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Novel polymersomes based on amphiphilic graft polyphosphazenes and their encapsulation of water-soluble anti-cancer drug

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ABSTRACT

A series of amphiphilic graft polyphosphazenes with hydrophilic weight fraction ranging from 0.40 to 0.55 were synthesized. These copolymers could self-assemble into distinct aggregates in aqueous solutions. Spherical micelles were observed for the copolymer sample with higher hydrophilic weight fraction. However, when the hydrophilic weight fractions decreased to less than 0.50, vesicle-like polymersomes were formed. Doxorubicin hydrochloride (DOX·HCl), a water-soluble anti-cancer drug, was successfully loaded into the aqueous core of polymersome, which was clearly observed by transmission electron microscopy. The in vitro release of DOX·HCl from polymersome carries further confirmed its encapsulation. In addition, the cytotoxicity of DOX against HepG2 cells was significantly enhanced via polymersome delivery. These results suggest that amphiphilic graft polyphosphazenes could be used for the delivery of water-soluble drugs as polymersome vehicles.

1. Introduction

Polymersomes are self-assembled vesicles of amphiphilic copolymers that are currently being developed by many research groups for fundamental insights into the nature of self-assembled states as well as for drug delivery applications [1,2]. Most of the investigations about polymersomes have been focused on block copolymers. Polymers with other kinds of architectures were not studied until very recently Lee et al. [3] first proclaimed their research on polymersome formation by comb-like traditional graft copolymers. However, there are still lacks of research work about drug delivery of polymersomes based on graft copolymers.

Polyphosphazene is a class of novel biocompatible and biodegradable polymers with an inorganic main chain and two active chloride groups on each repeat unit. These chloride groups can be readily substituted by other small molecule or macromolecules, therefore, multi-functionalized polyphosphazenes with tunable physicochemical/biological properties could be easily obtained by simply varying the graft units [4]. This is a unique advantage of polyphosphazene compared to conventional block copolymer which usually can only be functionalized on the end of polymer chain. Pioneer researchers have studied the biomedical application of polyphosphazenes such as nano-fibers [5] and hydrogels [6]. Our group has been devoting our attention to the micellization of amphiphilic polyphosphazenes and developed several thermosensitive amphiphilic polyphosphazenes for local drug sustained release [7,8]. Those drug carriers are more suitable for delivery of hydrophobic drugs, but not available for water-soluble substances.

In this investigation graft copolymers containing methoxy-poly(ethylene glycol) and ethyl-*p*-aminobenzoate side groups (PEG/EAB–PPPs) were synthesized and utilized to fabricate polymersomes for water-soluble anti-cancer drug delivery. In PEG/EAB–PPPs, the polymer backbone is highly flexible while the side groups are PEG and EAB molecules. Since the ratio of PEG to EAB was tunable, PEG/EAB–PPPs have the potential to construct nanostructures of interest. Moreover, anti-bodies and site-specific ligands can also be introduced into this class of copolymer, thus the functional copolymers obtained would exhibit high potentials to generate multi-functional carriers for efficient drug delivery especially for tumor target.

2. Experimental

2.1. Materials

Hexachlorocyclotriphosphazene (Acros Organics) was purified by sublimation at 80–90 °C. PEG (Fluka) was azeotropic distilled with benzene before use. Aluminum chloride (99%) was purchased from Acros Organics and used without further purification. Doxorubicin hydrochloride (DOX·HCl) was kindly supplied by Juhua

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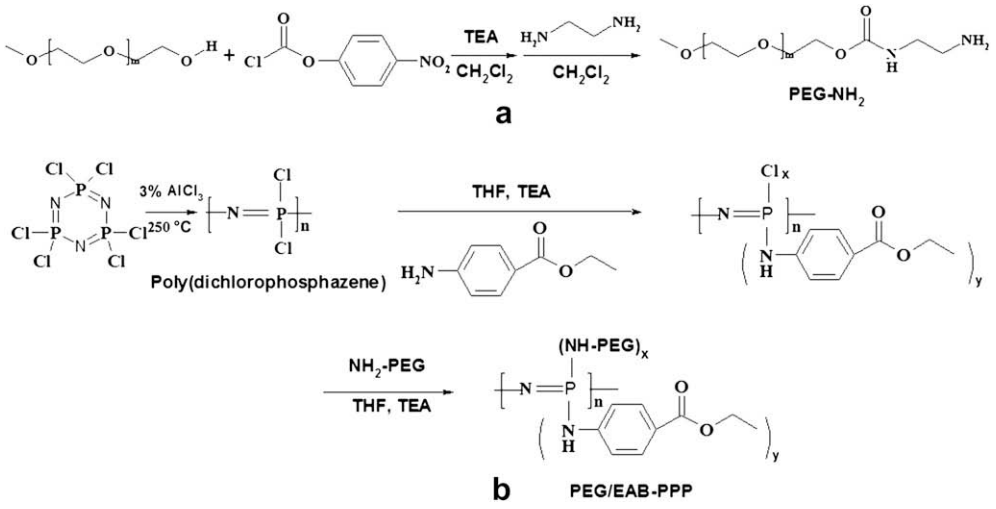

Scheme 1. Synthetic routes of (a) PEG–NH_2 and (b) PEG/EAB–PPP.

Group Pharmaceutical Factory (Zhejiang, China), and all other reagents were commercially available and used without further purification.

2.2. Synthesis of graft copolymer

The synthesis of PEG/EAB–PPPs graft copolymers involved the synthesis of a terminally functionalized PEG (Scheme 1a) and the final copolymers fabricated by substitution reaction between the terminal amino of PEG–NH_2/EAB and chloride atoms on poly(dichlorophosphazene) backbone which was prepared by a ring-open polymerization as illustrated in Scheme 1b. Poly(dichlorophosphazene) dissolved in THF solutions were firstly reacted with EAB, then excessive amount of PEG–NH_2 were added to make sure a completely substitution of chloride atoms on the backbone. The whole substitution procedures were conducted under extremely dry atmosphere and three kinds of copolymers with different chemical compositions were prepared by changing the feeding ratio of PEG/EAB, which were abbreviated as P-1, P-2 and P-3 respectively. The unreacted EAB and PEG–NH_2 were eliminated by precipitation in diethyl ether and dialysis against pure water respectively. More synthesize details can be found in our previous reports [8].

2.3. Drug encapsulation

Polymersomes for drug encapsulate were prepared by reversed emulsion and evaporation process. The polymer was first dissolved in chloroform (3 mg/ml), then an aqueous solution of ammonium sulfate (250 mM) was added into the polymer solution under agitation and the final water to oil ratio was 1:3. The mixture was sonicated until a stable emulsion appeared, afterwards, the chloroform was evaporated under vacuum and the aqueous solution obtained was dialyzed for 5 h. At the end, DOX·HCl was added and the solution was incubated for 1 h at 4 °C. Unloaded free DOX·HCl can be eliminated utilizing Sephadex G-50 Column and trehalose (5 wt%) was employed to keep drug carriers stable throughout the following freeze dry process.

2.4. Cell line and cytotoxicity evaluation

HepG2 cell lines were seeded in a 96 well culture plate and cultured for 24 h before polymersome addition. At predetermined time, the media were discarded and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added. The plates were incubated at 37 °C for 4 h. Then the intracellular metabolized product MTT formazan was retrieved by the addition of DMSO and incubation at room temperature for 10 min. The plates were read at 550 nm, and then the cell viability was calculated.

2.5. Characterization and measurements

^{1}H NMR spectra were recorded on an Avance DMX500 spectrometer using DMSO as the solvent at room temperature. The molecular weight of copolymers was determined by gel permeation chromatography (GPC) equipped with a Waters 515 HPLC Pump and a Waters 2410 refractive index detector, tetrahydrofuran was used as solvent with a flow rate of 1.5 ml/min at 40 °C and narrow disperse polystyrene as calibration standards. A UV–vis Spectrophotometer was used to determine the drug content and trace the drug release profile by monitoring the absorption at 483 nm. Confocal Laser Scanning Microscope (CLSM) images were taken with a Leica TCS SP Spectral Confocal Microscope (Leica, Germany). Transmission Electron Microscopy (TEM) images were obtained using a JEM 1230 operating at an acceleration voltage of 80 kV. The hydrodynamic diameter of micelles was determined by dynamic light scattering (DLS) (90 Plus Particle Size Analyzer, Brookhaven Instruments Co.). The scattering angle was kept at 90° and the wavelength in the vacuum was set as 633 nm during the whole experiment.

Table 1
Molecular characterizations of PEG/EAB–PPPs.

Polymer	PEG type	x[b]	y[b]	f_{PEG}[c] (w)	M_n	Mean particle size (nm)	PDI	Morphology
P-1	PEG_{2000}[a]	0.21	1.79	0.55	10 000	93	0.209	Micelle
P-2	PEG_{1100}	0.31	1.69	0.49	8960	305	0.196	Polymersome
P-3	PEG_{350}	0.54	1.46	0.40	6170	268	0.622	Polymersome

[a] The number means the molecular weight of PEG.
[b] Mole fraction of PEG and EAB on each repeat unit as shown in Scheme 1b ($x + y = 2.00$).
[c] The weight ratio of PEG in the copolymer which was calculated from ^{1}H NMR analysis.

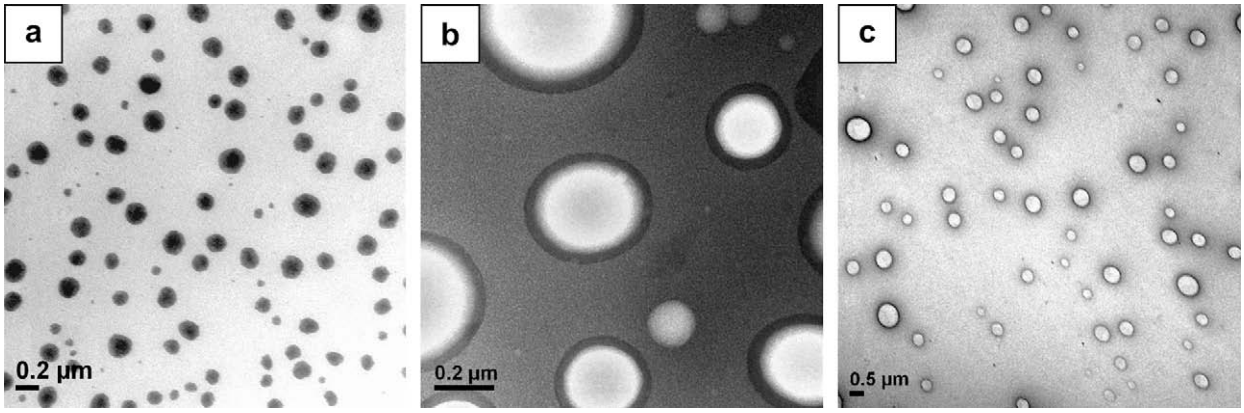

Fig. 1. TEM images of aggregates of (a) P-1, (b) P-2, and (c) P-3 in aqueous solution. The scale bars in (a) and (b) images represent 200 nm and the one in image (c) represents 500 nm.

3. Results and discussion

3.1. Polymer synthesis

PEG grafted polyphosphazenes have been previously studied by Sohn group [6], however, the drug carriers from their polymers are in hydrogel or solid precipitate form. Here, aiming to generate polymersomes from amphiphilic polyphosphazene copolymers, bulky structured EAB molecules were employed to enhance the rigidity of polyphosphazene backbones and three different PEG chains were used with molecular weight of 2000, 1100 and 350 respectively to regulate the weight fraction.

The formation of the expected graft copolymer was confirmed by ^{1}H NMR analysis. The weight ratio of PEG fraction can be calculated from ^{1}H NMR data by comparing the peak intensities of the methylene protons of the ethylene oxide units of PEG at 3.6 ppm to the methyl protons of EAB at 1.2 ppm (Table 1). Increasing molecular weight of PEG polymer resulted in the increase of PEG weight fraction f_{PEG} (w) in the resultant polymer.

$[NP(PEG)_x(EAB)_y]n$.

^{13}PNMR (DMSO): $\delta = 3.04$. ^{1}H NMR (DMSO): δ (ppm) = 3.5 (PEG, 4H, $-OCH_2CH_2$), 3.2 (PEG, 3H, $-OCH_3$), 7.2–7.6 (EAB, 4H, phenyl), 4.2 (EAB, 2H, $-OCH_2-$), 1.2 (EAB, 3H, $-CH_3$).

3.2. Self-assemble behavior

One of unique features of amphiphilic copolymers is their self-assemble behavior in water. In present work, the polymer aggregates in aqueous solution were prepared by precipitate-dialysis method using DMF. Self-aggregates were formed by dropping water to the DMF solution of copolymers, and the formation was completed by removal of DMF through extensive dialysis against water. The aggregate size of each copolymer was measured by DLS as shown in Table 1. The particle size suddenly increased from less than 100 nm to approximately 300 nm when the f_{PEG} (w) reduced from 0.55 to 0.49, particle size distributions are wild which can attribute to the polydisperse ($M_w/M_n > 2$) of the molecular weight of polyphosphazenes prepared by ring-open polymerization.

Fig. 1 shows the TEM images of the morphology of self-assemblies prepared using three different copolymers. TEM samples were prepared by dipping a TEM grid into a copolymer solution (1 wt%) and the extra solution was blotted with filter paper, polyphosphazene copolymers have good contrast, nanoparticles of polyphosphazenes can be directly observed as dark dots during TEM imaging [9], however, in this study polymersomes derived from polyphosphazenes are vesicle-shaped with thin bilayer structure, therefore negative staining was employed to enhance the contrast of TEM images. It can be seen from Fig. 1 that the graft copolymers with higher f_{PEG} (w) assembled into spherical micelles (Fig. 1a), while copolymers with f_{PEG} (w) of 0.49 and 0.40 formed larger vesicles (Fig. 1b and c). This hydrophilic fraction f related morphology transition similarly adhered to the principles established for amphiphilic diblock copolymers that decreasing f favors the vesicle formation. For diblock copolymers, the proper f generally falls in the range of 0.30–0.40 [10]. Besides, Lee [3] claimed that an f around 0.60 is the structure transition point of the graft copolymer based on poly(2-hydroxyethyl aspartamide). Indeed, the molecular structure of graft copolymer is much more complex than diblock copolymers. In this case, the bulky EAB molecules made the polyphosphazene backbones more rigid, which restricted the bending of backbone and facilitated the formation of bilayer structure. On the other hand, there are several hydrophilic PEG chains distributing on each backbone. They further confined the shrinkage of hydrophobic segments, which may explain the observed shift of structure transition point to around 0.50 in the case of graft polyphosphazene rather than 0.40 of traditional diblock copolymer, whose hydrophobic segment only conjuncted with one hydrophilic chain. Moreover, since the molecular weight and the degree of substitution (DS) are inevitably diverse, the differences in structure transition point between various graft copolymers are anticipated.

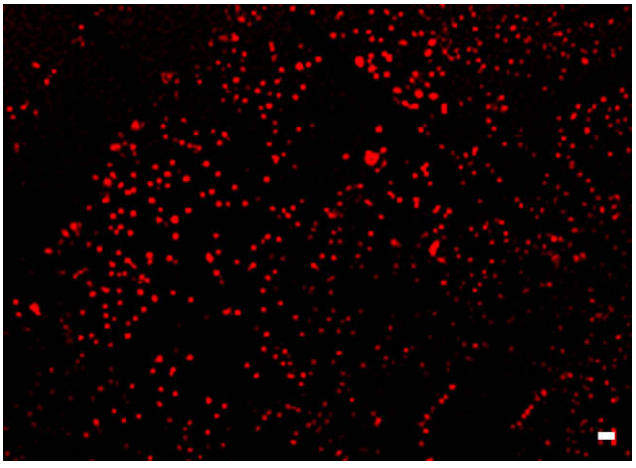

Fig. 2. CLSM image of DOX·HCL-loaded polymersomes. The scale bar is 5 μm.

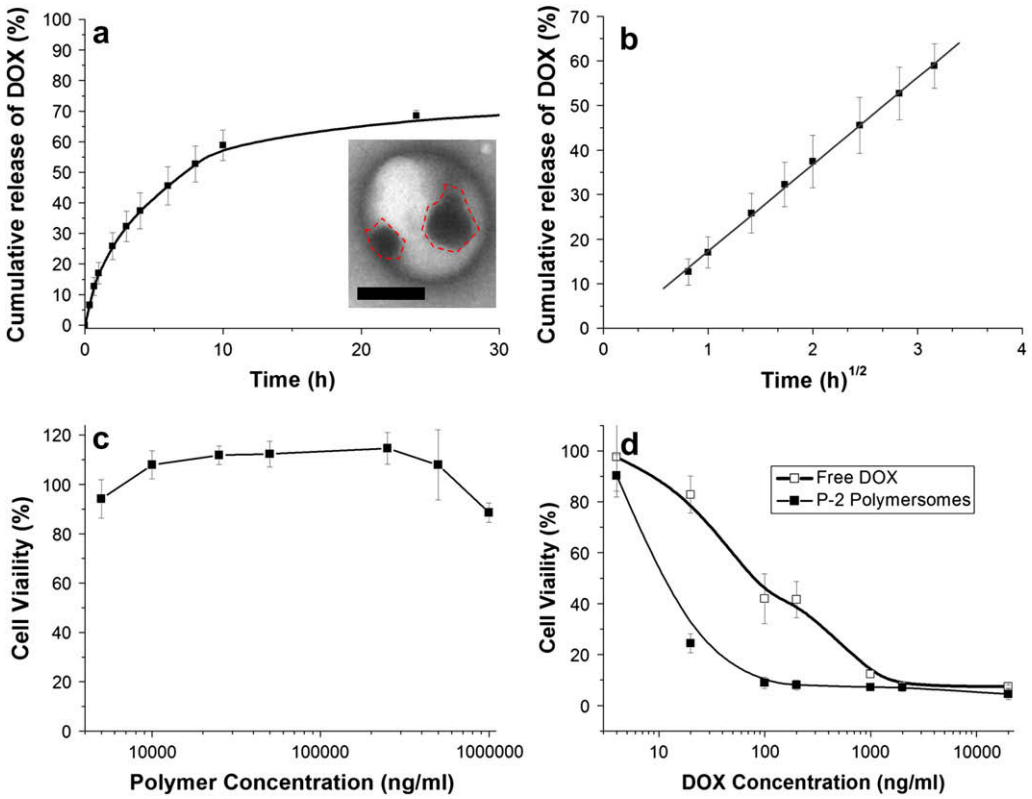

Fig. 3. (a) Drug release profile of DOX·HCl-loaded polymersomes from P-2 copolymer, insets is the TEM image of a single DOX·HCl-loaded polymersome (scale bar refers to 200 nm); (b) Higuchi model fitting of the in vitro drug release profile; (c) Cytotoxicity of P-2 copolymers only on HepG2 cells; (d) 48 h cytotoxicity of different DOX formulations on HepG2 cells.

3.3. Pharmaceutical studies

For drug delivery application, polymersomes with inner water core are suitable to be carriers of water-soluble drugs. Herein red fluorescence water-soluble anti-cancer drug DOX·HCl is encapsulated within preformed polymersomes by ammonium sulfate transmembrane gradients methods established for liposomes [11]. Sephadex G-50 Column is utilized to eliminate free DOX·HCl after encapsulation. And the drug loading content was found as 7.5% but the drug loading efficiency reached 87.2%. Then, the drug-loaded polymersomes are observed under confocal laser scanning microscope. We could observe drug-loaded polymersomes that are bright red dots as shown in the CLSM image (Fig. 2).

Drug release experiments were conducted using the dialysis method. Typically, 1.0 ml of polymersome solution in a concentration of 5 mg/ml was sealed in a dialysis bag (Spectrum MWCO 14 000) and was immersed in 20 ml of phosphate buffer solution (PBS) at pH 7.4 and incubated at 37 °C. This outside buffer solution was periodically withdrawn and replaced by fresh PBS, and then the released drug content was determined by UV–vis spectrometry from the sampling PBS. The in vitro release profiles of DOX·HCl from polymersomes based on amphiphilic polyphosphazenes is shown in Fig. 3a. The sustained release behavior was observed and in the middle stages of release curves from 10% to 60%. Drug release from micelles provided linear relationships for Higuchi plotting (Fig. 3b), indicating that Fickian diffusion played an important role during this release period and the drug burst release was effectively prevented in the beginning of drug release profile. In addition, DOX·HCl precipitates are visible in high-magnified TEM image of one drug-loaded vesicle in the inset of Fig. 3a, as observed previously with PEG-*b*-PLA polymersomes [12]. These phenomena further confirmed the drug encapsulation in the core of polymersomes but not adsorption on the polymersome surface.

One of the most intriguing aspects in this study is the cytotoxic effect of drug-loaded polymersomers. Fig. 3d shows a dose-dependent change in cytotoxicity of free DOX and polymersome formulation, which revealed that polymersome carriers dramatically enhanced the cytotoxicity of DOX against HepG2 cells, the IC50 value of free DOX is approximately 17-fold above the 9.6 ng/ml of DOX-loaded P-2 polymersome. This result is noticeable considering the fact that the copolymer itself does not exhibits significant cell proliferate suppression even in a very high concentration (Fig. 3c), suggesting that present polymeric carrier not only can encapsulate DOX but also can improve the therapeutic index of DOX, and drug concentration required for efficient treatment therefore may be reduced.

4. Conclusion

In summary, the results demonstrate that graft polyphosphazenes are able to construct polymersome, which can be employed as new vehicles for water-soluble drug delivery. To the best of our knowledge, this work represents the first polymersome system based on amphiphilic graft polyphosphazenes. It also emphasized that the hydrophilic ratio of copolymer was essential to accomplish polymersome morphology and enriched new experiment data to graft copolymer self-assembly theory. In addition the pilot pharmaceutical studies revealed that this class of copolymers exhibit high potentials to act as new vehicles for water-soluble drug delivery. The further study on in vivo biodistribution and anti-cancer effect of drug-loaded polymersomes is under going now.

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