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Thermosensitive Poly(N-isopropylacrylamide)-g - Polyamidoamine Dendrimer Derivatives: Preparation and the Drug Release Behaviors

Zheng Wang, Bo Zhou, Dong Liu, Xiang-pan Fan*

Summary: A series of thermally responsive dendritic core-shell polymers were prepared based upon dendritic polyamidoamine (PAMAM), modified with carboxyl end-capped linear poly(N-isopropylacrylamide) (PNIPAAm-COOH) in different ratios via an esterification process to obtain PNIPAAm-g-PAMAM. The graft ratio of PNIPAAm could be adjusted by changing the feed ratio of PAMAM-OH to PNIPAAm-COOH and was verified by ^{1}H NMR and gel penetration chromatography (GPC). The lower critical solution temperature (LCST) of PNIPAAm-g-PAMAM evaluated by UV-vis spectrophotometer was about 32 °C. Indomethacin (IMC) as a model drug was loaded in the thermosensitive polymer-grafted dendrimer derivative and its release behavior was studied below and above its LCST (27 °C vs 37 °C). Results showed that the LCST of PNIPAAm-g-PAMAM was around 32 °C compared with that of the pure PNIPAAm. The release behavior of the indomethacin entrapped in the internal cavities of the PNIPAAm-g-PAMAM showed that almost 77% of the drug was cumulatively released at 27 °C after 10 hours, whereas only 20% was released at 37 °C. The release rate of IMC from the IMC/PNIPAAm-g-PAMAM complex at 37 °C is significantly slower than that at 27 °C, which indicates that the PNIPAAm chains grafted on the surface of PAMAM dendrimer could act as a channel switching on-off button through expending or contracting in response to the temperature variation and could control the drug release by varying the surrounding temperature.

Keywords: drug release; esterification; polyamides; poly(N-isopropylacrylamide); thermosensitive polymer

Introduction

Compared with traditional linear polymers, dendrimers have more accurately controlled structures with a globular shape, a single molecular weight, and a large number of controllable peripheral functionalities,[1] which have been evaluated for many applications, such as drug delivery, gene transfection and imaging.[2–7]

Polyamidoamine (PAMAM) dendrimers are among the first dendrimers synthesized, characterized and commercialized. Lower generation ($G < 3$) PAMAMs can significantly increase the solubility of drugs poorly soluble in water because of the available internal cavities which are well-suited for host-guest interaction and encapsulation of guest molecules, whereas the higher generation ($G > 4$) PAMAMs have too many surface functional groups to release the drug freely.[3,4] Although the amine terminated PAMAM dendrimers are water-soluble, they are not well suited for medicinal applications because the toxicity of amine-terminated PAMAMs can lead to haemolysis.[5] Negatively charged carboxyl terminated dendrimers are less toxic, but they are unlikely to interact with or bind to the negative surface

Tianjin Key Laboratory for Modern Drug Delivery with High-Efficiency, Shool of Pharmaceutical Science and Technology, Tianjin University, Tianjin, 300072, P.R. China
E-mail: wangzheng2006@tju.edu.cn

of cells. The cytotoxicity and permeability of PAMAM dendrimers were found to be dependent on their concentration, number of generation, and surface charge.[8] Therefore, improving the properties of the dendrimers as drug carriers remains a challenge. A series of neutral water-soluble dendrimers have been synthesized by Twyman *et al.*[3] through reacting ester-terminated half-generation PAMAM dendrimers with TRIS (tris(hydroxymethyl) amino methane), and the host/guest properties were studied. The water-soluble dendrimers were capable of binding and solubilizing small acidic hydrophobic molecules, and releasing their hydrophobic guests on contact with a biological cell.

Although significant strides have been made in the design of drug delivery systems, developing a system that can eventually reach the desired target and deliver the drug in response to environmental stimuli such as temperature, light or pH remains an interesting area of research.[9] The synthesis of PAMAM dendrimer derivative with N-isopropylamide monomer on its surface has demonstrated that the dendrimer derivative could undergo a sharp transition by the dehydration of the peripheral moiety without a large conformational change of the whole molecule. However, no further studies were carried out to reveal the function of the thermosensitive dendrimer derivative as a drug carrier in controlled release of drug system.

A number of polymers are known to exhibit thermosensitive properties and the most thoroughly studies is poly(N-isopropylacrylamide) (PNIPAAm).[10–13] This polymer is highly soluble in water at low temperature, and becomes water-insoluble at temperature higher than 31-32 °C in that the polymer chains undergo a conformational transition from random coil to compact sphere, and simultaneously show a drastic phase change. It was reported[14] that PNIPAAm could act as a polymer shield in the size-dependent control of the binding of biotinylated proteins to streptavidin, making this kind of polymer be potential as a molecular switching on-off button in controlled drug release or protein separation.

In the present study, we designed a novel biocompatible dendrimer derivative by grafting the thermosensitive PNIPAAm chains to the surface of the PAMAM dendrimer with hydroxyl terminal groups (PAMAM-OH). The PNIPAAm chains can act as a channel switching on-off button through expending or contracting in response to the temperature variation. Indomethacin as a model hydrophobic drug was loaded in the internal cavities of the dendrimer derivatives, and the drug release could be controlled through opening or closing the channels of the drug dispersion by varying the ambient temperature.

Experimental Part

Materials

N-isopropylacrylamide (NIPAM) was purchased from ACROS Chemical Industries (USA) and was purified by recrystallization with hexane. 2,2′-azobisisobutyronitrile (AIBN) was purchased in China and used after recrystallization with methanol. 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (EDC•HCl) was purchased from China and used as received without further purification. All other reagents including ethylenediamine (EDA) and methyl acrylate (MA) were purchased from China and used after distillation. Indomethacin was obtained from Huasheng Pharmaceuticals Pvt. Ltd. China.

Synthesis of Lower Generation PAMAM with Ester Terminals

G 1.5 PAMAM dendrimers was synthesized from ethylenediamine (EDA) on the basis of repeatedly using two consecutive chain forming reactions, the Michael addition reaction and the amidation reaction as described elsewhere.[1,3] The obtained dendrimer was further purified by a Sephadex LH-20 column with methanol as the eluent. MS analysis: for a molecular structure of G 1.5 PAMAM dendrimer, the theoretic

calculated *m/z* is 1205.4, and the measured value was 1205.8. The ^{1}H NMR (CD_3Cl) spectrum of PAMAM G 1.5 with ester terminated: δ 2.26 (m, 8H, -CH_2CH_2CO-), 2.35 (m, 16H, -CH_2CH_2COOCH3), 2.40 (m, 12H, -CH_2CH_2N<), 2.62 (m, 24H, -CH_2CH_2CO-), 3.08 (m, 8H, -NHCH_2CH_2N<), 3.48 (s, 24H, -OCH_3).

Synthesis of G 2.0 PAMAM-OH (Scheme 1)

To a 3-neck round-bottomed flask equipped with a thermometer, dropping funnel and magnetic stirring, was added the solution of 4.1 g amino ethanol dissolved in 15 ml methanol, and then, 2.0 g of G 1.5 PAMAM dissolved in 30 ml methanol was added dropwise under vigorous stirring at room temperature for 30 min. The resulting solution was stirred at room temperature for 40 h under the nitrogen atmosphere. The excess amino ethanol and solvent were then removed under vacuum. The crude product was purified using a Sephadex LH-20 column with methanol as the eluent. Yield: 80%. MS analysis: for a molecular structure of G 2.0 PAMAM dendrimer, the measured m/z value was 1438.1, whereas the theoretic calculated m/z was 1437.7. ^{1}H NMR (D_2O) spectrum for PAMAM-OH G 2.0: δ 2.24 (m, 24H, -CH_2CH_2CO-), 2.43 (m, 12H, -CH_2CH_2N<), 2.63 (m, 24H, -CH_2CH_2CO-), 3.12 (m, 24H, -NHCH_2CH_2-), 3.44 (s, 16H, -CH_2CH_2OH).

Synthesis of PNIPAAm-COOH with MAA as a Chain-Transfer Agent (Scheme 1)

To a 3-neck round bottomed flask equipped with a thermometer and magnetic stirring, was added a solution of 4.0 g NIPAM in 32 ml methanol. Then 116 mg AIBN as initiators and 120 μl MAA were added successively. The reaction was performed at 60 °C under nitrogen atmosphere for 12 h. The obtained product was purified by repeated precipitation in THF/diethyl ether and dried in vacuum for 5 days. Yield: 70%. ^{1}H NMR (D_2O) spectrum of PNIPAAm-COOH: δ 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)$_2$). The average molecule weight was verified by ^{1}H NMR and GPC after purification by dialysis. GPC results: Mn = 2445 g/mol, Mw = 2655 g/mol, PDI = 1.1.

Synthesis of PAMAM Derivatives with Thermosensitive PNIPAAm Chains (Scheme 1)

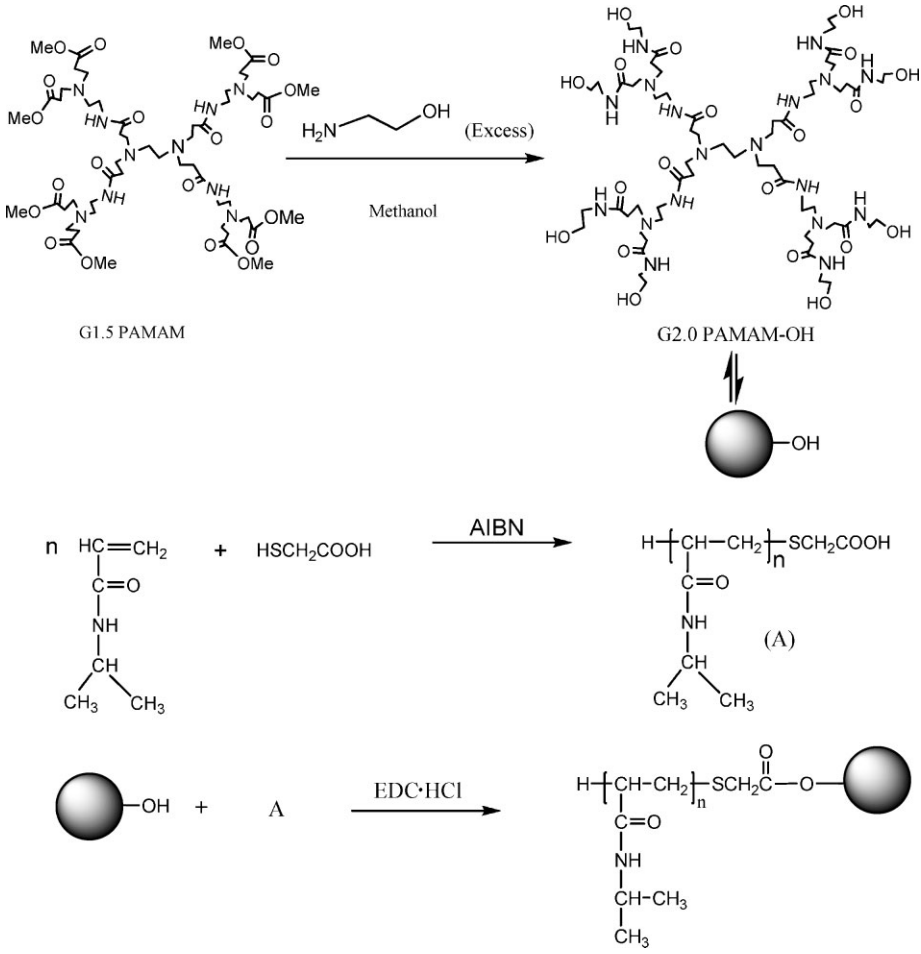
To a flask equipped with the magnetic stirring, were added PAMAM-OH (G 2.0) and PNIPAAm-COOH in 20 ml water under vigorous stirring at room temperature for 30 min, then EDC•HCl was added. The graft reaction was carried out for 24-96 h. After the reaction was completed, the mixture was dialyzed under vigorous stirring in a dialysis bag (molecular weight cut off: 7000) against distilled water for 72 h, and PNIPAAm-g-PAMAM was obtained after the dialyzed product was lyophilized.

Characterization

^{1}H NMR measurements were recorded on Varian INOVA-400 spectrometer, USA, at room temperature. UV spectroscopy measurements were preformed on Shimadzu UV-2550 model, Japan. MS measurements were carried out on Agilent 6310, USA.

The average molecular weights of PNIPAAm-COOH and PNIPAAm-g-PAMAM were determined by a gel-permeation chromatographic (GPC) system equipped with a Waters Ultrastyragel Columns separations module and Waters 2414 detector. THF was used as an eluant at a flow rate of 0.3 ml/min. Waters millennium module software was used to calculate molecular weight on the basis of a universal calibration curve generated by a polystyrene standard of narrow molecular weight distribution.

The optical absorbance of PNIPAAm-COOH and PNIPAAm-g-PAMAM solutions in pH 7.4 PBS solutions (1 mg/ml) at various temperatures was measured at 500 nm with CARY-100 UV-vis spectrometer (Varian) with an external constant temperature-controller. The sample cells were thermo-stated in a circulator bath at different temperatures from 30 to 35 °C prior to measurements. All measurements



Scheme 1.
Synthesis of G 2.0 PAMAM-OH, PNIPAAm-COOH and PNIPAAm-g-PAMAM.

were repeated for three times, and an average value was adopted. The LCST values of the sample solutions were defined as the temperature showing an optical transmittance of 50%.

Drug Loading and Release Studies of PNIPAAm-g-PAMAM

10 mg indomethacin (IMC) and 20 mg PNIPAAm-g-PAMAM derivative were dissolved in 2 ml methanol. The solution was kept sonicating at room temperature for 20 min, and then the solvent was volatilized at room temperature. Sample slices with IMC and PNIPAAm-g-PAMAM were dissolved in 5 ml distilled water, and centrifuged at 5000 rpm for 15 min, the clear supernatant was lyophilized and the drug loaded dendrimer-IMC conjugates were obtained.

To determine the amount of drug encapsulated in the complex, the dendrimer-IMC conjugate was weighed in a dialysis bag (molecular weight cut off: 7000) and dissolved in a 100 ml PBS (pH 7.4) solution. The solution was kept in a water-bath sonicator at room temperature for 2 h and then was measured by using UV absorbance at 320 nm to estimate the indomethacin content in the complex.

The sample slices loaded with IMC were sealed separately using a dialysis bag (molecular weight cut off: 7000). The bags were immersed into 100ml of pH 7.4 PBS solutions and 3 ml aliquots were withdrawn from the solution periodically. The volume of solution was held constant by adding the same volume of PBS solution after each sampling. The amount of IMC released from PNIPAAm-g-PAMAM was measured using UV absorbance at 320 nm. All release measurements were carried out in triplicate to each sample at 27 °C/37 °C, and an average value was adopted. The cumulative release of IMC was calculated by using the following equation.

$$\text{Cumulative release}(\%) = \frac{C_n \times V_0 + v\sum C_{n-1}}{W_0} \times 100\%$$

where W_0 is the weight of IMC in the polymer; C_n and $C_{n\text{-}1}$ are the concentration of IMC in buffer solution which was withdrawn for n and n-1 ($n > 0$) times, respectively; V_0 is the total volume of the PBS solution, and v is the volume of the aliquot each time withdrawn from the PBS solution.

Results and Discussion

Synthesis and Characterization of PNIPAAm-g-PAMAM

PAMAM dendrimer was synthesized from ethylenediamine (EDA) by repeatedly using two consecutive chain forming reactions: the Michael addition reaction and the amine reaction. The ester terminated half generation PAMAM dendrimer (G 1.5) was converted into dendrimer with hydroxyl surface (PAMAM-OH G 2.0) by amino ethanol. The structure of the resulted PAMAM-OH dendrimer was confirmed by ^{1}H NMR spectra.

As shown in Fig. 1, the single peak of -CH_3 (1) at 3.48 ppm disappeared, while a new group of peaks from -$HNCH_2C\boldsymbol{H}_2OH$ (2) at 3.43-3.45 ppm emerged showing the successful converting of –$COOCH_3$ into –OH group. In addition, the evidence of the peak -$CH_2C\boldsymbol{H}_2COOCH_3$- (3) at 2.33-2.36 ppm disappeared and the peak of -$CH_2C\boldsymbol{H}_2CONH$- (4) at 2.25-2.26 ppm enhanced could confirm the conversion. The chemical shifts of protons in $H_2NC\boldsymbol{H}_2CH_2OH$ (5), (6) at 2.53-2.55 ppm and 3.39-3.41 ppm were observed in the spectrum probably because the

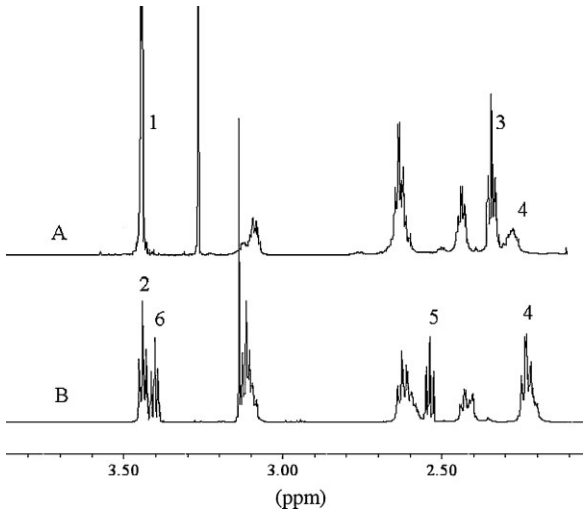


Figure 1.
^{1}H NMR spectra of PAMAM (G 1.5) (A) and PAMAM (G 2.0) (B) in D_2O.

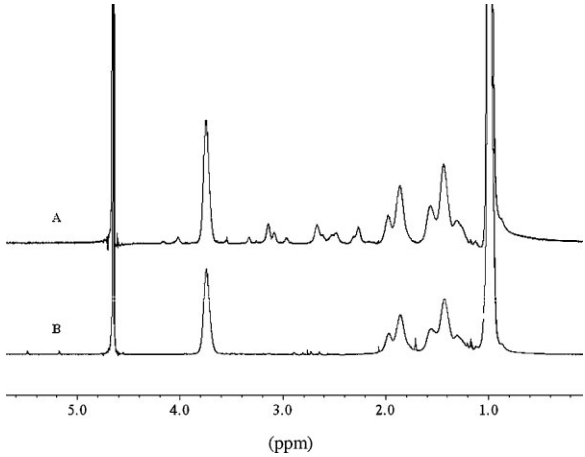


Figure 2.
^{1}H NMR spectra of PNIPAAm-g-PAMAM (A) and PNIPAAm (B) in D_2O.

internal cavities of the dendrimer derivatives contain some aminoethanol molecules and it is difficult to remove them thoroughly by dialysis during the preparation.

The thermosensitive PNIPAAm-COOH grafted on the PAMAM-OH (G 2.0) dendrimer was synthesized in the presence of EDC•HCl through esterification as shown in Scheme 1. The detail ^{1}H NMR spectra for the samples of PNIPAAm and PNIPAAm-g-PAMAM were shown in Fig. 2, where the chemical shift of -OCC***H***$_2$S- moved to the upfield obviously, and the PAMAM core could be found at 2.0-3.5 ppm in Fig. 2 (A), which indicates that the PNIPAAm with carboxyl terminal group was grafted on the PAMAM-OH dendrimer successfully. Since the proton signals in PAMAM-OH core might be shielded by that in the main chain of PNIPAAm-COOH, the signals would overlap to each other leading to the attenuation of the signal intensities, while the chemical shift of protons in PAMAM-OH terminal group did not move obviously.

In order to obtain the PNIPAAm-g-PAMAMs with different molecular weights, various feed ratio of reactants were selected as shown in Table 1. The graft ratio is calculated using the following formulation:

$$Graft\ ratio = \frac{M_w^1 - M_w^2}{M_w^3 \times 8} \times 100\%$$

where M_w^1 is M_w of product, M_w^2 is M_w of dendrimer (1438), M_w^3 is M_w of PNIPAAm (2655, PDI = 1.1).

As shown in Table 1, the graft ratio could be adjusted by changing the feed ratio of PAMAM-OH to PNIPAAm-COOH, which was as high as 76.6% when the molar ratio of reactants and reaction time increased (No.3). The result implies that about six PNIPAAm chains could be attached to each dendrimer molecule with eight end groups.

Table 1.
The reaction conditions and characteristics of PNIPAAm-g-PAMAM

No.	Molar ratio of –COOH/-OH	Time/h	M_w^1	M_w/M_n[a]	Graft ratio (%)
1	1:1	24	12507	–	50.0[b]
2	1:1	60	14818	2.16	69.7
3	1.5:1	96	16136	2.17	76.6

[a]Polydispersity of PNIPAAm-g-PAMAM; [b]measured by ^{1}H NMR, others were measured by GPC.

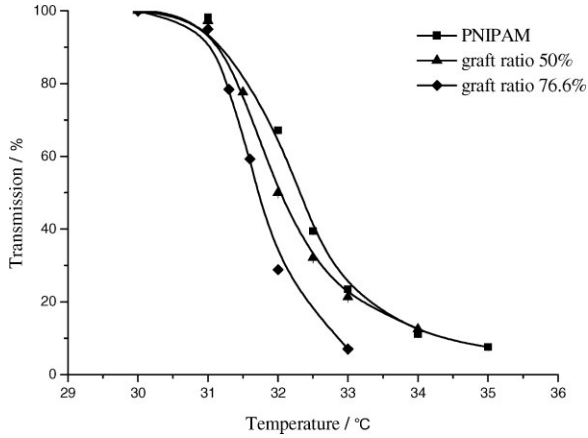


Figure 3.
Temperature effect on the light transmittance of the solutions of PNIPAAm-g-PAMAM with different graft ratios.

Thermosensitive Properties of PNIPAAm-g-PAMAM

Figure 3 showed the temperature effect on the light transmittance of the PNIPAAm-g-PAMAM with different graft ratios at 500 nm in PBS (pH 7.4) buffer solution. As shown in Fig. 3, the transmission of PNIPAAm-g-PAMAM solution was almost 100% when the temperature was lower than 31 °C. With the increase of temperature, the PNIPAAm-g-PAMAM solution changed sharply from transparency to opacity and its transmission decreased drastically, where a ''lower critical solution temperature'' (LCST) was called. It is well known that a hydrophilic/hydrophobic balance exists between the hydrophilic groups (-CONH-) and the hydrophobic groups (-$CH(CH_3)_2$) on PNIPAAm chains. The H-bonds between water molecules and the amide groups will make water molecules orient neatly around the isopropyl groups at the temperature below LCST resulting in the PNIPAAm chains soluble in the surrounding water. When the environmental temperature increases above the LCST, the hydrophilic/hydrophobic balance is broken and the H-bonds are destroyed leading to the strong interaction of the hydrophobic groups. The PNIPAAm chains grafted on the surface of the dendrimer would aggregate as a result of the dissociation of the hydrating water molecules from the PNIPAAm chains.

As shown in Fig. 3, the LCST of PNIPAAm-g-PAMAM was around 32 °C. It is well known that the LCST should descend with the increase of hydrophobicity of the polymers. Compared with PNIPAAm-COOH, the chain length of PNIPAAm-g-PAMAM was several times increased and the enhanced polymer chains could contribute to the hydrophobicity of the polymer derivative leading to a slight decrease of its LCST.

Drug Loading and Drug Release Profile *in vitro*

The internal cavities of the dendrimer derivatives can act as drug reservoirs to encapsulate drug molecules. Due to the thermosensitivity of the PNIPAAm component, it is expectable that PNIPAAm-g-PAMAM could be an intelligent drug carrier in the controlled release of drug systems. To investigate the potential application of PNIPAAm-g-PAMAM in the controlled drug release, the hydrophobic indomethacin (IMC) was selected as a model drug and was loaded in the PNIPAAm-g-PAMAM complex. The drug loading measured by UV-vis absorbance was about 17.5% and the *in vitro* release behavior of IMC from the PNIPAAm-g-PAMAM complex was examined in PBS

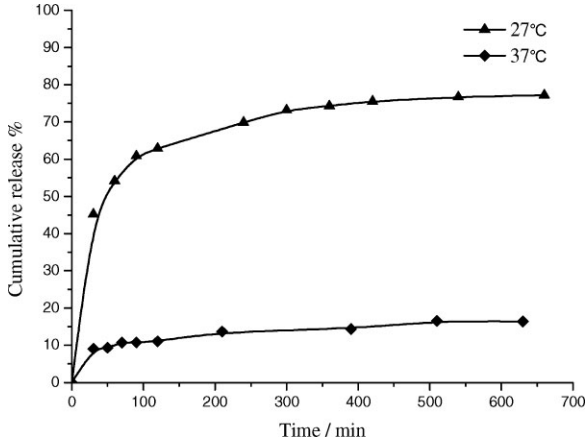


Figure 4.
Dissolution test of the IMC / PNIPAAm-g-PAMAM complex at 27 °C and 37 °C.

(pH 7.4) at 27 °C and 37 °C, respectively. Results were shown in Fig. 4.

The drug release profiles shown in Fig. 4 displayed the rapid release at the temperature below and above the LCST (27 °C vs 37 °C) during the first 30 min. After 30 min, only 9% of the drug was cumulatively released at 37 °C, whereas, 45% was released at 27 °C from the complex. Almost 77% of the drug was cumulatively released at 27 °C after 10 hours, while only 20% was released at 37 °C. The release of IMC from the IMC / PNIPAAm-g-PAMAM complex at 37 °C is significantly slower than that of at 27 °C. The reason might be that the drug aggregated in the second amines of PNIPAAm-g-PAMAM complex would be released rapidly because of the weak interaction between the molecules. However, the drug entrapped on the tertiary amines within the dendrimer cavities would be impeded to release due to the strong interaction of the drug with the tertiary nitrogen. As seen from Fig. 5, the cumulative release of the first 30 min was about half of that for 10 h. It was conjectured that IMC molecules loaded in PNIPAAm-g-PAMAM complex were not all entrapped in the cavities of PAMAM derivative, some IMC molecules might be grabbed by the random coils of the PNIPAAm chains grafting on the surface of PAMAM dendrimer derivative, leading to the rapid release of the drug during the first stage.

It is well known that PNIPAAm has a conformational transition from an expending coil to a compact globular in accordance with the variation of the surrounding temperature. The mechanism of the drug release behavior from the thermosensitive PNIPAAm-g-PAMAM dendrimer derivative was sketched in

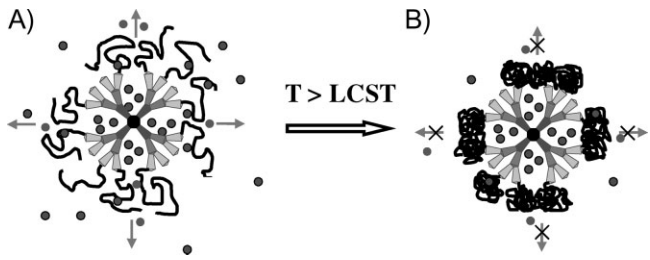


Figure 5.
Mechanism of drug release behavior of the thermosensitive dendrimer derivative.

Fig. 5: at the temperature above LCST, the PNIPAAm chains grafted on the surface of PAMAM take a compact globular shape shielding the release channel of the drug, which prevents the IMC releasing from the PNIPAAm-g-PAMAM complex; whereas at the temperature below LCST, the PNIPAAm segments show an expending coil conformation allowing the IMC to diffuse out slowly from the complex. The PNIPAAm chains could act as a channel switch on-off button through expending or contracting in response to the temperature variation. This thermosensitive drug carrier is potential to be used in the controlled drug release system.

Conclusion

Thermosensitive PNIPAAm-g-PAMAM dendrimer derivative was synthesized by grafting PNIPAAm-COOH to PAMAM dendrimer in the presence of EDC•HCl through esterification. The thermosensitive properties of the resulted dendrimer derivatives showed a little bit different from the linear PNIPAAm and the behaviors of the temperature controlled drug release were studied using indomethacin as a model drug. The results indicated that the release rate of the water-poorly soluble drug loaded in the PAMAM derivatives could be effectively controlled by varying the surrounding temperature. The PNIPAAm chain grafted on the PAMAM dendrimer could act as a channel switch on-off button through expending or contracting in response to the temperature variation. This novel PNIPAAm-g-PAMAM dendrimer derivative might be a promising strategy to control the drug release. Nevertheless, further investigations about the effect of the chain length of PNIPAAm on the thermo-properties of the dendrimer derivatives would be required for the controlled release of drugs.

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