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Research Article

The Investigation on Polyion Complex Micelles Composed of Diammonium Glycyrrhizinate/Poly(Ethylene Glycol)–Glycidyltrimethylammonium Chloride-Grafted Polyasparthydrazide

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Abstract. To prepare stable polyion complex (PIC) micelles, polyasparthydrazide (PAHy) modified with glycidyltrimethylammonium groups and methoxy poly(ethylene glycol) (mPEG) (mPEG-g-PAHy-GTA) was synthesized. The cytotoxicity of the polymer was evaluated by the methyl tetrazolium assay. The polymer entrapped the diammonium glycyrrhizinate (DG) and formed polyion complexes. The effect of pH value, grafting degree of mPEG, copolymer and drug concentration on the micelle formation was investigated by means of measuring entrapment efficiency and micelle size. *In vitro* DG release from the PIC micelles was detected by dialysis in various media of different ionic strengths. To examine the pharmacokinetic behavior of micelles *in vivo*, the time course of the drug in plasma was evaluated. The cytotoxicity of the polymer was very low. The results showed that entrapment efficiency can reach about 93%, and the mean particle size was almost 50 nm. The drug release rate decreased with a decrease in ionic strength of the release medium or an increase in the PEG grafting degree. Compared with DG solution, the AUC of DG micelles had a twofold increase. The smaller clearance and longer mean residence time of the DG micelles group compared with DG solution group showed that the DG loaded in PIC micelles can reduce drug elimination and prolong the drug residence time in the blood circulation. The results indicated that PIC micelles composed of mPEG-g-PAHy-GTA would be prospective as a drug carrier to the drugs which can be ionized in solution.

KEY WORDS: diammonium glycyrrhizinate; drug delivery systems; poly(ethylene glycol)–glycidyltrimethylammonium chloride-grafted polyasparthydrazide; polyion complex micelles.

INTRODUCTION

In recent years, polymeric micelles have been intensively studied and widely used as potential drug and gene carriers (1–3). The special core–shell structure ensures that polymeric micelles have many advantages, such as being able to solubilize hydrophobic drugs by chemical conjugation or physical entrapment (4), protect drugs from possible *in vivo* degradation, prolong the circulation time, and allow nano-size targeting of tumor sites or inflamed areas(1,5,6).

Also, there is some information about self-assembling micellar systems driven by ionic interactions of a charged copolymer with an oppositely charged drug that is polyion complex (PIC) micelles. Generally, the charged copolymer is soluble in aqueous medium, and its chemical structure is composed of a charged segment and a neutral hydrophilic segment. The charged part of the copolymer and oppositely charged drug generate an electrostatic attraction that results in an insoluble internal core and the hydrophilic segment surrounding the core as a corona (7–9). Since PIC micelles are prepared by simply mixing the drug and polymeric solutions, this approach eliminates the need for solvents. The interaction between drug and pendant groups on the polymer may lead to improve drug incorporation efficiency; ionic interactions also can make drug easily released from the micelles while in the case of covalent attachment and cleavage of the drug from the polymer (8).

To date, the source of polymers used as drug carriers is natural or synthetic (or artificially created). Poly(amino acids) would seem to have significant advantages over other polymers owing to their biodegradability, biocompatibility, low toxicity, and protein-like structure(10). Harada and Kataoka prepared micelles by means of ionic interactions between poly (ethylene glycol) (PEG)-polyaspartic acid and the protein lysozyme (11). Govender has also reported the formation of polymeric micelles driven by ionic interactions involving the di-block polymer PEG-polyaspartic acid and a small molecular weight drug, diminazene aceturate (12).

α,β-Polyasparthydrazide (PAHy), a derivative of polyaspartic acid, has been studied as a plasma expander and drug carrier due to its water solubility, nontoxicity, and biodegradation (13,14). The main chain of PAHy has many primary amines that make the polymer easily modified. The suitable modified polymer maybe has more good characteristics, such as improving drug loading, enhancing micelles stability, and so

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on. PAHy is a neutral polymer which cannot interact with drug by ion at physiological pH. Elisa Pedone used glycidyltrimethylammonium chloride to graft PAHy (PAHy-GTA) which can entrap DNA (15). Methoxy PEG (mPEG) is a good biocompatible material. Particles modified by PEG can improve the stability and prolong the circulation time in blood. PEG can shield the charge on particle surface and provide sufficient steric stabilization (16–20). The polyelectrolyte of PAHy-GTA and drugs may easily aggregate or combine with other molecules. To solve that problem, we intend to construct a novel polymer which is PAHy modified with PEG and glycidyltrimethylammonium groups (mPEG-g-PAHy-GTA).

The present study aimed at synthesizing a series of novel grafted copolymers composed of PAHy as the main chain, GTA and mPEG as the soluble side chain with different PEG grafting degrees and using it to form PIC micelles. PAHy-GTA was selected as the polycationic segment to allow the copolymer to interact ionically with an anion drug to be incorporated. PEG was used as the hydrophilic side chain to balance the molecular association forces and also provide steric stabilization of the micelles.

Glycyrrhizin, a major active component of licorice root, is used clinically for the treatment of chronic hepatitis, allergic disorders, and inflammation. It has a strong role in protecting the liver cell membrane and improving liver function. The poor solubility of glycyrrhizin provides limitation in use, while diammonium glycyrrhizinate (DG), a glycyrrhizin ammonia compound, is more soluble and more suitable for experiments and clinical use than glycyrrhizin (21). DG with three anions was chosen as a model drug to study the ability of mPEG-g-PAHy-GTA to form PIC micelles and load drugs.

MATERIALS AND METHODS

Materials

L-Aspartic acid (the purity was 99%) was obtained from Bio Basic, Inc. (Markham, Ontario, Canada) and used without further purification. mPEG (weight average molecular weight (Mw), 5,000) was obtained from Fluka (Buchs, Switzerland) and used without further purification. Diammonium glycyrrhizinate (molecular weight, 857) was purchased from Shanxi Dasheng Chemical Tech Co., Ltd. (Shanxi, China). Hydrazine hydrate was purchased from Tianjin Baishi Chemical Plant (Tianjin, China); sodium cyanoborohydride ($NaBH_3CN$) was purchased from Yingkou Sanzheng Chemical Tech Co., Ltd. (Liaoning, China); dialysis tubing (MWCO, 12,000–14,000 and 4,000–6,000) was purchased from Perbio Science UK, Ltd. (Chester, UK). All other chemicals were of analytical grade. Rats (male and female, 6 months old, 200±10 g) were kept under constant conditions with a controlled temperature (22±2°C), humidity (60±10%), and a light/dark cycle of 12 h.

Synthesis of Poly(Ethylene Glycol), Glycidyltrimethylammonium Chloride-Grafted-α, β-Polyasparthydrazide (mPEG, GTA-g-PAHy)

PAHy was synthesized from L-aspartic acid by a method reported previously (14). Briefly, polysuccimide (PSI) was prepared by polycondensation of L-aspartic acid at 180°C. The average Mw of PSI was 42,100 g/mol, and this was determined by viscosimetric method following the Mark–Houwink equation: $(\eta)=1.32\times10^{-2}\times M^{0.76}$. PSI was dissolved in DMF, hydrazine hydrate dissolved in DMF was added dropwise to the PSI solution, and the mixture was stirred at room temperature for 4 h. The precipitate was collected by filtration and repeatedly washed with acetone until the filtrate was neutral and then dried in a vacuum to obtain α,β-polyasparthydrazide (PAHy).

In order to obtain the PAHy-GTA, PAHy were dissolved in buffer solution (pH 8.5), then added a suitable amount of glycidyltrimethylammonium chloride water solution. The reaction was stirred for 24 h at 20°C. When the reaction has finished, the mixture was neutralized and dialyzed to remove the unreacted GTA. After dialysis, the product was concentrated by means of lyophilization. The structure of PAHy–GTA derivatives was characterized by Fourier transform infrared spectroscopy (FTIR) and proton NMR (^{1}H-NMR) analyses (15).

The synthetic procedure for poly(ethylene glycol), glycidyltrimethylammonium chloride-grafted–poly-α,β-polyasparthydrazide (mPEG-g-PAHy-GTA) is shown in Fig. 1. α-Aldehyde-ω-methoxy poly(ethylene glycol) (mPEG-CHO) was prepared by dimethyl sulfoxide-acetic anhydride oxidation from mPEG-5000 as reported previously (22). PAHy-GTA and mPEG-CHO (Table I) were dissolved in distilled water, respectively. The PAHy-GTA and mPEG-CHO solutions were mixed and stirred for 24 h at room temperature. After adding $NaBH_3CN$, the mixed solution was stirred for 48 h at room temperature. The resultant solution was purified on a Sephadex G-50 column (3.5 cm in inner diameter× 10.5 cm in length) using 0.05 mol/L phosphate buffer (pH 7.4) as the elution solvent. The high molecular weight fraction of the eluate was collected and dialyzed for 48 h against water (MWCO, 12,000–14,000). The purified solution was isolated by lyophilization and characterized by FTIR spectrophotometry and ^{1}H-NMR analysis.

The spectrum FTIR (KBr, centimeters) of PAHy is: 3,400–2,500 (−$\underline{NH_2}$), 2,930–2,850 (−$\underline{CH_2}$–, –$\underline{CH_3}$), 1,660 (−NH–$\underline{C=O}$), and 1,539 ($\underline{O=C}$–NH). Compared with PAHy, there were two obvious characteristic peaks 1,468 (−N($\underline{CH_3}$)$_3$) and 1,107 ($\underline{O–C}$) in the FTIR spectrum of mPEG-PAHy-GTA (Fig. 2a). The ^{1}H-NMR (δ/parts per million) of mPEG-PAHy-GTA: 2.3–2.7 ($C\underline{H_2}$ of amide of asparthydrazide), 3.19 (–N($\underline{CH_3}$)$_3$ of GTA), 3.65 (−O$C\underline{H_2}CH_2$–), and 4.61 ($C\underline{H}$ of amide of asparthydrazide; Fig. 2b). The molar ratio of GTA to the amino acid units of PAHy defined as the grafting degree (GD, mol%), (GD of GTA)=(trimethylammonium groups/the amino acid units). The degree of derivatization was 32%.

A series of the molar ratio of mPEG to the amino acid units of PAHy were prepared as described in Table I. Molecular weights were also given in Table I. The GD of mPEG-g-PAHy-GTA was calculated by comparing the integration area of the protons at 3.65 ppm with that at 4.61 ppm in ^{1}H-NMR according to the following equation:

$$GD = \frac{S_{3.65} \times N_b}{S_{4.61} \times N_a} \times 100\%$$

S is the integration area of the corresponding peaks; N_a is the proton number of –CH_2CH_2O– from mPEG and N_b is the proton number of –NH–C$\underline{H}$(CO)–CH– from PAHy-GTA.

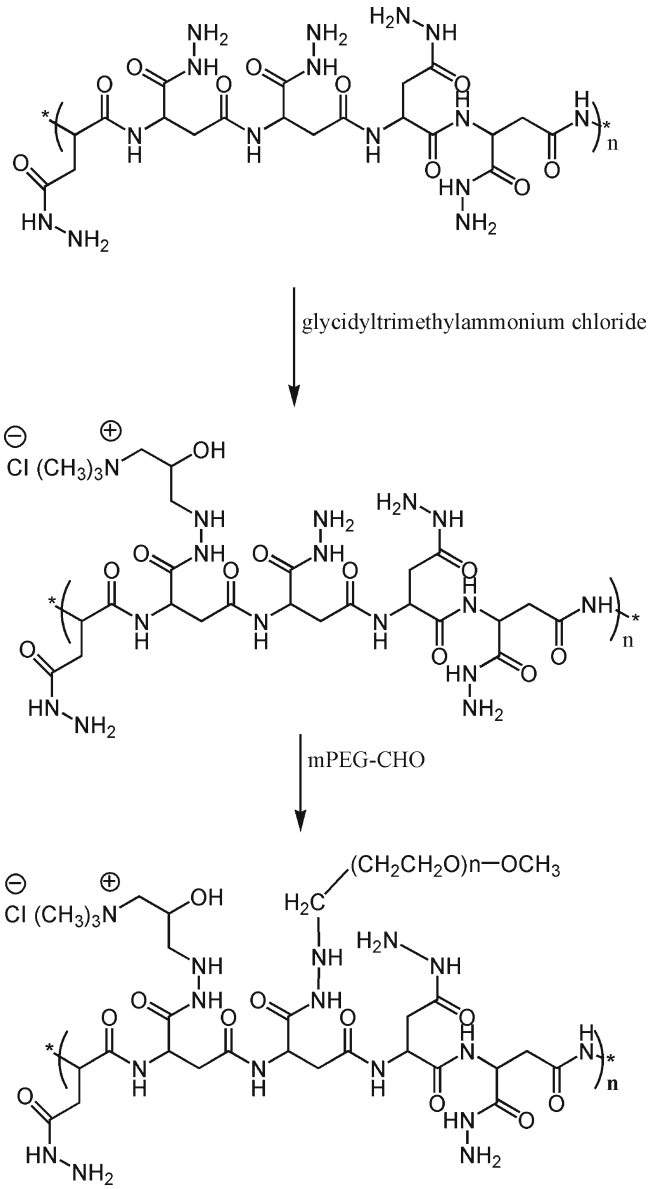

Fig. 1. ^{1}H-NMR spectra of mPEG-g-PAHy-GTA in D_2O (synthetic scheme for the preparation of mPEG-g-PAHy-GTA)

Cytotoxicity of Polymer

To evaluate the cytotoxicity of PAHy, PAHy-GTA, and mPEG-g-PAHy-GTA, HepG2 cells were used by methyl tetrazolium (MTT) assay. HepG2 cells were cultured in MEM media supplemented with 10% FBS, 1% NEAA, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2 humidified atmosphere. Cells were seeded in 96-well plates at a density of 5×10^3cells/well. After the cells were cultured for 24 h, the polymer solution was added to 96-well plates. The polymer concentration was 0.1–5 mg/ml. After incubation for 48 h at 37°C, 5 mg/ml MTT in PBS solution was added to each

Table I. The Preparation of a Series of Copolymers of mPEG-g-PAHy-GTA

Samples	P_1	P_2	P_3	P_4
PAHy-GTA (g)	4	4	4	4
mPEG (g)	12	24	48	72
$NaBH_3CN$ (g)	1.5	3	4.5	12.6
Grafting degree (mol%)	5.1%	11.9%	15.4%	17.8%
Molecular weight (g/mol)	189,374	326,822	405,368	440,295
Entrapment efficiency (%)	70.1	75.2	91.3	93.5
Drug loading rate (%)	27.1	29.5	33.1	35.9
Micelle size±SD (nm) (distribution)	142.8±13.9 (44.4±10.19)	125.9±12.1 (34.3±9.91)	79.2±11.5 (13.53±2.16)	75.4±1.08 (10.64±0.29)

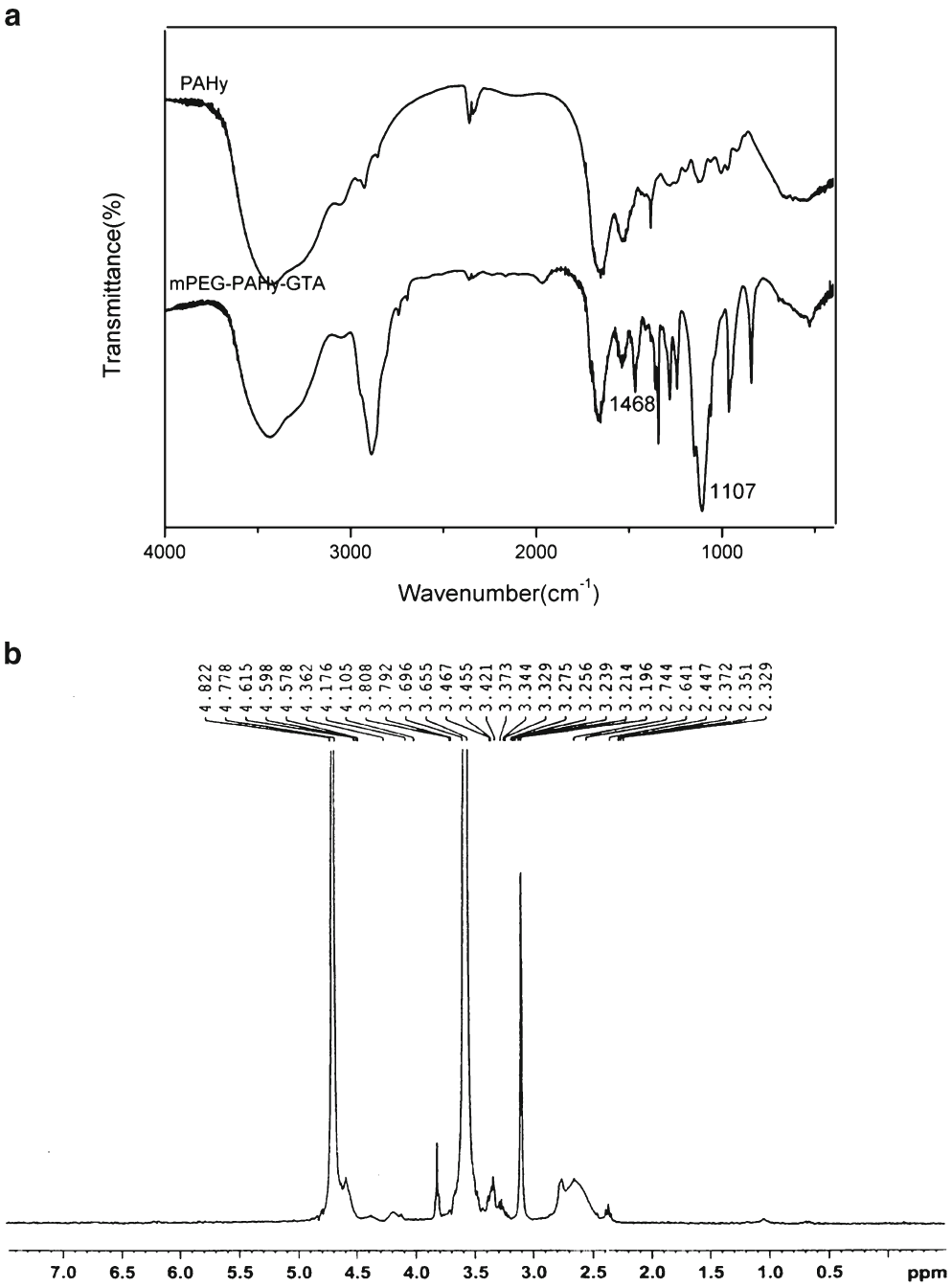

Fig. 2. FTIR spectra of PAHy and mPEG-g-PAHy-GTA

well. After further incubation for 4 h, the medium was removed, and 150 μl DMSO was added. After shaking for 10 min, the absorbance was examined at 490 nm by the plate reader spectrophotometer (Bio-Rad, USA). Cell viability was evaluated as the percentage of absorbance compared to control cells cultured with only culture medium.

Preparation of DG-Loaded PIC Micelles

The process of the preparation of DG-loaded PIC micelles was just like ionic gelation process (7). Briefly, mPEG-g-PAHy-GTA and DG were dissolved in 0.9% (*w*/*v*) physiological saline respectively, and the DG solution was added dropwise to the mPEG-g-PAHy-GTA solution under stirring. The mixture was stirred at room temperature to allow formation of PIC micelles, and then, the micelle solution was filtrated through a 0.22-μm Millipore membrane.

Three milliliters of sample was accurately measured and filtered through the ultrafiltration membrane (MWCO, 50,000). The filtrate was analyzed by UV spectrophotometry (Unico Instrument Co., Ltd, Shanghai, China) at 252 nm. The DG concentration in the filtrate is Cb (milligrams per milliliter), and the total DG concentration in sample is Ca (milligrams per milliliter). The entrapment efficiency (EE) of DG in the PIC micelles could be calculated by the equation: $EE = (Ca - Cb)/Ca \times 100\%$.

The PSD of the PIC micelles was assessed by photon correlation spectroscopy (dynamic light scattering) using a Nicomp™ 380 submicron particle sizer (Particle Sizing System, Santa Barbara, California, USA) at 25°C.

The Effect of Copolymer Concentration on the Formation of Micelles

Different amounts of P_4 were dissolved in physiological saline to obtain 5, 25, 40, 55, and 60 mg/ml solutions of mPEG-g-PAHy-GTA. Also, 100 mg/ml DG solution was obtained by dissolving DG in physiological saline. Then, 0.5 ml of DG solution was added slowly to 2.5 ml polymeric solution under gentle stirring, and stirred for 10 min at room temperature. Three batches of samples were prepared, and their micelle size and entrapment efficiency were determined.

The Effect of Drug Concentration on the Formation of Micelles

One hundred milligrams of P_4 was dissolved in 2.5 ml physiological saline. Different amounts of DG were dissolved in physiological saline to obtain 10, 50, 100, 150, and 200 mg/ml solutions of DG. DG solutions with different concentrations were added dropwise to the polymer solution under gentle stirring, and stirring was continued for 10 min. Three batches of samples were prepared, and their micelle size and entrapment efficiency were determined.

The Effect of pH Value on the Formation of Micelles

One hundred milligrams of P_4 was dissolved in 2.5 ml phosphate buffer at various pH values ranging from 3 to 8. Additionally, 50 mg DG was dissolved in 0.5 ml solution at the same pH value. DG solution was added dropwise to the polymeric solution at the same pH value under stirring, and then gently stirred for 10 min at room temperature. The micelle size and entrapment efficiency were determined.

Transmission Electron Microscopy Analyses

Transmission electron microscopy (TEM; JEM-1200EX, Japan) was used to observe the morphology of micelles. A drop of freshly prepared micelles solution was placed on a carbon-coated copper grid. Then, the sample was stained by 1% phosphotungstic acid (*w*/*v*) and dried at room temperature. The samples were observed using TEM at an accelerating voltage of 60 kV.

In Vitro Drug Release Study

In vitro, the DG release from the PIC micelles was determined using the dialysis bag method (8). PIC micelle solution (containing 100 mg P_4 and 50 mg DG) prepared under the optimized conditions was sealed in dialysis bags (MWCO, 4,000–6,000). Then, the bags were dialyzed against different release media at (37±0.5)°C. At specified time intervals (0.5, 1, 2, 4, 6, 8, 12, 24, and 36 h), 3 ml of the release media was removed and replaced with an equal volume of the fresh release medium. The samples were analyzed by UV spectrophotometry. Amount of DG was calculated using an appropriate calibration curve. The release experiment with DG solution was also carried out as a control. DG solution was obtained by dissolving an equivalent amount of the drug alone (50 mg DG) in 3 ml water solution, and the drug release of the DG solution was measured the same as PIC micelle samples. The drug release data obtained were used to construct the release profile of DG in different release media.

Pharmacokinetics Study

All rats were provided by the Animal Center of Shenyang Pharmaceutical University. The experimental protocol was

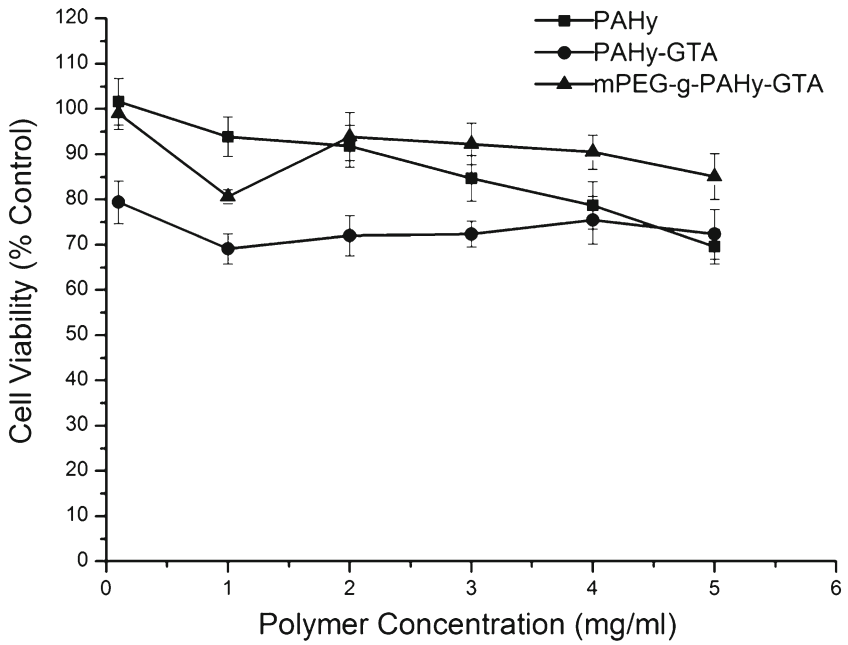

Fig. 3. Cytotoxicity of PAHy, PAHy-GTA, mPEG-g-PAHy-GTA against HepG2 cells

approved by the Ethics Review Committee for Animal Experimentation of Shenyang Pharmaceutical University. A total of 12 rats were randomly divided into two groups, one group serving as the solution group and the other as the micelle group. The rats were fasted for 12 h before administration, but had free access to water.

The DG-loaded PIC micelle solution (containing 100 mg mPEG-g-PAHy-GTA and 50 mg DG) was prepared. The micelle solution was administered to the rats in the micelle group at a dose of 20 mg/kg by intravenous injection into the tail vein. As a control, DG solution at the equivalent dose was also administered to the rats in the solution group by intravenous injection. At 0.16, 0.5, 1, 2, 4, 6, 8, 12, and 24 h, blood samples (approximately 400 μl) were taken by retrobulbar puncture and transferred to heparinized tubes. The samples were centrifuged, and 200 μl of plasma was removed and stored at −20°C until assay. Two hundred microliters of acetonitrile was added to the thawed plasma to precipitate the proteins by vortexing for 90 s and centrifuging for 15 min at 4000 rpm, and then, 20 μl supernatant was analyzed by Hitachi high-performance liquid chromatography (HPLC) consisting of L7000 pump and L7420 UV–VIS detector (Japan). The HPLC system was equipped with reversed phase column (Kromasil C18, 150×4.6 mm, 5 μm) at room temperature. The mobile phase was acetonitrile/tri-distilled water/triethylamine/acetic acid (35:61:2:2). The flow rate was 1.0 ml/min. The eluent was monitored with UV detector at 252 nm.

RESULTS AND DISCUSSION

Synthesis and Characterization of mPEG-g-PAHy-GTA

mPEG-g-PAHy-GTA was synthesized via the reaction of the primary amino group of PAHy-GTA with the aldehyde group of mPEG in the presence of $NaBH_3CN$. The remaining $NaBH_3CN$, free mPEG, and other by-products were completely removed by Sephadex G-50 column chromatography and dialysis. Based on this, the FTIR spectrum of mPEG-g-PAHy-GTA exhibited an ether absorption peak at 1,106.8 cm^{-1} which was not seen with PAHy, and the ^1H-NMR spectrum of mPEG-g-PAHy-GTA showed a specific proton of the methyl and methylene groups of mPEG and the methenyl groups of PAHy, which indicated that mPEG was successfully attached to PAHy-GTA (Fig. 2).

Cytotoxicity of Polymer

The cytotoxicity of PAHy, PAHy-GTA, and mPEG-g-PAHy-GTA was evaluated. Because of cationic charges, PAHy-GTA was a little toxic than PAHy. After PEGylation, cytotoxicity was decreased. The reason is that PEG chains can shield the effect of the positive charge (Fig. 3).

Table II. Effect of the Co-Polymeric Concentration on Entrapment Efficiency and Micelle Size

Concentration of P_4 (mg/ml)	Entrapment efficiency (%)	Micelle size±SD (nm) (distribution)
5	77.1	51.1±1.65 (11.9±2.47)
25	86.9	65.9±1.74 (14.1±1.83)
40	91.8	72.3±1.43 (11.6±1.78)
55	95.2	84.2±5.69 (29.7±3.61)
60	97.8	95.6±19.23 (41.2±7.19)

Table III. Effects of DG Concentrations on Entrapment Efficiency and Micelle Size

Concentration of DG (mg/ml)	Entrapment efficiency (%)	Micelle size±SD (nm) (distribution)
10	96.9	50.1±1.97 (16.8±1.45)
50	95.3	56.3±1.38 (15.4±1.61)
100	91.8	72.3±1.43 (11.6±1.78)
150	88.2	79.6±1.73(16.2±6.73)
200	75.9	86.7±2.34 (20.8±7.49)

Effect of PEG Grafting Degree of Copolymer on Micelle

Since PEG is hydrophilic, this will result in a DG/copolymer complex self-assembling to form micellar-type constructs. It has been reported that the transfer between copolymers and micelles in similar systems is dependent on various factors, one of which is the PEG corona density (23). The density and thickness of the hydrophilic PEG corona would also affect the size and physical stability of the micelles. Thus, it was necessary to investigate the effect of PEG grafting degree of the copolymers on the micellar size, entrapment efficiency, and loading capacity. The data are summarized in Table I. The micelle size decreased from 142.8 to 75.4 nm as the PEG grafting degree increased from 5.1% to 17.8%. Moreover, the micelle size distribution decreased. Although the size of micelle that was constructed with the copolymer having lower PEG grafting degree was greater, the entrapment efficiency and drug loading rate of the micelle were lower. In fact, during the experiment, it was observed that some floccus would generate in the micelle system composed of copolymer having lower PEG grafting degree. Some floccus was strained off and resulted in the lower entrapment efficiency and drug loading rate. It is presumed that the fewer PEG chains could not completely cover the complex core, and the primary smaller self-assembly system might continue to form loose secondary aggregates, which would lead to greater micelle size and its distribution. Based on

Table IV. Effects of pH on Entrapment Efficiency, Drug Loading Rate, and Micelle Size

pH	Entrapment efficiency (%)	Micelle size±SD (nm) (distribution)
10.0	93.4	55.2±5.5 (13.3±4.41)
9.0	92.9	56.7±4.57 (12.11±0.98)
8.0	92.5	57.2±3.03 (8.74±0.91)
7.0	93.1	56.9±1.08 (11.1±0.84)
6.0	93.9	61.2±1.89 (10.37±0.84)
5.0	96.8	73.2±2.83 (12.34±0.67)
4.0	98.5	85.3±3.64 (29.79±5.75)
3.0	–	–

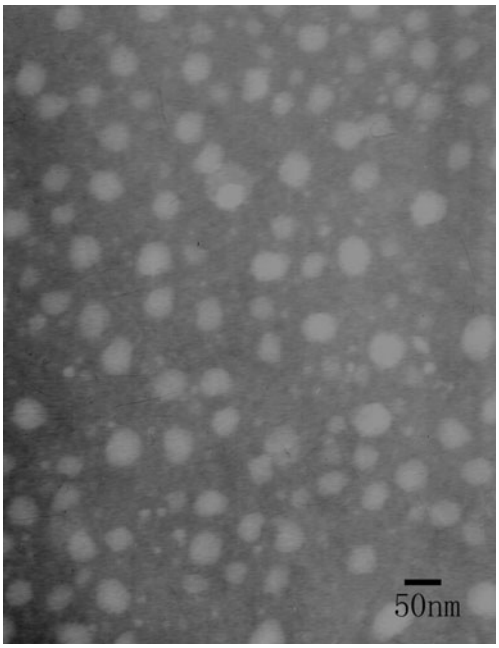

Fig. 4. TEM photograph of DG-loaded PIC micelles

entrapment efficiency, loading capacity, and micelles size, we chose P_4 in the following experiment.

Effect of the Concentration of Copolymer on Micelles

For a fixed DG concentration (100 mg/ml), the micelle size and entrapment efficiency increased with an increasing concentration of mPEG-g-PAHy-GTA (P_4) (as shown in Table II). With the increase of the copolymer concentration, copolymers provided more cationic site to capture ionized drug molecules. Thus, the entrapment efficiency increased. It should be noted that the micelle size and its distribution rapidly increased when the mPEG-g-PAHy-GTA concentration was greater than 40 mg/ml. The results that the entrapment efficiency slowly increased and micelle size distribution rapidly increased when the copolymer concentration was more than 40 mg/ml show that the 40 mg/ml of copolymer solution is an optimal concentration.

Effect of the DG Concentration on Micelles

The addition of 10, 50, 100, 150 and 200 mg/ml DG solutions were added to 40 mg/ml P_4 solutions respectively to prepare PIC micelles. The micelle size and entrapment efficiency of PIC micelles were shown in Table III. For given polymeric concentrations, the micelle size and its distribution increased, while the entrapment efficiency decreased with an increasing DG concentration. More drug molecules interacted with PAHy-GTA and formed a bigger and stronger insoluble complex core. The present findings are similar to those obtained in previous studies involving a PEG-P(asp) copolymer system with the concentration of polymer increasing from 5 to 25 mg, where the micellar size increased from approximately 25 to 50 nm for 5 mg/ml copolymer and from approximately 27 to 60 nm for 25 mg/ml copolymer, with an increase in the drug/monomer molar ratio (12).

Effect of pH Value of Medium on Micelles

Since the PIC micelles are formed by the ionic interaction of mPEG-g-PAHy-GTA and DG, it was presumed that mPEG-g-PAHy-GTA and DG must supply enough cations and anions, respectively. mPEG-g-PAHy-GTA can supply two kinds of cations, one is $-NH_2$ group in PAHy segment which is affected by pH value; the other is $-N(CH_3)_3$ group in GTA segment which is not influenced by

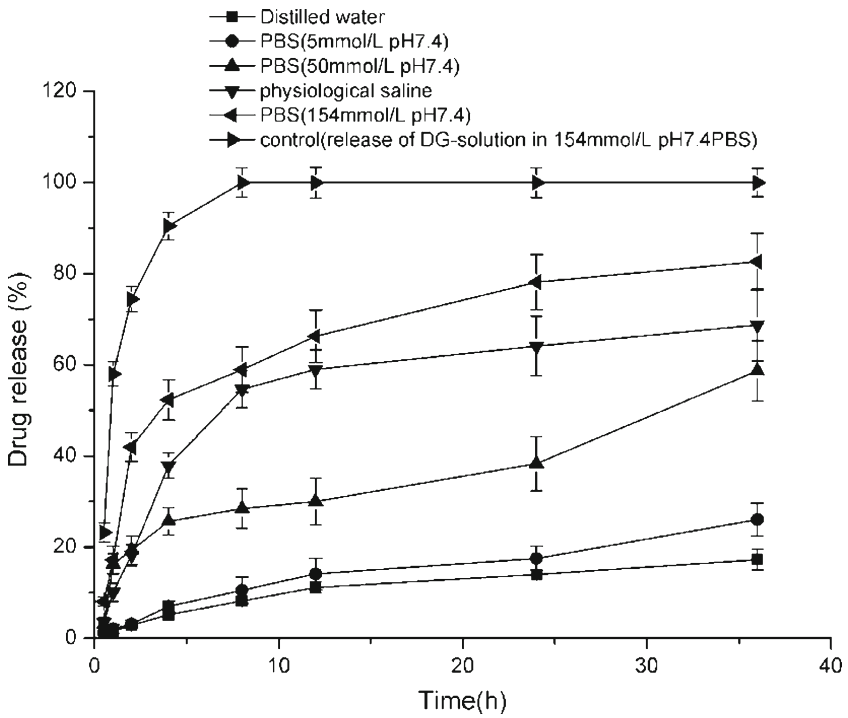

Fig. 5. *In vitro* drug release profile of DG-loaded PIC micelles at different ionic strengths

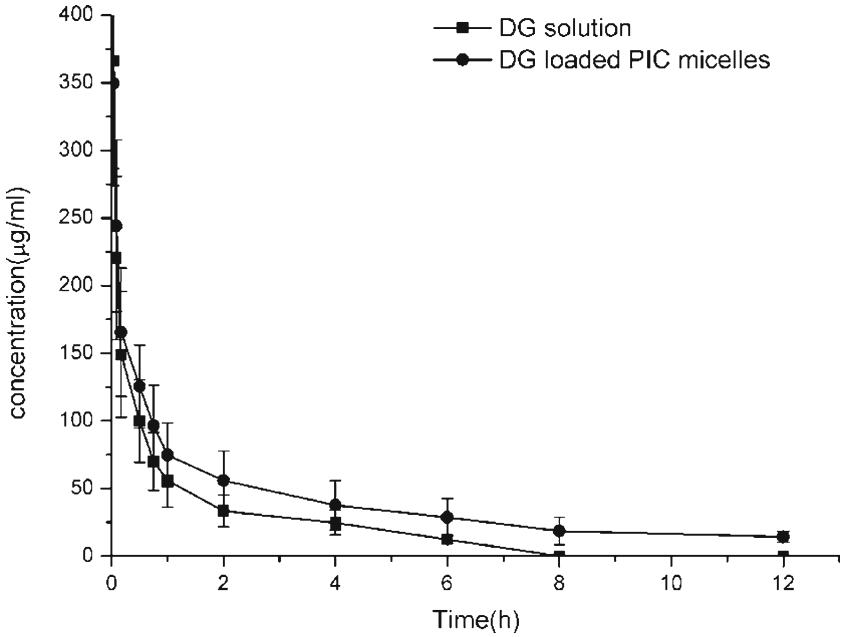

Fig. 6. Curves of DG plasma concentration *versus* time after i.v. administration

pH value. DG provide carboxyl acid group. If the pKa value of the carboxylic acid groups is taken as 4.0, the carboxyl acid group would be 95.2% ionized in pH 5.3 phosphate buffer but would be only 0.99% ionized when the pH of the solution was gradually reduced to pH 2. The effect of pH and a consequent different degree of ionization would be reflected in the micellar size and entrapment efficiency. Therefore, it is possible to determine the pH value range of micelle formation by measuring these data. The results were summarized in Table IV.

In the pH range of 10.0 to 6.0, the entrapment efficiency and particle size had almost no change compared with pH 6.0, about 93.9% and 61.2±1.89 (10.37±0.84) nm, respectively, because the $-NH_2$ in PAHy was almost not ionized and DG can be fully ionized. From 6.0 to 4.0, both of the entrapment efficiency and particle size were increased compared with pH 6.0. The $-NH_2$ can provide more protons, so polymer can incorporate more DG. Below pH 4.0, the entrapment efficiency and particle size were near zero. Because DG could not be fully ionized, the micelle cannot be formed. Finally, based on the pH value of the plasma, we chose 7.0 to prepare the micelles.

According to the experimental results above, the formulation and preparation of the DG-loaded PIC micelles were determined as 100 mg P_4 dissolved in 2.5 ml physiological saline. Then, 50 mg DG dissolved in 0.5 ml physiological saline was added slowly under magnetic stirring (50–100 rpm), then gently stirred for 10 min at room temperature to produce the PIC micelles. Three batches of samples were prepared and investigated *in vitro* and *in vivo*.

The Morphology of Micelles

The morphologies of the PIC micelles composed of mPEG-*g*-PAHy-GTA and DG were investigated by TEM. The shape of the PIC micelles was close to sphere without aggregation or accumulation, as seen in Fig. 4.

In Vitro Release Study

Since the micelles prepared in this study are held together by electrostatic interactions, it was unsurprising that the micelles dissociate in the presence of excess added salt (24,25). Before drug was released from PIC micelles, the PIC micelles needed to dissociate. Therefore, it was presumed that any factors affecting the dissociation of the micelles might affect the drug release, and one of these was the ion strength. *In vitro* DG release from PIC micelles was carried out using the dialysis method in release media with different ion strengths. Figure 5 shows the release profiles of DG-loaded PIC micelles in distilled water, physiological saline, and pH 7.4 PBS at different concentrations.

Within the first 2 h, 41.98±3.19% of DG was released in PBS (154 mmol/L, pH 7.4) and 82.66±25% within 36 h. In physiological saline, PBS (pH 7.4, 50 mmol/L), PBS (pH 7.4, 5 mmol/L) and distilled water, DG were released 68.77±7.98%, 58.66±6.64%, 26.04±3.61%, and 17.25%±2.26% separately within 36 h. At the initial stage of release, DG adsorbed on the surface of micelles was exchanged by the ions in the release medium, which led to the burst release. DG was released very slowly from the DG-loaded PIC micelles in distilled water because there were no other anions for exchanging with glycyrrhizic acid anions in the micelle core, resulting in its release except that glycyrrhizic acid anions

Table V. Pharmacokinetics Parameters of DG-Loaded PIC Micelles and DG Injections (n=6)

Parameters	DG micelles	DG injections
$AUC_{0-\infty}$ (mg/L*h)	571.189±197.405	282.905±94.472
$MRT_{0-\infty}$ (h)	6.294±1.992	2.230±0.619
$t_{1/2}$ (h)	5.201±1.890	1.556±0.982
CL (L/h/kg)	0.038±0.009	0.076±0.022
C_{max} (mg/L)	349.566±75.291	366.373±79.719

These values were significantly different from the solution (p<0.05)

attached to the interface of the core–shell structure diffused into the distilled water. For physiological saline and PBS (pH 7.4, 154 mmol/L) that have equal anion concentrations, glycyrrhizic acid anions were released faster in PBS (pH 7.4, 154 mmol/L) due to the stronger exchange capacity of the phosphate radical compared with that of chloridion. With an increase in the PBS concentration, the ionic strength of the release medium increased, and the release rate of anion glycyrrhizic acid also increased. It was concluded that the release behavior of glycyrrhizic acid from the PIC micelles correlated with the ionic strength and ionic species. Specifically, the stronger the exchange capacity of the negative ion in the release medium, the faster the glycyrrhizic acid anions were released from the micelle core. These results were similar to the report that the stability of polyion complexes is strongly influenced by the ionic strength of the medium: being destabilized by an increase in ionic strength due to electrostatic shielding (26).

PBS (154 mmol/L, pH 7.4) and physiological saline are more similar to physiological state compared to other release conditions, so we discussed kinetics of these two dissolution medium. The release data were fitted into first-order, Higuchi, Nibergull, Hixcon–Crowell, and Weibull equations. The regression results turned out that *in vitro* drug release in two kinds of dissolution medium fitted the Weibull distribution closely. The equations of release data of PBS (154 mmol/L, pH 7.4) and physiological saline were $\ln \ln(1/(1-Q/100)) = 0.063 \ln t - 1.566$ ($R=0.9560$) and $\ln \ln(1/(1-Q/100)) = 0.285 \ln t + 0.132$ ($R=0.9560$), respectively.

Pharmacokinetic Study

Polymeric micelles have significant advantages based on longer circulation of the loaded drug and an enhanced permeation effect. To examine the influence of the drug delivery system on the pharmacokinetic behavior of DG *in vivo*, the drug loaded in PIC micelles was intravenously injected into rats, and the time course of the drug in plasma was evaluated. Figure 6 shows the plasma concentration *versus* time profiles of DG after administration of DG-loaded PIC micelle solution and DG aqueous solution. The data for the drug plasma levels *versus* time were analyzed using DAS 2.0 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China). All statistical analyses were performed using SPSS (version 16.0). The Student's *t* test was used to analyze differences of the data between the micelle group and solution group. The pharmacokinetic parameters of the DG solution and DG–PIC micelle groups are summarized in Table V.

The $t_{1/2}$ of DG in the micelle group was found to be significantly longer compared with that of free DG (5.201 ± 1.890 and 1.556 ± 0.982 h, respectively), which indicates that the drug delivery system delays DG retention in the blood circulation. The ratios of $AUC_{0-\infty,\ micelle}$ to $AUC_{0-\infty,\ solution}$ of DG are about 202%; moreover, the smaller CL (0.038 ± 0.009 L/h/kg) and longer MRT for the DG micelles (6.294 ± 1.992 h) compared with the CL (0.076 ± 0.022 L/h/kg) and MRT (2.230 ± 0.619 h) for free DG showed that the micelles reduce drug elimination and prolong the residence time of DG in the blood circulation. Enzymes in blood or tissues can degrade DG, whereas micelles offer significant protection. DG loaded in PIC micelles would be useful in prolonging the DG residence in the blood circulation and reducing the side effects due to a prolonged release of PIC micelles after intravenous injection.

CONCLUSION

mPEG-g-PAHy-GTA with different PEG substituted ratios was successfully synthesized and characterized by FTIR and ^{1}H-NMR. Cytotoxicity of PAHy, PAHy-GTA, and mPEG-g-PAHy-GTA was investigated by MTT. The results showed that there was low cytotoxicity. mPEG-g-PAHy-GTA entrapped diammonium glycyrrhizinate (DG) and formed stable polyion complex micelles in suitable conditions. The micelles released DG in response to ionic strength. When the ionic strength of the release medium increased, the drug release rate also increased. DG-loaded PIC micelles significantly prolong the DG residence in the blood circulation. It should be noticed that the PIC micelles composed of mPEG-g-PAHy-GTA, as drug delivery vehicle, have a promising application.

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