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AMPHIPHILIC COPOLYMERS REDUCE AGGREGATION OF UNFOLDED LYSOZYME MORE EFFECTIVELY THAN POLYETHYLENE GLYCOL

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Abstract

Certain amphiphilic block copolymers are known to prevent aggregation of unfolded proteins. To better understand the mechanism of this effect, the optical properties of heat-denatured and dithiothreitol (DTT) reduced lysozyme were evaluated with respect to controls using UV-Vis spectroscopy, transmission electron microscopy (TEM) and circular dichroism (CD) measurements. Then, the effects of adding Polyethylene Glycol (8000 Da), the triblock surfactant Poloxamer 188 (P188), and the tetrablock copolymer Tetronic 1107 (T1107) to the lysozyme solution were compared. Overall, T1107 was found to be more effective than P188 in inhibiting aggregation, while PEG exhibited no efficacy. TEM imaging of heat-denatured and reduced lysozymes revealed spherical aggregates with on average 250–450 nm diameter. Using CD, more soluble lysozyme was recovered with T1107 than P188 with β -sheet secondary structure. The greater effectiveness of the larger T1107 in preventing aggregation of unfolded lysozyme than the smaller P188 and PEG points to steric hindrance at play; signifying the importance of size match between the hydrophobic region of denatured protein and that of amphiphilic copolymers. Thus, our results corroborate that certain multi-block copolymers are effective in preventing heat-induced aggregation of reduced lysozymes and future studies warrant more detailed focus on specific applications of these copolymers.

Keywords

COPOLYMER SURFACTANT; POLOXAMER; POLYETHYLENE GLYCOL; LYSOZYME; HYDROPHOBIC INTERACTION

1. INTRODUCTION

Protein conformation is stabilized by both intramolecular crosslinks and entropic interactions between regions of the protein with the solvent. These structures create kinetic

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barriers resistant to the conversion from folded proteins into aggregation-prone molten-globule forms [1, 2]. However, upon solvent exposure to hydrophobic domains of molten-globules to the aqueous solvent, aggregation between adjacent unfolded proteins becomes energetically favorable. Moreover, solvent exposed hydrophobic domains of some oligomers adversely affect the stability of native proteins through the solvent interactions [3], resulting in cytotoxicity [4, 5]. The cytotoxic effects of traumatic injuries, such as tissue burns, electrical shock, freezing and etc., are partially the result of protein unfolding and aggregation. Also, the accumulation of misfolded proteins in human cell environment has been implicated in approximately 50 clinical disorders [6], including Alzheimer's disease, Parkinson's disease and type II diabetes [7].

Several natural protective mechanisms exist within highly crowded cytoplasm as well as the extracellular space of the eukaryotic cells through which protein's hydrophobically exposed regions are sequestered and denatured proteins are properly refolded. In response to elevated temperature, a class of heat-shock proteins (HSPs) [8, 9] functions as molecular chaperones to bind unfolded proteins and prevent aggregation while catalyzing refolding of denatured proteins via favorable hydrophilic entropic pathways. Other molecular chaperones, such as clusterin [10] and degradation processes such as the ubiquitin-proteasome [11] and the autophagy [12] systems also function to sequester the aggregation-prone species and prevent accumulation of misfolded proteins.

Early efforts to rescue unfolded proteins from aggregation have included the usage of excipients/small molecules that function by transient association with non-native proteins and thereby sterically interfering with aggregation. Successful excipients have been low molecular weight detergents [13], block copolymer amphiphiles [14], polyethylene glycol (PEG) [15] and others [16]. More recently synthetic refolding additives made a come-back as effective agents for inhibiting protein aggregation and facilitating refolding of denatured proteins [17–19]. Also, by stabilizing the native states of the protein, some small molecules [20–22] and antibodies [23, 24] provided a potential for effective therapeutic intervention. Another parallel but slightly different paradigm for the chaperone system consisted of low molecular weight assistants known as “artificial chaperones.” Inspired by GroEL-GroES complex [25], a two-step method was devised where a detergent first captured the non-native protein to prevent aggregation, then a cyclodextrin was used to strip the protein-detergent complex and allow some proteins to refold [26, 27]. Since then, specific additives or the artificial chaperone strategy to refold various denatured proteins have been proposed [28, 29].

Because of the important role misfolded proteins play in human disease, we have chosen to investigate the potential of certain biocompatible multiblock amphiphiles as therapeutic agents to recover folded proteins. Poloxamer 188 (P188) and Tetronic 1107 (T1107) are amphiphilic surfactants containing hydrophilic poly(ethylene oxide) PEO and hydrophobic poly(propylene oxide) PPO blocks. P188 is a linear PEO-PPO-PEO triblock copolymer and T1107 is a four-armed PEO-PPO block copolymer with a hydrophobic core linked by ethylene diamine (Figure 1). A similarly sized to P188, purely hydrophilic PEG is employed as a control. Considerable attention has been given to these compounds because of their capability to restore the structural integrity of disrupted cell membranes [30–33]. Our

laboratory has shown that P188 has some capability to prevent aggregation of heat denatured lysozyme using differential scanning calorimetry, small angle x-ray scattering [34] and assist in its functional recovery [35]. However, much more needs to be done to elucidate the possible ways these copolymers can come to the rescue of misfolding proteins.

In this work, we used UV/Vis absorption spectroscopy, transmission electron microscopy (TEM), and circular dichroism (CD) to characterize the disaggregating capabilities of P188 and T1107. Lysozyme was denatured by reduction with dithiothreitol (DTT) and heat exposure. Light scattering at 400 nm was utilized to approximate the turbidity of aggregate solutions and TEM revealed the morphology of aggregates to be that of particulates. The detection of β -sheet in soluble protein after denaturing indicated the extent copolymer T1107 preserved a part of the secondary structure. The results suggested that T1107 was the most effective in limiting aggregation and comparison with PEG control showed that block copolymers prevented aggregation through hydrophobic interactions rather than crowding effects.

2. MATERIALS AND METHODS

2.1 Materials and Sample Preparation

Chicken egg white lysozyme (HEWL) was purchased from Sigma-Aldrich (St. Louis, MO). Dithiothreitol (DTT) in no-weight format (ThermoFisher Scientific, Grand Island, NY) was prepared fresh for each experiment. The polymers PEG (MW 8000, Sigma-Aldrich), P188 (BASF Corporation, Mt. Olive, NJ), and T1107 (BASF) were used as received. Stock solutions were prepared in 50 mM Tris-HCl with 100 mM NaCl, pH 7.4, and diluted to final concentrations of 15 μ M HEWL and 30 μ M polymer. The concentration of HEWL was determined using the extinction coefficient of $3.65 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 280nm. The denaturant DTT was added at final concentrations of 0, 0.05, 0.10, or 0.50 mM. Samples were placed in a preheated, Neslab RTE circulating water bath (Thermo) at 90°C for 30min, then cooled at room temperature for 30 min before vortex mixing and measurement.

2.2 Absorption Optical Spectroscopy

To measure the extent of protein aggregation, an increase in absorbance was measured using a Beckman DU 800 UV/Visible spectrophotometer (Fullerton, CA) with Beckman Tm cuvettes (325 μ l, 1 cm path length). Wavelength scans were conducted from 200–600nm and solvent-subtracted values at 400nm were herein compared. Measurements were collected at a scan speed of 600nm/min and at least 3 different solutions from separate days were scanned to obtain an average for each condition.

2.3 Transmission electron microscopy (TEM)

The sample was first passed through 0.22 μ m Millipore filter to remove unwanted debris. A 5 μ l drop of the solution was then placed on a carbon-coated Formvar grid (400-mesh) and blotted after 15 sec. A 5 μ l of 2% (w/v) uranyl acetate solution was applied to the grid to negatively stain the sample and after 15 sec, was blotted again with filter paper to remove excess solution. The resulting grids were examined using the FEI Tecnai F-30 transmission electron microscope (FEI, Hillsboro, OR) with an acceleration voltage of 300 kV.

2.4 Circular dichroism (CD)

Far-UV CD spectra were recorded using a Jasco J-1500 CD spectrometer (Jasco, Tokyo, Japan) and a 1 mm path length quartz cuvette. Spectra were collected from 180 to 260 nm with a scan speed of 100nm/min at 1nm bandwidth. Each spectrum was the average of 3 scans and was baseline-subtracted by Tris-HCl buffer, DTT and polymers where appropriate.

2.5 Statistics

Students T-tests were performed using R software (R Core Team).

3. RESULTS

3.1 UV/VIS Optical Spectroscopy

The lysozyme solutions treated with DTT and heated to 90°C/cooled to 20°C increased UV/Vis absorbance at all wavelengths between 200 and 600 nm (Figure 2). The absorbance at 400 nm was subsequently used to compare lysozyme aggregation as a measure of turbidity in the presence of PEG, P188, and T1107. DTT treatment without heating resulted in very little aggregation (Figure 3), as did heating alone. Thermal denaturation of lysozyme at 90 °C then putting it at 20 °C, 30 min each, in the presence of DTT resulted in visibly cloudy solutions with significantly higher absorbance at all DTT concentrations ($p < 0.001$ in all cases).

Inclusion of T1107 at double the molar concentration of lysozyme completely prevented heat-induced aggregation to produce soluble solution at all three concentrations of DTT compared to untreated controls: 0.05 mM ($p < 0.001$), 0.10 mM ($p < 0.001$), and 0.50 mM ($p < 0.001$). Treatment with P188 reduced aggregation compared to untreated controls at DTT concentrations: 0.05 mM ($p = 0.0001$), 0.10 mM ($p \approx 0.05$), and had no effect at 0.50 mM ($p = 0.94$). Treatment with PEG did not reduce aggregation compared to untreated controls: 0.05 mM ($p \approx 0.23$) and 0.10 mM ($p \approx 0.86$). At the PEG concentrations used, significant molecular crowding effect on lysozyme refolding would not be expected.

3.2 Aggregate size and morphology by Electron Microscopy

Aggregates were visualized using TEM. Figure 4a shows the solution containing native lysozymes. Individual particles were observed with diameters on the order of tens of nanometers, which were larger than native lysozyme's hydrodynamic radius of 1.9 nm and indicative of some aggregation or presence of debris as a result of imaging preparation. Lysozyme heated in the presence of 0.10 mM DTT without treatment (Figure 4b) and in the presence of 30 μ M P188 (Figure 4c) formed aggregates with diameters on the order of 250–450 nm, which in turn formed stringed and branching aggregates on the order of tens of microns. In contrast, TEM images of lysozyme treated with 0.10 mM DTT and heated in the presence of 30 μ M T1107 (Figure 4d) resembled the case for native lysozyme, an evidence of complete prevention of aggregates. Figures 4e and 4f are images of untreated and P188-treated lysozyme, respectively, heat-denatured in the presence of 0.05 mM DTT. Lower density of particles with diameters on the order of 10–300 nm (Figure 4f) demonstrated the ability of P188 to limit aggregation at low denaturant concentration.

3.3 T1107 retains partial secondary structure by CD

Far-UV CD was used to determine if treatment with T1107 retained the conformational structure of lysozyme following DTT and heat treatment. Figure 5 shows spectra of 15 μ M lysozyme heated in the presence of 0.05 mM DTT and 30 μ M polymer. The unheated native lysozyme spectrum without DTT reflected a mixed α -helix/ β -sheet, while heated lysozyme in control or in the presence of PEG produced near-zero ellipticity characteristic of denatured and aggregated proteins. Treatment with P188 or T1107 resulted in moderate ellipticity values, with spectra containing a single negative peak at $\sim$ 215 nm corresponding to β -sheet structures. The results suggested that denaturation by DTT treatment and heat produced visibly turbid solutions, which prevented correct spectral measurement, while treatment with block copolymers yielded soluble solutions either by preserving or catalyzing the reformation of β -sheet structures but not α -helical structures.

4. DISCUSSION

Protein aggregation characteristics such as the aggregation onset rate and final morphology of the aggregated state (i.e., amorphous precipitates or fibrils) depend on the properties of a protein's solution environment, including temperature, pH, salt type, and salt concentration, as well as the relative intrinsic thermodynamic stability of the protein native state [36]. Particularly, the solution conditions also affect two factors that dictate the kinetic formation of protein aggregates; conformational and colloidal stability. Conformational stability refers to the intrinsic conformational stability of the protein native state characterized by the free energy of unfolding and colloidal stability signifies the extent of intermolecular protein-protein interactions.

In this work, treatment with 0.05–0.50 mM DTT in addition to 90°C heating provided the colloidal instability the lysozymes needed to form aggregates. In the absence of DTT, the thermal energy unfolded lysozymes but with high refolding rate, it did not expose enough hydrophobic residues necessary to cause visible aggregation. Adding DTT disrupted intramolecular disulfide bonds, rearranged lysozyme conformation in such a way that perhaps increased the duration of lysozyme unfolding and caused intermolecular hydrophobic contact to be more likely. As a result, substantial lysozyme aggregated with DTT, turbid enough to be visible through a naked eye. It may not be that all disulfide bonds were involved since Cao et al. reported the difficulty of fully reducing all such bonds [37]. Nonetheless, we observed that 0.1–0.5 mM DTT was sufficient to maximize the detection of finite-sized aggregates from thermally cycled 15 μ M lysozyme using UV-Vis spectrophotometer and those conditions were then used to successfully analyze the effect of adding copolymers without potential artifact due to excessive DTT.

In reducing aggregation, T1107 was the most effective at all DTT concentrations. P188, being not as effective, showed greater influence in low 0.05 mM DTT concentration where not as much exposed hydrophobic side chains of denatured lysozyme were likely to aggregate; at all DTT concentrations used in these experiments, PEG essentially could not prevent aggregation. The implications of this result are two-fold. First, given the lack of hydrophobic domain in PEG and its lack of efficacy, the hydrophobic region must play an important role in T1107 and P188's ability to inhibit aggregation. Normally, hydrophobic

residues of the native protein are buried inside and shielded from interacting with outside hydrophilic solvent. Then, considering how protein's hydrophobic residues are most readily exposed to the solvent by the DTT and heat exposure, denatured protein's interaction with hydrophobic regions of the copolymer must curtail the protein's ability to aggregate. Entropic effect also favors such hydrophobic interactions because hydrogen bonded water molecules on the exposed hydrophobic residues of the protein are released into the bulk solvent upon binding to other copolymers. Thus, the presence of T1107 and P188 allows the exposed hydrophobic region of the denatured lysozyme to be attracted to the hydrophobic block of the copolymers and forms protein-copolymer complex that is soluble in solution. Second, considering the structural difference between T1107 and P188, there must exist an ideal architecture and size for each copolymer to have specific association with an unfolded protein of interest. Whereas P188 is a triblock copolymer, T1107 is a larger tetra block copolymer with a distinct ethylene-1,2-diamine structural core. Thus, steric factor could have very well played an important role in associating larger T1107 consisting of more hydrophobic branching domains with exposed hydrophobes of the unfolded lysozyme. Regarding, how big of a polymer with how much hydrophobicity is needed to effectively sequester unfolded lysozyme is unknown. The only study that implicated the degree of hydrophobicity to T1107 and P188's effectiveness has been in copolymers' other application, involving the protection of liposomes from peroxidation [38]. Thus, a systemic study extrapolating the role of each copolymer's structural component in preventing aggregation and refolding denatured proteins remains to be further explored.

Turbidity is a measure of aggregation. In this study, UV-Vis was used to detect light scattering off of large aggregates, whose resulting light signal was quantified by optical absorbance at a single wavelength. The reduction of light absorbance in UV-Vis (Figure 3) thus indicated that T1107 and P188 prevented aggregate formation, and this correlated well with the lower density of aggregates seen through TEM (Figure 4d,f). Moreover, TEM revealed aggregates formed by heating and cooling the reduced lysozyme had the shape of particulates. Krebs et al. proposed that formation of particulates that have relatively monodisperse spherical particle-like aggregates is a common property of all proteins under similar conditions [39]. They demonstrated that thermal denaturation of seven different low net charged (pH near each protein's isoelectric point) proteins resulted in formation of particulates. We observed similar particle-like aggregates even though the pH 7.4 of our experimental condition was not near lysozyme's pI of 11.35. This was feasible because the presence of salt ions in our system provided charge screening and reduced charge-charge repulsion, which made an apt environment for disordered aggregation. Second, the presence of DTT reduced many of the disulfide bonds that made intermolecular contact between hydrophobic regions more likely. Lastly, building on the two previous factors, thermal denaturation at 90 °C provided a strong kinetic impetus for a rapid and nonspecific aggregation upon cooling, which resulted in relatively particle-like/spherical aggregates.

Aggregation is a phenomenon known to enhance the underlying cross β -sheet structure in both amyloid fibrils and particulates, two well-known aggregate forms [6, 40–43 and 44]. The formation of β -sheet structure is known to be favored due to the universal propensity of proteins to form intermolecular hydrogen bonds in their backbone, making the structure stable and suitable for proliferation. Interestingly however, in our CD analysis, the case that

prevented aggregation the most with T1107, contained β -sheet rich secondary structure. Though the result may seem contradictory at first, the interpretation of it is not so simple. Because CD only measures secondary structure of the soluble protein, differing degree of solubility between samples prevents the comparison of total quantity of beta-sheets present in each sample. Large aggregates in heated and reduced lysozyme and that of PEG containing samples scattered light in CD analysis to produce very low signal. What this suggests is not that the samples lacked β -sheets but rather they were insoluble. It has also been shown that the particulates contain short fibril β -sheets [44]. Thus, in our study, we cannot simply jump to the conclusion that a signature of β -sheets means a greater aggregation-prone sample because CD detects β -sheets contained only in soluble protein. Rather, what we can make of β -sheets from T1107 containing soluble lysozymes is that T1107 most effectively preserved enough secondary structure of the β -sheet region, which prevented aggregation and kept the solution soluble. T1107 achieved this through superior hydrophobic binding of the unfolded lysozyme, which formed protein-copolymer complex that may dissociate at room temperature. Though this mechanistic understanding of how copolymers physically bind to unfolded protein cannot be directly visualized from our experiments, the consistent results from T1107 sample's low light scattering in UV-Vis, low density of aggregates in TEM and preservation of secondary structure in CD indicate such occurrence is highly likely. Whether the β -sheets are part of the original protein or rearrangement of whole new β -sheets is uncertain. Along with our desire to regenerate the entire original secondary structure, additional work is being planned to deduce a more complete pathway through which these block copolymers can be used to refold denatured proteins.

For future studies probing the refolding capability of these copolymers, one could utilize an alternative chemical denaturation method with different unfolding mechanism where various reagents can be used with greater technical flexibility. If one decides to use DTT or other reducing agent, further arrangement using additional pair of redox agents may be necessary to help refold the reduced proteins. At appropriate stages, borrowing from "artificial chaperone" strategy, cyclodextrin may prove useful in extracting the copolymers from protein-polymer complex to help proteins refold. Relatively recently, it has been shown that some of these copolymers could exert membrane sealing function by affecting the hydration dynamics at the interface of lipid membrane [45]. Also, high resolution NMR was used to deduce a possible role of water in thermal folding of hydrated lysozyme [46]. Thus, it is part of our plan to focus also on the role of polymers' hydrophilic components in their ability to alter water structure at protein-polymer interface and devise other future experiments to obtain a fuller picture of how these copolymers can come to the rescue of denatured proteins.

In this work, we have demonstrated the formation of particulate aggregates with thermal denaturation of reduced lysozyme and how they could most effectively be prevented by multiblock, biocompatible surfactant copolymer T1107 and somewhat less so by P188. This study provided valuable knowledge regarding protein-polymer interactions, which have important practical implications in medical, food storage and environmental applications. Efforts are underway in our laboratory to optimize the design of biocompatible copolymers that prevent aggregation and refold denatured proteins in different experimental conditions.

Finally, we believe that some of these multi-block copolymers that also happen to seal damaged membrane have unique potential to be used therapeutically for treating tissue trauma injury and facilitating body's recovery.

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Abbreviations

PEG	Polyethylene Glycol (8000 Da)
P188	Poloxamer 188
T1107	Tetronic 1107
DTT	Dithiothreitol
TEM	Transmission electron microscopy
CD	Circular dichroism

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Figure 1. Chemical bonding structures of (a) the tri-block co-polymer P188 ($a=80$, $b=27$) (b) the tetra-block co-polymer T1107 ($x=20$, $y=58$). PEG consists only of ethylene oxide (EO), and with reference to formula (a) above, PEG8000 ($a=182$, $b=0$).

Figure 2. Representative UV-Vis wavelength scan (200–600nm) of 15 μ M lysozyme, 0.05mM DTT (a) before heating (b–e) after heating to 90°C and cooling to 20°C. (a,b) No polymer (c) 30 μ M PEG8000 (d) 30 μ M P188 (e) 30 μ M T1107.

Figure 3.

UV-Vis absorbance (400nm) of 15μM lysozyme, 30 μM polymers with varying DTT concentrations before heating (□) and after heating to 90°C and cooling to 20°C (■). Solutions with (a) 0.05mM DTT (b) 0.1mM DTT (c) 0.5mM DTT. 15μM lyso (No DTT) denotes 15μM lysozyme dissolved in Tris-HCl buffer without DTT. Control refers to 15μM lysozyme with respective DTT concentration. Error bars are standard deviations of the average collected from three separate samples.

Figure 4.

TEM images of soluble and aggregating 15 μ M lysozymes. (a) Before heating (b–f) after heating to 90°C and cooling to 20°C. (a,d) scale bar 500nm (b,c,e,f) scale bar 1 μ m. All samples had 15 μ M lyso in Tris-HCl buffer with (a) no polymer (b) 0.1mM DTT (c) 0.1mM DTT, 30 μ M P188 (d) 0.1mM DTT, 30 μ M T1107 (e) 0.05mM DTT (f) 0.05mM DTT, 30 μ M P188.

90C, 20C, 15 μ M lyso, 0.05mM DTT

Figure 5.

Far-UV CD spectra of 15 μ M lysozyme, no DTT or 0.05mM DTT with no polymer (control), 30 μ M polymers (PEG8000, P188, T1107), all heated to 90°C then cooled to 20°C except for 15 μ M lysozyme with no DTT.