ethanol for 10 min each. The dried samples were sputter-coated with a thin film of gold for SEM imaging.

MTT Assay

The MTT assay was performed according to ISO10993-5.[28] All experiments were run eight times. Polymer solutions were prepared in serum supplemented tissue culture medium and sterilized by filtration (0.2 mm, Schleicher & Schuell, Dassel, Germany). Human Embryonic Kidney 293 (HEK 293) cells

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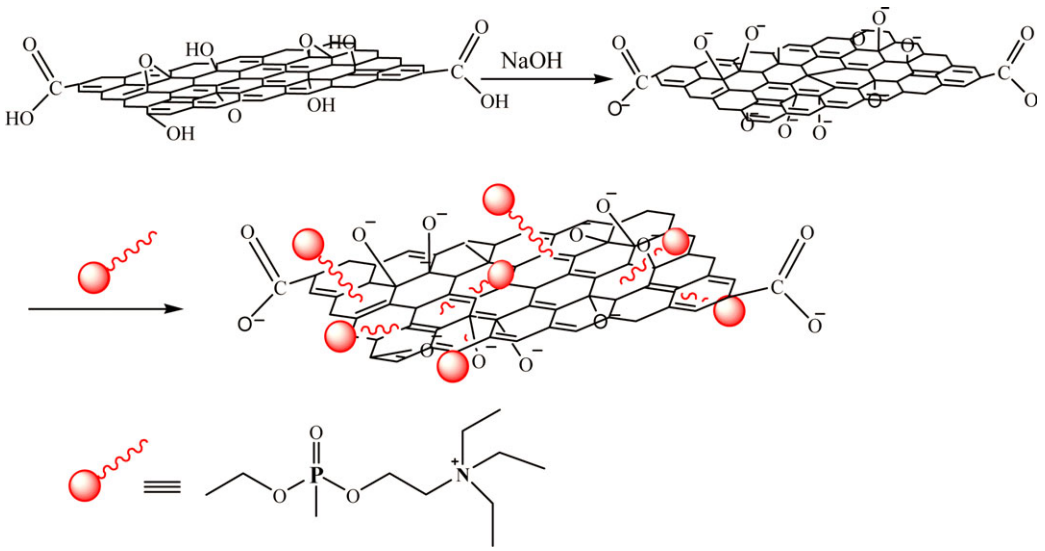

Figure 1. The preparation of PE/GeneO-MPC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(2×10^6 cells/mL) were suspended in DMEM containing 10% heat-inactivated FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin. The suspension (100 μL) was added to each well of a 96-well tissue culture plate and the cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After overnight cell culture, tissue culture medium was discarded and the extraction fluids were added. The incubation was performed for 24 h at 37°C. Twenty microliters of sterile filtered MTT stock solution in phosphate buffered saline (PBS) pH 7.4 (5 mg/mL) were added to each well reaching a final concentration of 0.5 mg MTT/mL. After 4 h unreacted dye was removed by aspiration, the insoluble formazan crystals were dissolved in 200 μL/well dimethylsulfoxide

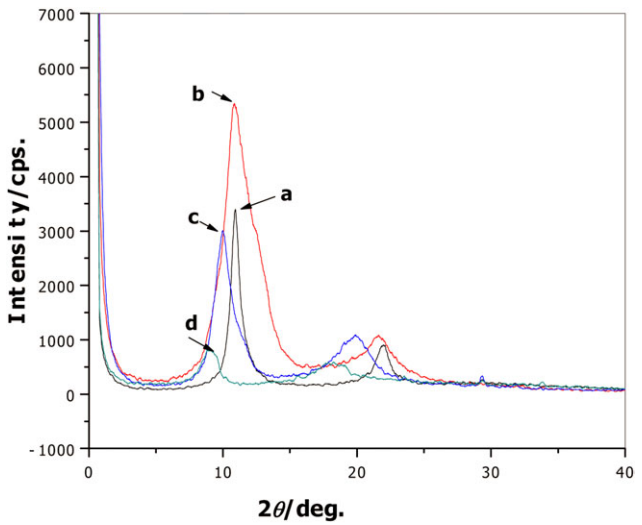

Figure 2. XRD patterns of GeneO-MPC complexes with different ratio of GeneO and MPC: (a) GeneO, (b) GeneO : MPC = 1 : 0.01, (c) GeneO : MPC=1 : 0.1, and (d) GeneO : MPC=1 : 1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

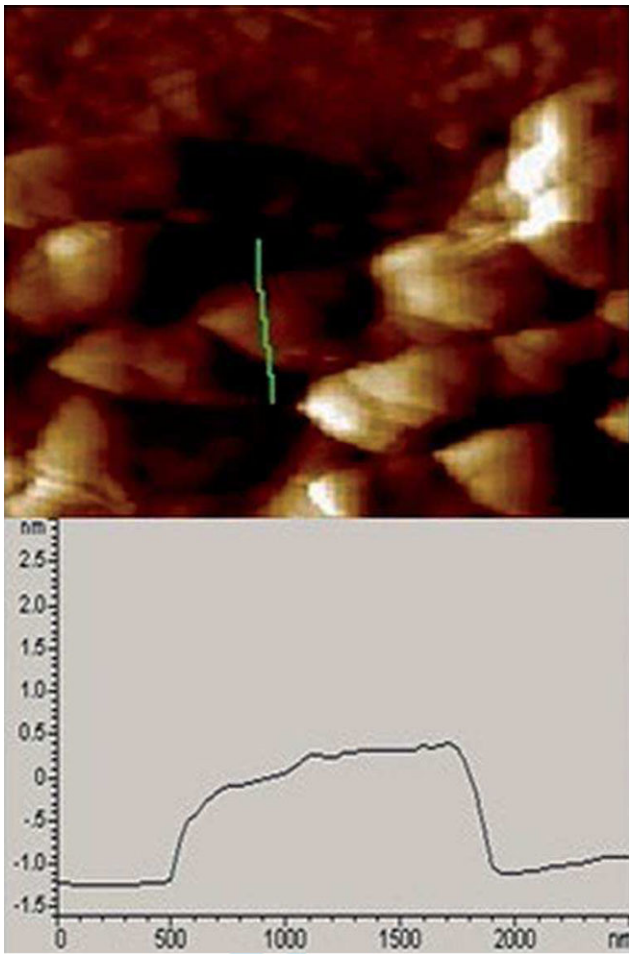

Figure 3. AFM of GeneO-MPC (GeneO : MPC = 1 : 1). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

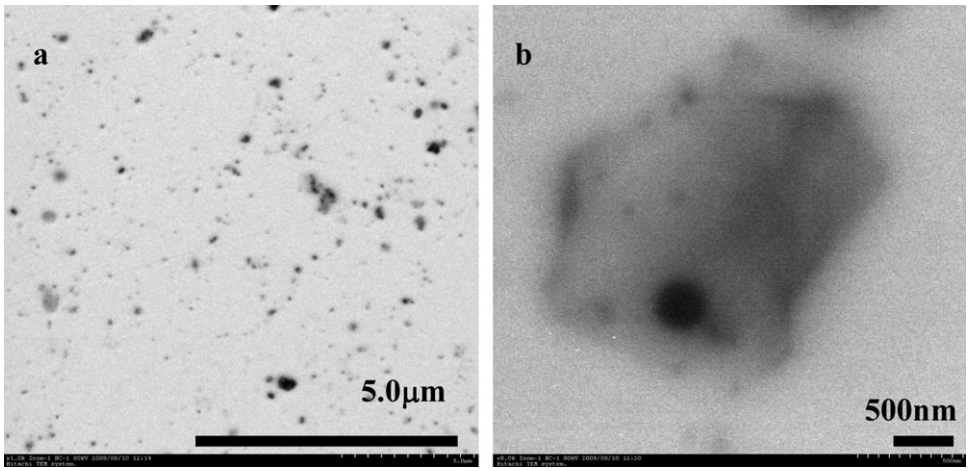

Figure 4. TEM of GeneO-MPC (GeneO : MPC = 1 : 1).

(DMSO) and measured spectrophotometrically by a BioTek synergy 2 Multi-Mode Microplate Reader at a wavelength of 490 nm (test). The test was accompanied by a blank control (MTT) and negative control (MTT+ cells).

RESULTS AND DISCUSSION

Preparation and Characterization PE/GeneO-MPC

In this study, we aim to first complex GeneO with MPC, and then use it as filler to obtain PE/GeneO-MPC composite (Figure 1). The composite should endow good mechanical properties and biocompatibility due to introduction of GeneO and MPC. The crystalline structures of GeneO and GeneO-MPC complexes were corroborated by XRD measurements (Figure 2). The (110) diffraction peak of GeneO [Figure 2(a)] appeared at 10.8 ° and the corresponding interlay space was 0.82 nm. It indicated that the interlay space of the resulting GeneO became larger than that of graphite. After exfoliation of MPC, the (001) XRD peak site of GeneO shifted to lower angles and the peak intensity decreased till disappeared with the increasing MPC content. The former result was attributed the increased amount of MPC, resulting of the increasing the interlay space. The latter was owed to MPC exfoliation result in the decreased crystallization degree.

The morphology of the composite was confirmed by AFM. The single layer of GeneO was about 0.5 μm × 1.5 μm with a thickness of 1–1.2 nm. These results were fairly agreement with the data reported in literature.[29,30] Figure 3 showed that GeneO-MPC remained a single-layer structure with a thickness of about 1 nm. MPC was dispersed on the layer and edges of GeneO, which indicated a successful loading of MPC onto GeneO layer.

TEM (a) showed that GeneO-MPC distributed homogeneously into PE by melting intercalation, revealing the well-scattered GeneO in PE. TEM (b) showed the single layer of GeneO-MPC with MPC loading on the surface and edge of GeneO, which was consistent to the AFM results (Figure 4).

Mechanical tests were performed on PE/GeneO-MPC nanocomposite film with GeneO-MPC loadings varying from 0 to 0.5 wt % (Table I). The mechanical properties of the PE/GeneO-MPC nanocomposite film were greatly enhanced with the increasing GeneO-MPC content. At 0.2 wt % GeneO-MPC content, a significant increased in the tensile strength and elongation at break. The tensile strength and elongation at break were increased by about 15.5 and 97.3%, respectively. From this point, further increase of GeneO-MPC content lead to a decrease in elongation at break and tensile strength. The aggregates of modified graphene oxide might result in poor mechanical properties of the polymer systems. Therefore, a hybrid film containing 0.2 wt % of the GeneO-MPC was prepared for further study.

Thermal stability is an important property when the nanocomposite morphology plays an important role. The thermogravimetric analysis (TGA) results of PE/0.2 wt % GeneO-MPC nanocomposite film and PDMS film are shown in Figure 5. The unparalleled ability of modified graphite oxide was found to boost the thermal stability of polymers. Evidently, we found that the onset of the thermal decomposition of PE/0.2 wt %

Table I. Mechanical Properties of PE and PE/GeneO-MPC Nanocomposite Materials

Sample	GeneO-MPC content (wt %)	Tensile strength (MPa)	Elongation (%)	Modulus (MPa)
PE/GeneO-MPC	0	10.0 ± 1.1	288.4 ± 23.5	55.3 ± 10.1
	0.02	11.5 ± 0.5	568.8 ± 36.0	53.9 ± 10.2
	0.2	12.3 ± 0.9	628.5 ± 6.7	72.4 ± 7.3
	0.5	13.9 ± 0.7	568.3 ± 71.2	72.2 ± 0.03

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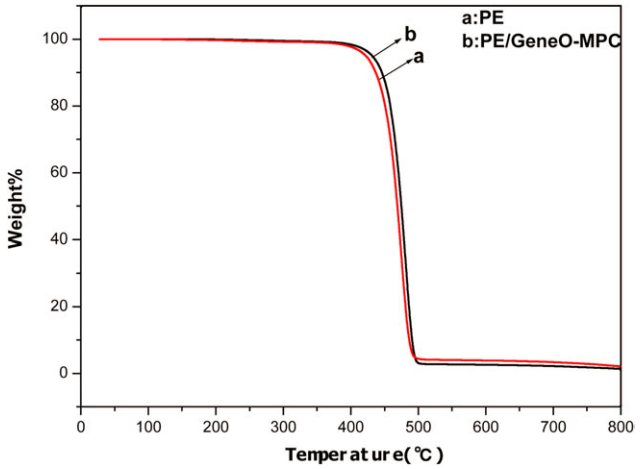

Figure 5. The TGA curves of PE (a) and PE/0.2 wt % GeneO-MPC nanocomposite film (b). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

GeneO-MPC nanocomposite film material greatly shifted towards a higher temperature range than that of neat PE. For PE/0.2 wt % GeneO-MPC nanocomposites, the initial temperature of thermal degradation increased by 10°C. Obviously, modified graphite oxide was effective in improving the thermal decomposition temperature of neat PE, which could be explained by good dispersion of modified graphite oxide particles in the PE matrix.

Hemolysis Ratio Test

Hemolysis is a common test for evaluating blood compatibility of biomaterials. All PE/GeneO-MPC composites showed a lower hemolytic degree as compared with PE (Figure 6). According to ASTM guideline, samples with hemolytic degree lower than 5% can be considered nonhemolytic. Therefore, PE/GeneO-MPC can be used as an antithrombogenic material.

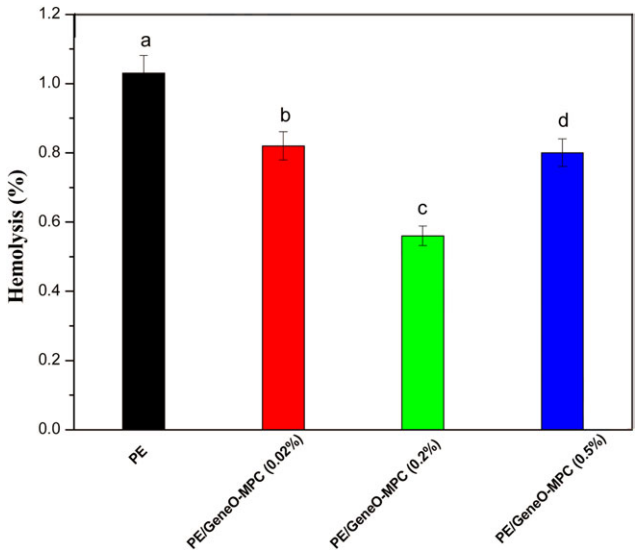

Figure 6. Hemolysis test of PE and PE/GeneO-MPC nanocomposite materials. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

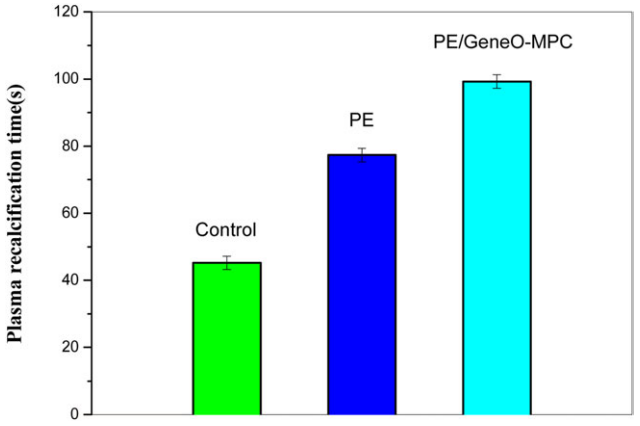

Figure 7. Recalcification time of controls, PE, and PE/GeneO-MPC with 0.2% GeneO-MPC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Plasma Recalcification Time

Coagulation is the culmination of a series of reactions, ultimately resulting in the thrombin-catalyzed transformation of fibrinogen into an insoluble fibrin clot. Thrombin is formed upon the convergence of the intrinsic and extrinsic path ways of coagulation. Viewed from the perspective of blood biomaterial interactions, the intrinsic path way is triggered by surface contact and causes a linear cascade of reactions, requiring Ca^{2+} ions to participate. As a result, plasma recalcification profiles serve as a measure of the intrinsic coagulation system. The absorbance increases as the plasma becomes more turbid, correlating with the formation of a clot. Recalcification times of control, PE and PE/GeneO-MPC are shown in Figure 7. The average recalcification time was 45, 77, and 92 s for control, PE and PE/GeneO-MPC, respectively. From above data, PE/GeneO-MPC showed a prolonged recalcification time as compared to PE.

Platelet Adhesion

Platelet adhesion experiment is commonly used to evaluate antithrombogenic properties of biomaterials. Figure 8 shows the SEM micrographs of platelet adhesion on the surface of PE and PE/GeneO-MPC. The amount of platelet adhered on blank PE was quantity. There was a serious platelet aggregation. However, the amount of platelet adhered on PE/GeneO-MPC was significantly reduced. From the SEM images, it can be seen that almost no activation of adhered platelets on the surface of PE/GeneO-MPC while a significant amount of adhered platelets were observed on PE surface. The platelet on the surface was a single round ball or with a small number of pseudopodia. This might be due to the biocompatibility of the GeneO-MPC. Such a lower platelet adhesion of GeneO-MPC nanocomposite showed antithrombogenic property much better. It might be due to the electrostatic repulsion between the negative charges of the platelet and modest MPC released from the film.

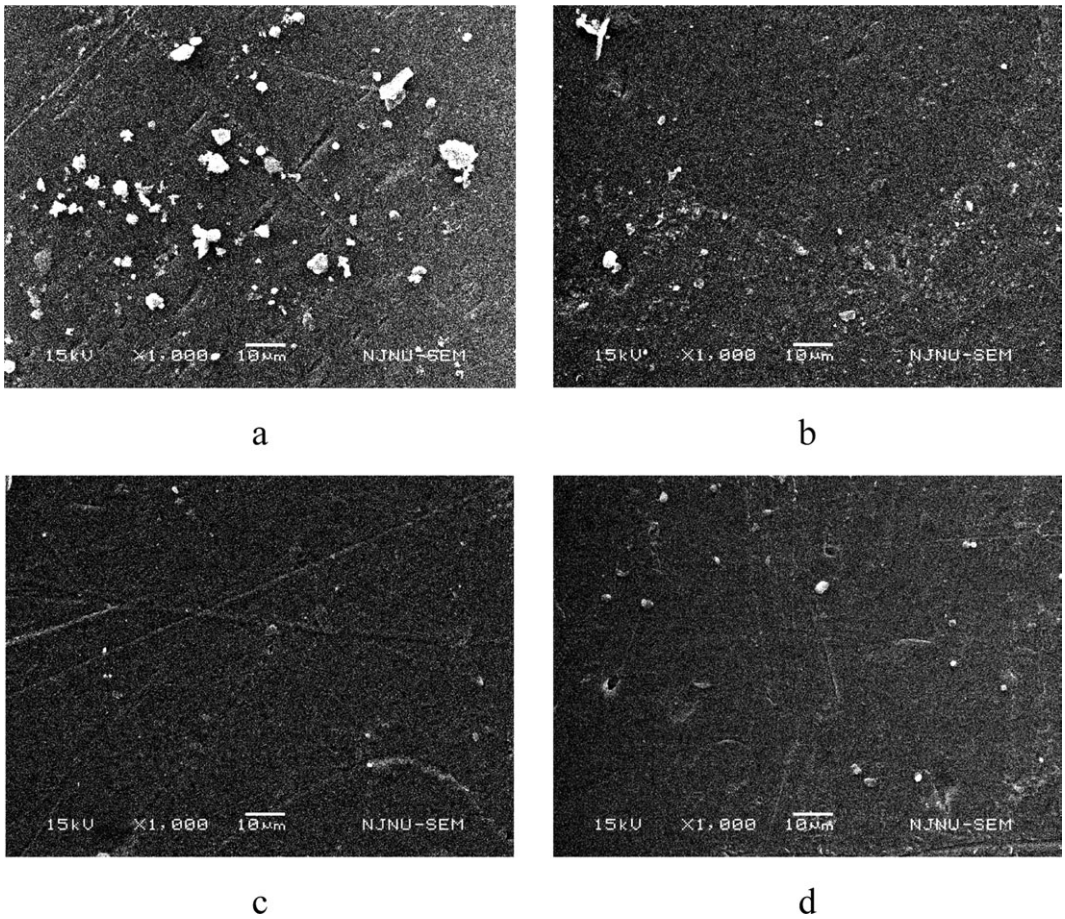

Figure 8. SEM for the morphologies of PRP contacted surfaces: (a) pristine PE, (b) PE/GeneO-MPC 0.02%, (c) PE/GeneO-MPC 0.2%, and (d) PE/GeneO-MPC 0.5%.

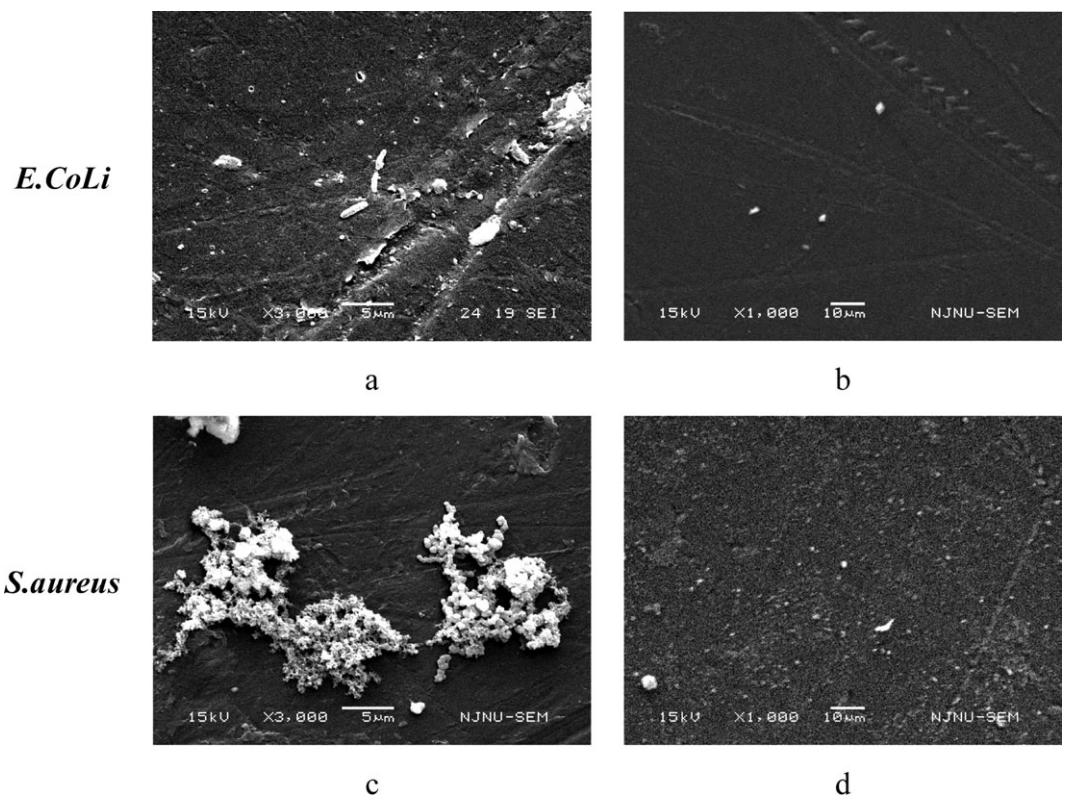

Figure 9. SEM for *E. coli* and *S. aureus* contacted surfaces of PE and PE/GeneO-MPC (0.2%): (a) pristine PE for *E. coli*, (b) PE/GeneO-MPC for *E. coli*, (c) pristine PE for *S. aureus*, and (d) PE/GeneO-MPC for *S. aureus*.

Bacterial Adhesion

Figure 9 shows the change in number of adherent *E. coli* and *S. aureus* on PE and PE/GeneO-MPC (0.2%) films. It showed that almost no activation of adhered *E. coli* and *S. aureus* on the surface of PE/GeneO-MPC while a significant amount of adhered bacteria were observed on PE surface. Similar antimicrobial activity of PE/GeneO-MPC was also effective against *S. aureus*. The reason for this phenomenon were probably as follows: (1) the bacteria adhered to materials was inactivated due to grapheme oxide which formed nanoscale film during reaction, and (2) the large number of —COO^- adsorbed cytoplasm of bacterial cells and engendered flocculation, which prevented the cells from the normal activities and killed bacteria. This property will be favorable to the applications of the novel antimicrobial material.

MTT Assay

MTT assays were performed to test the effects of polymer structure on the metabolic activity of cells. RGR of control PE, PE/GeneO-MPC (with different filling amount of GeneO-MPC in PE) were in Figure 10. It showed that the RGR of all polymers were outnumbered 80% compared with PE blank, which illustrated that all the modified polymers were no cytotoxic according to the grading standards of cytotoxicity (Table II). The experiments indicated that PE modified by GeneO-MPC had better cell compatibility.

CONCLUSIONS

In this study, GeneO-MPC complexes and PE/GeneO-MPC nanocomposites were prepared. The nanocomposite film not only maintained outstanding mechanical properties, but also exhibited excellent hemocompatibility and antimicrobial activity against *E. coli* and *S. aureus*. This novel material was expected to be eventually used in biomedical fields.

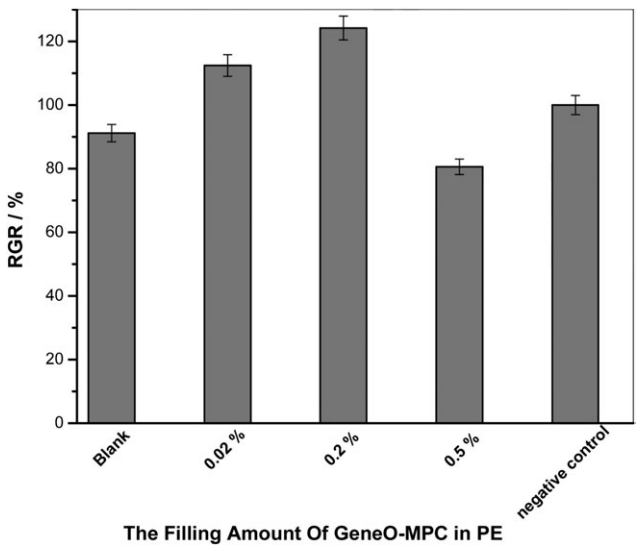

Figure 10. The cytotoxicity of PE/GeneO-MPC polymers measured by MTT assay after 24-h incubation with HEK293 cells (mean ± SD, $n = 8$).

Table II. The Grading Standards of Cytotoxicity

RGR (%)	Grading standards of cytotoxicity
≥100	0
75–99	1
50–74	2
25–49	3
1–24	4
0	5

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