Journal of Materials Chemistry

Cite this: *J. Mater. Chem.*, 2012, **22**, 11730

www.rsc.org/materials

PAPER

Biodegradable poly(ε-caprolactone)-*g*-poly(2-hydroxyethyl methacrylate) graft copolymer micelles as superior nano-carriers for "smart" doxorubicin release†

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Received 6th February 2012, Accepted 28th March 2012
DOI: 10.1039/c2jm30700f

Biodegradable micelles were prepared from poly(ε-caprolactone)-*g*-poly(2-hydroxyethyl methacrylate) (PCL-*g*-PHEMA) graft copolymers and investigated for controlled release of doxorubicin (DOX). PCL-*g*-PHEMA copolymers were readily obtained by controlled ring-opening copolymerization of acryloyl cyclic carbonate and ε-caprolactone, Michael-type conjugate addition reaction with cysteamine, coupling reaction with 4-cyanopentanoic acid dithionaphthalenoate (CPADN) *via* carbodiimide chemistry, and reversible addition–fragmentation chain transfer (RAFT) polymerization of 2-hydroxyethyl methacrylate (HEMA). ^{1}H NMR analyses showed that M_n of PHEMA ranged from 8.7, 16.3 to 33.8 kg mol^{-1}, in proximity to the design as well as those determined by gel permeation chromatography (GPC). Differential scanning calorimetry (DSC) revealed that all three PCL-*g*-PHEMA graft copolymers had depressed melting temperatures (T_m = 31.3–32.5 °C) and low crystallinities (X_c = 3.05–5.66%). Dynamic light scattering (DLS) showed that PCL-*g*-PHEMA formed monodisperse micelles with low polydispersity indexes of 0.04–0.16 and average sizes ranging from 80.5 to 179.7 nm depending on PHEMA chain lengths. These graft copolymers displayed low critical micelle concentrations (CMCs) of 0.051–0.151 μM. The micellar sizes decreased following loading with DOX while PDI remained low. Interestingly, *in vitro* drug release studies showed that DOX-loaded PCL-*g*-PHEMA micelles exhibited superior pH-responsive release behaviors, in which up to 94.5% of DOX was released in 3 d at pH 5.0 while DOX release was significantly slower at pH 7.4 (maximum 54.1% release in 3 d). MTT assays with HeLa cells demonstrated that DOX-loaded PCL-*g*-PHEMA micelles retained high anti-tumor activity with low IC_{50} (half inhibitory concentration) of 1.47–1.74 μg DOX equiv. mL^{-1} while PCL-*g*-PHEMA micelles were practically non-toxic up to a tested concentration of 80 mg mL^{-1}. These novel biodegradable PCL-*g*-PHEMA graft copolymer micelles with low CMC, small and tunable sizes, high drug loading, and pH-responsive drug release have emerged as superior nanocarriers for "smart" tumor-targeting drug delivery.

Introduction

In recent years, polymeric micelles self-assembled from amphiphilic copolymers have attracted much attention for drug delivery in that they offer several advantages such as enhancing water solubility of lipophilic drugs, prolonging circulation time, passive targeting to the tumor tissues *via* the enhanced permeability and retention (EPR) effect, decreasing side effects, and improving drug bioavailability.[1–5] Micelles based on amphiphilic di- or tri-block copolymers of poly(ethylene glycol) (PEG) or poly(ethylene oxide) (PEO) and aliphatic polyesters such as polylactide (PLA), poly(ε-caprolactone) (PCL) and poly(lactide-*co*-glycolide) (PLGA) are among the most studied due to their excellent biocompatibility, *in vivo* biodegradability and easy synthesis.[5–9] Biodegradable micelles have also been developed from amphiphilic graft copolymers with hydrophobic biodegradable polymers grafted to natural or synthetic polymers such as dextran,[10] chondroitin sulfate,[11] poly(vinyl alcohol),[12,13] thermosensitive poly(*N*-isopropylacrylamide) copolymer,[14] and poly-α,β-(*N*-(2-hydroxyethyl)-L-aspartamide).[15,16]

It is interesting to note that there are few reports on biodegradable graft copolymer micelles based on hydrophobic

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† Electronic supplementary information (ESI) available: ^{1}H NMR spectra of PCL(Acr) and PCL(Cys) as well as CMC determination plot. See DOI: 10.1039/c2jm30700f

biodegradable polymers grafted with hydrophilic polymers, likely due to challenging synthesis. Graft copolymer micelles offer several advantages over block copolymer micelles; for instance, they might have low CMC and thereby enhanced stability, micellar core and surface properties might be broadly adjusted by backbone length, graft density and graft length, and the presence of many hydrophilic grafts per macromolecule enables conjugation of high density targeting ligands for optimal tumor-targeting. Emrick,[17] Jérôme,[18] Cheng,[19] and Darcos[20] groups reported synthesis of PCL-*g*-oligopeptide, PCL-*g*-PEG, PLA-*g*-paclitaxel-PEG and PCL-*g*-PDMAEMA graft copolymers, respectively, *via* copper-catalyzed click reactions. Wooley *et al.* obtained PCL-*g*-PEO graft copolymers by conjugating PEO to ketone-functionalized PCL *via* a stable ketoxime ether linkage.[21] Coudane *et al.* reported preparation of amphiphilic PCL-*g*-poly(L-lysine), PCL-*g*-poly(4-vinylpyridine), PCL-*g*-poly(2-(*N*,*N*-dimethylamino)ethyl methacrylate) (PDMAEMA) and PCL-*g*-poly((*N*,*N*-dimethyl) acrylamide) graft copolymers by anionic polymerization or iodine transfer polymerization.[22–24] Dong *et al.* prepared PCL-*g*-PDMAEMA copolymers by atom transfer radical polymerization (ATRP) and investigated them for co-delivery of paclitaxel and DNA.[25,26] It should be noted that most of the above studies focus on synthetic chemistry and there is no systemic investigation on biocompatibility, micellization, drug loading, *in vitro* drug release and anti-tumor activity of graft copolymer micelles containing a biodegradable hydrophobic polymer backbone.

In this paper, we report on novel biodegradable graft copolymer micelles based on poly(ε-caprolactone)-*g*-poly(2-hydroxyethyl methacrylate) (PCL-*g*-PHEMA) as superior nanocarriers for "smart" release of doxorubicin (DOX) (Scheme 1). PHEMA is a biocompatible hydrophilic material widely used in biomedical areas including contact lenses and hydrogels.[27] PCL-*g*-PHEMA graft copolymers were facilely prepared by reversible addition–fragmentation chain transfer (RAFT) polymerization of 2-hydroxyethyl methacrylate (HEMA) in the presence of the PCL-*g*-CPADN macro-RAFT agent (CPADN: 4-cyanopentanoic acid dithionaphthalenoate). Unlike anionic polymerization, RAFT polymerization proceeds under very mild conditions, which minimizes degradation of biodegradable polymer backbones. In contrast to click reaction and ATRP polymerization, RAFT polymerization does not involve any toxic metal catalysts, which prevents possible toxicity.[28] We previously prepared PEG–PHEMA,[29] PDMAEMA–PCL–PDMAEMA,[30] PDMAEMA–SS–PEG–SS–PDMAEMA,[31] and PEG–PCL–PDEA[32] block copolymers employing controlled RAFT polymerizations. Moreover, unlike PEO and PEG, PHEMA has abundant hydroxyl functional groups that are amenable to covalent conjugation of different modalities including targeting ligands and fluorescent molecules. In this study, synthesis and micellization of PCL-*g*-PHEMA graft copolymers, loading and *in vitro* release of DOX, and anti-tumor activity of DOX-loaded PCL-*g*-PHEMA graft copolymer micelles were investigated.

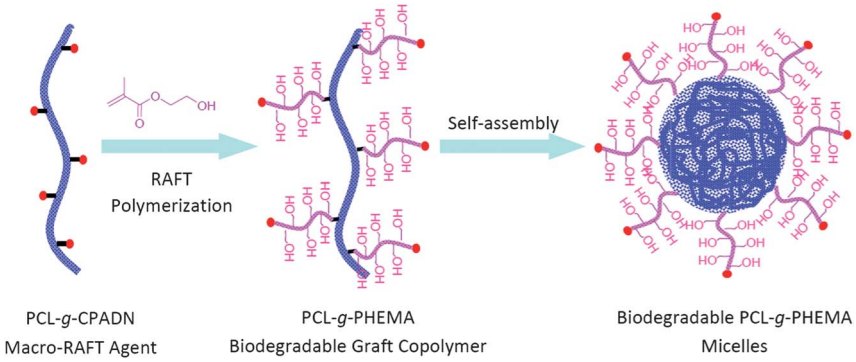
Experimental part

Materials

ε-Caprolactone (ε-CL, Alfa Aesar, 99%) was dried over CaH_2 and distilled under reduced pressure prior to use. Acryloyl cyclic carbonate (AC) was synthesized according to our previous report.[33] 2,2′-Azobisisobutyronitrile (AIBN, 98%, J&K) was recrystallized twice from hexane and methanol. Toluene and methylene dichloride were dried by refluxing over sodium wire and CaH_2, respectively, and distilled prior to use. 2-Hydroxyethyl methacrylate (HEMA, 95%, Fluka) was purified by passing through a basic alumina column before use. 4-Cyanopentanoic acid dithionaphthalenoate (CPADN) was synthesized according to the reported procedure.[34] Stannous octoate ($Sn(Oct.)_2$, 95%, Sigma), dicyclohexyl carbodiimide (DCC, 99%, Alfa Aesar), *N*-hydroxysuccinimide (NHS, 98%, Alfa Aesar), cysteamine hydrochloride (99%, Alfa Aesar), triethylamine (Et_3N, 99%, Alfa Aesar), *n*-hexanol (99%, Alfa Aesar), pyridine (99.5%), *N*,*N*-dimethyl formamide (DMF), diethyl ether, ethanol and doxorubicin hydrochloride (>99%, Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd.), and 2-(*N*-morpholino)ethanesulfonic acid (MES, 98%, Beike Suzhou) were used as received.

Synthesis of PCL-*g*-CPADN macro-RAFT agent

The PCL-*g*-CPADN macro-RAFT agent was obtained in three steps. Firstly, acryloyl-functionalized poly(ε-caprolactone),



Scheme 1 Illustration of biodegradable PCL-*g*-PHEMA graft copolymer micelles. PCL-*g*-PHEMA graft copolymers are facilely obtained by RAFT polymerization of 2-hydroxyethyl methacrylate (HEMA) using PCL-*g*-CPADN as a macro-RAFT agent. The properties of micelles (including size, drug loading level, and drug release rate) can be adjusted by PHEMA graft length.

denoted as PCL(Ac), was synthesized according to our previous report.[33] Typically, in a glove box under a nitrogen atmosphere, 0.333 mL of *n*-hexanol (0.75 M) and 0.625 mL of $Sn(Oct.)_2$ (0.1 M) stock solutions were quickly added to a stirred solution of ε-CL (4.503 g, 39.5 mmol) and AC (0.505 g, 2.5 mmol) in toluene (30 mL). The reaction vessel was sealed and placed in an oil-bath thermostatted at 110 °C for 24 h. The polymerization was terminated by adding two drops of acetic acid. A sample was taken for the determination of monomer conversion using ^{1}H NMR. The resulting copolymer was isolated by precipitation in cold diethyl ether, filtration and drying *in vacuo* at room temperature. Yield: 80%. ^{1}H NMR end group analysis revealed an M_n of 16.5 kg mol^{-1}. M_n(GPC) = 18.2 kg mol^{-1}, M_w/M_n(GPC) = 1.5. ^{1}H NMR (400 MHz, $CDCl_3$) of PCL(Ac): δ 5.88–6.42 (m, –C*H*=CH_2), 4.06 (m, –COOCH_2–), 2.30 (t, –COCH_2CH$_2$CH$_2$CH$_2$CH$_2$O–), 1.64 (m, –COCH$_2$CH_2CH$_2$CH_2-CH$_2$O–), 1.38 (m, –COCH$_2$CH$_2$CH_2CH$_2$CH$_2$O–), 1.2–1.3 (m, CH$_3$CH_2CH_2CH_2CH_2CH$_2$O–), 1.14 (s, CH_3C–), 0.8 (t, CH_3CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$O–).

Secondly, amine-functionalized PCL was synthesized by Michael-type conjugate addition of PCL(Ac) with cysteamine in the presence of pyridine. Briefly, PCL(Ac) (2.0 g, 0.848 mmol Ac), cysteamine hydrochloride (0.482 g, 4.24 mmol) and pyridine (0.335 g, 4.24 mmol) were reacted in 20 mL of DMF (final AC/SH/pyridine mole ratio of 1/5/5) at room temperature for 12 h. The product was isolated by precipitation from cold diethyl ether/ethanol, filtration and drying *in vacuo* at room temperature. In order to fully remove HCl, the product was re-dissolved in THF, treated for 1 h with triethylamine (two equiv. with respect to AC units), and filtered to remove the precipitates. PCL(Cys) was isolated by precipitation in cold diethyl ether, filtration, and drying *in vacuo*. Yield: 90%. ^{1}H NMR (400 MHz, $CDCl_3$) of PCL(Cys): δ 4.06 (m, –COOCH_2–), 2.92 (t, –CH_2NH$_2$), 2.79 (t, –CH_2COO–), 2.68 (t, –CH_2SCH$_2$–), 2.30 (t, –COCH_2CH$_2$CH$_2$CH$_2$CH$_2$O–), 1.64 (m, –COCH$_2$CH_2CH$_2$CH_2-CH$_2$O–), 1.38 (m, –COCH$_2$CH$_2$CH_2CH$_2$CH$_2$O–), 1.2–1.3 (m, CH$_3$CH_2CH_2CH_2CH_2CH$_2$O–), 1.14 (s, CH_3C–), 0.8 (t, CH_3CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$O–).

Finally, PCL-*g*-CPADN was prepared by conjugating CPADN to PCL(Cys) by carbodiimide chemistry. Briefly, CPADN (0.262 g, 0.796 mmol), *N*-hydroxysuccinimide (NHS) (0.137 g, 1.19 mmol) and anhydrous DCM (200 mL) were charged to a 500 mL round-bottom flask equipped with a magnetic stirrer. Under stirring at 0–4 °C a solution of DCC (0.487 g, 2.36 mmol) in 20 mL of DCM was dropwise added. The reaction was allowed to proceed at room temperature for 24 h. Then, 0.8 g of PCL(Cys) was added and allowed to react for another 24 h at room temperature. The reaction mixture was filtered, and the filtrate following concentration with rotary evaporator was precipitated in cold diethyl ether. The product was collected by filtration and drying *in vacuo*. Yield: 92%. ^{1}H NMR (400 MHz, $CDCl_3$) of PCL-*g*-CPADN: δ 7.49, 7.90, and 8.14 (–COCH$_2$CH$_2$–C(CN)(CH$_3$)–SCSC$_{10}H_7$), 4.06 (m, –COOCH_2–), 3.45 (t, –CH_2NHCO), 2.79 (t, –CH_2COO–), 2.5–2.68 (–CH_2SCH_2–, –NHCOCH_2–), 2.45 (–NHCOCH$_2$CH_2–), 2.30 (t, –COCH_2CH$_2$CH$_2$CH$_2$CH$_2$O–), 1.99 (s, –OCOCH$_2$CH$_2$C(CN)(CH_3)SCSC$_{10}$H$_7$), 1.64 (m, –COCH$_2$CH_2CH$_2$CH_2CH$_2$O–), 1.38 (m, –COCH$_2$CH$_2$CH_2CH$_2$-CH$_2$O–), 1.2–1.3 (m, CH$_3$CH_2CH_2CH_2CH_2CH$_2$O–), 1.14 (s, CH_3C–), 0.8(t, CH_3CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$O–).

Synthesis of PCL-*g*-PHEMA graft copolymers by RAFT polymerization

PCL-*g*-PHEMA graft copolymers were prepared by RAFT polymerization of HEMA using PCL-*g*-CPADN as a macro-RAFT agent. In a typical example, under a nitrogen atmosphere, HEMA (0.1 g, 0.77 mmol), PCL-*g*-CPADN (0.1 g, 5.3 μmol), AIBN (0.87 mg, 3.7 μmol) and DMF (1.5 mL) were added into a 10 mL Schlenk flask. The flask was sealed and placed into an oil bath thermostatted at 70 °C. The mixture proceeded with magnetic stirring for 24 h. The resulting copolymer was isolated by precipitation in cold diethyl ether, filtration and drying *in vacuo*. Yield: 93%. M_n (^{1}H NMR) = 35.5 kg mol^{-1}, M_n(GPC) = 38.3 kg mol^{-1}, M_w/M_n (GPC) = 1.9. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.80, 3.90, 3.57, 1.70–2.10, 0.70–1.10 (PHEMA), 4.06, 2.3, 1.64, 1.38 (PCL). In a similar way, we have also obtained PCL-*g*-PHEMA graft copolymers with M_n (^{1}H NMR) of 27.9 and 53.0 kg mol^{-1} by using HEMA/PCL-*g*-CPADN mole ratios of 72.6 and 290.6, respectively.

Characterization

The ^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using deuterated chloroform ($CDCl_3$) or deuterated dimethylsulfoxide (DMSO-d_6) as a solvent. The chemical shifts were calibrated against residual solvent signals. The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using THF or DMF as an eluent at a flow rate of 1.0 mL min^{-1} at 30 °C and a series of narrow polystyrene standards for the calibration of the columns. The thermal properties of PCL-*g*-PHEMA graft copolymers were studied using a differential scanning calorimeter (Perkin-Elmer DSC-7). The sample was heated to 130 °C at a rate of 20 °C min^{-1}, kept at 130 °C for 3 min, cooled to −80 °C at a rate of 80 °C min^{-1}, kept at −80 °C for 3 min, and then a second heating scan from −80 °C to 130 °C at a rate of 20 °C min^{-1} was recorded. The maximum of the endothermic peak was taken as the melting temperature.

The hydrodynamic sizes of micelles were determined using dynamic light scattering (DLS). Measurements were carried out at 25 °C using a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He–Ne laser using back-scattering detection. Data were analyzed using the associated Zetasizer software (Dispersion Technology Software v 5.00; Malvern). The instrument was standardized with 200 nm and 60 nm polystyrene beads and micelle hydrodynamic size was reported as the average of the three measurements with standard deviation. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 μL of 0.1 mg mL^{-1} DOX-loaded micelles on the copper grid.

Preparation of PCL-*g*-PHEMA graft copolymer micelles and determination of CMC

PCL-*g*-PHEMA graft copolymer micelles were prepared by a solvent exchange method. Briefly, to a stirred DMF solution

(1.0 mL) of PCL-*g*-PHEMA (1.0 mg mL^{-1}) was dropwise added 1.0 mL of D.I. water. The mixture was sonicated for 30 min and then extensively dialyzed against D.I. water for 24 h (MWCO 3500) at room temperature.

The CMC was determined using pyrene as a fluorescence probe. The concentration of graft copolymer was varied from 6.0×10^{-4} to 0.15 mg mL^{-1} and the concentration of pyrene was fixed at 0.6 μM. The fluorescence spectra were recorded using a FLS920 fluorescence spectrometer with the excitation wavelength of 330 nm. The emission fluorescence at 372 and 383 nm was monitored. The CMC was estimated as the cross-point when extrapolating the intensity ratio I_{372}/I_{383} at low and high concentration regions.

Loading of DOX into micelles

DOX-loaded micelles were prepared by dropwise addition of 1.0 mL of D.I. water to a mixture of PCL-*g*-PHEMA copolymer (1.0 mL, 2.0 mg mL^{-1} in DMF) and DOX (20 or 40 μL, 5.0 mg mL^{-1} in DMSO) under stirring at room temperature, followed by dialysis against D.I. water for 24 h at room temperature (Spectra/Pore® dialysis membrane, MWCO 3500, Spectrum Laboratories Inc., USA). The dialysis medium was changed five times. The whole procedure was performed in the dark. The amount of DOX was determined using fluorescence (FLS920) measurement (excitation at 480 nm and emission at 555 nm). For determination of drug loading content, lyophilized DOX loaded micelles were dissolved in DMSO and analyzed with fluorescence spectroscopy, wherein the calibration curve was obtained with DOX/DMSO solutions with different DOX concentrations.

Drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following formula:

$$\text{DLC (wt\%)} = (\text{weight of loaded drug/weight of polymer}) \times 100\%$$

$$\text{DLE (\%)} = (\text{weight of loaded drug/weight of drug in feed}) \times 100\%$$

Release of DOX from PCL-*g*-PHEMA micelles

The release profiles of DOX from PCL-*g*-PHEMA micelles were studied at 37 °C using a dialysis tube (MWCO 12 000–14 000) at 37 °C in two different media, *i.e.* MES buffer (pH 5.0, 10 mM) and phosphate buffer (pH 7.4, 10 mM). In order to acquire sink conditions, drug release studies were performed at low drug loading contents and with 0.7 mL of micelle solution dialysis against 20 mL of the same medium. At desired time intervals, 6 mL of release medium was taken out and replenished with an equal volume of fresh medium. The amount of DOX released was determined by using fluorescence (FLS920) measurement (excitation at 480 nm). The release experiments were conducted in triplicate. The results presented are the average data with standard deviations.

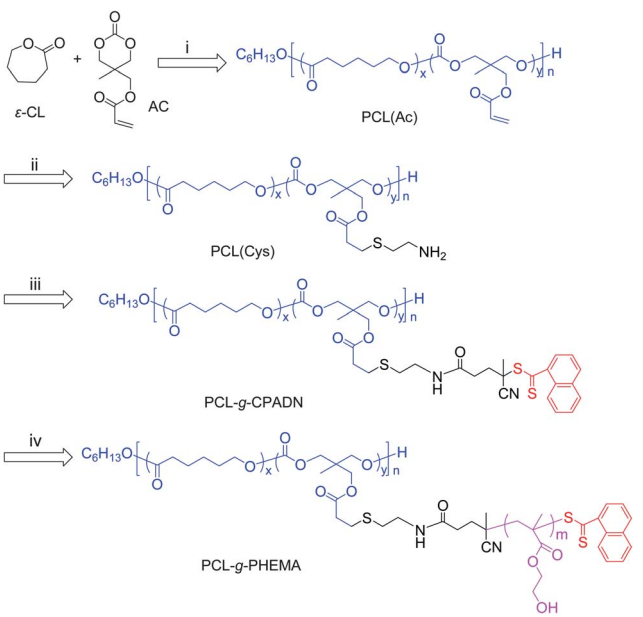
Cell viability assay

HeLa cells were seeded in 96-well plates (2×10^4 cells per well) using DMEM medium supplemented with 10% fetal bovine serum, antibiotics penicillin (50 IU mL^{-1}) and streptomycin (50 μg mL^{-1}) for 24 h. The media was aspirated. The cells were incubated with blank PCL-*g*-PHEMA graft copolymer micelles (10, 20, 40 or 80 mg mL^{-1}), DOX-loaded PCL-*g*-PHEMA graft copolymer micelles or free DOX (DOX dosage: 0.5–10 μg mL^{-1}) in 100 μL of complete DMEM medium for 72 h at 37 °C in a humidified 5% CO_2-containing atmosphere. The medium was aspirated, then 150 μL of a stock solution containing 0.1 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) in PBS was added and incubated for another 4 h. The PBS was aspirated, the MTT-formazan generated by live cells was dissolved in 150 μL of DMSO, and the absorbance of each well at a wavelength of 490 nm was measured using a microplate reader. The relative cell viability (%) was determined by comparing the absorbance at 490 nm with control wells containing only cell culture medium. Data are presented as average ± SD ($n = 4$).

Results and discussion

Synthesis of biodegradable PCL-*g*-PHEMA graft copolymers

PCL-*g*-PHEMA copolymers were readily prepared in four steps (Scheme 2). Firstly, acryloyl-functionalized PCL, denoted as PCL(Ac), was obtained by ring-opening copolymerization of cyclic acryloyl carbonate (AC) and ε-caprolactone (ε-CL) at a monomer-to-initiator ratio of 168/1 and an AC mole feed ratio of 5.95 mol% in toluene at 110 °C using *n*-hexanol as an initiator and stannous octoate as a catalyst, as in our previous report.[33] ^{1}H NMR displayed clearly resonances at δ 5.6–6.4 attributable to intact acryloyl protons (Fig. S1A†). The AC content in PCL(Ac) was determined to be 5.1 mol% by comparing integrals of signals at δ 5.6–6.4 and δ 2.30 (methylene protons next to the carbonyl group of PCL). GPC showed a unimodal distribution with a moderate M_w/M_n of 1.5 and an M_n of 18.2 kg mol^{-1}, in close



Scheme 2 Synthesis of biodegradable PCL-*g*-PHEMA graft copolymers. Conditions: (i) $Sn(Oct.)_2$, toluene, 110 °C, 24 h; (ii) cysteamine hydrochloride, pyridine, DMF, r.t., 12 h; Et_3N, THF, r.t.,1 h; (iii) CPADN, NHS/DCC, r.t., 24 h; and (iv) HEMA, DMF, 70 °C, 24 h.

agreement with that determined by ^{1}H NMR end group analysis (M_n = 16.5 kg mol^{-1}).

Secondly, amine-functionalized PCL, referred to as PCL(Cys), was synthesized by Michael-type conjugate addition of PCL(Ac) with cysteamine hydrochloride in DMF at room temperature in the presence of pyridine followed by full deprotonation with triethylamine. ^{1}H NMR revealed complete disappearance of peaks assignable to acryloyl groups, occurrence of new signals corresponding to cysteamine moieties (δ 2.5–3.0), and importantly the integral ratio between resonances at δ 2.88 (methylene protons neighboring to the carbonyl group of AC units) and δ 2.77 (methylene protons of cysteamine moiety next to the amine group) close to the theoretical value of 1 : 1, indicating quantitative functionalization of PCL(Ac) with cysteamine (Fig. S1B†).

Thirdly, the PCL-*g*-CPADN macro-RAFT agent was prepared from PCL(Cys) and CPADN *via* carbodiimide chemistry. ^{1}H NMR showed that signals at δ 2.95 assignable to the methylene protons neighboring to the amine group of PCL(Cys) completely vanished (Fig. 1A). In addition to peaks assignable to PCL(Cys) as well as CPADN (δ 7.49–8.14 and δ 2.44–2.67), a new resonance owing to methylene protons next to the amide group was detected at δ 3.49. The integral ratio between signals at δ 3.49 and 1.99 (methyl protons of CPADN) was close to the theoretical value of 2 : 3, confirming successful synthesis of the PCL-*g*-CPADN macro-RAFT agent.

Finally, PCL-*g*-PHEMA graft copolymers were obtained with high yields through RAFT polymerization of HEMA using PCL-*g*-CPADN as a macro-RAFT agent. The results of copolymerization are summarized in Table 1. ^{1}H NMR displayed clearly peaks characteristic of both PHEMA (δ 4.80, 3.90, 3.57, 1.70–2.10, 0.70–1.10) and PCL chains (δ 4.06, 2.3, 1.64, 1.38) (Fig. 1B). ^{1}H NMR analysis comparing the intensities of signals at δ 2.26 (methylene protons neighboring to the carbonyl group of PCL) and 3.57 (methylene protons next to the ester bonds of PHEMA) showed that M_n of PHEMA ranged from 8.7, 16.3 to 33.8 kg mol^{-1} (copolymers denoted accordingly as PCL-*g*-PHEMA(8.7k), PCL-*g*-PHEMA(16.3k), and PCL-*g*-PHEMA(33.8k), respectively), which were in proximity to the design as well as those determined by GPC (Table 1). Importantly, GPC revealed that all three PCL-*g*-PHEMA copolymers displayed a unimodal distribution, in which low molecular weight fractions due to PCL and PHEMA homopolymers were not detected. It is evident that RAFT polymerization furnishes controlled PCL-*g*-PHEMA graft copolymers.

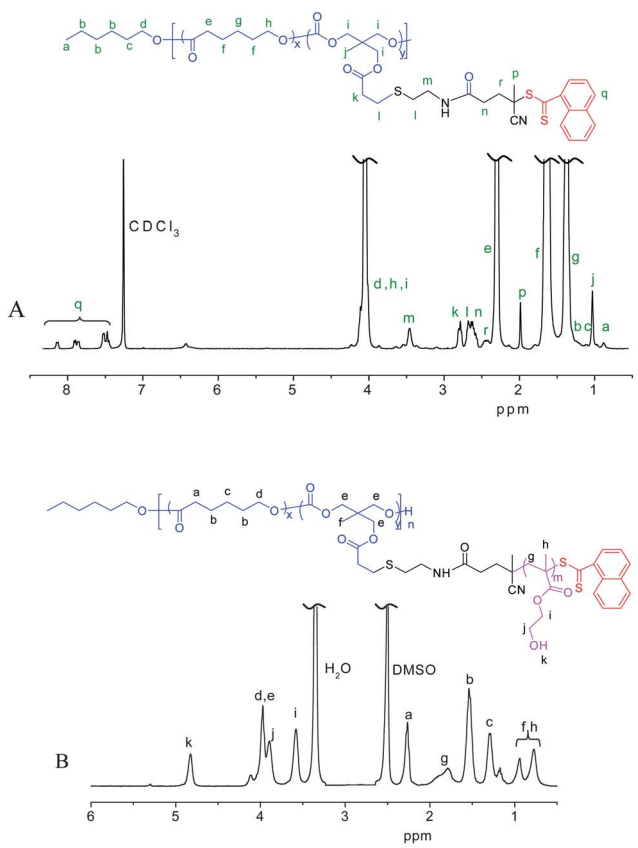


Fig. 1 ^{1}H NMR spectra (400 MHz) of PCL-*g*-CPADN in $CDCl_3$ (A) and PCL-*g*-PHEMA (Table 1, Entry 2) in DMSO-d_6 (B).

Differential scanning calorimetry (DSC) analyses showed that all PCL-*g*-PHEMA graft copolymers exhibited a single depressed melting temperature (T_m) of 31.3–32.5 °C, which was significantly lower than that of the PCL homopolymer (T_m = 60.2 °C)[35] as well as PCL(Ac) (T_m = 44.5 and 52.7 °C) (Table 2). The low T_m of PCL-*g*-PHEMA (<37 °C) indicates that these graft copolymers are in a rubbery state at body temperature, which may result in improved drug permeation and release. It is known that drug permeability is critically dependent on the physical state (crystalline or glassy *versus* amorphous) of matrices.[8] In accordance, PCL-*g*-PHEMA graft copolymers showed also a low crystallinity (X_c = 3.05–5.66%), which decreased with increasing PHEMA graft length (Table 2). In comparison, a high X_c of 47.5% was reported for PCL homopolymer[35] and 25.9% for PCL(Ac) (Table 2). Hence, grafting of PHEMA suppresses the melting point of PCL and disrupts its crystallization. The low PCL crystallinity is beneficial for high drug loading since only the amorphous hydrophobic regions in the micellar cores likely accommodate drug molecules.[36]

Formation and cytotoxicity of PCL-*g*-PHEMA graft copolymer micelles

PCL-*g*-PHEMA graft copolymer micelles were prepared by solvent exchange methods. Dynamic light scattering (DLS) measurements showed that all PCL-*g*-PHEMA graft copolymers formed mono-disperse nano-sized micelles with low polydispersities (PDIs) of 0.04–0.16 (Fig. 2). Notably, micelle sizes increased from 80.5, 108.2 to 179.7 nm with increasing M_n of PHEMA from 8.7, 16.3 to 33.8 kg mol^{-1} (Table 1). This increase of micelle size with increasing PHEMA graft length is most likely due to existence of significant steric hindrance between PHEMA shells.

The CMC of PCL-*g*-PHEMA graft copolymers was determined using pyrene as a fluorescence probe (Fig. S2†). The results showed that PCL-*g*-PHEMA graft copolymers had low CMCs of 0.051–0.151 μM (Table 1), which are significantly lower than that reported for PEG-*b*-PCL block copolymers.[38] It should further be noted that the CMC of PCL-*g*-PHEMA copolymers decreased with increasing PHEMA graft lengths (Table 1). These results confirm that amphiphilic graft copolymers based on a hydrophobic backbone form more stable micelles with a low CMC as compared to block-type copolymers.

Table 1 Synthesis and micellization of PCL-*g*-PHEMA graft copolymers[a]

Entry	Copolymer	M_n (kDa) Design	^{1}H NMR[b]	GPC[c]	M_w/M_n GPC[d]	Micelle size (nm)[d]	PDI[d]	CMC (μM)[e]
1	PCL-*g*-PHEMA(8.7k)	19.2–9.6	19.2–8.7	28.4	2.1	4.2	0.16	0.151
2	PCL-*g*-PHEMA(16.3k)	19.2–19.2	19.2–16.3	38.3	1.9	3.9	0.09	0.110
3	PCL-*g*-PHEMA(33.8k)	19.2–38.4	19.2–33.8	54.2	2.2	2.7	0.04	0.051

[a] RAFT polymerization conditions: $[CPADN]_0/[AIBN]_0 = 10/1$ (mol/mol), DMF, 70 °C, 24 h. [b] Determined by ^{1}H NMR analysis by comparing the intensities of signals at δ 2.26 (methylene protons neighboring to the carbonyl group in PCL) and 3.57 (methylene protons next to the ester in PHEMA). [c] Determined by GPC (DMF containing 0.05 M LiBr as an eluent, at a flow rate of 0.8 mL min^{-1}, 30 °C, polystyrene standards). [d] Size and PDI of micelles were determined by DLS. [e] Determined using pyrene as a fluorescence probe.

Table 2 DSC characterization of PCL-*g*-PHEMA graft copolymers

Entry	Polymer	T_m (°C)	ΔH_m (J g^{-1})	X_c (%)[a]
1	PCL(Ac)	44.5/52.7	5.2/30.8	25.9
2	PCL-*g*-PHEMA(8.7k)	31.3	3.7	5.66
3	PCL-*g*-PHEMA(16.3k)	31.6	2.5	4.93
4	PCL-*g*-PHEMA(33.8k)	32.5	1.0	3.05

[a] X_c (%) $= \Delta H_m/(\text{wt\% PCL} \times \Delta H_m^0) \times 100\%$, wherein $\Delta H_m^0 = 139$ J g^{-1}.[37]

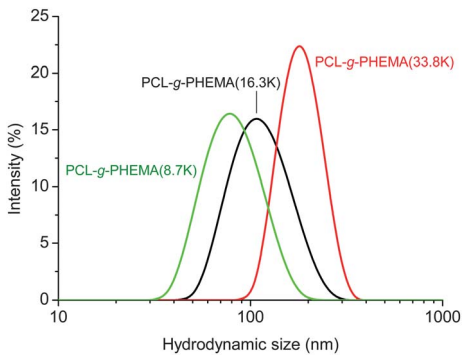


Fig. 2 The size distribution profiles of PCL-*g*-PHEMA graft copolymer micelles determined by DLS at a concentration of 1.0 mg mL^{-1} in PB buffer (20 mM, pH 7.4) at 25 °C.

The *in vitro* toxicity of PCL-*g*-PHEMA graft copolymer micelles was evaluated in HeLa cells using MTT assays. The cells were incubated with micelles for 72 h at varying concentrations from 10 to 80 mg mL^{-1}. The results revealed that all three PCL-*g*-PHEMA graft copolymer micelles were practically non-toxic (cell viability = 85–102%) up to a tested concentration of 80 mg mL^{-1} (Fig. 3), indicating that PCL-*g*-PHEMA micelles have excellent biocompatibility. The low cytotoxicity and intrinsic biodegradability of PCL-*g*-PHEMA micelles render them particularly interesting for further *in vivo* applications.

Loading and *in vitro* release of DOX

In the following, loading and release of DOX (a potent hydrophobic anticancer drug) from PCL-*g*-PHEMA graft copolymer micelles were investigated. The theoretical DLC was set at 5 or 10 wt%. The results showed that DOX loading levels were highly dependent on PHEMA graft lengths, in which PCL-*g*-PHEMA(8.7k) micelles displayed the lowest drug loading efficiency

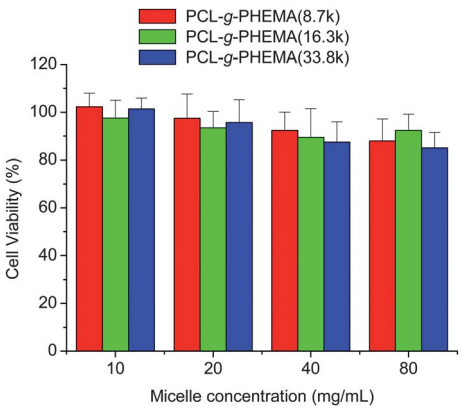


Fig. 3 Cytotoxicity of PCL-*g*-PHEMA graft copolymer micelles at varying concentrations of 10, 20, 40 and 80 mg mL^{-1}. HeLa cells were incubated with micelles for 72 h. The cell viability was determined by MTT assays ($n = 4$).

(DLE) while PCL-*g*-PHEMA(16.3k) micelles gave the best loading efficiency (Table 3). For example, at a theoretical DLC of 5 wt%, DOX loading efficiencies of 40.8%, 80.0%, and 74.2% were observed for PCL-*g*-PHEMA(8.7k), PCL-*g*-PHEMA(16.3k) and PCL-*g*-PHEMA(33.8k) micelles, respectively. Increasing theoretical DLC to 10 wt% resulted in slightly decreased DLE. For instance, a DLE of 76.8% was obtained for PCL-*g*-PHEMA(16.3k) micelles at a theoretical DLC of 10 wt%.

Interestingly, loading of DOX into PCL-*g*-PHEMA micelles resulted in smaller particle sizes while maintaining a low PDI of 0.09–0.18 (Table 3). As an example, the size distribution profiles of PCL-*g*-PHEMA(16.3k) micelles loaded with 7.68 wt% DOX are presented in Fig. 4. DLS showed that DOX-loaded PCL-*g*-PHEMA(16.3k) micelles had an average size of 82.5 nm (Fig. 4A), which was *ca.* 25.7 nm smaller than that of corresponding empty micelles. The TEM micrograph displayed a spherical morphology with an average micelle size of *ca.* 60 nm (Fig. 4B). The smaller size observed by TEM as compared to that determined by DLS is most likely due to shrinkage of hydrophilic shells upon drying samples. It should further be noted that micelle size decreased with increasing drug loading levels (Table 3). For example, average sizes of 90.6 nm and 82.5 nm were observed for PCL-*g*-PHEMA(16.3k) micelles at DLC of 4.0 wt% and 7.68 wt%, respectively.

The *in vitro* drug release from DOX-loaded PCL-*g*-PHEMA graft copolymer micelles was performed at 37 °C under two

Table 3 Characterization of DOX-loaded PCL-*g*-PHEMA graft copolymer micelles

Entry	Copolymers	Theoretical drug loading content (wt%)	DLC (wt%)[a]	DLE (%)	Size[b] (nm)	PDI[b]
1	PCL-*g*-PHEMA(8.7k)	5	2.04	40.8	80.0	0.09
		10	3.76	37.6	77.9	0.15
2	PCL-*g*-PHEMA(16.3k)	5	4.00	80.0	90.6	0.12
		10	7.68	76.8	82.5	0.18
3	PCL-*g*-PHEMA(33.8k)	5	3.71	74.2	175.6	0.14
		10	6.13	61.3	156.5	0.10

[a] Determined by fluorescence measurement (excitation at 480 nm). [b] Measured by DLS at a concentration of 1.0 mg mL^{-1} in PB buffer (20 mM, pH 7.4) at 25 °C.

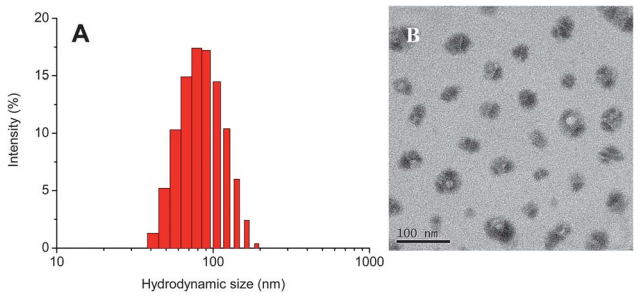


Fig. 4 The size distribution profiles of DOX-loaded PCL-*g*-PHEMA(16.3k) micelles (7.68 wt% DOX, Table 3, Entry 2) determined by DLS (A) and TEM (B).

different conditions, *i.e.* pH 7.4 (phosphate buffer, 10 mM) and pH 5.0 (MES buffer, 10 mM). The results showed sustained release of DOX over a period of 4 d (Fig. 5). No burst release was observed at both pHs. It is interesting to note that release of DOX from all three graft copolymer micelles was significantly faster at pH 5.0 than at pH 7.4. For example, approximately 16.7% and 44.6% DOX were released in 12 h, while 48.5% and 84.6% in 3 d from DOX-loaded PCL-*g*-PHEMA(16.3k) micelles at pH 7.4 and 5.0, respectively (Fig. 5). This pH-dependent release of DOX was also observed for PEG-*b*-PCL diblock copolymer micelles, though with a much lower drug release rate

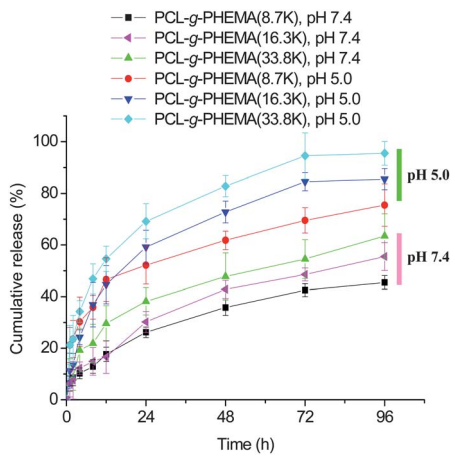


Fig. 5 *In vitro* drug release from DOX-loaded PCL-*g*-PHEMA graft copolymer micelles at pH 7.4 (phosphate buffer, 10 mM) or pH 5.0 (MES buffer, 10 mM) at 37 °C.

(approximately 30% release in 3 d and 65% in 35 d).[36] The faster release of DOX from PCL-*g*-PHEMA graft copolymer micelles under acidic conditions is likely due to improved water solubility of DOX following protonation as well as better drug permeability and/or enhanced degradation of graft copolymer micelles as compared to PEG-*b*-PCL diblock copolymer micelles. It is remarkable to note that PCL-*g*-PHEMA graft copolymer micelles exhibited pH-responsive drug release profiles comparable to those reported for rapidly acid-degradable PEG-*b*-polycarbonate block copolymer micelles,[39,40] supporting that polymer architecture plays a critical role in controlled drug release.[41] It should further be noted that drug release rate increased with increasing PHEMA graft lengths, in which 69.5%, 84.6% and 94.5% of DOX was released in 3 d at pH 5.0 from PCL-*g*-PHEMA(8.7k), PCL-*g*-PHEMA(16.3k) and PCL-*g*-PHEMA(33.8k) micelles, respectively (Fig. 5). This is likely because micelles with longer PHEMA grafts have a less densely packed core and therefore better drug permeability. The combination of small particle size, decent drug loading, and rapid drug release at endosomal pH renders PCL-*g*-PHEMA graft copolymer micelles, in particular PCL-*g*-PHEMA(16.3k) and PCL-*g*-PHEMA(33.8k) micelles, highly interesting for intracellular DOX release.

Anti-tumor activity of DOX-loaded PCL-*g*-PHEMA micelles

The *in vitro* cytotoxicity of DOX-loaded PCL-*g*-PHEMA graft copolymer micelles was investigated in HeLa cells by MTT assays. PCL-*g*-PHEMA(16.3k) and PCL-*g*-PHEMA(33.8k) micelles were selected for further studies due to their optimal DOX loading as well as superior pH-responsive drug release behaviors. The cells were incubated for 72 h with DOX-loaded PCL-*g*-PHEMA micelles or free DOX. The results showed that both DOX-loaded PCL-*g*-PHEMA micelles showed pronounced cytotoxic effects (Fig. 6). For example, significantly reduced cell viabilities of about 42.1% and 39.2% were observed for cells treated at a DOX dosage of 2.5 μg mL^{-1} with DOX-loaded PCL-*g*-PHEMA(16.3k) and PCL-*g*-PHEMA(33.8k) micelles, respectively. At a higher DOX dosage of 10 μg mL^{-1} under otherwise the same conditions, cell viabilities further decreased to about 19.2% and 18.5%, which was similar to that observed for free DOX (18.3% cell viability). The IC_{50} (*i.e.*, inhibitory concentration that produces 50% cell death) was determined to be 1.74 and 1.47 μg of DOX equiv. mL^{-1} for DOX-loaded PCL-*g*-PHEMA(16.3k) and PCL-*g*-PHEMA(33.8k) micelles, respectively, which was somewhat higher than that for free DOX

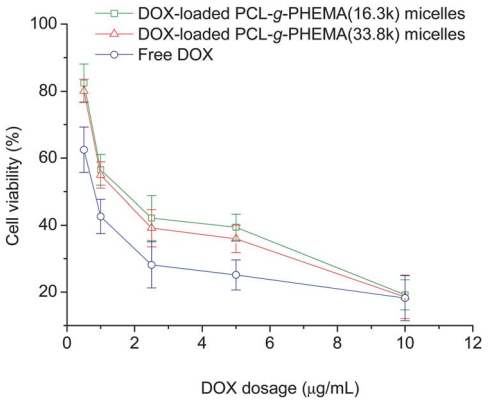


Fig. 6 Anti-tumor activity of DOX-loaded PCL-*g*-PHEMA graft copolymer micelles as a function of DOX concentrations. HeLa cells were incubated with DOX-loaded micelles or free DOX for 72 h. The cell viability was determined by MTT assays ($n = 4$).

($IC_{50} = 0.86$ μg mL^{-1}) (Fig. 6) but significantly lower than those reported for DOX-loaded non-targeted block copolymer micelles[42] as well as endosomal pH-activatable PEG-*g*-DOX prodrugs.[43] DOX-loaded PCL-*g*-PHEMA(33.8k) micelles revealed slightly lower IC_{50} than PCL-*g*-PHEMA(16.3k) counterparts, indicating that the drug release rate plays a more significant role than micelle size. The anti-tumor activity of DOX-loaded PCL-*g*-PHEMA micelles might be further enhanced by installing a tumor-targeting ligand that facilitates specific cellular uptake.[44,45] These novel biodegradable PCL-*g*-PHEMA graft copolymer micelles, therefore, possess several favorable properties including excellent biocompatibility, low CMC, small particle size, high drug loading, as well as pH-responsive drug release, which render them a highly promising alternative to biodegradable block copolymer micelles for tumor-targeting drug delivery.

Conclusions

We have demonstrated that novel biodegradable graft copolymer micelles based on poly(ε-caprolactone)-*g*-poly(2-hydroxyethyl methacrylate) (PCL-*g*-PHEMA) are superior nanocarriers for efficient delivery and release of doxorubicin into cancer cells. They offer several unique features as drug carriers: (i) there is no limitation in backbone length due to their intrinsic biodegradability, (ii) micelle properties including micelle size, core and surface properties, and drug permeability can be elegantly tuned by backbone length, graft density and graft length, (iii) they exhibit lower CMC and thereby higher stability than block copolymer micelles, (iv) remarkably, they rapidly release doxorubicin in response to mildly acidic pH mimicking that of endo/lysosomal compartments, achieving enhanced anti-tumor activity as compared to common biodegradable block copolymer micelles, and (v) the presence of many hydrophilic grafts per macromolecule enables conjugation of high density targeting ligands, which coupled with favorable exposing of ligands at the outer surface may present superior recognition and/or targetability. Moreover, PCL-*g*-PHEMA graft copolymers can be readily synthesized by controlled RAFT polymerization. These biodegradable PCL-*g*-PHEMA graft copolymer micelles are highly promising as an alternative to biodegradable block copolymer micelles for the development of "smart" nano-carriers for tumor-targeted drug delivery.

Acknowledgements

This work is financially supported by research grants from the National Natural Science Foundation of China (NSFC 51103093, 51173126, 20974073, 50973078 and 20874070), the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (11KJB150013), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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