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Preparation and characterization of *N*-phthaloyl-chitosan-*g*-(PEO-PLA-PEO) as potential drug carrier

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To improve the drug-loading capacity and control the initial release of amphiphilic drug carriers, a series of *N*-phthaloyl-chitosan-*g*-(PEO-PLA-PEO) compounds were synthesized with well-defined structures. The self-assembly behavior of copolymers in aqueous solutions was confirmed by various techniques such as fluorescence spectrometry, dynamic light scattering, and transmission electron microscopy. The results demonstrated that the micellization behavior of graft copolymers was different from that of their linear counterparts. The micelle sizes of the graft copolymers could be tuned with the chemical compositions as well as temperature. Furthermore, when hydrophobic indomethacin was loaded into the micelles, the graft copolymer micelles trapped more indomethacin than PEO-PLA-PEO micelles, facilitating the in vitro control of the initial burst release of the drug. Drug release could be controlled in vitro by simply altering the EO/LA ratio of the grafting chains. The graft copolymer showed low cytotoxicity to 293T cells, indicating its great potential application for drug delivery.

Introduction

In aqueous solutions, amphiphilic block copolymers poly(ethylene oxide)–poly(lactic acid)–poly(ethylene oxide) (hereafter (PEO-PLA-PEO)) assemble to form micelles with a hydrophobic core and a hydrophilic shell, which is a promising strategy for their use as drug carriers in the treatment of diseases.[1] This kind of material can solubilize hydrophobic agents in the hydrophobic core, while the hydrophilic shell facilitates prolonged circulation in the bloodstream.[2] It also accumulates in solid tumors through the enhanced permeability and retention effect, which is characterized by leaky blood vessels and impaired lymphatic drainage in tumor tissues.[3] Moreover, the size, stability, loading capacity, and release kinetics of drugs can be modulated using these micelles by tailoring the constituent block chains. These degradable block copolymers have received particular attention because they do not accumulate in the body.[4] However, the low drug-loading capacity limits the application of PEO-PLA-PEO.

Recently, several architectures and modifications have been explored to broaden the application of PLA-based copolymers.[5,6] For example, the acrylate functional group was incorporated into PEO-PLA-PEO to prepare highly cross-linked polymers. Cross-linking not only increased the mechanical property of the polymers but also controlled the water uptake stability, permeability, and degradation behavior.[5] However, it is not clear whether the residual cross-linking agent, initiator, and monomer cause potential toxicity and whether the cross-linking may decrease the degradability of polymers.

Chitosan, a natural linear polysaccharide, has recently received considerable attention as a biomaterial with potential clinical application because of its nontoxicity, good biocompatibility, biodegradability, low immunogenicity, and biological activity.[7–16] Various techniques have been developed to improve the aqueous solubility of chitosan, among which phthaloylation is regarded as the most common technique for solubilization as well as protection for the preparation of various multifunctional derivatives.

It is expected that chitosan grafts with PEO-PLA-PEO can improve the drug-loading capacity without the loss of initial biocompatibility and biodegradability of the block copolymers. In addition, the nonlinear structure could lead to unique

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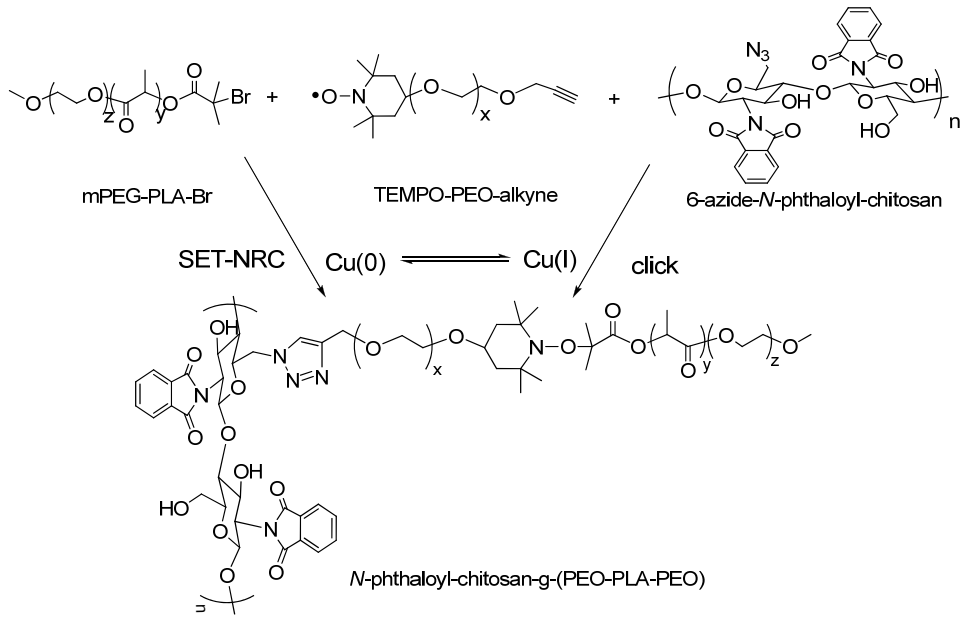
properties compared with their linear counterparts.[17,18] The current synthetic route of PEO-PLA-PEO involves coupling PEO-PLA using hexamethylene diisocyanate (HMDI) as a coupling agent.[19] Due to the nonreactive terminal functional group and the symmetric structure of PEO-PLA-PEO, it is difficult to obtain chitosan-based graft copolymers with PEO-PLA-PEO grafting chains. Previously, we reported a novel well-defined graft copolymer consisting of a chitosan backbone and amphiphilic PEO-PLA-PEO grafting chains through a nanosized Cu(0)-catalyzed one-pot strategy combining "click" chemistry and single electron transfer-nitroxide radical coupling (SET-NRC) reaction (Scheme 1).[20]

The effects of decoration with the grafting chains are very important, and controlled release of drugs from these polymeric carriers is closely related to the structure and the shape of the carriers. This paper describes the physical properties of the graft copolymers compared to those of the linear PEO-PLA-PEO. By characterization of the micelle behaviors, we assess the viability of these new materials as potential drug carriers.



Scheme 1. Synthesis of *N*-phthaloyl-chitosan-*g*-(PEO-PLA-PEO) via "click" and "SET-NRC" reaction.

Experimental

1. Materials

N-phthaloyl-chitosan-*g*-(PEO-PLA-PEO) and PEO-PLA-PEO were synthesized as previously described.[19,20] Pyrene and indomethacin (IMC) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China) and were used after recrystallization twice in ethanol. All other chemicals were obtained from commercial sources and used without further purification.

2. Characterization of copolymer micelles

The critical aggregation concentrations (CACs) of synthesized copolymers were determined by fluorescence measurements using pyrene as a fluorescent probe at 25°C. Briefly, copolymer tetrahydrofuran (THF) solutions with different concentrations were injected into 5-mL pyrene aqueous solution (5.0×10^{-7} M). The solutions were stirred for 12 h to reach the solubility equilibrium of pyrene, after which the THF was evaporated. The steady-state fluorescence of pyrene in solutions was measured using a Perkin-Elmer LS-55 spectrofluorometer in the range 350–480 nm. The excitation wavelength was set at 339 nm. The excitation and emission bandwidths were both 1 nm.

Transmission electron microscopy (TEM, JEM-1200EX, JEOL) measurements were performed at an acceleration voltage of 120 kV. For the observation of the micelles, a dro

of copolymer solution (0.5 mg/mL) was deposited onto a carbon-coated copper electron microscopy grid and dried at 25 ^{o}C.

The micelle size and size distribution (0.5 mg/mL) were measured by dynamic light scattering (DLS) using a Delsa Nano particle analyzer at a scattering angle of 165^{o} at different temperatures. The temperature was controlled by the automatic temperature-controlled accessory, and the sample cell was thermostated for 10 min prior to measurement.

3. Drug-loading capacity and *in vitro* release behavior from the micelles

The drug-loading capacity was determined according to a previously described method.[21] Graft and block copolymer solutions were dissolved in phosphate-buffered saline (PBS) containing 5% (v/v) ethanol at a concentration of 0.5 mg/mL, and IMC was employed as the model hydrophobic drug. To prepare IMC/copolymeric micelles, 10 mg of IMC was added into 20 mL copolymer solutions. These solutions were dialyzed (MWCO 3500) in 100 ml PBS/ethanol solvent for 24 h to remove free IMC. The dialysate was collected, and the IMC content in the dialysate (C_f) was measured by a UV spectrophotometer (Shimadzu UV-2550) at 320 nm using a calibration curve constructed from a series of IMC solutions with standard concentrations.[22] Each measurement was repeated three times. The drug-loading capacity (DL) was defined as the weight percentage of the loaded drug based on the feed amount and was calculated by

$$DL = \frac{10 - C_f}{10} \times 100\% \quad (1)$$

where C_f represents the weight of free IMC.

(10−C_f) represents the weight of IMC loaded in the micelle.

The drug-release profiles from micelles were measured as follows: the dialysis bags (MWCO 3500) containing 10 mL IMC-loaded micelle solutions were directly immersed into 50 mL PBS containing 5% (v/v) ethanol solvent in a conical beaker, which was then placed on a constant-temperature vibrator to maintain an internal temperature of 37 ^{o}C. A volume of 4 mL solution was withdrawn from the dialysate at predetermined time intervals, and then 4 mL fresh solvent was introduced into the dialysate to make the volume constant after each sampling. The concentration of IMC was measured with a UV spectrophotometer, as mentioned above. Each measurement was repeated three times.

4. Cytotoxicity assay

The cytotoxicity of *N*-phthaloyl-chitosan-*g*-(PEO-PLA-PEO) was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using 293T cells. The graft copolymer was diluted with a cell culture medium to obtain a concentration of 1 mg/mL. The 293T cells were seeded in a 96-well plate at a density of 1 × 10^5 cells per well and grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum and antibiotics penicillin (100 IU/mL) and streptomycin (100 μg/mL) at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. The cells were then incubated with the culture medium containing the graft copolymer for predetermined intervals. Next, the medium was replaced with 10 μL MTT, and the cells were further incubated for another 4 h. The absorbance of the colored medium was measured at 570 nm using a microplate reader. Each measurement was repeated three times.

Results and discussion

1. Characterization of copolymer micelles

The solubility of *N*-phthaloyl-chitosan-*g*-(PEO-PLA-PEO) was tested in several solvents including water. Chitosan (degree of deacetylation 80%; viscosity-average molecular weight 200 kDa) is insoluble in water at neutral and basic pH and other common solvents because of the strong hydrogen bonds. However, the graft copolymers are soluble in water, dimethylformamide (DMF), dimethylsulfoxide (DMSO), and THF. Scheme 1 shows the synthetic route and the chemical structure of *N*-phthaloyl-chitosan-*g*-(PEO-PLA-PEO). The synthesis and characterization results were reported in our previous paper.[20] The composition data of all samples summarized in Table 1 were reasonably deduced from the precursors. The efficiencies of the coupling reactions were greater than 90%, as determined by Fourier transform infrared (FTIR) and electron spin resonance (ESR) spectra. All samples were abbreviated according to the structure and EO/LA value of amphiphilic copolymers (Table 1).

Pyrene is widely used as a hydrophobic fluorescent probe to characterize the aggregation phenomenon in amphiphilic

copolymer solutions. The emission intensity at 373 nm is particularly an excellent index of the polarity around pyrene, with the emission increasing with the decrease of hydrophilicity.[23,24] The intensity of pyrene emission at 373 nm (I_{373}) in *N*-phthaloyl-chitosan-*g*-(PEO-PLA-PEO) aqueous solutions was plotted against their concentrations at 25 °C, as shown in Fig. 1. At low concentrations (ln *C*= –4), the value of I_{373} was relatively low in all samples. This suggested that pyrene still dissolved in the polar microenvironment. At 10^{-4}–10^{-3} g/mL (ln *C*= –4 to –3) of all copolymer solutions, I_{373} showed an abrupt increase, which suggested that pyrene experienced a change from a polar environment to a nonpolar environment with the formation of micelle aggregates. This assembly phenomenon was due to the aggregation of amphiphilic PEO-PLA-PEO chains. Thus, 1.66×10^{-4} g/mL (ln *C* = –3.78) was determined as the CAC of G-2.4, which was lower than that of the other samples (4.17×10^{-4} g/mL for G-3.6, 6.79×10^{-4} g/mL for G-9.5, and 6.61×10^{-4} g/mL for B-3.4). Compared to the CAC of the graft copolymers, the values decreased as the portion of the hydrophobic block increased in the grafting chains.[25] The grafting ratio was 43%, which meant that there was almost one grafting chain in two repeating glucan units. The chain segments of PEO-PLA-PEO grafting on the chitosan backbone are close to each other, thus the interactions between the hydrophilic and hydrophobic chains occur more easily than those of the separate block copolymer of PEO-PLA-PEO, resulting in a lower CAC value for G-3.6 than for B-3.4, even though the grafting chain of G-3.6 had a structure similar to that of B-3.4. Fig. 2 shows the TEM images of aggregates of B-3.4 and G-3.6, which are observed as sphere-like micelles. The core–shell structure of G-3.6 can also be observed.

The average hydrodynamic diameters of copolymer micelles (0.5 mg/mL) measured by DLS at different temperatures were used to investigate temperature-responsive behavior (Fig. 3). At 25 °C, the micelles exhibited a unimodal size distribution, and the average size of these polymeric micelles was ~100–200 nm (Fig. 3a). Particles with diameters more than 200 nm can be frequently removed by the reticuloendothelial system.[26] From this viewpoint, the introduction of chitosan does not cause the loss of its potential abilities for pharmaceutical applications. Interestingly, the size of the graft copolymer micelles did not depend on the length of grafting chains, but depended on the EO/LA ratio of the amphiphilic chains. The average hydrodynamic diameter of G-2.4 micelle was 217nm，which was almost two times that of G-3.6 and G-9.5. This could be a thermodynamic process driven by the hydrophobic interaction of the grafting chains. Generally, for amphiphilic copolymer micelles, increasing the ratio of the hydrophobic block to the hydrophilic one would increase the micelle size[27]; PEO did not have any effect on the structures of the nano-aggregates in solution except for making the aggregates soluble in the solution phase. So the larger size of G-2.4 than G-3.6 and G-9.5 probably resulted from the more hydrophobic nature of G-2.4.[28,29]

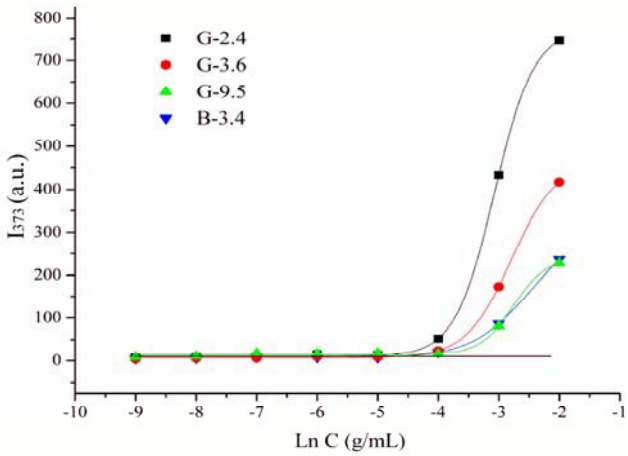


Fig. 1. Relationship between the fluorescence intensity of four copolymer aqueous solutions at 373 nm (I_{373}) and their concentrations at 25 °C.

Table 1 Constitution and micelle size of chitosan graft copolymers and block copolymer

Sample	M_n[1]	EO/LA[1]	Grafting ratio	Composition[2]	Before drug loading		After drug loading	
					average micelle size (nm)	PDI	average micelle size (nm)	PDI
G-2.4	5,208	2.4	43%	*N*-phthaloyl-chitosan-*g*-(PEO_{42}-PLA_{36}-PEO_{44})	216±4	0.45	307±6	0.31
G-3.6	15,583	3.6	43%	*N*-phthaloyl-chitosan-*g*-(PEO_{113}-PLA_{70}-PEO_{118})	118±2	0.44	127±5	0.37
G-9.5	11,292	9.5	43%	*N*-phthaloyl-chitosan-*g*-(PEO_{113}-PLA_{23}-PEO_{106})	97±2	0.61	110±4	0.47
B-3.4	14,579	3.4	-	$mPEO_{109}$-PLA_{68}-$mPEO_{109}$	140±4	0.39	128±4	0.35

[1]M_n and the EO/LA value were determined from the ^{1}H NMR spectra of copolymers in $CDCl_3$. M_n represented the number-average molecular weight of the branch chains.

[2]Compositions of copolymers were inferred from the structure of the precursors.

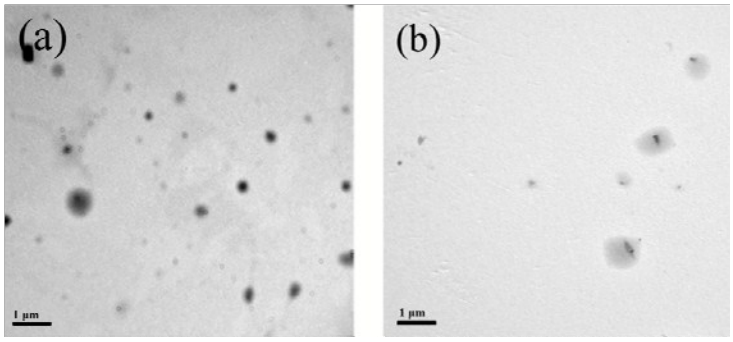


Fig. 2. TEM images of (a) B-3.4 and (b) G-3.6 at 25 °C.

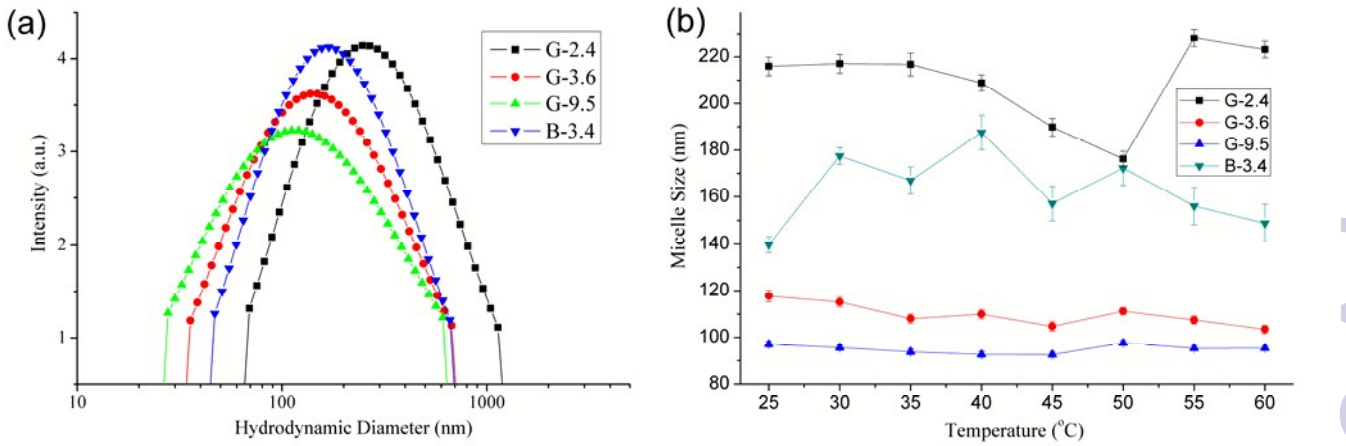


Fig. 3. (a) Effect of grafting chains on the micelle diameters at room temperature. (b) Temperature-dependent transition of the micelle diameters.

Grafting the thermosensitive PEO-PLA-PEO polymers onto chitosan would make the product thermosensitive. As shown in Fig. 3b, the size of B-3.4 micelles fluctuates more widely as the temperature rises, which was attributed to the dehydration of the PEO block, and it not only caused the shrinking of micelles but also led to the entanglements between micelles.[30] The stability of micelles formed by pure block polymers is not as good as that of the micelles formed by graft copolymers. Although B-3.4 and G-3.6 copolymers had a similar structure as that of the block copolymers, the introduction of chitosan in G-3.6 played a role as a physical linkage and constrained the movements of amphiphilic PEO-PLA-PEO chains. For the G-2.4 micelle, the average hydrodynamic diameter decreased slowly from 210 to 180 nm as the temperature increased from 35 ^{o}C to 50 ^{o}C, which was relevant to the dehydration of PLA. The hydrogen bonds between PEO chains and water molecules were broken at the same time. The average hydrodynamic diameter underwent a sharp increase above 50 ^{o}C because the hydrophilicity of the micelles reduced with the rise in temperature, which caused the micelles to come together to form bigger aggregates. In addition, the size transition of the graft copolymer micelles was dependent on the grafting chain compositions. Polymers with more hydrophobic chains were more sensitive to temperature. All these findings provided evidence that graft copolymers formed micelles in the aqueous solution.

2. Drug loading capacity and release profiles

IMC was employed as a model drug to investigate the drug-loading capacity (DL) and release profiles of polymeric micelles. IMC may be encapsulated by noncovalent hydrophobic interactions. Generally, IMC released into the solution phase is mainly dependent on the diffusion rate. It is possible that some of the trapped IMC would be removed with a low concentration of IMC in the medium. Adding some IMC in the dialysate may reduce the release of trapped IMC from the micelles. However, it was not easy to remove the remaining free IMC in the micelles. Considering the similar DL results of the two methods and the drug-release experiments, we decided to use the former method to study the DL of polymeric micelles. The DL of B-3.4 micelle was 17.6%; however, those of G-2.4, G-3.6, and G-9.5 were 85.4%, 56.8%, and 43.2%, respectively, which were much higher than the DL of B-3.4 micelle (Fig. 4). The larger amount will render a persistent release of IMC, which could be attributed to the fact that IMC may have hydrogen bond interactions with chitosan chains besides the hydrophobic interactions. In addition, considering that the G-2.4 micelle size was almost double that of the other graft copolymers at room temperature, it can encapsulate more drug in the hydrophobic region. It is reported that the PEO length affected the loading efficiency, showing that a long-chain PEO prevented a hydrophobic drug encapsulating into the micelles.[31] This may be another reason for the highest DL of the G-2.4 micelle. Most of the drug-loaded micelles became smaller because of the hydrophobic interactions between IMC and the hydrophobic core of these polymers (Fig. 5). However, encapsulating so much IMC in the hydrophobic core of G-2.4 micelles resulted in a large size.

Considering the poor water solubility of IMC, we used PBS containing 5% (v/v) ethanol as the release medium. The release behavior of IMC from different micelles was evaluated at 37 ^{o}C (Fig. 6). The initial burst releases of IMC both in B-3.4 and graft copolymer micelles were observed in the first 3 h, which might be the result of the localization of a small portion of IMC in the outer shell or the interfaces between the inner core and outer shell of micelles.[25] Obviously, decoration of chitosan can improve the initial burst release. For the B-3.4 micelle, the cumulative release amount was ~45% in 24 h because of the water-swelling micelle structure. All the copolymer micelles showed a sharp release of up to 10% in the first hour. After that, the release rates of the graft copolymer micelles were less than that of B-3.4 micelle. As seen from previous results, the graft copolymer formed large and compact micelles due to the hydrophobic interactions and entanglement of the chitosan chains. The slow IMC release was caused by the effects of chitosan decoration. The release rate from G-9.5 was higher than that from G-3.6 and G-2.4. According to the results, the higher ratio of EO/LA caused the stronger hydrophilicity of the copolymer, resulting in faster drug release. Thus, the constitution of grafting chains played an important role in the drug release. These results confirmed that *N*-phthaloyl-chitosan-*g*-(PEO-PLA-PEO) can be used as a potential drug carrier through the modulation of its chemical constitution.

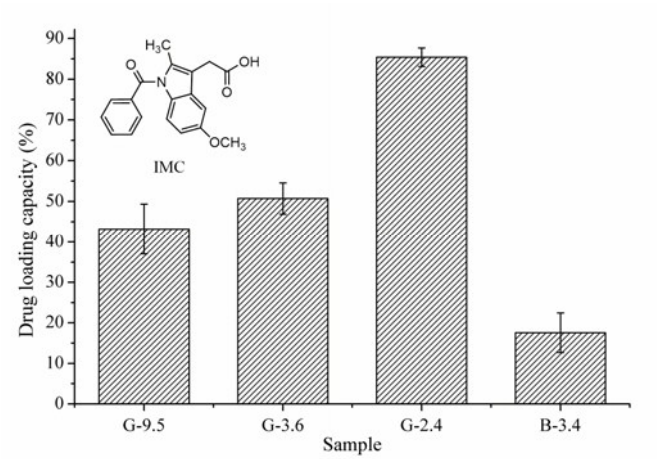


Fig. 4. IMC loading capacities of copolymer micelles (each sample was measured three times).

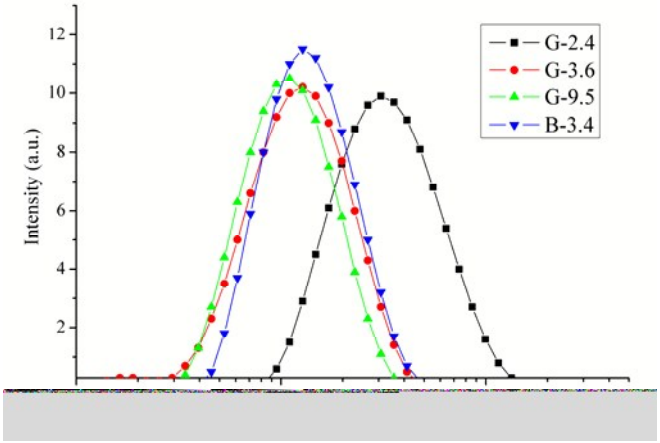


Fig. 5. Diameters of the micelles after drug loading at room temperature.

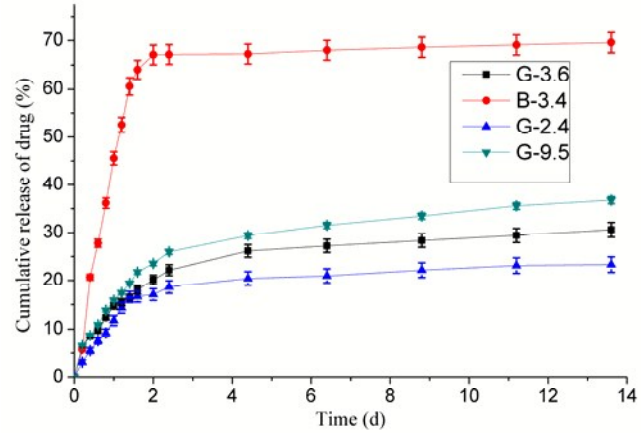


Fig. 6. *In vitro* drug release profiles of IMC-loaded micelles at 37 °C (each sample was measured three times).

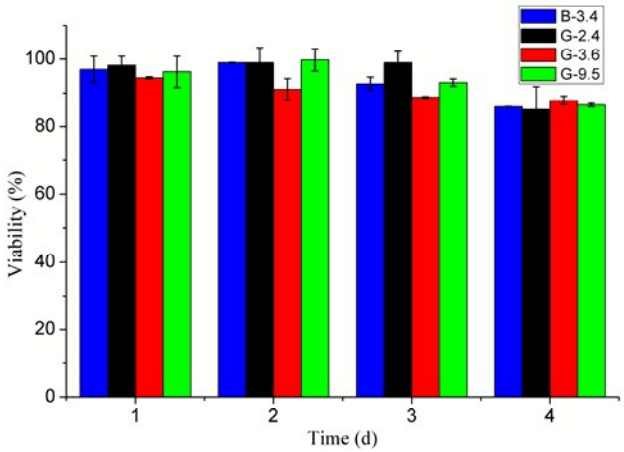


Fig. 7. Cytotoxicity of the micelles (1 mg/mL) after incubation with 293T cells (each sample was measured three times).

3.Cytotoxicity of micelles

During the deprotection of the *N*-phthaloyl group, the PLA block degraded intensively with hydrazine hydrate. Therefore, we used *N*-phthaloyl-chitosan-*g*-(PEO-PLA-PEO) as a drug carrier without the deprotection of the *N*-phthaloyl group. The cytotoxicity of these micelles was tested by using the 293T cells so as to evaluate their suitability for biomedical application. As shown in Fig. 7, the micelle showed very low cytotoxicity to 293T cells; nearly 90% cells were viable even at a high concentration (1 mg/mL) after 4 days of incubation. It can be concluded that *N*-phthaloyl-chitosan-*g*-(PEO-PLA-PEO) consisting of *N*-phthaloyl groups, PEO, and PLA blocks and chitosan backbones, has excellent biocompatibility.

Conclusions

PEO-PLA-PEO-modified chitosan derivatives with well-defined structures were synthesized, which showed good aqueous solubility. The formation of micelles was confirmed by various techniques such as fluorescence spectrometry, DLS, and TEM. Under similar EO/LA values, the graft copolymer had lower CAC at 25 °C than PEO-PLA-PEO block copolymer. In addition, chitosan backbone played a role of physical linkage in polymeric micelles and prevented them from disaggregation. By changing the structure and grafting chain constitution, the average size of the micelle could be easily controlled in the range 100–200 nm with a unimodal size distribution. Moreover, these graft copolymer micelles were thermosensitive. Temperature-dependent size transitions could be achieved

through modulating the constitution of the grafting chains. Furthermore, *in vitro* drug-release behavior indicated that the graft copolymer micelles could trap more hydrophobic drug than PEO-PLA-PEO micelles and that the initial burst release of the drug could be improved. The release profile could be well controlled by tuning the structure of the graft copolymers, and the micelles showed very low cytotoxicity to 293T cells, suggesting that *N*-phthaloyl-chitosan-*g*-(PEO-PLA-PEO) has a promising potential for biomedical applications.

Acknowledgments

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Preparation and characterization of *N*-phthaloyl-chitosan-*g*-(PEO-PLA-PEO) as potential drug carrier

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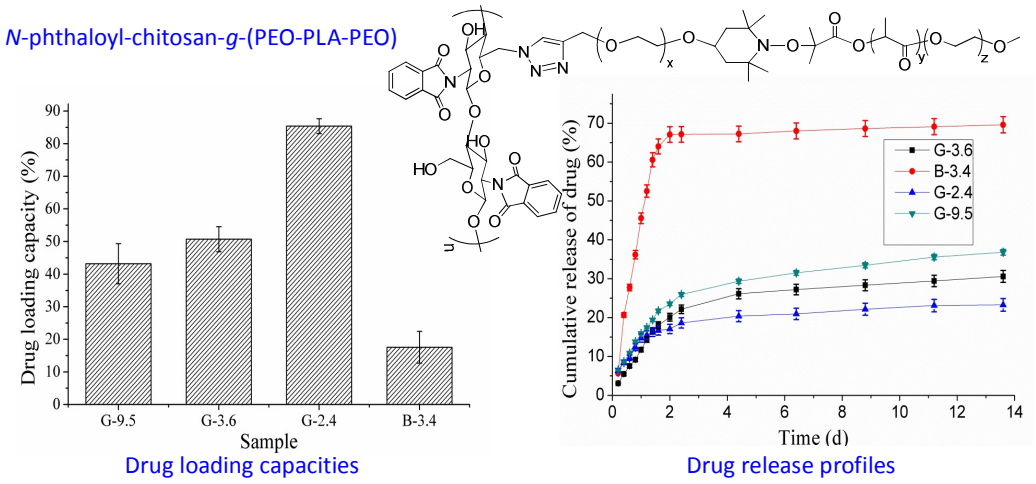

Synthesis of *N*-phthaloyl-chitosan-*g*-(PEO-PLA-PEO) and its drug loading capacities and drug release profiles of IMC.