Cytotoxicity and Hemocompatibility of a Family of Novel MeO-PEG-Poly (D,L-Lactic-*co*-glycolic acid)-PEG-OMe Triblock Copolymer Nanoparticles

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ABSTRACT: A family of newly synthesized monomethoxy (polyethylene glycol)-poly (D,L-lactic glycolic acid)-monomethoxy(polyethylene glycol) (MeO-PEG-poly (D,L-lactic-*co*-glycolic acid)-PEG-OMe, PELGE) biodegradable polymers are candidates for intravenous nanoparticle drug, because of their merits of biocompatibility and blood compatibility, and their capability of escaping from the endothelium system (RES) and adsorbing proteins. In the current research, relationships between composition, cytotoxicity, and hemocompatibility of a series of blank PELGE nanoparticles were investigated. Cytotoxicity on Chang cell lines was investigated using the methyl thiazolyl tetrazolium (MTT) assay. Human and rabbit blood were used in studies of red blood cell hemolysis, whole blood clotting time, plasma recalcification profiles, and red blood cell form and appearance in whole blood. The results suggested that the molecular weight of PEG used in the synthesis of polymers influenced their characteristics. Generally, as the molecular weight of PEG increased, increased cytotoxicity and hemocompatibility were observed. The RGR (relative growth rate) of PELGE nanoparticles synthesized with PEG 550 was above 70%, while that of PELGE nanoparticles synthesized with PEG 750 and PEG 2000 was in the range of 55–105% and 36–87% respectively. For PELGE nanoparticles synthesized with PEG 550, most hemolysis values were in the range of 1–3%, while for PELGE nanoparticles synthesized with PEG 750 and PEG 2000 hemolysis values were 1–2% and below 2%, respectively. None of the nanoparticles caused changes in red blood cell form or appearance. Based on the results, 12 kinds of PELGE were chosen for further studies. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 113: 2933–2944, 2009

Key words: copolymers; nanoparticles; composition-biocompatibility relationship; cytotoxicity; hemocompatibility

INTRODUCTION

Nanoparticles have been widely used as carriers to convey a sufficient dose of drug to the targeted lesion. Various polymers have been used in drug delivery research as they can effectively deliver a drug to a target site and thus increase its therapeutic benefit, while minimizing side effects.[1] Among them, poly(D,L-lactic and glycolic acid) (PLGA) has been a very attractive material for nanoparticles because of its well-documented safety and tissue compatibility.[2] However, PLGA suffers from a limitation as an intravenous drug delivery system because of its lack of compatibility with cells and blood.[3] To solve this problem, a family of monomethoxy (polyethylene glycol)-poly(D,L-lactic-*co*-glycolic acid)-monomethoxy (polyethylene glycol) (MeO-PEG-poly(D,L-lactic-*co*-glycolic acid)-PEG-OMe, PELGE) copolymers has been synthesized by our research team, because it was reported that monomethoxy PEG (MeO-PEG)-modified biodegradable polymers may be used for intravenous drug delivery, especially for nanoparticle drug delivery.[4] This kind of inconsistent block copolymer has a microphase-separated structure. As an amphiphilic network polymer, PELGE has a hydrophobic core and a hydrophilic shell. In a nonpolar medium like air, hydrophilic MeO-PEG aggregates in the core; while in physiological solutions, the polymer molecules reconstruct, with MeO-PEG being located in the surface. This makes the material biocompatible and blood compatible, and helps it to effectively escape from the endothelium system (RES) and adsorb proteins.[4] All together 45 kinds of novel PELGE have been

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synthesized in our laboratory with different lactic acid (LA) content, glycolic acid (GA) content, and PEG molecular weight and content, which are biodegradable polymer candidates for intravenous nanoparticle drug delivery systems.

Recently, it was reported that the composition of PLGA-PEG polymers affects the physicochemical characteristics, *in vitro* stability, and biodistribution of nanoparticles made from them.[5] However, the effect of composition on the cytotoxicity and hemocompatibility of PELGE nanoparticles and the biocompatibility of PELGE nanoparticles for clinical use have seldom been reported.

The purpose of this study was to evaluate the toxicity level, composition–cytotoxicity relationship, hemocompatibility, and composition–hemocompatibility relationship of a family of blank nanoparticles prepared from novel PELGE in order to determine the influence of LA/GA ratio, PEG molecular weight, and PEG content on cytotoxicity and blood compatibility, and to select the most biocompatible polymers. According to one report,[6] although the PLGA-mPEG nanoparticles had blood persistence and RES avoidance properties, the concentration of PLGA-mPEG nanoparticles in liver was much higher than in other organs eventually. Therefore, the cytotoxicity of PELGE nanoparticles on liver cells was investigated. Human Chang liver cells line,[7,8] which derived from normal human liver tissue, was used to measure the cytotoxicity of PELGE nanoparticles. Also, this work includes the interaction of PELGE nanoparticles with human whole blood, plasma, and rabbit red blood cells in an attempt to capture clotting and hemolysis processes.

EXPERIMENTAL

Materials

The triblock copolymer, MeO-PEG–PLGA–PEG-MeO (PELGE), was synthesized as described previously.[9,10] The composition of the polymers is illustrated in Table I.

TABLE I
Compositions of the Family of PELGE

No.	Molecular weight of PEG	PEG (%)	LA/GA ratio
1	550	5	8 : 2
2	550	10	8 : 2
3	550	15	8 : 2
4	550	20	8 : 2
5	550	5	7 : 3
6	550	10	7 : 3
7	550	15	7 : 3
8	550	20	7 : 3
9	550	5	6 : 4
10	550	10	6 : 4
11	550	15	6 : 4
12	550	20	6 : 4
13	550	5	5 : 5
14	550	10	5 : 5
15	550	15	5 : 5
16	750	5	8 : 2
17	750	10	8 : 2
18	750	15	8 : 2
19	750	20	8 : 2
20	750	5	7 : 3
21	750	10	7 : 3
22	750	15	7 : 3
23	750	20	7 : 3
24	750	5	6 : 4
25	750	10	6 : 4
26	750	15	6 : 4
27	750	20	6 : 4
28	750	5	5 : 5
29	750	10	5 : 5
30	750	15	5 : 5
31	750	20	5 : 5
32	2000	5	8 : 2
33	2000	10	8 : 2
34	2000	15	8 : 2
35	2000	20	8 : 2
36	2000	5	7 : 3
37	2000	10	7 : 3
38	2000	15	7 : 3
39	2000	20	7 : 3
40	2000	5	6 : 4
41	2000	10	6 : 4
42	2000	15	6 : 4
43	2000	5	5 : 5
44	2000	10	5 : 5
45	2000	15	5 : 5

Preparation and characterization of blank PELGE nanoparticles

Blank PELGE nanoparticles were prepared using a water-in-oil-in-water (w/o/w) emulsion solvent extraction/evaporation technique.[11] In a typical procedure, PELGE (20 mg) was dissolved in a mixture of ethyl acetate and acetone (9 : 1, v/v, 2.0 mL) and 0.2 mL distilled water was then added in. A primary water-in-oil emulsion was prepared by sonication using a microtip probe sonicator (JY92-II ultrasonic processor, Ningbo Scientz Biotechnology Co., Ltd., China) at an energy output of 60 W for 10 s five times. This primary emulsion was then poured into 1 mL of an aqueous solution of Pluronic F-68 (3%, w/v) and probe sonicated at 100 W for 10 s three times. The double emulsion was diluted in 2 mL Pluronic F-68 solution (3%, w/v) and the organic mixture was rapidly removed by evaporation under reduced pressure (Büchi, R-144 rotavaporator, Switzerland). The *z*-average diameters of nanoparticles were in the range 70–180 nm with a polydispersity index (PDI) value of about 0.23 calculated according to the International Standard on Photon Correlation Spectroscopy (PCS) using a Zetasizer Nano ZS90 (Malvern Instruments Ltd.,Malvern, UK).

TABLE II
The Mean and Standard Deviation of the RGR

No. of PELGE	RGR (%)	No. of PELGE	RGR (%)	No. of PELGE	RGR (%)
1	93.423 ± 2.785	16	78.708 ± 1.936	31	58.546 ± 0.182
2	91.916 ± 2.949	17	65.335 ± 1.879	32	50.051 ± 0.287
3	105.855 ± 5.223	18	89.390 ± 1.567	33	44.668 ± 2.357
4	107.389 ± 1.002	19	67.390 ± 1.495	34	46.217 ± 0.212
5	78.143 ± 1.602	20	74.407 ± 2.971	35	44.653 ± 2.062
6	92.302 ± 1.098	21	76.290 ± 1.136	36	45.722 ± 1.141
7	117.413 ± 0.833	22	71.559 ± 0.537	37	48.918 ± 1.852
8	132.902 ± 1.054	23	77.837 ± 0.317	38	48.687 ± 0.459
9	130.399 ± 0.514	24	42.269 ± 3.618	39	48.634 ± 0.851
10	84.968 ± 0.627	25	63.844 ± 1.657	40	52.626 ± 2.066
11	94.736 ± 0.827	26	75.940 ± 2.075	41	51.590 ± 3.154
12	81.593 ± 1.177	27	78.708 ± 1.936	42	57.481 ± 0.924
13	82.508 ± 1.145	28	97.349 ± 1.523	43	56.772 ± 0.877
14	96.199 ± 1.208	29	65.448 ± 1.057	44	55.387 ± 3.588
15	104.184 ± 2.860	30	54.117 ± 1.206	45	40.379 ± 0.707

The nanoparticles were then lyophilized and stored at 4°C before further analysis. Each lyophilized sample of the PELGE nanoparticle was weighed and dissolved in physiological saline forming a solution whose concentration was 400 μg/mL. These solutions were sterilized using sterile 0.22 μm membrane filters before being assayed for cytotoxicity.

Cell culture

Human Chang liver cells[7,8] were cultured at 37°C in a 95 : 5% O_2:CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA.), which was supplemented with 10% bovine serum, 100 U/mL Penicillin, and 100 μg/mL streptomycin.

Methyl thiazolyl tetrazolium (MTT) test

MTT assays were carried out with 45 kinds of blank PELGE nanoparticles at a concentration of 400 μg/mL. The MTT assay is founded on the ability of living cells to reduce a water-soluble yellow dye, 3-(4,5-dimethylthiazole-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) to purple formazan crystals by mitochondrial succinate dehydrogenases.[12,13] Upon addition of Dulbecco's modified Eagle's medium (DMEM), the purple product resulting from the dissolution of formazan crystals is formed. The absorbance is then measured for each sample and compared with the absorbance of the negative control. Chang cells were seeded in 96-well culture plates at a density of 3×10^4 cells/well (no cells were seeded in the blank control wells). The medium was replaced after 24 h with 100 μL of fresh medium and 100 μL of the family of blank nanoparticles and physiological saline (for negative control and blank control). Plates were incubated for 48 h with PELGE blank nanoparticles at 37°C in a humidified CO_2 atmosphere. Twenty microliters of 5 mg/mL of MTT solution (Sigma, USA) in phosphate-buffered saline (PBS) were added to each well for 4 h of incubation. At the end of the incubation time, the medium was discarded and replaced with 150 μL DMSO (Sigma, USA). Optical density (OD) data of converted dye was measured at a wavelength of 490 nm using a 96-well plate reader (Bio-Rad Model 550, USA). The relative growth rate (RGR) was calculated using the following equation, where OD_{smaple}, OD_{blank}, and $OD_{negative}$ are the absorbance of the test samples, blank control, and negative control, respectively.

$$\mathrm{RGR}\ (\%) = \frac{\mathrm{OD_{sample}} - \mathrm{OD_{blank}}}{\mathrm{OD_{negative}} - \mathrm{OD_{blank}}} \times 100$$

Blank PELGE nanoparticles blood compatibility analysis

Red blood cell hemolysis study (RBCHS)

All 45 kinds of PELGE nanoparticles were dissolved in 0.9% saline at a concentration of 2 mg/mL. Red blood cells were obtained from rabbit blood by centrifugation (20 min, 2000 × *g*). After the supernatant plasma was discarded, the erythrocytes were washed three times with 0.9% saline in order to remove serum proteins. An erythrocyte stock dispersion (ESD) with a fixed hemoglobin concentration (1 : 5 centrifuged erythrocytes, saline solution) was prepared from the washed erythrocytes using 0.9% saline solution. One hundred microlitres of the ESD were added to 1 mL sample, shaken and incubated at 37°C for 1 h. After centrifugation (5 min, 3000 × *g*) in order to remove intact erythrocytes and debris,

TABLE III
The Mean and Standard Deviation of the Absorbance at Every Time Point in the Whole Blood Clotting Time Test

	Time point				
No. of PELGE	5 min	15 min	25 min	35 min	45 min
Control	3.287 ± 0.089	0.500 ± 0.027	0.325 ± 0.013	0.136 ± 0.008	0.125 ± 0.004
1	3.240 ± 0.040	0.845 ± 0.007	0.442 ± 0.034	0.244 ± 0.015	0.174 ± 0.014
2	3.169 ± 0.068	0.150 ± 0.013	0.145 ± 0.005	0.182 ± 0.004	0.162 ± 0.016
3	3.240 ± 0.040	0.429 ± 0.009	0.278 ± 0.164	0.125 ± 0.002	0.156 ± 0.015
4	3.193 ± 0.042	0.272 ± 0.010	0.166 ± 0.016	0.158 ± 0.025	0.230 ± 0.065
5	3.229 ± 0.058	1.795 ± 0.014	1.244 ± 0.020	1.040 ± 0.086	0.789 ± 0.010
6	3.156 ± 0.039	1.369 ± 0.015	0.739 ± 0.010	0.171 ± 0.025	0.156 ± 0.007
7	3.213 ± 0.022	0.958 ± 0.007	0.708 ± 0.067	0.661 ± 0.004	0.273 ± 0.009
8	3.239 ± 0.039	1.601 ± 0.007	1.511 ± 0.027	1.279 ± 0.015	0.710 ± 0.015
9	3.363 ± 0.055	1.310 ± 0.040	1.270 ± 0.052	0.864 ± 0.015	0.365 ± 0.018
10	3.311 ± 0.049	0.707 ± 0.073	0.739 ± 0.034	0.869 ± 0.015	0.134 ± 0.016
11	3.280 ± 0.051	0.926 ± 0.001	1.264 ± 0.052	0.158 ± 0.008	0.179 ± 0.011
12	3.049 ± 0.014	2.322 ± 0.030	0.183 ± 0.008	0.146 ± 0.010	0.155 ± 0.002
13	3.058 ± 0.043	1.294 ± 0.021	1.253 ± 0.012	0.358 ± 0.014	0.201 ± 0.008
14	2.947 ± 0.070	2.482 ± 0.057	0.180 ± 0.004	0.155 ± 0.010	0.140 ± 0.008
15	3.056 ± 0.000	1.820 ± 0.033	0.442 ± 0.020	0.161 ± 0.025	0.172 ± 0.015
16	3.097 ± 0.089	0.626 ± 0.018	0.147 ± 0.012	0.188 ± 0.008	0.130 ± 0.008
17	3.112 ± 0.037	1.543 ± 0.039	0.310 ± 0.006	0.133 ± 0.005	0.143 ± 0.012
18	3.111 ± 0.066	1.458 ± 0.037	0.505 ± 0.023	0.168 ± 0.029	0.266 ± 0.003
19	3.333 ± 0.102	1.075 ± 0.118	0.763 ± 0.019	0.872 ± 0.030	0.163 ± 0.012
20	3.014 ± 0.191	2.661 ± 0.033	0.445 ± 0.084	0.290 ± 0.006	0.350 ± 0.153
21	2.921 ± 0.054	0.766 ± 0.026	0.530 ± 0.016	0.559 ± 0.018	0.171 ± 0.011
22	2.902 ± 0.182	2.076 ± 0.034	0.322 ± 0.011	0.155 ± 0.019	0.161 ± 0.012
23	2.909 ± 0.079	1.862 ± 0.098	0.974 ± 0.006	1.087 ± 0.034	0.689 ± 0.045
24	2.661 ± 0.232	0.902 ± 0.004	0.589 ± 0.020	0.562 ± 0.021	0.348 ± 0.026
25	2.743 ± 0.205	0.751 ± 0.004	0.555 ± 0.015	0.577 ± 0.017	0.192 ± 0.011
26	2.909 ± 0.127	1.549 ± 0.041	1.029 ± 0.082	0.643 ± 0.030	0.808 ± 0.004
27	3.017 ± 0.057	0.804 ± 0.024	0.154 ± 0.015	0.156 ± 0.020	0.183 ± 0.013
28	3.134 ± 0.000	0.458 ± 0.026	0.843 ± 0.004	0.570 ± 0.013	0.103 ± 0.004
29	3.159 ± 0.071	1.025 ± 0.018	0.991 ± 0.074	0.485 ± 0.023	0.110 ± 0.005
30	3.167 ± 0.000	0.372 ± 0.014	0.667 ± 0.117	0.142 ± 0.003	0.145 ± 0.036
31	3.167 ± 0.034	0.924 ± 0.023	0.787 ± 0.026	0.535 ± 0.018	0.117 ± 0.013
32	3.171 ± 0.104	0.153 ± 0.095	0.276 ± 0.104	0.327 ± 0.127	0.165 ± 0.052
33	3.153 ± 0.034	0.239 ± 0.005	0.217 ± 0.009	0.223 ± 0.003	0.097 ± 0.005
34	3.034 ± 0.026	0.338 ± 0.011	0.178 ± 0.006	0.133 ± 0.003	0.097 ± 0.005
35	2.993 ± 0.036	0.623 ± 0.003	0.504 ± 0.014	0.175 ± 0.009	0.109 ± 0.008
36	2.845 ± 0.009	3.003 ± 0.080	1.837 ± 0.062	0.717 ± 0.024	0.386 ± 0.012
37	2.867 ± 0.029	1.556 ± 0.095	0.675 ± 0.026	0.739 ± 0.008	0.670 ± 0.057
38	3.014 ± 0.048	2.280 ± 0.124	0.875 ± 0.016	0.575 ± 0.014	0.212 ± 0.008
39	2.951 ± 0.074	2.919 ± 0.310	1.976 ± 0.111	0.706 ± 0.025	0.770 ± 0.043
40	2.836 ± 0.177	1.834 ± 0.129	1.354 ± 0.122	1.148 ± 0.012	0.498 ± 0.014
41	2.774 ± 0.127	3.146 ± 0.063	0.818 ± 0.102	0.128 ± 0.002	0.170 ± 0.015
42	2.975 ± 0.057	3.165 ± 0.039	1.649 ± 0.045	1.120 ± 0.196	0.154 ± 0.021
43	2.946 ± 0.059	3.133 ± 0.047	1.082 ± 0.057	0.852 ± 0.018	0.330 ± 0.035
44	2.898 ± 0.012	3.092 ± 0.119	1.212 ± 0.375	0.883 ± 0.020	0.150 ± 0.023
45	2.988 ± 0.024	3.004 ± 0.015	1.264 ± 0.034	0.748 ± 0.024	0.226 ± 0.013

the optical density of the supernatant was measured by UV spectroscopy (Cary 100 Conc UV–visible spectrophotometer, Varian, USA) at 542 nm against blank samples. Results were determined in relation to control samples of 0% hemolysis (in 0.9% saline solution) and 100% hemolysis (in distilled water).[14–17] The percent hemolysis was calculated as follows,[16] where OD of test sample, OD(−) and OD(+) are the absorbance of the test samples, solution of 0% hemolysis and a solution of 100% hemolysis, respectively.

$$\text{Hemolysis}\ (\%) = \frac{\text{OD of test sample} - \text{OD}\,(-)\,\text{control}}{\text{OD}\,(+)\text{control} - \text{OD}\,(-)\,\text{control}} \times 100$$

Quantification of whole blood clotting time

Blood was drawn from healthy adult volunteers by venipuncture into acid citrate dextrose (ACD) anticoagulant vacutainer tubes. Ten microlitres of each of 45 kinds of PELGE blank nanoparticles (dissolved in

0.9% saline at a concentration of 2 mg/mL) were added to individual wells of 24-well plates (one well for each of five time points) and 0.9% saline was used as a negative control. The clotting reaction was activated by the addition of 2.5 mL $CaCl_2$ (0.1*M*) to the 25 mL sample of ACD blood. A 100 μL volume of the activated blood was carefully added to each PELGE blank nanoparticles sample. All samples were incubated at room temperature for 5, 15, 25, 35, and 45 min. At the end of each time point, the samples were incubated with 3 mL of distilled water for 5 min. Each well was sampled in triplicate (200 μL each) and transferred to a 96-well plate. The red blood cells that were not trapped in a clot were lysed by the addition of distilled water, thereby releasing hemoglobin into the water for subsequent measurement. The concentration of hemoglobin in solution was assessed by measuring the absorbance at 542 nm using a 96-well plate reader. The size of the clot is inversely proportional to the absorbance value. The experiments were carried out in triplicate.[18,19] The paired samples *t*-test of SPSS software version 13.0 was used to analyze the difference between the test samples and the control at every sampling time point.

Measurement of plasma recalcification profiles

Blood was drawn from healthy adult volunteers by venipuncture into ACD anticoagulant vacutainer tubes, and was subsequently centrifuged at 2000 × *g* for 10 min to obtain platelet poor plasma (PPP). Ten microlitres of each of 45 kinds of PELGE blank nanoparticles samples (dissolved in 0.9% saline at a concentration of 2 mg/mL) were placed in a 96-well plate with 0.9% saline as the negative control, and 100 μL of citrated PPP was added to each well. Then 100 μL of 0.025*M* $CaCl_2$ was added to each well (except the negative control). The plate was immediately placed in a 96-well plate reader, where the kinetics of the clotting process due to recalicification were monitored by measuring the absorbance at 405 nm (every 30 s for 45 min) at 37°C. In calculating the mean absorbance at each time point, three replicate wells were averaged per sample. The clotting time to reach half maximal absorbance was calculated and analyzed.[18]

Red blood cell form and appearance in whole blood

ACD-anticoagulated human blood (50 μL) was incubated for 20 min at 37°C with blank nanoparticle solution samples (50 μL) in 0.9% saline at final concentrations of 1 and 10 mg/mL of polymer in the blood. Whole blood incubated with saline was used as a negative control. After incubation, the red blood cells were examined by invert microscopy (Axiovert 40 CFL, Carl Zeiss, Oberkochem, Germany) using wet mounted slides at 400× magnification. Images were captured using a computer software system (Axio Vision release 4.1, Carl Zeiss, Oberkochem, Germany).

RESULTS

Composition–cytotoxicity relationship of blank PELGE nanoparticles

Cytotoxicity was calculated from the MTT assay. The RGR of PELGE nanoparticles synthesized with PEG 550 was above 70%, while that of PELGE nanoparticles synthesized with PEG 750 and PEG 2000 was in the range of 55–105% and 36–87% respectively. Origin 7.0 software was adopted to plot the results. Cytotoxic effects of blank nanopartciles made from PELGE with different ratios of LA/GA on Chang cells are shown in three-dimensional graphs to illustrate the effect of molecular weight and percentage of PEG on cytotocixity (Fig. 1). In every graph, the response model was mapped against two experimental factors, the molecular weight and percentage of PEG, while the ratio of LA/GA in the synthesized polymers and concentration of blank nanoparticles were kept constant at a fixed level. Responses at 4 levels of LA/GA ratio were investigated.

Visual inspection clearly shows that at all ratios of LA/GA there was a PEG molecular weight-dependent reduction of RGR generally, which indicated that cytotoxicity of PELGE nanoparticles increased as the molecular weight of PEG increased [Fig. 1(A–D)].

Figure 1(A) illustrates that when the ratio of LA/GA in the polymers was kept at 5 : 5, the PEG molecular weight-dependent reduction of RGR was confirmed, the only exception being when PEG percent was 5%. This indicated that the cytotoxicity of PELGE nanoparticles was affected by the molecular weight of PEG used in the synthesis of PELGE polymer, and that cytotoxicity increased when higher molecular weight PEG was used.

When PEG of 750 and 2000 molecular weight were adopted, cytotoxicity of PELGE nanoparticles increased as the percentage of PEG increased. But the reverse trend was observed with nanoparticles synthesized with PEG of 550 molecular weight. Those phenomena indicated that PEG with higher molecular weight increased cytotoxicity, but when the molecular weight was 550, increasing the percentage of PEG reduced the cytotoxicity. Generally, the primary determiner of cytotoxicity was the molecular weight of PEG when LA/GA ratio was 5 : 5. At LA/GA ratio of 6 : 4 [Fig. 1(B)], there was also an approximate PEG molecular weight- and

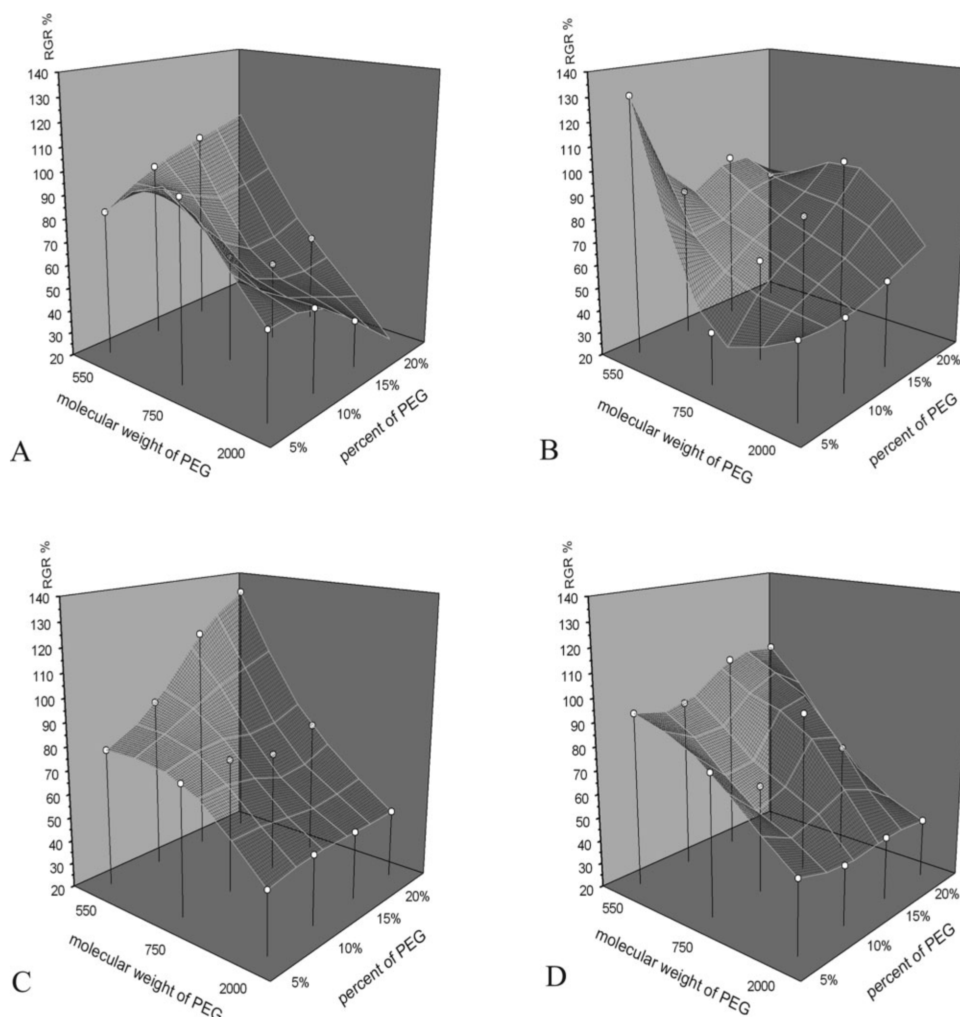


Figure 1 Three-dimensional plot of the effect of molecular weight and percent of PEG on cytotoxicity of blank PELGE nanoparticles (at 400 μg/mL) at four constant ratios of LA/GA in the polymer. (A) LA/GA = 5 : 5. (B) LA/GA = 6 : 4; (C) LA/GA = 7 : 3; (D) LA/GA = 8 : 2. The data were averaged over three experiments. (The mean and standard deviation of the RGR was listed in Table II).

percentage-dependent change of RGR. The response surface graph shows that the significant parameter was the percentage of PEG. With a PEG molecular weight of 750 and 2000, there was an obvious PEG percent-dependent decrease of cytotoxicity. With a molecular weight of 550, a rough PEG percent-dependent increase was observed instead. As molecular weight increased, there was only a slight increase in nanoparticles cytotoxicity, with some exceptions. At a LA/GA ratio of 7 : 3 [Fig. 1(C)], the molecular weight-dependent increase of cytotoxicity of blank PELGE nanoparticles was obvious. With a fixed molecular weight at 750 or 2000, altering the percentage of PEG hardly produced any change in the cytotoxicity. But enhancing the percent of PEG whose molecular weight was 550 resulted in reduction of cytotoxicity of blank nanoparticles. Also, a molecular weight-dependent increase of cytotoxicity of blank PELGE nanoparticles could be observed when the ratio of LA/GA was kept at 8 : 2 [Fig. 1(D)], and the effect of change in the percent of PEG was not significant.

Blank nanoparticles blood compatibility analysis

Red blood cell hemolysis study

Hemolysis of blood is a problem associated with the bio-incompatibility of nanoparticles for injection. *In-vitro* erythrocyte-induced hemolysis is considered to be a simple and reliable measure for estimating blood compatibility of materials.[17] The effect of PELGE nanoparticles on hemolytic activity was evaluated. The concentration of nanoparticles was fixed at 2 mg/mL. Deionized water and saline solution were used as a positive and negative control, respectively. Hemolysis results illustrated in Figure 2 showed the hemolysis produced in all PELGE

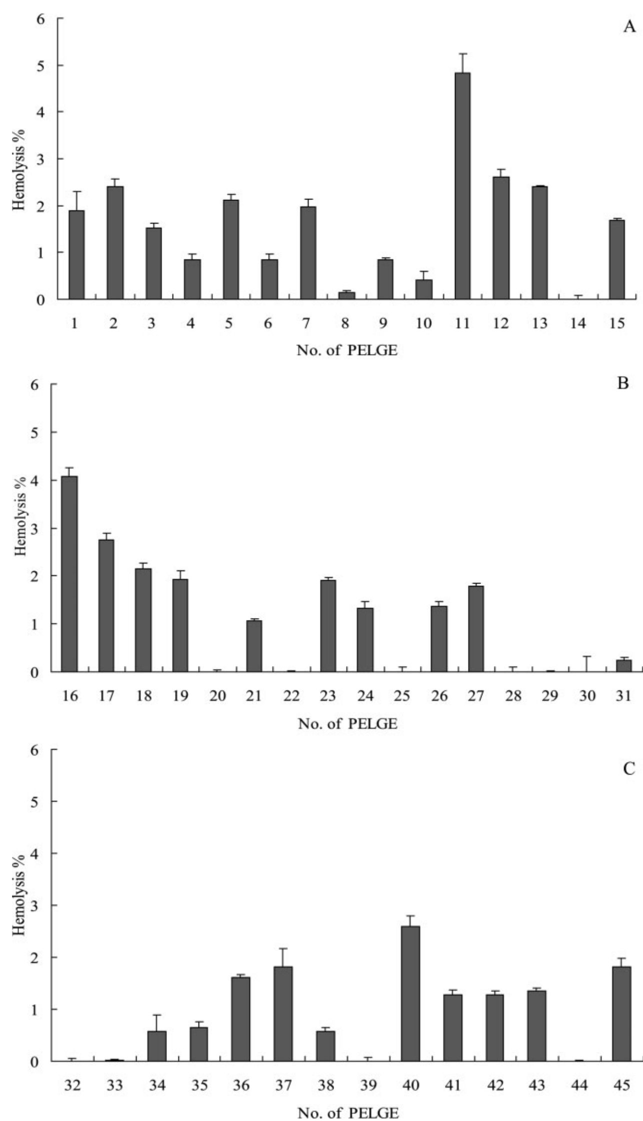


Figure 2 Percent hemolysis of red blood cells following incubation with blank nanoparticles made from PELGE synthesized with PEG of different molecular weight. (A) with PEG of molecular weight 550; (B) with PEG of molecular weight 750; (C) with PEG of molecular weight 2000. Red blood cells samples exposed to complete hemolysis with water were used to normalize the data. The number in the legend is the number of PELGE materials listed in Table I. The data were averaged over three experiments.

nanoparticle systems after 1-h incubation. It has been reported that up to 5% hemolysis is permissible for biomaterials.[16] The largest observed hemolytic activity was lower than 5% which indicates a wide safety margin in blood-contacting applications and suitability for intravenous administration.

Whole blood clotting time

Figure 3 depicts the blood clotting profiles on the 45 tested PELGE blank nanoparticles, and the 0.9% saline used as negative control. The absorbance of hemolyzed hemoglobin solution varies with time. Higher absorbance indicates better clot resistance. The best blood compatibility of materials is demonstrated by the longest clotting time. The time at which the absorbance equals 0.1 is generally defined as clotting time.[4]

Among the blank nanoparticles made from PEG of different molecular weights, visual inspection illustrated clearly that most of them had clotting dynamic curves above the negative control curve, which indicated that blood compatibility was significantly improved (Fig. 3). However, there were nine

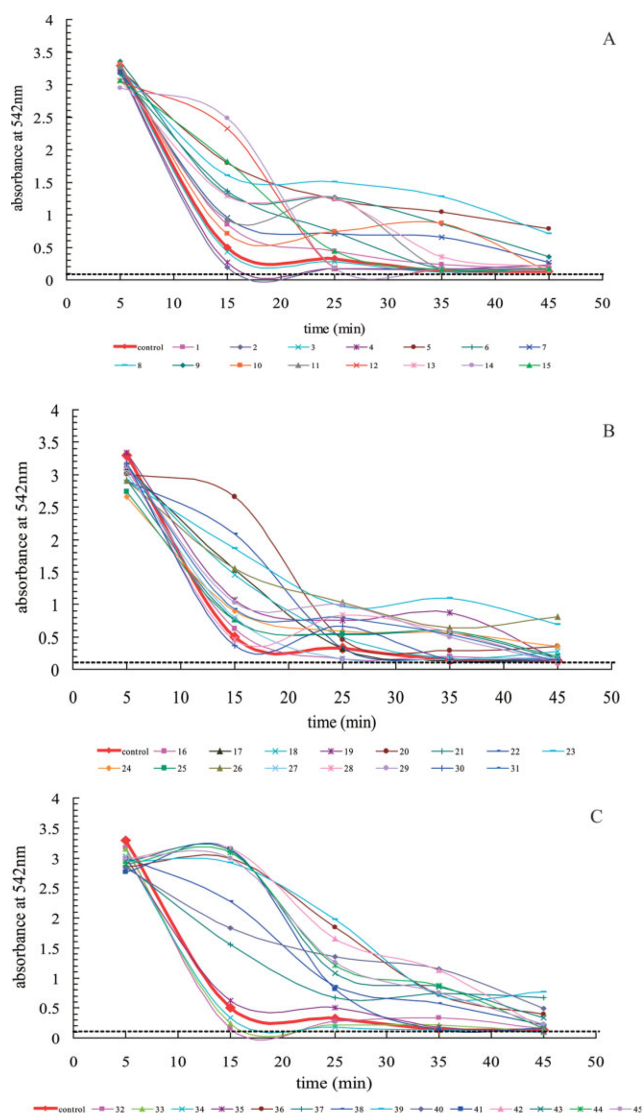


Figure 3 The whole blood clotting time of blank nanoparticles made from PELGE synthesized with PEG of different molecular weight. (A) with PEG of molecular weight 550; (B) with PEG of molecular weight 750; (C) with PEG of molecular weight 2000. The number in the legend is the number of PELGE materials listed in Table I. The data were averaged over three experiments. (The mean and standard deviation of the absorbance was listed in Table III). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE IV
Spss Analysis Results of the PELGE Nanoparticles whose Clotting Curves were Under that of Control

No. of PELGE	Paired samples test at every sampling time points				
	Min 5	Min 15	Min 25	Min 35	Min 45
2	3.169 ± 0.068	0.150 ± 0.013[a]	0.145 ± 0.005[a]	0.182 ± 0.004[a]	0.162 ± 0.016
4	3.193 ± 0.042	0.272 ± 0.010[a]	0.166 ± 0.016[a]	0.158 ± 0.025	0.230 ± 0.065
12	3.049 ± 0.014[a]	2.322 ± 0.030[a]	0.183 ± 0.008[a]	0.146 ± 0.010	0.155 ± 0.002[a]
14	2.947 ± 0.070[a]	2.482 ± 0.057[a]	0.180 ± 0.004[a]	0.155 ± 0.010	0.140 ± 0.008
16	3.097 ± 0.089	0.626 ± 0.018[a]	0.147 ± 0.012[a]	0.188 ± 0.008[a]	0.130 ± 0.008
27	3.017 ± 0.057	0.804 ± 0.024[a]	0.154 ± 0.015[a]	0.156 ± 0.020	0.183 ± 0.013[a]
32	3.171 ± 0.104	0.153 ± 0.095[a]	0.276 ± 0.104	0.327 ± 0.127	0.165 ± 0.052
33	3.153 ± 0.034	0.239 ± 0.005[a]	0.217 ± 0.009[a]	0.223 ± 0.003[a]	0.097 ± 0.005[a]
34	3.034 ± 0.026	0.338 ± 0.011[a]	0.178 ± 0.006[a]	0.1330.003	0.097 ± 0.005[a]

[a] Corresponds to a $p < 0.05$ in comparison to control.

exceptions, whose clotting curves were below that of the control. The results of SPSS analysis of the difference between the curves of those samples and the control are listed in Table IV. It was obvious that at 15 and 25 min, almost all those samples were statistically significantly different from the control; while at 5, 35, and 45 min, most of them showed no significant difference. That indicated that those nine kinds of PELGE nanoparticles induced an increase in the clotting rate at the time periods of 15–25 min. These results indicated that those nine kinds of PELGE polymers do not exhibit the desired blood compatibility.

Measurement of plasma recalcification profiles

The clotting process consists of both intrinsic and extrinsic pathways which ultimately lead to clot formation. The intrinsic cascade is initiated when blood contacts a surface, while the extrinsic pathway is initiated upon vascular injury which leads to exposure of tissue factor (TF).[14] Classical biochemistry of the intrinsic clotting pathway suggests that a 'pro-coagulant stimulus', starting with exposure of plasma to a test surface, potentiates a cascade of events that culminates in the release of thrombin (Factor IIa).[14] In the progression of the intrinsic pathway of clot formation, calcium is involved as an activating factor. Therefore, plasma recalcification profiles serve as a measure of the intrinsic coagulation system and can be used to investigate whether the blank nanoparticle surface would trigger this blood-biomaterial interaction. Typical recalcification clotting profiles and clotting rates for 45 kinds of PELGE blank nanoparticles are shown in Figure 4. The absorbance increases as the plasma becomes more turbid, which suggests the formation of a clot. A rightward shift of the curve indicates an overall slower clot time; whereas a leftward shift of the curve indicates a faster clot time.[14] Citrated PPP (without the addition of $CaCl_2$) served as a negative control, as it does not clot within the experimental time. The time required to reach half maximal absorbance (half-max time) was calculated as a measure of clotting time for each blank nanoparticle.[18] Most blank nanoparticles made from PELGE synthesized with PEG whose molecular weight was 2000 have a less coagulative surface as evidenced by a rightward shift in the curves, and the longest half-max time relative to all other nanoparticles tested. There was also a notable trend that the higher the molecular weight of PEG, the more the recalcification clotting profiles moved rightwards. Three-dimensional graphs showing the effect of molecular weight and percent of PEG on half-max time of blank PELGE nanoparticles plasma recalcification curve at a fixed ratio of LA/GA in the polymer (Fig. 5) illustrated that at all ratios of LA/GA, there was a PEG molecular weight-dependent increase in half-max time generally. This indicated that the clotting time of nanoparticles made from PELGE with higher molecular weight of PEG was longer than that of the others [Fig. 5(A– D)].

Red blood cell form and appearance in whole blood

Red blood cell interaction with polymers is particularly important for the use of polymers in *in-vivo* applications. Interaction with erythrocytes would negatively influence the safety and applicability of polymer–drug conjugates. Aggregation, crenation, and hemolysis are indicators of interaction and incompatibility of polymers with red blood cells.[19] The blank nanoparticles of our newly synthesized polymers were incubated with ACD anticoagulated whole blood at two concentrations (1, 10 mg/mL) and effects on erythrocyte hemolysis, shape, and aggregation were observed microscopically (Fig. 6). The nanoparticles did not produce any measurable hemolysis even at high concentration (10 mg/mL). Moreover, the red cells were morphologically

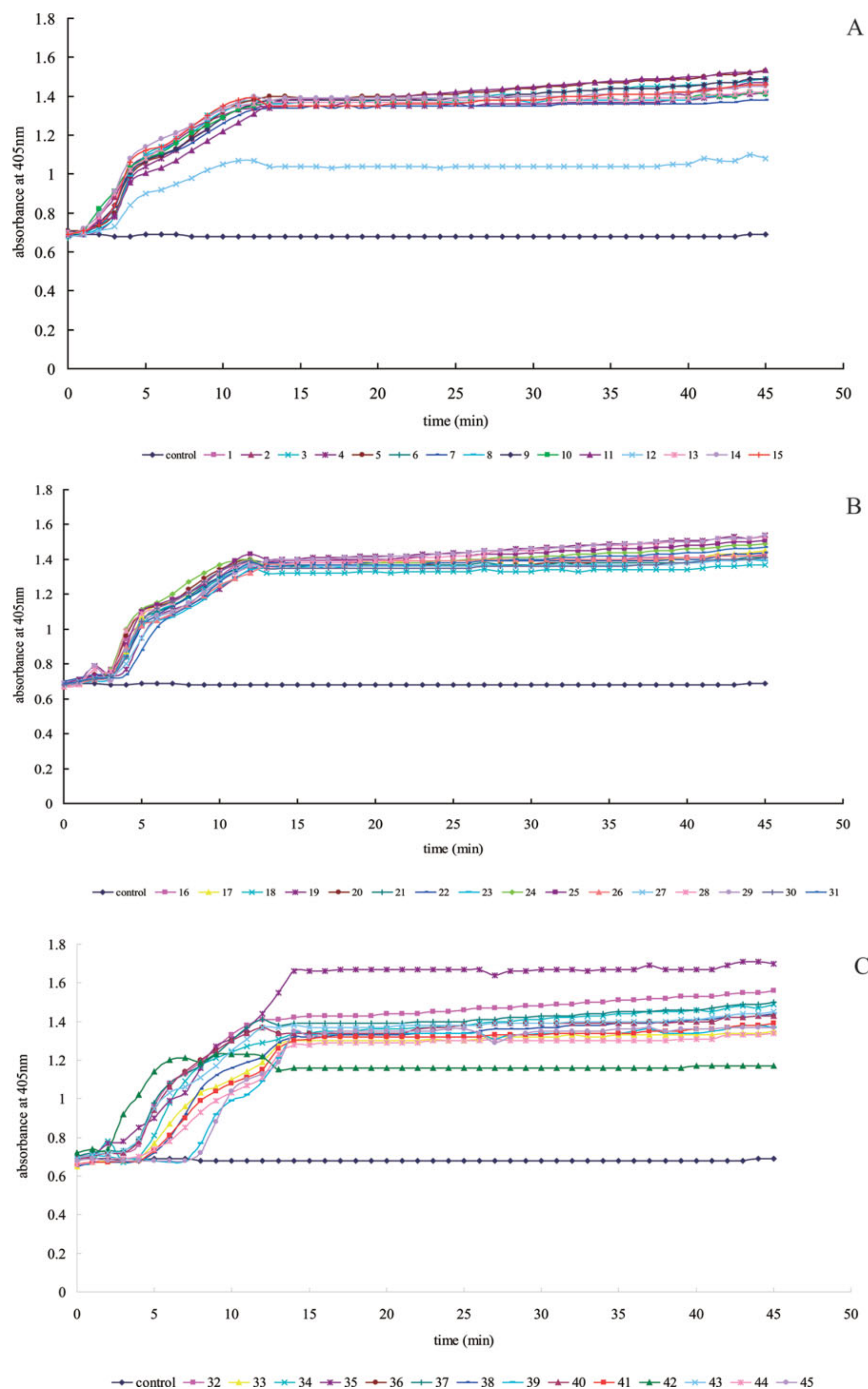


Figure 4 The plasma recalcification profiles of blank nanoparticles made from PELGE synthesized with PEG of different molecular weights. (A) with PEG of molecular weight 550; (B) with PEG of molecular weight 750; (C) with PEG of molecular weight 2000. The number in the legend is the number of PELGE materials listed in Table I. The data were averaged over three experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

normal [Fig. 6(B,C)] at 1 mg/mL and 10 mg/mL, similar to cells in saline controls [Fig. 6(A)]. No remarkable erythrocyte aggregation was observed in the presence of PELGE blank nanoparticles. All 45 kinds of blank nanoparticles yielded similar results.

DISCUSSION

The cytotoxicity and hemocompatibility of novel MeO-PEG-poly (D,L-lactic-*co*-glycolic acid)-PEG-OMe triblock copolymers were evaluated *in vitro* to determine their potential utility as biodegradable

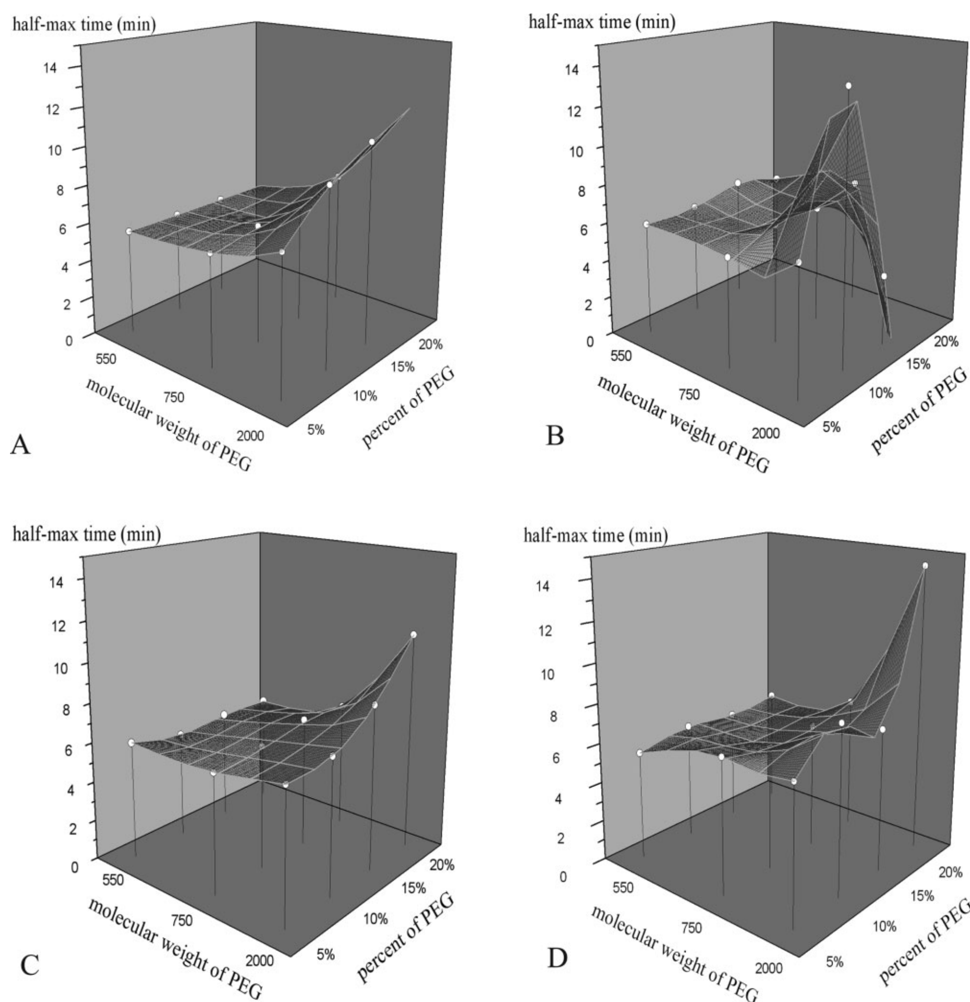
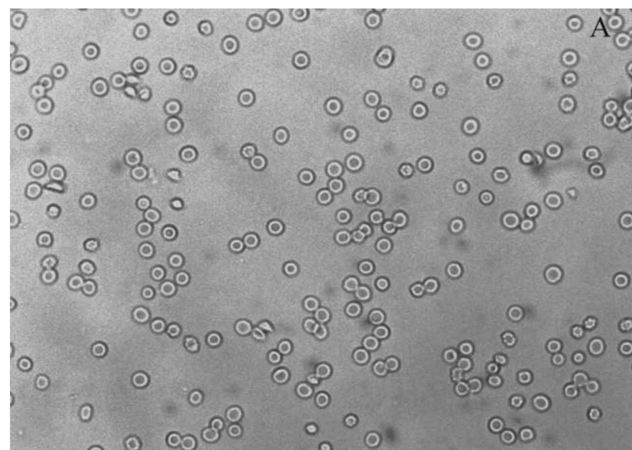
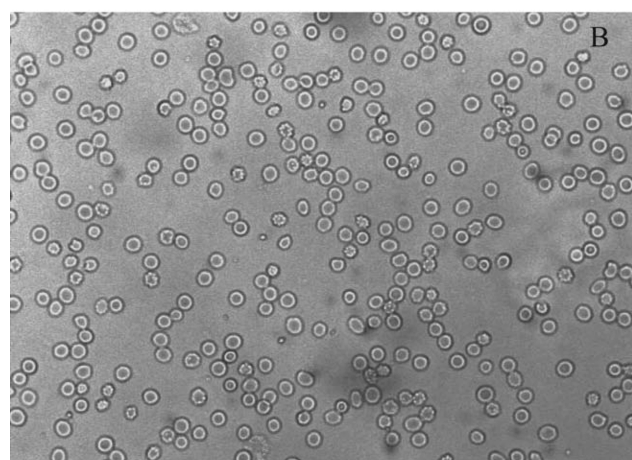


Figure 5 Three-dimensional profiles of the effect of molecular weight and percent of PEG on the half-max time of plasma recalcification for different blank PELGE nanoparticles while keeping ratio of LA/GA in the polymer constant at four levels. (A) LA/GA = 5 : 5. (B) LA/GA = 6 : 4; (C) LA/GA = 7 : 3; (D) LA/GA = 8 : 2. The data were averaged over three experiments.

polymers for intravenous nanoparticle drug delivery. A general composition–cytotoxicity relationship was observed, which suggested that the molecular weight of PEG applied in the synthesis of those polymers would influence their characteristics. Generally, the higher the molecular weight of PEG used, the more cytotoxic the polymer was. The reason for this trend may be that during the 48 h incubation at 37°C, some of the PELGE nanoparticles may have degraded producing an acidic condition in the medium, which would depress the growth of cells. Avgoustakis et al.[20] reported that the final *in vitro* degradation products of PLGA–mPEG nanoparticles are oligomers of LA and GA, LA, GA, and mPEG. Therefore, the more PELGE nanoparticles degraded, the more acidic the medium would become, and as a result the lower the relative growth rate (RGR) would be. PEG with larger molecular weight has a longer chain, which would cause instability in the synthesized PELGE polymers [reference?]. That might serve as the explanation for why the increase of PEG molecular weight produced such remarkable decrease of RGR of the PELGE nanoparticles. And the rapid nanoparticle degradation *in vivo* would result in rapid polymer removal from the body.[20] This hypothesis is consistent with the researches done by Delphine et al.,[21] who proved that microspheres made of a copolymer of PLGA and PEG released more teverelix than PLGA microspheres, since the PEG fragments may act as a 'water pump'.

Also, there was a composition–hemocompatibility relationship of PELGE blank nanoparticles according to the experiment results. For PELGE nanoparticles synthesized with PEG 550, most hemolysis values were in the range of 1–3%, while for PELGE nanoparticles synthesized with PEG 750 and PEG 2000 hemolysis values were 1–2% and below 2%, respectively. Zhu et al., who used PEG to modify chitosan derivatives,[22] reported that the reduction of hemolysis is related to the molecular weight of PEG, and





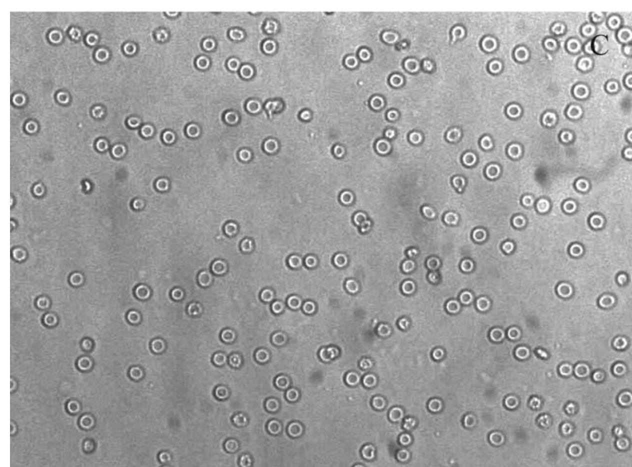


Figure 6 Optical micrographs of human red blood cells after 10 min incubation with PELGE nanoparticles at different concentrations in whole blood. All images are at 400-magnification. (A) saline control; (B) blank PEGLE nanoparticles (PEG molecular weight 550, content 15%, LA/GA = 6 : 4) at 1 mg/mL; (C) blank PEGLE nanoparticles (PEG molecular 2000, content 15%, LA/GA = 7 : 3) at 10 mg/mL. All other samples yielded similar results; these two were chosen as representative.

TABLE V
The Polymers having Better Biocompatibility

No. of PELGE	Molecular weight of PEG	PEG (%)	LA/GA ratio
1	550	5	8:2
3	550	15	8:2
5	550	5	7:3
7	550	15	7:3
8	550	20	7:3
9	550	5	6:4
10	550	10	6:4
11	550	15	6:4
13	550	5	5:5
15	550	15	5:5
18	750	15	8:2
28	750	5	5:5

that PEG 2000 is preferable to smaller molecular weight PEG as its comparatively long chain can shield the polymer more efficiently.

Furthermore, the results of measurement of plasma recalcification profiles suggested a PEG molecular weight-dependent increase of half-max time generally, which indicated that the clotting time of nanoparticles made from PELGE with a higher molecular weight of PEG was longer than the others. These results are consistent with the observation of Wang et al., who investigated the blood compatibility of PET grafted with PEG of different molecular weights.[23] They proved that activation of the intrinsic blood coagulation system is suppressed by PEG grafting and the extent of suppression is relative to the length of the grafted chain, because the hydrophilic polymers in the diffused layer exert steric repulsion to proteins that reach the surface. Steric repulsion is due to the loss of configurational entropy resulting from volume restriction and/or osmotic repulsion between the two overlapping polymer layers.[23] Therefore, according to Wang et al., the improvement of blood compatibility is related to the PEG molecular chain length. Experimental results showed that among PEG with molecular weights of 200, 1000, 6000, and 10,000, the best blood compatibility was achieved with the grafted PEG having a molecular weight of 6000.[23]

CONCLUSION

The results of investigation of the composition–cytotoxicity relationship and composition–hemocompatibility relationship of PELGE blank nanoparticles suggested that the molecular weight of PEG applied in the synthesis of those polymers would influence their characteristics. As the PEG molecular weight increased, increased cytotoxicity and hemocompatibility were observed. The percentage of PEG and the ratio of LA/GA in the polymer did not have any

influence. The red blood cell hemolysis assay indicated that blank nanoparticles did not cause hemolysis. And after incubation with the series of nanoparticles, no change in red blood cell form or appearance could be observed under the microscope. Based on our results, 33 polymers were deleted from the candidate list of potential materials for intravenous nanoparticle drug delivery systems. They were polymers which had high cytotoxicity (RGR<80%) and which produced significant rapid clotting compared with the control. Table V lists the remaining 12 candidate polymers, among which the most outstanding PELGE polymers for clinical use will be chosen after further experiments.

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