PELGE Nanoparticles as New Carriers for the Delivery of Plasmid DNA

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Biodegradable monomethoxy(polyethyleneglycol)–poly(lactide-co-glycolide)–monomethoxy(poly-ethyleneglycol) (PELGE) copolymers were synthesized by ring-opening polymerization to formulate plasmid DNA loaded nanoparticles. A double emusion method with polyvinyl alcohol as the emulsifier in the external aqueous phase was employed to prepare nanoparticles. The effects of monomethoxypoly(ethyleneglycol) (mPEG) segments in the polymer on particle size, zeta potential, encapsulation efficiency and *in vitro* release were investigated. It was found that the introduction of a certain amount of hydrophilic mPEG segments in the copolymer chains could improve the affinity of copolymer with plasmid DNA and enhance the emulsification ability of the copolymer. Thus DNA loaded nanoparticles with smaller particle sizes and higher encapsulation efficiencies were obtained by using PELGE copolymer as the matrix.

Key words mPEG–PLGA–mPEG (PELGE); nanoparticle; gene delivery

Gene therapy is becoming a promising approach for the treatment of disease with the more understanding of the genetic basis of disease. A key challenge in the gene therapy approach is to develop safe and efficient vehicles for delivering therapeutic genes into body cells.[1)] Non-viral vectors, although generally not as efficient as viral vectors, are attractive because they are associated with fewer safety concerns and are easier to produce. In addition, they are potentially less immunogenic and have fewer restrictions on their capacity to carry DNA.[2)] Among non-viral vectors, nanoparticles and microparticles formulated using poly(DL-lactide-co-glycolide) (PLGA) and polylacide (PLA) polymers are recently been investigated because of their sustained release characteristics, biocompatibility, and biodegradability, and their ability to protect DNA from degradation.[3)] Hedley *et al.* have reported that oral administration of plasmid DNA encapsulated in PLGA microspheres could obtain both humoral and mucosal immune responses.[4)] Furthermore, many articles published have shown sustained expression of DNA was achieved by PLGA nanoparticle and microparticle mediated delivery.[5—7)]

However, the hydrophobic property of PLA and PLGA limit their advantages. To enhance the affinity between polymer molecules and DNA molecules, poly(DL-lactide-co-polyethylene glycol) (PELA) has been investigated as a gene delivery system and satisfactory results have been achieved owing to the introduction of certain amount of hydrophilic domains, poly(ethylene glycol) (PEG) in the molecular chain of polyester.[8,9)] Nevertheless, PEG modified PLGA polymer has not been reported as the nanoparticle matrix for gene delivery up to now. In present study, we synthesized mPEG–PLGA–mPEG three block copolymers (PELGE) and prepared plasmid DNA (pDNA) loaded nanoparticles with PELGE as the carrier. The characterization of the PELGE nanoparticles was studied and compared with PLGA napoparticles.

Experimental

Materials DL-Lactide and glycolide were recrystallized twice form ethyl acetate and dried under high vacuum at room temperature before use. Monomethoxypoly(ethyleneglycol) (mPEG, molecular weight 2000), stannous octoate and hexamethylene diisocyanate (HMDI) were obtained form Sigma. PLGA (molecular weight 8000—10000) was from Shandong Institute of Medical Instrument (China). Polyvinyl alcohol (88% hydrolyzed, molecular weight 22000) was purchased from Acros Organics (U.S.A.). The plasmid pORF lacZ (3.54 kb) was purchased from Invivogen (U.S.A.). Qiagen Giga Endo-free plasmid purification kit was purchased from Qiagen (CA, U.S.A.). All the other chemicals and reagents used were of the analytical grade obtained commercially.

Synthesis of PELGEs The synthesis of PELGE polymers has been already described.[10)] Briefly, varied amount of lactide and glycolide crystals and specified amount of mPEG 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 obtained copolymers were dissolved in dichloromethane (DCM) and then precipitated in excess methanol for purification. Afterwards, 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. The synthesized PELGEs had different compositions. The molar ratio of lactic to glycolic acid moieties was 80 : 20, 70 : 30 and 50 : 50, respectively. The mPEG content was 5%, 10% and 15%, respectively. The identity and purity of the copolymers were examined by IR and nuclear magnetic resonance (^{1}H-NMR) spectroscopy. The composition of the copolymers was determined from the integrals of the peaks in the ^{1}H-NMR spectra. Their molecular weight and molecular distribution were determined by gel permeation chromatography (GPC).

Encapsulation of the Plasmid within PELGE Nanoparticles pORF-lacZ plasmid DNA was isolated and purified from DH5-α *E. coli* using the Qiagen Giga Endo-free plasmid purification kit. DNA concentration and purity were quantified by UV absorbance at 260 and 280 nm on a GBC UV cintra 10e Spectrophotometer.[11)] The plasmid DNA was encapsulated into PELGE nanoparticles using a double-emulsion solvent-evaporation technique.[12)] In brief, a volume of 100 μl DNA solution (1.18 mg/ml) was emulsified into a solution of PELGE in 1 ml of dichloromethane by probe sonication for 15 s. Then, 2 ml of an aqueous polyvinyl alcohol (PVA) solution (2%, w/v, if not mentioned specifically) were added to this emulsion. The w/o/w emulsion was obtained by sonicated again for 15 s and pored into 8 ml of the same PVA solution under moderate magnetic stirring. The magnetic stirring was maintained for 4 h at room temperature to allow solidification of the nanodroplets and elimination of the organic solvents. The nanoparticles were finally collected by centrifugation at 40000×g for 60 min at 4 °C. The sediments were washed twice to remove PVA and unentrapped DNA, resuspended in sterile water, and lyophilized for about 48 h.

Particle Size and Zeta Potential Measurements Diameter and surface charge of nanoparticles were measured by photon correlation spectroscopy (PCS) (Zetasizer Nano ZS90, Malvern instruments Ltd., U.K.) with a 50 mV laser.[11)] One milligram of nanoparticles was diluted by 3 ml of 10 mM Hepes buffer and added into the sample cell. The measurement time was set to 2 min (rapid measurement) and each run consisted of 10 subruns. The measurements were done at 25 °C at an angel of 90°. The size distribution follows a lognormal distribution. The zeta potential was measured at least three times at appropriate concentrations of samples.

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Morphological Characterization of Nanoparticels Negative stain electron micrographs of nanoparticles were taken using a JEM-100SX electron microscope.[13] Samples were prepared by placing a drop of nanoparticle suspension onto a copper grid and air-drying, followed by negative staining with a drop of 2% aqueous solution of uranyl acetate for contrast enhancement. The air-dried samples were then directly examined under the transmission microscope.

DNA Integrity In order to study the integrity of the pDNA, 15 mg of nanoparticles were suspended in 225 μl TE. Then, 750 μl of chloroform was added to the suspension and the mixture was vortexed for 1 h. After centrifugation at 20000×g for 10 min at 4 °C, the water phase was collected. Agarose (0.8%, w/v) gel electrophoresis was performed (100 V for 1 h) and visualized under UV light after ethidium bromide staining.

Determination of Encapsulation Efficiency The encapsulation efficiency, which refers to the amount of plasmid encapsulated into the nanoparticles as compared to the amount used in the encapsulation process, was determined by measuring the amount of plasmid that was not encapsulated and, therefore, remained in the supernatant upon centrifugation of the nanoparticle suspension. The concentration of plasmid was assessed by fluorescence spectrophotometer (Shimadzu RF-5301, Japan) dyed with the fluorophores Hoechst 33258.[11] In detail, the concentration of DNA in the supernatant was determined by fluorescence with the supernatant from the control nanoparticles formulated without DNA as a blank. The amount of DNA loaded in the nanoparticles was calculated from the standard curve of DNA prepared in supernatant obtained from control nanoparticles. Three determinations of three different batches were examined to give an average value.

***In Vitro* Release of Plasmid DNA from Nanoparticles** The release of plasmid DNA was studied by incubating the nanoparticles (10 mg) in 1.0 ml of TE buffer (pH 7.4) at 37 °C under horizontal shaking. At predetermined time intervals, the supernatant was collected by centrifugation (40000×g for 60 min). The nanoparticles were resuspended in the same volume of fresh medium and incubated again under the same conditions. Plasmid DNA released into the supernatant was quantified by fluorescence spectrophotometer as mentioned above.

Results and Discussion

Synthesis of PELGEs Stannous octoate has been approved by the U.S. FDA for surgical and pharmacological application. This compound is one of the most widely used initiators. It provides high reaction ratio, and high molar mass even under relatively mild conditions. The identity and purity of the copolymers were investigated by IR and ^{1}H-NMR spectroscopy. A typical IR spectrum of a mPEG–PLGA–mPEG copolymer and of pure PLGA copolymer and of pure mPEG were analyzed. The major peaks assigned to the structure of mPEG–PLGA–mPEG were: 2900—3000 cm^{-1} (C–H stretching), 1750 cm^{-1} (ester C=O stretching), and 1080 cm^{-1} (O–CH_2 stretching). The comparision of the IR spectrum of mPEG–PLGA–mPEG with that of mPEG confirmed that the reaction between PLGA and mPEG had been effective. It is characteristic that the broad absorption band at 3500 cm^{-1} in the spectrum of mPEG, assigned to O–H stretching, was practically eliminated from the spectrum of mPEG–PLGA–mPEG, indicating that the free hydroxyl groups of mPEG had reacted with the carbonyl groups of lactide/glycolide (Fig. 1).

The composition of the copolymers was determined from the integrals of the peaks in the ^{1}H-NMR spectra. ^{1}H-NMR spectra of PELGE triblock copolymers with their chemical structure are presented in Fig. 2. The average molecular weights of the PELGEs determined by GPC ranged from 8000 to 12000.

Morphology of Nanoparticles From the micrographs (Fig. 3), it could be found that DNA loaded nanoparticles with PLGA/PELGE as carriers were spherical in shape and almost uniform with the average particle size about 300 nm. No obvious difference was found between PLGA nanoparticles and PELGE nanoparticles, which confirmed using PELGE copolymer to prepare DNA loaded nanoparticles did not adversely influence particle morphology.

DNA Integrity The integrity of the encapsulated pDNA would have a significant effect on the *in vivo* activity of the nanoparticles and was therefore an important consideration during the encapsulation process development.[14] The energy used in the encapsulation process was a compromise between that required producing nanoparticles of a suitable size and that, which damaged the pDNA. The process described here was optimized to reduce denaturing of the pDNA whilst still retaining the small particle size of nanoparticles. As shown in Fig. 4, each DNA band was integrated as a volume. Supercoiled plasmid is believed to be the most efficient form for gene expression, followed by relaxed DNA, and then nicked DNA.[15] The preparation method described here, despite the

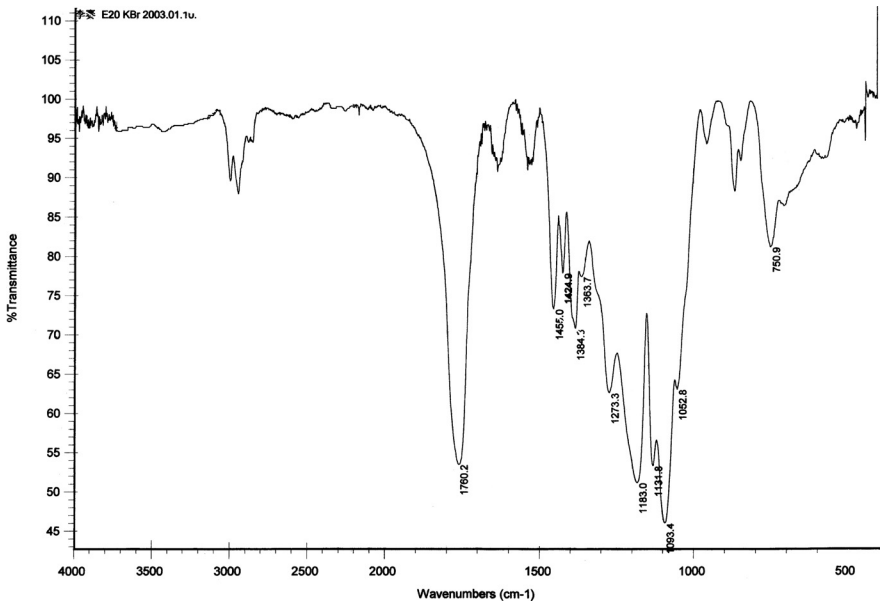


Fig. 1. IR Spectra of PELGE with LA : GA Molar Ratio of 80 : 20 and 10% mPEG

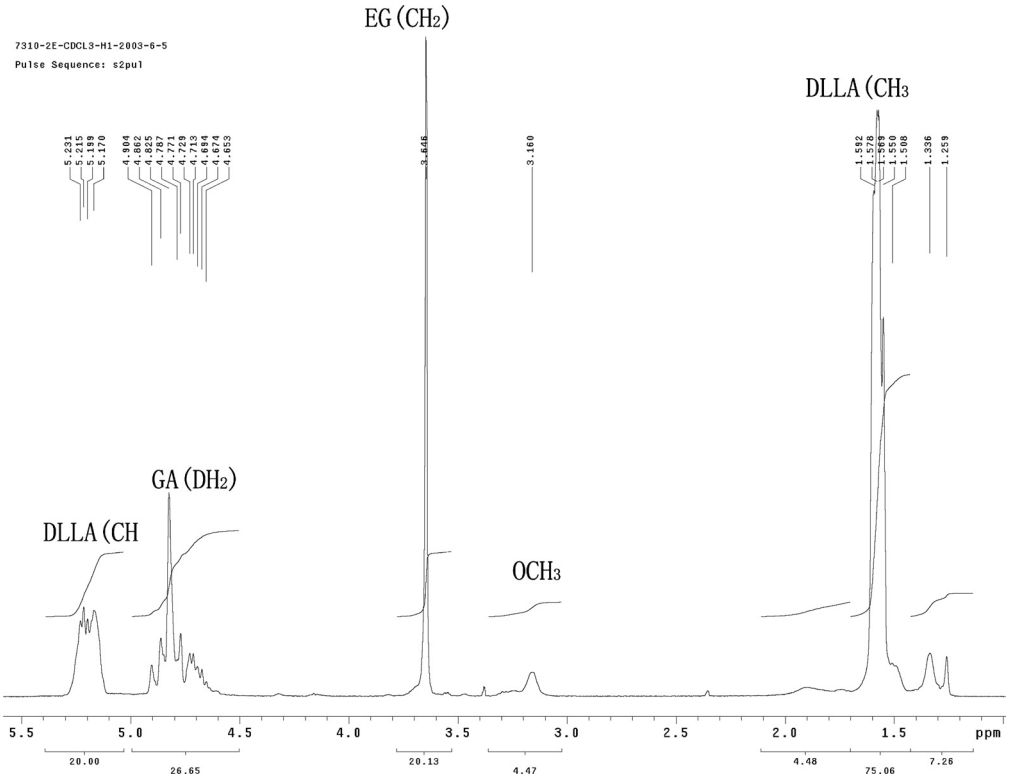
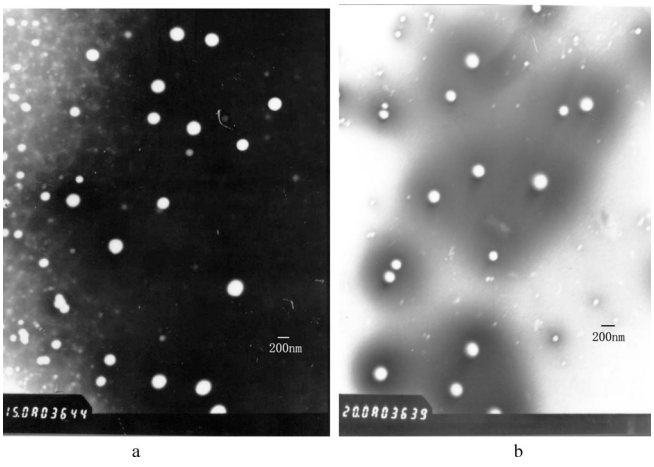


Fig. 2. ^{1}H-NMR Spectra of PELGE in $CDCL_3$

The composition of this copolymer was LA : GA ratio of 70 : 30 and 10% mPEG.



a b

Fig. 3. Electronic Transmission Microscopy of pDNA Loaded PLGA Nanoparticles (a) (×15000) and pDNA Loaded PELGE (D3) Nanoparticles (b) (×20000)

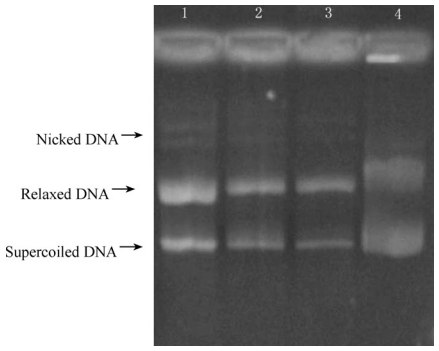


Fig. 4. Agarose Gel Electrophoresis of pDNA Recovered from Three Formulations of Nanoparticles

Lanes 1—3 show the recovered pDNA from D3, B2 and A2 nanoparticles, respectively. Lane 4 contains control (unencapsulated) pDNA.

decreased supercoiled content, did not result in pDNA fragmenting.

Influence of Polymer Matrix on the Particle Sizes The average diameter of nanoparticles prepared by different copolymers was between 290 to 340 nm with the polyindex of 0.15—0.32. Table 1 shows that the particle size of nanoparticles decreased as the mPEG content in the PELGE copolymer increased. This is because the copolymers have both the hydrophobic group and the hydrophilic group, which provides them the emulsification capabilities.[8] With the increase of mPEG content in the copolymer, the polymer would have stronger emulsification capability, resulting in decreased sizes of nanoparticles.

Influence of Polymer Matrix on the Zeta Potential The zeta potential of nanoparticles was in the range of −19 to −34 mV in 10 mM Hepes buffer (pH 7.4). It has been reported that PLA–PEG nanoparticles had higher zeta potential than PLA nanoparticles due to the presence of the PEG chains on the nanoparticle's surface.[9] In the present study, the zeta potential of nanoparticles was also found to increase slightly when PELGE triblock copolymers were used as the matrix in comparison with PLGA as the matrix. In PLGA nanoparticles, both the lactic acid and glycolic acid segments contribute to the surface charge. While in PELGE nanoparticles, the carboxyl groups of PLGA were condensed with the hydroxyl groups of the mPEG. As a result, the amount of free lactic acid and free glycolic acid segments decreased,

Table 1. Physical Characterization of the Nanoparticles Formulated Using PLGA/PELGE of Different Compositions ($n=3$)

Sample	Lactide : glycolide ratio	mPEG content (%)	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Encapsulation efficiency (%)
A0	80 : 20	0	337.1±14.4	0.18±0.01	−33.8±0.9	59.97±4.42
A1	80 : 20	5	320.6±13.1	0.22±0.02	−27.7±2.9	59.52±5.70
A2	80 : 20	10	305.6±9.0	0.15±0.01	−25.5±1.3	61.60±3.59
A3	80 : 20	15	312.0±9.6	0.20±0.02	−26.8±1.2	68.07±5.93
B0	70 : 30	0	335.8±11.3	0.25±0.02	−30.4±2.6	55.99±7.21
B1	70 : 30	5	317.6±5.9	0.31±0.03	−24.6±3.4	61.93±4.98
B2	70 : 30	10	296.8±12.2	0.19±0.01	−24.5±2.3	64.24±4.25
B3	70 : 30	15	293.3±10.3	0.21±0.02	−22.9±2.7	60.27±3.74
D0	50 : 50	0	325.2±15.4	0.25±0.02	−30.3±3.7	69.34±5.85
D1	50 : 50	5	312.0±6.5	0.32±0.04	−27.3±1.6	69.98±5.47
D2	50 : 50	10	292.1±12.8	0.26±0.03	−19.2±2.8	76.14±3.25
D3	50 : 50	15	291.3±9.2	0.17±0.02	−22.7±2.6	72.64±4.47

Data represented as mean±S.D.

Table 2. Physical Characterization of the Nanoparticles Formulated Using Different Concentrations of PVA ($n=3$)

Sample	mPEG content (%)	PVA concentration (%)	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Encapsulation efficiency (%)
D0	0	0.5	353.2±12.9	0.36±0.03	−32.1±1.6	45.64±3.18
D0	0	2.0	325.2±15.4	0.25±0.02	−30.3±3.7	69.34±5.85
D0	0	5.0	294.4±7.3	0.19±0.01	−29.7±1.8	74.23±3.56
D3	15	0.5	303.4±5.2	0.20±0.02	−24.4±1.2	67.74±5.14
D3	15	2.0	291.3±9.2	0.17±0.02	−22.7±2.6	72.64±4.47
D3	15	5.0	261.4±5.9	0.21±0.03	−23.3±3.2	80.56±6.32

Data represented as mean±S.D.

which led to the enhanced zeta potential of the PELGE nanoparticles. Furthermore, the mPEG on the surface of the PELGE nanoparticles could prevent the lactic acid and the glycolic acid from ionizing.

Influence of Polymer Matrix on the Encapsulation Efficiency It could be found from Table 1 that the encapsulation efficiency enhanced accordingly with the increase of mPEG contents in the copolymer. This may be due to that the existence of the hydrophilic mPEG segments in the copolymer chains improved the affinity of copolymer with pDNA and led to easier dispersion of pDNA in the internal water phase to form nanoparticles.[9] However, as for the copolymer with LA : GA ratio of 70 : 30 and 50 : 50, the encapsulation efficiency decreased when the mPEG content above 10%. The underlying reason was the large content of mPEG in the copolymer decelerated the precipitation of copolymer, which contributed to the increased possibility of pDNA diffusing out of the polymeric matrix during nanoparticle preparation. Therefore, the optimal mPEG content for these polymers was 10% to achieve the highest pDNA encapsulation efficiency.

Influence of Emulsifier (PVA) Concentration on Particle Size PVA is a commonly used emulsifier in the formulation of nanoparticles, mainly because the nanoparticles formed are smaller and uniform in size and are easy to redisperse in buffer or saline.[16] Table 2 demonstrated that nanoparticles formulated with higher concentration of PVA (5% w/v) had smaller particle sizes compared with those formulated with lower concentration of PVA. Moreover, it was noted that when mPEG content was as high as 10%, the nanoparticles using the copolymers (lactide : glycolide; 50 : 50) as matrix could be smaller than 300 nm with 2% (w/v) PVA as emulsifier. In comparison, the PVA concentration need to be no less than 5% (w/v) to form PLGA nanoparticles smaller than 300 nm. As mentioned above, the size differences were due to the emulsification ability of the copolymers.

Influence of Emulsifier (PVA) Concentration on Encapsulation Efficiency It was found that nanoparticles formulated with 0.5% w/v PVA had lower encapsulation efficiency than those formulated using 2% and 5% w/v PVA. This could be because of the stabilizing effect of PVA on the water-in-oil emulsion. A stable water-in-oil emulsion provided a higher mass transferring resistance, thus the amount of DNA molecules diffusing into the external water phase from the internal water phase was reduced during the second emulsification. In addition, when the polymer solution precipitated to form nanoparticles, the surfactant could promote compatibility between the hydrophilic DNA molecules and the hydrophobic polymer network, which was preferential for DNA to locate within the nanoparticle matrix.

***In Vitro* Release Study** As shown in Fig. 5, the *in vitro* release of pDNA from PLGA/PELGE nanoparticles all appeared to have two components: a rapid releasing phase followed by a sustained releasing phase. As it has been explained for PLGA nanoparticles, the first phase is attributed to the surface located molecules, while the second phase corresponds to the release following the degradation-erosion of the particles.[6] However, the block copolymer showed a double release mechanism: diffusion release duo to mPEG segments and degradation-erosion release duo to PLGA seg-

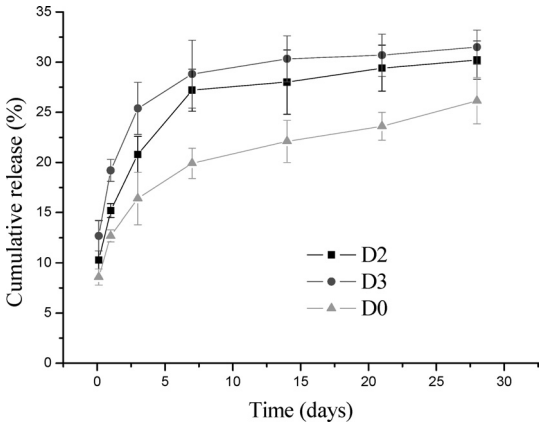


Fig. 5. *In Vitro* Release of Plasmid DNA from PLGA/PELGE Nanoparticles

The data points represent the mean±S.D. of three experiments.

ment. The aqueous pores or channels created by mPEG segments would accelerate the release of pDNA from nanoparticles during the rapid releasing phase. Moreover, it was found in Fig. 5 that with the increased mPEG contents in the copolymer more pDNA could release from nanoparticles in the first releasing phase because more aqueous pores or channels could form. Therefore, the optimal releasing profile for intracellular transfection of PELGE nanoparticles could be obtained by adjusting the ratio of PLGA/mPEG.

Conclusions

In this work, plasmid DNA was encapsulated in PLGA/PELGE nanoparticles by a double-emulsion solvent-evaporation method without compromising its structural and functional integrity. The effects of mPEG segments in the polymer on particle size, zeta potential, encapsulation efficiency and *in vitro* release were investigated. In conclusion, the introduction of a certain amount of hydrophilic mPEG segments in the copolymer chains could improve the affinity of copolymer with plasmid DNA and enhance the emulsification ability of the copolymer. Thus DNA loaded nanoparticles with smaller particle sizes and higher encapsulation efficiencies were obtained by using PELGE copolymer as the matrix.

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