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Y-shaped block copolymer (methoxy-poly(ethylene glycol))$_2$-b-poly(L-glutamic acid): preparation, self-assembly, and usage as drug carriers

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Y-shaped amphiphilic block copolymers, (methoxy-poly(ethylene glycol))$_2$-block-poly(L-glutamic acid) ((mPEG)$_2$-PGA) and its precursor (methoxy-poly(ethylene glycol))$_2$-block-poly(γ-benzyl-L-glutamate) ((mPEG)$_2$-PBG), were prepared in three steps: (1) Macroinitiator (methoxy-poly(ethylene glycol))$_2$-NH$_2$ ((mPEG)$_2$-NH$_2$) was synthesized by coupling two methoxy-poly(ethylene glycol)s with serinol and diisocyanate. (2) (mPEG)$_2$-PBG was synthesized by ring opening polymerization of γ-benzyl-L-glutamate-N-carboxyanhydride initiated with the macroinitiator ((mPEG)$_2$-NH$_2$); (3) The protective benzyl groups in (mPEG)$_2$-PBG were removed to obtain (mPEG)$_2$-PGA. The properties of both (mPEG)$_2$-PBG and (mPEG)$_2$-PGA were characterized by $^1$H NMR, FT-IR, GPC, and DLS. In aqueous solution (mPEG)$_2$-PBG tends to form more stable micelles compared to linear mPEG-PBG copolymer. The size of (mPEG)$_2$-PBG decreases with increasing length of hydrophobic PBG in (mPEG)$_2$-PBG. Paclitaxel and cisplatin were grafted onto (mPEG)$_2$-PGA to form (mPEG)$_2$-PGA-PTX (MPTX) with a grafting ratio of near 90 % and (mPEG)$_2$-PGA-Pt (MPt) conjugates with a loading efficacy of 15% (w/w). MPTX can greatly improve the solubility of PTX. Both conjugates can self-assemble into micelles with a mean diameter of about 50 nm and show enhanced anti-cancer activity against MCF-7, HeLa, and SMMC cell lines. The in vivo anticancer evaluation in mice shows MPt showed a desirable antitumor activity and deduced the system toxicity. Therefore, both MPTX and MPt have a great potential as a polymer drug in cancer chemotherapy.

Introduction

Poly(ethylene glycol)-containing amphiphilic block copolymers (PEG-PE), such as copoly(carbonate (PEG-PC), copoly(amo acid) (PEG-PAA) have been synthesized and employed as drug carriers$^{1-3}$. With the use of these polymeric drug carriers, it is possible to effectively prolong the circulation time of clinical anti-cancer drugs, to shield the recognition by immune system, to escape the capture of reticuloendothelial system (RES), and to accumulate at tumor site via the enhanced permeability and retention (EPR) effect$^{4,5}$. Among these polymer carriers, linear diblock or triblock copolymers have been extensively investigated and some of them have entered clinical trials and shown encouraging clinical results$^6$.

Recently, thanks to the development of synthetic methods, such as atom transfer radical polymerization (ATRP), reversible addition fragmentation chain transfer (RAFT) and “click chemistry” reactions, and so on, miktoarm amphiphilic block copolymers have been designed and synthesized such as mPEG-(PLA)$_n$ ($n=1, 2$ or $4$; mPEG: mPEG$_{2k}$ or mPEG$_{4k}$; PLA: atactic or isotactic) to evaluate the architecture and chemical composition effect on the micelles formation and stability. Compared to other copolymers, mPEG$_{2k}$-(PD,LLA)$_2$ was able to form monodisperse and stable micelles$^10$. Sun et al. investigated the effect of solvent and the relative length of the PBG on the self-assembly of Y-shaped copolymer, poly(L-lactide)$_2$-b-poly(γ-benzyl-L-glutamate). At a fixed composition of copolymer, the copolymer formed a transparent gel in toluene, while became homogeneous dispersion containing nano-scale fibrous aggregates in benzyl alcohol$^{11}$.

In the past few decades, linear amphiphilic PEG-PAA copolymers, mostly PEG-b-poly(aspartic acid), PEG-b-poly(glutamic acid) (PEG-PGA), and PEG-b-poly(lysine)
have been successfully used to physically encapsulate or chemically bond anti-cancer drugs, such as cisplatin, oxaliplatin, paclitaxel and doxorubicin, etc.  

Compared with other drug carriers, PEG-PAAs have their specific features: (1) Many amino acids can be chosen and available easily; (2) Several amino acids contain functional groups and special functionalization is not needed; (3) Some amino acids can exist in different charge states such as neutral in protected form and positive or negative in deprotected form; (4) Amino acids have different isoelectric potentials and their solubility is pH dependent; (5) Chiral products (mPEG)PGAγPTX, XYOTAX selfγassemble behaviors and drug delivery, it can be concluded that PGAγPt were synthesized. The selfγassembly behaviors of (PDI) of the polymers were determined by size exclusion chromatography (SEC), which was performed with a Waters 1525 fitted with two columns (Styragel HT3 and HT4, 7.8 × 300 mm), with THF as mobile phase at a flow rate of 1.0 mL/min and an operating temperature of 35 °C. PS standards were used for calibration. Fourier transform infrared (FTIR) spectra were recorded on a Bio-Rad Win-IR instrument using potassium bromide (KBr) method. The size of micelles was measured by DLS measurements carried out with a DAMN EOS instrument equipped with a He-Ne laser at the scattering angle fixed at 90°. The micelle solution of about 0.5 mg/mL in water was filtered through a 0.45 µm of filter membrane before measurement. The morphologies of the nanoparticles were confirmed by using transmission electron microscopy (TEM) measurement on a JEOL JEM-1011 transmission electron microscope with an accelerating voltage of 100 kV. A drop of the nanoparticle solution (1 mg/mL) was deposited onto a 230-mesh copper grid coated with carbon, and was air-dried at room temperature.

Preparation of 2-(tert-butoxycarbamino)-1,3-propanediol (N-Boc-Serinol, NBS)

2-(tert-butoxycarbamino)-1,3-propanediol (N-Boc-Serinol) was synthesized according to the reference. Briefly, serinol (2-amino-1,3-propanediol) (9.1g, 0.1 mol) was dissolved in EtOH (90 ml), and then diγtertγbutyl dicarbonate (23.9 g, 0.11 mol) was dropped. The solution was stirred for 1 hour at room temperature, and the solvent was removed under reduced pressure. The yellow solid was recrystallized from hexane/ EtOAc to give NBS as 17.1 g of white solid (yield: 83 %).  

H NMR (400 MHz, DMSO): δ= 5.25 (1H, N=NH). A typical synthetic procedure for (mPEG)2γPBG was added. The solution was stirred at 70 °C under nitrogen for 1 hour, and then NBS (0.29 g, 1.5 mmol) was added. The solution was stirred for 2 h and then precipitated in cold ether. The precipitate was collected and dried to obtain 14.5 g white product (mPEG2γPBG (yield: 85%).

Synthesis of macroinitiator mPEG5k-NH2–mPEG5k γNH2 (m(mPEG)2γPBG)

Dried mPEG5k (15.0g, 3 mmol) and HDI (0.53 g, 3.15 mmol) were added into a dried flask, 20ml 1,4-dioxacyclohexane and 0.1 g Sn(Oct)2 were added. The solution was stirred at 70 °C under nitrogen for 1 hour, and then NBS (0.29 g, 1.5 mmol) was added. The solution was stirred for 2 h and then precipitated in cold ether. The precipitate was collected and dried to obtain 14.5 g white product (m(mPEG)2γPBG) (yield: 91.7 %). To a 20 ml solution of (mPEG)2γNHBoc (10.0 g) in dichloromethane, 20 ml trifluoroacetic acid was added. The solution was stirred in ice bath for 1 h and poured into cold ether. The precipitate was filtered out and dried. The product was dissolved in water and neutralized by saturated aqueous sodium bicarbonate. The pH value of the solution was adjusted to 7-8. The solution was dialyzed against water using a dialysis bag (MWCO 3500 g/mol) for 2 days and frozen-dried to obtain 8.5 g of white product (m(mPEG)2γNHBoc) (yield: 85%).

Synthesis of Y-shaped copolymer mPEG5k-PBG–mPEG5k γNHBoc–mPEG5k γNHBoc (m(mPEG)2γPBG)

Three kinds of (mPEG5kγPBG with different PBG lengths, i.e., (mPEG5kγPBG, (mPEG5kγPBG, and (mPEG5kγPBG, were prepared by theROP offL-NCA initiated by (mPEG5kγNHBoc). A typical synthetic procedure for (mPEG5kγNHBoc–PBG was as follows: Dried (mPEG5kγNHBoc (3.0 g) as a macroinitiator and BLC-NCA (1.5 g) were dissolved in 5 ml dry DMF. The solution was stirred at 25 °C for 72 h and then precipitated in cold ether. The precipitate was collected and...
Synthesis of (methoxy-poly(ethylene glycols))<sub>2</sub>-b-poly(L-glutamic acid) (mPEG<sub>35</sub>-PGA)<sup>5</sup>

(mPEG<sub>35</sub>)<sub>2</sub>-PBG (1.5 g) was dissolved in a mixed solution of 6 ml of CF<sub>3</sub>COOH and 2 ml of 33 wt% HBr/acetic acid. The solution was stirred at room temperature for 2.5 h and poured into excessive ether. The precipitate was filtered out and dried to obtain white product (mPEG<sub>35</sub>)<sub>2</sub>-PGA.<

**Synthesis of (mPEG<sub>35</sub>)<sub>2</sub>-PGA-PTX prodrug (MPTX)**

To a 20 ml solution of (mPEG<sub>35</sub>)<sub>2</sub>-PGA<sub>35</sub> (200 mg, 0.012 mmol) and PTX (70 mg, 0.08 mmol), DCC (105 mg, 0.51 mmol) and DMAP (30 mg, 0.25 mmol) were added. The solution was stirred at room temperature for 8 h, and then precipitated in a mixture of cold ether/ethanol. The product was collected and dried. PTX content in the prodrug was determined by <sup>1</sup>H NMR. The PTX content and its graft efficacy were calculated according to the following formula: PTX (wt%) = (weight of PTX/weight of prodrug) × 100% and PTX graft efficiency (wt%) = (weight of PTX in prodrug/weight of PTX in feed) × 100%, respectively.

**Synthesis of (mPEG<sub>35</sub>)<sub>2</sub>-PGA-Cisplatin prodrug (MPT)**

Cisplatin (150 mg, 0.5 mmol) was dissolved into 20 ml water, and then AgNO<sub>3</sub> (170 mg, 1.0 mmol) was added, the solution was stirred under dark for 8 h. To the clear solution obtained by removal of the white precipitate formed via filtration, was added (mPEG<sub>35</sub>)<sub>2</sub>-PGA<sub>35</sub> (300 mg, 0.018 mmol). The solution was stirred under dark for 8 h, and then dialyzed against water using a dialysis bag (MWCO 3500 g/mol) for 1 day and frozen-dried to obtain 450 mg of white product MPT.

**Cell culture**

MCF-7, HeLa, and SMMC cell lines were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China and were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma), and the culture medium was replaced once every day.

**In vitro MTT assay**

MCF-7 cells harvested in a logarithmic growth phase were seeded in 96-well plates at a density of 10<sup>3</sup> cells/well and incubated in RPMI 1640 for 24 h. The medium was then replaced by various drug formulations of PTX, (mPEG<sub>35</sub>)<sub>2</sub>-PGA<sub>35</sub>, and (mPEG<sub>35</sub>)<sub>2</sub>-PGA<sub>35</sub>-PTX. To test the cytotoxicity of the free drug carrier, just (mPEG<sub>35</sub>)<sub>2</sub>-PGA<sub>35</sub> was used to treat the cells and its amount was equal to that used in (mPEG<sub>35</sub>)<sub>2</sub>-PGA<sub>35</sub>-PTX. All of the drugs with PTX were modulated to a series of final equivalent PTX concentrations ranging from 0.005 to 50 µg/ml. The incubation of each drug formulation was continued for 48 h or 72 h. Then, 20 µL of MTT solution of 5 mg/ml in PBS was added and the plates were incubated for another 4 h at 37 °C, followed by removal of the culture medium containing MTT and addition of 150 µL of DMSO to each well to dissolve the formazan crystals formed. Finally, the plates were shaken for 10 minutes, and the absorbance of formazan formed was measured at 492 nm by a microplate reader. The cell viability against HeLa and SMMC lines was determined by the similar way.

**In vivo evaluation**

**Animal model preparation.**

Chinese Kunming (KM) female mice were obtained from Jilin University, China (56–84 days old, 20–25 g in weight) and maintained under required conditions. All animal studies were conducted in accordance with the principles and procedures outlined in “Regulations for the Administration of Affairs Concerning Laboratory Animals”, approved by the National Council of China on October 31, 1988, and “The National Regulation of China for Care and Use of Laboratory Animals”, promulgated by the National Science and Technology Commission of China, on November 14, 1988. To develop the tumor xenografts, H22 cells were injected into the lateral aspect of the right anterior limb of the mice (5×10<sup>6</sup> cells in 0.1 ml PBS). After the tumor volume reached 100–200 mm<sup>3</sup>, the hair of the mice was removed with a sodium sulfide solution (80 g/L in 30 vol% aqueous alcohol).

**Anti-tumor efficacy**

Thirty two KM mice bearing H22 tumor nodules were randomly divided into four groups for (a) normal saline (control); (b) cisplatin, 3 mg Pt/kg; (c) MPT, 3 mg Pt/kg and (d) MPT, 6 mg Pt/kg. Before injection, all the mice were marked and weighed, and the length and width of the tumors were measured as the initial size on day 1. The day of the first injection was designated as day 1. For groups (b) to (d), designed doses of Pt were intravenously injected via tail vein on day 1, day 3, and day 5, separately. For group (a), the mice were injected with equivalent volume of normal saline. The tumor size was measured every other day and the tumor volume (V) was calculated by the formula of V=ab<sup>2</sup>/2, where a and b were the length and width of tumor, respectively.

**Statistics**

The data were expressed as the mean ± standard deviation (SD). The Student's t-test was used to determine the statistical difference between various experimental and control groups. Differences were considered statistically significant at a level of p < 0.05.

**Results and Discussion**

**Synthesis of Y shaped amphiphilic copolymers**

In order to prepare Y shaped amphiphilic block polymer, that is, methoxy-poly(ethylene glycol)-block-poly(γ-benzyl-L-glutamate) ((mPEG<sub>35</sub>)<sub>2</sub>-PBG), two main synthetic strategies were adopted. One is that the coupling reaction between isocyanate group and hydroxyl group is always adopted to
prepare copolymers containing accurate structure, such as hydrogels and drug carriers \textsuperscript{19,20}. The other is the ring-opening polymerization (ROP) of corresponding amino acid NCA monomers initiated by primary amines. Various polyethylene glycol-containing copolypeptides with well controlled degrees of polymerization (DP) have been synthesized by this way \textsuperscript{21,22}, which helps to the preparation of the accurate structure of drug carriers.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{\textsuperscript{1}H NMR spectra of (mPEG\textsubscript{3k})\textsubscript{2}-NHBoc (A) and (mPEG\textsubscript{3k})\textsubscript{2}-NH\textsubscript{2} (B) in CDCl\textsubscript{3}.}
\end{figure}

In this work, three Y-shaped (mPEG\textsubscript{3k})-PBG and (mPEG\textsubscript{3k})-PGA copolymers with different PBG or PGA lengths were prepared in three steps as shown in Scheme 1: (1) Synthesis of macroinitiator (mPEG\textsubscript{3k})\textsubscript{2}-NH\textsubscript{2}. Briefly, mPEG\textsubscript{3k} was reacted with HDI to form mPEG\textsubscript{3k}-NCO, then two

\begin{align*}
&\text{(1)} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{OH} + \text{HDI} \quad \xrightarrow{75^\circ C} \quad \text{O} \quad \text{N} \quad \text{C} \\
&\text{mPEG\textsubscript{3k}} \quad \text{NCO} + \\
&\text{HOC} \quad \text{H} \quad \text{O} \quad \text{NHBoc}
\end{align*}

(2)

\begin{align*}
&\text{mPEG\textsubscript{3k}} \quad \xrightarrow{15.7 \text{ min}} \quad \text{mPEG\textsubscript{3k}} \quad \text{NH} \\
&\text{HOC} \quad \text{H} \quad \text{O} \quad \text{NHBoc}
\end{align*}

(3)

\begin{align*}
&\text{mPEG\textsubscript{3k}} \quad \xrightarrow{15.7 \text{ min}} \quad \text{mPEG\textsubscript{3k}} \quad \text{NH} \\
&\text{HOC} \quad \text{H} \quad \text{O} \quad \text{NHBoc}
\end{align*}

Scheme 1 Synthetic routes of (mPEG\textsubscript{3k})\textsubscript{2}-NH\textsubscript{2}, (mPEG\textsubscript{3k})\textsubscript{2}-PBG and (mPEG\textsubscript{3k})\textsubscript{2}-PGA.

The structures of (mPEG\textsubscript{3k})-NHBoc and (mPEG\textsubscript{3k})\textsubscript{2}-NH\textsubscript{2} were confirmed by \textsuperscript{1}H NMR and FT-IR. The \textsuperscript{1}H NMR spectra of (mPEG\textsubscript{3k})\textsubscript{2}-NHBoc and (mPEG\textsubscript{3k})\textsubscript{2}-NH\textsubscript{2} are shown in Fig. 1. As shown in Fig. 1A, peaks a at 3.38 ppm and b at 3.62 ppm are assigned to the protons of CH\textsubscript{2}O and -OCH\textsubscript{2}CH\textsubscript{2}O units of mPEG of (mPEG\textsubscript{3k})\textsubscript{2}-NHBoc. The peaks marked with c (3.14 ppm), d (1.47 ppm) and e (1.32 ppm) are assigned to the protons of methylene units of HDI. Peak h at 1.42 ppm is assigned to -OH of (CH\textsubscript{2})\textsubscript{3} unit in NBS. After the removal of the protecting group (Boc) in (mPEG\textsubscript{3k})-NHBoc, peak h at 1.42 ppm disappeared completely in Fig. 1B, indicating successful synthesis of macroinitiator (mPEG\textsubscript{3k})\textsubscript{2}-NH\textsubscript{2}.

FT-IR spectra of (mPEG\textsubscript{3k})\textsubscript{2}-NHBoc and (mPEG\textsubscript{3k})\textsubscript{2}-NH\textsubscript{2} are shown in Fig. 2B and 2C. Compared to that of mPEG5k in Fig. 2A, the peaks at 1721 cm\textsuperscript{-1} (C=O stretching) and 1533 cm\textsuperscript{-1} (amide II mode) in (mPEG\textsubscript{3k})\textsubscript{2}-NHBoc appeared and the peak at 3475 cm\textsuperscript{-1} (OH stretching) disappeared, indicating successful synthesis of (mPEG\textsubscript{3k})\textsubscript{2}-NHBoc. After the removal of Boc group in (mPEG\textsubscript{3k})\textsubscript{2}-NHBoc, the peak at 3352 cm\textsuperscript{-1} appeared in Fig. 3C and was assigned to free NH\textsubscript{2} group of (mPEG\textsubscript{3k})\textsubscript{2}-NH\textsubscript{2}.

The structure of (mPEG\textsubscript{3k})\textsubscript{2}-NHBoc can also be proved by GPC. As shown in Fig. 3 and Table 1, mPEG\textsubscript{3k} and mPEG\textsubscript{10k} gave GPC peaks at 17.1 and 15.7 min, respectively, while that of (mPEG\textsubscript{3k})\textsubscript{2}-NHBoc was at 15.7 min, close to that of mPEG\textsubscript{10k}. This is understandable, because (mPEG\textsubscript{3k})\textsubscript{2}-NHBoc is two (mPEG\textsubscript{3k})\textsubscript{2} coupled with an HDI-seritol-HDI linkage. Of course, the molecular weight values determined by GPC were not exactly 5k or 10k, but their two-fold relationship did exist (8.4 vs. 16.4 kg/mol, Table 1). Moreover, the unimodal peak shape of (mPEG\textsubscript{3k})\textsubscript{2}-NHBoc and the narrow distribution (PDI=1.02) in Fig.
Fig. 3 The GPC traces of mPEG3k (A), PEG10k (B), (mPEG3k)2-NHBoc (C) and (mPEG3k)2-PBG35 (D).

Table 1 GPC results of mPEG3k, PEG10k, (mPEG3k)2-NHBoc, mPEG3k-PBGs and (mPEG3k)2-PBGs

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mn (kg/mol)</th>
<th>PDI</th>
<th>Mn* (kg/mol)</th>
<th>DP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPEG3k</td>
<td>8.44</td>
<td>1.02</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>mPEG10k</td>
<td>16.2</td>
<td>1.02</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>(mPEG3k)2-NHBoc</td>
<td>16.4</td>
<td>1.02</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>(mPEG3k)2-PBG14</td>
<td>14.8</td>
<td>1.03</td>
<td>11.7</td>
<td>13/12</td>
</tr>
<tr>
<td>(mPEG3k)2-PBG20</td>
<td>12.7</td>
<td>1.08</td>
<td>12.5</td>
<td>19/19</td>
</tr>
<tr>
<td>(mPEG3k)2-PBG35</td>
<td>15.5</td>
<td>1.05</td>
<td>14.1</td>
<td>32/31</td>
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<tr>
<td>mPEG3k-PBG14</td>
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<td>1.05</td>
<td>6.7</td>
<td>13/12</td>
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<tr>
<td>mPEG3k-PBG20</td>
<td>12.6</td>
<td>1.06</td>
<td>7.2</td>
<td>17/12</td>
</tr>
<tr>
<td>mPEG3k-PBG35</td>
<td>17.5</td>
<td>1.07</td>
<td>9.1</td>
<td>32/32</td>
</tr>
</tbody>
</table>

a) The molecular weight determined by 1H NMR.

b) The degree of polymerization (DP) of PBG and PGA was calculated by 1H NMR.

c) The degree of polymerization (DP) of HNBr 33 wt% in acetic acid was used as a deprotection reagent to remove the benzyl groups in (mPEG3k)2-PBG35. After deprotection, the peaks at 5.03 and 7.25 ppm assigned to the benzyl group in PBG disappeared almost completely in Fig. 4B, indicating complete removal of the protective groups.

The degree of polymerization (DP) of BLG in (mPEG3k)2-PBG can be calculated by comparing the relative intensities of peak l and peak b in Fig. 4A. The DP of PGA in (mPEG3k)2-PGA is calculated by a similar way. The DP data of PBG and PGA are listed in Table 1. It is clear that the DP of PBG of (mPEG3k)2-PBG is well in accordance with feed composition, indicating almost complete conversion of BLG-NCA during the ROP of BLG-NCA. The little DP reduction of (mPEG3k)2-PBG after deprotection demonstrates that CF3-COOH/HBr/acetic acid has no harmful effect on the degradation of the main chain of PBG segment of (mPEG3k)2-PBG. Relatively, the molecular weight obtained by GPC is larger than that determined by 1H NMR (table 1), but Y-shaped copolymers shows low correction factor, and linear copolymer gives higher results. This result indicated the difference of various topologies.

Fig. 4 1H NMR spectra of (mPEG3k)2-PBG35 in CDCl3 (A) and (mPEG3k)2-PGA35 (B) in CDCl3/CF3COOD (v/v, 1:1).

The FT-IR spectra of (mPEG3k)2-PBG35 and (mPEG3k)2-PGA35 are shown in Fig. 2D and 2E. In comparison with that of (mPEG3k)2-NH2 in Fig. 2C, the peaks at 3297 cm⁻¹, 1651 cm⁻¹ and 1549 cm⁻¹ appeared and they are characteristic of NH stretching and amide I and amide II modes of PBG, respectively, indicating the formation of the polypeptide block. Moreover, the absorption peaks at 750 cm⁻¹ and 700 cm⁻¹ are attributed to the phenyl groups in the PBG block. The disappearance of these two peaks from Fig. 4E implies successful removal of the benzyl groups, and the red shift of the peak (C=O stretching) from 1734 cm⁻¹ to 1727 cm⁻¹ and the blue shift of the peak (amide I mode) from 1651 cm⁻¹ to 1655 cm⁻¹ in Fig. 2E with respect to those in Fig. 2D can be ascribed to the enhanced hydrogen bonding between repeat units in PGA of (mPEG3k)2-PGA35. The molecular weight of (mPEG3k)2-PBG was characterized by GPC. As shown in Fig. 3 and Table 1, the GPC of (mPEG3k)2-PBG35 (Curve D) showed even less retention time compared to that of (mPEG3k)2-NHBoc (Curve C) and the Mw of (mPEG3k)2-PBGs thus obtained were in the range of 12.7~15.5 kg/mol, indicating different hydrodynamic behaviors of them from that of linear PEG counterparts. Nevertheless, the PDIs of (mPEG3k)2-PBGs calculated from GPC profiles were less than 1.08 (Table 1),...
indicating absence of side reactions and approximately living polymerization of BLG-NCA under the polymerization conditions employed. In conclusion, Y-shaped block copolymers (mPEG<sub>5k</sub>γPBG) and (mPEG<sub>5k</sub>γPGA) were successfully synthesized.

**Self-assembly of copolymers in aqueous solution**

A steady-state fluorescent spectroscopic method using pyrene as a probe was used to study self-assembly of linear and Y-shaped copolymers in aqueous solution. It is well known that the CMC values reflect the stability of micelles, i.e., a low CMC value means a high stability of micelles. It was reported that linear mPEG<sub>5k</sub>-PBGs with the different molar ratio of PBG (14.9 to 4.8%) self-assembled into micelles in water, their CMC values ranged from 7.8 to 19.9 mg/L. As listed in Table 2, the CMC values of (mPEG<sub>5k</sub>γPBG) are in the range from 4.1 to 4.9 mg/L, close to those of mPEG<sub>5k</sub>-PBGs, indicating that (mPEG<sub>5k</sub>γPBG) can be a competent drug carrier.

**Table 2 Parameters of linear and Y-shaped micelles**

<table>
<thead>
<tr>
<th>Copolymers</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>CMC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mPEG&lt;sub&gt;5k&lt;/sub&gt;γPBG&lt;sub&gt;14&lt;/sub&gt;)</td>
<td>123</td>
<td>80</td>
<td>0.15</td>
</tr>
<tr>
<td>(mPEG&lt;sub&gt;5k&lt;/sub&gt;γPBG&lt;sub&gt;20&lt;/sub&gt;)</td>
<td>117</td>
<td>82</td>
<td>0.16</td>
</tr>
<tr>
<td>(mPEG&lt;sub&gt;5k&lt;/sub&gt;γPBG&lt;sub&gt;35&lt;/sub&gt;)</td>
<td>99</td>
<td>75</td>
<td>0.18</td>
</tr>
<tr>
<td>mPEG&lt;sub&gt;5k&lt;/sub&gt;γPBG&lt;sub&gt;14&lt;/sub&gt;</td>
<td>108</td>
<td>75</td>
<td>0.17</td>
</tr>
<tr>
<td>mPEG&lt;sub&gt;5k&lt;/sub&gt;γPBG&lt;sub&gt;20&lt;/sub&gt;</td>
<td>87</td>
<td>60</td>
<td>0.18</td>
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<tr>
<td>mPEG&lt;sub&gt;5k&lt;/sub&gt;γPBG&lt;sub&gt;35&lt;/sub&gt;</td>
<td>88</td>
<td>62</td>
<td>0.16</td>
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From Table 2, it can be seen that the size of (mPEG<sub>5k</sub>γPBG) is two times that in mPEG<sub>5k</sub>-PBG; resulting in a larger size of micelles. Comparatively, the size of mPEG<sub>5k</sub>-PBG and (mPEG<sub>5k</sub>γPBG) determined by TEM is smaller than those determined by DLS because of the micelle shrinkage during TEM sample preparation. The size of (mPEG<sub>5k</sub>γPBG) ranges from 75 nm to 82 nm, and that of mPEG<sub>5k</sub>-PBG is in the range from 60 to 62 nm. This is in agreement with DLS measurements.

**Synthesis and characterization of (mPEG<sub>5k</sub>γPGA-PTX and (mPEG<sub>5k</sub>γPGA-Cisplatin conjugates**

Polymer produgs based on mPEG-PGA has been widely investigated and some of these polymer produgs have entered preclinical studies. These polymer produgs can enhance the solubility of hydrophobic drugs, such as paclitaxel, camptothecin, prolong the circulation time of loaded drugs, reduce system toxicity caused by anti-cancer small molecule drugs, and improve the quality of patients’ lives. In this work, for further exploring the potential of (mPEG<sub>5k</sub>γPGA-PTX on the field of polymer produgs, Paclitaxel (PTX) and Cisplatin (Pt) were selected as model drugs and loaded into (mPEG<sub>5k</sub>γPGA to prepare (mPEG<sub>5k</sub>γPGA-PTX and (mPEG<sub>5k</sub>γPGA-Pt conjugates, the in vitro and in vivo anti-cancer activities of them were further investigated.

Paclitaxel (PTX) is widely used in treatment of advanced and refractory ovarian, breast, lung, and head and neck cancers. Because of its low water solubility, it is usually administrated in the dose form of Taxol, i.e., a suspension in Cremophor EL (a polyoxyethylene castor oil / anhydrous ethanol (1:1) solution). Cremophor EL leads to severe side effects and lowers the antitumor efficacies of PTX. In order to overcome these side effects, polymer–drug conjugates have been developed. For example, poly(L-glutamic acid)–paclitaxel conjugate (PGA-PTX, XYOTAX<sup>TM</sup> CT2103), now under phase III clinical trials, presents improved water solubility, self-assembles into ~80 nm nanoparticles in aqueous solution, and shows enhanced anti-cancer activity and targeted accumulation at tumor sites with the help of both cathepsin B and the enhanced permeability and retention (EPR) effect, which improve the efficacy of chemotherapy and reduce the side effects as compared to free Taxol. Considering that PEG has merits of good water solubility, non-immunogenicity, chain mobility and long blood circulation time through prevention of renal elimination and avoidance of receptor-mediated protein uptake by cells of the reticuloendothelial system (RES), PTX was conjugated to (mPEG<sub>5k</sub>γPGA for the preparation of (mPEG<sub>5k</sub>γPGA-PTX conjugates. The structure, morphology, and in vitro anti-cancer efficacy of (mPEG<sub>5k</sub>γPGA-PTX were further investigated.

By virtue of the reactivity of hydroxyl groups in the 2′ position of PTX, it was conjugated onto (mPEG<sub>5k</sub>γPGA with the help of DCC and DMAP to form an ester linkage. The structure of (mPEG<sub>5k</sub>γPGA-PTX was characterized by <sup>1</sup>H NMR. As shown in Fig. 6, the <sup>1</sup>H NMR spectrum (6A) of (mPEG<sub>5k</sub>γPGA-PTX contains all feature peaks of PTX (6B) and (mPEG<sub>5k</sub>γPGA-PTX (6C) except that of the proton (4.80 ppm) at the 2′ position of PTX, indicating successful synthesis of...
(mPEG)₂-PGA-PTX. The content of PTX in (mPEG₃₅k)₂-PGA₃₅-PTX is about 20%, and the coupling efficiency of PTX was calculated to be 90% by using the relative integral areas of the protons of benzyl groups of PTX between 7.34 and 8.03 ppm with respect to that of the protons of -CH₂CH₂O- units of mPEG at 3.53 ppm. Similarly, (mPEG)₂-PGA-Pt conjugate was prepared by virtue the coordination of the carboxyl groups in PGA with respect to that of the protons of γC_H conjugate was determined by ICP-MS to be 15% (w/w). The Pt content in the conjugate was determined by ICP-MS to be 15% (w/w).

The cytotoxicity of MPTX, and MPt against MCF-7, HeLa and SMMC cell lines was evaluated by MTT assay in comparison with that of (mPEG)₂-PGA₃₅, PTX, and Pt. As shown in Fig. 8, the cell viability of (mPEG₃₅k)₂-PGA₃₅ treated cells was higher than 90% no matter at 48 or 72 h, indicating very low toxicity of (mPEG)₂-PGA as a drug carrier material. As shown in Fig. 9, the cell viability showed obvious concentration dependence and cell line dependence for PTX, MPTX, Pt, and MPt. Based on the concentration dependence, IC₅₀ was calculated and collected in Table 3. Firstly, given a formulation, IC₅₀ of MPTX varied from less than 0.005 μg/ml for MCF-7 to 1.24 μg/ml for HeLa cells, approximately corresponding to that of pure PTX; while IC₅₀ of MPt varied from 9.11 μg/ml for MCF-7 to 1.76 μg/ml for SMMC cells, approximately in parallel to that of pure Pt. This is understandable, because every anti-cancer drug is not effective to all cancers. Secondly, for each cell line tested, MPTX and MPt displayed comparable or a little bit less cytotoxicity than pure PTX and Pt, respectively. Because MPTX and MPt as nano-micelles have other advantages over PTX and Pt, e.g., reduced systemic toxicity, this comparable cytotoxicity implies possibility for them to be used as effective drug delivery systems.

Fig. 6 ¹H NMR spectra of (mPEG₃₅k)₂-PGA₃₅-PTX (A) in DMSO/CF₃COOD (v/v, 1:1), PTX in CDCl₃ (B) and (mPEG₃₅k)₂-PGA₃₅ (C) in CDCl₃/CF₃COOD (v/v, 1:1)

(mPEG₃₅k)₂-PGA₃₅-PTX and (mPEG₃₅k)₂-PGA₃₅-Pt were allowed to self-assemble into nano-micelles (MPTX and MPt) in aqueous solution. TEM micrographs in Fig. 7A and 7B revealed the spherical shape of the micelles formed. The average particle size was about 50 nm for MPTX and about 45 nm for MPt. It implies that although PGA is hydrophilic to some extent, the conjugate PGA₂-PTX or PGA₂-Pt as a whole is indeed hydrophobic. Therefore, the mPEG segments forms the outer shell and the hydrophobic PGA-PTX or PGA-Pt segment forms the core of micelles. To confirm this structure, the frozen-dried MPTX micelles were re-dissolved in D₂O for ¹H NMR measurement. As shown in Fig. 7C, the peak at 3.64 ppm assigned to the protons of -CH₂CH₂O- units remained, while the characteristic signals associated with the PGA-PTX segment were all absent.

Fig. 7 TEM images of (mPEG₃₅k)₂-PGA₃₅-PTX (A) and (mPEG₃₅k)₂-PGA₃₅-Pt (B) particles. (C) ¹H NMR spectrum of frozen-dried (mPEG₃₅k)₂-PGA₃₅-PTX micelles in D₂O.

In vitro Antitumor activity of MPt

Cisplatin (abbr. as Pt) is a first line anti-cancer drug for treatment of many malignancies, such as ovarian, bladder, non-small-cell lung and liver cancers. H22 is a well-known cell line obtained from human liver carcinoma. In this work, H22 bearing Kunming

Fig. 8 In vitro cytotoxicity of (mPEG₃₅k)₂-PGA₃₅ for 48 h and 72 h against MCF-7 cell line.

In vivo Antitumor activity of MPt

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mice were treated i.v. three times at 2-day intervals at the same dose of 3 mg and 6 mg Pt/kg for cisplatin and MPt. The relative tumor volume (RTV) vs. time and the relative body weight vs. time are shown in Fig. 10. As shown in Fig. 10A, the RTV increment of four groups took an order of Saline >> MPt (3 mg Pt/kg) > Pt (3 mg Pt/kg) > MPt (6 mg Pt/kg). For example, on the 14th day, the RTV values were 343.9±25.2, 81.5±10.7, 34.7±8.0, and 16.9±3.8, respectively. Notably, increasing the dose of MPt from 3 mg Pt/kg to 6 mg Pt/kg led to enhanced anti-cancer efficacy while similar increase in cisplatin dose caused death of all test mice on the 4th day post first injection, indicating the lower systemic toxicity of MPt than cisplatin. This conclusion was supported by the body weight measurement. As shown in Fig. 10B, the relative body weight took an order of Saline >> MPt (3 mg Pt/kg) > MPt (6 mg Pt/kg) > Pt (3 mg Pt/kg) on day 14.

**Table 3** IC\(_{50}\) values of MPTX, PTX, MPt, and Pt against MCF7, HeLa, and SMMC cell lines

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC(_{50}) (µg/ml)</th>
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<tr>
<td>MPTX</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>PTX</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>MPt</td>
<td>9.11</td>
</tr>
<tr>
<td>Pt</td>
<td>5.28</td>
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</table>

**Fig. 9** MTT assay results at 48 h: (A) MPTX and PTX for MCF7; (B) MPTX and PTX for HeLa; (C) PTX and PTX for SMMC; (D) Pt and cisplatin for MCF7; (E) Pt and cisplatin for HeLa; (F) MPt and Pt for SMMC.

**Fig. 10** Relative tumor volume (A) and relative body weight (B) of the H22 cancer bearing mice as a function of time. (a) saline; (b) cisplatin (3 mg Pt/kg); (c) MPt (3 mg Pt/kg); (d) MPt (6 mg Pt/kg)

**Conclusions**

A novel strategy for preparing Y-shaped amphiphilic (mPEG)\(_2\)PBG copolymer containing two hydrophilic arms and one hydrophobic arm was successfully developed, that is, to prepare (mPEG)\(_2\)PBG by using a macroinitiator with a middle primary amino group (mPEG)\(_2\)-NH\(_2\) to initiate the ROP of γ-benzyl-L-glutamate-N-carboxyanhydride. Due to its amphiphilic nature, (mPEG)\(_2\)-PBG can self-assemble into micelles and can encapsulate hydrophobic drugs. After removal of the benzyl groups on the PBG segment, the copolymer became (mPEG)\(_2\)-PGA containing free carboxyl groups. By virtue of these carboxyl groups, (mPEG)\(_2\)-PGA can self-assemble into micelles and can encapsulate hydrophobic drugs. Therefore, polymer nano-prodrug based on (mPEG)\(_2\)-PGA has a potential in the treatment of solid-tumors.

**Acknowledgements**

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Notes and References


