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Preparation of DHAQ-loaded mPEG-PLGA-mPEG nanoparticles and evaluation of drug release behaviors *in vitro*/*in vivo*

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Abstract This study describes the preparation and the evaluation of biodegradation monomethoxy (polyethylene glycol)-poly (lactide-co-glycolide)-monomethoxy (polyethyleneglycol) (mPEG-PLGA-mPEG, PELGE) nanoparticles (PELGE-NP) containing mitoxantrone (DHAQ) as a model drug. PELGE copolymers with various molar ratios of lactic to glycolic acid and different molecular weights and various content mPEG were synthesized by ring-opening polymerization. mPEG with weight-average molecular weight (Mw) 2000 or 5000 was introduced as a hydrophilic segment into a hydrophobic PLGA. A double emulsion method with dextran70 as stabilizer in the external aqueous phase was used to prepare the nanoparticles. The drug entrapment efficiencies were more than 80% and the mean diameters of the nanoparticles were less than 200 nm. Various PELGE was studied as biodegradable drug carriers and there *in vitro*/*in vivo* release profiles were examined. It was found that drug loading, polymer molecular weight, copolymer composition and end group modifications were critical factors affecting the *in vitro*/*in vivo* release properties. The amount of drug released increased as the mPEG contents increased and the molar ratios of lactic acid decreased *in vitro*. The intravenous (i.v.) administration of mPEG-PLGA–mPEG nanoparticles of DHAQ in mice resulted in prolonged DHAQ residence in systemic blood circulation compared to the intravenous administration of PLGA nanoparticles.

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1. Introduction

Nanoparticles have been widely studied for drug targeting issues in drug delivery. Nanoparticles in intravenous injection have attracted considerable interest to achieve these objectives. The fate of nanoparticles after intravenous injection is greatly influenced by their interaction with the biological environment and their physicochemical properties. The effect of nanoparticle size has been shown to be of primary importance [1]. An extremely small-sized carrier for the intravenous injection to prevent the carriers clogging up in vessels is generally demanded [2]. The carrier should also be biodegradable and biocompatible. Although various polymers can be used to prepare nanoparticles, polymers used to nanoparticles for intravenous injection are significantly limited because of regulatory approval. Among them, poly (DL-lactide –co-glycolide) (PLGA) is one of the most widely used biodegradation polymers to make micro- or nanoparticles for controlled drug delivery systems [3]. However, they are not the optimal carriers for intravenous drug delivery applications. Many problems still remain such as long-term incompatibility with blood cells and uptake by the reticuloendothelial system (RES), and non-zero-order release behaviors [2]. One of the limiting factors in this field is their thrombogenic property. Therefore, the surface modification of the nanoparticles with a blood compatible material is important in order to improve the potential of nanoparticles in the intravenous injection drug delivery systems. Poly (ethylene glycol) (PEG) modified biodegradable polymers may be used for the intravenous drug delivery [4]. It has been suggested that mPEG modified nanoparticles provides

protection against interaction with the blood components, which induce removal of the foreign particles from the blood [5]. It prolongs, thus, their circulation in the blood stream. In consequence, modified nanoparticles may function as circulation depots of the administered drugs. Slowly releasing drugs into plasma, and thus altering their concentration profiles can achieve obvious therapeutically benefits. In present study we have synthesized mPEG-PLGA-mPEG polymers with biocompatibility, blood compatibility, drug compatibility and suitable biodegradation kinetics. DHAQ is a very potent anticancer drug. However, the full therapeutic exploitation of DHAQ is limited by its toxicity in healthy tissues. Our objective is to investigate the possibility of a more selective delivery of DHAQ to tumor cells using PELGE nanoparticles as DHAQ carrier for i.v. administration. In this work we prepared PELGE nanoparticles by using the emulsion-solvent evaporation technique (w/o/w) with DHAQ as the model drug. We also evaluated the *in vitro* drug release from PELGE nanoparticles and the *in vivo* drug release in blood after the i.v. administration of PELGE nanoparticles loaded with DHAQ to mice.

2. Materials and methods

2.1. Materials

Lactic acid (LA) and glycolic acid (GA) was obtained from Shandong medical appliance factory, China. Beckman culter centrifugation (Allegra X-22R centrifuge), CS501 super constant water bath box (Shanghai Yue-xin scientific instrument manufactory), GBC UV cintra 10e Spectrophotometer, scanning electron microscopy(SEM) (Hitachi S-450, Japan); Chemicals and reagents used were of analytical grade obtained commercially. Japanese white rabbits were received from Sichuan Industrial Institute of Antibiotics (P.R. China).

2.2. Synthesis of PELGEs

Varied amount of lactide and glycolide crystals and specified amount of mPEG were accurately weighed and put in 25-ml glass ampoules. Catalyst stannous octoate (dissolved in hexane) was added at a concentration of 0.05% by weight of the feed and the tubes were evacuated. Then the tubes were heated in an oil bath at 150°C for 5 h. The resulting copolymers were purified by dissolving them in dichloromethane (DCM) and then precipitating them in excess methanol. The purified copolymers were dried under vacuum. The coupling reaction of diblock copolymers was preformed with hexamethylene diisocyanate (HMDI) in toluene at 60°C for 12 h, followed by refluxing for 6 h. The triblock copolymers were purified by methanol precipitation of polymer from methylene chloride using diethyl ether. 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).

The twelve kinds of copolymers were selected to prepare drug-loaded nanoparticles. PELGE copolymers with varying composition, whose mPEG content was 5% or 10% or 15% or 20% (w/w) and LA: GA molar ratio was 80:20 or 70:30 or 50:50, were polymerized in our laboratory. mPEG with weight-average molecular weight (Mw) 2000 or 5000 was introduced as a hydrophilic segment into a hydrophobic PLGA (Table 1).

2.3. Preparation of nanoparticle

The nanoparticles loaded with DHAQ were fabricated using the double emulsion method with dextran70 as surfactants in the external aqueous phase. An aqueous solution of DHAQ was emulsified in 1 ml acetone/DCM, in which 10 mg of the copolymer had been dissolved, using probes sonication at 360 w for 30 s. This w/o emulsion was transferred to an aqueous solution of dextran70 and the mixture was probe sonicated at 200 w for 30 s. The resulting w/o/w emulsion was gently stirred at room temperature until the evaporation of the organic phase was completed. Orthogonal experiment design was adopted to select the optimal scheme by the experiment of the singular factor. The following six factors: the concentrations of PELGE, the ultrasonic time, the concentrations of dextran70, the volume ratios of inner water phase to external water phase and the stirring time, were selected to investigate the influence of the factors on particle sizes.

2.4. Determination of the DHAQ entrapment efficiency

The DHAQ-loaded PELGE nanoparticle colloid was centrifuged (12,000 × g for 20 min) and the supernatant was taken for measurement of the drug concentration using a UV spectrophotometer (Cintra 10e,GBC Scientific equipment) at 611 nm. The loading efficiency were calculated with the following equation: loading efficiency (wt%) = [(amount of remaining drug in nanoparticles)/(initial feeding amount of drug)] × 100.

2.5. Characteristic of nanoparticles

The morphological examination of nanoparticles was performed using a transmission electron microscope (TEM,

Table 1 Characteristics of the nanoparticles involved in the degradation studies

Experiment number	LA: GA ratio	mPEG contents (% (W: w))	MPEG Mw	PELGE Mw	Particle Size (nm)	Entrapment drug (%)
a1	70:30	5%	2000	11233	63	92.07
a2	70:30	10%	2000	10427	68	86.78
a3	70:30	15%	2000	13539	63	86.2
A2	70:30	10%	5000	1423.5	98	95.74
b1	80:20	5%	2000	12254	65	95.93
b2	80:20	10%	2000	12806	61	97.40
b3	80:20	15%	2000	9945	63	98.13
B2	80:20	10%	5000	1358	105	99.24
d1	50:50	5%	2000	10127	169	90.95
d2	50:50	10%	2000	9562.5	128	85.82
d3	50:50	15%	2000	11059	115	86.89
D2	50:50	10%	5000	1269.8	118	94.21

JEM 1200 FXII, Jeol Ltd, Tokyo, Japan) following negative staining with sodium phosphotungstate solution (0.2%, w/v). The samples were diluted with distilled water and measured at room temperature with a scattering angle of 90° and the size distribution of nanoparticles was determined by laser diffractometry (Mastersize/2000, Malvern).

2.6. Study of the in vitro release of DHAQ from the nanoparticles

The size of the nanoparticles involved in the drug release studies *in vitro* was from 60 nm to 200 nm (Table 1).

Nanoparticle samples, enclosed in dialysis bags, were incubated in 20 ml saline at 36.5 ± 1°C under mild agitation in a water bath. At given time intervals, 1 ml samples were withdrawn form the incubation medium. The samples were replaced by equal volume of the fresh incubation medium. The samples were centrifuged at 12,000 × g for 10 min and the amount of DHAQ in the supernatants was measured by high-performance liquid chromatography (HPLC). The HPLC system (Chiyoda-Ku, Tokyo, Japan) consisted of an SPD-10A variable UV-VIS detector, a Model FCV-12AH column-switching valve, and a set of Model LC-10AT liquid chromatograph including two pumps, a manometric module and a dynamic mixer from Shimadzu. The mobile phase consisted of methanol/0.2 Mol ammonium acetate buffer (48/52) solution. A Shimpack ODS column (150 × 4.6 mm, 5 μm) was eluted with the mobile phase at a flow rate of 1.0 mL/min. The eluate was monitored by measuring the absorption at 599 nm at sensitivity of AUFS 0.01 at 30°C. The Class VP V5.0 software was employed for the date analysis.

In a preliminary study, the total amount of DHAQ in the whole suspension after the release test was measured, and it was confirmed that DHAQ was stable during the release test and extracting procedures from nanoparticles.

2.7. Evaluation of the in vivo release of DHAQ from the nanoparticles

The DHAQ entrapped in PELGE (LA: GA molar ratio is 80:20, MPEG content is 10% or 20%) nanoparticles and PLGA (LA: GA molar ratio is 80:20) nanoparticles were evaluated for intravenous administration. West China Experimental Animal Center of Sichuan University supplied the mice weighing 18–20 g. Three per group, were injected in the tail vein with 0.5 ml of DHAQ nanoparticles (20–50 μg DHAQ, 3 mg polymer) or 100 μl of a DHAQ solution in saline (20 μg DHAQ). At predetermined time intervals, the mice were sacrificed and blood samples (0.3–0.5 g) were obtained. The percentage DHAQ dose in blood was calculated taking into consideration that the blood constitutes 7% of the body weight [6, 7]. Subsequently, plasma was separated by centrifugation (12,000 g, 10 min) for the DHAQ concentration analysis. The amount of DHAQ in the supernatants was measured by high-performance liquid chromatography (HPLC).

3. Results and discussion

3.1. The nanoparticles preparation

Orthogonal design was applied to optimize the preparation technology on the basis of the single factor evaluation. The optimal conditions for preparation nanoparticle were as follows: 10 mg/ml was the concentration of PELGE, the acetone/DCM ratio was 4:1(v/v), the concentration of dextran70 was 1% and the volume ratio of O/W was 1/8 (v/v).

The PELGE nanoparticles were prepared by a double emulsion method, where the mPEG fraction migrated to the surface of the nanoparticles forming a protective cover. The particles had increased hydrophilicity and decreased

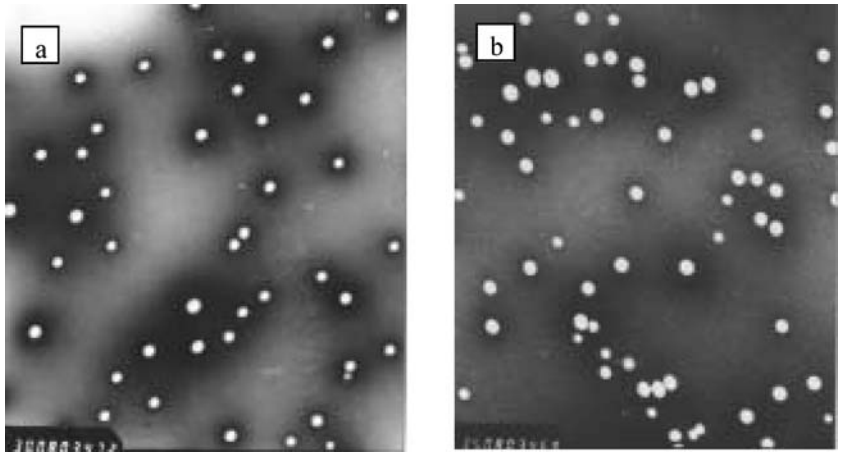

Fig. 1 TEM micrograph of PELGE nanoparticles containing DHAQ (a) 30×; (b) 35×.

surface charge (as determined by measuring their surface zeta potentials) and were sterically stabilized particles. Nanoparticles were formed by "solvent-evaporation" process, in which the remaining dichloromethane was removed from the system, makes the droplets solidify and finally form polymeric nanoparticles [8].

3.2. Characteristic of nanoparticles

A transmission electron microphotograph of freeze-dried nanoparticles prepared with the acetone/DCM system was shown in Fig. 1. The PELGA and PELGE nanoparticles were spherical, discrete particles without aggregation, and smooth in surface morphology, with a diameter of less than 200 nm.

Another characteristic of polymeric nanoparticles that is of extreme interest is zeta potential. The potential at the nanoparticle surface, called zeta potential (ξ) can be a useful tool for characterizing colloid drug delivery systems. They can give information to predict the stability of the colloidal solution. In the case of charged particles, as the zeta potential increases, the repulsive interactions will be larger leading to the formation of more stable particles with a more uniform size distribution. A physically stable Nan suspension solely stabilized by electrostatic repulsion will have a minimum zeta potential of 6.30 mV [9, 10]. This stability is important in preventing aggregation. The zeta potential was −5.8 mV (Zetasizer 2000, Malvern). The negative zeta potential PELGE nanoparticles is lowered, increasing the nanoparticles stability [11].

3.3. Factors affecting nanoparticals particles size

The size of the particles is a very important parameter, because it is one of the factors controlling the kinetics of drug release. The formation of the nanoparticles is affected by a number of factors. In our condition, the main variables that influence the nanoparticulating process and the final nanoparticle product are: (1) the polymer concentration, composition, and molecular weight; (2) the organic solvent; (3) the concentration and nature of the emulsifier; (4) the stirring speed in the emulsification process; and (5) the volume ratios of the inner water phase to external water phases [12].

3.3.1. The effect of organic solvent on the particle size

Generally, in self-assembling nanoparticulate systems such as the core-shell type nanoparticles (mPEG-PLGA-mPEG nanoparticles), the mechanism of nanoparticle formation is believed to be the hydrophobic interaction between the hydrophobic domains of the block copolymers [1, 13]. Fessi *et al*. [14] reported that the origin of the mechanism of nanoparticles formation could be explained in terms of interfacial turbulence or spontaneous agitation of the interface between two equilibrated liquid phases involving flow, diffusion, and surface processes. Although the mechanism is not fully understood at present, it was thought that the principle of PELGE nanoparticles formation might be based on a mechanism similar to the Fessi *et al*. method.

The physical and chemical properties of the organic solvent in the organic phase can greatly affect the emulsification and sphere-formation property. The selected initial solvent used to dissolve the copolymer had a slight affect on the size of the nanoparticles, but had an evident effect on the drug loading. Dichloromethane has certain solubility in water, after the primary emulsion formation, dichloromethane gradually dissolves and disperses into water through the organic-water interface and volatilizes through the water-air interface. In these processes, organic drops gradually solidify to form microspheres. Because the solubility of dichloromethane in water is very low, the solidification is very slow and therefore, it is very easy to form particles with good sphere shape. But the structure of the microspheres is porous due to the slow evaporation of the dichloromethane. The solubility of acetone in water is high. If there is only acetone in the organic

phase, acetone in the organic particles will dissolve into the water phase so quickly that it cannot form intact microsphere and therefore, irregular shaped particles will form. When organic phase is composed of water-soluble solvent acetone and water-insoluble dichloromethane, nano-sized emulsion particles can form gradually and precipitate because the dispersion of the water-soluble solvent results in the reduction of the surface tension and thus, the reduction of the particle sizes. The increase of the concentration of the water-soluble solvent will result in significant decrease of the size of the nanoparticles. In this study, we have used mixed dichloromethane and acetone solvent to fabricate nano-sized microspheres to obtain intact nano-spheres without porous on the surface while enhancing their drug loading ability.

3.3.2. The effect of molecular weight on the particle size

In the same fabrication condition, when the molecular weight of the mPEG chain is the same, the micro-particle size increases with the molecular weight of PLGA chain; when the molecular weight of the PLGA chain is the same, microparticle size will decrease when the molecular weight of mPEG chain increases. This is because that organic phase-water surface tension of the emulsion system will affect the micro-particle size. The higher water solubility the polymer is, the less the surface tension between water phase and polymer solution, which results in smaller particle sizes. Experiment result indicates that the water-solubility of the copolymer decreases with the increase of the molecular weight of PLGA while the diameter of the microspheres increases with the increase of the molecular weight of PLGA; the increase of the molecular weight of mPEG enhances its water solubility while decreases the particle size.

3.3.3. The effect of the concentration of dextran70 on the nano-particle size

The amount of stabilizer used will also have an effect on the properties of the nanoparticles. Most importantly, if the concentration of the stabilizer is too low, aggregation of the polymer droplets will occur. Alternatively, if too much of the stabilizer is used, the drug incorporation could be reduced due to interaction between the drug and the stabilizer. However, when the stabilizer concentration is between the 'limits', adjusting the concentration can be a means of controlling nanoparticle size [10]. Result shows that the particle size of the colloid solution is between 60–200 nm when the concentration of dextran70 is in the range of 0.5%∼2%(w/w). The increase of the concentration of dextran70 is in favorable to the fabrication of small sized nano-particles. However, when the concentration is higher than 2%, the increase of the concentration of dextran will not affect the particle size, but the drug loading ability decreases. In the present study a 1% concentration of dextran 70 was observed to provide the optimal values.

3.3.4. The effect of the concentration of the polymers on the nano-particle size

This study demonstrates that the concentration of the vector materials affects the precipitation rate of nano-particles in the nanoparticles formation process. When the concentration of PELGE or PELGA is between 10–30 mg/ml, the nano-particle size will increase with the increase of the concentration of the polymers. The concentration of polymer in the organic phase increases, and this increase of concentration of the polymer will result in the coagulation of the polymer. In the same time, with the increase of the concentration of the polymer in the organic phase, the viscosity of the organic phase increases. This high viscosity slows down the rapid dispersion of the polymer. Therefore, it is impossible to produce small equal-sized particles. The polymers will coagulate to form relatively large particles, which result in the increase of the size of the nano-particles.

3.3.5. The effect of the volume ratio of water phase to organic phase on the nano-particle size

The organic/aqueous phase ratio played a predominant role. The nanoparticle formation was dependent on the rate of diffusion of the organic solvent into the aqueous phase, thus influencing polymer precipitation. When o/w ratio (v/v) decreases from 1/3 to 1/8, the diameter of the nano-particle decreased from 2.063 micron to 62 nm. The organic phase disperses to form small particles under emulsifier. If the concentration of the particles in the dispersion medium of water is very low, the viscosity of the solution will be very low and the emulsion will be evenly distributed. Therefore, the chance for particles to get coagulated will be low and relatively stable dispersion system can be obtained. But when the o/w ratio (v/v) decreases to a certain degree, there are no such changes on the particle size.

3.3.6. The effect of other factors on the nano-particle size

The rate of solvent removal by evaporation strongly influences the characteristics of the final nanoparticles. Very rapid solvent evaporation may cause local explosion inside the droplets and lead to formation of porous structures on the nanoparticles surface.

The stirring time has no effect on the particle size of PELGA-NP. But long time stirring will be unfavorable to the stability of the emulsion. The stirring time will be 3 to 4 h to evaporate all the dissolvent when dichloromethane was used as the only dissolvent. While when acetone and

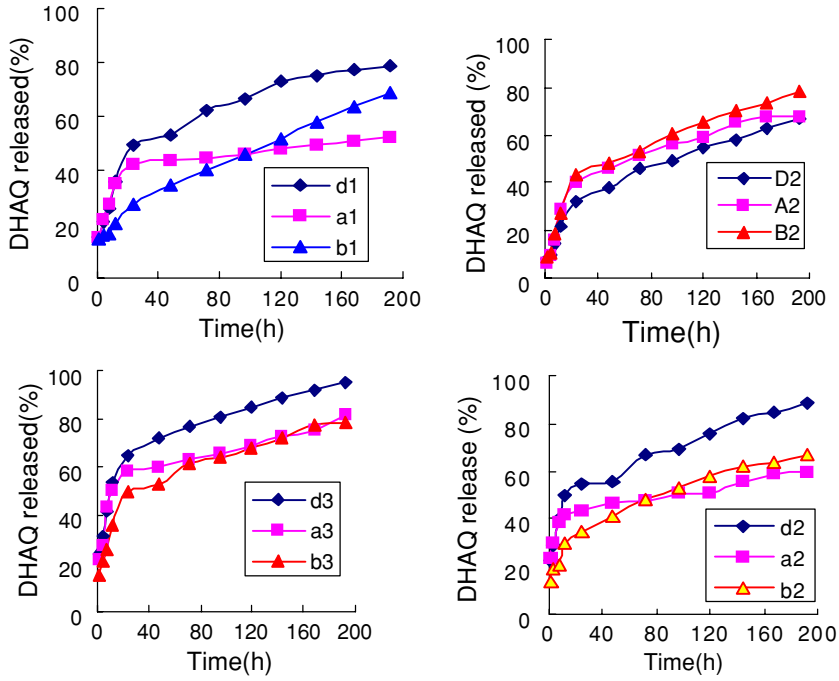

Fig. 2 The cumulative amount of DHAQ released from the nanoparticle with different LA: GA ratio PELGE formulations as a function of time.

dichloromethane were used as mixed dissolvent, due to the slow evaporation rate of acetone, 5 h of stirring is needed to evaporate all the organic dissolvent.

3.4. Drug release behavior of PELGE nanoparticles

The advantages of biodegradable drug delivery systems are: (1) maintenance of constant plasma drug concentration for a desired period of time, especially useful for treating chronic diseases; (2) reduced dosing times and improved therapeutic effect; (3) elimination of side effects by locally targeting a drug to its acting sites. Drug release can be controlled by several means, depending on the design of the delivery system and the way the drug exists in the system, the type of polymer used, polymer degradation. Polymer degradation plays a very important role in the release of therapeutic agents from biodegradable drug delivery systems.

The block copolymer showed a double release mechanism: diffusion release duo to MPEG segment and degradation-caused release duo to PLGA segment. The effects of these two pathways can be balanced by tailoring the ratio of PLGA/MPEG to such an extent that the degradation–caused release could just compensate for the declined amount resulted from diffusion release and a whole zero-order kinetics can be actually achieved.

PELGE-NP containing DHAQ were tested for *in vitro* release at 36.5 ± 1°C. Figs. 2–4 display the plot of the data expressed as the cumulative amount of DHAQ released from the PELGE nanoparticle formulations as a function of time. The formulations displayed different release profiles. The DHAQ was released from nanoparticles in a biphasic way characterized by an initial burst effect, followed by a continuous and slower release.

DHAQ release from PELGE nanoparticles is shown in Fig. 2 with the different LA:GA ratios. As shown in Fig. 2, at the same MPEG contents and molecular weight, we found that the higher the lactide ratio, the slower the drug release. Nanoparticle with LA: GA molar ratio of 50:50 would release DHAQ faster than that of other nanoparticles. The drug delivery profiles are dependent on chemical composition of PELGE. We found the drug release profile agreed well with that of the polymer degradation. We also found that after the initial burst, the release rate was dominated mainly by the degradation of the copolymer. The nanoparticels prepared with a higher GA content exhibited a faster release rate.

At the same LA: GA molar ratio, the higher release rate of the nanoparticles with a high mPEG content may be attributed to their increased hydrophilicity. These results were shown in Fig. 3. At the same LA: GA molar ratio and MPEG contents, an increased molecular weight of MPEG resulted in increased the drug release due to increased hydrophilic of the copolymers. These results were shown in Fig. 3.

The drug release rates from other type of PELGE nanoparticles appeared to be similar. The lower the molar mass, the faster the degradation rate. Drug release profiles are also affected. We found that the lower the molar mass, the faster the drug release. Moreover, the initial burst increased with decreasing molar mass. When the polymer has a slow degradation rate, the predominant mode of drug release is theoretically diffusion through the matrix. With fast-degrading

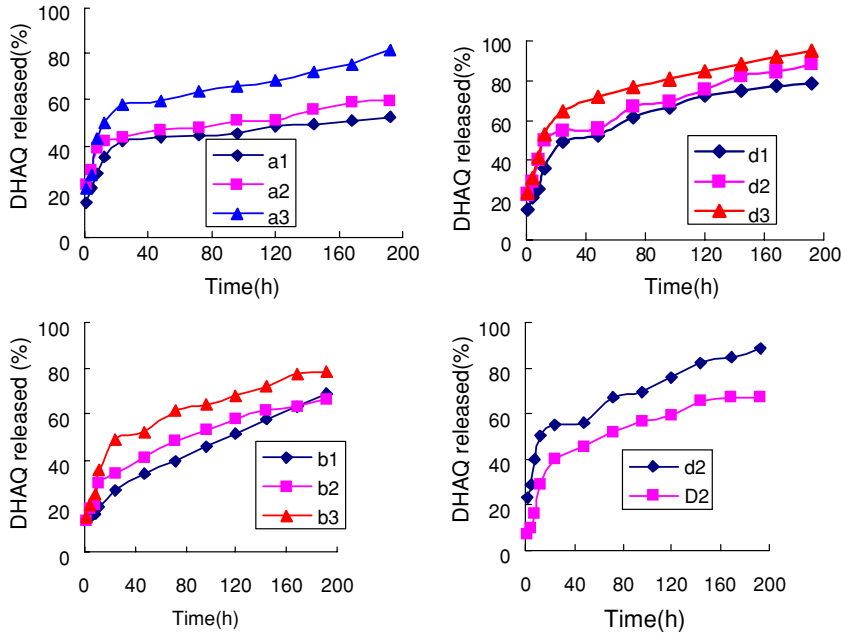

Fig. 3 the cumulative amount of DHAQ released from the nanoparticle with different content mPEG in PELGE formulations as a function of time.

polymers, the drug release can occur through diffusion and concomitant release of drug duo to the matrix degradation [15].

3.5. Concentration of DHAQ in the blood circulation

The core of these particles consists mainly of the degradable PLGA chains, while the mPEG blocks are located mainly on the surface [16, 17]. These mPEG chains on the surface with a hydrophilic, flexible and non-ionic polymer suppress the adsorption of plasma proteins, which is thought to be the initial step towards the uptake of the particles by cells of the reticuloendothelial system (RES) or mononuclear phagocytic system (MPS). The larger amount of mPEG on the surface of nanoparticles could more effectively change the surface properties suitable for avoiding the RES uptake [18]. mPEG–PLGA-mPEG nanoparticles showed initial high blood-circulating levels compared with PLGA nanoparticles. PLGA-NP was quickly removed from circulation. In fact, DHAQ in blood at 1 h after intravenous administration of mPEG–PLGA-mPEG nanoparticles was about 10-fold that observed for the PLGA nanoparticles. DHAQ loaded in PLGA nanoparticles was quickly removed from the circulation. On the contrary, DHAQ loaded in mPEG–PLGA-mPEG nanoparticles exhibited a markedly delayed blood clearance. It could be seen that the DHAQ in blood remained higher after 24 h compared with that of PLGA nanoparticles (Fig. 4).

This result confirmed the idea of forming particles with a satiric MPEG barrier that would prevent their rapid uptake by mononuclear phagocyte system and improve their circulatory half-life. The surface modification of the nanoparticles could have the potential benefit for intravenous injectable drug delivery.

4. Conclusions

The PELGE nanoparticles were prepared by a double emulsion method. The optimal conditions for preparation nanoparticle were as follows: the concentration of PELGE was 10 mg/ml, the acetone/DCM ratio was 4:1(v/v), the concentration of dextran70 was 1% and the volume ratio of O/W was 1/8 (v/v). The drug-loading contents and loading efficiency were dependent on the lactide: glycolide ratios. The resulting showed the higher the lactate ratio, the higher the drug loading. The DHAQ releases rate was slower at higher lactide: glycolide ratios. Also, the release rate

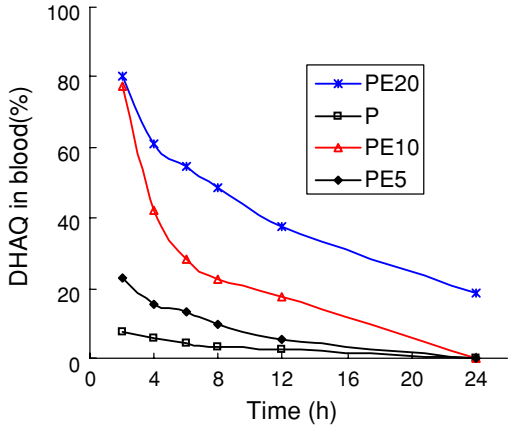

Fig. 4 The percentage DHAQ in blood stream: LA: GA ratio was 80:20, the contents of mPEG (P:0%; PE5: 5%; PE10: 10%; PE20: 20%).

of DHAQ from the PELGE nanoparticles with high drug-loading nanoparticles was slower than that from lower drug-loading nanoparticles. Our results showed PELGE was able to prolong the circulation of the particulate drug carriers with less RES uptake. The PLEGE copolymers were suitable candidates to manufacture nanoparticles with increased blood half-life.

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