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Confinement of proteins by thermoresponsive dendronized polymers†

Yi Yao,  Jintao Yang, Wen Li * and Afang Zhang *

The crowding environment created by host polymers plays crucial roles in manipulating interactions with proteins and modulating their bioactivity. Here, we report our investigation on the interactions between polymers and proteins in the confined microenvironments constructed by oligoethylene glycol (OEG)-based dendronized polymers due to the crowded OEG dendrons. Several important characteristics of these dendronized polymers, including their aggregation state, charge state and combination form with biomolecules, were revealed to be the main factors decoding the polymer–protein interactions. To examine the effects of encapsulation and shielding from the dendronized polymers for the biomacromolecules, the guest proteins were combined through either bioconjugation, electrostatic complexation, or just physical mixing. The unprecedented thermoresponsiveness of the dendronized polymers provides tunable crowding and hydrophobicity of the microenvironment conveniently through their thermally induced aggregation, resulting in regulation of the activities for the proteins. This kind of dendronized polymer with structural and topological features is a promising candidate for the construction of intelligent artificial microenvironments to tunably confine biomacromolecules.

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Introduction

Biotransformation in cells is often accomplished in confined microenvironments created by crowded biomacromolecules in the vicinity.^{1,2} The secondary and tertiary structures of biomacromolecules, and even the ways that they associate with each other, will be affected by molecular crowding, resulting in their different behaviours in biological processes.^{3–5} Moreover, the adverse interactions between the active sites of proteins and crowded molecules could lead to pronounced effects on their folding states, and cause the loss of their activities directly.^{4,6} Various crowding agents, such as synthetic or natural molecules (polymers,⁷ polysaccharides,⁸ liposomes,⁹ etc.), have been used to examine the influence on the folding and stability of proteins caused through their weak hydrophobic interactions.^{4,8,10,11} The factors affecting the interactions among proteins and crowding agents mainly include: (1) structural features of the macromolecules, such as chain length,¹² hydrophobicity,^{13,14} and even concentration;¹⁵ (2) protein properties, such as amino acid sequence/hydrophili-

city, and molecular weight;¹⁶ and (3) solution conditions, such as temperature and pH.^{16,17}

Macromolecules used for biological activity protection should have conformational freedom of the chains in aqueous solutions, like a randomly coiled coil, resulting in the formation of an energetically stable hydration shell wrapped around the proteins and hanging freely in the solution.^{13,18} For synthetic biomolecule stabilization agents, such as the widely used polyethylene glycols (PEGs),³ their suitable amphiphilicity has been found to be a key parameter that determines their states in aqueous solutions, dictating their activity protection capacity.^{18,19} PEG analogues are being continuously developed for achieving macromolecules with suitable amphiphilicity.²⁰ Among them, one kind of comb-shaped polymer carrying oligoethylene glycol (OEG) pendants and a methacrylate backbone has attracted wide attention due to its adjustable amphiphilicity, which can be achieved through changing the chain length of the OEGs.⁷ Compared to their linear OEG counterparts, dendritic OEGs have more advantages in activity protection because of the multi-valency, topological cooperativity, and, most importantly, the densely packed dendritic OEG moieties providing a crowding effect in nanometre dimensions for efficient shielding.^{4,13,20,21}

Recently, we found that OEG-based dendronized polymers exhibit unprecedented thermoresponsiveness, and have shown advantages in forming confined environments at molecular levels for activity protection of biomacromolecules through

International Joint Laboratory of Biomimetic and Smart Polymers, School of Materials Science and Engineering, Shanghai University, Nanchen Street 333, Shanghai 200444, China. E-mail: wli@shu.edu.cn, azhang@shu.edu.cn

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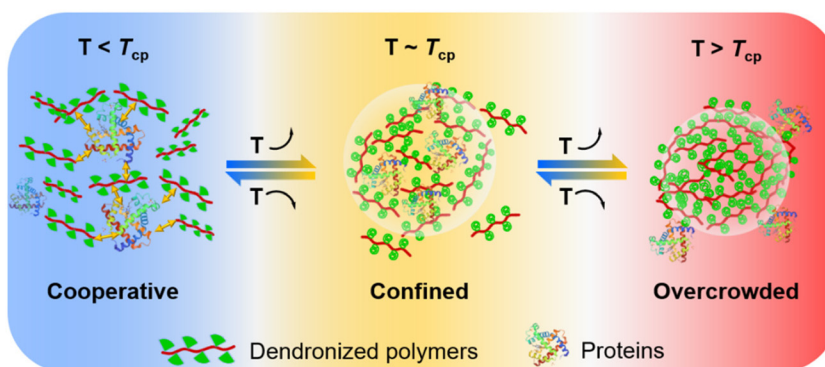


Fig. 1 Schematic representation of the possible interaction modes between the dendronized polymers and proteins below, around, and above their cloud points (T_{cp}).

cooperation interactions and shielding effects.^{4,10,22} Herein, the interactions between dendronized polymers and proteins in aqueous solutions were investigated in detail. The OEG-based dendronized homopolymers (PG1), copolymers (PG1S), and dendronized chitosans (DCSs) were selected as the models to examine the confinement of proteins through physical mixing, covalent conjugation, or electrostatic complexation (Fig. 1). Moreover, the effects on the hydrophobic interactions between the dendronized polymers and the proteins during thermally induced aggregation of the polymers were revealed by various measurements. On this basis, the protein activity regulation capability of these dendronized polymers was explored under different conditions.

Results and discussion

Preparation of dendronized polymer–protein conjugates or complexes and their thermoresponsive behaviour

OEG-based dendronized homopolymers (PG1), copolymers (PG1S), and DCSs were selected to examine the confinement of proteins through physical mixing, covalent conjugation, and electrostatic complexation, respectively. Their molecular structures are shown in Fig. S1A.† The synthesis of PG1²³ and DCSs²⁴ was performed according to previous reports. Dendronized copolymer PG1S pendant with three-fold dendritic OEG and a 4-nitrophenyl active ester was synthesized through free radical polymerization as shown in Fig. S1B.† Homopolymers and copolymers with similar molecular weights (the M_n s of PG1 and PG1S are 2.3×10^5 and 2.2×10^5 , respectively, Table S1†) were prepared to reduce the possible effects of molecular size on the interactions.²⁵ Here, the 4-nitrophenyl active ester in MS=S was designed for covalent conjugation of proteins *via* amidation. The copolymerization ratio ($[M3]/[MS=S]$) for PG1S was designed to be 21.5 (Table S1†), which can endow the polymer with a cloud point (T_{cp}) around physiological temperature. DCSs with a dendron coverage of around 55% were prepared, aiming to keep a suitable amount of positive amino pendants in the CS backbones for complexation with the negatively charged proteins, and

simultaneously a proper amount of OEG dendron pendants to provide sufficient shielding effects to form a micro-environment along the polymer chains.

Formation of copolymer–protein conjugates (PG1S-Mb) from PG1S and myoglobin (Mb) was followed by UV/vis measurement. The absorbance at 420 nm changed obviously after the addition of Mb to the solution of PG1S (Fig. S3A†). Moreover, the hydrodynamic radii (R_h s, intensity radii) increased twice after the addition of Mb to PG1S as shown in Fig. 2A. Both results prove that the conjugation of Mb to PG1S was successful. The loading ability of the polymers for proteins was further evaluated. The loading efficiency increased dramatically with the increase of the polymer concentration of PG1S, and exceeded 90% when the mass ratio of MS=S to proteins was over 16 (Fig. 2B). Through isothermal titration calorimetry (ITC) measurements, the binding stoichiometry (n) of

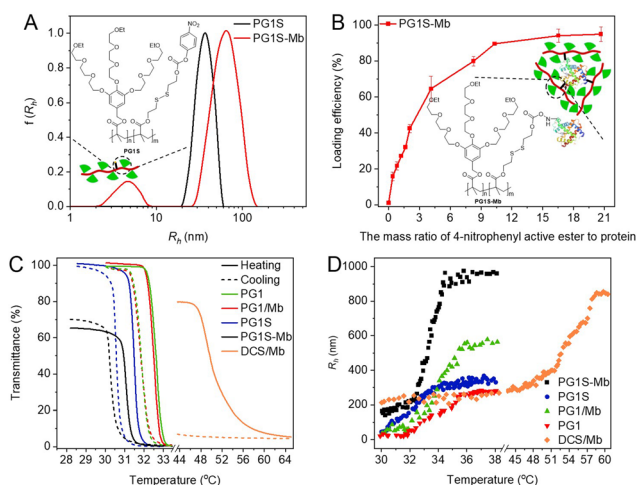


Fig. 2 (A) Plots of R_h from PG1S and PG1S-Mb. 25 °C, $C = 0.05$ mg mL^{-1} . The size polydispersities were 0.40 and 0.59, respectively. (B) Loading efficiency of PG1S for Mb at different concentrations. (C) Plots of transmittance vs. temperature for PG1S, PG1S-Mb, PG1, PG1/Mb, and DCS/Mb. $C = 2.5$ mg mL^{-1} . (D) Plots of R_h for PG1S, PG1S-Mb, PG1, PG1/Mb, and DCS/Mb between 30 and 60 °C, respectively. $C = 0.05$ mg mL^{-1} .

blue-shifting compared to **PG1** (located at 2885 cm^{-1}). In addition, there were several new peaks at $1300\text{--}1050\text{ cm}^{-1}$ (corresponding to the stretching vibration of the ether bond --C--O--) for **PG1S-Mb** when compared with the other samples, shown as Fig. S6A.† These results indicate that there are indeed hydrophobic interactions between the polymer backbone or dendritic OEGs and proteins.³⁰ The FT-IR spectra of **PG1S-Mb** were further recorded at different temperatures. As shown in Fig. 3B, the peak shape in the range of $1260\text{--}1030\text{ cm}^{-1}$ gradually became smoother with increasing temperature. The peak shape of **PG1S-Mb** almost overlapped with those of **PG1S** at temperatures above $40\text{ }^{\circ}\text{C}$, which is much higher ($>10\text{ }^{\circ}\text{C}$) than their T_{cp} s (Fig. S6B†). This indicates that the enhanced hydrophobic interactions between the polymer chains due to the extensive dehydration should have reduced their interactions with the proteins, which could eventually cause exposure of the active center of the proteins through collapse and aggregation of the polymer chains.²⁸ This also suggests that the hydrophobic interactions between the dendritic OEGs and proteins changed dynamically during thermally induced aggregation of the polymers. This is a valuable finding, implying that hydrophobic interactions between dendronized polymers and proteins can be regulated through their characteristic thermoresponsiveness, resulting in the adjustment of the confinement of the proteins.

The quartz crystal microbalance with dissipation technique (QCM-D) was used to provide information on the adsorbed mass and the viscoelastic properties during the interactions between the dendronized polymers and the proteins at different temperatures.³¹ The gold chip was modified by the proteins before testing according to the method reported previously (Fig. S7A†).³² The frequency shift (Δf) for the adsorption process of **PG1** took 5.3 h to reach equilibrium at $25\text{ }^{\circ}\text{C}$ (Fig. 4A, red curve), but changed continuously at $38\text{ }^{\circ}\text{C}$ (Fig. 4B, red curve). The adsorbed mass Δm (calculated accord-

ing to Sauerbrey model and Δf) of **PG1** on Mb at a given time (5.3 h) was 30.98 and $1971.71\text{ ng cm}^{-2}$ at 25 and $38\text{ }^{\circ}\text{C}$, respectively. The energy dissipation (ΔD) of **PG1/Mb** at 5.3 h was 0.461×10^{-6} at $25\text{ }^{\circ}\text{C}$ (Fig. 4A, blue curve), which increased dramatically to 157.67×10^{-6} at $38\text{ }^{\circ}\text{C}$ (Fig. 4B, blue curve). In addition, the Δf of **DCS/Mb** (at 42 min) was found to be -16.8 at $25\text{ }^{\circ}\text{C}$, which decreased to -31.2 Hz at $60\text{ }^{\circ}\text{C}$. The above results suggest that both **PG1** and **DCS** show different affinities to the protein at temperatures below and above their T_{cp} s, which remarkably influenced their interactions.³³ The dendronized polymers adsorbed on the protein to form a relatively stiff layer with low dissipation capacity at temperatures below the T_{cp} (Fig. 4C). This is comparable to properties reported for OEG-alkanethiol self-assembled monolayers (SAMs) on gold or silver surfaces.^{34–36} Differently, as the temperature increases above the T_{cp} , the adsorption layer of dendronized polymers on the protein showed viscoelastic and strongly dissipating properties, which are comparable to those of the widely studied PEG-based brush assemblies.^{31,37,38}

Influence on protein secondary structure in the presence of dendronized polymers

Circular dichroism (CD) was employed to monitor the effect of dendronized polymers on the secondary structures of the proteins. There is a weaker CD signal at $210\text{--}220\text{ nm}$ (mainly from α -helical structures) in the spectrum of free Mb (Fig. 5A, curve in light pink).³⁹ For **PG1/protein** (including Mb, BSA, and LYZ, Fig. S8A and B†), the negative CD signal near 210 nm was enhanced with the increase of the polymer concentration. This should be mainly due to the enhanced interactions of the proteins with **PG1**. The hydrophobicity of the densely crowded dendritic chains from **PG1** created a hydrophobic microenvironment,^{27,39} resulting in weakening solvation of the proteins, and accordingly enhancing their chiral conformation.^{40,41}

Possible effects of thermally induced aggregation of **PG1** on the helical conformation of the proteins were further monitored. As shown in Fig. 5B, the intensities of the negative (near 210 nm) and positive (near 220 nm) Cotton effects from the CD spectra of **PG1/Mb** decreased with increase of the solution temperature. The spectra of **PG1/protein** (Fig. S8C and D†) were almost overlapping with those of the free protein when

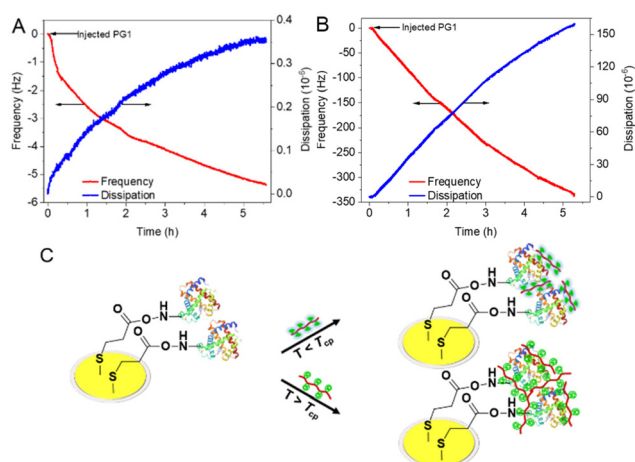


Fig. 4 Changes of frequency Δf (red curves) and dissipation ΔD (blue curves) in a QCM cell (modified by Mb) over time after injection of **PG1** at (A) $25\text{ }^{\circ}\text{C}$ and (B) $38\text{ }^{\circ}\text{C}$. (C) Possible interactions between the proteins and the polymers.

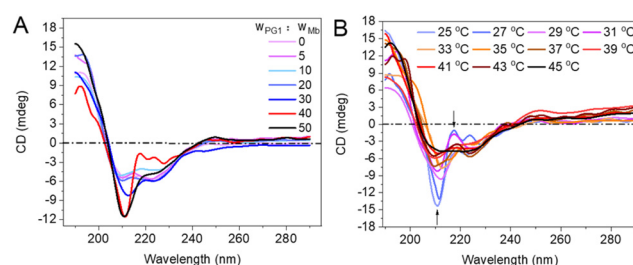


Fig. 5 Far-UV CD spectra of Mb from **PG1/Mb** (A) with different concentrations of polymer, and (B) at different temperatures (polymer/protein = 40, w/w).

the temperature well exceeds ($>40\text{ }^{\circ}\text{C}$) its T_{cp} . This is further evidence that thermally induced aggregation of the polymer changed the hydrophobic interactions between **PG1** and Mb.^{39,42}

Activity regulation of the proteins by the dendronized polymers

In myoglobin, the interactions between the protein side chain and heme result in a strong absorption near 410 nm and will determine its activity.³⁹ Hence, the absorption spectra of **PG1/Mb** solutions were recorded at different temperatures to explore the effect of dendronized polymers on the bioactivity. As shown in Fig. S9A,[†] the absorbance near 410 nm in spectra of Mb at 25 $^{\circ}\text{C}$ only showed a slight decrease (from 0.45 to 0.41) when the concentration of **PG1** was 50 times higher than that of the protein. The spectra of **PG1/Mb** were overall upward shifted when the temperature was higher than 35 $^{\circ}\text{C}$ ($>T_{\text{cp}}$) without changing their shape (Fig. S9B[†]), and can be restored to their original state after temperature recovery to 25 $^{\circ}\text{C}$. This process can be repeated multiple times (Fig. S9C[†]), indicating that the thermally induced aggregation process of the dendronized polymers hardly caused any destruction of the protein.^{39,43}

The catalytic activity of Mb within the mixture was further investigated at different temperatures, and the results are shown in Fig. 6A. The activity of Mb from all samples increased linearly with the temperature between 25–45 $^{\circ}\text{C}$ from 30% to 61% for Mb, 40% to 73% for **PG1/Mb**, 42% to 79% for **DCS/Mb**, and 41% to 90% for **PG1S-Mb**, respectively. Compared to other groups, the activity of **PG1S-Mb** increased more obviously between 30–37 $^{\circ}\text{C}$. For **DCS/Mb**, it increased obviously from 79% to nearly 100% between 45–60 $^{\circ}\text{C}$. These temperatures correspond to their T_{cp} s, indicating that around the phase transitions, the proteins exhibited the highest activities. However, the activity of the free proteins, **PG1/Mb** and

PG1S-Mb decreased obviously to lower than 34% with further increase to 60 $^{\circ}\text{C}$. We ascribe these abnormal increases of activity to the thermally induced dehydration of the dendritic OEGs from the polymers, which collapsed and aggregated to form hydrophobic microenvironments to enhance the hydrophobic interactions between the polymer and proteins. This leads to enhancement of the enveloping and shielding capability for the polymer chains towards Mb, simultaneously preventing the solvation of Mb, which is helpful to the binding of the proteins to the substrate. Moreover, the activity of the samples decreased at temperatures much higher than their T_{cp} s. This should be due to the collapsing between the polymer chains, which caused the exposure of the active center of the proteins, eventually resulting in the loss of their activity.²⁸ Differently, in the case of **DCS/Mb**, the effective electrostatic interactions between the CS mainchains and Mb was supportive of confinement on the proteins and regulation of their bioactivity. This complexation of the protein by **DCS** would make it benefit from the shielding effect from dendritic OEGs even at high temperature.

The bioactivity protection capability of the polymer was further explored at different temperatures for different time intervals. As shown in Fig. 6B, the activity for the free protein, **PG1/Mb**, **PG1S-Mb**, and **DCS/Mb** after being kept at 25 $^{\circ}\text{C}$ for 12 h was 41%, 42%, 76%, and 97%, respectively. Notably, the bioactivity of the protein in the cases of **PG1S-Mb** and **DCS/Mb** was almost two times higher than the other cases, indicating better protection capability of the protein. Furthermore, after being kept at 60 $^{\circ}\text{C}$ for 3.5 h, the bioactivity of the protein in the cases of free Mb and **PG1/Mb** only remained 6% and 11% (Fig. 6C), respectively, indicating significant loss of activity at elevated temperatures. In contrast, the bioactivity in the case of **DCS/Mb** was over 2 times that for **PG1S-Mb** at a high temperature, and was found to be 34% and 61% for **PG1S-Mb** and **DCS/Mb**, respectively. This indicates that the **DCS**s showed much better protection capability towards the protein at high temperature. This is understandable since the aggregated dendronized polymers provided a more hydrophobic and crowded microenvironment, which protected the proteins from strong solvation and was more suitable for proteins to maintain their high activities, especially at elevated temperatures.^{44,45} On the other hand, the effective electrostatic interactions between the CS mainchains and Mb form stable complexes, which should be favourable for Mb to compensate against solvation at high temperature.

Conclusions

The interactions between thermoresponsive polymers and proteins within the confined microenvironments created by OEG-based dendronized polymers were investigated. The proteins were combined with the polymers through physical mixing, bioconjugation or ionic complexation, aiming at examining possible effects of the forms of linkage of the proteins on the confinement. The thermally mediated aggregation of the den-

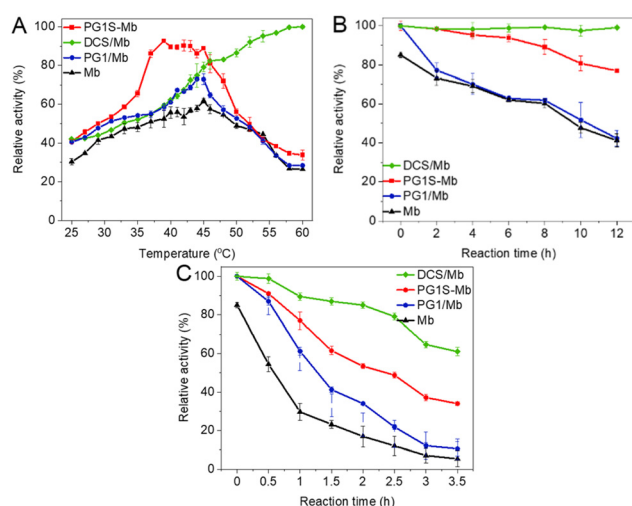


Fig. 6 Quantitative relative activity of Mb from different samples at different temperatures (A), as well as after being kept at (B) 25 $^{\circ}\text{C}$ and (C) 60 $^{\circ}\text{C}$ for different time intervals.

dronized polymers and their combination forms with biomolecules were found to play an important role in modulating the polymer–protein interactions. The confinement of proteins by the dendronized polymers is better achieved through their covalent conjugation and electrostatic complexation. This favours the enhancement of cooperative, encapsulation, and shielding effects from the dendritic OEGs. The crowding level and hydrophobicity of the microenvironment can be adjusted conveniently during the thermally induced aggregation of the polymers, which has exhibited advantages for activity regulation of the biomacromolecules. Due to the suitable number of amino pendants in the CS backbone to provide binding sites for complexation with proteins, and a proper amount of OEG dendron pendants to provide sufficient shielding effects to form a microenvironment along the polymers, **DCS** exhibited the best activity protection capability towards the proteins. We therefore believe that these stimuli-responsive polymers are promising for the construction of a bionic system to ensure biomacromolecule activity.

Experimental

Materials and reagents

The OEG-based dendronized methacrylate monomer (**M3**),²³ dendronized homopolymer (**PG1**),²³ dendronized chitosans (**DCSs**),²⁴ and comonomer carrying a 4-nitrophenyl active ester (**MS=S**)²⁹ were synthesized according to previous reports. Myoglobin (Mb), bovine serum albumin (BSA), and lysozyme (LYZ) were purchased from Sigma-Aldrich. Dithiobis (succinimidylpropionate) (DTSP) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Tokyo Chemical Industry, Japan. Other reagents were purchased at reagent grade and used without further purification. Doubly deionized water was used in all experiments.

Synthesis of PG1S

M3 (200 mg, 0.284 mmol), **MS=S** (5.5 mg, 0.014 mmol), and AIBN (1.0 mg) were dissolved in dry DMF (0.3 mL) in a Schlenk tube. The solution was deoxygenated and stirred at 65 °C for copolymerization. After 4 h, the mixture was cooled to room temperature. Then, the copolymer was purified by column chromatography (silica gel, DCM as eluent) to yield the copolymer as a colourless oil (125 mg, 61%). ¹H NMR (500 MHz, DMSO-*d*₆, Fig. S2†): δ = 1.04 (br, CH₃), 1.25–1.31 (m, CH₂), 3.49–3.67 (m, CH₂), 3.99 (br, CH₂), 4.78 (d, CH₂), 6.56 (br, Ar-H), 7.56 (br, Ar-H), 8.25 (br, Ar-H).

Preparation of dendronized copolymer–protein conjugates and measurement of the loading efficiency for the proteins

The preparation of copolymer–protein conjugates was achieved according to a method reported previously.^{46,47} PB solution (pH = 8.0) containing Mb (0.5 mg mL^{−1}) was mixed with **PG1S** at different mass ratios (at 25 °C) and stirred for 6 h. Then, the mixed solution was separated by using a filter membrane

(0.1 μm). Meanwhile, the content of free protein remaining in the filtrate was determined by the Bradford method, to determine the loading efficiency of **PG1S**. The loading efficiency was calculated according to the following formula (1):

$$\text{Loading efficiency (\%)} = \left(1 - \frac{A_b}{A_0}\right) \times 100\% \quad (1)$$

where *A*₀ and *A*_b are the solution absorbance at 595 nm of proteins initially added into the solution and remaining in the filtrate, respectively.

Characterization

¹H NMR spectra were recorded on a Bruker ADVANCE NEO 500 M (¹H: 500 MHz) spectrometer at 60 °C, and chemical shifts were reported as values (ppm) relative to TMS.

Gel permeation chromatography (GPC) measurements were carried out on a Waters GPC e2695 instrument with a 3 column set (Styragel HR3 + HR4 + HR5) equipped with a refractive index detector (Waters 2414), and DMF (containing 1 g L^{−1} LiBr) as eluent at 45 °C. The calibration was performed with poly(methyl methacrylate) standards in the range of Mp = 2580–981 000 (Polymer Standards Service-USA Inc.).

UV/vis absorption spectra (between 350 and 450 nm) and turbidity measurements were performed on a JASCO V750 UV/vis spectrophotometer with a thermo-controlled bath. Polymer solutions were placed in the spectrophotometer (path length 1 cm) under the heating or cooling rate of 0.2 °C min^{−1}. *T*_{cp} is determined at which the transmittance had reached 50% of its initial value at λ = 700 nm. The concentration of the polymer is 2.5 mg mL^{−1}. The samples were all prepared in buffer solution (PBS, pH 7.0, 50 mM).

DLS measurements were performed on DynaPro Nanostar (Wyatt Technology Corporation). The solutions of the polymers and their mixtures with proteins were placed under the heating or cooling rate of 0.2 °C min^{−1}. All *R*_hs were calculated according to the intensity.

Fluorescence experiments were carried out on a Horiba Jobin Yvon Fluorolog^R-3 with FluorEssence. The spectra of all samples were obtained with the addition of ANS (30 μM) at the excitation conditions of 350 nm and emission was scanned between 410 and 650 nm with a 4 nm band-pass of the excitation and emission slits.

FT-IR spectra were recorded using a Nicolet Is50 spectrophotometer with a diamond/ZnSe universal ATR sampling accessory. The spectra of all samples in deuterioxide were obtained in transmission mode from 400–4000 cm^{−1} at a resolution of 4 cm^{−1} averaging 64 scans. All samples were prepared in D₂O.

CD spectra were recorded in the far-UV region using a JASCO-815 spectrometer (Jasco, Japan). The CD spectra were obtained in the range between 190 and 290 nm. Quartz 0.5 cm path length cells were used for all CD experiments. The recording parameters were: scan speed 200 nm min^{−1}, response time 4 s, slit width 1 mmol L^{−1}, bandwidth 4.0 nm, and step resolution 0.2 nm. From 3 to 6 scans were run for each sample.

QCM-D measurements were carried out on a Q-Sense E4 (Q-Sense, Sweden) equipped with a multichannel pump (IPC Ismatec SA, Switzerland). The grafting of protein to the gold cells (5 MHz) was performed according to the method reported previously.³¹ Firstly, the bare gold surface was exposed to a DMSO solution of 2 mg mL⁻¹ DTSP. DTSP forms SAMs spontaneously through covalent interactions between the sulfur group and the metal surface. Then, the surface was rinsed with pure DMSO several times and dried in a nitrogen stream. In the next step, the gold cells with SAMs was immersed in a PBS solution of Mb (10 mL, 0.1 mg L⁻¹) in an ice bath for 6 h. The gold cells were further purified by deionized water several times. Subsequently, the cells were installed into their corresponding channels. The PBS (for the acquisition of the base line) and dendronized polymers were injected successively, and the changes of Δf and ΔD were recorded over time. The changes of the resonance frequency (Δf) of the crystal reflect the adsorbed mass, while the energy dissipation (ΔD) provides information on the viscoelastic properties of the adsorbed layer.³¹ $\Delta m = C \times \Delta f$, where Δm = adsorbed mass [ng cm⁻²], $C = -17.7$ ng (cm² Hz)⁻¹.

ITC measurements were performed on an isothermal titration calorimeter (TA instruments, TAM IV), to evaluate the thermodynamics of the binding process between the samples and Mb aqueous solutions. The instrument was calibrated electrically with a precision better than ($\pm 0.1\%$). The **PG1** or **DCS** in the syringes was titrated into the solutions containing myoglobin. The titrating solution was automatically added in aliquots of 4.974 μ L with a 30 min interval between each injection, and the system was stirred at 60 rpm with a gold propeller. Each experiment was repeated at least 3 times, and the heat evolved per injection was integrated using the software especially designed for TAM IV. The data was analysed using the ITC software with the “independent” model and Boltzmann formula $y = A_2 + (A_1 - A_2)/(1 + \exp((x - x_0)/dx))$ was used for curve fitting.

Mb activity

The activity data of Mb was obtained according to its reaction rate with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 1 mg mL⁻¹) and H₂O₂ (0.3%) as the substrate within 1 min. The Mb solution (0.05 mg mL⁻¹) was mixed with different concentrations of **PG1** or **DCS** and kept (mass ratio of polymers to protein from 10 to 50) at 25 °C and 60 °C for different time intervals. Subsequently, 1 mL of solution of **PG1/Mb** or **DCS/Mb** was mixed with ABTS (1 mL) and H₂O₂ (1 mL), and the absorbance changes of the solutions at 405 nm were tracked with the stirring speed at 200 rpm for 1 min immediately. Then, their activity was calculated through formula (2).

$$U = \frac{V\Delta A}{M\Delta t} \quad (2)$$

where V is the volume of the mixed solutions in mL; ΔA is the absorbance change of the solution at 405 nm before and after

the reaction; Δt is the change of time (1 min); M is the mass of protein in mg.

Author contributions

Y. Y. and J. Y. conducted all experiments. W. L. and A. Z. designed and directed the project. Y. Y. wrote the draft, and W. L. and A. Z. cowrote the paper. All authors discussed the results, commented on the manuscript, and have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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