Artificial chaperones based on thermoresponsive polymers recognize the unfolded state of the protein

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Abstract

The chaperone-like activity of thermoresponsive polymers based on poly(dimethylaminoethyl methacrylate) (PDMAEMA) was studied on two different proteins, glyceraldehyde-3-phosphate dehydrogenase and chicken egg lysozyme. The polymers do not interact with the folded protein at room temperature but form a complex upon heating to either protein unfolding or polycation phase transition temperature. A PDMAEMA-PEO block copolymer with a dodecyl end-group (d-PDMAEMA-PEO) as well as PDMAEMA-PEO without the dodecyl groups protected the denatured protein against aggregation in contrast to PDMAEMA homopolymer. No effect of the polymers on the enzymatic activity of the client protein was observed at room temperature. The polymers also partially protected the enzyme against inactivation at high temperature. The results provide a platform for creation of artificial chaperones with unfolded protein recognition which is a major feature of natural chaperones. The thermoresponsive polymers can be also used for reversible sedimentation of the folded enzyme.

KEYWORDS: thermoresponsive polymer; artificial chaperone; protein aggregation; protein-polyelectrolyte complexes; molecular recognition; poly(dimethylaminoethyl methacrylate); protein stabilization; chaperone.

Abbreviations

AIBN, Azobisisobutyronitrile; CD, circular dichroism; d-, dodecyl; DMAEMA, dimethylaminoethyl methacrylate; CPA, 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid; CTA, chain transfer agent; DLS, dynamic light scattering; DSC, differential scanning calorimetry; FTIR, fourier-transform infrared spectroscopy; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPC, gel permeation chromatography; MWCO, molecular weight cut-off; PDMAEMA, poly(dimethylaminoethyl methacrylate); PEO, polyethylene oxide; PEO-dodecyl, poly(ethylene glycol) methyl ether 2-(dodecylthiocarbonothioylthio)-2-methylpropionate; RAFT, reversible addition-fragmentation chain transfer; ^{1}H-NMR, proton nuclear magnetic resonance.

Introduction

Searching for a way for protection of enzymes against aggregation is of special interest for modern biochemistry, bioengineering, and biotechnology because of a growing usage of proteins. Since the use of natural molecular chaperones is very complicated and often impossible at hard conditions, artificial chaperones can be used for this purpose. Among the suggested strategies to create artificial chaperones, on can mention the use polysaccharide gels [1,2], detergents [3] or polymeric micelles [4,5] with following action of cyclodextrins, as well as cyclodextrins themselves [6]. These approaches are very promising though cyclodextrin itself can affect the proteins [7,8]. Another way is the use of liposomes [9]. Finally, charged molecules are very promising as artificial chaperones: anionic surfactants [10] and amphipathic anionic nanoparticles [11] or cationic nanostructured hydrogel [12]. An easy release of the

bound protein under increasing salt concentration or shift in pH value is an important advantage of the use of charged polymers, i.e. polyelectrolytes.

Synthetic polyelectrolytes of different nature can be an efficient tool to protect proteins against aggregation and to reactivate protein from pre-formed aggregates [13,14]. Indeed, protein aggregation can be suppressed by addition of polyanion [15,16] or polycation [17] depending on the protein charge. Linear [13] and dendrimer [18–20] polyelectrolytes were also suggested as a prospective way to treat amyloid aggregation. The protective power of the polyelectrolyte was shown to depend on the nature of the charged groups (sulfated and sulfonated polymers are more efficient than polyphosphates [21] and polycarboxylates [15]), amount of the hydrophobic groups in the polymer chain [22], and degree of polymerization [15,21]. Furthermore, protection of the protein against aggregation can be achieved even on a “wrong” side of the pH scale, when the protein and the polyelectrolyte are similarly charged [23], since the binding is driven by “properly” (oppositely to the polymer) charged patches on the protein surface [24,25].

Thus, the mentioned polymers can be considered as artificial chaperones. It is noteworthy that chaperone-like activity of them is comparable to the activity of the natural chaperones and sometimes can be even much higher, as is the case for long chains of poly(styrene sulfonate) at high temperature [14]. One of the major problem is that many polyelectrolytes are prone to induce denaturation of the bound protein. Thus, relatively hydrophobic sulfated or sulfonated polymers efficiently protect the protein against thermal aggregation but cause the enzyme inactivation [26–28]. The use of polyanions with higher degree of polymerization as well as more hydrophilic polymers is a possible way to decrease negative influence on the protein structure. However, all polyelectrolytes efficiently interacting with the protein at room temperature can alter its structure and behavior [29–31]. It is especially important for sulfated and sulfonated polymers, enhanced efficiency of which seem to be associated with tight binding with the protein [21].

On the other hand, natural chaperones are capable of recognition of unfolded or misfolded state of the protein. For example, the members of chaperonins family which were found in all domains of life [32] are known to bind unfolded protein via the inner part of the cavity and then release the folded protein [33,34]. Other chaperones exhibit different functions, including passive suppression of protein aggregation as is the case of many small heat shock proteins [35], disruption of pre-formed protein aggregates [36], and transfer of the unfolded/misfolded protein to proteasomes for further degradation [35,37]. Together these proteins compose a very complicated chaperone system of cell. Despite promiscuous functions, all chaperones can recognize unfolded/misfolded state of the protein. In the present work, we attempt the use of thermoresponsive polymers to get a kind of molecular recognition of the unfolded state of the protein. The change in hydrophobic/hydrophilic ratio around collapse point can be useful for regulation of interaction with proteins. Non-charged thermoresponsive polymers were suggested for protein refolding [5,38,39]. To gain high anti-aggregation activity, we selected thermoresponsive polyelectrolyte, poly(dimethylaminoethyl methacrylate), which is known to possess phase transition and change the solubility in water solution at near-physiological temperature [40–43]. We hypothesize that the interaction of the polymer with the protein should depend on the temperature and on the state of the protein since the interaction of the polymer with itself changes when heated because of the change in the hydrophobic and electrostatic contribution. To stabilize the complexes in solution after collapse of the polymer, we synthesized block copolymers of poly(dimethylaminoethyl methacrylate) and poly(ethylene oxide) aiming to get small particles or micelles at high temperature.

Materials and methods

Synthesis of the polymers

Materials. All solvents were from Sigma-Aldrich and used as received unless otherwise stated. Monomer 2-(Dimethylamino)ethyl methacrylate (DMAEMA, Acros organics) was run through an anhydrous alumina column (Al_2O_3, Merck) and distilled in vacuum to remove the inhibitors.

Azobisisobutyronitrile (AIBN, Fluka) was recrystallized from methanol and dissolved into 1, 4-Dioxane (VWR). Chain transfer agents (CTA) 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPA) and Poly(ethylene glycol) methyl ether 2-(dodecylthiocarbonothioylthio)-2-methylpropionate (PEO-dodecyl, avrg. M_n 1100) were used as received and bought from Sigma-Aldrich.

Synthesis of PDMAEMA. The polymers were synthesized via one-pot reversible addition-fragmentation chain transfer (RAFT) polymerization.

1 g DMAEMA (6.35 mmol), 11.7 mg CPA (0.042 mmol) were weighed into a 25 mL round bottom flask followed by addition of 3.335 mL AIBN / dioxane solution (concentration 0.0042 mmol/mL, 0.014 mmol) and 1.665 ml dioxane measured with a precision pipette. The flask was sealed and the reagents stirred until dissolved / homogenized. Three freeze-thaw cycles were conducted to remove oxygen and flask filled with nitrogen. The polymerization was initiated by immersing the reaction flask into an 80 °C oil bath. The reaction was left to stir in the bath for 6 h and was stopped by placing the flask into ice-water bath for 10 minutes and opening the seal to let oxygen in. 5 mL of deionized water was added to the reaction mixture and stirred until homogenized. The resulting mixture was then moved to a dialysis membrane with MWCO 3500 and dialyzed against de-ionized water for 2 days changing the dialysis solvent at least 2 times a day. The dialyzed product was freeze dried and the final product was obtained.

Synthesis of d-PDMAEMA-PEO. The synthesis of d-PDMAEMA-PEO was conducted in the same manner as the synthesis of PDMAEMA with the only differences being the CTA used and the molar-ratio of the initiator.

1 g DMAEMA (6.35 mmol), 46.2 mg PEO-dodecyl (0.042 mmol) were weighed into a 25 mL round bottom flask followed by addition of 1 mL AIBN / dioxane solution (concentration 0.0042 mmol/mL, 0.0042 mmol) and 4 ml dioxane measured with a precision pipette. The rest of the synthesis was performed as described in the synthesis of PDMAEMA.

End-group modification of d-PDMAEMA-PEO. The modification of dodecyl end-group was performed with a modified version of the method developed by Perrier et al. [44]

0.1 g of d-PDMAEMA-PEO (from which end groups 0.0016 g, 0.00624 mmol) and 0.0154 g of AIBN (0.0936 mmol) was weighed into a 25 ml flask and the flask was sealed with a septum. The mixture was then purged with argon for 5 minutes and 5 ml of dried dioxane was added through the septum with an argon washed needle. The reagents were dissolved and the mixture was stirred with argon bubbling for 35 min at room temperature. The flask was then placed into oil bath at 80 °C with argon bubbling for additional 5 min and then left to react for 19 h. The reaction was stopped by opening the flask to air and immersing into a cold water bath.

The product was purified by precipitation to cold n-hexane 3x. The purified product was then dissolved in methanol, solvents evaporated in vacuum and dried at 130 °C for 1 h to get rid of the last solvent traces.

Proteins

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was isolated from rabbit skeletal muscle and purified by Scopes method [45]. The purified protein was characterized by specific activity of 80 µmol NADH/min per mg of the protein. Before experiments, suspension of GAPDH in ammonium sulfate was dialyzed against the 1000-fold volume of 10 mM HEPES buffer, pH 7.6 or 7.0.

Chicken egg **lysozyme** was purchased from Sigma-Aldrich.

Concentration of the proteins was measured spectrophotometrically using $A_{280}^{0.1\%}$ value of 1.0 and 2.3 for GAPDH and lysozyme respectively.

Methods

Proton nuclear magnetic resonance (^{1}H NMR). ^{1}H NMR spectra were recorded with a Bruker Avance III 500 spectrometer to determine the polymerization conversion and the structure- and purity of the synthesized polymers.

Fourier-transform infrared spectroscopy (FTIR). FTIR spectra were recorded from solid samples with a PerkinElmer Spectrum One spectrometer to verify the structure of synthesized polymers.

Gel permeation chromatography (GPC). GPC measurements were conducted with Waters Acquity system and columns running THF with 1% of tetra-n-butylammonium bromide. The columns were calibrated with poly(methyl methacrylate) standards.

Dynamic light scattering (DLS). Dynamic light scattering measurements were performed using a Brookhaven Instruments BI-200SM goniometer, a BI-9000AT digital correlator, and a Coherent Sapphire 488-100 CDRH laser operating at wavelength of 488 nm. Scattering angle was 90°. The measurement cell was thermostated at 22°C.

Isothermal titration calorimetry. ITC experiments were performed using a Microcal VP-ITC calorimeter at 25°C and 65°C. A solution of protein (GAPDH 0.5 g/l or lysozyme 0.17 g/l) was titrated by successive 20-µl injections of the 1 g/l d-PDMAEMA-PEO solution. The time intervals between the injections were 5 min. To compare the heat effect with a heat effect of the dilution of the polymers, he same polymer solutions were titrated into the buffer. All samples were degassed before the experiment. Data were analyzed using MicroCal Origin 7.0 software. The binding isotherm at 65°C was fitted with "one set of sites" model after subtraction of the reference data.

Differential scanning calorimetry (DSC). DSC measurements were performed on Microcal VP-DSC microcalorimeter. Polymer concentration was 1 mg/ml, protein concentration was 0.2–1.0 mg/ml; scanning rate was 1°C/min. All samples were degassed before measurement. The chemical baseline was calculated and subtracted separately for each individual peak with the use of Arina 2 software (Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Russia).

Enzyme activity assay. Enzymatic activity of **GAPDH** was determined spectrophotometrically from the increase in the absorption at 340 nm due to NADH accumulation in the forward reaction. A 1-ml cuvette was filled with the reaction mixture (100 mM potassium phosphate, 100 mM glycine, 5 mM EDTA, 1 mM NAD^+ and 1 mM glyceraldehyde-3-phosphate, pH 8.9). The reaction was started by the injection of 2–5 µg of the protein, and the increase in the absorption was recorded for 10–20 s. The amount of the

protein catalyzed the transformation of 1 μmol of NAD^+ per minute was taken as the unit of the enzymatic activity. The specific activity was the number of activity units per 1 mg of the protein. GAPDH activity in the presence of the polymers was measured after 30-min incubation of the mixture containing GAPDH 0.5 mg/ml and polyanion 1.0 mg/ml in 10 mM HEPES buffer, pH 7.6. The observed activity values were normalized to the activity of native GAPDH. All experiments were carried out at 25 °C using an Implen NanoPhotometer instrument.

For measurement of thermal inactivation of GAPDH, the samples of free GAPDH and its mixtures with PDMAEMA-PEO or d-PDMAEMA-PEO were incubated at 55°C in 10 mM HEPES buffer, pH 7.6, and aliquots were taken for the measurement as described above. Concentration of GAPDH was 0.5 mg/ml; concentration of the copolymers was 1 mg/ml.

Enzymatic activity of **lysozyme** was determined from decrease in absorbance of cell suspension due to addition of the enzyme. The *E. coli* CR63 cells treated by freeze were used as a substrate. A 2.5 μg of the protein incubated with the polymers or alone was mixed with 150 μl of cell suspension, and optical density was measured at 400 nm for 15 min using a VersaMax microplate reader (Molecular Devices, USA). Negative control (buffer without enzyme) was subtracted from sample measurements. The activity values determined as a slope of linear part of the time dependence were averaged among at least three measurements and normalized to the activity of intact lysozyme.

SDS-PAGE. SDS-PAGE was run according to the standard procedure [46]. The mixtures of were heated up 75°C and cooled down. The samples as well as a reference solution of non-heated GAPDH were diluted twice with sample buffer and heated 5 min at 95°C.

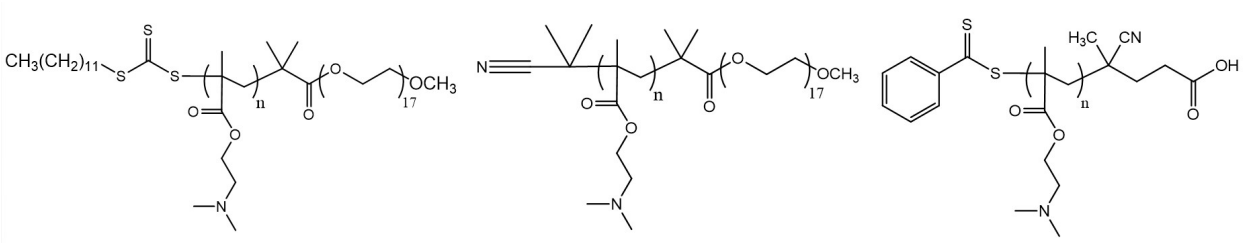
CD measurements. CD measurements in far UV were conducted using a JASCO J-815 CD spectrometer equipped with a PTC-423S/15 Peltier-type temperature control system. Spectra were recorded in the range from 200 to 260 nm and were corrected by subtracting the buffer baseline. A 0.5-mm cell was used, each point was measured for approximately 0.5 s. Protein concentration was 1 g/l.

Transmittance measurements. Temperature dependence of transmittance was measured using a JASCO J-815 CD spectrometer equipped with a PTC-423S/15 Peltier-type temperature control system.

The transmittance was measured at wavelength of 400 nm. All measurements were carried out with the use of 10 mm cuvettes. The heating rate was 1 °C/min. The start transmittance value at 25°C was set as 100%.

Results

Three polymers were compared: PDMAEMA homopolymer, PDMAEMA-PEO block copolymer with a dodecyl tail (d-PDMAEMA-PEO) and PDMAEMA-PEO block copolymer, where the hydrophobic dodecyl tail was removed. Structures and abbreviations are presented on the Scheme 1; see also Table S1 and Figure S1 for NMR characterization. Data for supporting information available at request (NMR spectra etc.). First, the phase transition temperatures of the polymers were measured by turbidimetry upon heating. A pronounced concentration dependence of the phase transition was observed in water but not in HEPES buffer at pH 7.6 (Figure S2). However, the polymers in HEPES buffer, pH 7.6, showed clearly different phase transition temperature (Figure 1, black lines). PDMAEMA homopolymer solution became cloudy after heating up to 75°C, showing phase transition at 63.5°C, whereas d-PDMAEMA-PEO became only slightly turbid at temperatures above 61°C and PDMAEMA-PEO stayed transparent up to 75°C. In all cases, the phase transition behavior of pure polymer solutions was completely reversible. It is noteworthy that d-PDMAEMA-PEO and PDMAEMA-PEO form multi molecular aggregates (Figure 2B) [47]. Apparent presence of large particles in solution of homopolymer hPDMAEMA may be due to insufficient ionic strength resulting in a polyelectrolyte effect [48].



d-PDMAEMA-PEO	**PDMAEMA-PEO**	**PDMAEMA**
block copolymer with dodecyl tail	block copolymer	homopolymer

Schema 1. Formulas of the polymers.

Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and chicken egg lysozyme were selected as model proteins for investigation of interaction with the polymers. Pure GAPDH denatures and aggregates forming large precipitating flakes approximately at 45°C (Figure 1, blue line). In the presence of d-PDMAEMA-PEO, the aggregation was retarded, and transmittance of the system at 58°C was around 40% (Figure 1A). Heating up to the temperature of the polymer phase transition resulted in further aggregation and transmittance decreased down to zero. However, the latter stage was reversible and when cooled the mixture became partially transparent again, and the transmittance during the second heating coincided with the second step of the first heating.

As for the other synthetic polymers, no aggregation of GADPH was observed in presence of PDMAEMA-PEO up to 75°C (Figure 1B), and PDMAEMA did not influence the GAPDH aggregation: the protein completely aggregated forming flakes and then precipitated that caused increase in the transmittance (Figure 1C).

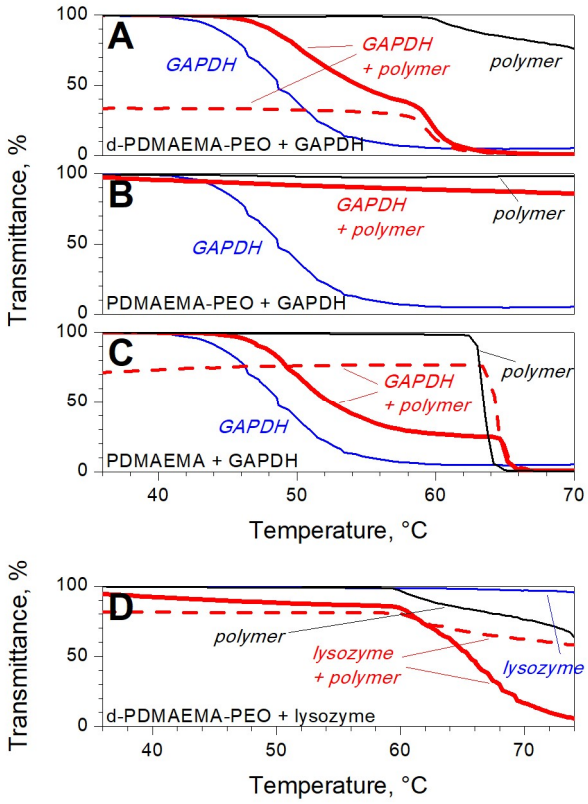


Figure 1. Temperature dependence of transmittance of free polymer solution (black line), free GAPDH (A-C) or lysozyme (D) solutions (blue line) and its mixtures (red lines). Solid and dashed lines for the mixtures represent first and second heating respectively. A. d-PDMAEMA-PEO and GAPDH, concentration was 1 g/l and 0.1 g/l respectively. Line 5 represents the same experiment as line 3 but the heating stopped at 57°C. B. PDMAEMA-PEO and GAPDH, concentration was 1 g/l and 0.1 g/l respectively. C. PDMAEMA and GAPDH, concentration was 0.5 g/l and 0.1 g/l respectively. D. d-PDMAEMA-PEO and lysozyme, concentration was 1 g/l and 0.5 g/l respectively. 10 mM HEPES buffer, pH 7.6.

Figure 2A shows a photo of heated up to 75°C and cooled down the pure GAPDH solution, and mixtures of GAPDH and PDMAEMA-PEO or d-PDMAEMA-PEO. Obviously, both copolymers suppressed GAPDH thermal aggregation and prevented formation of large flakes. Indeed, the solution of GAPDH and PDMAEMA-PEO was still completely transparent after heating, and the hydrodynamic diameter of the particles was 110 nm (Figure 2B-4). However, the hydrodynamic diameter of the particles formed after heating the GAPDH and d-PDMAEMA-PEO mixture was 500 nm (Figure 2B-5). These relatively large complexes were stable but prone to sedimentation after even gentle centrifugation. Aiming to reduce the size of the complexes and improve their stability, we tested particles formed under different conditions (Figure 2B). Smaller complexes with the diameter of ~180 nm were formed at lower concentration of the polymer, namely after heating of the mixture of GAPDH 0.1 g/l and d-PDMAEMA-PEO 0.1 g/l (Figure 2B-6). These complexes were much more stable than in the case of higher concentration of the polymer and did not sediment due to centrifugation at 5000g. SDS-PAGE indicates the presence of GAPDH in the soluble phase of GAPDH/d-PDMAEMA-PEO and GAPDH/PDMAEMA-PEO mixture but not of free GAPDH after 30-min incubation at 75°C, cooling down and centrifugation (Figure 2C). These results suggests a chaperone-like activity of the polymers, i.e. prevention of the thermal aggregation of the protein.

Since PDMAEMA is known as a weak pH-responsive polycation [40,43], we also tested the antiaggregation activity of d-PDMAEMA-PEO at lower pH where the polycation part of the polymer is more charged than at pH 7.6. The size of the complexes formed after the heating the GAPDH/d-

PDMAEMA-PEO mixture up to 75°C followed by cooling down was 100 nm suggesting an efficient suppression of the aggregation (Figure 2B-7). It is noteworthy that no aggregation was observed in HEPES buffer, pH 7.0 in the mixture of GAPDH and d-PDMAEMA-PEO as well as PDMAEMA, though larger complexes (300 nm) were formed in the latter case (data not shown).

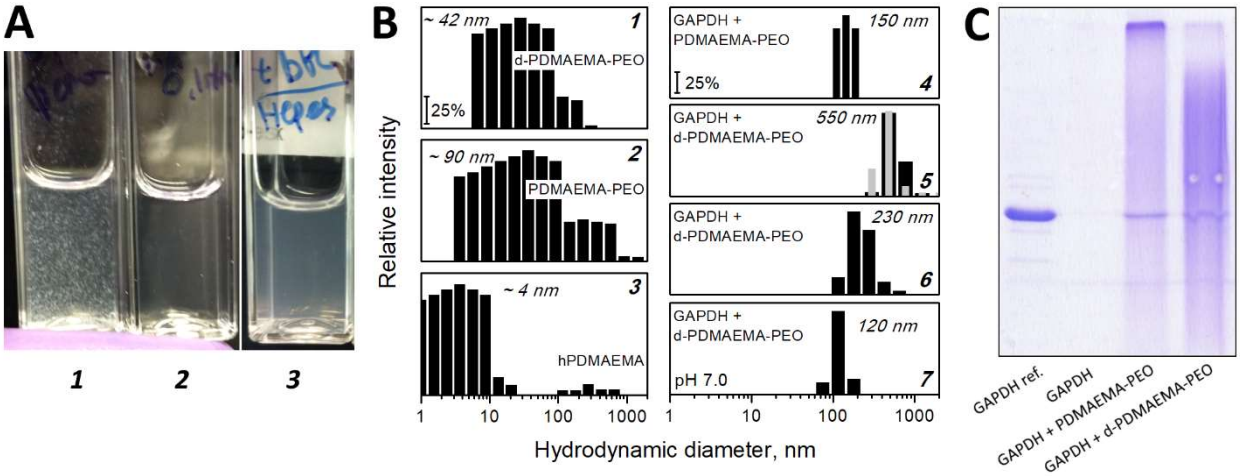


Figure 2. A. Photo of the solutions heated up to 75°C with a heating rate of 1°C/min in CD spectrometer and cooled down: free GAPDH 0.1 g/l (1), GAPDH 0.1 g/l + PDMAEMA-PEO 1.0 g/l (2), and GAPDH 0.1 g/l + d-PDMAEMA-PEO 1.0 g/l (3). B. Hydrodynamic diameter of free polymers in solution (1–3), and its mixtures with GAPDH heated up to 75°C and cooled down: GAPDH 0.1 g/l + PDMAEMA-PEO 1.0 g/l (4), GAPDH 0.1 g/l + d-PDMAEMA-PEO 1.0 g/l (5), GAPDH 0.1 g/l + d-PDMAEMA-PEO 0.1 g/l (6), GAPDH 0.1 g/l + d-PDMAEMA-PEO 0.5 g/l (7). 10 mM HEPES buffer, pH 7.6 (1–6) or 7.0 (7). Gray bars in 5 correspond to the sample heated up to 57°C (see Figure 1A, line 5). C. SDS-PAGE of GAPDH and the supernatant of samples incubated for 30 min at 75°C, cooled down and centrifuged: GAPDH 0.1 g/l, GAPDH 0.1 g/l + PDMAEMA-PEO 0.1 g/l, and GAPDH 0.1 g/l + d-PDMAEMA-PEO 0.1 g/l.

To gain insight into the aggregation processes, DSC experiments were performed (Figure 3). Beside the positive enthalpy signals originating from the denaturation of GAPDH (asterisks in Figure 3A), remarkable negative peak corresponding to GAPDH aggregation was observed in the DSC curve (arrows in Figure 3A). Addition of either of the copolymers changed the peak. In the presence of d-PDMAEMA-PEO, the peak became smooth and shifted to the lower temperature. In all likelihood, this peak reflect co-

aggregation and formation of the stable complexes of protein and polymer. In the presence of PDMAEMA-PEO, the negative peak was even higher and shifted to the higher temperature. Since the solution was transparent up to 75°C in contrast to heated free GAPDH solution (Figure 1B), interaction of the polymer and GAPDH occurred before the negative peak, and therefore this peak seem to reflect additional complexation of co-aggregation due to phase transition of the polymer.

As for the positive peak corresponding to the protein denaturation, no remarkable changes were observed after addition of copolymers (see peaks below 60°C in Figure 3B-C). However, the peak corresponding to the collapse of the copolymers changed. Thus, addition of GAPDH 0.2 g/l slightly shifted the peak to higher temperature (from 63.4 to 64.0°C), and addition of GAPDH 1 g/l shifted the peak for 5°C (Figure 3B). Furthermore, the signals of the first and the second heating with GAPDH in the mixture differed from each other in contrast to completely reversible phase transition of pure d-PDMAEMA-PEO. This indicates additional binding or structural rearrangement induced by the copolymer phase transition. As for the PDMAEMA-PEO, it is difficult to conclude from comparison of curves corresponding to free PDMAEMA-PEO and its complex with GAPDH because the peaks corresponding to collapse of the polymer are small and wide (Figure 3C).

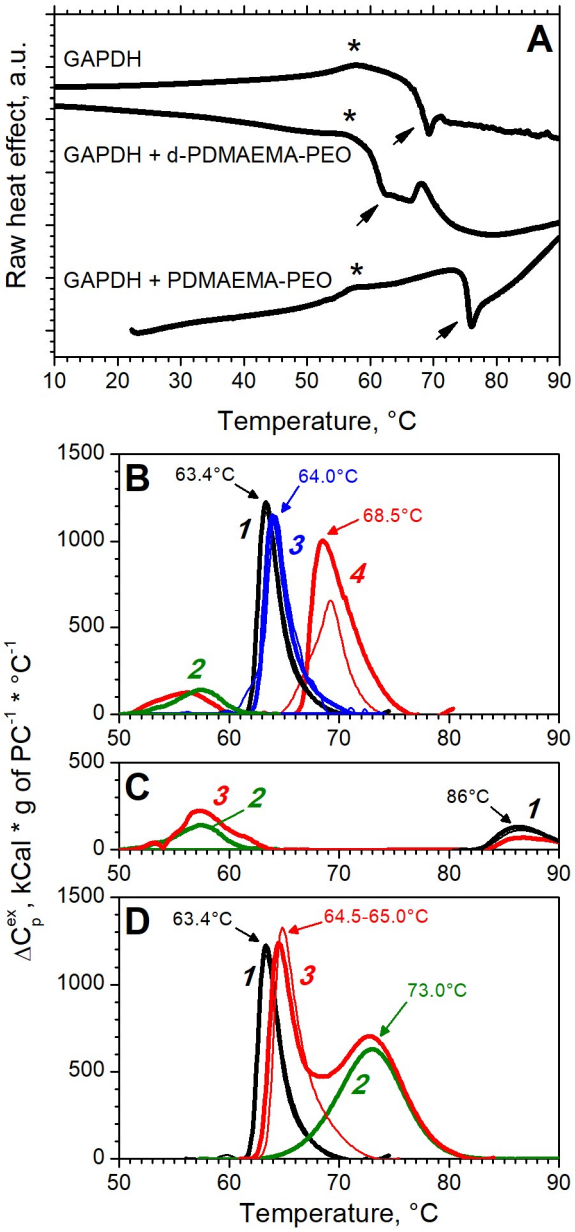


Figure 3. DSC curves of the polymers and proteins mixtures. A. raw DSC curves showing protein aggregation, asterisks and arrows represent the protein denaturation and aggregation peaks respectively. Concentration of the polymers 1 g/l, GAPDH 1 g/l. B. Temperature dependence of the excess heat capacity of d-PDMAEMA-PEO (line 1), GAPDH (2), and their mixture with different concentration of GAPDH, 0.2 g/l (3) and 1 g/l (4). Concentration of the polymer is 1 g/l. C. Temperature dependence of the excess heat capacity of PDMAEMA-PEO 1 g/l (line 1), GAPDH 1 g/l (2), and their mixture in the same concentrations. D. Temperature dependence of the excess heat capacity of d-PDMAEMA-PEO 1 g/l (line 1), lysozyme 0.5 g/l (2), and their mixture in the same concentrations. In B-D panels, thick and thin lines correspond to the first and the second heating respectively.

In order to further probe the effects of the polymer phase transition on the interaction, different thermal treatments were made. Copolymer d-PDMAEMA-PEO was selected because of clear phase transition point. First, the same transmittance experiment with d-PDMAEMA-PEO 1 mg/ml and GADPH 0.1 mg/ml was made with the heating stopped at 57°C, which is lower than phase transition temperature of the d-PDMAEMA-PEO (Figure 1A). The size of the formed complexes was the same as in the case of heating up to 75°C, where the polymer underwent phase transition (Figure 2B, gray bars in 5). Therefore, phase transition of the polymer is not necessary for the protein protection against aggregation.

However, it is unclear whether the polymer interacts with native GAPDH at room temperature or the interaction starts after the protein denaturation. We adopted ITC for direct measurement of the binding parameters at 25°C. No difference was observed between titration of GAPDH solution and the buffer with the d-PDMAEMA-PEO copolymer (Figure 4A). Therefore, we can conclude that the polymers most likely bind only with the unfolded state of the protein but do not interact with a folded state.

Unfortunately, ITC experiments with GAPDH at temperature higher than d-PDMAEMA-PEO phase transition temperature are not possible due to the aggregation of free GAPDH. It is also difficult to shift phase transition temperature of the polymer without effect on the interaction as the degree of ionization of PDMAEMA changes with changes in conditions. Attempts to shift polycation phase transition temperature were unsuccessful: if it occurs at lower temperature (as in the presence of phosphate ions), large aggregates are formed instead of small soluble complexes (transmittance decreases down to zero). The best way is to alter pH, but it will change all interactions because of the change in the degree of PDMAEMA ionization. Therefore, egg white lysozyme, a protein with higher melting temperature than the phase transition temperature of the used polymers was chosen for the studies.

First, the interaction of the polymers and lysozyme were studied by measuring transmittance changes due to heating. Transmittance of the free lysozyme solution was constant up to 75°C suggesting the absence of aggregation. However, the curves corresponding to free d-PDMAEMA-PEO and its mixture with lysozyme differed unambiguously indicating the binding (Figure 1D). Mixture of lysozyme and d-

PDMAEMA-PEO became turbid after the phase transition of the polymer and transparent again after cooling. The second heating was accompanied with transmittance decrease almost the same as in case of free d-PDMAEMA-PEO, indicating an additional interaction of the polymer with unfolded lysozyme due to first heating.

The interaction was then studied with ITC. Lysozyme solution was titrated with d-PDMAEMA-PEO at two different temperatures, below or above the phase transition temperature of the polymer (Figure 4B). At 25°C, the difference between d-PDMAEMA-PEO titration into lysozyme and the control titration into the buffer was negligible, suggesting no interaction. Completely different picture was observed at 65°C: the protein/polymer titration isotherm remarkably differed from the control indicating the interaction. Apparent association constant was 2.4 ± 1.1 μM^{-1}. In other words, d-PDMAEMA-PEO binds the folded form of the protein at temperature higher than phase transition temperature.

This suggestion is corroborated also by DSC data (Figure 3D). In the presence of lysozyme, DSC peak corresponding to the phase transition of the polymer shifted to 2°C higher temperature indicating formation of the complex. It is noteworthy that the protein denaturation starts at approximately 65°C, but the polymer's peak on the second heating of the mixture coincides with the peak on the first heating suggesting that protein denaturation is not necessary for the interaction.

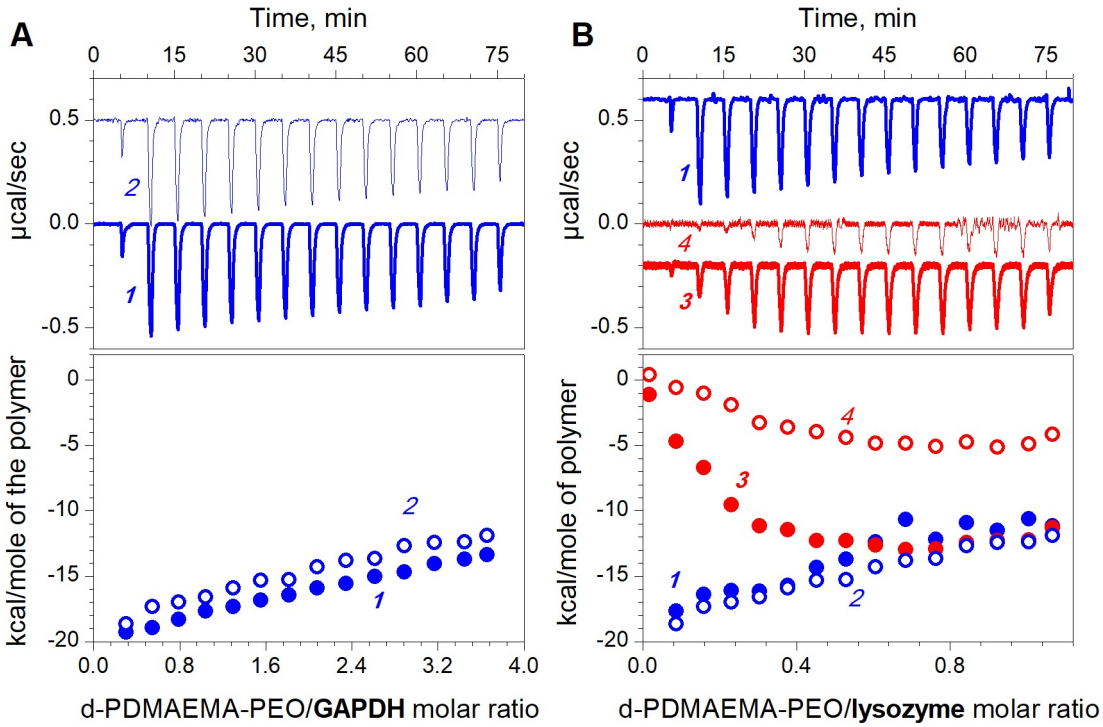


Figure 4. ITC data for GAPDH (A) and Lysozyme (B) titration with d-PDMAEMA-PEO. Top – raw data; bottom – heat effect of the each successive injections versus molar ratio of the reagents. Curve 1 and 2 (blue) represent titration at 25°C, curves 3 and 4 (red) – at 65°C. Thick lines and filled circles (1, 3) correspond to the protein solution titration with the polymer, thin lines and empty circles (2, 4) – to the control titration of the polymer into the buffer.

Then the influence of the polymers on the protein structure at different temperature was analyzed. At room temperature, no significant decrease of the GAPDH enzymatic activity was observed (Figure 5A). At 55°C, also no significant influence of the copolymers on thermal inactivation of GAPDH was observed (Figure 6, left). Noteworthy, 20-min incubation of the mixture of GAPDH and d-PDMAEMA-PEO at 65°C resulted in partial inactivation with residual activity of 10% in contrast to free GAPDH which was almost completely inactivated Figure 6, right). Besides, far UV CD spectra of free GAPDH and GAPDH and the presence of the d-PDMAEMA-PEO also were the same, and difference in CD appeared after the start of thermal denaturation of the protein (Figure 5B). These results corroborate a conclusion that the polymers do not interact with folded GAPDH but bind unfolded form of GAPDH, inducing its additional structural rearrangement.

As for the lysozyme, no influence of the polymers on the enzymatic activity was observed at room temperature as well as after heating up to 65°C and cooling down (Figure 5C). A slight inactivation was observed after the heating up to 75°C, but the activity was equal in all four samples, regardless the presence of the polymers. It is noteworthy that the difference in CD spectra which can indicate interaction appeared at 55-70°C, that is the temperature of phase transition of d-PDMAEMA-PEO. Therefore, changes of spectra corroborate the interaction of the protein with collapsed polymer.

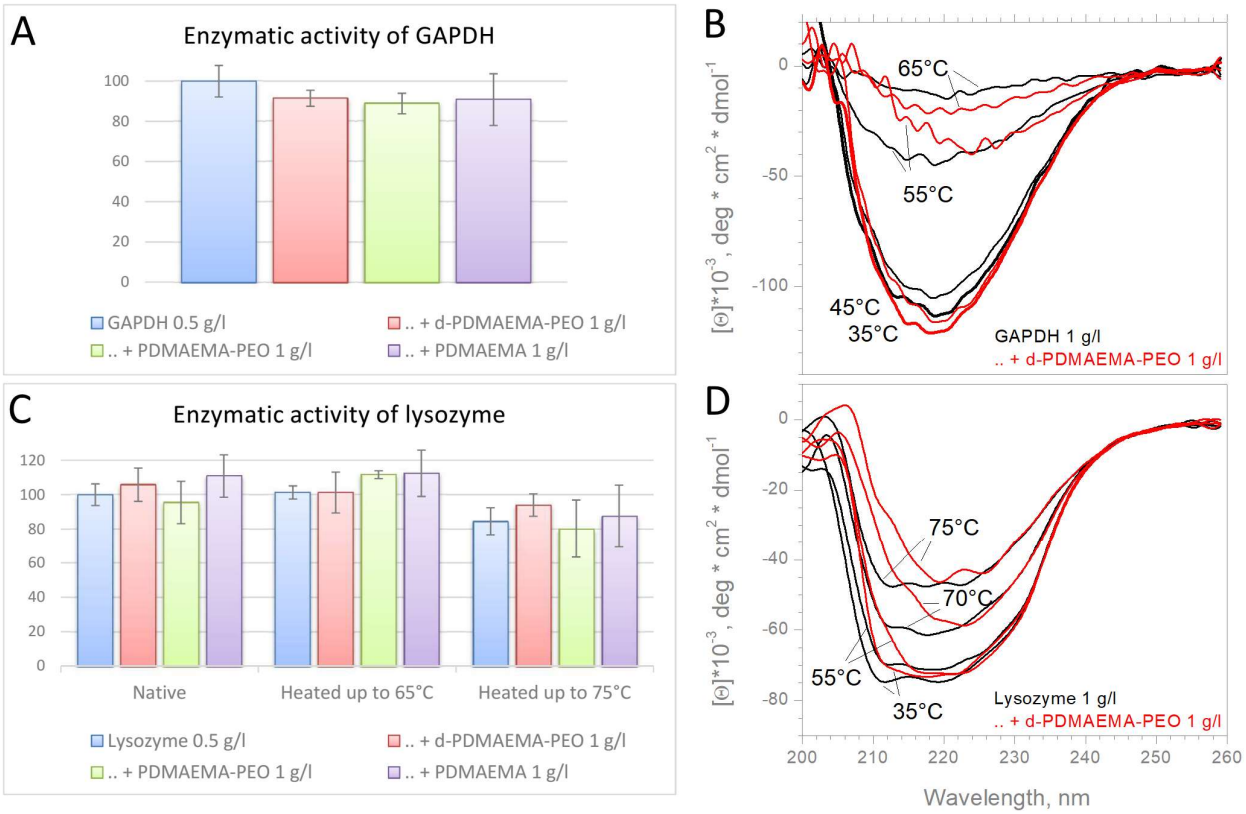


Figure 5. Enzymatic activity (A, C) and CD spectra (B, D) of GAPDH (A–B) and lysozyme (C–D). A. Enzymatic activity of GAPDH in free form and in the presence of the polymers. B. CD spectra of GAPDH in a free form (black lines) and in the presence of d-PDMAEMA-PEO (red lines) at different temperatures. C. Enzymatic activity of lysozyme in free form and in the presence of the polymers before and after heating up to 65°C and 75°C. D. CD spectra of lysozyme in a free form (black lines) and in the presence of d-PDMAEMA-PEO (red lines) at different temperatures.

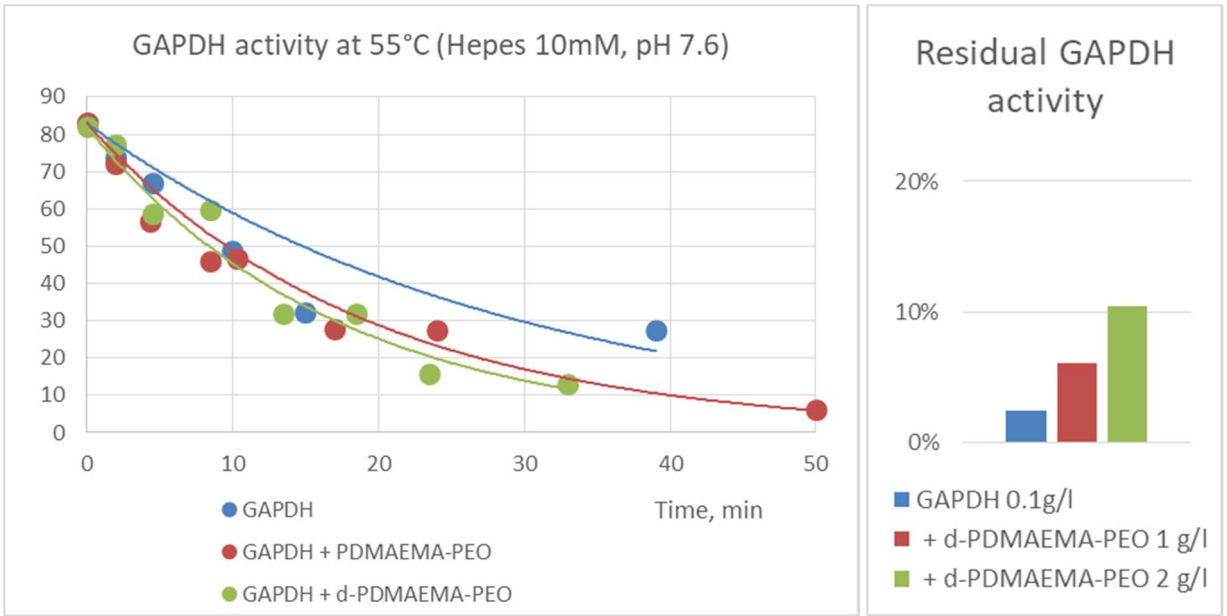


Figure 6. Left: Thermal inactivation of GAPDH in a free form and in the presence of the PDMAEMA-PEO or d-PDMAEMA-PEO at 55°C. Concentration of GAPDH was 0.5 mg/ml; concentration of the copolymers was 1 mg/ml. 10 mM HEPES buffer, pH 7.6. Curves represent fit with exponential decay. Right: Residual activity of GAPDH in a free form and in the presence of the d-PDMAEMA-PEO in different concentration after 20-min incubation at 65°C.

Discussion

We have investigated interaction of thermoresponsive polymers with two different proteins. Three different polymers were studied: homopolymer PDMAEMA and two copolymers PDMAEMA-PEO and d-PDMAEMA-PEO having a dodecyl end-group. When dispersed in water of HEPES buffer at pH 7.6 the polymer with dodecyl end-group and PEO block forms micelles at room temperature and aggregated above its phase transition temperature. Copolymer without the dodecyl tail (PDMAEMA-PEO) was very soluble and did not undergo aggregation due to heating, though according to DSC undergoes phase transition in HEPES buffer at pH 7.6.

When mixed with protein solutions, the PDMAEMA polymers seem not interact with native protein but efficiently bind unfolded proteins (Figure 7, top). Thus, no interaction was observed in the isothermal titration calorimetry experiments (Figure 4A), and zero effect on the protein structure and activity was shown at room temperature for both tested proteins. In contrast, the polymers interact with denatured protein. The interaction is evident from the transmittance measurements of the heated GAPDH mixtures, the data on structural changes of the protein (see CD spectra of GADPH, Figure 5B) as well as by the shifting of the DSC peaks corresponding to phase transition of the polymer (Figure 3B). Phase transition of the polymers is not necessary for the interaction since the polymers influence the protein aggregation at lower temperature than the phase transition, and the size of the complexes formed after

the heating of the GAPDH and d-PDMAEMA-PEO mixture up to 57°C and up to 75°C is the same (Figure 2B, panel 5).

On the other hand, collapsed polymers seem to interact with folded protein at high temperature (Figure 7, bottom). Thus, according to the isothermal titration calorimetry data (Figure 4B), d-PDMAEMA-PEO interacts with lysozyme at 65°C when the protein is folded. The shift of the polymer's DSC peak in the presence of lysozyme (Figure 3D) as well as changes in CD spectra of the protein in the presence of the polymer (Figure 5D) also shows that the polymer binds the folded protein at the temperature higher than phase transition point of the polymer.

The tested polymers have been shown to protect the unfolded protein against aggregation and partially against inactivation. Homopolymer PDMAEMA did not exhibit such protective activity, being included into aggregates of denatured GAPDH. In contrast, both copolymers (PDMAEMA-PEO and d-PDMAEMA-PEO) stabilized GAPDH against aggregation. Hydrophilic PEO provides steric stabilization of multimolecular aggregates upon heating forming core-shell structures. Dodecyl tail provides binding between hydrophobic moieties within such cores. Thus, small particles were formed in the presence of PDMAEMA-PEO and aggregation to slightly turbid solution occurred only in the course of heating up to 80-90°C. Relatively large but soluble complexes (100 – 500 nm depending on concentrations and conditions) were formed in the presence of d-PDMAEMA-PEO. In the other words, the polymers based on thermoresponsive polycation have been shown to share chaperone-like activity which consist in the binding of unfolded or misfolded protein and protection against aggregation (small heat shock protein-like activity [35,49,50]).

Summarizing above, we can consider PDMAEMA-based polymers as a promising platform for creation of artificial chaperones which recognize the unfolded state of the protein. There is no interaction of the PDMAEMA copolymers and the protein at room temperature when the enzyme is folded and active, which is a significant advantage compared with other artificial antiaggregant polymers. Indeed, the enzyme is intact and active, and its behavior does not change in the presence of the polymer. Protein

denaturation caused for example by elevated temperature will not result in the aggregation because of interaction of the unfolded enzyme molecules with the chaperone-like polymer. Furthermore, the polymer exhibited a certain protection of the enzyme against inactivation when the mixture was quickly heated up to the temperature higher than phase transition of the polymer (Figure 6, right). At lower temperature, such protective effect was not observed most likely because the polymer interacts only with denatured enzyme which is already inactive. Summarizing above, we conclude that PDMAEMA-based polymers share chaperone-like activity with zero influence on the folded enzyme.

Another prospective application of the thermoresponsive polymers with enzyme is reversible precipitation and dissolution controlled by temperature. The complexes of the folded lysozyme and collapsed polymers can be softly separated using centrifugation, then cooled down and stored or transferred to a different buffer. The same is true for the complexes of unfolded protein and uncollapsed polymer, which reversibly aggregate at high temperature because of collapsing of PDMAEMA. Therefore, the proposed polymers can be used to purify the native state of the protein: the mixture of denatured and native protein can be treated with polymer and then centrifuged. Free native protein will be in supernatant while denatured protein binding the polymer and forming large complexes will be in the sediment and then can be removed. Finally, these artificial chaperones can help misfolded protein to renature. Indeed, misfolded protein has to undergo further denaturation to reach correct folding, and the proposed polymers should protect the unfolded state against aggregation in the same manner as natural chaperones do. A few reversible cycles of heating/cooling could enable renaturation.

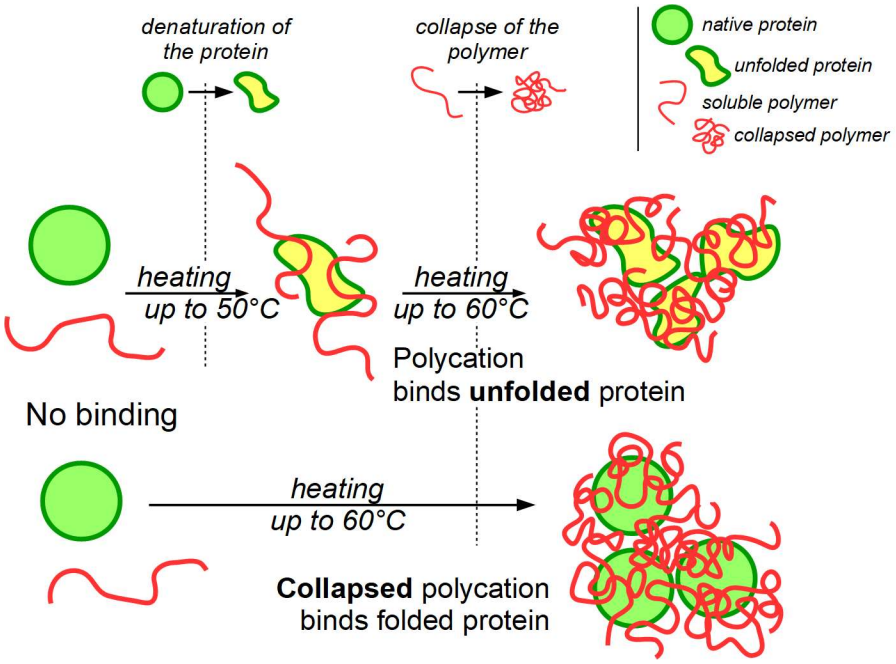


Figure 7. Scheme of two cases of polymer interaction with the protein: polycation binds unfolded protein, or collapsed polycation binds folded protein.

Conclusions

Thermoresponsive polymers based on poly-(dimethylaminoethyl methacrylate) were shown to interact with unfolded state of the protein at elevated temperature. At room temperature no interaction with the protein was observed, but the polymers interacted with folded protein at temperature higher than phase transition point of the polymer. The tested polymers did not induce any structural changes to the protein and their enzymatic activity was preserved below the native protein denaturation temperature. Copolymers of poly(dimethylaminoethyl methacrylate) and polyethylene oxide were found to protect the protein against thermal aggregation, and partially against thermal inactivation. Therefore the proposed polymers can be considered as a promising platform for creation of artificial chaperones which are able to recognize unfolded state of the protein as natural chaperones do.

Acknowledgements

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Supporting Information

NMR data and spectra of the polymers, temperature dependence of transmittance of d-PDMAEMA-PEO solutions in water and in 10 mM HEPES buffer, pH 7.6, and thermal inactivation of GAPDH in the presence of the copolymers.

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Supplementary Materials

Artificial chaperones based on thermoresponsive polymers recognize the unfolded state of the protein

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ABBREVIATIONS: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPC, gel permeation chromatography; PDMAEMA, poly(dimethylaminoethyl methacrylate); PEO, polyethylene oxide.

Table S1. Molar mass data on the PDMAEMA-based polymers.

PDMAEMA
M_n theoretical based on conversion = 13913 g/mol GPC (M_n, PDI) = 10100 g/mol, 1.24
d-PDMAEMA-PEO
M_n theoretical based on conversion = 13834 g/mol GPC (M_n, PDI) = 17000 g/mol, 1.27
PDMAEMA-PEO
M_n calculated assuming full deprotection = 16790 g/mol

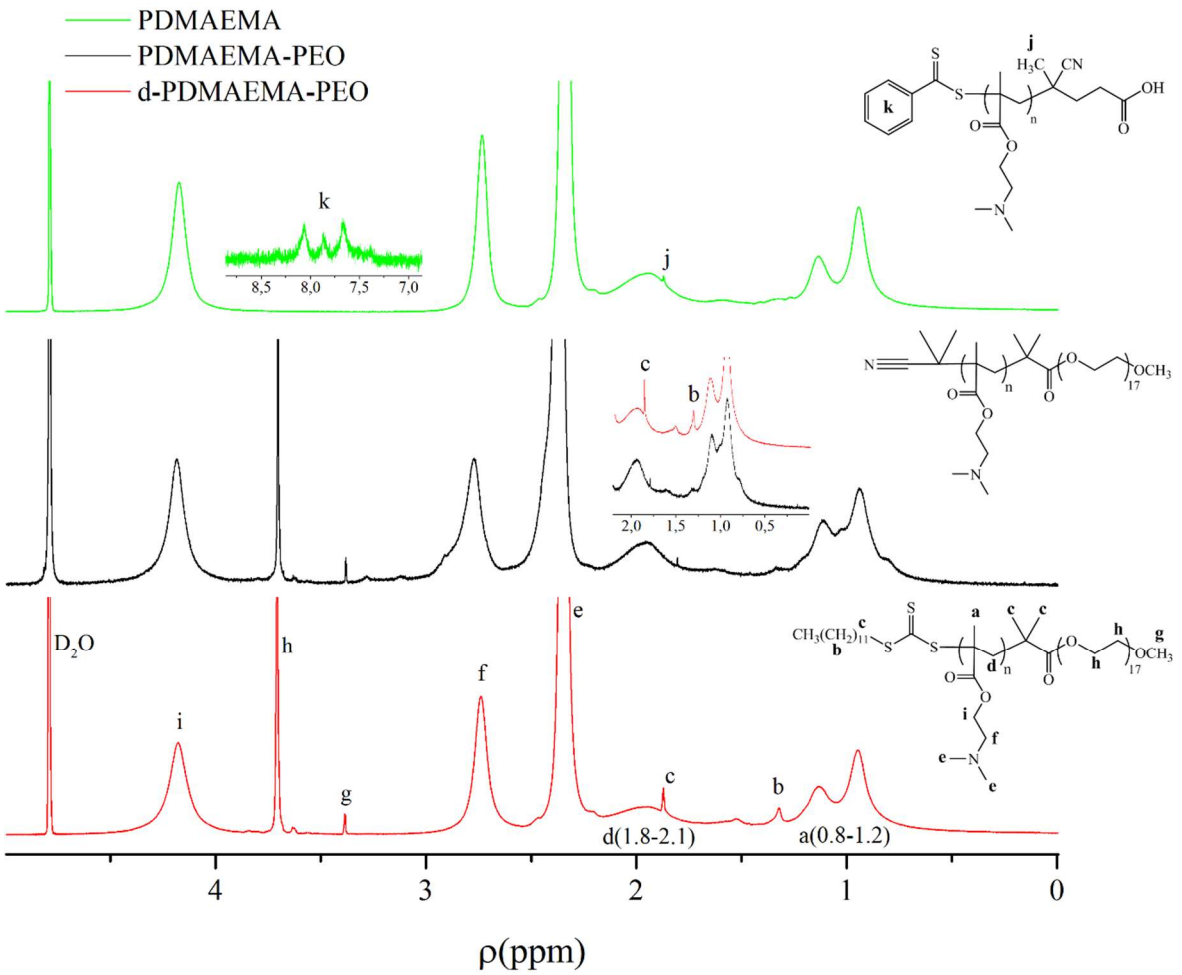

Figure S1. ^{1}H NMR spectra of PDMAEMA (top, green), PDMAEMA-PEO (middle, black), and d-PDMAEMA-PEO (bottom, red).

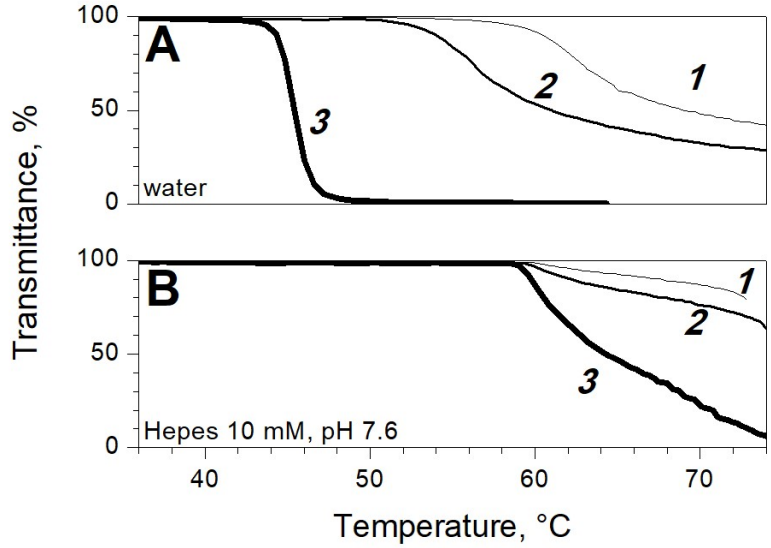

Figure S2. Temperature dependence of transmittance of d-PDMAEMA-PEO solutions in water (A) and in 10 mM Hepes buffer, pH 7.6 (B) with different concentration of the polymer: 0.5 g/l (curve 1), 1 g/l (2), and 2.5 g/l (3).