

Functional Amphiphilic Poly(2-oxazoline) Block Copolymers as Drug Carriers: the Relationship between Structure and Drug Loading Capacity

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Electronic Supplementary Information

Abstract Poly(2-oxazoline) (POx) is a kind of polymeric amides that can be viewed as conformational isomers of polypeptides with excellent cyto- and hemo-compatibility, and is promising to be used as drug carriers. However, the drug loading capacity (DLC) of POx for many drugs is still low except several hydrophobic ones including paclitaxel (PTX). Herein, we prepared a series of amphiphilic POx block copolymers with various functional groups, and investigated the relationship between functional structures and the DLC. Functional POxs with benzyl, carboxyl, and amino groups in the side-chain were synthesized based on a poly(2-methyl-2-oxazoline)-block-poly(2-butyl-2-oxazoline-co-2-butenyl-2-oxazoline) (PMeOx-*P*(*n*BuOx-co-ButenOx), PMBEOx) precursor, followed by click reaction between vinyl and the 2-phenylethanethiol, thioglycolic acid and cysteamine. Using thin-film hydration method, eight commonly used drugs with various characteristics were encapsulated within these functional POx polymers. We found that amine-containing drugs were more easily encapsulated by POx with carboxyl groups, while amine functionalities in POx enhanced the loading capacity of drugs with carboxyl groups. In addition, π - π interactions resulted in enhanced DLC of most drugs, except several hydrophobic drugs with aromatic to total carbon ratios less than 0.5. In general, we could successfully encapsulate all the selected drugs with a DLC% over 10% using properly selected functional POxs. The above results confirm that the DLC of polymeric carriers can be adjusted by modifying the functional groups, and the prepared series of functional POxs provide an option for various drug loadings.

Keywords Poly(2-oxazoline); Polymeric micelles; Drug loading; π - π Interaction; Electrostatic interaction

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INTRODUCTION

Nanocarriers based on polymers have been investigated for decades to improve the delivery efficiency of drugs with poor water solubility.^[1–6] There are two major methods for preparing polymeric nanomedicines: covalent conjugation and physical encapsulation.^[7–14] Covalent conjugation is a common method for loading hydrophobic drugs, which links drugs and polymers together through covalent bonds. The covalent bonds between the drugs and the polymers can be designed to be cleavable at desired stimulus conditions,^[15,16] and the drug release rate is determined by the dissociation rate of the formed covalent bonds. Different from covalent conjugation, physical encapsulation can preserve the original forms of drugs, and is more widely considered as a potential strategy in the loading of

poorly water-soluble drugs for clinical use due to its simple preparation process. An ideal drug carrier should possess several desirable characteristic features such as high drug loading capacity (DLC, >10 wt%), high encapsulation stability, long blood circulation, and so on. However, most of currently applied polymers for physical encapsulation of drugs are still unsatisfactory,^[17] and there is still a need in developing new materials or tailoring the chemical structures of drug carriers for personalized drug loading.

Poly(2-oxazoline)s (POxs) have been investigated for decades as biomaterials due to excellent cyto- and hemo-compatibility^[18] and superior immune camouflage properties.^[19,20] The possibility of realizing tailor-made properties by manipulating functional groups on the side chains of 2-oxazoline monomers makes POx a promising candidates for satisfying various biomedical needs.^[21] Kabanov *et al.* described a high drug loading tri-block polymeric nanomicelle based on poly(2-methyl-2-oxazoline)-block-2-butyl-2-oxazoline-block-2-methyl-2-oxazoline) (P(MeOx-*b*-BuOx-*b*-MeOx)).^[22] As a result of the polar and hydrated amide func-

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tionality in each repeating unit, P(MeOx-*b*-BuOx-*b*-MeOx) presented a unique polar/hydrophobic interaction with drugs. This polymeric micelle system exhibited an extraordinary high encapsulate capacity of up to 50 wt% for paclitaxel (PTX). Besides PTX, docetaxel, etoposide, and bortezomib could also be highly efficiently encapsulated in the P(MeOx-*b*-BuOx-*b*-MeOx) (with DLC% over 27.9 wt%, 26.6 wt%, and 23.8 wt%, respectively).^[23,24] However, the loading capability of P(MeOx-*b*-BuOx-*b*-MeOx) to many other drugs is poor. For example, the DLC% of P(MeOx-*b*-BuOx-*b*-MeOx) to aclacinomycin A and wortmannin were both less than 5 wt% (4.6 wt% and 1 wt%), while the DLC% to imiquimod and SN38 were even less than 1 wt%.^[25] Thus, there is still much room for improving the capability of POx for wide application in drug encapsulation.

Several recent studies have proved that introducing specific functional groups into polymeric drug carriers can improve the DLC and the stability by enhancing the interactions between carriers and the loaded cargos. Electrostatic interactions,^[26,27] hydrogen bonding,^[28] and π - π stacking^[29] have been used as the driving forces to improve the encapsulation capability of various drugs. For example, Li *et al.* reported that anionic polymer methoxy poly(ethylene glycol)-block-poly(L-glutamic acid) (mPEG-*b*-PLG) could form complex with cationic drug doxorubicin (DOX) and the DLC% could increase to 32.1 wt% with drug loading efficiency of almost 100%.^[30] Lv *et al.* reported an amphiphilic anionic copolymer methoxy poly(ethylene glycol)-block-poly(L-glutamic acid-co-L-phenylalanine) (mPEG-*b*-P(Glu-co-Phe)) for DOX encapsulation and obtained a DLC% of 21.7 wt% through both electrostatic and π - π interactions.^[31] Shi *et al.* synthesized an amphiphilic block copolymer comprising the aromatic monomer *N*-2-benzoyloxypropyl methacrylamide as a hydrophobic building block. The π - π interaction significantly increased the stability, loading capacity, and therapeutic index of drug-loaded polymeric micelles.^[32] Besides, electronic donor-acceptor coordination has also been used for enhancing the DLC of drugs. Lv *et al.* synthesized an amphiphilic copolymer decorated with pendant phenylboronic acid as electron acceptor unit, which constructed donor-acceptor coordination with primary amines containing drugs like DOX, and resulted in an ultrahigh DLC.^[33] These non-covalent interactions provide a new direction for improving the DLC and stability by introducing polymer-drug interactions.

Herein, we synthesized a series of functional amphiphilic POx copolymers for various drug loadings and evaluated the relationships between the introduced functional groups and the DLC. The fundamental copolymer PMBEOx was synthesized by a living cationic ring-opening polymerization (CROP) of three monomers 2-methyl-2-oxazoline, 2-butyl-2-oxazoline, and 2-(3-butenyl)-2-oxazoline, and the functional groups (including benzyl, carboxyl, and amino groups) were introduced into the polymer *via* a thiol-ene “click” reaction. The DLC against eight drugs of different structures were evaluated.

EXPERIMENTAL

Materials and Methods

Valeric acid, ethyl chloroformate, triethylamine (TEA), bis(2-

chloroethyl)amine hydrochloride, potassium *tert*-butoxide, allylactic acid and ethyldiisopropylamine (DIPEA) were purchased from Sigma-Aldrich Co., Ltd. (Shanghai, China). PTX and DOX were purchased from Beijing Huafeng United Technology Co., Ltd. (Beijing, China). Celecoxib, tranilast and imiquimod were purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). BLZ945 was purchased from Shanghai Bixi Chemical Co., Ltd. (Shanghai, China). Olaparib was purchased from Liverpool ChiroChem Co., Ltd. (Taizhou, China). Obeticholic acid was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Taizhou, China). *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropionophenone (Irgacure 2959, I2959), cysteamine, thioglycolic acid, 2-phenylethanethiol, methyl trifluoromethylsulfonate (MeOTf) and 2-methyl-2-oxazoline (MeOx) were purchased from Energy Chemical Co., Ltd. (Beijing, China). All other reagents and solvents were obtained from Sinopharm Group Co., LTD. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV 300 NMR spectrometer at room temperature. The spectra were calibrated using the solvent signals (CDCl₃ 7.26 ppm, DMSO-*d*₆ 2.50 ppm). Fourier transform infrared spectroscopy (FTIR) was recorded on a Bio-Rad Win-IR instrument using KBr method. Raman spectrum were obtained from Nicolet 960 Raman Spectrometer. Gel permeation chromatography (GPC) analyses were conducted on a Waters 2414 system equipped with Ultrahydrogel™ linear column and a Waters 2414 refractive index detector (eluent: 1 mol/L NaNO₃ aqueous; eluent: 1.0 mL/min; temperature: 35 °C, standard: poly(ethylene glycol)). High performance liquid chromatography (HPLC) was equipped with a Waters e2695 HPLC system, Waters 2487 two-channel fluorescence detector and a Symmetry C18 column (Waters, Milford, MA, USA).

Monomer Synthesis

Synthesis of *N*-(2-chloroethyl)pentanamide (**1a**)

A solution of valeric acid (25.0 g, 244.8 mmol) and TEA (54.5 g, 538.5 mmol) in tetrahydrofuran (THF) (300.0 mL) was cooled to 0 °C. After a dropwise addition of ethyl chloroformate (25.6 g, 235.5 mmol), the reaction solution was slowly warmed to room temperature and stirred for 1 h. After that, the mixture was cooled to 0 °C again, 2-chloroethylamine hydrochloride (34.1 g, 293.7 mmol) was added into the mixture and stirred for another 12 h. The solvent was evaporated by rotary evaporator, and the residue was dissolved in dichloromethane and washed by water for 3 times. The solution was dried over sodium sulfate and the solvent was evaporated. The product **1a** was obtained as a dark brown oily liquid (17.2 g, 105.1 mmol, yield: 45.1%). ¹H-NMR (CDCl₃, 300 MHz, δ , ppm) 3.70 (4H, s, CH₂CH₂N), 2.25 (2H, t, CH₂CO), 1.65 (2H, m, CH₂CH₂CO), 1.47 (2H, m, CH₃CH₂), 0.92 (3H, t, CH₃CH₂).

Synthesis of 2-butyl-2-oxazoline (**1b**, BuOx)

N-(2-chloroethyl)pentanamide (**1a**, 13.6 g, 83.4 mmol) was dissolved by 50.0 mL dry THF. A solution of potassium *tert*-butoxide (12.2 g, 108.4 mmol) in 80.0 mL THF was slowly added under a nitrogen atmosphere. The reaction mixture was stirred at 70 °C for 48 h. Then, the salt was removed by filtration and the solvent was evaporated. The product **1b** was purified by distilling under vacuum to obtain colorless liquid (6.5 g, yield: 62.4%). ¹H-NMR (CDCl₃, 300 MHz, δ , ppm) 0.94 (3H, t, CH₂CH₃),

1.38 (2H, m, CH₂CH₃), 1.63 (2H, m, CH₂CH₂CH₃), 2.31 (2H, t, CH₂CH₂CH₂CH₃), 3.81 (2H, t, CH₂N), 4.24 (2H, t, CH₂O). ¹³C-NMR (CDCl₃, 300 MHz, δ, ppm) 13.71 (CH₂CH₃), 22.15 (CH₂CH₂CH₃), 27.55 (CH₂CH₂CH₃), 28.09 (CH₂CH₃), 54.32 (CH₂N), 67.21 (CH₂O), 168.11 (OC=N). ESI MS: *m/z*: calculated 127.1, found 127.8 and 129[M+H⁺].

Synthesis of *N*-(2-chloroethyl)-4-pentenamide (**2a**)

Allylacetic acid (20.0 g, 199.8 mmol), NHS (34.5 g, 300.0 mmol), and EDC (46.0 g, 239.6 mmol) were dissolved in 250.0 mL of dry dichloromethane in a dry flask. The mixture was stirred for 2 h at room temperature. Meanwhile, 100 mL dichloromethane solution of bis(2-chloroethyl) amine hydrochloride (46.4 g, 400.0 mmol) and DIPEA (56.8 g, 439.5 mmol) was prepared and added dropwisely into the flask above. After that, the reaction mixture was stirred at room temperature under a nitrogen atmosphere for 12 h. After reaction, the mixture was washed for 3 times with water, the organic layer was dried over sodium sulfate, and the solvent was evaporated to give the product **2a** as yellowish oil (18.2 g, yield: 56.6%). ¹H-NMR (CDCl₃, 300 MHz, δ, ppm) 2.23–2.38 (4H, m, CH₂CH₂O), 3.65 (4H, m, CH₂CH₂Cl), 4.92 (2H, m, CH₂=CHCH₂), 5.75–5.83 (1H, m, CH₂=CH), 6.16 (1H, bs, NHCO).

Synthesis of 2-(3-butenyl)-2-oxazoline (**2b**, ButenOx)

2a (17.5 g, 100 mmol) and potassium *tert*-butoxide (14.5 g, 130 mmol) were dissolved in dry THF. Then the mixture was allowed to stir at 60 °C under a nitrogen atmosphere for 48 h. After filtering salt and evaporating the solvent, the product **2b** was purified by distilling under vacuum to obtain colorless oil (10.1 g, yield: 72.6%). ¹H-NMR (CDCl₃, 300 MHz, δ, ppm) 2.41 (4H, s, CH₂CHCH₂CH₂), 3.84 (2H, t, CH₂N), 4.24 (2H, tr, OCH₂), 5.05 (2H, m, CH₂=CHCH₂), 5.79–5.93 (1H, m, CH₂=CH). ¹³C-NMR (CDCl₃, 300 MHz, δ, ppm) 27.17 (CH₂CHCH₂CH₂), 30.11 (CH₂CHCH₂), 54.12 (CH₂N), 66.91 (CH₂O), 115.39 (CH₂CHCH₂), 136.54 (CH₂CH), 167.1 (OC=N). ESI MS: *m/z*: calculated 125.08, found 125.9 and 126.9[M+H⁺]. IR: ν_{C-H}: 3120 cm⁻¹, ν_{C=C}: 1650 cm⁻¹.

Synthesis of Polymers

Synthesis of PMeOx₇₀-*b*-P(*n*BuOx₁₇-*co*-ButenOx₁₀) (PMBEOx)

Prior to the reaction, the polymerization flask was dried by heating under vacuum for at least 1 h. A solution of methyl trifluoromethylsulfonate (32.0 mg, 0.2 mmol, MeOTf) in dry acetonitrile (10.0 mL) was prepared under dry argon. 2-Methyl-2-oxazoline (1.2 g, 14.0 mmol, MeOx) was added into the solution at room temperature and stirred at 70 °C for 24 h under an argon atmosphere. Once the mixture was cooled to the room temperature, the monomers for the second block, 2-butyl-2-oxazoline (**1b**, 432.0 mg, 3.4 mmol, BuOx) and 2-(3-butenyl)-2-oxazoline (**2b**, 250.0 mg, 20.0 mmol, ButenOx), were added and the mixture was stirred at 70 °C for another 48 h. The resulting solution was cooled to room temperature and treated with methanolic NaOH (1 mol/L) to terminate the reaction. The product were purified *via* dialysis for 2 days against distilled water and then recovered by lyophilization. Yield: 1.7 g, 73.2%.

Synthesis of thioglycolic acid-grafted PMBEOx (PMBEOx-COOH), 2-phenylethanethiol-grafted PMBEOx (PMBEOx-Ph) and cysteamine-grafted PMBEOx (PMBEOx-NH₂)

PMBEOx (910.0 mg, 1.0 mmol C=C) was dissolved in 10.0 mL of dry DMF in a quartz bottle, and argon was bubbled through the solution for 10 min. Thioglycolic acid (1.8 mg, 20.0 mmol) and

Irgacure 2959 (0.2 mg, 1.0 mmol) were added into the mixture, and argon was kept bubbling for 20 min. The reaction mixture was then sealed and subjected to 365 nm ultraviolet irradiation for 60 min. The product was purified *via* dialysis for 2 days against distilled water and then PMBEOx-COOH was recovered by lyophilization. Yield: 640.2 mg, 69.8%.

PMBEOx-Ph and PMBEOx-NH₂ were synthesized according to a similar protocol described above by using 2-phenylethanethiol and cysteamine to replace thioglycolic acid, respectively. The isolated yields were 72% (PMBEOx-Ph) and 68% (PMBEOx-NH₂), respectively.

Drug Encapsulation

Drug loaded POx micelles were prepared using thin-film method. In brief, drugs and polymers were solubilized in common volatile solvent. The solution was mixed with a designed drug polymer ratio and solvent was removed to obtain a thin solid film. The film was then hydrated with deionized (DI) water to give a micellar solution. Insoluble drug was removed *via* filtration (0.22 μm pore size). Only the transparent supernatant was used for the following experiments.

Drug encapsulation with thin-film method

Appropriate amounts of PTX (2.0 mg) and polymer (8.0 mg) were solubilized in minimum amounts of ethanol and mixed thoroughly. The ethanol was removed by the rotary evaporator (45 °C, 20 min). The dried film was re-dissolved in proper amounts of 5.0 mL DI water and heated at 45 °C for 5 min. Insoluble PTX was removed *via* filtration (0.22 μm pore size).

Tranilast (drug/POx: 2.0/8.0 mg), celecoxib (drug/POx: 2.0/8.0 mg), olaparib (drug/POx: 2.0/8.0 mg), BLZ945 (drug/POx: 2.0/8.0 mg), obeticholic acid (drug/POx: 2.0/8.0 mg), imiquimod (drug/POx: 2.0/8.0 mg), and DOX (hydrochloride firstly removed with TEA, drug/POx: 2.0/8.0 mg) were encapsulated in a similar protocol to PTX, except for imiquimod and olaparib dissolved in acetonitrile.

Drug loading content evaluated with HPLC

The DLC% of most drugs was evaluated with HPLC. The sample was diluted using mobile phase and injected (20.0 μL) into the HPLC system. For PTX, celecoxib, BLZ945, imiquimod, olaparib, tranilast, a mixture of acetonitrile /water (80/20, V/V) was used as mobile phase. Detection wavelength and retention time were 227 nm and 3.5 min for PTX, 254 nm and 4.3 min for celecoxib, 250 nm and 3.2 min for BLZ945, 245 nm and 6.4 min for imiquimod, 265 nm and 2.7 min for olaparib, 330 nm and 2.8 min for tranilast, respectively. For obeticholic acid, the mobile phase was a mixture of 0.1 wt% *o*-phosphoric acid aqueous solution and acetonitrile (80/20, V/V), the detection wavelength was 190 nm and the retention time was 8.3 min.

Drug loading content evaluated with ultraviolet-visible (UV-Vis) spectroscopy

The DLC% of DOX was analyzed on UV-Vis spectroscopy. The sample aqueous solution was mixed with DMF (1/1, V/V), and UV-Vis absorption spectra were measured from 200 nm to 500 nm with a 1 nm step. The detection wavelength was 490 nm. Loaded drug concentration was quantified against corresponding free drug calibration curves.

DLC and Drug loading efficiency (DLE) calculations

Following equations were used to calculate DLC and DLE of

various drugs in drug-loaded POx micelles:

$$\text{DLC}(\%) = \frac{M_{\text{drug}}}{M_{\text{drug}} + M_{\text{excipient}}} \times 100\% \quad (1)$$

$$\text{DLC}(\%) = \frac{M_{\text{drug}}}{M_{\text{drug feed}}} \times 100\% \quad (2)$$

M_{drug} and $M_{\text{excipient}}$ were the mass of the solubilized drugs and polymers in the solution, respectively. $M_{\text{drug feed}}$ was the amount of drugs feed in the preparation of the micelle formulation.

Size and Stability Measurement

The hydrodynamic diameters of the obtained micelles were determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS. Briefly, each sample was diluted with DI water to a final concentration of 1 mg/mL before measurement. The morphologies of the obtained micelles were measured by transmission electron microscope (JEOL JEM-1011) with an accelerating voltage of 100 kV.

In Vitro Cytotoxicity Assay

The murine breast cancer 4T1 cells were used to carry out the *in vitro* studies. 4T1 cells were cultured with Roswell Park Memorial Institute (RPMI) 1640 (containing 10% fetal bovine serum (FBS), 50 U/mL penicillin and 50 U/mL streptomycin) and incubated at 37 °C with 5% CO₂.

Four POxs, namely PMBEOx, PMBEOx-Ph, PMBEOx-COOH, and PMBEOx-NH₂, were compared using 4T1 cell line. Briefly, cells were seeded in 96-well plates at a density of 8000 cells/well for 12 h prior to POxs treatment. Cells were treated for 24 h or 48 h with respective POxs each prepared at a series of dilutions in the full medium. The absorbance of each well was measured at 490 nm on a Bio-Rad 680 microplate reader. The relative cell viability (%) was determined through comparing the absorbance values of sample wells with those of control wells.

Hemolysis Assay

The hemolytic activities of the four POxs were tested using rabbit red blood cells (rRBCs). Fresh rabbit blood was washed with phosphate buffered saline (PBS) for three times and the collected rRBCs were diluted to 4% with PBS at pH=7.4. Four POxs were diluted to concentrations ranging from 31.25 µg/mL to 1000 µg/mL in PBS by a two-fold gradient dilution, using 1% Triton X-100 in PBS as the positive control. After mixing an equal volume of rRBCs suspension and POxs solution, the 96-well plate was incubated at 37 °C for 2 h. PBS was used as the blank. After centrifugation at 2000 r/min for 5 min, 80 µL of the

supernatant in each well was transferred to another 96-well plate and the OD values were collected to calculate the percentage of hemolysis from the Eq. (3) as follows, and plotted against polymer concentration to give the dose-response curves of hemolysis.

$$\text{Hemolysis}(\%) = \frac{A_{405}^{\text{polymer}} - A_{405}^{\text{blank}}}{A_{405}^{\text{control}} - A_{405}^{\text{blank}}} \times 100\% \quad (3)$$

RESULTS AND DISCUSSION

Synthesis of 2-Butyl-2-oxazoline and 2-(3-Butenyl)-2-oxazoline

Typically 2-oxazolines are synthesized *via* direct synthesis from non-activated carboxylic acids with Wenker method.^[34] The hydrophobic monomer BuOx (**1b**) was synthesized in a three-step reaction, starting from the commercially available valeric acid (Fig. 1a). In the first step, the valeric acid was activated by ethyl chloroformate. Then the activated acid reacted with 2-chloroethylamine hydrochloride in the presence of TEA yielding intermediate compounds **1a**. In the last step, the monomer **1a** was obtained by reacting with potassium *tert*-butoxide. The chemical structure of the final product was confirmed by ¹H-NMR (Fig. S1 in the electronic supplementary information, ESI), ¹³C-NMR (Fig. S2 in ESI) and electrospray-ionization spectrum (ESI, Fig. S3 in ESI).

Since the vinyl groups can be employed as a versatile group in transforming into many functional groups such as benzyl, carboxyl and amine *via* thiol-ene click reaction. We used ButenOx (**2b**) as the precursor functionalized monomer to synthesize functionalized POxs. ButenOx (**2b**) was synthesized in a two-step reaction, and started from the commercially available allylacetic acid (Fig. 1b). The first step included an activation of the 4-pentenoic acid with EDC and NHS. The activated acid was then reacted with 2-chloroethylamine hydrochloride in the presence of DIPEA to form the amide **2a**. Cyclization was achieved in the presence of potassium *tert*-butoxide to give the final product **2b**. The chemical structure of the final product was confirmed with ¹H-NMR (Fig. S4 in ESI), ¹³C-NMR (Fig. S5 in ESI), FTIR (Fig. S6 in ESI) and ESI (Fig. S7 in ESI).

Synthesis and Characterization of PMBEOx

The well-defined amphiphathic di-block copolymer PMBEOx was prepared by living CROP. An electrophile can initiate the polymerization by the formation of an oxazolinium species. Because of a weakened CO-bond, the “activated” monomer is

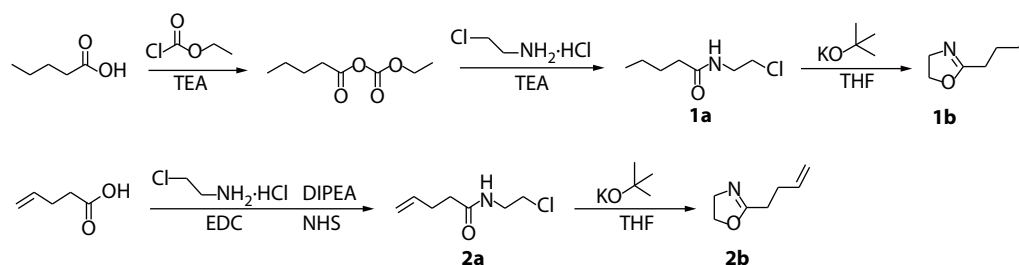


Fig. 1 Synthesis routes to 2-butyl-2-oxazoline (**1b**) and 2-(3-butenyl)-2-oxazoline (**2b**).

prone to undergo a nucleophilic attack by the nitrogen atom of another monomer. The resulting propagating species is growing either as long as the monomer is available or until the addition of a nucleophile to the reaction mixture, which terminates the polymerization (Fig. 2a).

Here we choose methyl trifluoromethylsulfonate (MeOTf) as the initiator, and methanolic NaOH (1 mol/L) as the terminator. Since the polymerization is a living cationic polymerization, the molecular weight of POx products can be precisely controlled by adjusting the molar ratio of the monomers to initiators. In the polymerization of hydrophilic block PMeOx, the initial concentrations of monomer and initiator were $[M]_{\text{Me}}=1.40$ mol/L and $[I]=0.02$ mol/L, respectively. For the hydrophobic block P(BuOx-co-ButenOx), the initial concentrations of the monomers were $[M]_{\text{Bu}}=0.40$ mol/L and $[M]_{\text{Buten}}=0.22$ mol/L. The degree of polymerization of MeOx, BuOx and ButenOx in the obtained copolymers was determined via the analysis of $^1\text{H-NMR}$ spectrum (Fig. 2b). The number of repeating units of PMBEOx was determined according to the peak integration ratio of the methylene protons on the polymer backbone (i, $\delta=3.2\text{--}3.5$ ppm) and the methyl protons of the methyl group (h, $\delta=3.10$ ppm) at the end of the polymer chain. Meanwhile, the composition of the hydrophobic block P(*n*BuOx-co-ButenOx) was analyzed by comparing the peak integration ratio of the methyl protons in BuOx unit (a, $\delta=0.95$ ppm) or the methylene protons in ButenOx unit (j, $\delta=4.78$ ppm) to the methyl protons of the methyl group (h, $\delta=3.10$ ppm) at the end of the polymer chain. Based on the integration ratio between i, a, j and h, the degrees of polymerization of MeOx, BuOx, and ButenOx were calculated to be 70, 17, and 10, and the corresponding transformation rates were 100%, 85% and 91%, respectively. GPC was also

used to evaluate the obtained POx. As shown in Fig. 2(c), the monomodal and quite symmetric elution curve proved the polymer was successfully synthesized, and polydispersity index (PDI, M_w/M_n) of PMBEOx was 1.39.

Synthesis and Characterization of PMBEOx-Ph, PMBEOx-COOH, and PMBEOx-NH₂

Photo-initiated thiol-ene click chemistry was utilized for adjusting the side chain of PMBEOx with benzyl, carboxyl, or amino groups. PMBEOx reacted with 2-phenylethanethiol, thioglycolic acid or cysteamine with Irgacure 2959 as the photoinitiator at room temperature, respectively (Fig. 3).

The structures of the obtained PMBEOx-Ph, PMBEOx-COOH, and PMBEOx-NH₂ were confirmed by $^1\text{H-NMR}$, FTIR, Raman and GPC. As shown in Fig. 4(a), complete disappearance of the proton signals was attributed to the double bond (j, $\delta=4.78$ ppm; k, $\delta=5.75$ ppm), the appearance of the characteristic proton signals was attributed to the 2-phenylethanethiol, thioglycolic acid, and cysteamine (PMBEOx-Ph: o, $\delta=7.34$ ppm; PMBEOx-COOH: q, $\delta=2.67$ ppm; PMBEOx-NH₂: t, $\delta=3.21$) demonstrated the successful and complete modification to the side chains of PMBEOx. As shown in Fig. 4(b), all the elution curves of the polymers were monomodal and quite symmetric, and PDI of PMBEOx-Ph, PMBEOx-COOH, and PMBEOx-NH₂ were 1.37, 1.40, and 1.41, respectively. The FTIR spectra proved the successful synthesis of PMBEOx-Ph and PMBEOx-COOH as characteristic C—H contraction vibration of phenyl group and carboxyl group at 707 and 1718 cm^{-1} appeared (Fig. 4c). As for PMBEOx-NH₂ (HCl), a clearly broad absorption peak at 2500–3200 cm^{-1} could be observed in the FTIR spectrum, which demonstrated the existence of NH₂. The disappearance of the vinyl double bond signal (1610 cm^{-1}) in the Raman spectrum of PMBEOx and modified POxs also

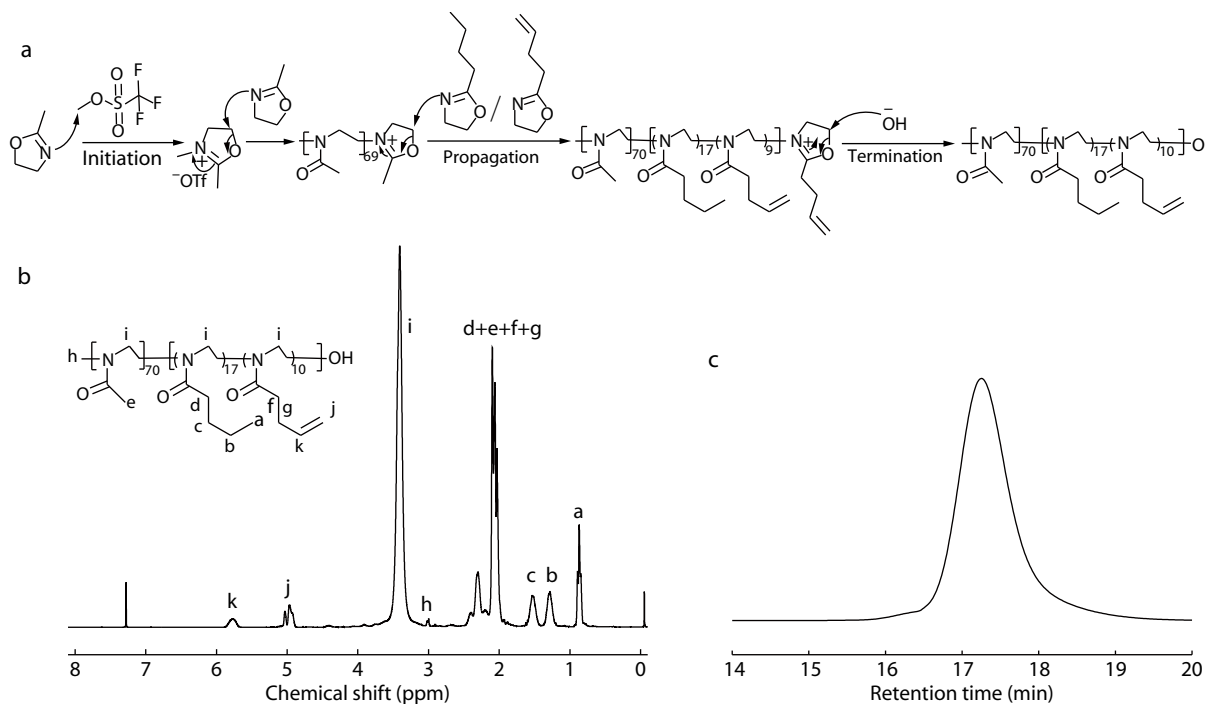


Fig. 2 (a) Schematic illustration of the mechanism of CROP of 2-oxazolines; (b) $^1\text{H-NMR}$ spectrum of PMBEOx (CDCl_3 , 300 MHz); (c) GPC curve of PMBEOx.

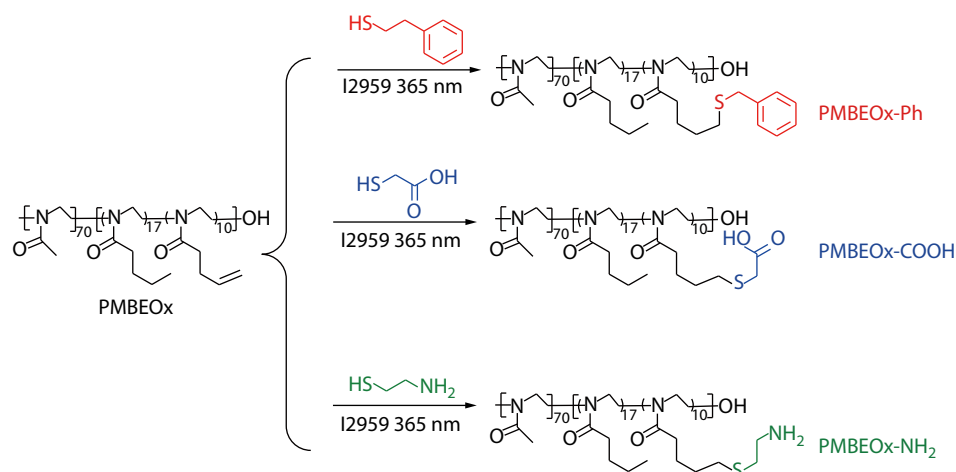


Fig. 3 Schematic illustration of the preparation methods for PMBEOx-Ph, PMBEOx-COOH, and PMBEOx-NH₂.

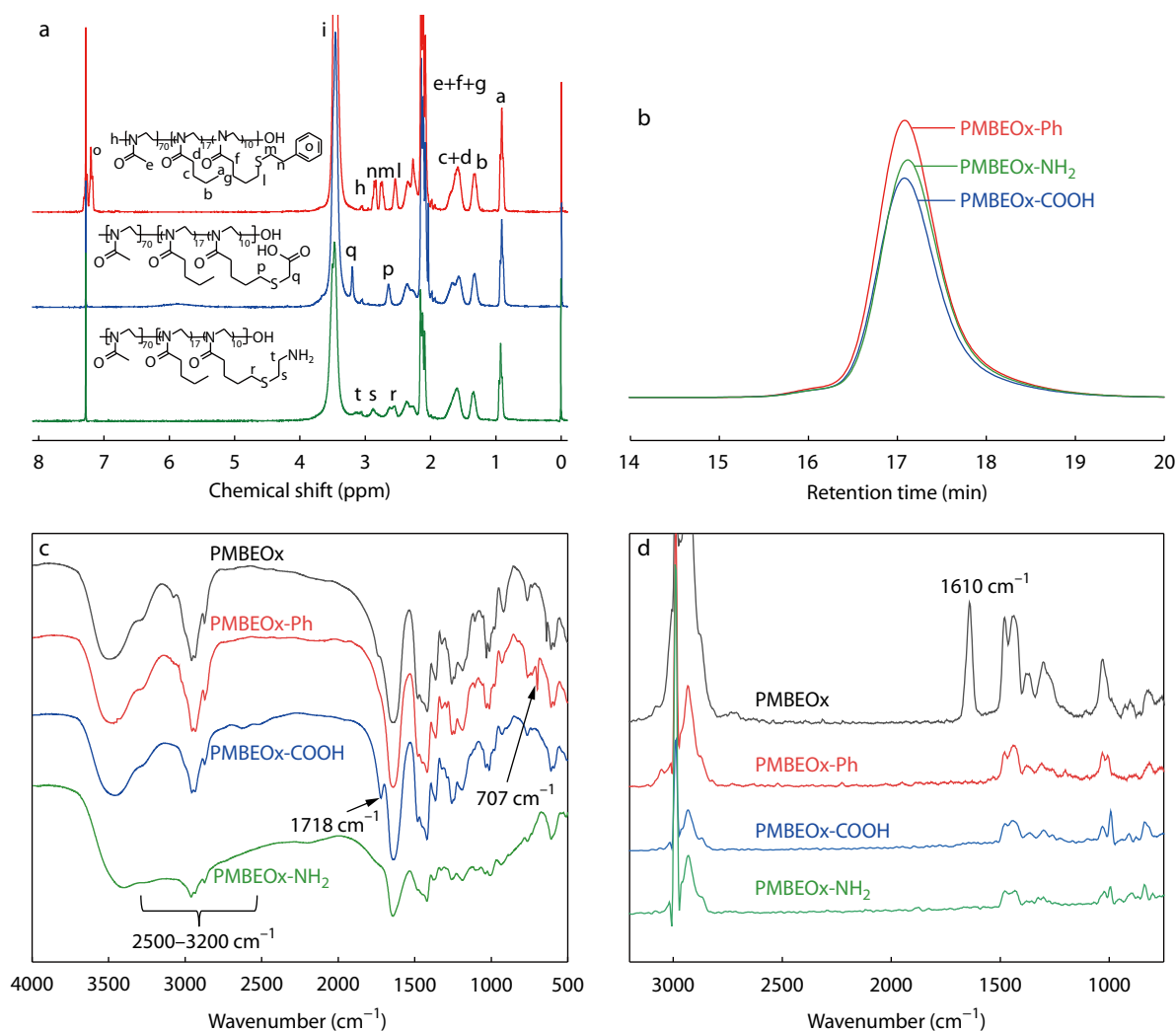


Fig. 4 (a) ¹H-NMR spectra of PMBEOx-Ph, PMBEOx-COOH and PMBEOx-NH₂ (CDCl₃, 300 MHz); (b) GPC curves of PMBEOx-Ph, PMBEOx-COOH and PMBEOx-NH₂; (c) FTIR spectra and (d) Raman spectra of PMBEOx, PMBEOx-Ph, PMBEOx-COOH and PMBEOx-NH₂.

confirmed the complete conversion of the vinyl into modified groups (Fig. 4d). The above results confirmed the successful synthesis of the three kinds of functional POxs.

Cyto- and Hemo- Compatibility Evaluation

The cyto-compatibility of the POxs was evaluated by MTT assay with 4T1 cells. Cell viability of 4T1 cells was evaluated after

incubating with functional POxs for 24 or 48 h. As shown in Fig. S8 (in ESI), over 90% of survival rate was observed at a concentration as high as 1000 µg/mL in 48 h, suggesting good cyto-compatibility of these materials.

Hemo-compatibility of synthetic materials is often analyzed on the basis of their activity to lyse mammalian red blood cells, which is implied by its hemolysis properties. As indicated in Fig. S9 (in ESI), all the POxs induced very low hemolysis up to 500 µg/mL. These results can be cited as evidence of the good hemo-compatibility of these POxs as potential drug-carriers for *in vivo* application.

Drug Encapsulation Study

We evaluated the DLC of the obtained functional POxs for eight commercially available small molecule drugs with various characteristic structures (Fig. 5a). All drugs were encapsulated with a thin-film method with a feeding drug ratio of 20 wt%. As shown in Fig. 5(b) and Table 1, similar to previous reports, PMBEOx with butyl and butenyl showed very good encapsulation ability for PTX, with DLC of 17.7 wt% and DLE of 88.5%. In addition, PMBEOx also showed good encapsulation

ability for olaparib (DLC=18.9 wt%, DLE=94.5%). However, PMBEOx did not perform well in encapsulating other drugs such as celecoxib, tranilast and imiquimod with unsatisfactory DLC of only 4.9 wt%, 2.2 wt% and 1.8 wt%, respectively.

Previous studies have shown that drugs with relatively higher aromatic content result in a higher encapsulation efficiency in nano-carrier with aromatic moieties. The aromatic content can be calculated by the ratio of the carbon atom numbers in the aromatic system (C_{arom}) and the total carbon atom numbers in the hydrophobic compounds (C_{total}), with $C_{\text{arom}}/C_{\text{total}}$ over 0.5 defined as a high ratio of aromatic carbon atoms.^[34] Therefore, we evaluated the relationship between the aromatic content and the encapsulation efficiency of the tested drugs. In comparison with PMBEOx, PMBEOx-Ph was more suitable for encapsulating DOX, celecoxib and imiquimod, resulting in a much higher DLC of 10.7 wt%, 10.1 wt%, 6.8 wt%, respectively. More significantly, the introduction of aromatic groups into the copolymer showed an obvious improvement for the loading ability of celecoxib (4.2 wt% versus 10.1 wt%) and DOX (6.1 wt% versus 10.7 wt%). While, as for

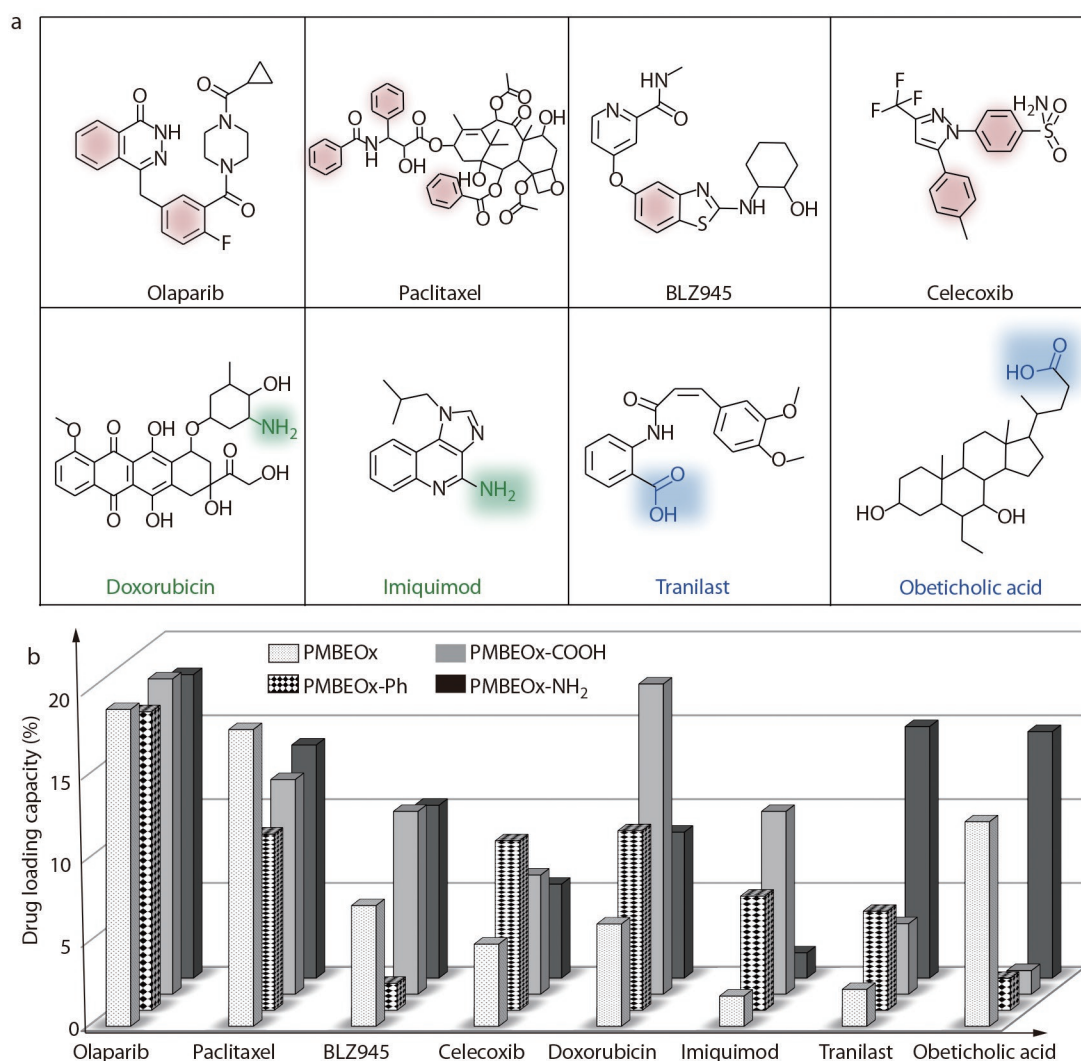


Fig. 5 (a) Chemical structures of the selected drugs; (b) Columns showing the DLC of various drugs in PMBEOx, PMBEOx-COOH, PMBEOx-NH₂ and PMBEOx-Ph.

Table 1 DLC (%) and DLE (%) of various drugs in functional POxs.

Drug	PMBEOx		PMBEOx-Ph		PMBEOx-COOH		PMBEOx-NH ₂	
	DLC (%)	DLE (%)	DLC (%)	DLE (%)	DLC (%)	DLE (%)	DLC (%)	DLE (%)
Olaparib	18.9	94.5	17.8	89.0	18.8	94.0	18.1	90.5
Paclitaxel	17.7	88.5	10.5 ^a	52.5 ^a	12.8	64.0	13.9	69.5
BLZ945	7.2	36.0	1.6 ^a	8.0 ^a	10.9	54.5	10.3	51.5
Celecoxib	4.9	21.0	10.1 ^b	50.5 ^b	7.1	35.5	5.6	28.0
Doxorubicin	6.1	30.5	10.7 ^b	53.5 ^b	18.5 ^b	92.5 ^b	8.7	43.5
Imiquimod	1.8	9.0	6.8	34.0	10.9 ^b	54.5 ^b	1.5	7.5
Tranilast	2.2	11.0	5.9	29.5	4.2	21.0	15.0 ^b	75.0 ^b
Obeticholic acid	12.2	61.0	1.5	9.5	1.4	7.0	14.7 ^b	73.0 ^b

^a Blue represents the interaction between POxs and drugs could decrease the drug encapsulation. ^b Red represents the interaction between POxs and drugs could enhance the drug encapsulation.

drugs with the ratio of aromatic carbon to total carbon less than 0.5, like PTX and BLZ945, the introduction of an aromatic groups into PMBEOx did not improve the drug encapsulation ability, but will dampen DLC (10.5 wt% versus 17.7 wt% for PTX, 1.6 wt% versus 7.2 wt% for BLZ945). To sum up, we found that celecoxib ($C_{\text{arom}}/C_{\text{total}}=0.8$) and DOX ($C_{\text{arom}}/C_{\text{total}}=0.56$) with higher ratios of the aromatic carbon atoms were correlated with high DLC in PMBEOx-Ph (celecoxib:DLC_{PMBEOx-Ph}=10.1 wt%; DOX:DLC_{PMBEOx-Ph}=10.7 wt%), while PTX ($C_{\text{arom}}/C_{\text{total}}=0.38$) and BLZ945 ($C_{\text{arom}}/C_{\text{total}}=0.3$) with lower ratios of the aromatic carbon atoms were correlated with low DLC in PMBEOx-Ph (PTX:DLC_{PMBEOx-Ph}=10.5 wt%; BLZ945:DLC_{PMBEOx-Ph}=1.6 wt%).

Then we evaluated the DLC of POxs with cationic or anionic charges in the side chains and compared them with PMBEOx. For tranilast and obeticholic acid with a carboxyl group, the DLC in PMBEOx-NH₂ was superior to other PMBEOx polymers. The DLC of tranilast in PMBEOx-NH₂ achieved nearly seven times as high as that in the unmodified PMBEOx (15 wt% versus 2.2 wt%). For obeticholic acid, both PMBEOx-NH₂ and PMBEOx formulation displayed a reasonably good loading capacity, and the DLC of PMBEOx-NH₂ showed a slight advantage over that of PMBEOx (14.7 wt% versus 12.2 wt%). Similar to that, we found an obvious improvement in the DLC of amino group containing drugs (DOX and imiquimod) when using PMBEOx-COOH as the drug encapsulation carrier. Compared to the PMBEOx, the DLC% of DOX and imiquimod in PMBEOx-COOH are significantly improved to 18.5 wt% and 10.9 wt%, respectively. This result proved that the electrostatic interaction between functional POxs and drugs could greatly enhance the DLC.

In summary, DLC of drugs with high aromatic carbon atoms ratio (celecoxib and DOX) and charged groups (tranilast, obeticholic acid, DOX and imiquimod) could be perfectly encapsulated into the POx carriers after matching the appropriate functional POxs, which indicated the importance of introducing functional groups into the polymers in drug loading.

Size and Stability of POx Micelles

Hydrodynamic sizes of the drug-loaded POx micelles were measured with DLS. As summarized in Table S1 (in ESI) the diameter of most of the drug-loaded micelles are between 30 and 55 nm, which is in consistent with the representative TEM

images showing the uniform nanostructures of the obtained POx micelles (Fig. S10 in ESI). However, PTX and DOX loading in PMBEOx and PMBEOx-COOH leads to obvious size increase. The stability of these drug-loading micelles at 7 days post preparation was also measured. While most of the micelles kept stable in aqueous solutions, there are precipitations observed in the PTX-PMBEOx-Ph, PTX-PMBEOx-NH₂, BLZ945-PMBEOx-Ph and tranilast-PMBEOx-COOH. Importantly, the sizes are stable in the properly matched carriers with both high DLC and stability.

CONCLUSIONS

In conclusion, we reported a series of functional POx copolymers based on PMBEOx and evaluated their capability for various drug loadings. Functional POxs were synthesized via post-modification on the side-chains of PMBEOx through the thio-ene click reaction. Based on the drug encapsulation study with these functional POxs, we found that functional groups showed a direct influence on the DLCs of various drugs. π - π interactions will improve the DLC in the drugs with high aromatic contents (>0.5), while decrease the DLC in drugs with low aromatic contents (<0.5). Electronic interactions between the oppositely charged segments in the functional POxs and the drugs could obviously improve the DLC. Collectively, the DLC of all chosen drugs could be more than 10 wt% after matching the appropriate functional POxs. These results were instructive for drug encapsulation and the series of functional POxs presents available carriers for various drugs encapsulation.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://doi.org/10.1007/s10118-021-2547-6>.

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