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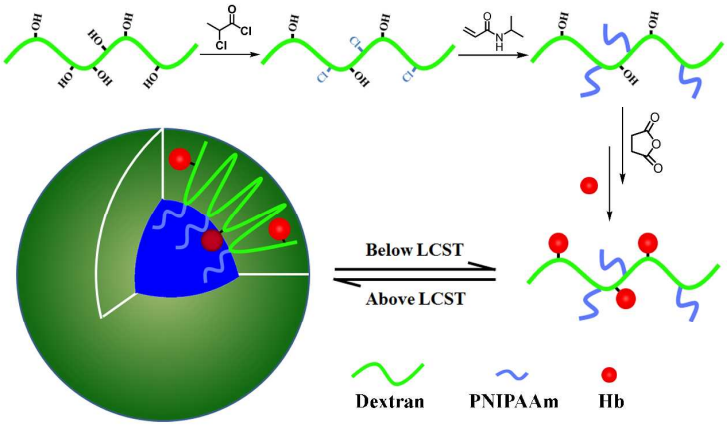
Graphical Abstract

Dextran-based Thermo-Responsive Hemoglobin-Polymer Conjugates with Oxygen-Carrying Capacity

Shasha Wang, Fang Yuan, Gaojian Chen*, Kehua Tu, Hongjun Wang, Li-Qun Wang*

Graft copolymer dextran-g-poly(NIPAAm) were synthesized via SET-LRP and covalently attached to bovine hemoglobin to form thermo-responsive protein-polymer conjugates as novel oxygen carrier.



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Dextran-based Thermo-Responsive Hemoglobin-Polymer Conjugates with Oxygen-Carrying Capacity

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The potential toxicity towards human kidney of hemoglobin (Hb), when used directly, has severely limited its application as red blood cell substitutes and in cancer treatments. In this work, a novel hemoglobin-polymer conjugate was prepared by a reaction between the lysine amino groups of Hb and carboxyl groups of a copolymer, Poly(*N*-isopropylacrylamide) grafted carboxylated dextran (HOOC-Dex-g-PNIPAAm), which was synthesized by single electron transfer living radical polymerization (SET-LRP) and post-carboxylation. The conjugate was characterized by FTIR, ^{1}H NMR, SDS-PAGE, DLS, fluorescence spectroscopy and TEM. Results showed it had a relatively low critical micelle concentration (CMC) and could form stable spherical nanoparticles upon heating above LCST. The redox activity and gas-binding capacity of the Hb conjugate were subsequently confirmed by UV-vis spectroscopy, indicating the retention of Hb bioactivity after conjugation. Furthermore, dextran with different number of PNIPAAm chains were synthesized as comparison. It revealed that at 37 °C, the temperature above LCST, the conjugation of Hb to the copolymer Dex-g-PNIPAAm could improve the stability of Hb which increased with the number of PNIPAAm chains.

Introduction

Protein-polymer conjugates formed by covalently linking synthetic polymers with proteins have proved to enhance the bioactivity of proteins because of the nature of the synthetic component.[1, 2] In particular, stimuli-responsive bioconjugates have received world-wide attention due to its potential to impart proteins with the responsive behaviour.[3-5] Thermo-responsive based bioconjugates are one of the most important component of stimuli-responsive conjugates. Thereinto, poly(*N*-isopropylacrylamide) (PNIPAAm) has been widely adopted to synthesize various "smart conjugates" since they can exhibit a reversible phase transition with temperature in aqueous solution.[6, 7] For example, Chen *et al.* carried out a visible light induced RAFT polymerization of NIPAAm from protein at ambient temperature while the protein retained its bioactivity and presented an efficient on–off-switchable property.[8]

Hemoglobin is a major protein in mammalian blood, and is responsible for storage and transport of O_2 and other gaseous ligands in Red blood cells (RBCs)[9, 10]. Due to the unique capacity of carrying O_2, Hb has become a potential candidate as RBC substitute for use in transfusion medicine. However, Hb extracted from the blood cells couldn't be used directly[11]. That's because the Hb tetramer would quickly dissociates into dimers which are highly toxic to human kidney.[10] Aiming at stabilizing Hb in a tetrameric form or in a polymeric form, many acellular hemoglobin-based O_2 carriers (HBOCs) have been developed as blood substitutes for the clinical and preclinical application, such as HemAssist (DCLHb)[12], Hemospan[13], and Pyridoxylated Hb (PHP)[14] and so on. They can overcome some limitations[15] existing in modern transfusion, such as limited availability of blood donors, difficulty in blood type matching, possible infection by hepatitis viruses and HIV, and limited storage time. In addition, HBOCs have been also demonstrated a great potential to change the tumor hypoxia microenvironment because of which the tumor cells could be more resistant to standard chemotherapy and radiotherapy, more invasive and metastatic, resistant to apoptosis, and genetically unstable.[16] As the fast rising of bio-nanotechnology, nano-scale HBOCs have attracted a great deal of interests in recent years. Huang *et al.* synthesized a series of amphiphilic graft copolymers poly(ethylene oxide-co-allyl glycidyl ether)-g-poly(3 -caprolactone) (PEAG-g-PCL) which could self-assemble into polymersomes in aqueous solution and encapsulate Hb.[17] While Kumar's group presented another protein nanoparticle consisting of met-hemoglobin conjugated with polyacrylic acid (PAA) and demonstrated that these conjugates were highly resistant to deactivation by steam sterilization due to the soft hydrophilic polymer chains which could facilitate the refolding of the denatured Hb.[18]

Dextran, a water soluble polysaccharide, is an optimal molecular model for the synthesis of well-defined polymer derivatives due to its low branching and excellent biocompatibility.[19, 20] It can be not only applied as an antithrombotic to reduce blood viscosity or as a plasma volume expander in hypovolaemia, but also designed as various polymeric nanocarriers for controlled drug delivery[21-23].

In this work, a novel hemoglobin-polymer conjugate was prepared by a reaction between the lysine amino groups of Hb and carboxyl groups of copolymer, Poly(*N*-isopropylacrylamide) grafted carboxylated dextran (HOOC-Dex-g-PNIPAAm), which was synthesized by single electron transfer living radical polymerization (SET-LRP)[24, 25] and post-carboxylation. The introduction of switchable hydrophobicity (PNIPAAm) has conferred our dextran-based hemoglobin conjugate thermo-responsive behaviours, controlled oxygen-carrying capability and a stronger stability in air.

Experimental

Materials

Dextran (Mr~40000, from Sigma) was dried under vacuum at 110 °C for several hours before use to remove the bound water. Tris(2-dimethylaminoethyl)amine (Me_6TREN) was prepared according to the literature procedure[26] with slight modification. N-isopropylacrylamide (NIPAAm, Aldrich, ≥99%) was recrystallized from hexane and stored in a refrigerator. 2-Chloropropionyl chloride (CPCl, 97%) and 4-Dimethylamino pyridine (DMAP, ≥99%) purchased from Aldrich were used without further purification. Bovine Hemoglobin was purchased from Shanghai Yuanju Biological Technology Co. Ltd. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 98.5%) and Succinic anhydride (98%) were from Aladdin Industrial Co. and used as received. Dimetbylformamide (DMF) and all other solvents were purchased from Sinopharm Chemical Reagent Co. and purified according to standard methods.

Characterization

FTIR spectra were recorded on a Bruker Vector 22 FTIR spectrometer. ^{1}H NMR spectra were registered in D_2O at 25 °C using a Bruker DMX-500 NMR spectrometer operating at 500 MHz. Molecular weight and molecular weight distribution were obtained by Waters PL-GPC-50 instrument using THF as the eluent after calibration with standard polystyrene. The particle size and polydispersity were measured by dynamic light scattering (DLS) using a Brookhaven 90 plus particle size analyzer. The morphology of particles was monitored using a JEOL JEM-1200EX transmission electron microscope (TEM) operating at an acceleration voltage of 80 kV. After being incubated for 12 h at 37 °C, the samples (1 mg/mL in water) were deposited onto carbon coated copper grids and left to dry in a drying oven at 37 °C. Steady-state fluorescence excitation spectra were recorded on a HITACHI F-4500 luminescence spectrometer equipped with a temperature controller. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a PowerPac HC electrophoresis apparatus and gels were photographed using an EC3 imaging system. UV-vis spectrum were collected using a Shimidazu UV-1800 spectrophotometer equipped with a temperature-controlled sample holder.

Synthesis of Dex-Cl_x-g-PNIPAAm$_n$

Copolymers of dextran grafted with poly(NIPAAm) were prepared according to a previously published procedure[19] with the following modifications: 1) dextran macroinitiators with different degree of substitution (DS) of chlorine (Dex-Cl_x) were prepared by reacting dextran with various amounts of CPCl, where x is the number of 2-chloropropionyl chloride introduced per 100 glucose units of dextran; 2) In all polymerizations here we used [NIPAAm]:[Dex-Cl_x]:[CuCl]:[Me6TREN]= 60:1:1:1, and hence the grafted poly(PNIPAAm) chains had almost the same length and DP (degree of polymerization) which was independent on DS of Dex-Cl_x. ^{1}H NMR spectra in D_2O and FT-IR were utilized to confirm the formation of macroinitiators Dex-Cl_x and copolymers named Dex-Cl_x-g-PNIPAAm$_n$, where n is the number average degree of polymerization of grafted PNIPAAm chains. In addition, number average molecular weight and polydispersity of PNIPAAm grafted chains, isolated from dextran as previously reported[19], were obtained by GPC.

Carboxylation of Dex-Clx-g-PNIPAAm$_n$

Incorporation of carboxyl groups to Dex-Cl_x-g-PNIPAAm$_n$ was carried out by partial esterification of the hydroxyl groups of dextran backbone with succinic anhydride. Dex-Cl_x-g-PNIPAAm$_n$ (3.7 mmol), previously dehydrated under vacuum at 110 °C, was dissolved in 10 mL anhydrous DMF containing LiCl (20 mg/mL) at 90 °C. Then succinic anhydride (185.15 mg, 1.85 mmol) and pyridine (146.34 mg, 1.85 mmol) were added. The mixture was stirred at 80 °C for 3 h, remaining soluble throughout the process. After that, 25 mL ice water and 0.2 mL concentrated hydrochloric acid were added to stop the reaction and remove residual succinic anhydride and pyridine. After dialysis (MWCO = 3500) against deionized water and lyophilization, the carboxyl groups modified dextran copolymers was obtained and named as $(COOH)_y$-Dex-Cl_x-g-PNIPAAm$_n$, where y was calculated from the ^{1}H NMR spectrum as described in the results and discussion section.

Synthesis of hemoglobin-polymer conjugates

The covalent conjugation of Hb to $(COOH)_y$-Dex-Cl_x-g-PNIPAAm$_n$ was performed via a standard carbodiimide chemistry[18, 27]. The calculated $(COOH)_y$-Dex-Cl_x-g-PNIPAAm$_n$ with 0.5 mmol carboxyl group was dissolved in a mixture of 4 mL deionized water and 0.5 mL Na_2HPO_4 buffer (200mM, pH 7). EDC (95.8 mg, 0.5 mmol) was added as the coupling agent and the mixture was stirred at 0 °C for 15 min. And then Hb (50 nmol) dissolved in 0.5 mL Na_2HPO_4 buffer was dropwise added. The mixture was then allowed to reacted at 25 °C for 24 h and exhaustively dialyzed (MWCO = 100 kD) against deionized water for several days to obtain the final conjugates named as Hb-Dex-Cl_x-g-PNIPAAm$_n$.

LCST and CMC of Dex-Clx-g-PNIPAAm and Hb-Dex-Clx-g-PNIPAAm

The LCST values were determined as the onset temperatures of the hydrodynamic diameter (Dh) versus temperature curves, which were obtained by DLS at a constant polymeric concentration 1 mg/mL. Temperature increased ranging from 15 to 40 °C and the solution was allowed to equilibrate for 10 min at a fixed temperature prior to analysis. Each experiment was repeated three times at least and a liner analysis was used to fit the data.

Critical micelle concentration (CMC) was obtained by the reference method using pyrene as a fluorescence probe. Various concentration of polymeric solutions, ranging from10×10^{-4} to 0.5 mg/mL, were prepared in a series of 10 mL volumetric flasks containing a certain amount of pyrene making sure that the pyrene concentration in the final sample solutions was 6×10^{-7} mol/L. The samples were incubated for 12 h at 37 °C prior to measurement. The fluorescence excitation spectra (300-350 nm) of pyrene at 37 °C were monitored using an emission wavelength of 372 nm and excitation bandwidth of 3 nm. Thus the CMC values were determined as the onset concentration of the plot of intensity ratio (I_{339}/I_{335}).

Redox activity

The ferric iron (Fe^{3+}) present in met-hemoglobin can be reduced by suitable reductant to the ferrous state (Fe^{2+}) which is essential for oxygen transport. Hence, the hemoglobin-polymer conjugates in this work have been treated by sodium dithionite to test their redox activity. In detail, calculated amount of sodium dithionate was added to solutions of the three hemoglobin conjugates (containing 1 μM protein) and the absorbance spectra were recorded. After that, pure oxygen was bubbled through the solutions and the oxidation was monitored via UV-vis spectroscopy until the Soret absorbance band remained unchanged.

Gas-binding capacity

In addition to its ability of binding oxygen, hemoglobin could also react with other gases including carbon monoxide (CO), nitric oxide (NO) *etc*. In this work, the combinations of CO and hemoglobin were carried out by flowing CO over solutions of the three hemoglobin-polymer conjugates (containing 1 μM protein), referring to a previously published procedure[15]. Then the conjugates combining oxygen (O_2-Hb) were obtained by bubbling O_2 to 1 mL of the solutions of CO stabilized Hb conjugate (CO-Hb) under visible light irradiation. Native Hb (1μM) served as a control and experienced the same processes. The change of characteristic adsorption spectra in the whole process were monitored via UV-vis spectrophotometry at 25 and 37 °C, respectively. And the kinetic data on oxygen binding and half time ($t_{1/2}$) at which 50% CO-Hb were converted to O_2-Hb were recorded to compare the gas binding ability of the three hemoglobin-polymer conjugates.

Stability of the oxygenated hemoglobin-polymer conjugates

The stability of the three oxygenated hemoglobin-polymer conjugates was tested by exposing the O_2-Hb solutions to air at 25 and 37 °C to convert the oxy-Hb to its met-form gradually. The oxidation process was monitored by UV-vis spectrophotometry at intervals. When the absorbance of 405 nm reached the maximum value, the hemoglobin in the three conjugates was considered to have been converted to its met-form. The oxidation curve and $t_{1/2}$ (the time at which 50% of the Hb molecules were oxidized) were used to compare the stability of the three oxygenated hemoglobin-polymer conjugates.

Results and discussion

Synthesis and characterization of hemoglobin-polymer conjugates

In order to obtain thermo-responsive hemoglobin-polymer conjugates, dextran with poly(NIPAAm) and carboxylic acid groups in the side chain was synthesized following the route in Fig. 1a. Macromolecular initiator was firstly introduced to the dextran chain by reacting with CPCl. Then the NIPAAm monomers were polymerized via a SET-LRP mechanism to yield a copolymer, Dex-Cl_x-g-$PNIPAAm_n$. Graft polymerization of NIPAAm onto dextran side chains in DMF/water solvent mixture using CuCl/Me_6TREN as the catalyst system was initially considered as a normal atom transfer radical polymerization (ATRP) mechanism.[19] However, recent investigations have indicated that the polymerization of NIPAAm is more likely a SET-LRP mechanism since CuCl would disproportionate in water in the presence of Me_6TREN[24, 28, 29]. In our case, once Me_6TREN in water solution was added to a Schlenk flask containing CuCl, a red suspension appeared immediately at the bottom of the flask which could be ascribed to the disproportionation of Cu(I) into Cu(0) and Cu(II) in DMF/H_2O. When the macromolecular initiator was added, the 'visible' sedimentation of Cu(0) transformed into 'invisible' Cu(0) nanoparticles to activate the initiator and dormant chains as well[30]. As a result, the polymerization of NIPAAm onto dextran chains is possibly a SET-LRP mechanism. Then carboxyl groups were incorporated to the backbone of Dex-Cl_x-g-$PNIPAAm_n$ copolymers by partial succinoylation of the hydroxyl groups of dextran. The final hemoglobin-polymer conjugates were obtained by covalent linking of the carboxyl groups of $(COOH)_y$-Dex-Cl_x-g-$PNIPAAm_n$ to the lysine amino groups of met-Hb.

Copolymers of dextran grafted with poly(NIPAAm) were characterized by FTIR and ^{1}H NMR. As shown in Fig.1b, the characteristic absorption peak of the C=O stretching vibration appeared in the FTIR spectrum at 1740 cm^{-1}, indicating that CPCl was grafted onto dextran hydroxyl groups by esterification reaction. Similarly, the peak located at 1536 cm^{-1} was ascribed mainly to the N-C=O stretching vibration, suggesting the successful polymerization of NIPAAm. Further verification of the synthesis process was established by ^{1}H NMR (Fig. 1c). The characteristic chemical shift of protons of the methyl group located at 1.65 ppm revealed that the initiating group was introduced onto dextran side chains. As listed in Table 1, three macroinitiators with different degree of substitution (DS) were obtained by reacting dextran with various amounts of CPCl. The signals c, d, e and f in the spectrum of Dex-g-PNIPAAm could be ascribed to the PNIPAAm chains. And the ratio of the integral of the methylene protons of PNIPAAm at 1.51 ppm to that of the anomeric proton of dextran at 4.9 ppm could be used to calculate the degree of polymerization (DP) and number average molecular weight (Mn) of the grafted PNIPAAm chains. As shown in Table 1, three copolymers of dextran grafted with poly(NIPAAm) with different DS but approximately similar DP were synthesized, meaning that they have roughly equal PNIPAAm chain length. Meanwhile, the measurement of GPC for the isolated PNIPAAm (Fig. S1, Supporting Information), obtained by hydrolysis of dextran backbone of the grafted copolymers, also confirmed that they had approximately same number average molecular weight. Besides, polydispersity, lower than 1.13, verified the controllable nature of the graft polymerization of NIPAAm onto dextran chains

In order to obtain Hb-polymer conjugates, carboxylic acid groups were introduced to the dextran chain. As verified by ^{1}H NMR (Fig. 1c), the presence of the resonance g at 2.68-2.58 ppm due to the methylene protons proved that carboxyl groups were introduced to Dex-g-PNIPAAm. For the three different copolymers, there existed little distinction in the grafting degree of monosuccinate groups named COOH% (Table 1), which was calculated from the ratio of the integral of the resonance g to that of the signal a. After being partially succinoylated with succinic anhydride, the copolymers were covalently conjugated to met-Hb, resulting in the appearance of the signal h at 2.89 ppm. Since the sum of the integral of h and g in the spectrum of Hb-Dex-g-PNIPAAm was close to the integral of g for HOOC-Dex-g-PNIPAAm, we reasoned that the characteristic peak of the methylene protons of monosuccinate groups after conjugated with Hb had shifted to 2.89 ppm. In addition, a conventional Tris-glycine SDS-PAGE procedure was also performed to double confirm the successful synthesis of the conjugates (Fig. S2, Supporting Information).

Table 1 Characterization of PNIPAAm grafted from Dex-Cl with different degree of substitution

Copolymers	[Dex]/ [CPCl]	Ds[a]	[NIPAAm]/ [Dex-Cl_x]	Conv.[b]	n	Mn (NMR)[a]	Mn (GPC)[c]	PDI	$(COOH)_y$ (NMR)[a]
Dex-Cl_{10}-g-$PNIPAAm_{60}$	20/100	10	60/1	100%	60	6790	10211	1.10	25
Dex-Cl_{22}-g-$PNIPAAm_{55}$	40/100	22	60/1	91%	55	6223	9760	1.12	30
Dex-Cl_{30}-g-$PNIPAAm_{54}$	50/100	30	60/1	90%	54	6110	9180	1.13	28

[a] Determined by ^{1}H NMR in D_2O.
[b] Conversion of NIPAAm monomer calculated by ^{1}H NMR in D_2O.
[c] Mn of the isolated PNIPAAm obtained by hydrolysis of the grafted copolymers and determined by GPC.

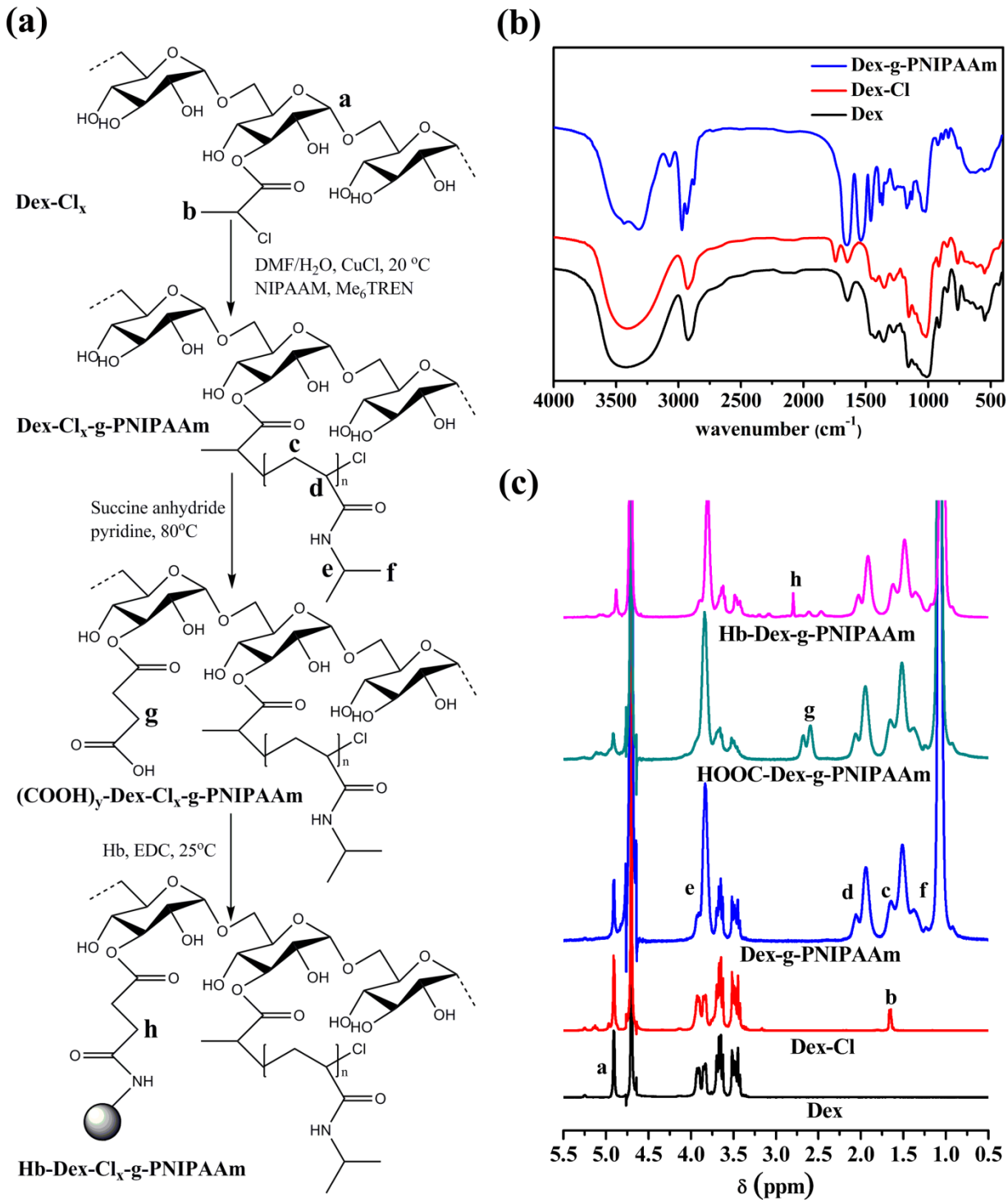

Fig. 1 (a) Scheme for the synthesis of hemoglobin-polymer conjugates; (b) FTIR spectra of dextran, Dex-Cl_{10} and Dex-Cl_{10}-g-PNIPAAm over the region 4000-400 cm^{-1}; (c) ^{1}H NMR spectra of dextran, Dex-Cl_{10}, Dex-Cl_{10}-g-PNIPAAm, HOOC-Dex-Cl_{10}-g-PNIPAAm and Hb-Dex-Cl_{10}-g-PNIPAAm in D_2O.

Thermo-responsive properties of copolymers and conjugates

The copolymers of dextran grafted with PNIPAAm displayed a reversible change of solubility in response to temperature, denoted by lower critical solution temperature (LCST) and critical micelle concentration (CMC).

Above LCST, PNIPAAm underwent a transition from hydrophilic to hydrophobic, accounting for the changes of solution properties such as surface tension[31], transmittance[32] and particle size[33]. Therefore, The LCST of the dextran copolymers grafted with PNIPAAm was determined by DLS. The curves of particle size and polydispersity (PDI) versus temperature for both Dex-Cl_x-g-PNIPAAm and Hb-Dex-Cl_x-g-PNIPAAm were shown in Fig. 2.

Taking Dex-Cl_{10}-g-PNIPAAm as an example, at temperature below 33 °C the hydrodynamic diameters were between 10 to 20 nm, indicating that the graft copolymers should be molecularly dissolved in aqueous solution. When the temperature rises above 33 °C, the particle size soared to as high as 100 nm while PDI decreased significantly to a minimum value of 0.06. That was due to the self-assembly of copolymers, generating micelles with good dispersion consisted of PNIPAAm as the hydrophobic core and dextran as the hydrophilic shell[33]. As the temperature continued to rise, the hydrodynamic diameters slowly decreased and reached a plateau probably due to the progressive dehydration and shrinking of PNIPAAm core. The intersection of the horizontal line and the straight line passing through the inflection point of the curve was considered as the LCST values as listed in Table 2. It was obvious that the degree of substitution had limited impact on the LCST and hydrodynamic diameters either for Dex-Cl_x-g-PNIPAAm or for Hb-Dex-Cl_x-g-PNIPAAm, which was consistent with the previous report[19].

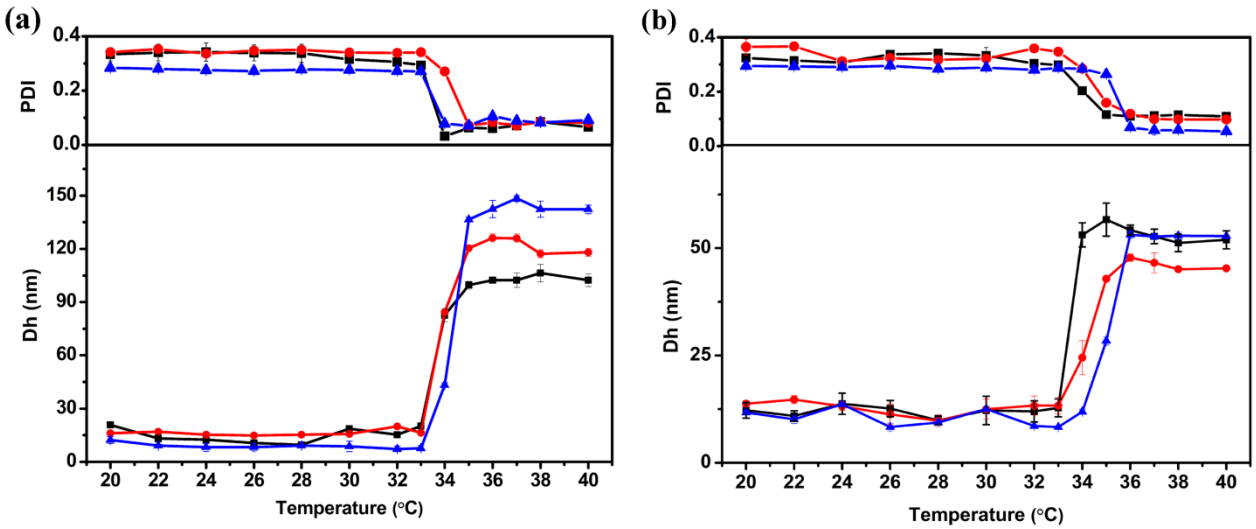

Fig. 2 Temperature dependence of Dh (on the bottom)and PDI (on the top) measured by DLS experiments in aqueous solution (1mg/mL) for Dex-Cl_x-g-PNIPAAm (a) and Hb-Dex-Cl_x-g-PNIPAAm (b). Coplymers with different degree of substitution are labeled using different symbol: Dex-Cl_{10} by square, Dex-Cl_{22} by circle and Dex-Cl_{30} by triangle.

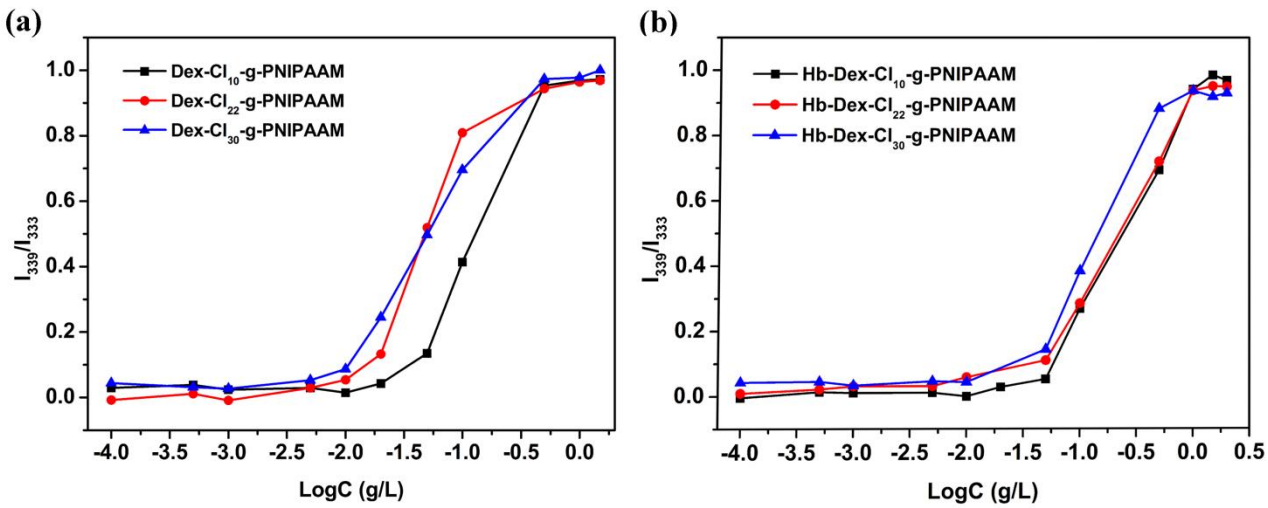

Fig. 3 Plots of intensity ratio (I_{339} /I_{333}) vs. log C for Dex-Cl_x-g-PNIPAAm (a) and Hb-Dex-Cl_x-g-PNIPAAm (b)

The CMC values of copolymers was quantified through fluorescence spectroscopy by using pyrene as the probe, which was sensitive to surrounding environment[34, 35]. The ratio of I_{339}/I_{335} was measured at a series of polymer concentration at 37 °C to characterize the polarity of microenvironment in the presence of pyrene[35]. As shown in Fig. 3, the small I_{339}/I_{335} values at lower concentrations indicated pyrene existed only in aqueous solution since the copolymers were hydrophilic. However, after reaching a certain concentration, the I_{339}/I_{335} ratio increased significantly because pyrene assembled into the PNIPAAm hydrophobic core. The onset concentration of the burst of I_{339}/I_{335} could be observed roughly as the CMC values as listed in table 2. CMC depends on the proportion of the hydrophobic chain to the hydrophilic chain. For the Dex-Cl_x-g-PNIPAAm copolymers with the increase of DS, the CMC values decreased since the number of PNIPAAm grafted chains increased correspondingly. For the Hb-polymer conjugates, the CMC values were relatively larger compared with that of Dex-Cl_x-g-PNIPAAm copolymers due to the increased hydrophilicity by the introduction of Hb.

Table 2 Thermo-responsive properties determined by DLS and fluorescence spectroscopy

Copolymers	LCST (°C)	Dh (nm) at 37 °C	CMC at 37 °C
Dex-Cl_{10}-g-PNIPAAm	32.9	142	0.035
Hb-Dex-Cl_{10}-g-PNIPAAm	33	53	0.046
Dex-Cl_{22}-g-PNIPAAm	33	156	0.016
Hb-Dex-Cl_{22}-g-PNIPAAm	33.3	47	0.043
Dex-Cl_{30}-g-PNIPAAm	33.2	175	0.008
Hb-Dex-Cl_{30}-g-PNIPAAm	33.9	52	0.036

Morphology

TEM was used to determine the size and morphology of the nanoparticles of dextran-based copolymers grafted with poly(NIPAAm) formed at 37 °C. As shown in Fig. 4, the images of Dex-Cl_{10}-g-PNIPAAm and Hb-Dex-Cl_{10}-g-PNIPAAm both showed a lot of isolated and uniform spherical nanoparticles. However, the average size of the Dex-Cl_{10}-g-PNIPAAm particles was estimated to be 60 nm, larger than that of Hb-Dex-Cl_{10}-g-PNIPAAm (~30 nm). This distinction was also observed in the DLS studies which were shown in the inset graph. The two particles differed quite a bit in the hydrodynamic diameters which were ~100 nm and ~50 nm respectively, probably because the hydrogen-bond interactions between residual carboxyl and amide bonds might make the PNIPAAm core shrink and tight[36]. In addition, the average size obtained by TEM was much smaller than by DLS, on account of rapid dehydration in drying process for preparation of TEM specimens.[37]

The results of DLS, TEM and fluorescence spectroscopy showed that the Hb-(Dex-g-PNIPAAm) conjugates could form spherical nanoparticles with relatively low CMC, which indicated that the thermo-responsive nature of PNIPAAm has been conferred to the conjugates.

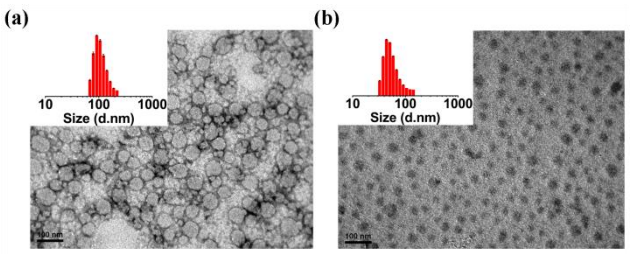

Fig. 4 TEM images and size distribution(in the upper left inset) of Dex-Cl_{10}-g-PNIPAAm (a) and Hb-Dex-Cl_{10}-g-PNIPAAm (b).

Redox activity

As is well known, heme, an Fe^{2+} protoporphyrin IX coordinating with the sub-unit of Hb, is crucial to its intrinsic capacity of transporting O_2. However, upon exposure of Hb to O_2 for a long term, the heme will be oxidized irreversibly to inactive Fe^{3+} state which is totally unable to bind dioxygen. Therefore, the reduction activity is fundamental when evaluating whether or not the polymer-Hb conjugate can perform as an oxygen carrier [27]. Taking Hb-Dex-Cl_{10}-g-PNIPAAm as an example (Fig. 5), the Fe^{3+} form of methemoglobin (met-Hb) could be reduced to its ferrous state with sodium dithionite which was confirmed by a red shift of the Soret band from 405 nm to 427nm accompanied with a change of solution colour from brown to pale green. When pure oxygen was bubbled into the solutions, the colour changed from pale green to bright red and finally back to brown. These changes indicated that the deoxy-Hb-polymer conjugates (State 2 in Fig. 5) were transformed to the oxygen-binding state (State 3 in Fig. 5) with a blue shift of the maximum absorption peaks to 412 nm. After continuous exposure to O_2, the Fe^{2+} form of Hb was expected to revert to its Fe^{3+} state which could be concluded by the reversal of the Soret band to initial state. Similar circumstance occurred in other two Hb-polymer conjugates with different DS (Fig. S3, Supporting Information). These conjugates managed to keep the ability of Hb from being reduced, therefore very likely holding the ability to transport oxygen.

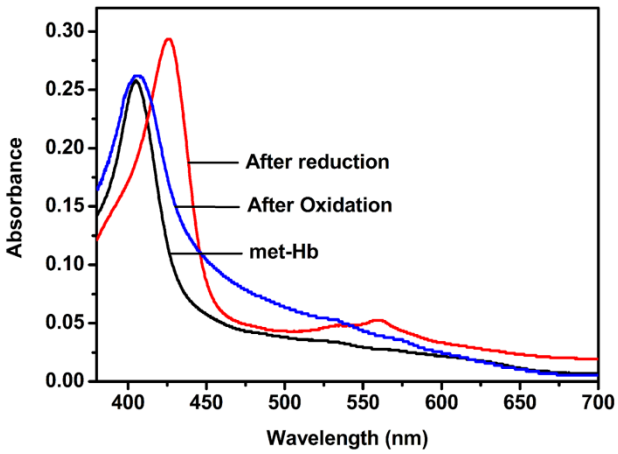

Fig. 5 Redox activity of Hb-Dex-Cl_{10}-g-PNIPAAm indicated by the shift of Soret bands from state 1 to 3.

Gas-binding capacity

In addition to its ability of binding oxygen, hemoglobin could also react with other gases including CO, NO *etc.*. Generally, the formation of CO-Hb prevented hemoglobin from combining with oxygen since the former had a more affinity towards Hb. However, the CO molecule bound to Hb could be displaced by O_2 if its concentration was high enough[15, 17]. As shown in Fig. 6, the met-Hb in ferric state could be transformed into the ferrous CO-Hb in the presence of ascorbic acid under carbon monoxide atmosphere, accompanied with a red shift of the Soret band from 405 nm to 419 nm. Then the O_2-Hb was obtained by bubbling O_2 to the solutions of CO-Hb, generating a blue shift of the Soret band from 419 nm to 412 nm which is the characteristic adsorption peak O_2-Hb. Similar circumstance occurred in other two Hb-polymer conjugates with different DS (Fig. S4, Supporting Information).

The kinetic data on oxygen binding for the native Hb and the three Hb-Dex-g-PNIPAAm conjugates with different DS were showed in Fig. S5 (supporting information). The half-life $t_{1/2}$, at which 50% of CO-Hb was converted to its oxygenated form, were used to compare the oxygen binding ability of CO-Hb in the experimental group and the control group. As shown in Table 3 and Fig. S5, the rate of oxygen binding for Hb-Dex-Cl_x-g-PNIPAAm increased with the number of PNIPAAm chains, probably because that the increased molecular mass might provide a better protection to O_2 from off-loading[38]. While the half life of native CO-Hb seemed close to Hb-Dex-Cl_{30}-g-PNIPAAm with the highest DS. These results indicated that the conjugation of Hb to Dex-g-PNIPAAm had

somewhat affected its affinity to oxygen, but this effect could be eliminated with the increase of DS.

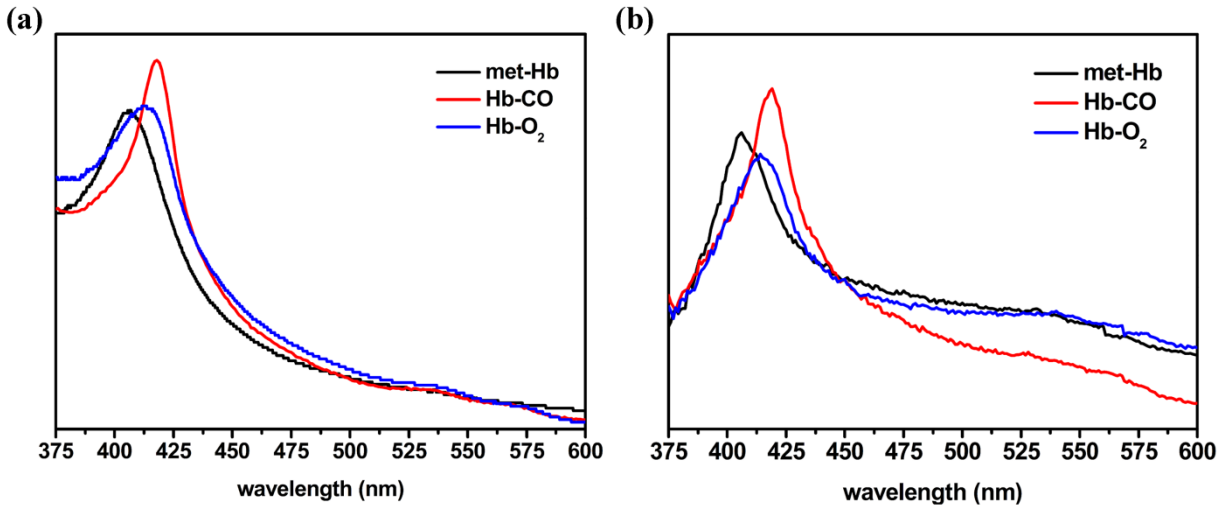

Fig. 6 UV–vis spectra of Hb-Dex-Cl_{22}-g-PNIPAAm under various atmospheres at 25 °C (a) and 37 °C (b).

Autoxidation of O_2-Hb

As is well known, in the absence of antioxidant, O_2-Hb is gradually oxidized to met-Hb state in air which is fatally unable to deliver oxygen to tissues[39, 40]. The autoxidation processes of O_2-Hb in the three Hb-Dex-g-PNIPAAm conjugates were characterized by the plot of O_2-Hb percentage versus time (see Fig. 8). The O_2-Hb percentage was defined as 100% when the maximum absorption peak in UV-vis spectra located at 412 nm (the characteristic adsorption peak of O_2-Hb). Then the percentage dropped over time since the Fe^{2+} protoporphyrin in O_2-Hb was gradually oxidized to its inactive Fe^{3+} state (met-Hb) in air. When the maximum absorption peak shifted to 405 nm, the content of O_2-Hb was denoted as 0. The half-life $t_{1/2}$ obtained from the fitted oxidation curves were used to compare the stability of O_2-Hb in the experimental group and the control group. As listed in Table 3, at room temperature, the autoxidation rate of native Hb was much faster than all of the three Hb-polymer conjugates with the longest half-life. Conversely, at higher temperature, the autoxidation rate of native Hb was slower than the two Hb-Dex-Clx-g-PNIPAAm conjugates with higher DS. The kinetic behaviours of Hb-Dex-Cl_x-g-PNIPAAm were totally different from that of pegylated Hb[41] which showed higher autoxidation rate compared with native Hb both at room temperature and 37 °C. The unique properties of Hb-Dex-Cl_x-g-PNIPAAm at higher temperature were determined by their special structure as a result of the thermo-sensitivity. At 37 °C, the temperature above LCST, the aggregation of PNIPAAm chains seemed to provide a better protection to the O_2-Hb molecules and thus improved their stability in air. And the stability of O_2-Hb in Hb-Dex-Cl_x-g-PNIPAAm would increase with the number of PNIPAAm chains.

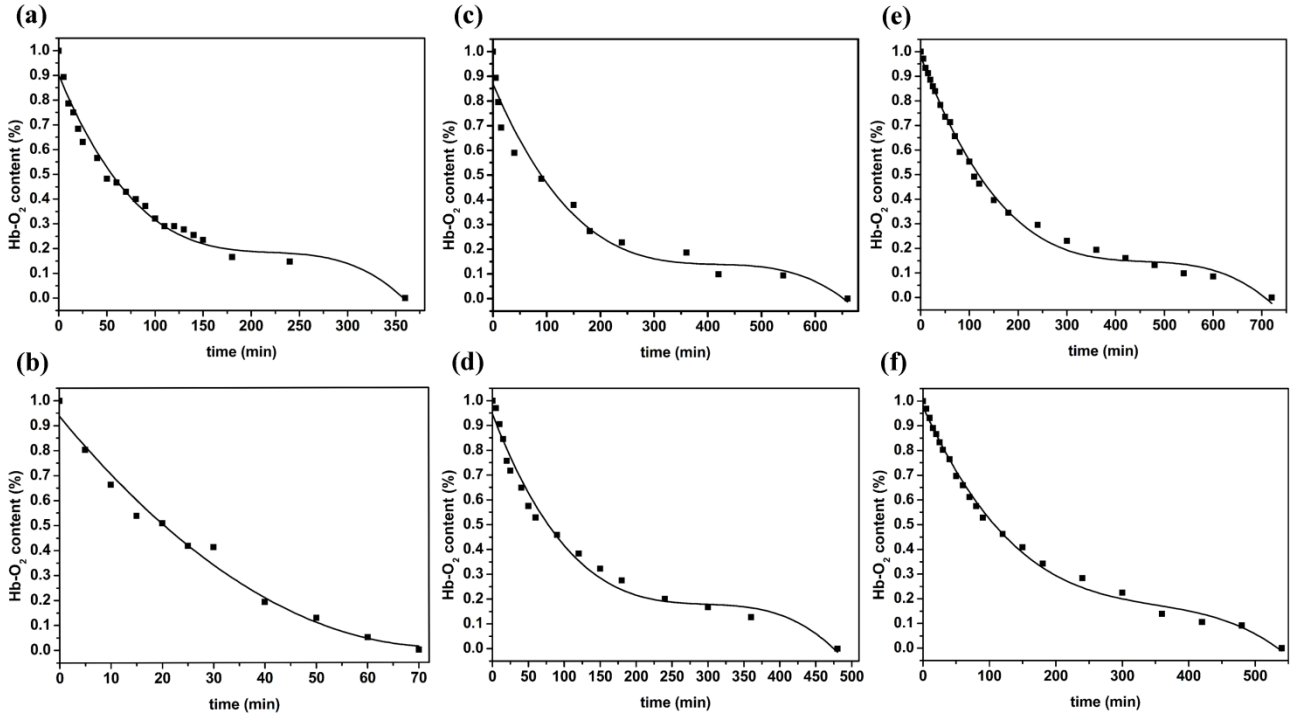

Fig. 7 Stability tests of O_2-Hb in Hb-Dex-Cl_{10}-g-PNIPAAm at 25 °C (a) and 37 °C (b), Hb-Dex-Cl_{22}-g-PNIPAAm at 25 °C (c) and 37 °C (d), Hb-Dex-Cl_{30}-g-PNIPAAm at 25 °C (e) and 37 °C (f).

Table 3 Kinetic data on oxygen binding and autoxidation

Hb-polymer conjugates	Oxygen binding ($t_{1/2}$, s)[a]		Autoxidation ($t_{1/2}$, min)[b]	
	25 °C	37 °C	25 °C	37 °C
Hb-Dex-Cl_{10}-g-PNIPAAm	363	217	55	21
Hb-Dex-Cl_{22}-g-PNIPAAm	279	149	90	77
Hb-Dex-Cl_{30}-g-PNIPAAm	175	119	119	107
native Hb	176	127	304	59

[a] the time at which 50% of CO-Hb was converted to O_2-Hb.
[b] the time at which 50% of O_2-Hb was autoxidized in air.

Conclusions

In summary, we have successfully synthesized a novel thermo-responsive protein-polymer conjugate composed of bovine haemoglobin (Hb) and a copolymer HOOC-Dex-g-PNIPAAm which was prepared via a SET-LRP mechanism and post carboxylation. Upon heating above LCST, the conjugate could form isolated uniform spherical nanoparticles with a relatively low CMC. Simultaneously, the Hb-polymer conjugate was confirmed to have redox activity and gas-binding capacity under different atmosphere. Furthermore, we also demonstrated that the Hb-Dex-Cl_x-PNIPAAm had a unique property to improve the stability of O_2-Hb above LCST, probably due to the thermo-sensitivity of grafted PNIPAAm chains. And the stability of O_2-Hb in Hb-Dex-Cl_x-g-PNIPAAm would increase with the number of PNIPAAm chains.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Supplementary data, including SDS-PAGE, redox activity and UV–vis spectra of conjugates. See DOI: 10.1039/b000000x/

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