Polymer Chemistry

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Synthesis of degradable bifunctional dendritic polymers as versatile drug carriers

Cite this: *Polym. Chem.*, 2013, **4**, 812

Xinpeng Ma,[b] Qihang Sun,[b] Zhuxian Zhou,[b] Erlei Jin,[b] Jianbin Tang,[a] Edward Van Kirk,[c] William J. Murdoch[c] and Youqing Shen*[ab]

Received 20th September 2012
Accepted 12th October 2012

DOI: 10.1039/c2py20771k

www.rsc.org/polymers

Dendritic polymers have shown great potential as drug carriers due to their precise chemical makeup, nanosized structures and high density of surface functionalities, but most dendrimers bear functionalities only on the periphery, limiting their utility as drug-delivery carriers. Herein, we report synthesis of biodegradable bifunctional dendritic polymers with acrylate termini and interior hydroxyl groups. These bifunctional dendritic polymers are nontoxic and biodegradable, offering a versatile platform for various biomedical applications. As a proof of concept, the fourth-generation dendritic polymer was PEGylated on the periphery, and the anticancer drug camptothecin was tethered in its interior, forming a well-defined core–shell-structured dendritic polymer conjugate with a high drug loading capacity (up to ~17.4 wt%).

1 Introduction

Dendritic polymers are perfect nanosized platforms for numerous applications,[1–5] particularly in pharmaceuticals as drug[6–8] and gene carriers[9,10] owing to their internal cavities for drug encapsulation,[11–13] copious surface functional groups for conjugations,[14–18] and perfect sizes for tumor targeting *via* the enhanced permeability and retention effect.[19–21]

Dendrimers are synthesized by multistep reactions of multifunctional monomers.[22–25] Generally, all the functional groups are used to construct the dendrimers, and thus there are no reactive groups left in the interior. Therefore, most dendrimers have nonreactive interiors, serving simply as the skeleton of the nanostructure, and reactive peripheries with functional groups such as amine, carboxylic acid, hydroxyl, or unsaturated double bonds available for functionalization.[5,13] PAMAM and triazine dendrimers are typical examples.[25–27] Thus, drugs, peptides, targeting groups, or polymer chains are generally introduced on the peripheries to obtain dendrimer–drug conjugates.[5,13,28–32] Given the various systems developed from these dendrimers, there are still some limitations in drug delivery. In these dendrimer conjugates, drug moieties tethered to the periphery can contact and thus interact with blood components such as the lipophilic domains of proteins in the blood, causing opsonization. The drug-loading contents of the tethered hydrophobic drugs also cannot be high, generally several percent, to keep the dendrimer conjugates water soluble.[33] When the dendrimer interiors are used to encapsulate drugs, the low drug-loading content and burst release are the two major problems.[3,34,35]

Interior-and-periphery bifunctional dendrimers can make full use of the nanostructures.[36] One approach to prepare such bifunctional dendrimers is to use core-forming compounds or monomers containing functional group(s) that do not participate in dendrimer synthesis.[37,38] For instance, Antoni *et al.* synthesized a dendrimer with interior and peripheral groups using trifunctional AB_2C monomers (A, carboxylic acid; B, hydroxyl group; and C, acetylene or azide group).[39] The C groups (acetylene or azide) in the monomer did not participate in the dendrimer synthesis reaction (esterification), remaining within the interior. The other approach is to generate functional groups from the dendrimer synthesis reactions.[40,41] For instance, taking advantage of the amine/epoxy reaction producing a hydroxyl group, Hawker and coworkers synthesized dendrimers with hydroxyl-functionalized interiors very efficiently.[42]

We proposed interior and peripheral bifunctional polyester dendrimers as versatile and biodegradable drug carriers whose interior functional groups might be used for drug conjugation while the periphery might be PEGylated for stealth properties. Herein, we report a convenient synthesis of these bifunctional dendritic polyesters with a high density of functionalities *via* sequential thiol/acrylate Michael addition reaction from A_3 (triacrylates) and B_2C_2 (dithiothreitol, C═OH) types of monomers. The thiol/acrylate reaction was chosen for its fast and quantitative reaction, considered as a new type of click reaction.[43,44] This made the synthesis of each generation fast and complete, simplifying the purification. The resulting dendritic

[a]*Key Laboratory of Biomass Chemical Engineering of Ministry of Education, and Center for Bionanoengineering Zhejiang University, Hangzhou 310027, China. E-mail: shenyq@zju.edu.cn; Fax: +86 571-87953993; Tel: +86 571-87953993*

[b]*Department of Chemical & Petroleum Engineering, University of Wyoming, Laramie, Wyoming 82071, USA*

[c]*Department of Animal Science, University of Wyoming, Laramie, Wyoming 82071, USA*

polymers had peripheral acrylate groups and interior hydroxyl groups. They consisted of hydrolyzable ester bonds, and thus were biocompatible and biodegradable.[45] To demonstrate their drug carrier abilities, the fourth-generation dendritic polymer was PEGylated with PEG2k on the periphery and an anticancer drug camptothecin (CPT) was tethered in its interior, forming a well-defined core–shell structured dendritic polymer conjugate with a high drug-loading capacity (~17.4 wt%).

2 Experimental section

2.1 Materials

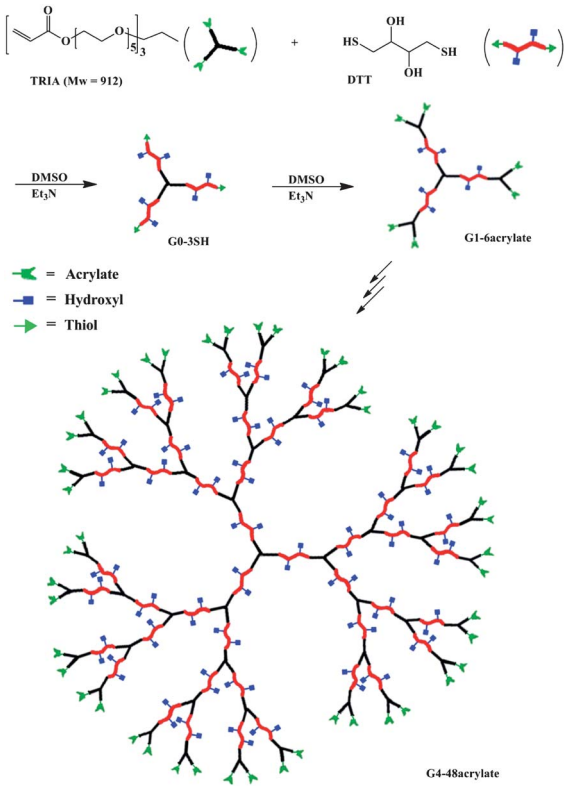
Trimethylolpropane ethoxylate triacrylate (Mn 912, TRIA), DL-dithiothreitol (DTT, ≥98%), triethylamine, (TEA, ≥99%), (*S*)-(+)-CPT (95%), mono-*tert*-butyl succinate (97%), trifluoroacetic acid (99%), thioglycolic acid (≥98%), *N*-(3-dimethylamino-propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, ≥98%), sulfuric acid (95.0–98.0%), 4-(*N*,*N*-dimethylamino)pyridine (DMAP, ≥99%), poly(ethylene glycol) methyl ether (PEG2k, M_n ~ 2000), 2,2-dimethoxy-2-phenylacetophenone (DMPA, 99%) and anhydrous dimethyl sulfoxide (DMSO, 99.9%) were purchased from Sigma-Aldrich. Anhydrous dichloromethane (99.96%) and ethyl ether (99.0%) came from Fisher Scientific (Pittsburgh, PA). All chemicals were used as received.

2.2 Instrumentation

Gel permeation chromatography (GPC) was performed on a Waters SEC equipped with a Waters 2414 refractive index detector and a PD2000 dynamic laser light scattering detector with 15° and 90° scattered light collecting angles, and two 300 mm Solvent-Saving GPC Columns (molecular weight ranges: 5×10^2 to 3×10^4, 5×10^3 to 6×10^5) set at 30 °C. THF with 3% v/v triethylamine was used as the eluent at a flow rate of 0.30 ml min^{-1}. Data were recorded and processed using the Waters software package. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance DRX-400 spectrometer using $CDCl_3$ or DMSO-d_6 as the solvent. Chemical shifts were reported downfield from 0.00 ppm using TMS as an internal reference. A Beckman System Gold® high-performance liquid chromatography (HPLC) device was equipped with a Jasco FP-2020 UV detector and a Phenomenex C18 column (4.6 × 250 mm, 5 μm) at 25 °C. The mobile phase was 50/50 acetonitrile–water (Fisher, HPLC grade) at a flow rate of 1.0 ml min^{-1}. A wavelength of 366 nm was used to detect CPT. The sizes of the dendrimers were determined using a Nano-ZS Nanosizer (Malvern Instruments., Worcestershire, UK) with a laser light wavelength of 632.8 nm and a scattering angle of 173°.

2.3 Synthesis of the core (G_0-3SH) (Scheme 1)

DTT (1.54 g, 10 mmol) and TEA (0.1 ml) were dissolved in CH_2Cl_2–DMSO (10/1 v/v) mixed solvent (60 ml). TRIA (0.75 g, 0.83 mmol) was dropped into the solution with stirring at room temperature and the solution was stirred at room temperature for 6 hours. The resulting solution was poured into cold ether. The precipitate was isolated and dried under vacuum as a colorless oil (1.07 g). ^{1}H-NMR (DMSO-d_6, 400 MHz): δ_{ppm}: 4.10 (m, 6H, CH_2COO), 3.89–3.48 (m, 62H, CH_2CH_2O and C*H*(OH)), 3.26–3.20 (m, 6H, OCH_2C), 2.73 (m, 6H, SHCH_2), 2.61 (m, 12H, CH_2SCH_2 and CH_2COO), 1.31 (m, 2H, CCH_2CH$_3$), 0.81 (t, 3H, J = 7.2 Hz, CCH$_2$CH_3).



Scheme 1 Synthesis of degradable bifunctional dendritic polymers from TRIA and DTT.

2.4 Synthesis of the first-generation with acrylate termini (G_1-6acrylate)

TRIA (6.39 g, 7.08 mmol) and TEA (0.1 ml) were dissolved in CH_2Cl_2–DMSO (10/1 v/v) mixed solvent (60 ml). G_0-3SH (0.80 g, 0.59 mmol) in 10 ml CH_2Cl_2 was dropped into the solution with stirring at room temperature and the solution was stirred at room temperature for 6 hours. The resulting solution was poured into ether and the precipitate was isolated and dried under vacuum as a colorless oil (2.26 g). PDI: 1.19. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ_{ppm}: 6.33 (d, 6H, J = 16.8 Hz, HC*H*=CH), 6.17 (m, 6H, HCH=C*H*), 5.92 (d, 6H, J = 10.0 Hz, *H*CH=CH), 4.19 (m, 24H, CH_2COO), 3.62–3.49 (m, 230H, OCH_2CH_2O and C*H*(OH)), 3.28–3.20 (m, 24H, OCH_2C), 2.71 (m, 6H, COCH$_2$CH_2S), 2.63–2.53 (m, 18H, SCH_2CH(OH) and COCH_2CH$_2$S and CH_2COO), 1.33 (m, 8H, CCH_2CH$_3$), 0.81 (t, 12H, J = 7.2 Hz, CCH$_2$CH_3).

2.5 Synthesis of the first-generation with thiol-termini (G_1-6SH)

G_1-6SH was synthesized from G_1-6acrylate (0.70 g, 0.17 mmol) and DTT (0.75 g, 4.87 mmol) following the same procedure used in the G_0-3SH synthesis, yielding a colorless oil (0.84 g). ^{1}H-NMR (DMSO-d_6, 400 MHz): δ_{ppm}: 4.11 (m, 24H, CH_2COO), 3.90–3.49

(m, 248H, OCH_2CH_2O and CH(OH)), 3.26–3.20 (m, 24H, OCH_2C), 2.71 (m, 12H, SHCH_2), 2.64–2.53 (m, 36H, CH_2SCH_2 and CH_2COO), 1.32 (m, 8H, CCH_2CH$_3$), 0.81 (t, 12H, J = 7.2 Hz, CCH$_2$CH_3).

2.6 Synthesis of the second-generation with acrylate termini (G_2-12acrylate)

G_2-12acrylate was synthesized from G_1-6SH (0.55 g, 0.11 mmol) and TRIA (2.38 g, 2.64 mmol) following the same procedure used in the G_1-6acrylate synthesis, yielding a colorless oil (1.03 g). PDI: 1.22. ^{1}H-NMR (DMSO-d$_6$, 400 MHz): δ_{ppm}: 6.33 (d, 12H, J = 17.6 Hz, HCH=CH), 6.17 (m, 12H, HCH=CH), 5.94 (d, 12H, J = 10.4 Hz, HCH=CH), 4.19 (m, 60H, CH_2COO), 3.61–3.48 (m, 578H, OCH_2CH_2O and CH(OH)), 3.28–3.20 (m, 60H, OCH_2C), 2.71 (m, 18H, COCH$_2$CH_2S), 2.64–2.52 (m, 36H, SCH_2CH(OH) and COCH_2CH$_2$S), 2.14 (t, 20H, J = 8 Hz, CH_2COO), 1.33 (m, 20H, CCH_2CH$_3$), 0.79 (t, 30H, J = 6.8 Hz, CCH$_2$CH_3).

2.7 Synthesis of the second-generation with thiol-termini (G_2-12SH)

G_2-12SH was synthesized from G_2-12acrylate (0.65 g, 62.5 μmol) and DTT (0.46 g, 3.0 mmol) following the same procedure used in the G_1-3SH synthesis, yielding a colorless oil (0.69 g). ^{1}H-NMR (DMSO-d$_6$, 400 MHz): δ_{ppm}: 4.11 (m, 60H, CH_2COO), 3.88–3.49 (m, 600H, OCH_2CH_2O and CH(OH)), 3.27–3.20 (m, 60H, OCH_2C), 2.69 (m, 24H, SHCH_2), 2.58 (m, 120H, CH_2SCH_2 and CH_2COO), 1.36 (m, 20H, CCH_2CH$_3$), 0.82 (t, 30H, J = 7.2 Hz, CCH$_2$CH_3).

2.8 Synthesis of the third-generation with acrylate termini (G_3-24acrylate)

G_3-24acrylate was synthesized from G_2-12SH (0.69 g, 56.3 μmol) and TRIA (2.38 g, 2.70 mmol) following the same procedure used in the G_1-6acrylate synthesis, yielding a colorless oil (1.31 g). PDI: 1.27. ^{1}H-NMR (DMSO-d$_6$, 400 MHz): δ_{ppm}: 6.33 (d, 24H, J = 16.8 Hz, HCH=CH), 6.20 (m, 24H, HCH=CH), 5.94 (d, 24H, J = 10.4 Hz, HCH=CH), 4.20 (m, 132H, CH_2COO), 3.63–3.48 (m, 1274H, OCH_2CH_2O and CH(OH)), 3.28–3.20 (m, 132H, OCH_2C), 2.73 (m, 84H, COCH$_2$CH_2S), 2.65–2.48 (m, 84H, SCH_2CH(OH) and COCH_2CH$_2$S), 1.30 (m, 44H, CCH_2CH$_3$), 0.80 (t, 66H, J = 6.8 Hz, CCH$_2$CH_3).

2.9 Synthesis of the third-generation with thiol-termini (G_3-24SH)

G_3-24SH was synthesized from G_3-24acrylate (0.53 g, 23.0 μmol) and DTT (0.34 g, 2.21 mmol) following the same procedure used in the G_1-3SH synthesis, yielding a colorless oil (0.56 g). ^{1}H-NMR (DMSO-d$_6$, 400 MHz): δ_{ppm}: 4.13 (m, 132H, CH_2COO), 3.79–3.47 (m, 1322H, OCH_2CH_2O and CH(OH)), 3.26–3.20 (m, 132H, OCH_2C), 2.72 (m, 48H, SHCH_2), 2.61 (m, 264H, CH_2SCH_2 and CH_2COO), 1.31 (m, 44H, CCH_2CH$_3$), 0.79 (t, 66H, J = 7.2 Hz, CCH$_2$CH_3).

2.10 Synthesis of the fourth-generation with acrylate termini (G_4-48acrylate)

G_4-48acrylate was synthesized from G_3-24SH (0.56 g, 20.9 μmol) and TRIA (1.81 g, 2.01 mmol) following the same procedure used in the G_1-6acrylate synthesis, yielding a colorless oil (0.92 g). PDI: 1.33. ^{1}H-NMR (DMSO-d$_6$, 400 MHz): δ_{ppm}: 6.33 (d, 48H, J = 17.6 Hz, HCH=CH), 6.20 (m, 48H, HCH=CH), 5.94 (d, 48H, J = 9.6 Hz, HCH=CH), 4.19 (m, 396H, CH_2COO), 3.67–3.44 (m, 2666H, OCH_2CH_2O and SCH(OH)), 3.28–3.20 (m, 396H, OCH_2C), 2.71 (m, 180H, COCH$_2$CH_2S), 2.66–2.52 (m, 180H, SCH_2CH(OH) and COCH_2CH$_2$S), 1.26 (m, 92H, CCH_2CH$_3$), 0.78 (t, 138H, J = 6.8 Hz, CCH$_2$CH_3).

2.11 Synthesis of camptothecin succinate (CPT–COOH) (Scheme 2)

CPT (1.05 g, 3.02 mmol), mono-*tert*-butyl succinate (1.74 g, 3.62 mmol), EDC (1.15 g, 6.03 mmol), and DMAP (0.74 mg, 6.03 mmol) were mixed in anhydrous dichloromethane (250 ml). The solution was stirred at room temperature for 24 h. The solution was washed with water and the organic layer was separated and dried over anhydrous sodium sulfate. The solution was concentrated to 10 ml and was poured into an excess of ethyl ether. The precipitate was isolated and dried under vacuum. CPT–CH$_2$CH$_2$COOBoc was obtained as a yellowish powder with a yield of 99%. ^{1}H-NMR (DMSO-d$_6$, 400 MHz): δ_{ppm}: 8.68 (s 1H), 8.16 (m, 2H), 7.87 (m, 1H), 7.71 (m, 1H), 7.18 (s, 1H), 5.49 (s, 2H), 5.27 (s, 2H), 2.77 (m, 2H), 2.47 (m, 2H), 2.17 (tetra, J = 7.2 Hz, 2H), 1.31 (s, 9H), 0.95 (t, J = 7.2 Hz, 3H).

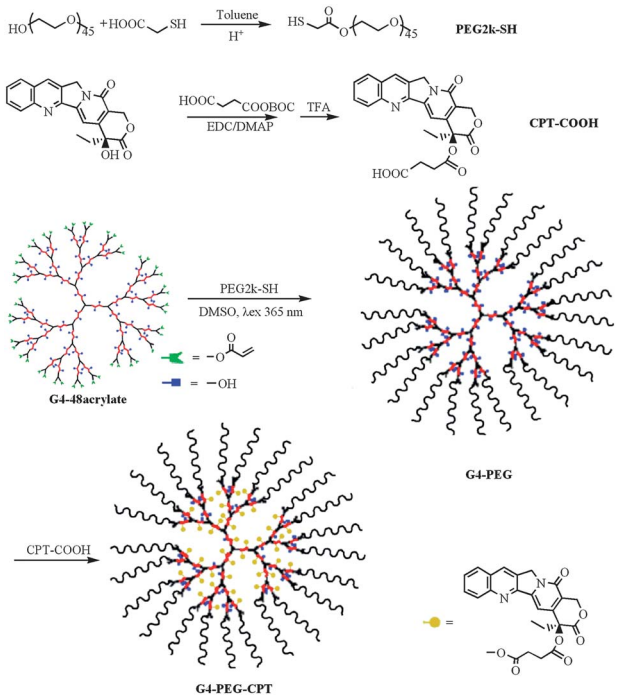
The CPT–CH$_2$CH$_2$COOBoc (1.51 g) and trifluoroacetic acid (TFA, 10 m.) were dissolved in CH_2Cl_2 (10 ml) and stirred for 2 hours. The solvent and TFA were removed under reduced pressure and the residue was poured into ether. The precipitate was isolated and dried under vacuum. The product was obtained as a yellowish powder with a yield of 97%. ^{1}H-NMR (DMSO-d$_6$, 400 MHz): δ_{ppm}: 8.67 (s 1H), 8.16 (m, 2H), 7.85 (m, 1H), 7.70 (m, 1H), 7.13 (s, 1H), 5.46 (s, 2H), 5.18 (s, 2H), 2.49 (m, 2H), 2.17 (tetra, J = 7.2 Hz, 2H), 0.91 (t, J = 7.2 Hz, 3H).

2.12 Synthesis of PEG2k-SH (Scheme 2)

PEG2k (10 g) and thioglycolic acid were dissolved in 50 ml toluene, and one drop of concentrated sulfuric acid was added to the solution. The reaction solution was refluxed overnight, cooled to room temperature and then poured into ether. The precipitate was isolated and purified by reprecipitation (500 ml × 3). Finally, the product was obtained and dried under vacuum as a white solid (10.4 g). ^{1}H-NMR (DMSO-d$_6$, 400 MHz): δ_{ppm}: 4.29 (t, J = 4.8 Hz, 2H), 3.82–3.45 (m, 180H), 3.37 (s, 3H), 2.05 (m, 2H).

2.13 Synthesis of the fourth-generation dendrimer with CPT-conjugated interior and PEGylated periphery (G_4-CPT-PEG, 9.61 wt% of CPT) (Scheme 2)

G_4-48acrylate (0.5 g), PEG2k-SH (2.0 g) and DMPA (0.13 g) were dissolved in 5 ml DMSO. The mixture was radiated by UV lamp (λ_{ex} 365 nm) for 30 minutes. ^{1}H-NMR showed that all the



Scheme 2 Surface PEGylation and interior conjugation of CPT of G_4.

acrylate groups reacted with PEG-SH and the mixture was poured into the solvent (THF–ether, 1 : 1 v/v). The product, G_4-PEG2k was isolated and dried under vacuum. ^{1}H-NMR ($CDCl_3$, 400 MHz): 4.28 (b, 492H), 3.76–3.23 (m, 11306), 2.87 (m, 90H), 2.77 (m, 90H), 2.70 (m, 180H), 1.27 (tetra, 92H, $J = 7.2$ Hz), 0.82 (b, 138H).

G_4-PEG2k (0.50 g), CPT–COOH (0.13 g, 0.3 mmol), EDC (0.067 g, 0.36 mmol) and DMAP (0.043 g, 0.36 mmol) were dissolved in 30 ml CH_2Cl_2 and stirred at room temperature for 24 h. The reaction solution was dialyzed (Spectra/Pro MWCO = 3500) against chloroform (500 ml × 3) for 24 hours. The solution in the dialysis bag was dried, producing 0.57 g of the G_4-CPT-PEG2k. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ_{ppm}: 8.65 (b, 46H), 8.10 (b, 92H), 7.83 (b, 46H), 7.68 (b, 46H), 7.10 (b, 46H), 5.48 (b, 46H), 5.28 (b, 46H), 4.10 (b, 588), 3.77–3.43 (m, 11306), 3.25 (m, 396H), 2.86 (m, 180H), 2.76 (m, 188H), 2.70 (b, 272H), 2.58 (b, 92H), 2.11 (b, 92H), 1.33 (m, 194H), 0.90 (b, 138H), 0.76 (b, 138H).

2.14 Synthesis of interior fully CPT-conjugated G4-PEG (G_4-CPT-PEG, 17.4 wt% of CPT)

G_4-48acrylate (0.50 g), CPT–COOH (0.78 g, 1.8 mmol), EDC (0.402 g, 2.16 mmol) and DMAP (0.258 g, 2.16 mmol) were dissolved in 10 ml CH_2Cl_2 and stirred at room temperature for 24 hours. The reaction solution was dialyzed (Spectra/Pro MWCO = 3500) against chloroform (500 ml × 3) for 24 h. The solution in the dialysis bag was dried under vacuum and gave 0.57 g of the G_4-CPT_{90}. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ_{ppm}: 8.48 (b, 90H), 8.25 (b, 180H), 7.96 (b, 90H), 7.83 (b, 90H), 7.67 (b, 90H), 6.46 (m, 48H), 6.20 (m, 48H), 5.86 (m, 48H), 5.68 (b, 90H), 5.41 (b, 90H), 4.32–4.19 (m, 576H), 3.75–3.46 (m, 4052H), 3.31 (b, 396H), 2.84 (m, 90H), 2.65 (m, 90H), 2.53 (b, 90H), 2.46 (b, 180H), 2.28 (b, 180H), 1.37 (m, 272), 0.99 (b, 270H), 0.83 (b, 138H).

G_4-CPT_{90} (0.5 g), PEG2k-SH (2.0 g) and DMPA (0.13 g) were dissolved in 5 ml DMSO. The mixture was radiated by UV lamp (λ_{ex} 365 nm) at the wavelength of 365 nm for 30 minutes. ^{1}H-NMR showed that all the acrylate groups reacted with PEG-SH. The mixture was poured into the THF–ether (1 : 1 v/v) solvent. The precipitate was dried under vacuum and obtained G_4-CPT-PEG (1.10 g) with 17.4 wt% of CPT as determined by ^{1}H-NMR spectrum. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ_{ppm}: 8.67 (b, 90H), 8.15 (b, 180H), 7.86 (b, 90H), 7.69 (b, 90H), 7.11 (b, 90H), 5.46 (b, 90H), 5.25 (m, 90H), 4.10 (b, 672H), 3.89 (b, 276H), 3.77–3.26 (m, 11300H), 3.21 (b, 576H), 2.71 (m, 90H), 2.59 (m, 90H), 2.34 (b, 180H), 2.12 (b, 180H), 1.26 (m, 180H), 1.15 (m, 92H), 0.90 (b, 270H), 0.77 (b, 138H).

2.15 *In vitro* cytotoxicity assay

The cytotoxicity assay was carried out using the (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (MTT) cell proliferation assay kit (ATCC, Manassas, VA) according to the modified manufacturer's protocol. SKOV-3 cells were cultured in medium (Invitrogen Corp., Carlsbad, CA) for at least two weeks before use. They were then seeded onto 96-well plates at a density of 10 000 cells per well and incubated for 24 h. The original medium (200 μl) was removed and replaced with the G4-CPT-PEG (9.61 wt% of CPT), G4-PEG, or free CPT solutions at different concentrations and incubated for 24 h. The medium in each well was then replaced with fresh cell culture medium and further incubated for 24 h. MTT reagent (10 μl) was then added to each well and incubated for 6 h until purple precipitates were visible. Finally, the detergent reagent (100 μl) was added to each well, and the plates were incubated at 37.0 °C for 18 h until all the crystals were dissolved. The absorbance intensity at 570 nm was recorded and the cytotoxicity was expressed as a percentage of the control.

3 Results and discussion

To synthesize dendrimers with interior functionalities, the monomer(s) must contain either protected functional groups or functional groups that do not participate in the dendrimer synthesis reactions. DTT contains two highly reactive thiol groups and two hydroxyl groups. Thiol–ene reaction, as a complement of click chemistry, has been widely used in polymer modification, dendrimer synthesis, and other bioconjugate systems.[46–48] Michael addition reactions of thiols with acrylates and other electron-deficient enes are particularly efficient but simple.[49] For instance, in the presence of triethylamine, a thiol reacts with acrylate with a quantitative yield under mild conditions.[43,50,51] The hydroxyl group does not react with acrylates under the same conditions. Thus, no protection/deprotection is needed when using the DTT–acrylate reaction to prepare interior hydroxy-functionalized dendritic polymers.

We chose the readily available A_3-type monomer TRIA[52] (Mn 912) and DTT (B_2C_2 type) as a pair of monomers. TRIA was first used as the core-forming compound to react with an excess of

DTT (thiol/acrylate = 4) and obtained the thiol-terminated core G_0-3SH. It was then reacted with an excess of A_3 monomer TRIA (acrylate/SH = 4) to produce the acrylate-terminated first generation (G_1-6acrylate) with 6 hydroxyl groups in the interior. Alternating addition of the two monomers produced the fourth-generation dendritic polymer (G_4-48acrylate) with ninety interior hydroxyl groups at a high overall yield (62%). The synthesis strategy is shown in Scheme 1.

Due to the equal reactivity of the two thiol groups in DTT and the three acrylate groups in TRIA, an excess of a monomer had to be used to avoid crosslinking to create a low-polydispersity dendritic polymer. The solvent was also found to play an important role in the synthesis. The reaction of thiol/acrylate was too fast in DMSO and thus caused crosslinking even in the presence of a great excess of DTT. The reaction was slow in dichloromethane. Thus, a mixed solution of dichloromethane–DMSO at the ratio of 10 : 1 was found to give an optimal reaction rate. Most reactions could be completed within 6 hours at room temperature. The thiol/acrylate ratio was subsequently optimized. To prepare the core G_0-3SH, the DTT amounts at the thiol/acrylate molar ratio ranging from 2 : 1 to 4 : 1 were tested. The GPC traces of the crude reaction mixtures are showed in Fig. 1. The peak at 20.8 min corresponds to G_0-3SH. At the thiol/acrylate ratio of 2 : 1, the reaction solution had a shoulder at 20 minutes, suggesting that some DTT molecules reacted with two TRIAs (Fig. 1a and b). At the thiol/acrylate ratio of 4 : 1, the intensity of the shoulder peak reduced and the polydispersity of G_0-3SH (Fig. 1c) was close to that of TRIA (Fig. 1d). Thus, a minimal thiol/acrylate ratio of 4 : 1 was needed to prepare the core G_0-3SH. Subsequently, the G_0-3SH was added to 4-equivalent TRIA (molar) and the first generation, G_1, with six acrylates (G_1-6acrylate) was obtained. At high generations, a DTT/acrylate or TRIA/SH ratio of at least 4 was found necessary to get low-polydispersity products. Though an excess of the monomer in each step was needed, it was much less than is used in PAMAM dendrimer synthesis.

The efficiencies of the reactions were confirmed by ^{1}H-NMR (Fig. 2). After 6 hours stirring of TRIA and DTT (DTT–acrylate = 4/1), the characteristic peaks of acrylate (5.8–6.5 ppm) disappeared (Fig. 2a). After the reaction of TRIA with G_0-3SH at a TRIA/SH ratio of 4 for 6 h at room temperature, the characteristic peak of the thiol group at 2.15 ppm (Fig. 2a) also disappeared, producing G_1-6acrylate (Fig. 2b).

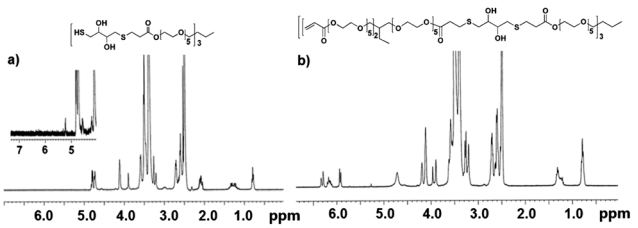


Fig. 2 The ^{1}H-NMR spectra of G_0-3SH (a) and G_1-6-acrylate in DMSO-d_6 (b).

The purification of the dendritic polymers was simple precipitation. The core G_0-3SH needed to be precipitated in cold ether (−20 °C) due to its low molecular weight. Other dendritic polymers easily precipitated in ether to remove the unreacted TRIA or DTT at room temperature. The GPC traces of the four dendritic polymer generations are shown in Fig. 3. Their molecular weights gradually increased as the generation increased from 1 to 4, and were very close to their calculated values. For example, the measured molecular weight of the third-generation G_3-24acrylate was 26.1 kDa, close to its theoretical value of 25.1 kDa. There was almost no trace of small molecules, which would appear at 23 min. This indicates that the monomers were efficiently removed by precipitation. Their polydispersity was around 1.2, close to that of the monomer TRIA, although the polydispersity of the fourth generation was slightly broader.

While the dendritic polymers had thiol or acrylate functional groups on their periphery, they also contained hydroxyl groups in their interiors and thus the total number of functional groups increased quickly with the generation. For instance, the fourth-generation had 48 peripheral acrylate groups and 90 interior hydroxyl groups, totalling 138 functional groups. More importantly, the two types of functional groups are so different as to allow for selective modifications on the surface and the interior.

The dendritic polymers are composed of hydrolysable beta-thioester bonds. Thus, they are biodegradable and biocompatible, making them suitable for biomedical applications. The application of the bifunctionalities in drug delivery was demonstrated by using the interior hydroxyl groups for drug conjugation and the peripheral acrylates for PEGylation (Scheme 2). This interior drug conjugation not only avoids burst drug release, but also hides the drug inside the dendritic polymer, preventing its interaction with serum proteins generally found in drug conjugation on the dendrimer periphery.[3]

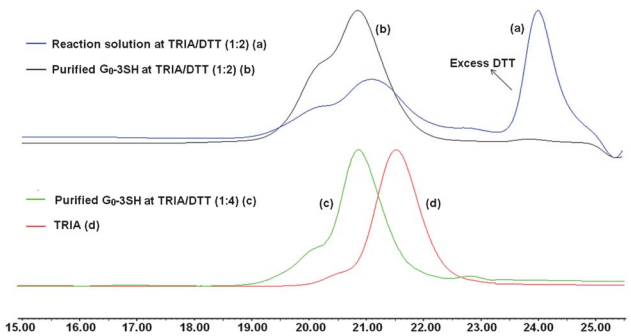


Fig. 1 The GPC traces of G_0-3SH obtained at different TRIA/SH ratios.

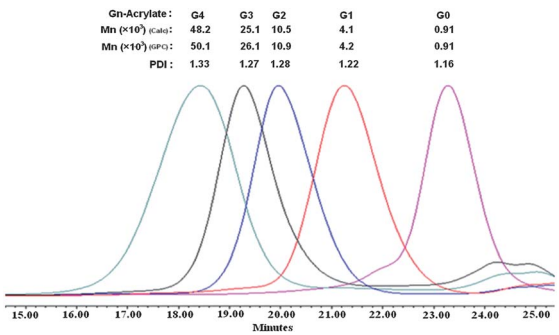


Fig. 3 GPC traces of the dendritic polymers from G_0 to G_4.

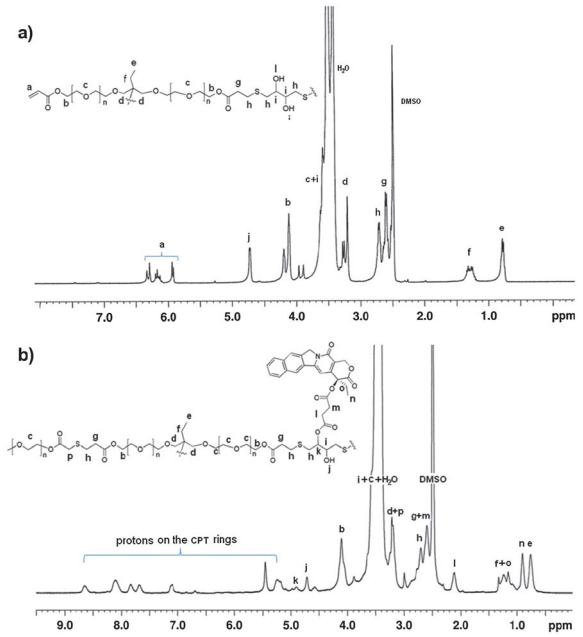


Fig. 4 The ^{1}H-NMR spectra of the G_4-48acrylate (a) and G_4-CPT-PEG with 9.61 wt% CPT in DMSO-d_6 (b).

PEG of molecular weight of 2 kDa with a terminal thiol group (PEG2k-SH) was first synthesized by esterification from PEG2k and thioglycolic acid in the presence of a catalytic amount of sulfuric acid. An excess of PEG2k-SH (2 equivalents) was reacted with the G4-48acrylate by UV radiation at 365 nm in the presence of 5 wt% of a photoinitiator, DMPA, or direct Michael addition catalyzed by TEA at room temperature (data not shown). Both approaches could complete the reaction to produce PEGylated G_4 (G_4-PEG) as confirmed by complete disappearance of the characteristic peaks of the acrylate at 6.4 ppm to 5.8 ppm in the ^{1}H-NMR spectra and the peaks at 310–400 nm in the UV spectra. The UV radiation method required a less excess of PEG2k-SH (Michael addition needs 4 equivalents) and thus simplified the purification process.

Hydroxyl groups in the interior of G_4-PEG were reacted with CPT–COOH (Scheme 2). The signal of the hydroxyl group at 4.7 ppm reduced after the reaction with CPT–COOH, suggesting the formation of CPT–COO– esters (Fig. 4, peak j). The conjugation was characterized by ^{1}H-NMR spectroscopy (Fig. 4) and UV spectra (Fig. 5). The CPT content in G_4-CPT-PEG was calculated from the integrations of methyl groups in the

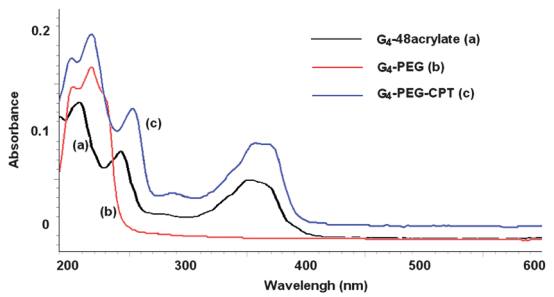


Fig. 5 The UV spectra of G_4-48acrylate (a), G_4-PEG (b) and G_4-CPT-PEG (c).

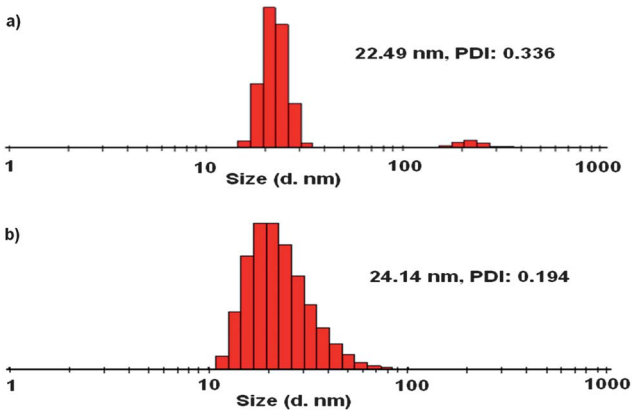


Fig. 6 Dynamic laser light scattering (DLS) data of G_4-CPT-PEG conjugated with 9.61 wt% CPT (a) and 17.4 wt% CPT (b) (0.1 mg ml^{-1}).

dendritic backbone (Fig. 4b, peak e) and CPT (Fig. 4b, peak n) and confirmed by UV spectra. Under the reaction conditions slightly more than half of the hydroxyl groups were conjugated with CPT, giving a CPT content of 9.61 wt%, probably due to the steric effect of the tethered PEG chains. To conjugate CPT to all the hydroxyl-groups to achieve the theoretical CPT content, 16.6 wt%, G_4-48acrylate was reacted with CPT–COOH first, and then reacted with PEG2k-SH for peripheral PEGylation. However, the resulting CPT content was 17.4 wt%, because the PEGylation efficiency was about 91.5%.

The G_4-CPT-PEG with 9.61 wt% or 17.4 wt% CPT was very water soluble. Its average size in DI water was 22.5 nm (Fig. 6a) or 24.2 nm (Fig. 6b), respectively. Thus, the PEGylated dendritic polymer with interior-conjugated CPT (G_4-CPT-PEG) was unimolecular micelles with a core–shell structure, which is expected to have a long blood circulation.[53] This is advantageous over linear PEG, to which only one or two drug molecules can be conjugated. For example, Pegamotecan (Enzon Pharmaceuticals, Inc.), a PEG-CPT conjugate with two CPT molecules at the two ends of a PEG diol with a molecular weight of 40 kDa, had a drug loading content as low as 1.7 wt%.[54] Compared to conventional micelles made from amphiphilic block copolymers, such drug-conjugated dendritic

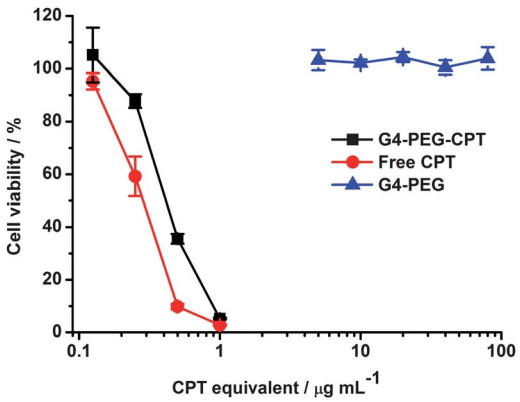


Fig. 7 The cytotoxicity of free CPT, G_4-CPT-PEG (9.61 wt% CPT) and G_4-PEG to SKOV-3 ovarian cancer cells estimated by MTT assay. Cells were exposed to the indicated drug or polymers for 72 h in medium. Data represent mean ± s.d., $n = 5$.

unimolecular micelles also have advantages of fixed drug loading without burst release, small micelle size, well-defined structure, and infinite stability.[55]

We compared the cytotoxicity of free CPT, G_4-PEG2k, and G_4-CPT-PEG (9.61 wt% CPT) to SKOV-3 ovarian cancer cells using the MTT assay. The cells were treated for 72 h and then post-cultured for 24 h to allow the damaged cells to undergo apoptosis. The results are presented in Fig. 7. G_4-PEG2k is not toxic even at high doses. The IC_{50} of the CPT in the G_4-CPT-PEG to SKOV-3 cells was 0.499 μg ml^{-1}, slightly higher than that of free CPT (0.293 μg ml^{-1}), indicating that the CPT conjugated in the dendritic interior could be released inside the cell. Thus, this unique dendritic polymer conjugate merits further study as a carrier for cancer drug delivery.

4 Conclusions

We demonstrated a convenient synthesis of interior-and-peripheral-bifunctionalized dendritic polymers. These bifunctional dendritic polymers are nontoxic and biodegradable, offering a versatile platform for various biomedical applications. As a proof of a concept, the fourth-generation product was surface-PEGylated with PEG2k and CPT was tethered in its interior, forming a core–shell dendritic polymer with a well-defined structure and a high drug loading capacity (~17.4 wt%). The new type dendritic polymer–drug conjugate behaved like a unimolecular micelle with good water solubility and stability. Thus, this conjugate is very promising as a drug delivery carrier.

Acknowledgements

We thank the National Science Foundation of China (21090352), the National Fund for Distinguished Young Scholars (50888001), Ministry of Education (20110101130007), the Program for Changjiang Scholars and Innovative Research Team of the University of China, and the U.S. Department of Defense (BC090502) for financial support.

Notes and references

1 D. Astruc, E. Boisselier and C. T. Ornelas, *Chem. Rev.*, 2010, **110**, 1857.
2 B. V. Basavaraj, S. Furtado, S. Bharath, R. Deveswaran, S. Abharam and V. Madhavan, *J. Pharm. Res.*, 2009, **2**, 970.
3 L. M. Kaminskas, V. M. McLeod, C. J. H. Porter and B. J. Boyd, *Mol. Pharmaceutics*, 2012, **9**, 355.
4 J. Satija, V. V. R. Sai and S. Mukherji, *J. Mater. Chem.*, 2011, **21**, 14367.
5 A. R. Menjoge, R. M. Kannan and D. A. Tomalia, *Drug Discovery Today*, 2010, **15**, 171.
6 Y. Li, H. He, X. Jia, W. Lu, J. Lou and Y. Wei, *Biomaterials*, 2012, **33**, 3899.
7 T. C. Wan, D. K. Tosh, L. Du, E. T. Gizewski, K. A. Jacobson and J. A. Auchampach, *BMC Pharmacol.*, 2011, **11**, 11.
8 I. J. Majoros, A. Myc, T. Thomas, C. B. Mehta and J. R. Baker, *Biomacromolecules*, 2006, **7**, 572.
9 H. Y. Nam, K. Nam, M. Lee, S. W. Kim and D. A. Bull, *J. Controlled Release*, 2012, **160**, 592.
10 Y. Tang, Y. Li, B. Wang, R. Lin, M. Dongen, D. M. Zurcher, X. Gu, M. M. Banaszak, G. Liu and R. Qi, *Mol. Pharmaceutics*, 2012, **9**, 1812.
11 M. Fang, J. Zhang, Q. Wu, T. Xu and Y. Cheng, *J. Phys. Chem. B*, 2012, **116**, 3075.
12 Y. Wang, X. Cao, R. Guo, M. Shen, M. Zhang, M. Zhu and X. Shi, *Polym. Chem.*, 2011, **2**, 1754.
13 J. B. Wolinsky and M. W. Grinstaff, *Adv. Drug Delivery Rev.*, 2008, **60**, 1037.
14 S. Chandra, S. Dietrich, H. Lang and D. Bahadur, *J. Mater. Chem.*, 2011, **21**, 5729.
15 S. Zhu, L. Qian, M. Hong, L. Zhang, Y. Pei and Y. Jiang, *Adv. Mater.*, 2011, **23**, H84.
16 A. B. Witte, C. M. Timmer, J. J. Gam, S. K. Choi, M. M. Banaszak Holl, B. G. Orr, J. R. Baker and K. Sinniah, *Biomacromolecules*, 2012, **13**, 507.
17 R. Iezz, B. R. Guru, I. V. Glybina, M. K. Mishra, A. Kennedy and R. M. Kannan, *Biomaterials*, 2012, **33**, 979.
18 H. Zong, T. P. Thomas, K. H. Lee, A. M. Desai, M. Li, A. Kotlyar, Y. Zhang, P. R. Leroueil, J. J. Gam, M. M. Banaszak-Holl and J. R. Baker, *Biomacromolecules*, 2012, **13**, 982.
19 Y. Matsumura and H. Maeda, *Cancer Res.*, 1986, **46**, 6387.
20 H. Maeda, L. W. Seymour and Y. Miyamoto, *Bioconjugate Chem.*, 1992, **3**, 351.
21 H. Maeda, J. Wu, T. Sawa, Y. Matsumura and K. Hori, *J. Controlled Release*, 2000, **65**, 271.
22 D. A. Tomalia, H. Baker, J. Dewald, M. Hall, G. Kallos, S. Martin, J. Roeck, J. Ryder and P. Smith, *Polym. J.*, 1985, **17**, 117.
23 C. J. Hawker and J. M. J. Frechet, *J. Am. Chem. Soc.*, 1990, **112**, 7638.
24 M. V. Walter and M. Malkoch, *Chem. Soc. Rev.*, 2012, **41**, 4593.
25 J. Lim and E. E. Simanek, *Adv. Drug Delivery Rev.*, 2012, **64**, 826.
26 S. El Kazzouli, S. Mignani, M. Bousmina and J. P. Majoral, *New J. Chem.*, 2012, **36**, 227.
27 G. S. Yu, Y. M. Bae, H. Choi, B. Kong, I. S. Choi and J. S. Choi, *Bioconjugate Chem.*, 2011, **22**, 1046.
28 H. Zong, T. P. Thomas, K. H. Lee, A. M. Desai, M. H. Li, A. Kotlyar, Y. H. Zhang, P. R. Leroueil, J. J. Gam, M. M. B. Holl and J. R. Baker, *Biomacromolecules*, 2012, **13**, 982.
29 Y. Shen, Z. Zhou, M. Sui, J. Tang, P. Xu, E. A. Van Kirk, W. J. Murdoch, M. Fan and M. Radosz, *Nanomedicine*, 2010, **5**, 1205.
30 W. Cao, J. Zhou, Y. Wang and L. Zhu, *Biomacromolecules*, 2010, **11**, 3680.
31 L. Ye, K. Letchford, M. Heller, R. Liggins, D. Guan, J. N. Kizhakkedathu, D. E. Brooks, J. K. Jackson and H. M. Burt, *Biomacromolecules*, 2011, **12**, 145.
32 Y. Wang, X. Cao, R. Guo, M. Shen, M. Zhang, M. Zhu and X. Shi, *Polym. Chem.*, 2011, **2**, 1754.

33 R. Duncan and L. Izzo, *Adv. Drug Delivery Rev.*, 2005, **57**, 2215.
34 Y. Wang, R. Guo, X. Y. Cao, M. W. Shen and X. Y. Shi, *Biomaterials*, 2011, **32**, 3322.
35 A. K. Patri, J. F. Kukowska-Latallo and J. R. Baker, *Adv. Drug Delivery Rev.*, 2005, **57**, 2203.
36 S. Hecht, *J. Polym. Sci., Part A: Polym. Chem.*, 2003, **41**, 1047.
37 C. Ornelas and M. Weck, *Chem. Commun.*, 2009, 5710.
38 B. Yeniad, H. Naik, R. J. Amir, C. E. Koning, C. J. Hawker and A. Heise, *Chem. Commun.*, 2011, **47**, 9870.
39 P. Antoni, Y. Hed, A. Nordberg, D. Nystrom, H. V. Holst, A. Hult and M. Malkoch, *Angew. Chem., Int. Ed.*, 2009, **48**, 2126.
40 A. P. Goodwin, S. S. Lam and J. M. J. Frechet, *J. Am. Chem. Soc.*, 2007, **129**, 6994.
41 R. J. Amir, L. Albertazzi, J. Willis, A. Khan, T. Kang and C. J. Hawker, *Angew. Chem., Int. Ed.*, 2011, **50**, 3425.
42 T. Kang, R. J. Amir, A. Khan, K. Ohshimizu, J. N. Hunt, K. Sivanandan, M. I. Montanez, M. Malkoch, M. Ueda and C. J. Hawker, *Chem. Commun.*, 2010, **46**, 1556.
43 N. Wang, A. Dong, M. Radosz and Y. Shen, *J. Biomed. Mater. Res., Part A*, 2008, **84**, 148.
44 Z. Chen, J. Tang, Y. Shen and X. Wang, *Sci. Sinica Chim.*, 2011, 281.
45 N. Feliu, M. V. Walter, M. I. Montanez, A. Kunzmann, A. Hult, A. Nystrom, M. Malkoch and B. Fadeel, *Biomaterials*, 2012, **33**, 1970.
46 C. E. Hoyle and C. N. Bowman, *Angew. Chem., Int. Ed.*, 2010, **49**, 1540.
47 A. B. Lowe, *Polym. Chem.*, 2010, **1**, 17.
48 K. L. Killops, L. M. Campos and C. J. Hawker, *J. Am. Chem. Soc.*, 2008, **130**, 5062.
49 J. W. Chan, C. E. Hoyle, A. B. Lowe and M. Bowman, *Macromolecules*, 2010, **43**, 6381.
50 X. Ma, J. Tang, Y. Shen, M. Fan, H. Tang and M. Radosz, *J. Am. Chem. Soc.*, 2009, **131**, 14795.
51 N. Wang, A. Dong, H. Tang, E. A. Van Kirk, P. A. Johnson, W. J. Murdoch, M. Radosz and Y. Shen, *Macromol. Biosci.*, 2007, **7**, 1187.
52 D. R. Swanson, B. Huang, H. G. Abdelhady and D. A. Tomalia, *New J. Chem.*, 2007, **31**, 1368.
53 J. W. Yoo, E. Chambers and S. Mitragotri, *Curr. Pharm. Des.*, 2010, **16**, 2298.
54 R. B. Greenwald, *J. Controlled Release*, 2001, **74**, 159.
55 Q. Sun, M. Radosz and Y. Shen, *J. Controlled Release*, 2012, DOI: 10.1016/j.jconrel.2012.05.042.