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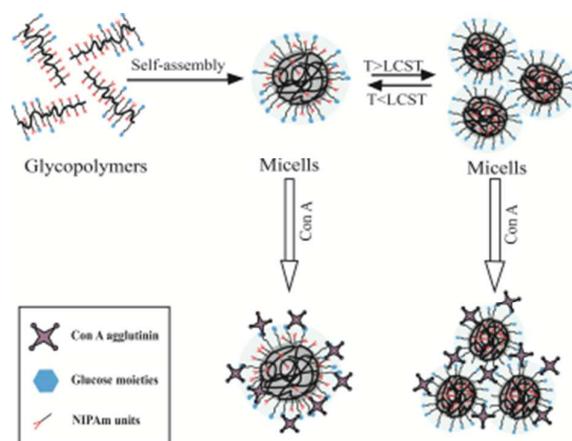


Illustration showing the lectin recognition of thermoresponsive block glycopolymer micelles.

Lectin Recognizable Thermoresponsive Double Hydrophilic Glycopolymer Micelles by RAFT Polymerization

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Kan Sun,^a S. W. Annie Bligh,^b Hua-li Nie,^a Jing Quan,^{*a} and Li-min Zhu^{*a}

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Thermoresponsive double hydrophilic block glycopolymers poly(*N*-isopropylacrylamide-*co*-6-O-vinyladipoyl-D-glucose)-*b*-poly(*N*-isopropylacrylamide) (P(NIPAm-*co*-OVAG)-*b*-PNIPAm) were prepared by a combination of enzymatic synthesis and reversible addition-fragment chain transfer (RAFT) polymerization protocols using P(NIPAm-*co*-OVAG) as macro-RAFT agent. All the precisely synthesized glycopolymers were characterized by nuclear magnetic resonance spectroscopy and gel permeation chromatography. Detections using laser light scattering and transmission electron microscopy revealed that the block glycopolymer was able to self-assemble into micelles with various sizes and sphere morphologies in aqueous solutions. The glucose pendants in the glycopolymers had obvious interaction with lectin, Concanavalin A (Con A), and their capacity of interaction with Con A was controlled by the number of glucose units in the glycopolymers. The block glycopolymer micelles have excellent biocompatibility with pig iliac endothelial cells using the MTT assay, however the glycopolymer-Con A micelles could be used to induce apoptosis in human hepatoma SMMC-7721 cells.

Introduction

Functional double hydrophilic block copolymers (DHBCs) containing at least one functional unit such as temperature-responsive, light-sensitive, and pH-sensitive monomer have recently attracted significant interest for potential applications in drug delivery systems¹⁻⁴, switchable catalysts⁵, and gene delivery vectors⁶. Thermoresponsive DHBCs are one of the most important candidates of biomaterials in DHBCs and have capability of reversible micellization and dissociation responding to the change of temperature. When the temperature is above the lower critical solution temperature (LCST) of the thermoresponsive copolymer, the DHBCs self-assemble into micelles. Inversely, the formed micelles dissociate into aqueous solution when the temperature is below the LCST. Many studies have been focused on the connection of thermoresponsive DHBC with other functional monomer such as sugar-functional monomer, which are deemed to be promising for protein binding^{7,8}.

The glycopolymers that contain sugar pendant groups have attracted chemists to investigate for their biomedical applications and protein recognition⁹⁻¹¹. They have been used to mimic biological systems such as binding lectins through multipoint interactions^{12,13}. As a lectin, concanavalin A (Con A) shows high specificity for α -D-glucose and α -D-mannose, and can induce cancer cell death¹⁴⁻¹⁷. The protein recognition is closely related to the sugar moiety of the

glycopolymer, for example, a glycopolymer containing galactose can recognize peanut agglutinin (PNA)⁴⁴.

The architecture of glycopolymer plays an crucial role in its interaction with proteins^{18,19}. The precisely synthesized glycopolymers with great potential for use as novel biomaterials can be produced by controlled polymerization technique¹⁸⁻²⁰. RAFT polymerization as one of the most useful and amenable polymerization techniques can easily generate polymeric systems and control structures for a wide-variety of polymers with defined end and pendant functionalities, controlled molecular weights, narrow polydispersities and low toxicity using mild conditions²¹⁻³⁵. Miura et al reported the advantage of RAFT polymerization was that it provided precise glycopolymer characteristics such as molecular weights with narrow polydispersities and random saccharide spacing²⁰.

In this study we aimed to obtain a series of well-defined thermoresponsive double hydrophilic block glycopolymers which could be used to recognize protein in a controlled fashion and not toxic. Thermoresponsive glycopolymers were fabricated through polymerization of a sugar-functional monomer 6-O-vinyladipoyl-D-glucose (OVAG) and a temperature sensitive monomer NIPAm via controlled RAFT polymerization using S-1-dodecyl-S'- (α , α' -dimethyl- α'' -acetic acid) trithiocarbonate (DDATC) as chain transfer agent (CTA) and 2,2'-Azo-bis-iso-butyronitrile (AIBN) as initiator. The synthesis and characterization of well-defined thermoresponsive block glycopolymers poly(NIPAm-*co*-OVAG)-*b*-poly(NIPAm) by

RAFT polymerization using poly(NIPAm-co-OVAG) as a macroagent are presented. The binding ability of these glycopolymers to Con A, their thermoresponsiveness, their cell biocompatibility in free and bound lectin forms with pig idiliac endothelial (PIE) cells and human hepatoma SMMC-7721 cells are also reported.

Experimental

Materials.

N-isopropylacrylamide (NIPAm) (98%, Tokyo Chemical Co. Ltd.) was purified by recrystallization in n-hexane. Alkaline protease from bacillus subtilis (EC 3.4.21.14, a crude preparation of alkaline serine protease, powder, 100 U/mg) was purchased from the Wuxi Xue Mei Technological Co.Ltd. (Wuxi, PR China). Dimethyl Formamide (DMF) was purchased from the Sinopharm Chemical Reagent Co. (Shanghai, PR China). 2,2'-Azo-bis-iso-butyronitrile (AIBN; Sinopharm Chemical Reagent Co., Ltd., Shanghai, PR China, 97%) was purified by recrystallization from a 95% water solution. Adipic acid was purchased from the Sinopharm Chemical Reagent Co. (Shanghai, PR China). S-1-dodecyl-S'- (α , α' - dimethyl- α'' -acetic acid) trithiocarbonate (DDATC) was purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd.. 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) was procured from the Nanjing Robiot Co. (Nanjing, China). Concanavalin A (Con A) was obtained from Sigma-Aldrich (Shanghai, China). Fluorescently-labeled Con A (FITC-Con A) was purchased from Vector Laboratories (Burlingame, CA, USA). Dimethyl sulfoxide (DMSO) was purchased from the China National Medicines Corporation Ltd. (Beijing, China). Dulbecco's modified Eagle medium (DMEM), penicillin-streptomycin and Trypsin-EDTA solutions were obtained from Gibco (Tulsa, OK, USA). All solvents used in this work were of analytical grade and were dried by storing over activated 4Å molecular sieves for 24 h prior to use. All

other reagents were used as received. Water was distilled before use.

Syntheses.

Synthesis of 6-O-vinyladipoyl-D-glucose (OVAG). According to literatures, OVAG can be synthesized via the chemoenzymatic synthesis of carbohydrates^{36,37}.

RAFT polymerization of OVAG and NIPAm (Experiment 1 in Table 1). In a typical experiment, NIPAm (0.79 g, 7.00×10^{-3} mol) was first measured in a 25 mL one-neck round-bottom flask, and 25 mol % of OVAG (0.58 g, 1.75×10^{-3} mol) based on the amount of NIPAm used was introduced into the same flask. N,N-Dimethylformamide (DMF, 1.50 mL) was later used to dissolve the monomers in the flask. S-1-dodecyl-S'- (α , α' - dimethyl- α'' -acetic acid) trithiocarbonate (DDATC, 3.15×10^{-2} g, 8.75×10^{-5} mol) was finally added to the solution as chain transfer agent (CTA). The mixture was then degassed by three freeze-thaw cycles and sealed under vacuum. The polymerization was carried out at 70 °C for 12 h. The product was dialyzed into double distilled water for 24 h to remove residual monomers, the dialyzed solution was freeze-dried into slightly yellowish solids with a yield of 63%.

Chain extension polymerization of P(NIPAm-co-OVAG) with NIPAm (Experiment 4 in Table 1). Purified P(NIPAm-co-OVAG) from experiment 1 (0.30 g, 6.31×10^{-6} mol, $M_n(\text{GPC})=4.75 \times 10^4$ g mol⁻¹, $M_w/M_n=1.26$) was measured into a 25 mL one-neck round-bottom flask followed by addition of NIPAm (0.22 g, 1.95×10^{-3} mol). The mixture was dissolved in DMF (1 mL) and degassed by three freeze-thaw cycles and sealed under vacuum. The polymerization was carried out at 70 °C for 8 h. The diblock copolymer was dialyzed into double distilled water for 24 h to remove residual monomers, the dialyzed solution was freeze-dried to yield a fine slightly yellowish powder.

Table 1. RAFT polymerization of glycopolymers

Experiment	Polymer ^a	[NIPAm] ₀ /[OVAG] ₀ /[CTA] ₀ /[AIBN] ₀ ^{b,c}	Yield ^d (%)
1	PNO-1	80/20/1/0.05	63
2	PNO-2	90/10/1/0.05	67
3	PNO-3	93/7/1/0.05	75
4	PNO- <i>b</i> -PN-1	300/0/1/0.25	71
5	PNO- <i>b</i> -PN-2	300/0/1/0.25	76
6	PNO- <i>b</i> -PN-3	300/0/1/0.25	80

^a PNO and PNO-*b*-PN were respectively shorted for P(NIPAm-co-OVAG) and P(NIPAm-co-OVAG)-*b*-PNIPAm.

^b [NIPAm]₀, [OVAG]₀, [AIBN]₀ and [CTA]₀ represent the initial concentrations of monomers, initiator and chain transfer agents (CTA), respectively.

^c In the synthesis of P(NIPAm-co-OVAG), CTA was S-1-dodecyl-S'- (α , α' - dimethyl- α'' -acetic acid) trithiocarbonate (DDATC), and P(NIPAm-co-OVAG) was macroCTA in the polymerization of P(NIPAm-co-OVAG)-*b*-PNIPAm.

^d Yield was determined by gravimetric analysis.

Analytical Techniques.

Nuclear Magnetic Resonance (NMR) Spectra. All ¹H NMR spectra were recorded by dissolving 10 mg of purified samples in D₂O and using a Bruker DRX 400 MHz spectrometer (Bruker, Rheinstetten, Germany).

Gel Permeation Chromatography (GPC). The molecular weights (M_w and M_n) and molecular weight distributions of glycopolymers were determined by GPC measurements on a Waters LS measurement system using THF as the eluent, at a flow rate of 1.0 mL/min with a column temperature of 35 °C. The molecular weights (M_w and M_n) and molecular weight distributions (M_w/M_n) for the sample polymers were calibrated with standard polystyrene samples.

Critical Micelle Concentration (CMC) Measurements of Glycopolymers. The CMC of P(NIPAm-co-OVAG)-1 and P(NIPAm-co-OVAG)-*b*-PNIPAm-1 were characterized by the fluorescence spectra (QM / TM, PTI, USA) and pyrene as the fluorescence probe. Pyrene solutions ($10 \mu\text{L}$, $6.576 \times 10^{-4} \text{ M}$ in acetone) were added to the open tubes, and the acetone was evaporated overnight. 10 mL of glycopolymer solutions at different concentrations (2.0 , 1.0 , 0.5 , 0.1 , 0.01 and 0.001 mg/mL) were added to tubes containing evaporated pyrene. These solutions were stored at room temperature for 24 h to reach solubilization equilibrium. Fluorescence emission spectra were recorded from 350 to 500 nm with an excitation wavelength of 334 nm and a slit-width of 2 nm . The intensity ratio of I_{384}/I_{374} from the emission spectra was analyzed to obtain CMC of P(NIPAm-co-OVAG)-*b*-PNIPAm-1 and P(NIPAm-co-OVAG)-1.

Lower Critical Solution Temperature (LCST) Determination of Glycopolymers. The thermoresponsive abilities of glycopolymers were investigated by UV-vis spectrophotometer (Lambda 35, Perkin Elmer, USA). A thermostatically controlled cuvette was used, and the heating rate was $0.5 \text{ }^\circ\text{C}/\text{min}$. Absorbance through aqueous solutions of glycopolymer (1.0 mg/mL) at 500 nm was recorded at different temperatures. The LCST was defined as the temperature corresponding to a 50% of the maximum absorbance³⁷.

Laser Light Scattering (LLS) Measurement of Block Glycopolymers. P(NIPAm-co-OVAG)-*b*-PNIPAm-1 was dissolved in double distilled water to prepare a aqueous solution with glycopolymer concentration of 0.8 mg/mL . The sample was filtered through a $0.45 \mu\text{m}$ Millipore filter into a clean scintillation vial. Laser light scattering (LLS) system (BI-200SM, Brookhaven, USA), combined static laser scattering (SLS) and dynamic laser scattering (DLS), was used at $\lambda = 532 \text{ nm}$. To obtain average hydrodynamic radius (R_h) and average gyration radius (R_g) by LLS at different temperature, the filtered sample was heated at different temperatures for 15 min .

Transmission Electron Microscopy (TEM). The TEM micrographs were obtained by using a transmission electron microscope (JEM-2100, JEOL, Japan). The samples were prepared by casting an aqueous solution of glycopolymer micelle solution (0.4 mg/mL) onto a copper grid with thin films of Formvar and carbon successively at $38 \text{ }^\circ\text{C}$. The images taken were also used to obtain an average particle size using the equation $D_n = \sum n_i D_i / \sum n_i$. Thirty particles were each measured from the images and calculated using the equation, where D_n is the number-average diameter.

Lectin-binding assay.

Turbidity. The lectin agglutination activity of the glycopolymers micelles was evaluated by measuring the change in the turbidity of buffer solution because of the multipoint interaction between the glycopolymer and Con A. Buffer solution ($\text{pH}=7.4$) for lectin-binding assay was prepared by adding 10 mM HEPES, 0.15 M NaCl, 0.1 mM CaCl_2 , 0.01 mM MnCl_2 . The final mixed solutions contained 7 mg/mL glycopolymer and 2 mg/mL Con A. The turbidity was measured by using a UV-vis spectrophotometer (Lambda 35, Perkin Elmer, USA) at a wave-length of 600 nm . The

interaction tests were repeated at least three-times to ensure the measurement reproducibility.

Temperature influence on lectin-binding. The glycopolymers P(NIPAm-co-OVAG)-*b*-PNIPAm-1 and P(NIPAm-co-OVAG)-1 were dissolved in HEPES buffer solution ($\text{pH}=7.4$) to prepare solutions with concentration of 0.4 mg/mL . The samples were filtered through at a $0.45 \mu\text{m}$ Millipore filter into clean scintillation vials, and were heated at $25 \text{ }^\circ\text{C}$ and $37 \text{ }^\circ\text{C}$ for 15 min to measure the D_h . The Con A solution was added to glycopolymer solutions to form the final mixed solution containing 0.35 mg/mL glycopolymer and 0.15 mg/mL Con A. After 1 h , the mixed solution was heated at $25 \text{ }^\circ\text{C}$ and $37 \text{ }^\circ\text{C}$ for 15 min to measure the D_h .

Confocal microscopy. Fluorescein isothiocyanate Con A (FITC-Con A) was utilized to determine the protein recognition with glycopolymer micelles, and a confocal laser scanning microscope (Carl Zeiss LSM700, Jena, Germany) with four channels (405 , 488 , 555 , 634 nm) excitation was used. Glycopolymer and FITC-Con A all were dissolved in HEPES buffer solution ($\text{pH}=7.4$). Before the determination of confocal, the same volume of glycopolymers (7 mg/mL) and FITC-Con A (0.5 mg/mL) were mixed keeping at $25 \text{ }^\circ\text{C}$ for 2 h .

Cell viability study.

Cell culture. Pig iliac endothelial (PIE) cells were maintained as an exponentially growing monolayer in a DMEM high sugar medium supplemented with 10% fetal bovine serum, and 1% (v/v) penicillin-streptomycin solution. The cells were cultured at $37 \text{ }^\circ\text{C}$ in a humidified atmosphere containing $5\% \text{ CO}_2$. Cells were passaged every $1-2$ days. For passaging, the cells were trypsinized with $0.5/0.2\%$ (v/v) trypsin-EDTA solution. Human hepatoma SMMC-7721 cells were cultured in a 1640 medium supplemented with 10% fetal bovine serum, and 1% (v/v) penicillin-streptomycin solution, and the next is the same with above.

MTT assays. The cytotoxicity of the RAFT-based glycopolymers P(NIPAm-co-OVAG)-*b*-PNIPAm-1 and P(NIPAm-co-OVAG)-1 was evaluated by MTT assay. PIE cells were seeded into 96-well plates at a density of $20\,000$ cells per well and incubated for 24 h . The glycopolymers in PBS solutions were added into the cells (final concentrations were 1 , 5 , 10 , 25 , 50 , 100 mg/L). After incubation for another 24 h , $20 \mu\text{L}$ of MTT solution (5 mg/mL in PBS buffer) was added to each well. Four hours later, the medium was removed and the samples in the wells were air dried. $200 \mu\text{L}$ of DMSO was added to dissolve the formed crystals. The optical density of the solution was measured at 570 nm using a microplate reader (Thermo Multiskan MK3, Thermo Scientific Company, Waltham, UK). The PIE cells without any treatment were used as the control.

Further experiments of the cytotoxicity of glycopolymers binding Con A were performed in which a series of solutions with 1 g/L of P(NIPAm-co-OVAG)-*b*-PNIPAm-1 and different concentration of Con A (0 , 100 , 300 , 500 , 1000 mg/L) in HEPES buffer were initially incubated for 4 h . SMMC-7721 cells were seeded into 96-well plates at a density of $20\,000$ cells per well and incubated for 24 h . The glycopolymers binding Con A in HEPES buffer were added to the cells (final concentrations of Con A were 0 , 10 , 30 , 50 , 100 mg/L and glycopolymer was 100 mg/L in every well). They were

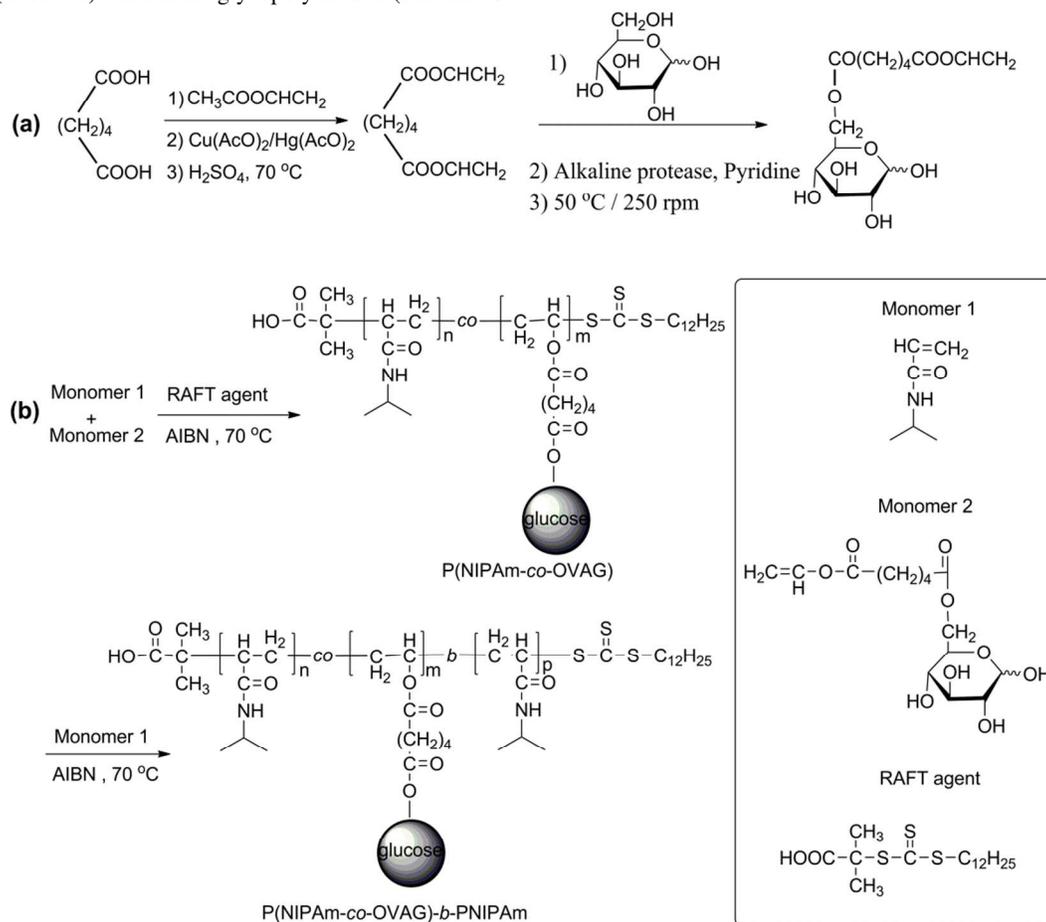
then assayed as above. The SMMC-7721 cells without any treatment were used as the control.

Results and Discussion

Polymer Synthesis.

Thermoresponsive block glycopolymers P(NIPAm-*co*-OVAG)-*b*-PNIPAm with glucose was facily synthesized by combining enzymatic synthesis with RAFT polymerization (Scheme 1) with good yields (Table 1). Diblock glycopolymers P(NIPAm-*co*-

OVAG)-*b*-PNIPAm were prepared by RAFT polymerization of NIPAm in DMF at 70 °C using P(NIPAm-*co*-OVAG) as a macro-RAFT agent, which was prepared by RAFT polymerization of NIPAm and OVAG with different feed molar ratios (Table 1). In the polymerization reaction, S-1-dodecyl-S'- (α , α' -dimethyl- α'' -acetic acid) trithiocarbonate (DDATC) was used as a RAFT agent because of its ability to control the reaction and its low toxicity property^[40]. The molar ratio of monomers to DDATC was fixed at 100:1. The conditions of the polymerization were listed in Table 1.



Scheme 1. a) The synthesis of 6-O-vinyladipoyl-D-glucopyranose (OVAG) by controllable chemoenzymatic reaction; b) The synthesis of P(NIPAm-*co*-OVAG)-*b*-PNIPAm by RAFT polymerization.

Table 2. Properties of the glycopolymers prepared by RAFT polymerization

Polymer ^a	M_n^b ($\times 10^4$)	M_w^b ($\times 10^4$)	PDI ^b	[NIPAm]/[OVAG] ^c	LCST (°C) ^d	$M_{n, \text{NMR}}^e$ ($\times 10^4$)
PNO-1	4.75	6.00	1.26	5.9	38.0	4.94
PNO-2	4.55	5.49	1.20	9.1	37.0	4.76
PNO-3	5.36	6.72	1.25	15.6	36.0	5.87
PNO- <i>b</i> -PN-1	7.42	9.17	1.23	9.7	36.5	7.95
PNO- <i>b</i> -PN-2	7.26	8.41	1.16	13.8	35.5	7.55
PNO- <i>b</i> -PN-3	7.79	9.27	1.19	20.3	33.0	8.36

^a PNO and PNO-*b*-PN were respectively shorted for P(NIPAm-*co*-OVAG) and P(NIPAm-*co*-OVAG)-*b*-PNIPAm.

^b M_n , M_w and PDI were determined by GPC.

^c [NIPAm]/[OVAG] represented the molar ratio of NIPAm and OVAG in the resultant glycopolymers and was determined by ¹H NMR.

^d LCST was determined by UV-vis spectroscopy.

^e M_{n,NMR} was determined by ¹H NMR.

The ¹H NMR spectra of glycopolymers are shown in Figure 1 a and b, which reveal the presence of NIPAm and glucose moieties and the absence of vinyl groups. Characterizing data are as follows. ¹H NMR of NIPAm (D₂O, 400 MHz) δ (ppm): 6.02 (qd, 2H, 1-H), 5.56 (dd, 1H, 2-H), 3.82 (m, 1H, 4-H), 1.00 (d, 6H, 5, 5'-H). ¹H NMR of OVAG (D₂O, 400 MHz) δ (ppm): 7.15 (dd, 1 H, 13-H), 5.25 (d, 0.46 H, 1-H_α), 4.85 (dd, 1 H, 14-H trans), 4.65 (dd, 1 H, 14-H cis), 4.55 (d, 0.54 H, 1-H_β), 4.25 (m, 2 H, 6-H), 3.85 (m, 0.46 H, 5-H), 3.60 (m, 3-H_α), 3.55 (m, 5-H_β), 3.40 (m, 2-H_β), 3.35 (m, 3-H_β), 3.30 (m, 4-H), 3.15 (m, 0.52 H, 2-H_α), 2.35 (m, 4 H), 1.55 (m, 4 H). In the ¹H NMR spectrum of P(NIPAm-co-OVAG)-b-PNIPAm-1,

the signals at δ 7.15 (dd, 1 H, 13-H) and 4.85 (dd, 1 H, 14-H trans), 4.65 (dd, 1 H, 14-H cis) of OVAG and 6.02 (qd, 2H), 5.56 (dd, 1H) of NIPAm has almost disappeared demonstrating polymerization has proceeded. In addition, the molar ratio of NIPAm and OVAG units in the resultant polymers was calculated based on the integral values of the signals at δ= 2.38-2.22 (2H of OVAG) and δ= 1.25-0.95 (6H of NIPAm) and the results are presented in Table 2. Meanwhile, when compared with P(NIPAm-co-OVAG)-1, the characteristic signals of NIPAm are enhanced because of the additional PNIPAm block. The calculated monomer ratio are basically consistent with the feed of the monomer ratio.

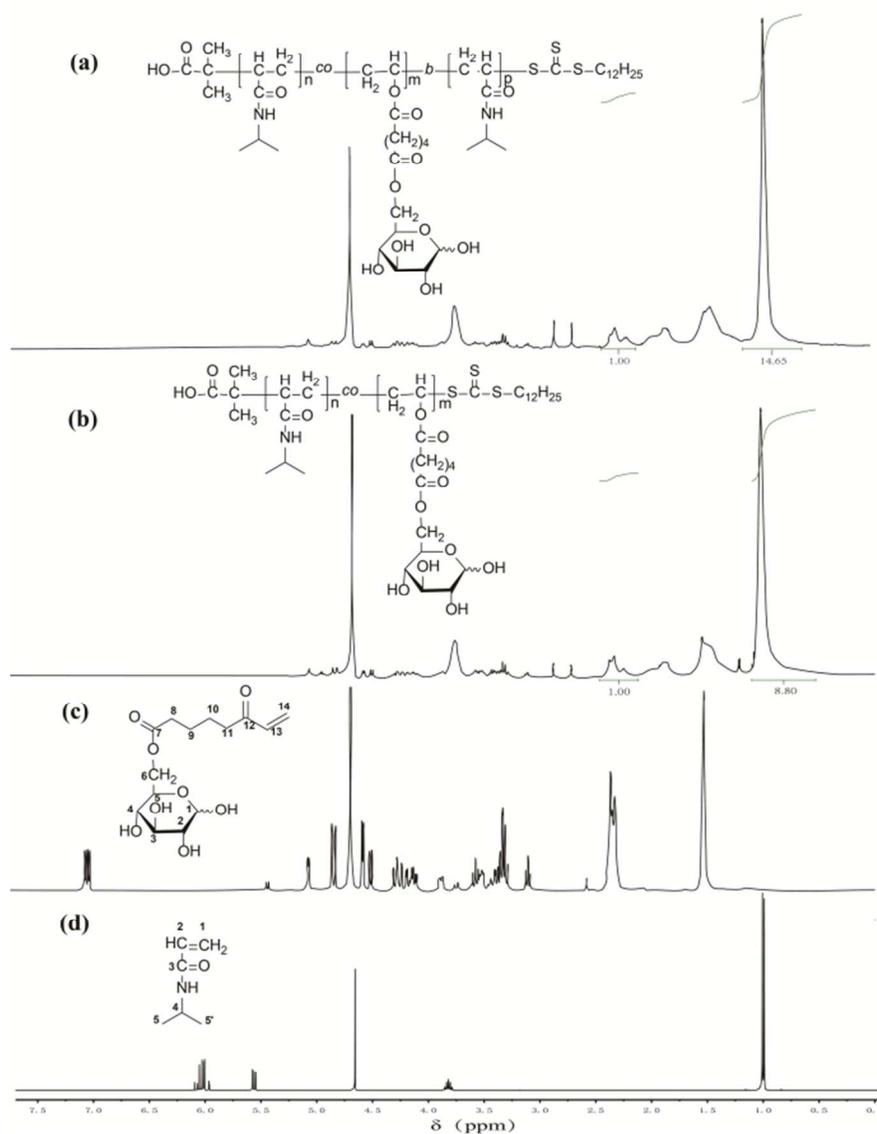


Figure 1. ¹H NMR spectra of (a) P(NIPAm-co-OVAG)-b-PNIPAm; (b) P(NIPAm-co-OVAG); (c) OVAG; (d) NIPAm in D₂O.

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The molecular weights (M_n and M_w) and molecular weight distributions (M_w/M_n) of glycopolymers measured by GPC in THF are presented in Table 2. Compared to the macro-RAFT agents, all the diblock glycopolymers eluted at higher molecular weights and a clear shift can be observed. Fig. 2 shows the M_n and M_w of P(NIPAm-co-OVAG)-*b*-PNIPAm-1 is 7.42×10^4 and 9.17×10^4 , M_w/M_n of P(NIPAm-co-OVAG)-*b*-PNIPAm-1 is 1.23. All the GPC traces of glycopolymers are symmetrical and unimodal, and the molecular weight distributions are relatively narrow. Because of the polydispersity of polymer, the values on M_n and M_w (and thus, also of M_w/M_n) are the values of samples with statistical means, which can't be considered as the real ones. Compared with the results by GPC, the $M_{n,NMR}$ (molecular weight determined by 1H NMR) is similar to the $M_{n,GPC}$, which is shown in Table 2. Meanwhile, in order to reduce the error caused by the structural difference between synthesized samples and standard sample, universal calibration curve was used. All data including M_n , M_w and M_w/M_n were the average numbers of three replicates.

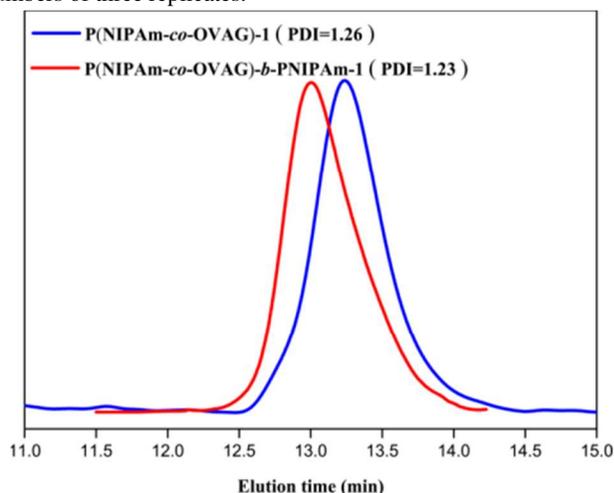


Figure 2. GPC traces of P(NIPAm-co-OVAG)-*b*-PNIPAm-1 (red curve) and P(NIPAm-co-OVAG)-1 (blue curve) in THF.

LCST Determination of Glycopolymers.

It has been reported that the LCST value of PNIPAm-based copolymer can be precisely designed to be in the range $32 \sim 40$ °C by adjusting the relative amount of monomers, and LCST value increases with the molar fraction of hydrophilic monomers in the copolymers³⁷. Fig. 3 shows the absorbance depends on temperature of the glycopolymers micellar solution. The LCST of diblock glycopolymer P(NIPAm-co-OVAG)-*b*-PNIPAm-1 at 1.0 mg/mL was 36.5 °C (Fig. 3). Overall, the LCST values of glycopolymers increase with increasing molar fraction of OVAG in the glycopolymers (Table 2).

Meanwhile, it can be found that phase transition of P(NIPAm-co-OVAG)-1 lies in the range of $35 \sim 41$ °C which is the widest in the glycopolymers studied. It is due to the increase of hydrogen bonds between hydroxyl groups of glucose and water which retard the process of phase transition with the increasing the molar ratio of OVAG. However, it also can be found that the range of phase transition of P(NIPAm-co-OVAG)-*b*-PNIPAm-1 is $35 \sim 38$ °C and it is narrower than that of P(NIPAm-co-OVAG)-1. The reason for this change is the increasing proportion of PNIPAm and the formation of a block, which has also been observed in other similar studies^{42,43}.

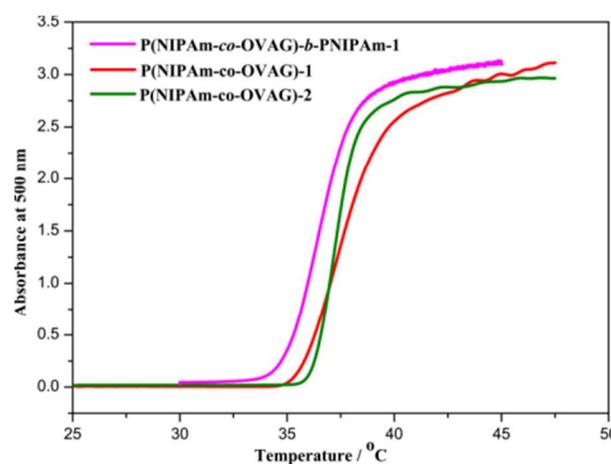


Figure 3. Turbidity curves recorded for 1.0 mg/mL solution of glycopolymers in water on UV-vis spectroscopy at 500 nm and the heating rate was 0.5 °C/min.

Self-Assembled Micellar Behavior.

Critical micelle concentration of glycopolymers. The critical micelle concentration (CMC) of glycopolymers is derived from steady-state fluorescent probe studies. Pyrene has been widely used as a fluorescence probe in the polymeric micellar solution³⁸. As long as polymeric micelles are formed, pyrene was preferentially distributed in the hydrophobic micelle cores, meanwhile, the surrounding for pyrene changed from polar to non-polar³⁷. In the fluorescence emission spectra of pyrene, the relative intensities of the first and third peak (I_1 at 374 nm and I_3 at 384 nm) are useful in detecting the critical micelle concentration (CMC) of polymeric micelles. The relative intensity ratio (I_3/I_1) is a function of copolymer concentration which can be used to determine the CMC of glycopolymers.

From the plot of the relative intensity ratio (I_3/I_1) versus glycopolymer concentration (Fig. 4), an abrupt increase in the relative intensity ratio (I_3/I_1) was observed with increasing glycopolymer concentrations, indicating formation of micelles and the transfer of pyrene into the hydrophobic core of the micelles. The

CMC values for P(NIPAm-co-OVAG)-b-PNIPAm-1 and P(NIPAm-co-OVAG)-1 were as low as 0.31 and 0.19 g/L, respectively (Fig. 4), providing evidence for an apparent stability of micelles, which is consistent with the literature³⁹.

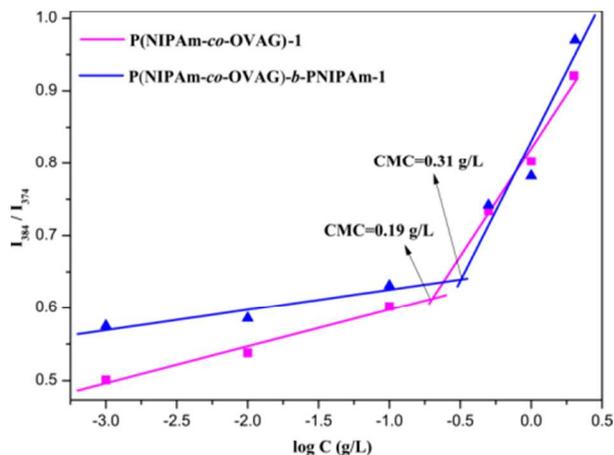


Figure 4. The critical micellar concentration (CMC) graphs of P(NIPAm-co-OVAG)-b-PNIPAm-1 (blue graph) and P(NIPAm-co-OVAG)-1 (purple graph). A series of concentrations are 2, 1, 0.5, 0.1, 0.01 and 0.001 mg/mL. The CMC values of P(NIPAm-co-OVAG)-b-PNIPAm-1 and P(NIPAm-co-OVAG)-1 in aqueous media are about 0.19 g/L and 0.31 g/L, respectively.

Thermoresponsive behavior of block glycopolymer micelles. Thermoresponsive double hydrophilic block copolymer could self-assemble into micelles and have been well characterized by Rangelov et al.^{5,40}. In order to investigate the thermoresponsive phenomenology of diblock glycopolymer micellization, temperature dependences of both average hydrodynamic radius (R_h) and average gyration radius (R_g) were investigated.

Fig. 5(a) shows the temperature dependence of R_h and R_g for aqueous solution of P(NIPAm-co-OVAG)-b-PNIPAm-1. On heating, both R_h and R_g hardly changed between 25 °C and 31 °C. Obviously, for the glycopolymer, when the temperature is lower than the LCST, the glycopolymer is molecularly soluble, R_h is typically less than 35 nm and R_g is less than 65 nm. However, at elevated temperature to 33 °C ~ 35 °C, R_h and R_g increased dramatically. This suggests the occurrence of the coil-to-globule transition of the glycopolymer and aggregation of globose nanoparticles is due to the increasing specific surface area. At even higher temperatures, R_h and R_g maintain almost constant.

In order to understand in detail the process of the chain conformation changes, the temperature dependence of R_g/R_h were also studied (Fig. 5(b)). R_g/R_h is nearly a constant (~1.6) in the heating process when temperature is lower than the LCST. Water is a good solvent for the block polymer P(NIPAm-co-OVAG)-b-PNIPAm-1, and glycopolymer is linear flexible chain coils in water. In the transition range of the heating process, R_g/R_h decreases sharply from 1.6 to 0.7 and remains ~0.7 when the temperature is higher than 35 °C. This indicates that PNIPAm block and PNIPAm chain in the block polymer are induced from the extended coil conformation to the collapsed compact globule.

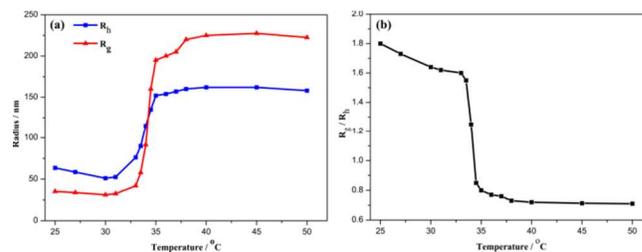


Figure 5. (a) The temperature dependences of average hydrodynamic radius (R_h , blue square) and average gyration radius (R_g , red circle) of P(NIPAm-co-OVAG)-b-PNIPAm-1 micelles in water at a concentration of 8×10^{-4} g/mL. (b) The temperature dependence of R_g/R_h for P(NIPAm-co-OVAG)-b-PNIPAm-1 micelles in water.

Transmission Electron Microscopy (TEM). Micelle morphology of the block glycopolymers was investigated by TEM (see Fig. 6), which shows the images observed from a sample-loaded TEM grid. The number-average diameter (D_n) of P(NIPAm-co-OVAG)-b-PNIPAm-1 is 12 ± 3 nm, which is measured from Fig. 6(c,d). It is evident that the block glycopolymers were dispersed as self-assembled micelles with uniform sizes and a regularly spherical shape. Compared to the size by TEM, the average hydrodynamic diameter (D_h) of the micelles by DLS is larger with the size at around 20 ± 7 nm (see Fig. 8). For P(NIPAm-co-OVAG)-1, the D_n is 25 ± 5 nm (Fig. 6(a,b)) and the D_h is 32 ± 10 nm (Fig. 8).

The smaller size observed from the TEM study may be due to the collapse of the free segment of the hydrophilic chain of the polymer as well as the dehydration of the polymer chain³⁸.

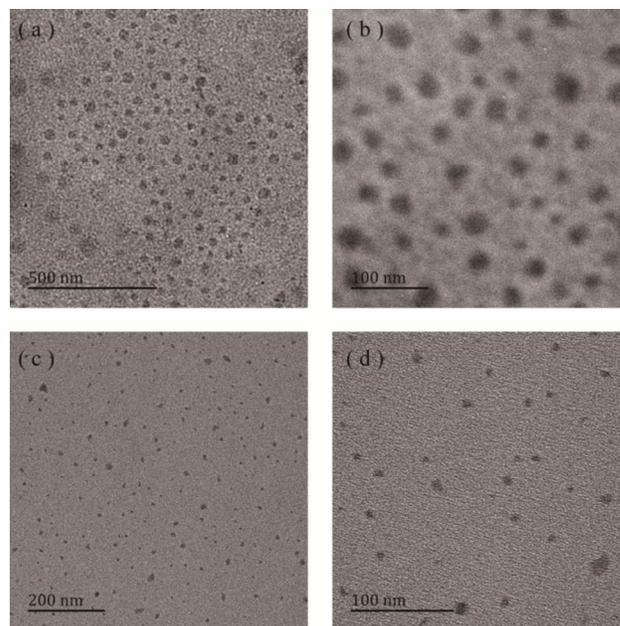


Figure 6. TEM images of micelles self-assembled from P(NIPAm-co-OVAG)-2 (a, b) and P(NIPAm-co-OVAG)-b-PNIPAm-1 (c, d) at low (a, c) and high (b, d) magnification.

Lectin-Binding Assay.

Structure effect on lectin-binding. It is well known that glycopolymers with a large quantity of glucose units are very attractive to serve as carriers for drug delivery or as systems for purification because of their ability to protein recognition^[41]. In order to prove that the bio-functionality of the glucose moieties is still active and not lost during the RAFT polymerization process and to understand how the protein-binding reaction would proceed in the biological system, the lectin-binding ability of the glycopolymer micelles was tested. Lectin Con A was used to characterize the specific binding with glucose moieties. The turbidity assay measures the changes in absorbance at a wavelength of 600 nm using UV/vis spectroscopy.

In Fig. 7, the turbidity increases with conjugation time to reach plateaus after 4 min and the maximum absorbance value increases with the portion of OVAG in P(NIPAm-co-OVAG). It proved that the more sugar moieties in glycopolymer and the greater recognizability to Con A. Multivalent arrays displayed by the glycopolymer allow high specific interactions between the glucose ligands on the polymers. Meanwhile, the maximum absorbance value of P(NIPAm-co-OVAG)-*b*-PNIPAm-1 is similar to P(NIPAm-co-OVAG)-2 because of analogical molar ratio of monomers. However, Fig. 7 also shows that glycopolymers contain the same portion of OVAG and have the different rate of increase of absorbance, P(NIPAm-co-OVAG)-*b*-PNIPAm-1 is a little faster than P(NIPAm-co-OVAG)-2. The reason of this phenomenon can be explained that the micellars of block glycopolymer may have more regular structure because of the existence of double block.

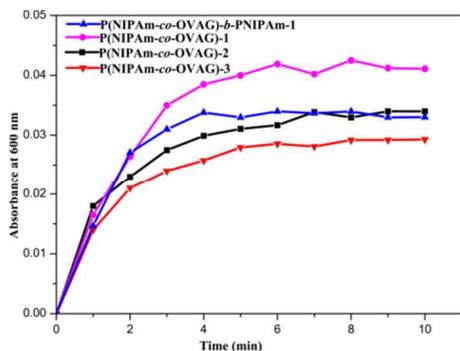


Figure 7. Turbidimetry assay experiments of P(NIPAm-co-OVAG)-1 (purple circle), P(NIPAm-co-OVAG)-2 (black square), P(NIPAm-co-OVAG)-3 (red down triangle), P(NIPAm-co-OVAG)-*b*-PNIPAm-1 (blue up triangle) micelles with Con A at 30 °C.

Temperature influence on lectin-binding. In addition to the turbidity test, dynamic light scattering measures were also conducted to study the size growth (Fig. 8). The particle sizes increased obviously when Con A was attached to the particles at different temperature. Since Con A has four binding sides, inter-micelles cross-linking occurred and led to increase of the average hydrodynamic diameters. Fig. 8 shows that the average hydrodynamic diameters (D_h) of P(NIPAm-co-OVAG)-2 increases by about 110% after the adding of Con A at 30 °C and 53% at 37 °C, and D_h of P(NIPAm-co-OVAG)-*b*-PNIPAm-1 increases by about 95% after the adding of Con A at 30 °C and 50% at 37 °C. When the temperature is lower than the LCST of glycopolymer, the

recognition is better than the temperature at 37 °C (above the LCST). Because random coil structure (under the LCST) of glycopolymer micelle is more flexible to bind lectin and more sugar moieties are exposed to bind lectin¹⁸. Besides, P(NIPAm-co-OVAG)-2 is similar to P(NIPAm-co-OVAG)-*b*-PNIPAm-1 in change of D_h at different temperature because of their similar molar ratio of monomers.

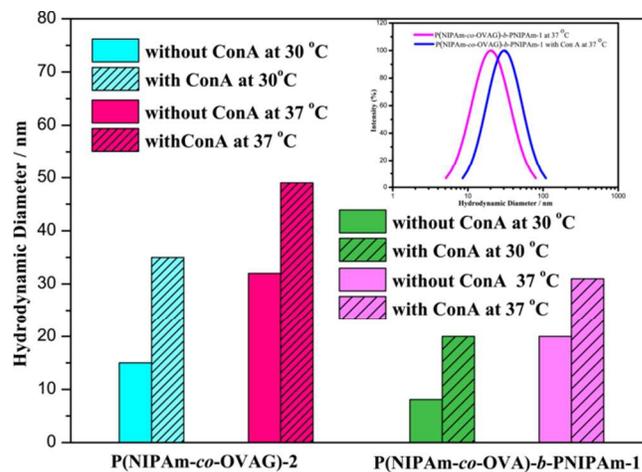


Figure 8. DLS experiments of the glycopolymers with Con A at different temperature.

Confocal microscopy. To be able to visualize the conjugation of Con A in the glucose functionalized polymeric micelle and confirm a successful conjugation, fluorescein isothiocyanate conjugated Con A (FITC-Con A) was employed. Figure 9 shows the interaction of glycopolymer with FITC-Con A. Fig. 9 (a) depicts P(NIPAm-co-OVAG)-1, in confocal mode, with green fluorescence indicative of FITC-Con A. Fig. 9 (b) depicts P(NIPAm-co-OVAG)-*b*-PNIPAm-1, in confocal mode, with green fluorescence indicative of FITC-Con A.

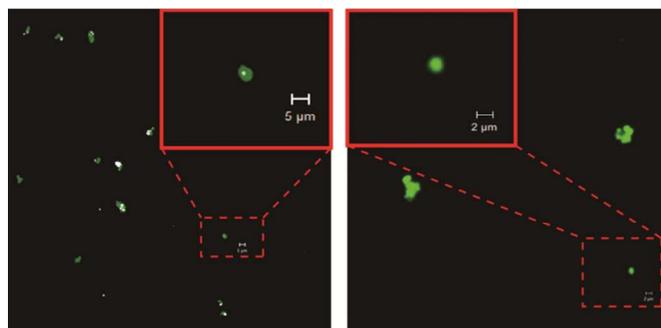


Figure 9. Confocal microscopy images of interaction of glycopolymer P(NIPAm-co-OVAG)-1 (a) and P(NIPAm-co-OVAG)-*b*-PNIPAm-1 (b) with FITC-Con A.

Cell Biocompatibility.

The cell biocompatibility of the synthesized glycopolymers was explored using the MTT assay, the results are shown in Fig. 10. In Fig. 10 (a), it is clear that the glycopolymer micelle solutions in different concentration have generally very good biocompatibility. The cell viability of glycopolymer-treated wells is the same or greater when compared to the untreated control group. It is

consistent with the work of Dakrong Pissuwan group that polymers with trithiocarbonate end group have a low cytotoxicity³⁵. To some degree, the glucose groups of glycopolymers can support the growth

of cells. The data in Fig. 10 (b) show the results of an analogous experiment but with Con A-loaded micelle solution. The cell viabilities after 24 h are reduced from the untreated control.

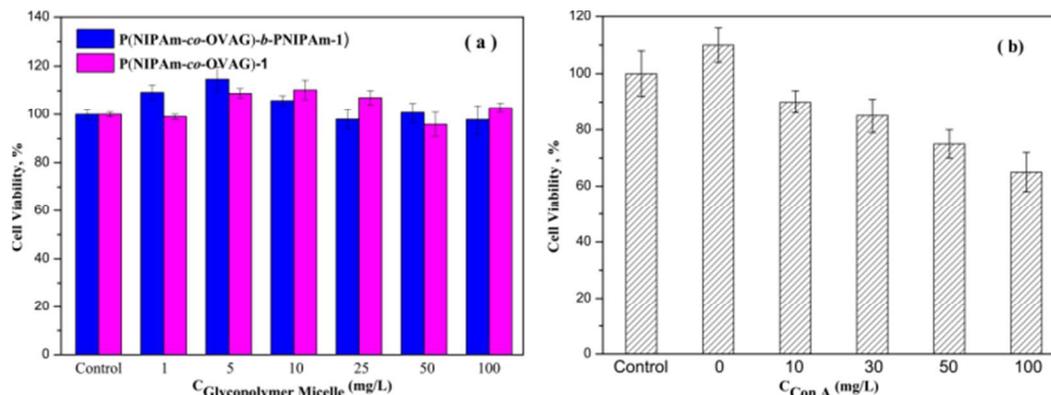


Figure 10. (a) Viability of PIECs after incubation with P(NIPAm-co-OVAG)-1 (purple bar) and P(NIPAm-co-OVAG)-b-PNIPAm-1 (blue bar) at various concentrations; (b) Viability of SMMC-7721 cells after incubation with P(NIPAm-co-OVAG)-b-PNIPAm-1 binding different concentrations Con A. The viability was measured by a MTT assay.

Conclusions

A series of multifunctional double hydrophilic block glycopolymers in the form of self-assembling micelles were successfully synthesized via controlled RAFT polymerization of a sugar-functional vinyl ester monomer and a thermoresponsive monomer. The conditions for preparing thermoresponsive P(NIPAm-co-OVAG)-b-PNIPAm diblock glycopolymers were effective. The LCST of the glycopolymer solution has been successfully controlled by changing the molar ratio of monomers in the reaction condition. In addition, the range of temperature transition can be decreased by adding the block of PNIPAm. The micelle structures formed by block glycopolymer were small and loose at low temperature, but large and compact spherical aggregations at temperature above the LCST. The protein recognition of the glucose moieties on the micelles were screened by using Con A. The ability of protein recognition not only related to the amount of sugar but also the structures of glycopolymers. The well-defined block glycopolymers had good cell biocompatibility and minimum cytotoxicity. When the glycopolymers were loaded with Con A, they were able to promote SMMC-7721 cell death.

A wide range of block glycopolymers can be prepared by the developed method described in this work. Given their temperature sensitive and protein recognition properties, they have potential applications in targeting cancer cells scaffolds, as drug release carriers and for clinical diagnosis.

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Notes and references

^a College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai, 201620, P.R. China.

^b Department of Life Sciences, Faculty of Science and Technology, University of Westminster, 115 New Cavendish Street, London W1W 6UW, UK.

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