Preparation and *In Vitro* Release of *D,L*-tetrahydropalmatine-Loaded Graft Copolymer Nanoparticles

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ABSTRACT: *D,L*-tetrahydropalmatine (THP)-loaded poly {[α-maleic anhydride-ω-methoxy-poly(ethylene glycol)]-*co*-(ethy cyanoacrylate)} (PEGECA) amphiphilic graft copolymer nanoparticles (PEGECAT NPs) were prepared by the nanoprecipitation technique. The effects of solvent property, temperature, copolymer composition, and drug feeding on the drug-loaded amount and size of PEGECAT NPs were investigated. The morphological structure of PEGECAT NPs was characterized by transmission electron microscopy (TEM), proton nuclear magnetic resonance (^{1}H NMR), and the size was measured by laser particle size analyzer (LPSA). *In vitro* release behaviors of drug from PEGECAT NPs were examined by high-pressure liquid chromatography (HPLC). The results demonstrate that PEGECAT NPs take on a spherical morphology with an inner core and outer shell before and after *in vitro* release. THP can be incorporated into the hydrophobic core of PEGECAT NPs and the drug-loaded amount is higher than 5%. The release of THP from PEGECAT NPs is initially fast and then slows down. The accumulated release is lower than 40% after 48 h. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 110: 3525–3531, 2008

Key words: *D,L*-tetrahydropalmatine; nanoparticles; amphiphilic graft copolymers; controlled drug delivery; *in vitro* release; poly{[α-maleic anhydride-ω-methoxy-poly (ethylene glycol)]-*co*-(ethy cyanoacrylate)}

INTRODUCTION

D,L-tetrahydropalmatine (THP) is an isoquinoline alkaloid isolated from Corydalis yanhusuo, a traditional Chinese herbal medicine, and its effective moiety is *L*-tetrahydropalmatine (*L*–THP).[1,2] THP is a white or yellowish crystal powder with bitterness. It is soluble in organic solvents such as ethanol, acetone, and acetonitrile, and insoluble in water and melts at 148°C.[3]

THP is a neural drug possessing analgesic and hypnotic effects without risk of addiction; furthermore it has been embodied in the country pharmacopoeia of China. THP does not belong to anesthesia abirritate drug or diminishing inflammation abirritate drug, which has been used to reinforce vital energy and alleviate pain such as headache, chest pain, hypochondriac pain, epigastric pain, abdominal pain, backache, arthralgia, dysmenorrheal, or trauma.[4–7] In recent years, THP has also been used to treat hypertension,[8,9] arrhythmia,[10–12] anxiolytic,[13] thrombus,[14] and acidity,[15] etc. Even though THP has been widely applied in the medical field, the hydrophobicity affects its therapeutic benefit and limits its application in the medical field. Importantly, there is no drug-controlled delivery system for THP. So developing a novel formulation of THP is indispensable.

Nanoparticles formed from amphiphilic copolymers have been exploited as the long-circulating carriers for hydrophobic drug.[16–24] In an aqueous environment, the hydrophobic moieties of the copolymer form the core of the nanoparticles, whereas the hydrophilic moieties form the corona or outer shell. The inner core serves as a microenvironment for the incorporation of lipophilic drugs, and the corona shell serves as a stabilizing interface between the hydrophobic core and the external medium.

The unique application of PEGylated polycyanoacrylate amphiphilic graft copolymers (PEGACA) in the biomedical field involves their role as carriers for

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drug delivery,[25–30] which is based on the nanoparticle-forming propensity of PEGACA in an aqueous medium through multimolecular association. The polymer nanoparticles are characterized by a core–shell structure, in which a segregated core of PACA moieties is surrounded by a dense palisade of PEG moieties. Diverse hydrophobic drug can be loaded into the core of nanoparticles with high efficacy. Because of the reduced interaction with biological components, the nanoparticles loading the hydrophobic drug have a long half-life in the blood compartment.

In our early work, poly{[α-maleic anhydride-ω-methoxy-poly(ethylene glycol)]-*co*-(ethy cyanoacrylate)} amphiphilic graft copolymers (PEGECA) had been prepared by radical polymerization from poly (ethylene glycol) macro-monomers and ethyl 2-cyanoacrylate (ECA), and PEGECA nanoparticles were prepared by the nanoprecipitation technique.[31] In this article, *D,L*-THP-loaded PEGECA nanoparticles (PEGECAT NPs) were prepared by nanoprecipitation technique and *in vitro* release behaviors were also investigated.

EXPERIMENTAL

Materials

D,L-THP was obtained from Tongrentang Co. (Tianjin, China). Commercial mPEG (M_n = 5000 g/mol) was purchased from Aldrich (St. Louis, MO). ECA was obtained from the Beijing East Chemical manufactory (Beijing, China). Maleic anhydride was supplied from the Tianjin First Chemical Reagent manufactory (Tianjin, China). Azobisisobutyronitrile (AIBN), toluene, dichloromethane (DCM), chloroform and acetone were purchased from the Tianjin Chemical Reagent Co. Acetic acid and ammonium acetate were supplied from the Tianjin Wendaxigui reagent manufactory (Tianjin, China). All reagents were analytical grade and used as received. Methanol and acetonitrile with chromatographic grade were purchased from MERCK (Germany).

Preparation of PEGECA

PEGECA prepared by radical polymerization was reported in our previous article.[31] In brief, mPEG (10 g) and maleic anhydride (300 mg) were added into toluene (50 mL) and the reaction was carried out at 65°C for 4 h to form the PEG macromonomer (PEGA). The purified PEGA (5 g), ECA (2.5 g), AIBN (1 wt% of ECA) and toluene (50 mL) were added into a reactor and the copolymerization was carried out at 60°C for 5 h. The obtained copolymer solution was precipitated in 300 mL of diethyl ether (precooled to 0°C) and filtered. Pure PEGECA1 was stored at 4°C prior to use. The molecular weights and compositions of the PEGECA copolymers prepared in this article are listed in Table I.

TABLE I
Molecular Weights and Compositions of PEGECA Copolymers

Sample	M_n (g mol^{-1})[a]	$M_{n,\mathrm{PECA}}$ (g mol^{-1})	$M_{n,\ \mathrm{mPEG}}$ (g mol^{-1})
PEGECA1	7400	2400	5000
PEGECA2	9300	4300	5000
PEGECA3	11600	6600	5000

Preparation of PEGECAT NPs

PEGECAT NPs were prepared by the nanoprecipitation technique. The drug was dissolved in the copolymer solution in acetone, i.e., 3-mg THP dissolved in 2-mL copolymer–acetone solution at 50-mg/mL concentration and then the mixture solution was added into 10-mL distilled water under magnetic stirring at ambient temperature and acetone was allowed to evaporate completely under agitation. The obtained dispersion was then centrifuged by a centrifuge (LD5-2A, Beijing, China) at 3000 rpm for 30 min in order to eliminate the aggregated particles. The supernatant was PEGECAT NP dispersion, which was directly used for transmission electron microscopy (TEM) and particle size distribution. The supernatant could also be frozen and lyophilized by a freeze-dryer system (LGJ-10, Beijing, China) to obtain the PEGECAT NPs freeze-dried powder, which can easily redisperse into water to form a nanoparticle dispersion.

Characterization of PEGECAT NPs

Differential scanning calorimetry (DSC) measurements were carried out with Diamond DSC (Perkin Elmer Co., USA). All the measurements were carried out at a heating rate of 10°C/min from 0°C to 200°C.

The TEM specimens for the PEGECAT NPs dispersions were observed under a JEM-100CX II instrument. The samples were prepared by adding a drop of the PEGECAT NPs dispersion on the Formvar-coated copper TEM grid, and then dyed by phosphatotungstic acid.

The size and distribution of PEGECAT NPs were determined by BI-90Plus laser particle size analyzer (LPSA, Brookhaven Instruments, USA). For all cases, λ of measurement was 678 nm, the angle of measurement was 90° and the temperature of measurement was 25°C.

The proton nuclear magnetic resonance (^{1}H NMR) was carried out on Varian Unity-Plus INOVA 500 with tetramethylsilane (TMS) as the internal

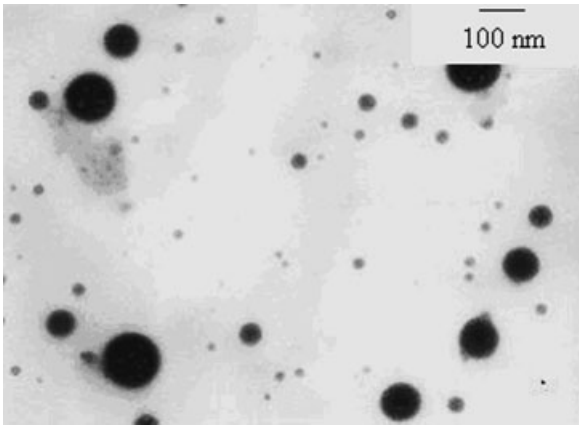


Figure 1 TEM micrograph of PEGECAT NPs. PEGECA3 was used as Carrier material. The THP-loaded amount was 6.19%. The samples were prepared by adding a drop of the PEGECAT NPs dispersion on the Formvar-coated copper TEM grid, and then dyed by phosphatotungstic acid.

standard. The PEGECAT NPs dispersion obtained by dispersing the PEGECAT NPs freeze-dried powder in D_2O was used for ^1H NMR measurement. The solutions of PEGECAT NPs and THP in $CDCl_3$ were also used for ^1H NMR measurement.

Measurement of drug-loaded amount of PEGECAT NPs

The content determination methods of THP include ultraviolet spectrophotometry,[32] TLC scanning,[33,34] polarimetry,[35] Fluorometry,[36,37] electrophoresis,[38,39] and high-pressure liquids chromatogram (HPLC),[40,41] etc. In the above methods, HPLC is adopted abroad due to its handy, precise, and reproducible nature.

In this article, the THP-loaded amount of PEGECAT NPs was determined by HPLC. PEGECAT NPs were dissolved in acetonitrile (5 mL) (a common solvent for both PEGECA and THP) and injected into a HPLC (Waters, USA) with a Hypersil ODS-2 (250 mm × 4.6 mm) C18 column. The mobile phase, composed of methanol, 7% acetic acid, and 4% ammonium acetate (43 : 28 : 29, v/v/v), was performed at a temperature of 25°C and at a flow rate of 1.0 mL/min. The THP peak was detected at 280 nm. The drug-loaded amount (wt %) was defined as the weight ratio of THP in PEGECAT NPs to preweighted PEGECAT NPs. Before this analysis, the standard curve of THP was calibrated by HPLC.

In vitro release of THP from PEGECAT NPs

About 3 mg PEGECAT NPs was well dispersed in a 2-mL phosphate buffer solution (PBS, pH 7.4) and then placed in a dialysis bag (12 Kda molecular weight cut-off), which was immersed in a conical flask containing 48 mL PBS (pH 7.4). *In vitro* release was preformed in an incubator shaker (SHZ-88, Jiangsu, China) at 130 rpm and 37°C. At the appropriate time intervals, a 10-mL aliquot of PBS outside the dialysis bag was removed to measure the amount of THP released from PEGECAT NPs. After the removal of each 10-mL aliquot, a 10-mL aliquot of fresh PBS was supplemented. The amount of THP in the release medium was determined by HPLC. The accumulated release was calculated as follows:

$$E_r = \frac{V_e \sum_{1}^{n-1} Ci + V_0 C_n}{m_{\text{drug}}}$$

Where E_r is the accumulated release (%), V_e is the sampling volume (10 mL), V_0 is the initial volume (50 mL), C_i and C_n are the THP concentrations (μg/mL), i and n are the sampling times, and m_{drug} is the mass of THP in PEGECAT NPs (μg).

RESULTS AND DISCUSSION

Morphology of PEGECAT NPs

Figure 1 shows the TEM micrograph of PEGECAT NPs. The micrograph indicates that PEGECAT NPs are of spherical morphology and discrete particles in aqueous medium. THP distributes in the nanoparticles.

Figure 2 illustrates a typical particle size distribution of PEGECAT NPs. As shown in Figure 2, the mean particle size of PEGECAT NPs is 82 nm, and the size polydispersity index is 0.254. This result shows that the particle size and size distribution

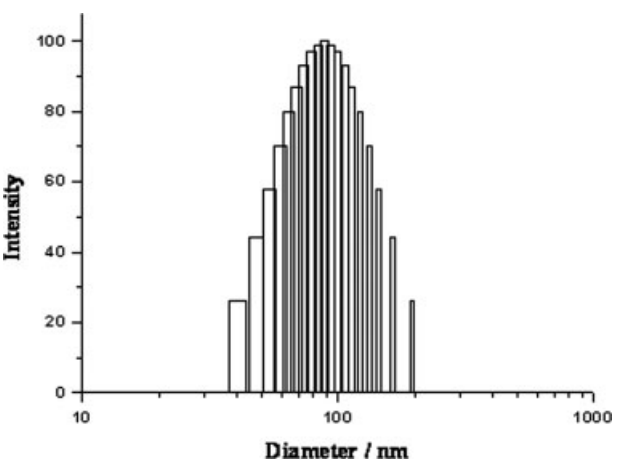
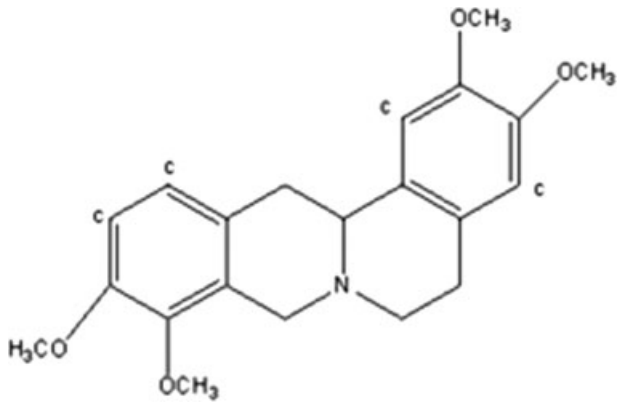


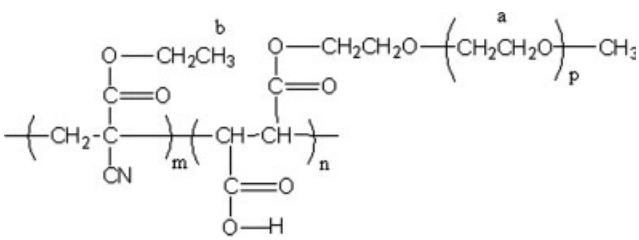
Figure 2 Particle size distribution of PEGECAT NPs in aqueous dispersion. PEGECA3 was used as Carrier material. The THP-loaded amount was 6.19%. For all cases, λ of measurement was 678 nm, the angle of measurement was 90°and the temperature of measurement was 25°C.



Scheme 1 Structure of *D,L*-tetrahydropalmatine.

measured by LPSA correspond well with the TEM result.

High-resolution ^{1}H NMR spectroscopy has often been employed to analyze the microstructure of polymer chains and the molecular motion of amphiphilic copolymers in solution.[42] We also estimated the core/shell structure of PEGECAT NPs by means of ^{1}H-NMR spectroscopy.[31] Structures of THP and PEGECAT are shown in Schemes 1 and 2. Comparing the spectra of THP in $CDCl_3$ of Figure 3(C), PEGECAT NPs in $CDCl_3$ of Figure 3(B) with that of PEGECAT NPs in D_2O in Figure 3(A), it can be seen that the only chemical shift peak of methylene proton in the PEG moieties is around 3.65 ppm (H_a), and the peaks 1.4 ppm (H_b) and 6.7 ppm (H_c) assigned to the protons of PECA moieties and THP disappear in Figure 3(A). These results indicate that the hydrophobic PECA moieties locate in the core of the nanoparticles and hydrophobic drug, THP, distributes in the core. For the drug-loaded nanoparticles of amphiphilic graft copolymers, it is presumed that there is hardly any interaction between the hydrophobic moieties, hydrophobic drug and water in aqueous solution. In water, it is believed that the amphiphilic copolymer exists as separated microphases of soluble PEG moieties with insoluble PECA moieties and THP. Obviously, the conformation of the amphiphilic graft copolymer and THP in $CDCl_3$ is of the mixed-microphase form, in which all of THP, mPEG and PECA moieties are dissolved, so all the ^{1}H NMR peaks of the protons



Scheme 2 Structure of PEGECA.

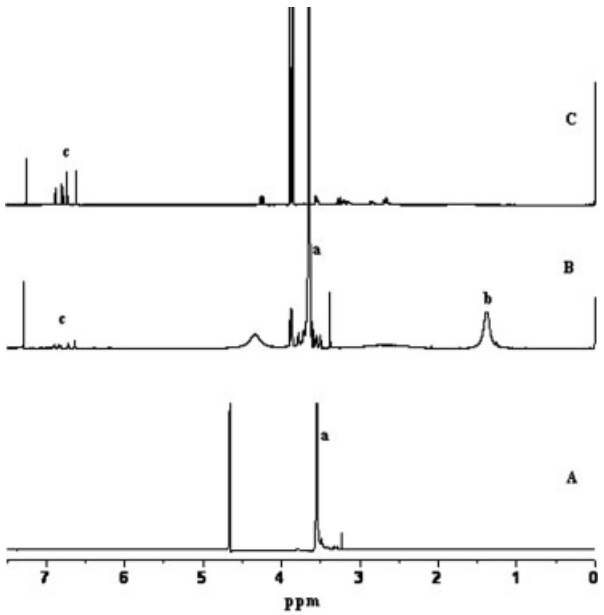


Figure 3 ^{1}H NMR spectra of THP and PEGECAT NPs. A: PEGECAT NPs, D_2O was used as the solvent; B: PEGECAT NPs, $CDCl_3$ was used as the solvent; C: THP, $CDCl_3$ was used as the solvent. PEGECA3 was used as the carrier material. The THP-loaded amount was 6.19%.

in PEGECA chains and THP appear in the ^{1}H NMR spectrum. In D_2O, the hydration reaction leads to the stretching conformation of PEG moieties into water phase and, at the same time, the hydrophobic aggregation between the PECA moieties occurs and THP is incorporated into the core formed by the PECA moieties. As a result, the drug-loaded hydrophobic cores covered by PEG moieties are formed and stably disperse in the water.

It can be seen from Figures 1 and 3 that PEGECAT NPs exist as core-shell-like spherical structure composed of a THP-loaded hydrophobic core of PECA and a hydrophilic outer shell of mPEG in water.

Evaluation of PEGECAT NPs

In our early work, we found that the average size of PEGECA formed in the mixed solvent of DCM and acetone increased with the amount of DCM increasing.[31] Therefore, DCM and acetone were chosen, respectively, as the hydrophobic solvent and the hydrophilic solvent to investigate the influence of solvent property on PEGECAT NPs. The results show that the stable dispersion is obtained and the average size of PEGECAT NPs is smaller than 100 nm when acetone is used as the solvent, but the stable dispersion cannot be obtained when DCM is used as the solvent. The effect of the solvent nature on the stability of the dispersion is attributed to the different PEGECA molecular assembly mechanisms when PEGECA molecules diffuse into water from

TABLE II
Effect of the Temperature on PEGECAT NPs[a]

T/°C	THP-feeding/%	THP-loaded amount/%	Entrapment efficiency/%	Diameter/nm	Polydispersion index
30	3.25	3.28	99.6	52	0.229
40	3.67	3.62	99.5	64	0.299
50	3.46	3.37	99.0	70	0.317

organic phase. DCM is a water-insoluble solvent whereas acetone is a water-miscible solvent. The formation of the nanoparticles using DCM as the organic solvent is a microphase inversion process, when the coagulation between particles and the leakage of THP may easily take place. In contrast, the formation of the nanoparticles using acetone as the organic solvent is a self-diffusion and precipitation process, when water can easily diffuse into the acetone phase, hydrate the PEG segments, and simultaneously make the PECA segments and THP precipitate from the liquid. Consequently, the hydrophilic segments stretch into water and the hydrophobic segments and THP associate into the internal core rapidly. Therefore, the stable nanoparticles dispersions can be easily obtained with acetone as an organic solvent.

As shown in Table II, the temperature can impact PEGECAT NPs. The results show that the THP-loaded amount increases and the entrapment efficiency slightly decreases with the temperature increase and the size and polydispersion index increase, simultaneously. When the temperature is raised, the rate of solvent evaporation increases accordingly whereas the solution viscosity drops, affecting the stability of PEGECAT NPs dispersion. The increment of the diameter and polydispersion index indicates that the coagulation between particles becomes obvious with the temperature rising.

The effects of the copolymer compositions on PEGECAT NPs were investigated and the results are shown in Table III. From Table III, it can be seen that the THP-loaded amount, entrapment efficiency, and diameter increase slightly with the increase of chain length of PECA moieties in the copolymers at the same THP feeding when the nanoparticle dispersions are stable. However, the largest THP-loaded amounts are strikingly different. We defined the largest THP-loaded amount as the THP-loaded amount of the THP-loaded nanoparticles in the supernatant after the large particles in the instable dispersion are eliminated by centrifuging. As shown in Table III, when PEGECA1, PEGECA2, and PEGECA3 are used as the carrier, the largest THP-loaded amounts are 3.56, 3.99, and 6.20, respectively. This phenomenon suggests that the compatibility of PECA segment with THP is enhanced with the increase in chain length of PECA segment in copolymers.

From Table III and Figure 4, it can be seen that with the THP-feeding increasing, the THP-loaded amount increases and then levels off. Simultaneously, the entrapment efficiency drops obviously when the THP-loaded amount reaches the maximum point. The effects of the THP-loaded amount on the size of nanoparticles are depicted in Figure 5. With the increase in the THP-loaded amount, the size of nanoparticles increases slightly.

In vitro release of PEGECAT NPs

The effects of the THP-loaded amount on *in vitro* release behavior were investigated with PEGECA3 as the carrier material and the results are exhibited in Figure 6. As shown in Figure 6, the release of THP is slow and the accumulated release is lower than 40% after 48 h. The drug release is controlled by two factors: one is diffusion of drug and the other is degradation of polymer. In our opinion, the low cumulative release is due to the restriction of diffusion of THP. The release rate of THP from PEGECAT NPs increases with the THP-loaded

TABLE III
Effects of the Copolymer Composition on PEGECAT NPs

Copolymer	THP-feeding/%	THP-loaded amount/%	Entrapment efficiency/%	Diameter/nm	Stability
PEGECA1	3.76	3.06	99.1	50	Good
PEGECA2	3.25	3.28	99.6	52	Good
PEGECA3	3.74	4.02	99.9	67	Good
PEGECA1	5.45	3.56	80.4	60	Bad
PEGECA2	5.36	3.99	78.5	55	Bad
PEGECA3	7.72	6.20	77.4	55	Bad

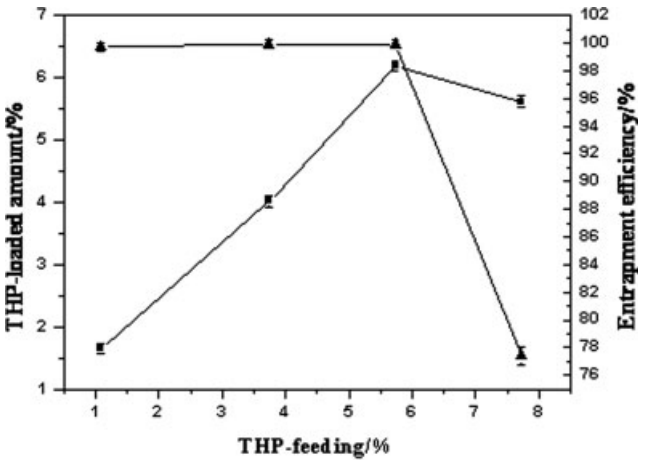


Figure 4 Effects of the THP feeding on the THP-loaded amount (A) and the entrapment efficiency (B) of PEGECAT NPs. PEGECAT NPs were prepared by the nanoprecipitation technique. PEGECA 3 was used as carrier, acetone was used as the organic solvent and the dosage was 2 mL, the water was 10 mL and the rev of magnetic stirring was 500 rpm.

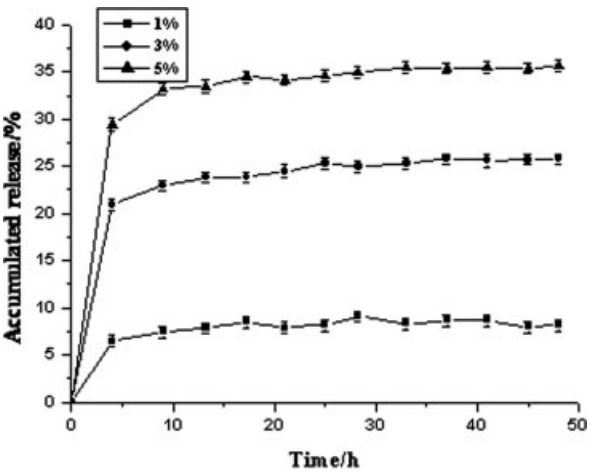


Figure 6 *In vitro* release profiles of THP from PEGECAT NPs with various THP-loaded amount. The THP-loaded amount was 1.57% (■), 4.02% (●), and 6.19% (▲), respectively. PEGECA3 was used as the carrier. The receptor liquid volume was 50 mL, the sampling volume was 10 mL, the rev of the incubator shaker was 130 rpm, the temperature was 37°C and the release time was 2 days.

amount increasing. A reasonable explanation of this phenomenon is that a homogeneous matrix was formed with the drug randomly distributed throughout the polymer particles at lower loadings. As shown in Figure 7, there is no melting peak at 148°C in the DSC thermograms of PEGECAT NPs when the THP-loaded amount is lower than 6.19%, which indicates that THP exists as amorphous form rather than crystal in PEGECAT NPs. Therefore THP in PEGECAT NPs directly diffuses into the receptor liquid and the controlling factor is the distance between the THP molecules. With increasing the THP-loaded amount, the distance between the THP molecules is so close that the diffusing channels in the core are more easily formed. Once the diffuse channels are formed, the THP molecules easily diffuse from PEGECAT NP into water.

The TEM micrograph of PEGECAT NPs after *in vitro* release is shown in Figure 8. The TEM micrograph displays that PEGECAT NPs were of spherical morphology all the same and the size hardly changes after *in vitro* release over a two-day period.

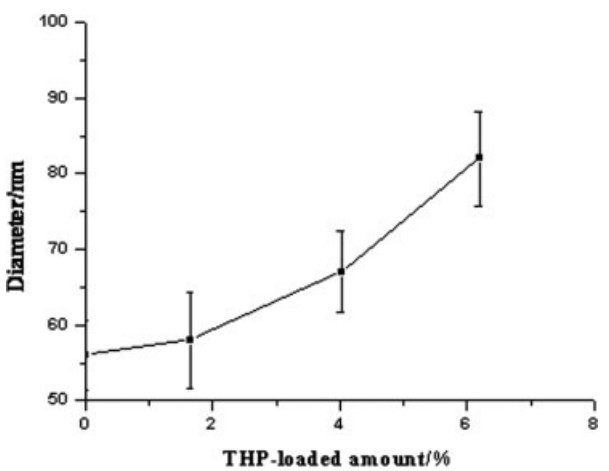


Figure 5 Effects of the THP-loaded amount on the size of PEGECAT NPs. PEGECAT NPs were prepared by the nanoprecipitation technique. PEGECA 3 was used as carrier, acetone was used the organic solvent and the dosage was 2 mL, the water was 10 mL and the rev of magnetic stirring was 500 rpm. The THP-loaded amounts were 0, 1.65%, 4.02%, and 6.19%, respectively.

CONCLUSIONS

PEGECAT NPs were prepared by the nanoprecipitation technique. PEGECAT NPs take on a spherical morphology with an inner core and outer shell before and after *in vitro* release. THP can be

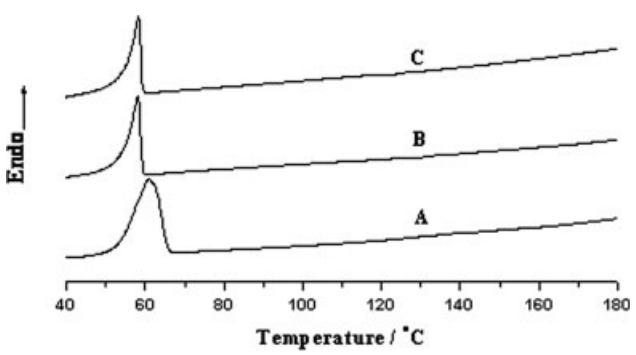


Figure 7 DSC thermograms of PEGECAT NPs. The THP-loaded amounts were 0 (A), 4.02% (B), and 6.19% (C), respectively. PEGECA3 was used as the carrier. All the measurements were carried out at a heating rate of 10°C/min from 0°C to 200°C.

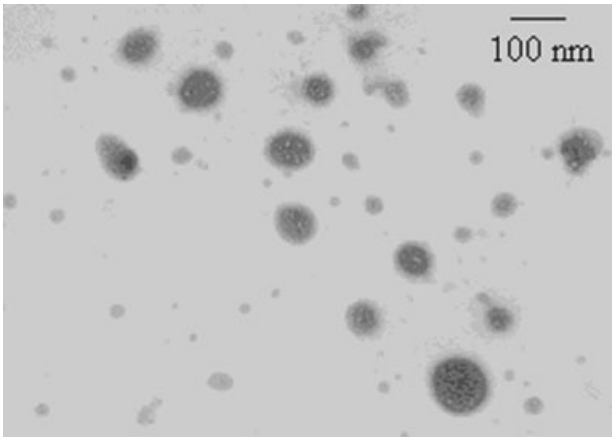


Figure 8 TEM image of PEGECAT NPs after *in vitro* release. The carrier material was PEGECA3 and the THP-loaded amount was 6.19%. The samples were prepared by adding a drop of the PEGECAT NPs dispersion on the Formvar-coated copper TEM grid, and then dyed by phosphatotungstic acid.

incorporated into the hydrophobic core of PEGECAT NPs and the drug-loaded amount is higher than 5%. The average size of PEGECAT NPs is lower than 100 nm. The *in vitro* release rate of THP from PEGECAT NPs is slow and the accumulated release is lower than 40% after 48 h. The *in vitro* release rate depends on the THP-loaded amount of PEGECAT NPs. The size hardly changes after *in vitro* release over a two-day period. From these results, we think that the PEGECA nanoparticles also have a hydrophobic drug-loaded ability.

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