International Journal of Pharmaceutics 409 (2011) 229–236

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

PEGylated thermo-sensitive poly(amidoamine) dendritic drug delivery systems

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ARTICLE INFO

Article history:
Received 23 November 2010
Received in revised form 2 February 2011
Accepted 4 February 2011
Available online 21 February 2011

Key words:
Dendrimer
PAMAM
Poly(N-isopropylacrylamide)
Poly(ethylene glycol)
Thermo-sensitive

ABSTRACT

Thermo-sensitive dendrimers hold promise in various biomedical and pharmaceutical applications due to their stimuli-responsive properties. However, for such systems there are still certain unaddressed issues e.g. the undesired toxicity, immunogenicity and short blood circulation time. PEGylation is a potential approach to solve these above problems. The aims of this study were to engineer PEGylated thermo-sensitive dendritic derivatives and to investigate their temperature sensitivity and drug release behaviour therein. Linear poly(N-isopropylacrylamide) (PNIPAAm) and methoxy poly(ethylene glycol) (MPEG) were attached to the surface of polyamidoamine (PAMAM) dendrimers to generate PAMAM–*g*–PNIPAAm and PAMAM–*g*–PNIPAAm–*co*–PEG. PAMAM–*g*–PNIPAAm exhibited the lowest critical solution temperature (LCST) of ca. 32 °C, whereas PAMAM–*g*–PNIPAAm–*co*–PEG showed a LCST of ca. 35 °C. Indomethacin was used as a model molecule to examine the drug release profiles from both types of dendritic polymers. Results showed that such thermo-sensitive PAMAM derivatives could manipulate drug release simply by controlling the temperature above or below the LCST. At 37 °C a prolonged drug release was obtained for both systems with less than 30% of drug was released over 12 h, whilst the release rate is much faster at 30 °C and ca. 90% of drug was released over 12 h. The results obtained suggest that these thermo-sensitive PAMAM derivatives could be potential drug delivery systems to achieve controlled drug release.

1. Introduction

Dendrimers are well-defined, hyper-branched, synthetic macromolecules which have a history of over 30 years (Buhleier et al., 1978). Unlike conventional linear and branched polymers, dendrimers possess a tree-like architecture emanating from a central core from which the repeating branches are built up via a stepwise fashion. The number of repeated branching cycles determines the generation of dendrimers (e.g. G1.0, G2.0, G3.0, etc.) and hence the size of the dendrimers. Despite the diversity of core molecule, repeating branches and end groups, dendrimers are often divided into three regions: the initiator core, the interior (building branches) and the exterior (surface terminal groups). This unique structure not only presents cavities (i.e. interior) for encapsulation guest molecule, but also confers dendrimers molecular uniformity and surface functionality (Jansen et al., 1994). The molecular uniformity is highly desirable for improving synthetic reproducibility and minimising experimental and therapeutic variability (Astruc et al., 2010). The abundance of differing functional surfaces impart dendrimers numerous unique properties, e.g. aqueous solubility and stimuli (temperature and pH, etc.) responsitivity. These properties make dendrimers favourable in many biomedical and pharmaceutical areas such as bio-imaging, tissue engineering, drug and gene delivery (Lee et al., 2005). By far poly(amidoamine) (PAMAM) dendrimer is the most intensively investigated.

In terms of drug delivery, the interior of PAMAM dendrimers exhibits high affinity for molecules with negative charges and is highly desirable as carriers of acidic drugs. It has been reported that PAMAM dendrimers could be efficient delivery systems with the benefits of enhanced drug solubility, prevention of drug degradation, increased circulation time, sustained/controlled drug release and potential drug targeting (Svenson, 2009). In addition, the advances in dendrimer surface engineering, i.e. the conjugation of functional groups to the chain ends of dendrimer surface, could provide stimuli-responsive properties to PAMAM dendritic delivery systems, which could add value to drug delivery efficiency and therapeutic efficacy (Kojima, 2010).

Among all the stimuli-responsive dendrimers, temperature-responsive systems are extremely promising with regard to drug delivery. Rendering dendrimers temperature-responsive usually involves grafting the terminal chain ends of dendrimers with temperature-sensitive groups, among which poly(N-isopropylacrylamide) is one of the best-known (Wei et al., 2007). PNIPAAm as a linear polymer is highly soluble in water at temperatures below ca. 32 °C, above which it becomes dehydrated and hence insoluble in water; this transition temperature is termed as lower critical solution temperature (LCST) (Zhang et al., 2004).

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doi:10.1016/j.ijpharm.2011.02.005

Previous studies have successfully attached PNIPAAm to the dendrimer surfaces. For example, PNIPAAm was conjugated to the surfaces of poly(propylene imine) dendrimers forming a temperature-sensitive shell (Kimura et al., 2000; Zheng and Pan, 2006). In the same way, PNIPAAm was also introduced to other types of dendrimers (You et al., 2004).

Despite the advantages of thermo-sensitive dendritic drug delivery systems, there are still concerns regarding their toxicity, immunogenicity, and circulation time upon dose administration (Jevprasesphant et al., 2003). However, PEGylation (covalent linking polyethylene glycol (PEG) polymer chains with other molecules) is a popular approach to address these problems (Astruc et al., 2010; Jiang et al., 2010; Wong et al., 2010). For instance, Jevprasesphant et al. (2003) reported that the cytotoxicity of PAMAM dendrimers could be significantly deceased via surface PEGylation due to the shielding of the positive charge on the dendrimer surface by the PEG chains. Wang et al. (2009) showed that conjugation with PEG could effectively reduce the PAMAM-induced cell apoptosis by attenuating the production of reactive oxygen species. Kojima et al. (2010) demonstrated that PEGylayed PAMAM dendrimers exhibited longer blood retention and lower accumulation in other normal organs such as the kidneys than the non-PEGylated ones. In addition, PEGylation strategy has also been used previously to render the metal nanoparticle delivery systems non-toxic (Boyer et al., 2010). However, to our best knowledge, surface PEGylation has not been utilised in thermo-sensitive dendrimer delivery systems such as PAMAM dendrimers with a PNIPAAm shell.

As such, the aims of this study were to attach both thermo-sensitive PNIPAAm and biocompatible PEG to the periphery of PAMAM engineering long-circulating functional dendritic delivery systems and to investigate the effect of surface modification on the *in vitro* release profile of a model drug, indomethacin.

2. Materials and methods

2.1. Materials

N-isopropylacrylamide (NIPAM) and 4-nitrophenyl chloroformate were purchased from ACROS Organics (NJ, USA). HPLC grade tetrahydrofuran, indomethacin and methoxy poly(ethylene glycol) (MPEG) with a molecular weight (MW) of 2000 was provided by Sigma–Aldrich Chemie Gmbh (Munich, Germany). Analytical grade methanol, hexane, dichloromethane, diethyl ether, dimethyl sulfoxide, ethylenediamine (EDA), triethylamine (TEA), methyl acrylate (MA), 2-mercaptoethanol, and 2,2′-azobisisobutyronitrile were sourced from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Double distilled water from laboratory supply was used in the whole study unless otherwise stated.

2.2. Methods

2.2.1. Synthesis of PAMAM dendrimers

PAMAM dendrimers with EDA cores were synthesized using Tomalia's divergent growth approach (Esfand and Tomalia, 2001). Two consecutive chain-forming reactions (the exhaustive Michael addition and the exhaustive amidation reaction) were repeated alternately during the dendrimer synthesis. In the present study, G1.5 and G2.0 PAMAM dendrimers were obtained.

2.2.2. Synthesis of PAMAM–g–PNIPAAm

NIPAM monomers were purified by recrystallization with hexane prior to use. PNIPAAm with a terminal hydroxyl group (i.e. PNIPAAm–OH) was prepared by radical telomerisation (a polymerization reaction) of NIPAM using 2,2′-azobisisobutyronitrile (recrystallization with methanol prior to use) as the radical initiator and 2-mercaptoethanol as the chain transfer agent (Choi et al., 2006). PNIPAAm–OH was then diluted with water and dialysed using a dialysis tube (MW cut-off: 3400) against water for 5 days. The solution in the dialysis tube was collected and freeze-dried to get PNIPAAm–OH.

PNIPAAm-4-nitrophenyl carbonate was synthesised by reacting PNIPAAm–OH with 4-nitrophenyl chloroformate (Fig. 1). 4-Nitrophenyl chloroformate (7.2 mmol) and triethylamine (7.2 mmol) were added to 100 ml dichloromethane solution containing 3.6 mmol PNIPAAm; the mixture was stirred for 48 h at ambient temperature ($25 \pm 2\,^{\circ}C$). The crude product was purified by recrystallization from diethyl ether to obtain PNIPAAm-4-nitrophenyl carbonate.

PNIPAAm-4-nitrophenyl carbonate (0.22 mmol) was put in a 10 ml solution of G2.0 PAMAM dendrimer (7 μmol) in dimethyl sulfoxide. The mixture was stirred for 7 days at ambient temperature, diluted with water and dialysed using a dialysis bag (MW cut-off: 14,000) against water for 36 h. The above crude compound was lyophilized and then eluted with water in a Sephadex G-75 column from Yuncheng, Inc. (Shanghai, China) to get the purified product of PAMAM–g–PNIPAAm (PAMAM dendrimers grafted with PNIPAAm).

2.2.3. Synthesis of PAMAM–g–PNIPAAm–co–PEG

The PAMAM dendrimers grafted with PNIPAAm and PEG (PAMAM–g–PNIPAAm–co–PEG) were synthesized via the formation of a urethane bond through the reaction of PAMAM–NH_2 with PNIPAAm-4-nitrophenyl carbonate and MPEG-4-nitrophenyl carbonate. MPEG-4-nitrophenyl carbonate was prepared by reacting MPEG with 4-nitrophenyl chloroformate and purified by recrystallization from diethyl ether (Yang et al., 2004) (Fig. 1). Briefly, PNIPAAm-4-nitrophenyl carbonate (0.22 mmol) and MPEG-4-nitrophenyl carbonate (0.11 mmol) were added to a solution of G2.0 PAMAM dendrimer (7 μmol) in dimethyl sulfoxide (10 ml); the mixture was stirred for 7 days at ambient temperature. Then the above solution was diluted with water and dialysed using a dialysis bag (MW cut-off: 14,000) against water for 36 h. The crude compound was lyophilized and then eluted with water in a Sephadex G-75 column to get the purified product of PAMAM–g–PNIPAAm–co–PEG.

2.2.4. FTIR and ^1H NMR

The dendrimer structures were analysed by Fourier transform infrared spectroscopy (FTIR) using a Tensor^{TM} 27 spectrometer (Bruker Optik GmbH, Ettlingen, Germany). Proton nuclear magnetic resonance spectroscopy (^1H NMR) was also employed to characterise and confirm the structure of dendrimers and relevant intermediate products. The measurements were recorded using a 400 MHz Varian spectrometer (Varian, Inc., Walnut Creek, CA, USA).

2.2.5. Gel permeation chromatography

The average molecular weights of PNIPAAm–OH and PAMAM derivatives were determined by a gel permeation chromatographic (GPC) system equipped with a Waters 2414 detector (Waters Corporation, Milford, MA, USA). Tetrahydrofuran was used as an eluent at a flow rate of 0.3 ml/min through a Waters Ultrastyragel® column. Peak analysis was performed using Waters Millenium software program to calculate the MW of dendrimers according to a universal calibration curve generated by a polystyrene standard of narrow molecular weight distribution.

2.2.6. LCST determination

The LCST of the dendrimers was determined by measuring the absorbance of aqueous dendrimer solution (1.0 mg/ml) at 500 nm using a Cary100 UV–vis spectrometer (Varian, Inc., Walnut Creek,

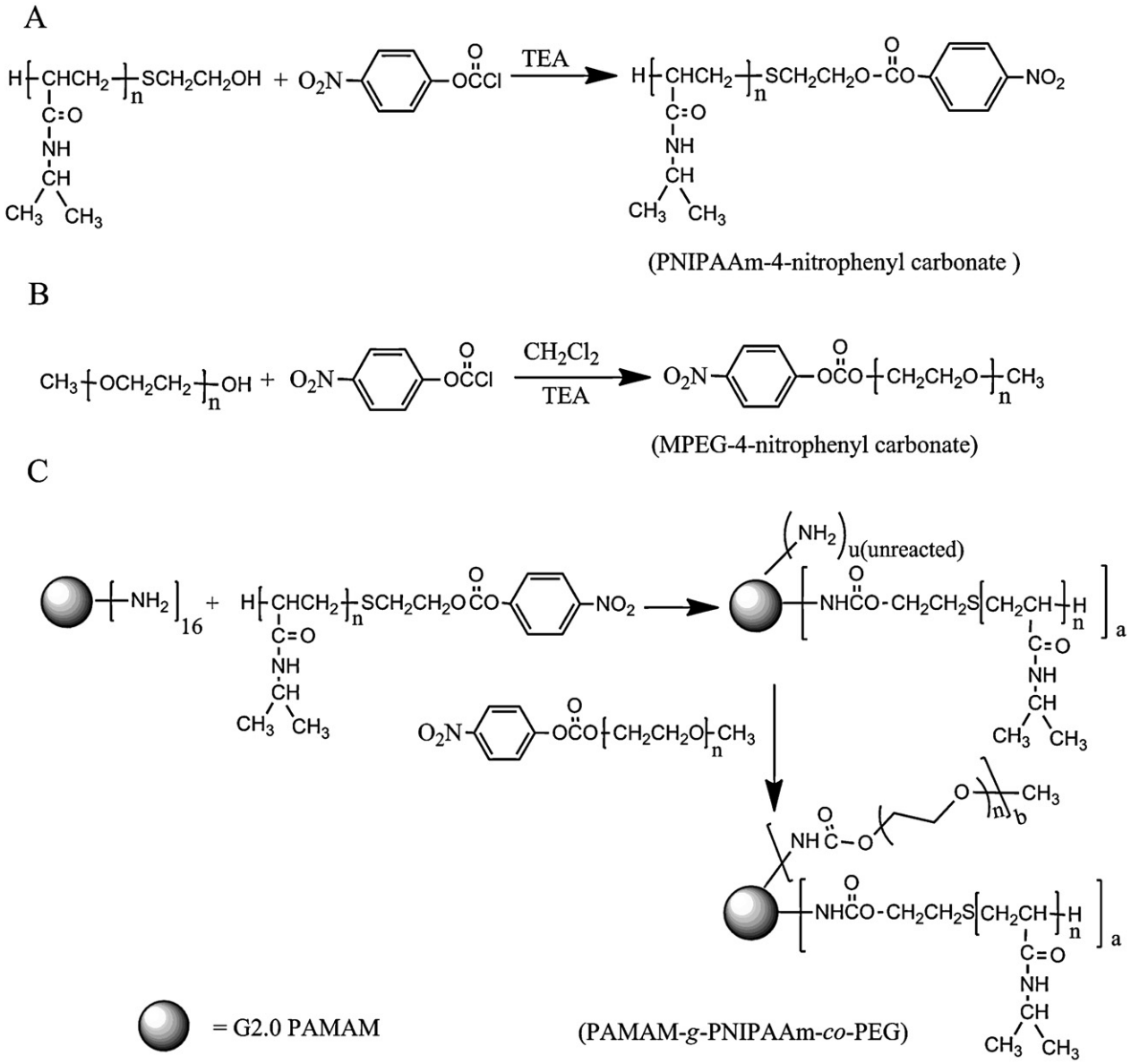

Fig. 1. Synthetic route for PNIPAAm and/or PEG grafted PAMAM dendrimers: (A) synthesis of PNIPAAm-4-nitrophenyl carbonate; (B) synthesis of MPEG-4-nitrophenyl carbonate; (C) synthesis of PAMAM–*g*–PNIPAAm and PAMAM–*g*–PNIPAAm–*co*–PEG. PAMAM, PNIPAAM, TEA, PEG and MPEG represent poly(amidoamine), poly(N-isopropylacrylamide), triethylamine, poly(ethylene glycol) and methoxy poly(ethylene glycol), respectively.

CA, USA) with an external temperature-controller. The sample cells were thermo-controlled in a circulator bath at predetermined temperatures prior to analysis. All measurements were performed in triplicate and the mean value was taken. The LCST values of the sample solutions were defined as the temperature at which the optical transmittance was 50% of the maximum value.

2.2.7. Drug loading

10 mg indomethacin and 20 mg PAMAM dendrimer or its derivatives (PAMAM–*g*–PNIPAAm and PAMAM–*g*–PNIPAAm–*co*–PEG) were dissolved in 2 ml methanol with gentle agitation. The solution was kept in a sonicator (Gongyi Yuhua, Inc., Zhengzhou, China) at ambient temperature for 30 min and then methanol was removed by evaporation. The solution samples were dissolved in 5 ml distilled water, and centrifuged at 10,000 rpm for 30 min. The supernatant was lyophilized and the indomethacin–dendrimer complexes were obtained. The drug loading was calculated by analysing the drug amount in the complex at 320 nm using a UV–vis spectrometer (Varian, Inc., Walnut Creek, CA, USA).

2.2.8. In vitro release studies

Indomethacin *in vitro* release experiments were performed using a dialysis method based on previous investigation (Kolhe et al., 2003). Indomethacin–dendrimer complexes were dissolved in a dialysis tube (MW cut-off: 7000) containing appropriate amount of phosphate buffered saline (PBS) solution (pH 7.4). The above solutions were incubated at either 30 °C or 37 °C with the presence of a gentle stirring (100 rpm/min). At pre-determined time points, 3 ml aliquots of the release media were taken and drug content therein was analysed by UV spectroscopy ($n = 3$). Meanwhile, the same volume of fresh PBS solution added to maintain the constant volume of the external media. The cumulative amount of drug released was plotted against time to plot the drug release profile.

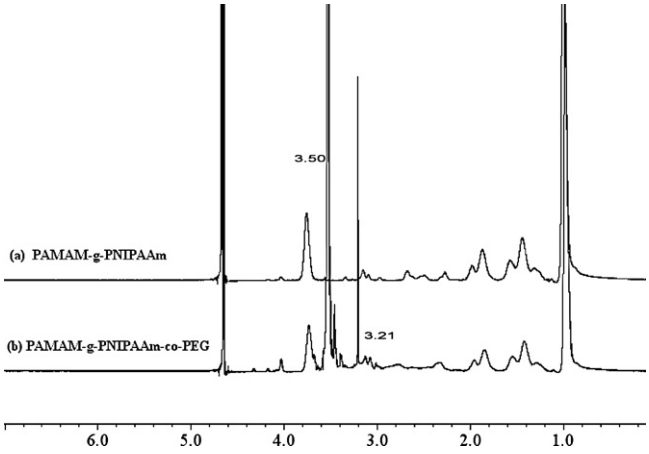

Fig. 2. ^{1}H NMR spectrum of (a) PAMAM–*g*–PNIPAAm and (b) PAMAM–*g*–PNIPAAm–*co*–PEG in D_2O. PAMAM, PNIPAAM and PEG represent poly(amidoamine), poly(N-isopropylacrylamide) and poly(ethylene glycol), respectively.

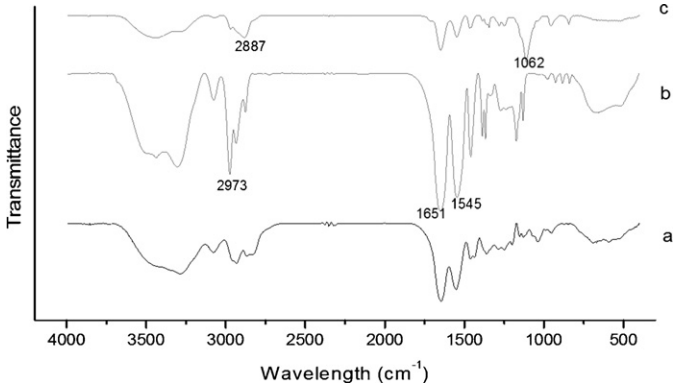

Fig. 3. FITR spectra of PAMAM dendrimer and its derivatives: (a) G2.0 PAMAM dendrimer; (b) PAMAM–*g*–PNIPAAm; (c) PAMAM–*g*–PNIPAAm–*co*–PEG. PAMAM, PNIPAAM and PEG represent poly(amidoamine), poly(N-isopropylacrylamide) and poly(ethylene glycol), respectively.

2.2.9. Statistical analysis

Statistical analysis of data was carried out using SPSS and a statistically significant difference was determined at a minimal level of significance of 0.05. Drug release data were analysed using Student's-*t* test.

3. Results

3.1. Synthesis of PAMAM–g–PNIPAAm–co–PEG

The proton chemical shift (δ) values (ppm) of PAMAM (G2.0) and its intermediate product (G1.5) were as follows. ^{1}H NMR (G1.5, D_2O): δ = 2.24–2.26 (24H, broad multiplet, –CH_2CH_2CONH–), δ = 2.36–2.39 (32H, broad multiplet, –CH_2CH_2COO–), δ = 2.42–2.47 (26H, broad multiplet, –$NHCH_2CH_2$N⟨), δ = 2.63–2.66 (56H, broad multiplet, –NCH_2CH_2CO–), δ = 3.10–3.14 (24H, broad multiplet, –CONHCH_2–), δ = 3.52 (48H, s, –OCH_3). ^{1}H NMR (G2.0, D_2O): δ = 2.24–2.27 (56H, broad multiplet, –CH_2CH_2CONH–), δ = 2.43–2.46 (26H, broad multiplet, –$NHCH_2CH_2$N⟨), δ = 2.53–2.56 (32H, broad multiplet, –$CH_2CH_2NH_2$), δ = 2.63–2.66 (56H, broad multiplet, –NCH_2CH_2CO–), δ = 3.05–3.08 (32H, broad multiplet, –$CH_2CH_2NH_2$), δ = 3.10–3.13 (24H, broad multiplet, –CONHCH_2–).

The proton chemical shift values of PNIPAAm–OH were 3.21 (–OCH_2–), 2.58 (–CH_2S–), 1.46–1.59 (⟩CH–CH_2–), 1.89–2.00 (⟩CHCO–), 3.77 (–NHCH⟨), 1.02 (–CH_3), respectively. The signals of PNIPAAm-4-nitrophenyl carbonate in the ^{1}H NMR ($CDCl_3$) were 1.03 (s, 6H, –NHCH (CH_3)$_2$), 1.46 (broad multiplet, 2H, –CHCH_2–), 1.89 (broad multiplet, 1H, –CHCH_2–), 3.78 (s, 1H, –NHCH (CH_3)$_2$), 7.30 (d, aromatic), 8.18 (d, aromatic), respectively. The formation of MPEG-4-nitrophenyl carbonate was also confirmed by ^{1}H NMR ($CDCl_3$): δ 3.38 (s, –OCH_3), 3.34–3.80 (m, –CH_2CH_2O–), 4.42 (m, –OCOOCH_2), 7.40 (d, aromatic), 8.28 (d, aromatic).

From the ^{1}H NMR spectrum of PAMAM–*g*–PNIPAAm (Fig. 2), the peaks at 1.46–1.59 (⟩CH–CH_2–), 1.89–2.00 (⟩CHCO–), 3.77 (–NHCH⟨), and 1.02 (–CH_3) were attributed to the PNIPAAm moiety at the shell of PAMAM–*g*–PNIPAAm and the signals at δ = 2.25–3.13 were attributed to the PAMAM moiety of PAMAM–*g*–PNIPAAm. The FTIR peaks at 1647–1651 cm^{-1} (C=O–N) and 2973 cm^{-1} (C–C) also partly verified the grafting of PNIPAAm to PAMAM–*g*–PNIPAAm (Fig. 3b). The ^{1}H NMR spectrum of PAMAM–*g*–PNIPAAm–*co*–PEG (Fig. 2) was similar to that of PAMAM–*g*–PNIPAAm, except for the signals at 3.50 ppm (–CH_2CH_2O–) and 3.21 ppm (CH_3O–) and both were attributed to the PEG moiety. The successful attachment of PEG to PAMAM–*g*–PNIPAAm–*co*–PEG was also confirmed by FTIR spectra (Fig. 3c) and evidenced by the peaks at 1050–1150 cm^{-1} (C–O) and 2850–3000 cm^{-1} (–CH_2).

3.2. Dendrimer characterisation

The molecular weights of PAMAM–*g*–PNIPAAm, PAMAM–*g*–PNIPAAm–*co*–PEG, PNIPAAm and PEG were determined by GPC (Table 1). For PAMAM–*g*–PNIPAAm–*co*–PEG, the grafted PNIPAAm/PEG chain ratio was calculated to be 1.3 based on the ratio of the integrated signals of the PNIPAAm moiety (δ 3.74, –NHC**H**⟨) to the signals of the PEG moiety (δ 3.50, –CH_2CH_2O–). The polydispersity index (PI) as a measure of molecular weight distribution was calculated by the ratio of weight-average molecular weight M_w to number-average molecular weight M_n. The PI value of PAMAM–*g*–PNIPAAm–*co*–PEG dramatically increased compared to that of PAMAM–*g*–PNIPAAm. However, the attaching of PEG to PAMAM–*g*–PNIPAAm resulted in a decrease of graft ratio (degree of substitution to the surface functional groups of PAMAM (–NH_2) from 75% to around 50% (Table 1). The LCST value of PAMAM–*g*–PNIPAAm was estimated to be 32 °C, which is almost equal to the linear PNIPAAm; however, due to the attachment of hydrophilic PEG moiety, the LCST value of PAMAM–*g*–PNIPAAm–*co*–PEG increased to ca. 35 °C (Fig. 4).

3.3. Drug loading and release

The indomethacin loading in PAMAM dendrimer and its derivatives was 0.21 mg/mg (PAMAM), 0.16 mg/mg (PAMAM–*g*–PNIPAAM), and 0.15 mg/mg (PAMAM–*g*–PNIPAAm–*co*–PEG), respectively. The average number of indomethacin molecules associated with PAMAM was 2, whilst this value increased to 10

Table 1
Gel permeation chromatography (GPC) results of PAMAM–*g*–PNIPAAm and PAMAM–*g*–PNIPAAm–*co*–PEGs (PAMAM, PNIPAAM and PEG represent poly(amidoamine), poly(N-isopropylacrylamide) and poly(ethylene glycol), respectively). M_n and M_w represent number-average and weight-average molecular weight, respectively and the polydispersity index was calculated by dividing M_w by M_n. The degree of substitution of PNIPAAm and PEG to the functional groups of PAMAM was indicated by graft ratio.

Polymer type	M_n	M_w	Polydispersity index	Total graft ratio
PNIPAAm	2130	2326	1.1	–
PEG	3198	3238	1.0	–
PAMAM–*g*–PNIPAAm	28,824	35,859	1.2	75%
PAMAM–*g*–PNIPAAm –*co*–PEG	22,916	33,875	1.5	49%

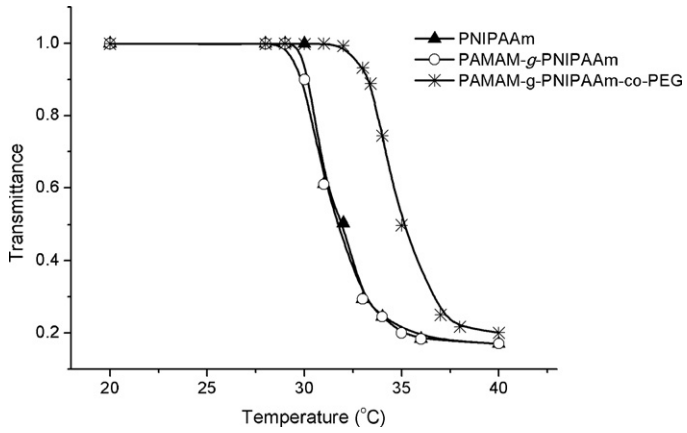

Fig. 4. The effect of temperature on the light transmittance of the aqueous solution of PNIPAAm and PAMAM derivatives: PNIPAAm (filled triangle) PAMAM–*g*–PNIPAAm (open circle); PAMAM–*g*–PNIPAAm–*co*–PEG (crossed lines). PAMAM, PNIPAAM and PEG represent poly(amidoamine), poly(N-isopropylacrylamide) and poly(ethylene glycol), respectively.

(PAMAM–*g*–PNIPAAm–*co*–PEG) and 13 (PAMAM–*g*–PNIPAAM), which indicated an improved drug encapsulation capability after dendrimer surface engineering. The *in vitro* drug release profile from PAMAM dendrimer was similar at 30 °C and 37 °C, which was evidenced by a burst release during the first six hours following by a slower release until plateaued (Fig. 5). The rate constant of drug release calculated by the simplified Higuchi model (Costa et al., 2001) at different temperatures during the first stage were 34.0 $h^{-1/2}$ at 30 °C and 34.3 $h^{-1/2}$ at 37 °C with no significant difference ($p > 0.05$). However, indomethacin release from surface-engineered dendritic polymers differed greatly at different temperatures (30 °C vs. 37 °C) (Fig. 6). The increase of release medium temperature to 37 °C led to a dramatic drop of release rate for both PAMAM–*g*–PNIPAAM and PAMAM–*g*–PNIPAAm–*co*–PEG. At lower temperature (30 °C), the drug release profile from both types of dendritic derivatives was analogous and the release rate constant during the first six hours (i.e. rapid release stage) was not significantly different ($p > 0.05$) with the corresponding values of 33.8 $h^{-1/2}$ (PAMAM–*g*–PNIPAAm–*co*–PEG) and 31.7 $h^{-1/2}$ (PAMAM–*g*–PNIPAAM). In the end of the release experiment, ca. 90% of drug were released from these dendritic derivatives. In contrast, at 37 °C indomethacin release

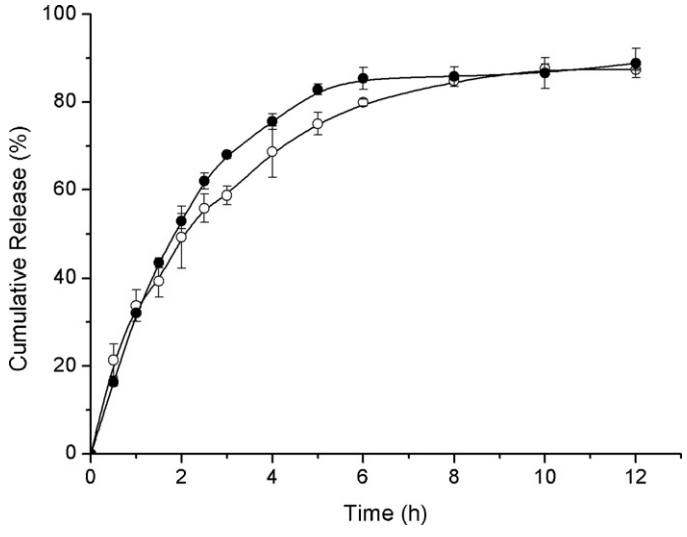

Fig. 5. Indomethacin release profiles from poly(amidoamine) (PAMAM) dendrimers at 30 °C (open circle) and 37 °C (filled circle).

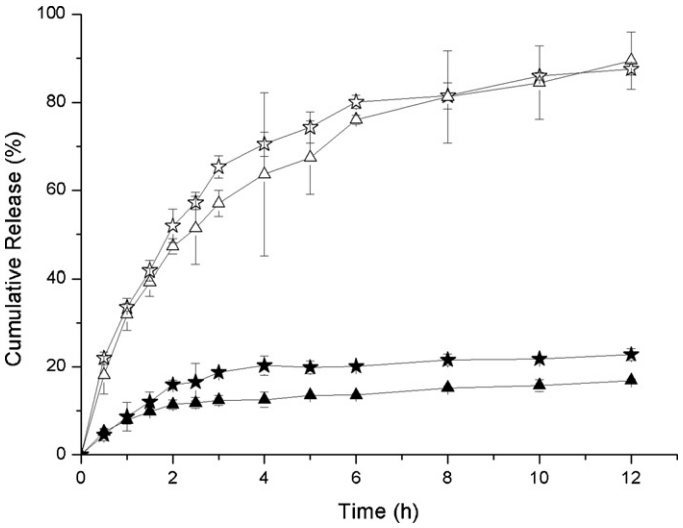

Fig. 6. Indomethacin release profiles from PAMAM–*g*–PNIPAAm at 30 °C (open triangle) and 37 °C (filled triangle) and from PAMAM–*g*–PNIPAAm–*co*–PEG at 30 °C (open star) and 37 °C (filled star). PAMAM, PNIPAAM and PEG represent poly(amidoamine), poly(N-isopropylacrylamide) and poly(ethylene glycol), respectively.

rate from PAMAM–*g*–PNIPAAm–*co*–PEG was faster compared to that from PAMAM–*g*–PNIPAAM. For example, during the burst release period, the mean value of drug release rate was 8.8 $h^{-1/2}$ (PAMAM–*g*–PNIPAAM) and 15.8 $h^{-1/2}$ (PAMAM–*g*–PNIPAAm–*co*–PEG), respectively. Nevertheless, from the second hour both profiles began to get plateaued; after 12 h ca. 17% of drug was released into the buffer medium from PAMAM–*g*–PNIPAAM and ca. 23% of drug was released from PEG-grafted carrier, i.e. PAMAM–*g*–PNIPAAm–*co*–PEG.

4. Discussion

The grafting of PNIPAAm to PAMAM confers the dendrimer thermo-sensitive property, which is promising in many biomedical and pharmaceutical applications such as 'intelligent' drug delivery. Similarly the conjugating of PEG to PAMAM could decrease the dendrimer's undesirable toxicity and increase its circulation time and stability. In current study, both PNIPAAm and PEG were successfully attached to the surface of PAMAM dendrimers (G2.0), which was confirmed by the ^{1}H NMR and FTIR analysis. The reason of selecting low generation PAMAM (G2.0) as a template is due to the fact that the toxicity of cationic PAMAM dendrimers becomes higher with increasing generation number and the hydrolytic degradation of high generation PAMAM at physiological temperatures are slow (Lee et al., 2005).

The G2.0 PAMAM had a M_n of 3256 and 16 surface groups ($-NH_2$). The GPC data (M_n: 28,824 M_w: 35,859 PI: 1.2, Table 1) showed that ca. 75% of dendrimer terminals were covered by PNIPAAm chains, which indicated that an average of 12 PNIPAAm arms were conjugated to each PAMAM dendrimer. After grafting, the molecular weight distribution of PAMAM–*g*–PNIPAAm remained narrow based on the PI value, which agreed well with previous investigation (You et al., 2004). However, as a result of the structure complexity of PAMAM–*g*–PNIPAAm–*co*–PEG molecule, it is difficult to control its mass distribution within a narrow range (PI: 1.5). The reason why PNIPAAm moiety could not cover all the functional $-NH_2$ groups of PAMAM was assumed to be the steric hindrance effect. Likewise, when attaching both PNIPPAm and PEG chains to the PAMAM surfaces, the total graft ratio was only round 50%. To increase the graft ratio, the synthesis conditions still need further optimisation, e.g. the sequential addition of PNIPAAm and PEG.

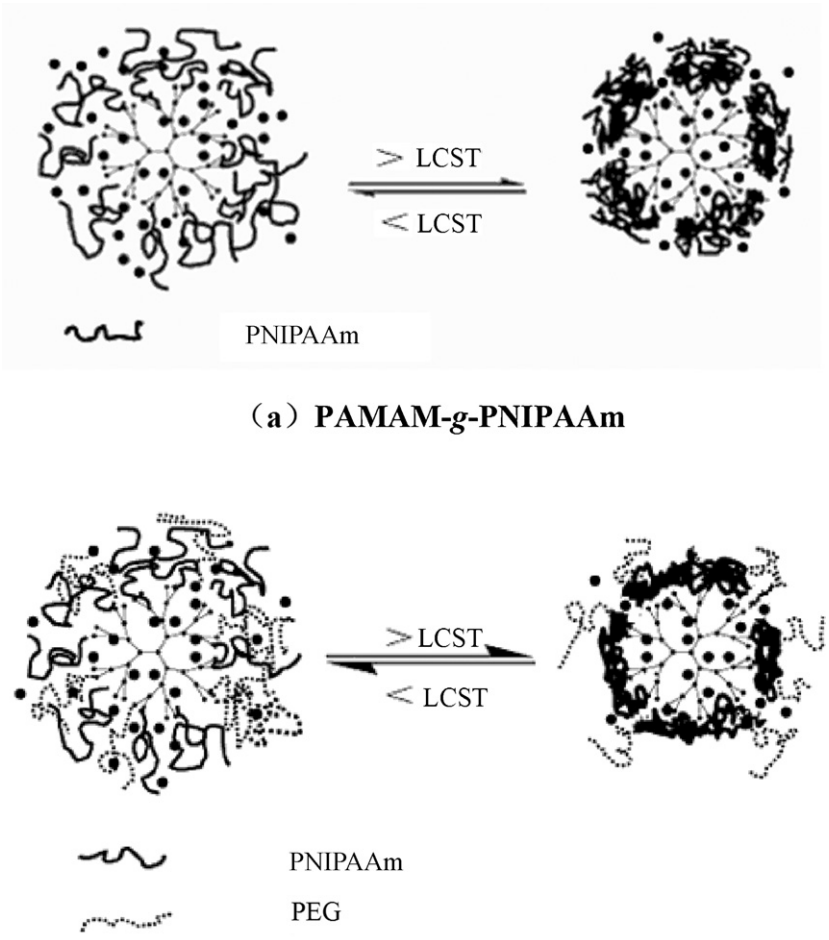

Fig. 7. Schematic illustration of the phase transition of PNIPAAm-bearing PAMAM dendritic derivatives PAMAM–*g*–PNIPAAm and PAMAM–*g*–PNIPAAm–*co*–PEG at temperature above the LCST (lower critical solution temperature). PAMAM, PNIPAAM and PEG represent poly(amidoamine), poly(N-isopropylacrylamide) and poly(ethylene glycol), respectively.

Although the LCST of thermo-sensitive polymers is related to the molecular weight, the extent is not significant. In terms of PNIPAAm, it has been reported that increasing its average molecular weight ca. 30 times only resulted in a LCST drop by ca. 2 °C (Schild and Tirrell, 1990). As such, the influence of PNIPAAm molecular weight on the thermo-sensitivity of PAMAM dendritic derivatives was not investigated in current study. Despite no great difference in LCST between PNIPAAm and PAMAM–*g*–PNIPAAm (both at ca. 32 °C), the attachment of linear PNIPAAm to PAMAM surfaces indeed rendered it temperature sensitive (Fig. 4). This phenomenon was thought due to the relatively lower generation of PAMAM dendrimer (G 2.0). It is expected that the density of terminal PNIPAAm groups near the periphery of low generation PAMAM is not high enough to enable the efficient intermolecular interaction between them, which has no significant influence on the dehydration behaviour of PNIPAAm upon temperature increase. Thus no dramatic LCST change was observed after conjugating PNIPAAm to PAMAM dendrimer surface. Such phenomenon also concurred with previous study (Haba et al., 2004).

Further grafting PEG chains to PAMAM–*g*–PNIPAAm resulted in an increase of LCST from 32 °C to 35 °C. It is well known that at below LCST the interaction between PNIPAAm and water such as hydrogen bonding is dominant, whilst above LCST the interaction between PNIPAAm is crucial to the transition of PNIPAAm chains to compact and collapsed conformations leading to the insolubility of PNIPAAm and hence its thermo-sensitive properties (You and Oupicky, 2006). For PAMAM–*g*–PNIPAAm–*co*–PEG, the introduction of PEG moiety not only increased the hydrophilicity of the dendritic derivative, but also diminished the packing density of PNIPAAm moiety. The total graft ratio of PAMAM–*g*–PNIPAAm–*co*–PEG is only around 50% compared to 75% of PAMAM–*g*–PNIPAAm. In addition, these partly grafted dendritic surfaces were shared by both PEG and PNIPAAm; the surface packing density (i.e. the degree of substitution) of PNIPAAm is greatly decreased. It has been reported that the loose packing of PNIPAAm in the dendrimer periphery is not favourable for their interaction and could decrease dehydration around these groups (Haba et al., 2007). In the same way, another independent work by Tono et al. (2006) demonstrated that the thermo-sensitive properties (i.e. LCST) of PAMAM dendrimers with hydrophobic peripheral phenylalanine groups could be tuned by introducing hydrophilic isoleucine moiety. Therefore, the LCST of PAMAM–*g*–PNIPAAm–*co*–PEG showed a 3 °C increase compared to that of PAMAM–*g*–PNIPAAm. Varying the ratio of PNIPAAm and PEG could adjust the LCST of PAMAM–*g*–PNIPAAm–*co*–PEG to around the physiological temperature (data not shown).

The loading capacity of surface-engineered dendrimers (i.e. PAMAM–*g*–PNIPAAm–*co*–PEG and PAMAM–*g*–PNIPAAm) was

much higher than that of PAMAM. This is attributed to the surface modification by PNIPAAm and PEG. As G2.0 PAMAM dendrimers show a relatively less packed periphery groups compared to higher generation ones, their ability of retaining the guest drug molecule within the dendrimer interior cavity is limited due to the ease of drug release from inside to outside. The approach of surface engineering generates a core–shell structure, which would form a barrier to prevent the escape of guest molecule leading to an enhanced loading capacity. Employing this method to improve the drug loading of PAMAM dendrimers has also been reported in previous investigations (Bhadra et al., 2003). Theoretically the drug could stay both inside and the outer surface of engineered PAMAM dendrimers. However, the drug localisation information in the PAMAM dendrimer and its derivatives still need further investigation.

The drug release profiles from PAMAM at temperatures below and above LSCT was similar (Fig. 5). The drug release is a diffusion controlled process from the dendrimer cavity to the release medium. However, for surface-engineered PAMAM derivatives, the influence of temperature on drug dissolution is remarkable. For instance, the cumulative drug release over 12 h from both types of surface-engineered dendrimers at 37 °C was less than 30% of that at 30 °C (Fig. 6). Such results concurred well with previous studies (Bhadra et al., 2003). This can be explained by the conformational transition of PNIPAAm from a hydrated coil to a dehydrated globule at temperatures above the LCST forming a drug release barrier (Haba et al., 2007). The difference of drug release behaviour from PAMAM–*g*–PNIPAAm and PAMAM–*g*–PNIPAAm–*co*–PEG could ascribe to the differing degree of substitution of PNIPAAm around the periphery of PAMAM dendrimers; due to the introduction of PEG, the packing of PNIPAAm in PAMAM–*g*–PNIPAAm–*co*–PEG was relatively loose than that without PEG, i.e. the degree of substitution of PNIPAAm decreased from ca. 75% to 30%. Therefore, upon temperature increase over the LCST, PNIPAAm developed a compact barrier layer over the surface of PAMAM–*g*–PNIPAAm; in contrast, a comparatively less packed barrier layer was created for PAMAM–*g*–PNIPAAm–*co*–PEG (Fig. 7). It is these barrier layers differing in compactness that determine the drug release rate and extent from surface-modified dendrimers at temperatures above the LCST. Hence, drug dissolution from PAMAM–*g*–PNIPAAm–*co*–PEG was faster compared to that from PAMAM–*g*–PNIPAAm at 37 °C. Despite the fact that PEGylation can slow down drug release (Bhadra et al., 2003; Haba et al., 2005), however, the influence of PEG on drug release was not apparent compared to that of PNIPAAm possibly as a consequence of the low degree of substitution (ca. 20%) to the low generation PAMAM (G2.0).

Nevertheless, due to the complicated structure of PAMAM–*g*–PNIPAAm–*co*–PEG, the toxicity profile of the novel functional dendrimer derivative might be different to that of conventional PAMAM. Further obtaining such information would be beneficial in improving or optimising the properties of PAMAM–*g*–PNIPAAm–*co*–PEG. For example, PEGylated thermo-sensitive dendrimers derived from medium or high generation PAMAM could be generated to manipulate the graft ratio of functional PNIPAAm and PEG groups, and achieve the controlled drug release if the toxicity is not a big issue.

5. Conclusions

Thermo-sensitive dendritic PAMAM–*g*–PNIPAAm and PAMAM–*g*–PNIPAAm–*co*–PEG were successfully synthesized. PNIPAAm confers both types of dendrimer derivatives thermo-sensitivity; however, due to the different packing density of PNIPAAm at the dendrimer surfaces the LCST of PAMAM–*g*–PNIPAAm–*co*–PEG was higher than that of PAMAM–*g*–PNIPAAm. The dendritic stimuli-responsive dendritic system was found not only to have improved drug-loading capability, but also to enable prolonged drug release via manipulating the environment temperature above the LCST. This novel 'intelligent' dendrimer would be a useful addition to the spectrum of drug delivery systems currently available. Future study should focus on the effect of PEGylation on the cytotoxicity of PAMAM–*g*–PNIPAAm–*co*–PEG, which would provide more valuable information to address the toxicity issue encountered in traditional thermo-sensitive dendrimers.

Acknowledgement

The authors acknowledge the financial support from the National Natural Science Foundation of China (20674055).

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