Polymer Chemistry

PAPER

A thermo-/pH-Responsive Hydrogel (PNIPAM-PDMA-PAA) with Diverse Nanostructures and Gel behaviors as a General Drug Carrier for Drug Release

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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The aim of this research was the development of thermo- or pH-responsive hydrogels based on H-bonds for the sustained release of the small-molecule model drug Methylene blue (MB). The thermo- or pH-sensitive triblock copolymer of PNIPAM-based or PAA-based was synthesized by sequential RAFT polymerization and hydrolysis. By tuning the temperature or pH, the copolymer was able to form diverse nanostructures, including core-shell micelles, electronegative or uncharged core-shell structures and 3D networks with the hydrophobic forces of PNIPAM or H-bonds between PAA and PNIPAM. Further, the reversible sol-gel transition of the copolymers was modulated by temperature (~37°C), pH (~5) and concentration (~7 wt%). The drug release potential was evaluated via in vitro injection of copolymer hydrogels in a simulated human body device. The copolymer hydrogels exhibited sustained drug release behavior and low instantaneous drug concentration. The MTT studies revealed that there is no noticeable cytotoxicity of NDB or NDA hydrogel against cell. Thus, the tunable drug release efficiency suggests the possibility of PNIPAM- and PAA-based environment responsive hydrogels being applied as drug carrier systems.

Introduction

Environment-responsive hydrogels are intelligent materials whose chemical or physical properties, like physical state, solubility and hydrophilic-hydrophobic balance, change distinctively under the stimulation of variable surrounding factors such as pH, temperature and salt concentration among others.¹⁻³ Due to the promising application prospects in tissue engineering, drug delivery and other biomedical fields, a lot of theoretical research and experimental applications have been reported for temperature or pH responsive hydrogels.⁴⁻⁸ Furthermore, compared with chemical environment-responsive hydrogels, which are covalently bonded into three-dimensional polymers networks in an irreversible way, physical

environment-responsive hydrogels are gaining interest due to the non-permanent physical forces (such as electrostatic interaction, hydrogen bonding, chain winding) at play in their formation.⁹⁻¹¹ Such hydrogels can be reversibly transformed from sol to gel by repeatedly changing the environment factor.¹² For instance, poly(N-isopropylacrylamide) (PNIPAM), a classic thermosensitive polymers with an obvious coil-globule transition at 32 °C (called the lower critical temperature, LCST), being hydrophilic below LCST or hydrophobic above it, has been used to fabricate thermo-responsive hydrogels.¹³ Ma et al. prepared P(NIPAM-co-PEI) hydrogels which have strong thermo-response as drug delivery models at human physiologic temperature (37°C).¹⁴ Indeed, the LCST can be easily adjusted by combining other hydrophilic (e.g. poly (acrylic acid) (PAA)) or hydrophobic polymers (e.g. poly (acrylate) (PtBA)) to be farther away from or closer to body temperature, further broadening the range of biological applications.¹⁵⁻¹⁷ In addition, due to the diverse human pH environment, pH-responsive polymers have also been drawing increased attention in the biomaterials field. pH-Responsive materials can respond to solution pH by undergoing structure and property changes such as surface, chain conformation or solubility.¹⁸ Among pH-responsive polymers, introducing acrylic acid with pKa of 4.28 into the PNIPAM network to make nanogels is an interesting option since PAA can modify the polarity and surface charge of the copolymer when the ionic

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* Electronic Supplementary Information (ESI) available: Supplementary Table S1-S3, Fig. S1-S7: ¹H NMR, GPC, PDI, DLS, Cloud point, Viscosity, SEM, Device

strength or pH is altered, being the pH responsiveness modulated by the PAA content.¹⁹

In this study, we report two novel stimuli-sensitive ABC triblock copolymer hydrogel as a general drug carrier for drug

Fig. 1 (A) The self-assembling process of PNIPAM-*b*-PDMA-*b*-PtBA and PNIPAM-*b*-PDMA-*b*-PAA triblock copolymer as temperature or pH change. The PDMA chains, PtBA, PNIPAM and PAA cores are marked bridge, B-core, N-core and A-core, respectively. (B) The drug release process under the induction of temperature or pH; the blue cylinder represents MB model drug.

Therefore, multi-functional polymer materials with PNIPAM and PAA (PtBA) can potentially be applied in the drug controlled-release field. Hoffman et al. found obvious H-bond between PNIPAM and protonated PAA when the pH was lower than 4.28 causing the change of the LCST of the polymers.²⁰ Li et al. prepared a P(AA-co-tBA)-*b*-PNIPAM copolymer via sequential atom transfer radical polymerization (ATRP) that forms thermo- and pH-responsive core-shell micelles in aqueous solution.²¹ These hydrogels have been extensively studied and used for drug delivery due to the fact that their properties are closely related to biological tissues and the delivery of small molecules can be easily controlled by altering the carrier's gel-sol state.²²⁻²⁴ Gao et al. studied microgels aggregated when P(NIPAM-co-AA) microgel and P(NIPAM-co-DMAPMA) microgel were mixed in different pH, which can be exploited to load (aggregation) and release (disaggregation) a model drug.²⁵ In our research, PDMA was selected as link chains with strong hydrophilicity so that the triblock copolymers exhibit improved micro and macro properties. Although the rapid thermo- and pH-response of triblock copolymers is advantageous for drug load and release, their high concentration of gelation avoids rapid drug release, which results in low gelation efficiency. Li et al. synthesized a triblock copolymer (PPO₄₃-PMPC₁₆₀-PNIPAM₈₁) by ATRP that presented low gelation efficiency where almost 20% polymer was required to be gel.²⁶ Elsewhere, high concentration of polymer solution was used in physical conditions, leading to undesirable side effects.²⁷ For this reason, the appropriate and low critical aggregation concentration (CAC) of hydrogels from triblock copolymers has attracted increasing attention.

release, including a thermo-responsive micelle-based PNIPAM-*b*-PDMA-*b*-PtBA (NDB) and a thermo- and pH-dual sensitive H-bond-based PNIPAM-*b*-PDMA-*b*-PAA (NDA), obtained via sequential reversible addition-fragmentation chain transfer (RAFT) polymerization. The micro and macro structures and drug release process of NDB and hydrolytic NDA triblock copolymer under different temperature and pH value in aqueous solution were firstly addressed as shown in Fig. 1(A). NDB triblock copolymer existed in aqueous solution as small core-shell micelles at room temperature due to the hydrophobic PtBA and hydrophilic PNIPAM. When the temperature is higher than the LCST of PNIPAM, NDB formed larger core-shell micelles at low concentrations or core-linking-core 3D network hydrogel at high concentrations due to the collapse of the hydrophilic PNIPAM chain. However, a more complex process was observed in the NDA system. Under alkaline conditions and low temperature, NDA could be dissolved in aqueous solution with unconstrained molecular chains. Changing temperature and pH at different sequences contributed to a series of complicated structures due to the thermo-sensitivity of PNIPAM, pH-sensitivity of PAA and the H-bond between the protonated PAA and PNIPAM. So we attempt to manipulate the reversible transition of gel-sol for NDB and NDA to the controlled release of methylene blue (MB) by adjusting temperature and pH (Fig. 1(B)). These triblock copolymer solutions are able to switch from solation to cross-linked gelation in lower CAC (7 wt%) after injection into the simulated human body device, driven by the thermo-responsive PNIPAM chains and the pH triggered hydrogen bonds between PNIPAM and PAA chains. We expect this

report can provide novel efficient controlled release hydrogels for tissue engineering and drug delivery methodology.

Experimental section

Materials

Unless otherwise specified, all reagents were purchased from China-Aladdin. N-isopropylacrylamide (NIPAM, 97%, Aladdin China) was recrystallized twice from mixtures of toluene and hexane (1/7 by volume). N, N-dimethylacrylamide (DMA, 99%, Aladdin China) was passed through alkaline alumina to remove inhibitors prior and store at -20°C . Tert-Butyl acrylate (tBA, 99%, Aladdin China) was distilled under reduced pressure to remove inhibitors prior at 80°C . 2, 2'-azobis (isobutyronitrile) (AIBN) was recrystallized from methanol. The chain transfer agents (CTA) 2-(2-cyanopropyl) dithiobenzoate (CPDB) was synthesized according to the reported procedures.²⁹ Trifluoroacetic acid (TFA, 99%, Aldrich China). 1, 4-dioxane, dichloromethane, diethyl ether, alkaline alumina were purified by standard methods before use.

Synthesis of PNIPAM-b-PDMA-b-PtBA by Sequential RAFT Polymerization

Scheme 1 shows the synthesis steps of PNIPAM-b-PDMA-b-PtBA (NDB) and PNIPAM-b-PDMA-b-PAA (NDA) by sequential RAFT polymerization and hydrolysis reaction. The polymerization degree (Dp) of each product was calculated by ^1H NMR CDCl_3 or GPC.

Synthesis of PNIPAM macro-RAFT agent: NIPAM (20 g, 177 mmol), CPDB (0.5433 g, 2.46 mmol) and 1, 4-dioxane were mixed into a 100 ml Schlenk flask. After degassing by three freeze-pump-thaw cycles, the AIBN (0.0908 g, 0.55 mmol) was injected into the reaction solution by a micro-injector. Then, the mixture was stirred for 24 h in 70°C oil bath. The flask was immersed in an ice bath to terminate the reaction. The crude product was precipitated twice in ice ether, then was dialyzed against distilled water by dialysis tubes with a molecular weight cut-off of 5000 Da for 48 h and freeze-dried. ^1H NMR (500 MHz, CDCl_3 , δ , ppm): 7.41 (para- C_6H_5 , 1H), 7.57-7.59 (meta- C_6H_5 , 1H), 7.96 (ortho- C_6H_5 , 1H), from -CPDB (-CTA); 1.14 ($-(\text{CH}_3)_2$, 6H), 4.01 ($-\text{CH}-$, 1H), 1.65-2.16 ($-\text{CH}-\text{CH}_2-$, 3H), from -PNIPAM. $M_{\text{nNMR}}=11992 \text{ g mol}^{-1}$, $\text{Dp}_{(\text{PNIPAM})}=106$. The Dp of polymers were also characterized by GPC, which were introduced in the following sections. Other RAFT

polymerizations were similar and are described in the following sections.

Synthesis of PNIPAM-b-PDMA macro-RAFT agent: CTA-PNIPAM (9.1001 g, 0.75 mmol); DMA (38.7005 g, 390.40 mmol), AIBN (0.0341 g, 0.21 mmol) and solvent (1, 4-dioxane, 80 ml). After three freeze-pump-thaw cycles, the mixture was stirred for 12 h in 70°C . The crude product was purified twice in 3-fold volume of n-hexane, then was dialyzed against distilled water for 48 h and freeze-dried. ^1H NMR (500 MHz, CDCl_3 , δ , ppm): Besides the H signals of CPDB and PNIPAM, 2.9-3.2 ($-\text{N}(\text{CH}_3)_2$, 6H); 1.65-2.16 ($-\text{CH}-\text{CH}_2-$, 3H), from PDMA. $M_{\text{nNMR}}=72858 \text{ g mol}^{-1}$, $\text{Dp}_{(\text{PDMA})}=614$.

Synthesis of PNIPAM-b-PDMA-b-PtBA Triblock Copolymer: CTA-PNIPAM-PDMA (16.1002 g, 0.22 mmol), tBA ((2.3101 g, 18.02 mmol), AIBN (0.0093 g, 0.05 mmol), and solvent (1, 4-dioxane, 32 ml). The mixture was stirred for 15h in 75°C , then the raw product was purified and characterized. ^1H NMR (500 MHz, CDCl_3 , δ , ppm): 1.42 ($-\text{C}(\text{CH}_3)_3$, 9H), part of 1.65-2.16 ($-\text{CH}-\text{CH}_2-$, 3H), from PtBA. $M_{\text{nNMR}}=82855 \text{ g mol}^{-1}$, $\text{Dp}_{(\text{PtBA})}=78$.

Acidic Hydrolysis of PNIPAM-b-PDMA-b-PtBA (NDB) Triblock Copolymer.

PNIPAM-b-PDMA-b-PtBA (8.5005 g, 0.10 mmol) was dissolved in CH_2Cl_2 (12.5 ml) at room temperature. Then, 7.5 ml of trifluoroacetic acid (CF_3COOH) was slowly added in reaction solution with ice bath and stirred in room temperature for 24 h. After the reaction, the volatiles were removed by distillation under reduced pressure, then the product was dissolved in water and dialysis for 48 h, freeze-dried.³⁰ ^1H NMR (500 MHz, $\text{DMSO}-d_6$, δ , ppm): Besides the signals of PNIPAM-PDMA-PtBA, 12.28 ($-\text{COOH}$, 1H) from PAA. $M_{\text{nNMR}}=78262 \text{ g mol}^{-1}$, $\text{Dp}_{(\text{PAA})}=75$.

Preparation for sustained-release system

At room temperature, NDB (0.21 g, 7 wt%) and MB (3mg, 0.1 wt%) were completely dissolved in PBS buffer solution (3ml) and configured as sustained-release system under $\text{pH}=7.4$, called NDB/MB. Similarly, the sustained-release systems of NDA were prepared in the same way but under different pH (e.g. 2, 4.6, 7.4), called NDA/MB. The ratio of NDB/MB or NDA/MB in the release system is explained in Fig. S8.

Drug Release in vitro

Scheme. 1 Schematic illustration of the synthesis of PNIPAM-b-PDMA-b-PAA by sequential RAFT polymerizations and subsequent hydrolysis.

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To simulate the human environment, all experiments were performed in PBS buffer solution. In the experiments, the sustained-release solution (3ml) was added into the injection hole of the experimental setup, and 3ml PBS buffer solution (the same pH as release solutions) were added to the right side of setup in the semi-permeable membrane with a molecular weight cutoff of 14,000 (Fig. S7). The temperature was set to 25 °C or 37 °C and with a stirring rate of low speed such as 80 rpm. Then, 1ml solutions were taken from the sampling hole of setup as UV test samples, simultaneously 1ml fresh PBS buffer solution also were added as supplements to guarantee constant volumes of sampling area. The released drug concentrations were measured by UV at a wavelength of 664nm, then combined with the standard curve of MB³¹ (Fig. S9).

Cytotoxicity studies

The cytotoxicity of NDB or NDA hydrogel against Human Embryonic Lung Fibroblast (HELFL) cell line, which was obtained from Cell Bank, Chinese Academy of Sciences (China), was assessed using MTT assay. In brief, the HELFL cells, which were washed twice with D-Hanks solution after remove the culture medium, were digested with 0.25% trypsin for 2 mins. Then, it were added the fresh cell culture medium containing 10% fetal bovine serum, were seeded into a concentration of 5×10^4 /ml in 96 well plates and incubated for 24h under standard conditions (5% CO₂, 37 °C). Thereafter, the cells were treated with NDB or NDA hydrogel at different concentration or pH for 48h. Next, after washing twice with D-Hanks solution, the cells were treated with 0.5mg/ml of MTT solution (0.1ml/hole) at 37 °C for 4h. The control cells were left untreated and the positive control cells were treated with 0.1% Triton-X 100. Then, the supernatant were aspirated gently, 0.1ml of DMSO was added to each well and the plate were shaken for 10 mins. The OD value of resulting solution was measured at 490 nm using a microplate reader.

Characterization

The Fourier Transform Infrared (FTIR) spectra were obtained on an AVATAR 370 FT-IR spectrometer (Nicolet, USA). The Dp of products were calculated by ¹H NMR signals recorded on a Bruker 500 MHz spectrometer using DMSO-d₆ or CDCl₃ as solvent or by gel permeation chromatography (GPC) was performed on a Waters Alliance e2695 GPC system. The turbidity behaviors of the triblock copolymer were measured at a wavelength of 500 nm by ultraviolet-visible spectrophotometer (UV-Vis, Agilent, USA).³² DLS measurements were performed on a Zetasizer Nano ZSP (Malvern Instruments Ltd, Malvern, U.K) with a He-Ne laser (633 nm, 4 mW) at a scattering angle of 173°. ³³ The hydrodynamic diameter (D_h) and the average particle size (Z-Ave) were calculated by the Stokes-Einstein equation and the cumulative distance method. The viscosity measurements were carried out using a LVDV-11 DV-II+Pro viscometer (Brookfield, USA) with 0.5 RPM and 64# spindle. ³⁰ Rheological experiments were conducted on a rotary rheometer DNR-3

Fig. 2 (a) FT-IR spectra and (b) ¹H NMR spectra in DMSO-d₆: (A) PNIPAM, (B) PNIPAM-*b*-PDMA, (C) PNIPAM-*b*-PDMA-*b*-PtBA, (D) PNIPAM-*b*-PDMA-*b*-PAA.

(TA, USA) in the temperature range of 25-70 °C. Micellar aggregates were examined using a JEOL JEM-1210 transmission microscope (TEM, Electron, Japan) at 200Kv accelerating voltage. Samples for TEM were prepared by dropping a polymer solution of different temperature and pH onto a 400-mesh copper grids pre-coated with a carbon film. During the preparation, all used tools must be preheated to the corresponding temperature. The sol and gel morphology were analysed by JSM-6010 LV scanning electron microscopy (SEM). The NDB and NDA samples were prepared by maintaining the polymer solution of 2 wt% and 7 wt% at different temperatures or pH for 30 min, then the samples were immediately frozen in liquid nitrogen and lyophilized. Finally, the dried sample was sprayed to be coated with a gold layer on the surface to form a SEM film.

Results and discussion

Synthesis and characterization of multi-responsive NDB and NDA copolymer

Compared with the conventional free radical polymerization, RAFT polymerization has the advantages of wide applicability, narrow molecular weight distribution and strong controllability in synthesizing block copolymers.^{34, 35} In this paper, PNIPAM, PDMA and PtBA were stepwise polymerized by 2-(2-cyanopropyl) dithiobenzoate (CPDB) as CTA to obtain the thermo-sensitive triblock copolymer PNIPAM-*b*-PDMA-*b*-PtBA (NDB) at 70-75 °C. After NDB hydrolysis, the thermo- and pH-dual sensitive triblock copolymer PNIPAM-*b*-PDMA-*b*-PAA (NDA) was obtained. The molecular structure, molecular weight and Dp were determined by FT-IR, ¹H NMR and GPC after purification for each products.

FT-IR spectrum of NDB, NDA and precursors are shown in Fig. 2(a). On the profile of PNIPAM, 1650 cm⁻¹ and 1550 cm⁻¹ are the stretching vibration of -C=O and the bending vibration peak of -NH from -CONH, respectively.³⁶ All spectra exhibit absorption bands at 3000-2800 cm⁻¹ from the stretching vibration of -CH₃ and -CH₂.³⁷ After embedding monomer DMA, the absorption peak at 1650 cm⁻¹ are significantly increased with the increasing molar ratio of -C=O from DMA. And the absorption peak around 1550 cm⁻¹ are attenuated with the decreasing molar ratio of -NH from NIPAM.³⁸ After adding with tBA, the stretching vibration of -COOR appeared in 1730 cm⁻¹.³⁹ After hydrolysis, the weaker peak indicates the formation of acrylic acid. More exquisite structures and Dp of all purified products are shown on ¹H NMR spectra as Fig. 2(b). The chemical shift of 7.41 ppm (meta-C₆H₅, 2H), 7.57-7.59 ppm (para-C₆H₅, 1H), 7.96 ppm (ortho-C₆H₅, 2H) are from the benzene ring of the chain transfer agent (CPDB); the chemical shift of 4.01 ppm (-CH-, 1H) is from the isopropyl hydrogen of PNIPAM.⁴⁰ Calculated the ratio of the integral peak area between the methine protons of PNIPAM (marked b) and the para-C₆H₅ protons of CPDB (marked 1), the Dp of PNIPAM was estimated to be about 104. The Dp (614) of PDMA is calculated by the integral area ratio of DMA (2.9-3.2 ppm (-N(CH₃)₂, 6H))/NIPAM (4.01 ppm (-CH-, 1H)) = 5.9. Similarly, the Dp of other polymers were also roughly calculated. The data of Mn, PDI were also detected by GPC and were summarized in Fig. S1. In general, all results above indicate that NDB, NDA triblock copolymers have been successfully synthesized with good

molecular weight distribution.

Fig. 4 TEM images of triblock copolymer nanoparticles (0.2 wt%): (a) NDB triblock copolymer at different temperature. (b) NDA triblock copolymer at different temperature or pH.

The Environmental Responsiveness of PNIPAM-*b*-PDMA-*b*-PtBA (NDB) and hydrolyzed PNIPAM-*b*-PDMA-*b*-PAA (NDA)

Micro self-assembly behaviors with thermo- or pH-Response of the Triblock Copolymer. As shown in Fig. 1, there is a complex self-assembly process for NDB and NDA triblock copolymer because of the monomers' unique property under different temperature or pH. Firstly, NDB evenly forms a spherical

Fig. 5 The sol-gel behaviors of NDB triblock copolymer: (a) Viscosity curves depend on temperature at different concentrations. (b) Temperature-dependent dynamic shear moduli (G' and G'') for NDB in aqueous solutions at $\gamma = 5\%$ and heating rate $1\text{ }^{\circ}\text{C}/\text{min}$. The 2 wt% NDB solution was measured at $\omega = 10\text{ rad/s}$ and 7 wt% NDB solution was measured at $\omega = 10\text{ rad/s}$. (c) SEM images of gelation behavior at different temperatures. The test concentration is 7 wt%.

micelle structure with PtBA-core, PDMA-PNIPAM-shell in aqueous solution at 25 °C (as DLS Fig. 3(a), TEM Fig. 4(a₁)). When temperature further increased and crossed LCST of PNIPAM, NDB gradually aggregates into the heterogeneous micro-particle distribution (45 °C) due to the role of hydrophobic PNIPAM, resulting in a significant variation for PDI from 0.196 to 0.329 (Table. S2(a)), and gradually forms constant PDMA-shell, PNIPAM-PtBA-core nanostructure because of the complete collapse of PNIPAM chains (60 °C)

Fig. 3 (a) Intensity distribution of hydrodynamic diameters of NDB triblock copolymer at 0.5 wt% as a function of temperature. (b) Temperature or pH response characteristics of NDA triblock copolymer at 0.5 wt%: b₁) The Z-average diameter (line A) and light scattering intensity (line B) at different pH, T=25 °C. (b₂, b₃) Intensity distribution of hydrodynamic diameters of NDA triblock copolymer at different temperature, pH=2 or pH=8.

(TEM Fig. 4(a₂)). In addition, TEM also shows that the structure changes from loose and small spherical micelles to tight and large aggregations with temperature increase (Fig. 4(a)). Furthermore, combined with Z-Average diameter, light scattering intensity and turbidity, the LCST of NDB triblock polymer is close to 40 °C (Fig. S1 and Fig. S2 (a)).

After the thermo-responsive NDB was hydrolyzed, a dual-responsive triblock copolymer is generated, called NDA, namely thermo- (PNIPAM block) and pH- response (PAA block).^{41, 42} When pH is lower than the pKa of PAA, the -NH- group of PNIPAM can combine with the -COOH group of PAA to form H-bond, resulting in plenty of micellar aggregations.⁴² Therefore, the larger Z-average diameter was monitored by DLS and the light scattering intensity also increased synchronously with pH decrease due to the tighter structure at the room temperature. When temperature increase at acidic conditions (e.g. pH=2), the particle size and size distribution does not obviously change, namely a narrow dispersion due to the closer aggregations at high temperature (shown in DLS Fig. 3(b₂) and TEM Fig. 4(b₁, b₂)). However, the redundant hydrophobic PNIPAM segments lead to slightly larger aggregations, so that the PDI gradually reduced and Z-Average diameter and light scattering intensity increased (Table. S2, Fig. S1 (b₁)). When pH increases to alkaline (e.g. pH=8) at low temperature, NDA showed small Z-average diameter (around 30nm) in solution in spite of full hydrophilia for three segments of NDA, probably due to: (1) the relatively high molecular weight of NDA that may result in each molecule being intertwined in a disorderly manner rather than completely dissolved in water in the form of small chains; or (2) according to the IR spectrum, trace amount of tBA remained after hydrolysis, resulting in hydrophobic aggregation (Fig. S1 (b₂)). Under higher temperature (e.g. 40 °C), the single peak, smaller PDI value and larger Z-Average diameter indicate that the free PNIPAM chains gradually collapsed to form new negative core-shell micelles (Fig. 3(b₃), Fig. S1 (b₂), Table. S2). After 50 °C, the downtrend of Z-average diameter and light scattering intensity are attributed to the tighter structure result from further shrinkage of PNIPAM. TEM images (Fig. 4(b₃, b₄)) also show that the structure changes from small size and sparse distribution of micelles at room temperature to large size and dense distribution at 60 °C, and the trends of size

and distribution are similar with DLS. These phenomena also were detected by turbidity (Fig. S2 (b)). Fig. S3 shows the variation of Z-average diameter values of the NDB or NDA micelles at 25 °C and 60 °C during several heating and cooling. However, the self-assembling nanostructure under lower pH or at higher temperature were difficultly monitored by DLS and are explained in sol-gel process.

Gel-Sol Behaviors with thermo- or pH-Response for Triblock Copolymer

Thermo-responsive behavior for NDB. In order to study the gelatinization behavior of the triblock copolymer, DLS was firstly applied to detect the micro self-assembling process of NDB induced by temperature at different concentrations (Fig. S4). At high temperatures (60 °C), there are new peaks on the larger hydrodynamic size region due to the crosslinked structure from the collapsed PNIPAM as the concentration increases. Further, when the concentration increases to a certain degree (e.g. 4 wt%), the crosslinked structure promotes the abrupt increase of viscosity when the temperature is above the LCST of PNIPAM (Fig. 5(a)). Interestingly, Fig. S5(a) reflected that the LCST obvious decreased and reached equilibrium at 8 wt%. Therefore, the 7 wt% is considered as the CAC of NDB. In addition, the significant change of viscosity implied that the phase transition (namely, sol-gel) occurred in a narrower temperature range (Fig. 5(b)). In detail, the loss modulus (G'') is always greater than storage modulus (G') because the micellar distance of NDB is too far to form crosslinked aggregations at low concentration (e.g. 2 wt%), namely sol state. When the concentration increased (e.g. 7 wt%), there is an obvious change in elasticity which viscous dominated ($G' > G''$) at high temperatures (higher than LCST), indicating that the transformation of sol-gel is successfully achieved. Moreover, the transition of sol-gel was reversible by repetitively heating and cooling. Besides, SEM revealed that the blocky structure at 25 °C and fibrous structure at 60 °C correspond to the flow state (non-crosslinking) and the free-standing hydrogel (crosslinking), respectively (Fig. 5(c)). Based on the above phenomenon, the gelation behavior of NDB can be effectively tuned by temperature and concentration.

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Fig. 6 The sol-gel behaviors of NDA triblock copolymer: (a₁) Viscosity curves depend on temperature at different concentrations, pH=3.5. (a₂) Viscosity curves of NDA (7 wt%) depend on pH at different temperature. (b) Temperature-dependent dynamic shear moduli (G' and G'') for NDA (7 wt%) with different pH at $\gamma = 50\%$, $\omega = 1 \text{ rad/s}$ and heating rate 1°C/min . (c) SEM images of gelation behavior at different temperatures, pH or concentrations.

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Thermo- and pH-dual responsive behavior for NDA. In the NDB system, the gelation is due to the hydrophobic interaction between PtBA and PNIPAM. However, as mentioned in the past report⁴¹, H-bond between PAA and PNIPAM will promote more complex self-assembly behavior in NDA. Under acidic conditions (e.g. pH=3.5), the weak H-bond interaction resulted in certain viscosity at low concentration. In particular, the significant increased viscosity due to the densely crosslinked structure under concentration higher than 4 wt%, namely gel state (Fig. 6(a), Fig. S5 (b)). However, it is difficult to determine the gel point of the mutation. Then, SEM showed the plate-like structure (namely, sol) at 2 wt% and alveolate 3D net structure (namely, gel) at 7 wt%, respectively (Fig. S6). However, some cross-linking already existed in sol state with plate-like structure due to unalterable H-bond at constant pH. In the high concentration, as the pH increased, lower viscosity was verified due to the continuously decreased H-bond amount from PNIPAM and gradually ionized PAA (Fig. S5 (C), Fig. 5(a₂)). Interestingly, there are two significant stages in the viscosity curve at pH 5. At the first stage (1#), as temperature elevated, the change of viscosity is attributed to the hydrophobic aggregations from partial free PNIPAM segments (without H-bond force with PAA, PNIPAM₁₀₄-*b*-PDMA₆₁₄-*b*-PAA₇₅). When temperature is further raised, the greater viscosity might be due to the denser crosslinked network. This is because the high temperature might break the H-bond between NIPAM and AA moieties³⁰, resulting in stronger H-bond among PAAs and hydrophobic force of PNIPAMs. Besides, SEM also intuitively revealed NDA underwent blocky, cluster and fibrous structure correspond to sol, soft gel and free-standing gel as temperature increases (Fig. 6(c₁, c₂, c₃)). In addition, rheometer also verified the similar sol-gel behavior with the same reason (Fig. 6(b)). Under alkaline conditions, the loss modulus is always higher than storage modulus ($G' > G''$), namely sol state. Under acidic condition, there is an effective cross from G'' to G' as temperature increases, but with a small gap. As shown in SEM, the blocky and alveolate structure respectively correspond to sol at pH=8 and weak gel at pH=2 (Fig. 6(c₄, c₅)). In general, the gelation behavior of NDA can be effectively tuned by temperature and pH.

In vitro stimuli-responsive drug release of MB

In this study, we developed a special release system to simulate the controlled release of model drug (methylene blue, MB) in vitro (Fig. S7). The drug release behaviors of MB (0.1 wt%) in environmental-responsive NDA or NDB system were estimated by the determination of cumulative release ratio (called CRR) and instantaneous drug concentration (called IDC) corresponding to the time, as shown in Fig. 7. It was found that the maximum concentration (20.08 μg/ml) of MB appeared at about 12h in the pure PBS system but then decreased sharply. When NDB hydrogel was introduced, the maximum concentration was reduced to 16.72 μg/ml and the prolonged release time might be due to the fact that NDB micelles hinder the release path of MB at 25°C. Further, the lower CRR and IDC mean that the 3D network NDB hydrogel

Fig. 7 The release curves of MB in different systems, w(MB)=0.1 wt% : (a) Cumulative release curve of MB (a₁) and instantaneous release curve of MB (a₂) in NDB hydrogel at different temperatures; (b) Cumulative release curve of MB (b₁) and instantaneous release curve of MB (b₂) in NDA hydrogel at different pH or temperature. The gray area corresponds to presumable effective and safe concentration area (ESC Area). When comparing the drug release efficiencies of each system, the initial unstable release process in first-hour was discarded because of a possible free drug release process.

can effectively delay the release of drug at 37°C (Fig. 7(a)). Similarly, the lower CRR and IDC also occur in the NDA hydrogel experiment at 37°C as pH decreases, because the tight structure crosslinked by H-bond between PNIPAM and protonated PAA can hinder the release of MB in the solution (Fig. 7(b)).⁴⁴ When the pH=4.6, the difference of CRR at different temperatures was due to the highly dense inter-micellar structure from residual PNIPAM at high temperatures. However, there are no significant variation of IDC and CRR due to the stable solation state in alkaline and gelation state in strong acidic solution before 37°C. Importantly, IDC curves showed that both of NDB and NDA hydrogel can significantly prolong the release time in presumable effective and safe concentration (ESC). Clearly, the release rate can be easily tuned by temperature or pH in NDB or NDA systems. Combined with the above phenomenon, we hope that such thermo- or pH-responsive hydrogel-based drug release platform can provide a general and efficient candidate for new drug carriers.

Cytotoxicity studies

To access the suitability of NDB or NDA hydrogel as a biomaterial for drug carrier, in this study, the cytotoxicity of drug free-NDB or NDA hydrogel against the cultured HELF cells was tested by MTT assay, which is based on the ability of succinate dehydrogenase in the mitochondria of viable cells to reduce the exogenous MTT to water-insoluble blue-violet crystal formazan. This metabolic activity of cells is an appropriate method to detect the viability of cells since dead cells do not show any mitochondrial dehydrogenase activity.⁴⁵ Fig.8 (a, b) shows HELF cells viability in the presence of NDB or NDA hydrogel at the concentration range of 1-9wt% for 48h. It could be seen that although there were significant different occurred in the cell viability of the cultured HELF cells with respect to control, this difference does not seem to be critical as cell viability was still higher than 80%. In addition, as shown

in Fig.8 (c), cell viability was also higher than 90% at different pH, indicating an excellent biocompatibility in different pH conditions. Based on these results, it can be proved that NDB or NDA hydrogel has no adverse influence against HELF cells, which can be a safe carrier to deliver the drug in biological field.

Fig.8 In vitro cytotoxicity of NDB or NDA hydrogel after incubation with cells for 48h at 37°C. Data was compared to control (*p<0.05, **p<0.01). (a)(b): 37°C, pH=7.4; (c) 37°C, 7wt%.

Conclusions

In this study, we synthesized a thermo-responsive PNIPAM₁₀₄-*b*-PDMA₆₁₄-*b*-PtBA₇₈ (NDB) triblock copolymer by RAFT consecutive polymerization that was then hydrolyzed to thermo- and pH-dual responsive PNIPAM₁₀₄-*b*-PDMA₆₁₄-*b*-PAA₇₅ (NDA) triblock copolymer. On account of the thermo-sensitive PNIPAM and the hydrophobic PtBA, DLS, turbidimetry and TEM revealed that NDB triblock copolymer underwent the change from core (PtBA)-shell (PDMA-PNIPAM) to core (PtBA)-shell (PDMA)-core (PNIPAM) structure as

temperature increased at low concentration. When the concentration increased to 6-7 wt%, the rheological behavior and SEM indicated the transition from flow state with blocky structure to free-standing hydrogel with fibrous structure at 42.5°C (phase transformation temperature). In addition, the hydrolyzed NDB, NDA triblock copolymer, also presented the transition of sol-gel (7 wt%) but the H-bond interaction between PNIPAM and PAA as the change of pH. Besides, the higher temperature can also reinforce the gel strength due to the hydrophobic aggregation of the free PNIPAM. Under the acidic condition, rheological behavior and SEM showed the sol (plate-like structure), soft gel (cluster structure) and free-standing gel (fibrous structure) at 25°C, 50°C and 70°C, respectively. However, once the pH was over 7, the NDA are difficult to reach gelation state before 70°C. Further, the controlled release of methylene blue, as a model drug, was applied in NDB and NDA hydrogels to simulate the drug release system in vitro. The cumulative and instantaneous release curves showed that the release time of MB in effective and presumably safe concentration for NDB or NDA systems can be significantly prolonged by increasing temperature or decreasing pH. The MTT studies revealed that there is no noticeable cytotoxicity of NDB or NDA hydrogel against HELF cell line. In sum, we demonstrate that such thermo- and pH-responsive injectable ABC triblock copolymer hydrogels have excellent prospects in biomedical science, drug delivery or other applied fields.

Conflicts of interest

There are no conflicts to declare

Acknowledgements

The authors are very grateful to Shanghai University for the support and help of this subject, and the University of Minho, Portugal for the SEM images used in this study.

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