Evaluation of Blood Compatibility of MeO-PEG-poly (D,L-lactic-*co*-glycolic acid)-PEG-OMe Triblock Copolymer

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ABSTRACT: The aim of the present work was to assess the blood compatibility of monomethoxy (polyethylene glycol)-poly (D,L-lactic-*co*-glycolic acid)-monomethoxy (polyethylene glycol) (MeO-PEG-PLGA-PEG-OMe, PELGE) triblock copolymer as a propriety material for intravenous use *in vitro*. Three different proportional triblock copolymers were synthesized. According to the International Standard Organization (ISO) and US Pharmacopoeia XXIII recommendations, siliconized glass tube was used as the negative control sample, while nonsiliconized glass tube was used as the positive control. The blood compatibility of the films of poly (D,L-lactic and glycolic acid) (PLGA) was evaluated by dynamic clotting time, activated partial thromboplastin time (APTT), and plasma recalcification time (PRT) measurements, platelet adhesion investigation, and hemolytic ratio analysis. The results revealed that blood compatibility of the materials was good. Nanoparticles made by this kind of materials might be promising for intravenous use. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 100: 1019–1023, 2006

Key words: evaluation; compatibility; copolymer

INTRODUCTION

Targeting delivery of drugs to the diseased lesions is one of the most important aspects of the drug delivery system (DDS).[1] Nanoparticles have been widely used as a carrier to convey sufficient dose of drug to the targeting lesion. But for the systemic administration, carriers should be biodegradable and biocompatible. Poly (D,L-lactic and glycolic acid) (PLGA) has been applied to a wide range of biomedical applications, such as suture material, artificial skin, bone repair, and drug delivery formulations.[2] Its degradation products, such as oligomers, lactic acid (LA), and glycolic acid (GA), are water soluble, and the breakdown products are finally eliminated from the body through the citrate cycle.[3] Thus, for a long time, PLGA has been a very attractive material for DDSs because of its well-documented safety and tissue compatibility.[4]

However, one great limitation of PLGA in intravenous DDSs is the lack of compatibility with cells and blood.[5] Wang et al.[6] had done platelet adhesion test of PLGA and reported that most platelets deposited onto the film of PLGA had developed pseudopodia.

One approach to solve these problems is to immobilize a biocompatible layer on the surface of the polymer to improve its blood–material interaction. Theoretically, the immobilization of special biologically active molecules can elicit some specific, predicable, and controlled responses from the blood.[7]

Polyethylene glycol (PEG) is a linear polymer with numerous hydroxyl groups, providing a propriety hydrophilic segment and opportunity of being further modified by some targeting reagent. Monomethoxy PEG (MeO-PEG)-modified biodegradable polymers may be used for the intravenous drug delivery.[8] It has been suggested that MeO-PEG-modified nanoparticles provide protection against interaction with the blood components, which induce removal of the foreign particles from the blood. Nowadays, MeO-PEGylated PLGA nanoparticles have been focused on for their prolonged circulation and controlled release property, seldom studies were done on the blood compatibility of the material itself. In the present study, we have synthesized MeO-PEG-poly (D,L-lactic-*co*-glycolic acid)-PEG-OMe triblock copolymer (PELGE) with characteristics of biocompatibility and checked their blood compatibility in vitro and ex vivo.

EXPERIMENTAL

Materials

PELGE was synthesized in our laboratory. PLGA (MW : 10,000; the ratio of LA to GA in the copolymer is 75 : 25), LA, and GA were obtained from Shandong

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Medical Appliance Factory, China. Beckman Coulter centrifuge (Allegra X-22R centrifuge) and CS501 super constant water bath were from Shanghai Yue-Xin Scientific Instrument Manufactory, China. GBC UV cintra 10e Spectrophotometer CO_2 critical point dryer (Hitachi HCP-2), high-vacuum gold–palladium sputter machine (Hitachi HUS-5GB), and scanning electron microscope (Hitachi *S*-450) were from Japan. Sodium citric acid, calcium chloride, and other chemicals and reagents used were of analytical grade and were obtained commercially. Japanese white rabbits were received from Sichuan Industrial Institute of Antibiotics (PRC). Human plasma was subscribed by a healthy 22-year-old man voluntarily. Actin-activated cephaloplastin reagent was home-made according to Ref. 9.

Synthesis of the polymer

The procedure of synthesis and isolation of the polymer was performed as reported previously.[10] Briefly, varied amount of lactide and glycolide crystals and specified amount of MeO-PEG were accurately weighed and put in 25-mL glass ampoules. Stannous octoate was added at a concentration of 0.05% by weight of the feed, and the tubes were evacuated. Then, the tubes were sealed and heated in an oil bath at 150°C for 5 h. The thus-obtained copolymers were purified by dissolving them in dichloromethane (DCM) and then precipitating them in excess methanol. The purified copolymers were dried under vacuum. Then, the coupling reaction of diblock copolymers was preformed with HMDI in toluene at 60°C for 12 h, followed by reflux for 6 h. The triblock copolymers were purified by methanol precipitation of polymer from methylene chloride using diethyl ether. Triblock copolymers of MeO-PEG-PLGA-PEG-OMe (PELGE) having 80–20 (PELGE82) or 70–30 (PELGE73) or 50–50 (PELGE55) molar ratio of LA to GA moieties and 15% mPEG with low-molecular-weight (about 10,000 Da) were synthesized. Their molecular weights and molecular distributions were determined by gel permeation chromatography. Average molecular weights were calculated using a series of polystyrene standards.[11]

Film preparation

In the hemolytic analysis and platelet adhesion test, polymers were dissolved in methylene chloride at a concentration of 20% (w/v), poured into a horizontal glassware, with solvent being evaporated in a current of dry air at room temperature for 24 h, followed by 24 h of vacuum drying, and then clipped into pieces (about $1 \times 1 \times 0.02$ cm^3). Films for the dynamic clotting time measurements, plasma recalcification time (PRT) measurements, and activated partial thromboplastin time (APTT) were prepared in a similar way, without clipping.

Hemolytic analyses[12,13]

The samples for hemolytic analysis were 5 g PLGA/MeO-PEG films with different proportion of LA/GA (80 : 20, 70 : 30, and 50 : 50, respectively). Three samples were prepared for each material.

Hemolytic activity was assessed by determining hemoglobin release under static conditions using the two-phase ISO/TR 7405–1984(f)[14] hemolytic test. Blood testing solution used was 4 mL of fresh rabbit blood mixed with an ACD medium (blood : 3.8% sodium citrate ratio is 4 : 1) and was diluted with 5 mL of 0.9% saline. In the first phase, each sample was incubated for 30 min in 10 mL sodium chloride solution (0.9% NaCl). Then, diluted fresh rabbit blood (200 μL) was added to the sample and incubated for another 60 min at 37°C ± 0.5°C without agitation. In the second phase, the immersion liquid was centrifuged at $750 \times g$ for 5 min and the optical density (OD) of the supernatant was read at 545 nm by a spectrophotometer. The positive reference (100% lysis) was blood/water mixture and the negative reference (0% lysis) was a blood/saline mixture. The OD value of positive should be 0.8 ± 0.3, while negative one less than 0.03. Each absorbance data point was obtained by measuring three samples and also the deviations of the three tests were determined. The hemolytic ratios of the samples were calculated as follows:

$$\text{Hemolytic ratio } (\%) = [(D_t - D_{nc})/D_{pc} - D_{nc}] \times 100\%$$

where D_t represents OD value of test sample and D_{nc} and D_{pc} stand for OD value of negative and positive controls, respectively. The samples were considered as hemolytic if the percent of hemolysis was $> 5\%$.[14]

Platelet adhesion test[11,13]

PLGA/MeO-PEG films with three different proportion of LA/GA were chosen for platelet adhesion test. The quantity and morphology for adherent platelets were examined as parameters for surface thrombogenicity studies. Blood was obtained from a healthy adult volunteer. Whole blood was collected in an ACD medium. After centrifugation ($100 \times g$ for 10 min at 25°C), red cells and platelets were speared and platelet-rich-plasma (PRP) was obtained. The samples were immersed into PRP and incubated at 37°C for 1 h. After rinsing, fixing, and critical-point drying, the specimens were coated with gold–palladium and examined using SEM and optical microscopy.

Dynamic clotting time measurements[13]

The kinetic method was adopted to study the thromboresistance property of the test materials. ACD blood was taken from rabbit using a plastic syringe, and 0.2 mL blood was immediately dropped onto a testing specimen. Then, 25 μL of 0.2 mol/L $CaCl_2$ aqueous solution was added to the specimen, agitated, and after a predetermined time, the specimen was transferred into a beaker containing 100 mL of distilled water and incubated for 5 min. The red blood cells, which had not been trapped in a thrombus, were hemolysed, and the free hemoglobin was dispersed in water. The concentration of free hemoglobin in the water was colorimetrically measured as the absorbance at 540 nm, with a spectrometer. The absorbance of the solution versus time was plotted against the contacting time of blood on the material surface. Each absorbance data point was obtained by measuring three samples and also the deviation of the three tests were determined. In addition to the three kinds of samples, siliconized and nonsiliconized glass tubes were also investigated as negative and positive controls for comparison.

PRT measurements[15]

We use Howell's method to perform the PRT measurements. The human whole blood containing 10% ACD was centrifuged at 3000 $\times$ g for 10 min to separate the blood corpuscles, and the platelet-poor plasma (PPP) obtained was used for the PRT experiments.

Interior surfaces of small glass tubes (10 $\times$ 100 mm^2) were cast with 1% w/v solution of PLGA/PEG in DCM, and dried for 3 days in vacuum at ambient temperature. Then, PPP (100 μL) was placed on the sample film attached to a watch glass tube, and incubated statically at 37°C. About 0.025 mol/L $CaCl_2$ aqueous solution (100 μL) was then added to the PPP, and the plasma solution was monitored for clotting by manually dipping a stainless-steel wire hook coated with silicone into the solution, to detect fibrin threads. Clotting times were recorded at the first fibrin formation on the hook. The experiment was carried out in triplicates and a mean value taken. In addition to the samples, siliconized and nonsiliconized glass tubes were also investigated as negative and positive controls for comparison.

Activated partial thromboplastin time[12,15]

Interior surfaces of small glass tubes (10 $\times$ 100 mm^2) were cast with 1% w/v solution of PLGA/MeO-PEG in DCM, and dried for 3 days in vacuum at ambient temperature. ACD plasma solution (PRP, 100 μL) and actin-activated cephaloplastin reagent (100 μL) was added to the sample film, followed by addition of 0.025 mol/L $CaCl_2$ solution (100 μL) after 5 min incubation. The clotting time of the plasma solution was observed as described in the in the PRT experiment. Siliconized and nonsiliconized glass tubes were also investigated as negative and positive controls for comparison.

TABLE I
Hemolytic Ratio of the Test Materials

Materials	OD values	Hemolytic ratios
Negative	0.0214 ± 0.0016	—
Positive	0.7244 ± 6E-5	—
$PELGE_{82}$	0.0333 ± 0.0002	1.69% ± 0.023%
$PELGE_{73}$	0.0517 ± 0.0001	4.3% ± 0.017%
$PELGE_{55}$	0.0441 ± 6E-5	4.31% ± 0.0058%

RESULTS

Hemolytic analyses

According to the ISO/TR 7405–1984(f), the samples were considered as hemolytic if the percent of hemolysis was $>$ 5%. Three tests were performed. All of the three materials induced a percent hemolysis less than 5%; therefore, we can assume that these three materials had no hemolytic effect on the human red cell suspension (Table I).

Platelet adhesion test

The morphology of the platelet adhered on PLGA/MeO-PEG in the platelet adhesion tests were not shown here. After 1 h of incubation, the platelet on sample film remain in separated state and no aggregation or pseudopodium development occurred, while the aggregation and pseudopodium development of platelets on the positive control films were much more obvious. This observation is consistent with the results obtained from the clotting time measurements, providing evidence for the improved blood compatibility by the MeO-PEG-modified PLGA films (results not showed).

Dynamic clotting time measurements

Figure 1 depicts the blood clotting profiles on the four tested materials. The absorbance of hemolysed hemoglobin solution varies with time. The higher the absorbance the better the thromboresistance was. The clotting tendency of PLGA/MeO–PEG film was much lower than that of the other positive control tested. The time at which the absorbance equals 0.1 is generally defined as clotting time. The best blood compatibility of materials can be achieved by the longest clotting time. Apparently, the clotting time of test materials was more or less higher than that of the positive control and even the negative one. This indicates that the blood compatibility is significantly improved by the formation of PLGA/MeO-PEG film.

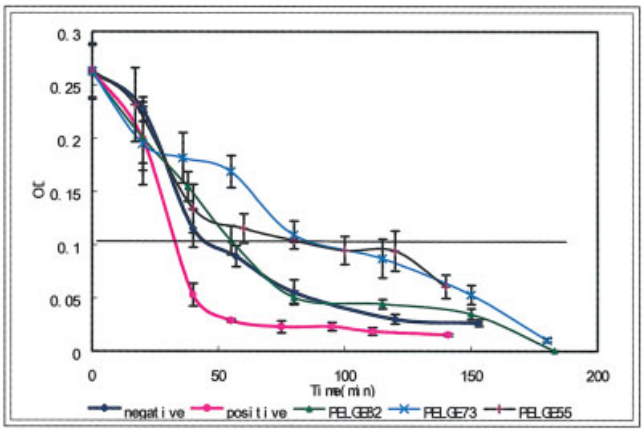

Figure 1 The dynamic clotting time curves of negative control, positive control, and test materials. Data are presented as mean ± standard deviation of three different experiments.

PRT measurements

The PRT of mPEG-modified PLGA is shown in Figure 2. PRT of PLGA/PEG was longer than that of the positive one.

Activated partial thromboplastin time

Three experiments were performed. All kinds of self-synthesized materials (PELGE) induced a significant increase in APTT (Table II), compared to that of the positive control.

DISCUSSION

This kind of inconsistent block copolymer has microphase-separated structure. As amphiphilic network polymers, PELGE was prepared as nanoparticle for target DDS, with a hydrophobic core and a hydrophilic shell. It was suitable for carrying different kind

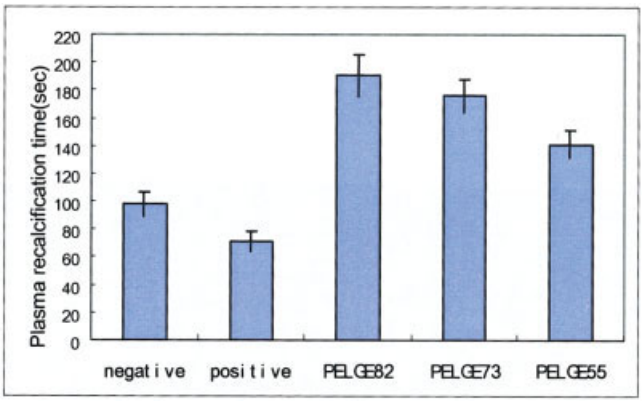

Figure 2 Plasma recalcification time of PLGA/PEG films, negative control, and positive control. Data are presented as mean ± standard deviation of three different experiments.

TABLE II
Mean and Standard Deviation of APTT after Contact with Test Materials

	No. of tests	APTT
Negative	3	51 ± 6.56
Positive	3	87 ± 2
$PELGE_{82}$	3	110 ± 6
$PELGE_{7}$	3	93 ± 7.09
$PELGE_{55}$	3	81 ± 5.57

of drugs, together with a "steal" character. In nonpolar medium like air, hydrophilic MeO-PEG aggregated in the core; while in physiological solutions, polymer molecules reconstructed, with MeO-PEG being located in the surface. This makes the material biocompatibile and blood compatibile, and helps it to effectively escape from endothelium system (RES) and adsorption of proteins.

After all, polymer MeO-PEG-PLGA- MeO-PEG is a promising drug carrier, which can overcome the shortcomings of traditional carriers. Researches on its further use may provide basis for its industrial and clinical use.

Until now, evaluation of blood compatibility is ongoing by cytology and histology methods. Nowadays, methods can only investigate the comprehensive results of the effects of biomaterials to organs, and there is a lack of device to check the real composition and structure of material's surface, so it is difficult to study the relation between the molecular structure of material's surface and blood compatibility.

Although many researchers have done much about the biological effect of these materials, the knowledge about them is superficial. Because of the complex of blood and reaction to cell/tissue, it is difficult to deduce the result of contact between materials and blood. Evaluation of blood compatibility is a comprehensive issue, for the reaction between material and blood is not simple. It is a procedure with mutually linked and relatively independent phases. It needs coresearch of more disciplines, such as hematology, engineering, material science, molecular biology, immunology, and genetics.

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