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Zwitterionic polymer as a novel inhibitor of protein aggregation

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We report the novel one-step synthesis of a zwitterionic polymer, polysulphobetaine, via living reversible addition fragmentation chain transfer (RAFT) polymerization. Lysozyme did not aggregate when heated in the presence of this polymer. Amyloid formation, the cause of many diseases, was also suppressed. The zwitterionic polymer was significantly more efficient than previously described inhibitors of protein aggregation. Lysozyme heated in the presence of polysulphobetaine retained its solubility and very high enzymatic efficiency, even after prolonged heating. The secondary structures of lysozyme change with increasing temperature, accompanied by an increase in β-structure. This change was prevented by mixing the polymer with lysozyme. ^{1}H-NMR before and after aggregation revealed the conformational changes taking place in the lysozyme: during aggregation, lysozyme is transformed into a random coil conformation, thus losing its secondary structure. Presence of the polymer facilitates retention of partial higher order structures and lysozyme solubility at higher temperatures. The high efficiency of the polyampholyte was ascribed to its ability to prevent collisions between aggregating species by acting as a molecular shield.

Introduction

Protein instability is an ongoing challenge in the field of biopharmaceutics. With physical and chemical deterioration, protein aggregation is one of the foremost causes of protein instability.[1] Protein aggregation leads to an array of deleterious effects, including mitochondrial dysfunction and cell death, and can trigger serious neurodegenerative conditions such as Alzheimer's, Parkinson's, prion disease, and amyotrophic lateral sclerosis (ALS).[2,3] Protein aggregation involves formation of fibrous structures with a β-sheet conformation of misfolded proteins, known as amyloids.[4]

One cause of protein aggregation may be the physical association of protein molecules with each other. When changes in primary structure are involved, this is known as physical aggregation; when it is a result of new bond formation, it is known as chemical aggregation.[1] Both phenomena can occur simultaneously, leading to the formation of soluble or insoluble aggregates.[5]

One of the most commonly used and widely studied proteins is hen egg white lysozyme (HEWL). Its complete primary[6] and three-dimensional structures[7] are known; thus, it is also commonly used as a model system for aggregation studies. HEWL is a single polypeptide chain of 129 amino acid residues with four intramolecular disulphide bridges.[8]

Many organic compounds (such as arginine,[9-11] proline,[12] and cyclodextrin[13]) and protein engineering techniques[14] have been used with some success to manage the refolding of proteins and inhibition of protein aggregation. Amphiphilic macromolecules have also been employed to inhibit amyloid formation for various proteins like HEWL.[15]

Polymers containing positive and negative groups (on different monomer units) are classified as polyampholytes[16, 17]. In contrast, zwitterionic polymers have anionic and cationic groups on the same monomer unit.[18] Due to the presence of a mixed charged state in zwitterionic polymers, they exhibit properties similar to those of proteins.[19]

Polyampholytes and zwitterionic polymers have unique antibiofouling[20] and cryoprotective properties,[21-24] which are strongly related to the interaction between polymer and water. They maintain the water structure[25] at the interface between polymer and material, due to the charge balance. Here, we propose the facile synthesis of a zwitterionic polymer via reversible addition fragmentation chain transfer (RAFT) polymerization and the efficient inhibition of protein aggregation.

Living polymerization, developed by Michael Szwarc in the 1950s[26, 27], has become an indispensable form of polymerization due to its potential to control various parameters such as architecture, molecular weight distribution, and functionality. This process eliminates transfer and termination reactions from chain growth polymerization. With the advent of living radical polymerization, also known as controlled radical polymerization (CRP)[28-33], it has become more convenient to react various types of monomers under different

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conditions. Of the three CRP types, namely, atom transfer radical polymerization (ATRP), RAFT polymerization, and nitroxide-mediated polymerization (NMP), RAFT is one of the newest and most versatile forms that does not require a transition metal, making polymers that are useful in biomaterial applications. RAFT involves degenerate reversible chain transfer[34] and is a robust method for developing complex macromolecular architectures.

In our previous research, we developed various polyampholytes with excellent cryoprotective properties that protect the cell membrane during freezing. These polyampholytes were biocompatible; however, their behaviour with respect to protein denaturation and folding has not been addressed. To our knowledge, there are few reports on the use of polymers for this purpose. Muraoka et al.[35] reported the use of the triangular-structured monodisperse polyethylene glycol to inhibit lysozyme aggregation, but synthesis requires 15 steps and various reagents. Tunacliffe et al. studied biological systems and found that Group 3 late embryogenesis abundant (G3LEA) proteins reduce aggregation of lysozymes during dehydration.[36] LEA proteins contain positive and negative charges, but are neutral or basic overall.[37] They have been employed as molecular shields and chaperones.[38-40]

In this study, we synthesized a novel zwitterionic polymer, polysulphobetaine (Poly SPB), which inhibits thermal aggregation of lysozyme with greater efficiency than the corresponding monomers. We also compared its efficiency with other reagents known to inhibit protein aggregation. To our knowledge, this is the first report of the use of a synthetic zwitterionic polymer as a protein aggregation inhibitor.

Experimental

Materials and Methods

Sulphobetaine was purchased from Osaka Organic Chemical (Osaka, Japan) and used without further purification. Lysozyme from chicken egg white, 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid, Thioflavin T (ThT), and *Micrococcus lysodeikticus* were purchased from Sigma-Aldrich. AIBN was purchased from Wako Pure Chemical Industries (Osaka, Japan). ND-195, ND-201, ND-211, and ND-256 were purchased from Anatrace, UK.

Synthesis of poly-SPB

SPB (Osaka Organic Chemical, Osaka, Japan), 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid, and AIBN were dissolved in methanol-water (3:1 v/v %). The solution was then purged with nitrogen gas for 1 h and stirred at 70°C (Fig. 1c). Samples were removed periodically (20 µL) and plunged in liquid nitrogen to quench the reaction and then the conversion at each reaction time was determined by 1H nuclear magnetic resonance (NMR) (Bruker AVANCE III 400MHz spectrometer, Bruker Biospin Inc., Switzerland). After 6 h, the reaction mixture was dialyzed against methanol and water for 24 h each with constant solvent exchange. The polymer was obtained after lyophilization. The molecular weight and distribution (polydispersity index, PDI) of the polymers were determined by gel permeation chromatography (GPC, column, BioSep-s2000; Phenomenex, Inc., CA, USA) and were measured using a Shimadzu high-performance liquid chromatography data system incorporating a refractive index detector. NaBr solution (pH 7.4, 0.1 M) was used as the mobile phase (flow rate, 1 mL/min) and Pullulan was used as the standard.

Thioflavin T assay

A stock solution of Thioflavin T (ThT) was prepared by adding 4 mg ThT to 5 mL PBS and filtered through a 0.22-µm filter. The stock solution was diluted by adding 1 mL stock to 50 mL PBS to produce the working solution.[41] Lysozyme solution in PBS was then mixed with various reagents and heated to 90°C for 30 min; then, 2 mL of this solution was mixed with 100 µL ThT and fluorescence was observed with an excitation wavelength of 440 nm and emission wavelength of 480 nm (JASCO FP-6500). Increased intensity corresponds to amyloid formation due to ThT binding with amyloid fibrils.

Residual enzyme activity

Lysozyme solution in PBS was mixed with poly-SPB solution to achieve a final lysozyme concentration of 0.1 mg/mL. The solution was heated at 90°C for 30 min. *Micrococcus lysodeikticus* (2 mL; 0.25 mg/mL in PBS) was placed in a quartz cuvette with 100 µL lysozyme-polymer solution and mixed well. Turbidity was measured by UV-Vis spectrophotometry (UV-1600PC, Shimadzu Corporation, Kyoto, Japan) at 600 nm from 0 to 6 min with constant stirring at room temperature.[42]

Transmission electron microscopy

Lysozyme (5 mg/mL) was dissolved in PBS with and without poly-SPB, and then incubated at 65°C for 14 days without agitation.[43] It was then diluted 10-fold for transmission electron microscopy (TEM). The sample (10 µL) was placed on a copper grid (NS-C15 Cu150P; Stem, Tokyo, Japan). The grids were negatively stained with 2% phosphotungstic acid (Sigma Aldrich, Steinheim, Germany) for 1 min and then washed with two drops of distilled water, blotted, and air-dried. TEM images were collected using a Hitachi H-7100 system (Hitachi, Tokyo, Japan) with an accelerating voltage of 100 kV.

Statistical Analysis

All data are expressed as the mean ± standard deviation (SD). All experiments were conducted in triplicate. The Tukey–Kramer test was used to compare the data shown in Figure 3.

Results & Discussion

Characterization

Polysulphobetaine was synthesized from the zwitterionic monomer 3-((3-acrylamidopropyl)-dimethylammonio)-propane-1-sulphonate by RAFT polymerization (Scheme 1). Polymers were characterized using various techniques.

^{1}H- and ^{13}C-NMR were used to analyse the chemical structure of the final product (polymer) obtained after purification (Fig. S1 and S2). Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (JASCO FT-IR-4200, Fourier Transform Infrared Spectrometer) revealed complete conversion of monomer to polymer with the loss of alkene stretching at around 1627 and 3270 cm^{-1} (Fig. S3) .Time-dependent NMR spectra were obtained after fixed intervals to calculate the rate of reaction and to ascertain the completion of reaction. Figure S4 shows that the reaction initially proceeds rapidly and almost all of the monomer is converted to the corresponding polymer, represented by disappearance of the vinyl peaks at 5.7 and 6.2 ppm. Kinetic study of the polymerization (Fig. 1) reaction revealed a first order kinetics, illustrated by a linear curve, representative of living polymerization[44]. Around 80% of the reaction was completed within the first 2 h and all the monomer was converted into the corresponding polymer within 6 h, indicating completion of the reaction.

A GPC curve showed that the polymer had a unimodal distribution with a PDI of around 1.18, which is within the range of living polymerization. M_n was around 5.5 kDa, which is consistent with the theoretical molecular weight, according to the feed ratio of 100:5:1 ([monomer]:[initiator]:[RAFT agent]).

Protein aggregation Inhibition

A clear solution was obtained when lysozyme was dissolved in PBS (pH 7.4, 3 mg/mL) alone. The solution was heated to 90°C for 30 min, which resulted in aggregation of lysozyme (Fig. 2a). Addition of SPB monomer (10% w/v) partially inhibited lysozyme aggregation (Fig. 2b), and addition of poly-SPB yielded a slightly yellowish solution due to the nature of the polymer in PBS. When this solution was heated to 90°C for 30 min, no aggregates were seen (Fig. 2c). This was verified by spectrophotometry, which showed no significant change in absorbance in the visible region (500 nm). This experiment clearly demonstrated that poly-SPB inhibits thermal aggregation of lysozyme.

Lysozyme aggregation was also observed by circular dichroism (CD) spectroscopy (JASCO J-820, CD spectrometer). When a solution of lysozyme in PBS (0.5 mg/mL) containing no polymer was heated to 90°C, it exhibited a considerable decrease in the intensity of CD bands around 205 (β-sheet) and 225 nm (α-helix), which corresponds to a loss of the lysozyme secondary structure[45-47] (Fig. 3) and complete unfolding upon heating. The electrostatic interaction of poly-SPB with lysozyme was observed by zeta-potential, which was recorded using a Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). This showed that poly-SPB has a slightly negative surface charge, which, combined with the positive charge of lysozyme, produces a weak and reversible electrostatic interaction (Table 1).

Amyloid fibril Formation

TEM revealed the formation of amyloid fibrils on heating of lysozyme (Fig. 4a). Fibrils were long, dense, and straight, representing amyloid morphology[48]. When poly-SPB was added to the lysozyme and heated, no fibril formation was observed. Hence, addition of poly-SPB completely blocked fibrillation (Fig. 4b).

Amyloid aggregation inhibition was quantitatively analysed using the Thioflavin T assay. Lysozyme in PBS containing poly-SPB showed very high inhibition of protein aggregation (amyloid fibril formation) with an efficiency that increased with increasing polymer concentrations. At 10% polymer concentration, ~96% inhibition was observed (Fig. 5a). This represented significantly greater efficiency than the monomer SPB and other commercially available reagents such as non-detergent sulphobetaines (NDSBs)[49-53] and L-arginine hydrochloride[54,55] (Fig. 5b).

Enzymatic activity

The enzymatic activity of lysozyme after heating was also studied by using gram-positive *Micrococcus lysodeikticus* to assess the ability of poly-SPB to inhibit thermal aggregation. When lysozyme acts on the cell suspension, the turbidity of the suspension decreases and sample absorbance undergoes a marked diminution. We monitored the reduction in turbidity caused by action of lysozyme on the cell suspension resulting in degradation of the bacterial cell wall as a decrease in absorbance at 600 nm (Fig. 6a). Residual lysozyme activity increased with increasing concentrations of poly-SPB: at 15% poly-SPB (w/v%), lysozyme retained more than 85% activity (Fig. 6b), which is greater than has been reported elsewhere.[35] This confirms that poly-SPB is an efficient inhibitor of lysozyme aggregation, which also prevents irreversible mis-folding.[42]

Preservation of secondary structure

ATR-FTIR

These studies revealed a change in the secondary structure of lysozyme upon aggregation. The amide I peak corresponding to a β-sheet[56] shifted to a lower-frequency band position (Fig. 7) suggesting an increase in β-structure in the fibrils.[57] Addition of poly-SPB prevented any change in secondary structure.

^{1}H-NMR

Conformational states of lysozyme before and after heating were analysed by ^{1}H-NMR. Due to the anisotropic magnetic fields of the aromatic or carbonyl groups, folded proteins show an extensive range of chemical shifts. On the other hand, a relatively narrow range of chemical shifts is shown by denatured proteins, due to their transformation to a random-coil conformation.[58] ^{1}H-NMR of lysozyme solution in D_2O exhibits a wide range of signals. After heating the solution to 90°C for 30 min, all the peaks disappeared (Fig. 8a). This finding suggests that, after heating, the lysozyme is transformed into a random coil state and has lost its secondary structure. In contrast, addition of monomer to the lysozyme aids in partial retention of the secondary structure (Fig. 8b). A mixture of lysozyme with poly-SPB resulted in many signals over a wide range of chemical shifts and almost all the peaks were retained (Fig.8c). The signals in the up-field and down-field regions persisted with considerable intensities, indicating that lysozyme remained dissolved and retained a partial higher-order structure. Most of the proton

signals can be assigned to their corresponding amino acids.[59] Signals observed in the high magnetic field region correspond to the amino acid residues located in the secondary structures[60, 61] (α-helix, β-sheet, and loop) and the retention of these signals even after heating reveals that poly-SPB stabilizes the partial higher-order structure of lysozyme, which in turn improves enzyme solubility at higher temperatures.

Protein aggregation or denaturation is generally caused by water stress.[62] Under extreme conditions such as heating, drying, and desiccation, proteins undergo severe dehydration due to hydrophobic aggregation of the unfolded or denatured states, thus leading to aggregation.[63] Because Poly-SPB shows weak and reversible interaction with proteins,[29] it acts as a molecular shield, reducing collisions between aggregating species and maintaining the water structure.[34]

Conclusion

In summary, we have shown that a zwitterionic polymer is an efficient inhibitor of thermal aggregation of lysozyme. To our knowledge, this is the first report of a zwitterionic polymer being used to inhibit protein aggregation. Poly-SPB stabilizes lysozyme and preserves its higher-order structure. The solubility of lysozyme in the presence of poly-SPB was retained even at higher temperatures, thus inhibiting lysozyme aggregation. Amyloid fibril formation was also suppressed. We believe that additional study of substituted poly-SPB, sulphobetaines with different alkyl chain lengths, and conversion into a nanogel may greatly improve the efficiency of this artificial molecular chaperone. Further studies are in progress to elucidate the mechanism by which aggregation is suppressed.

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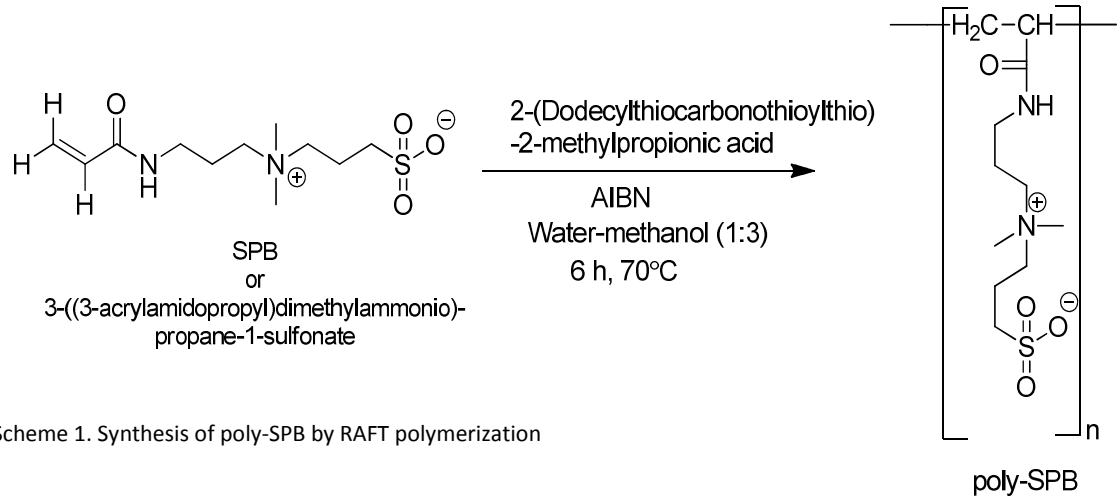


Scheme 1. Synthesis of poly-SPB by RAFT polymerization

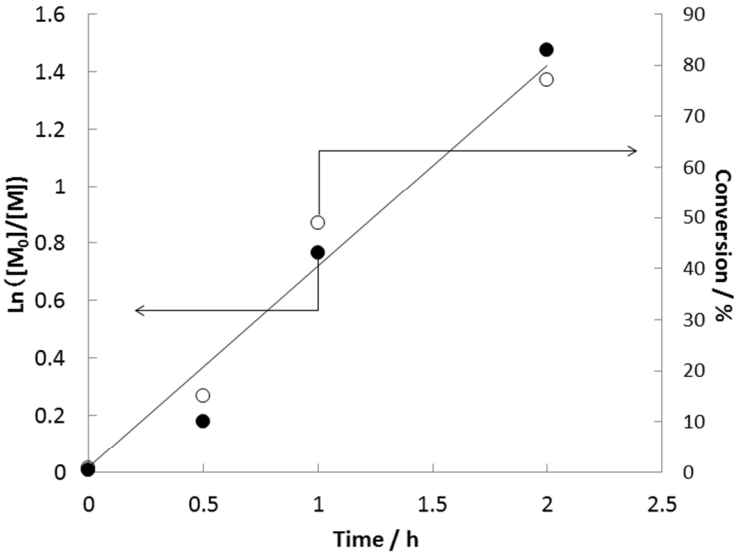


Fig. 1

Figure 1. Kinetic plot for the polymerization of SPB by RAFT polymerization, followed by ^{1}H NMR spectroscopy in D_2O.

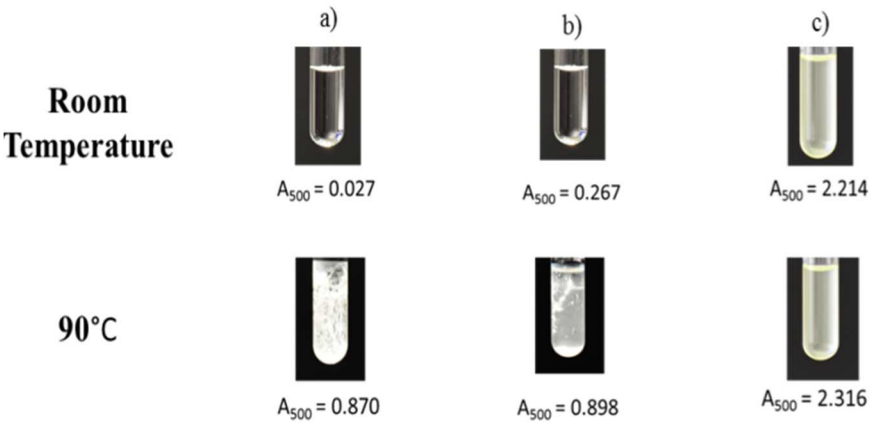


Figure 2. Photographs of lysozyme in PBS (3 mg/mL) at room temperature (top) and at 90°C (bottom); a) without additive; b) with 10% SPB; c) with 10% poly-SPB. Absorbance at 500 nm is shown below each image.

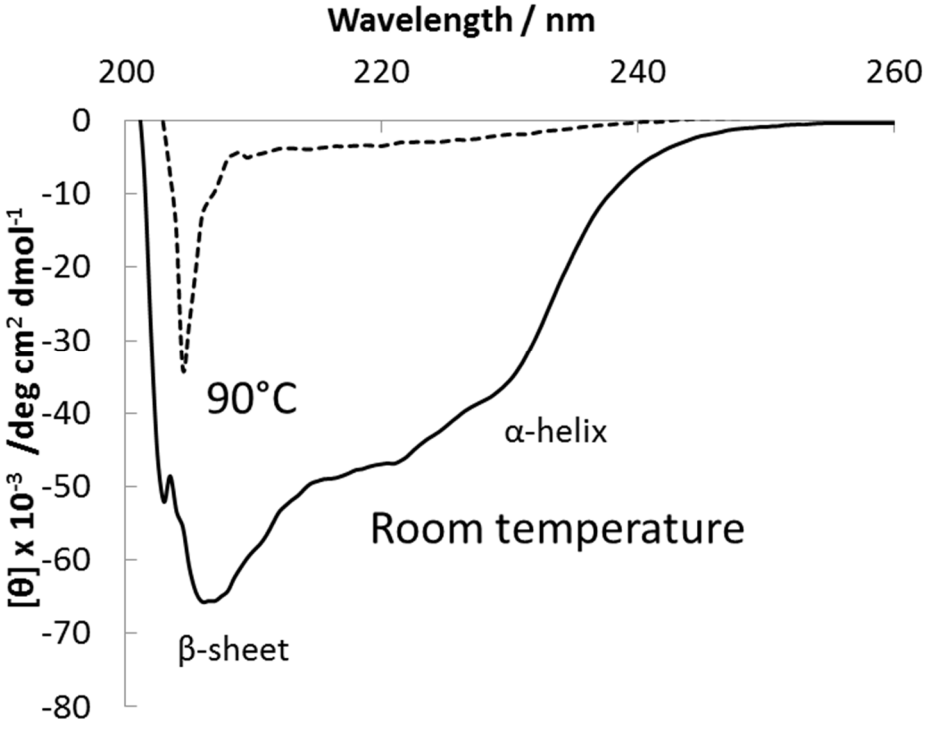


Figure 3. CD spectra of lysozyme (0.5 mg/mL) in PBS buffer at 30°C and 90°C.

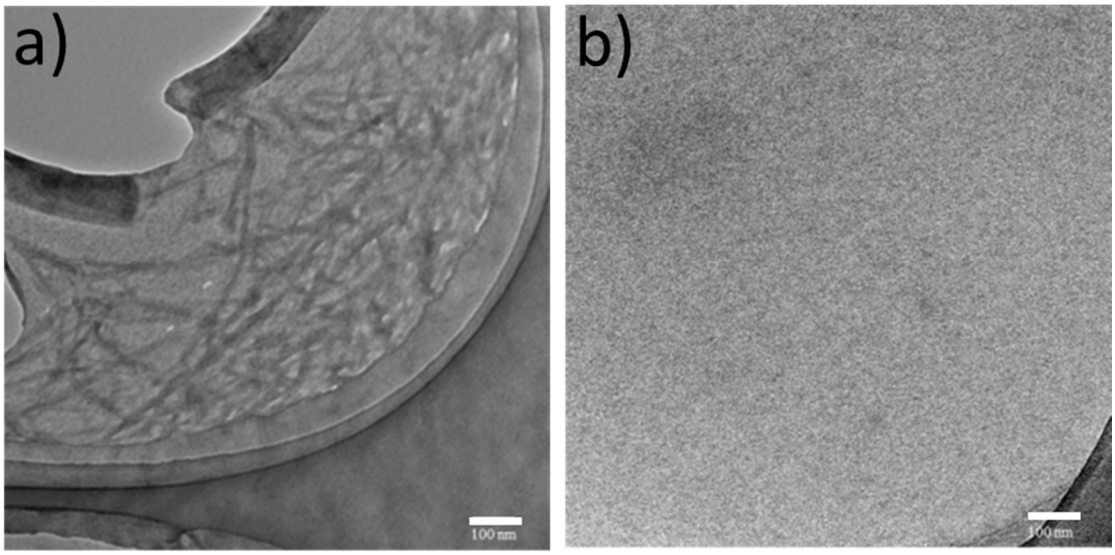


Figure 4. TEM images of lysozyme solution after incubation at 90°C a) in PBS alone, and b) with poly-SPB. Scale bars represent 100 nm.

Table 1. Zeta potential values at pH 7. (*Numbers in parenthesis indicate the final concentration (w/v) %).*

Sample	**Zeta Potential (mV)**
Lysozyme (0.33)	5.24
Poly-SPB (0.67)	-4.79
Lysozyme (0.33) + Poly-SPB (0.67)	-4.18

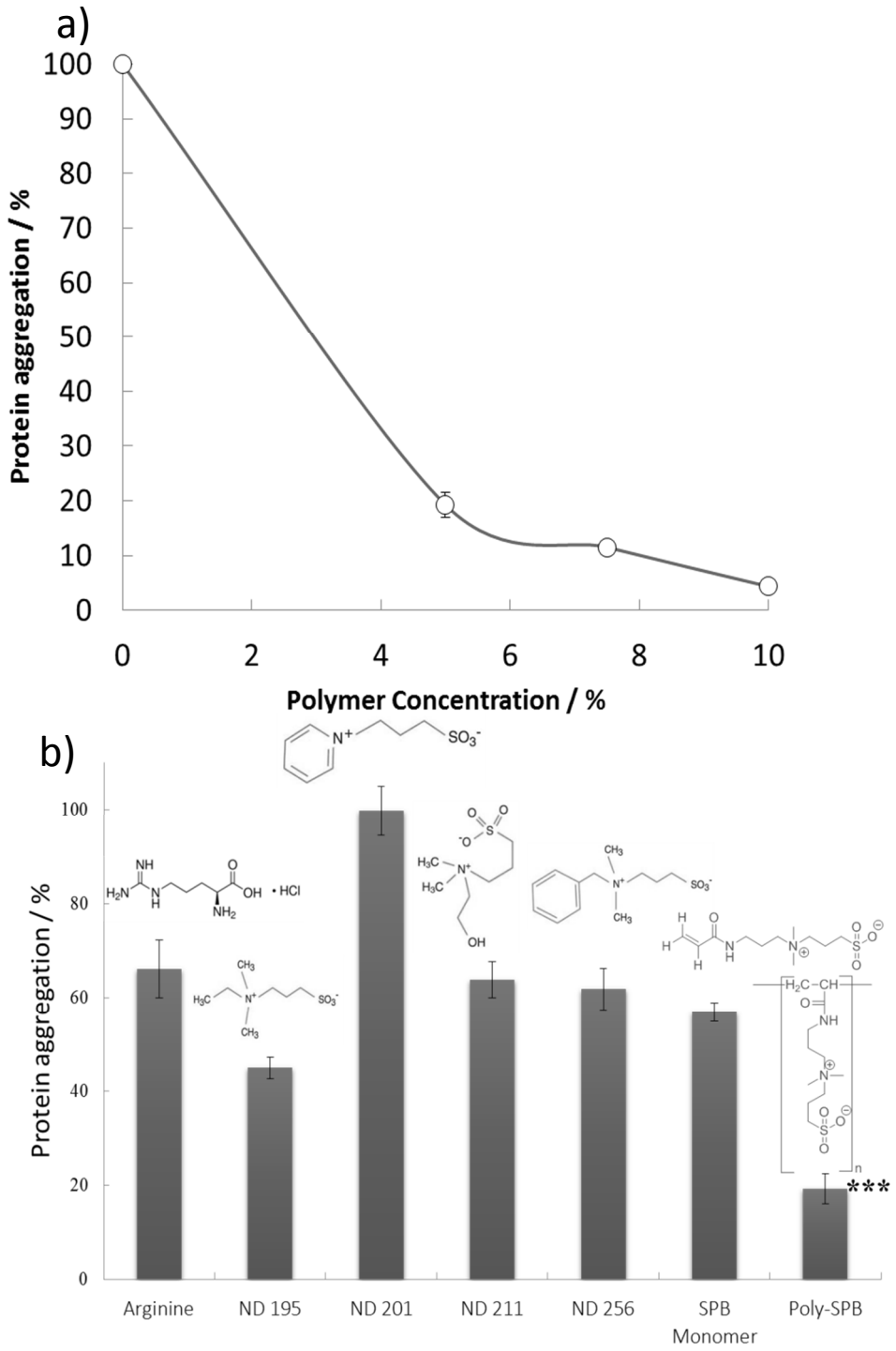


Figure 5. a) Protein aggregation of lysozyme (0.5 mg/mL) when heated to 90°C for 30 minutes a) in the presence of poly-SPB at various polymer concentrations, and b) in the presence of various reagents (5% w/v). Data are expressed as the mean ± SD of 3 independent experiments (5 samples each) ***P < 0.001 vs. all other reagents.

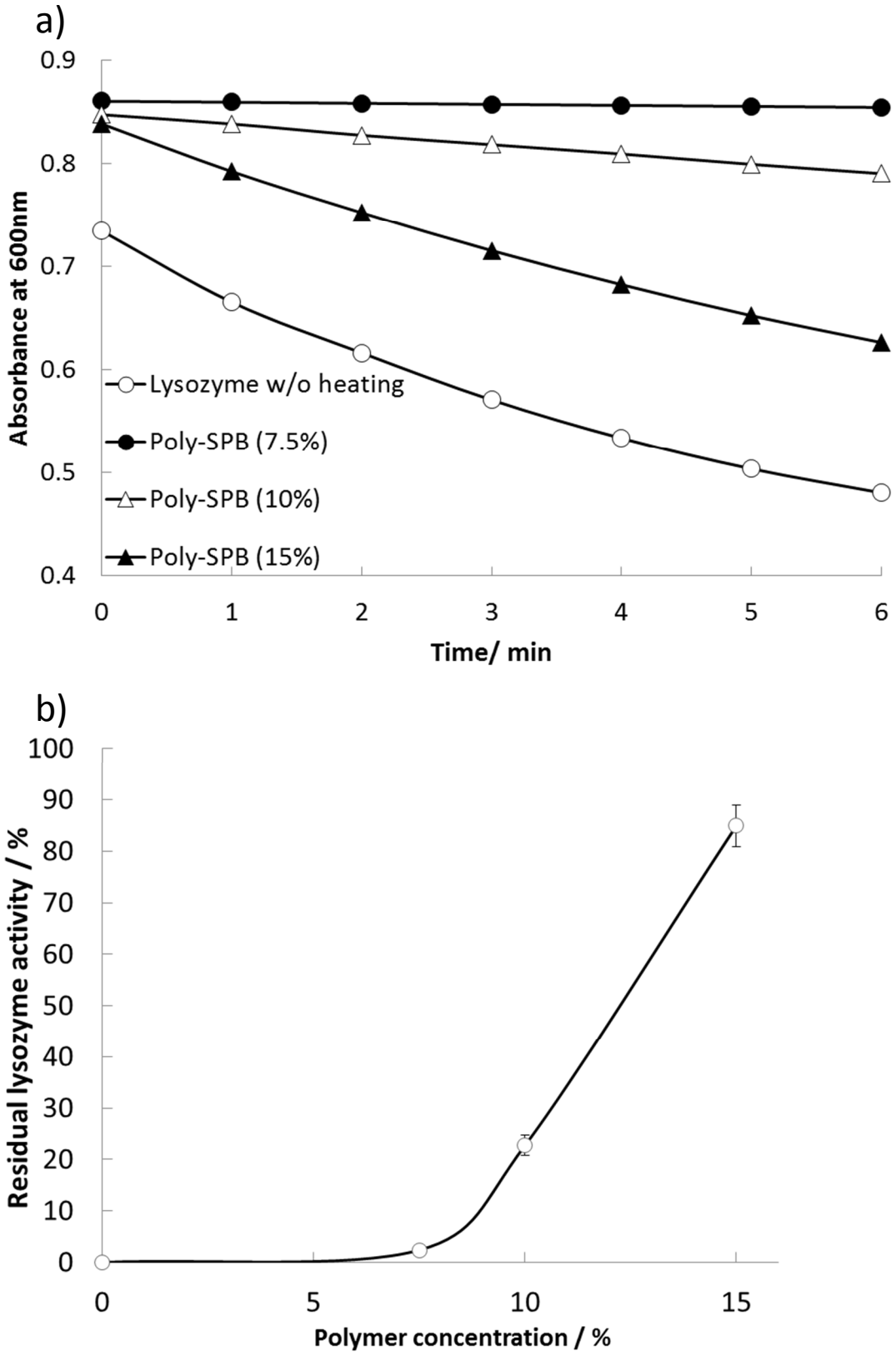


Figure 6. a) Rate of inactivation of lysozyme when heated to 90°C when lysozyme in PBS (open triangle, slope = 0.0418); with 15% poly-SPB (closed triangle, slope = 0.0355); with 10% poly-SPB (open circle, slope=0.0095) and with 7.5% poly-SPB (closed circle, slope = 0.001), and b) Enzymatic activity of lysozyme after treatment at 90°C in the presence of poly-SPB. Data are expressed as the mean ± SD of 3 independent experiments (5 samples each).

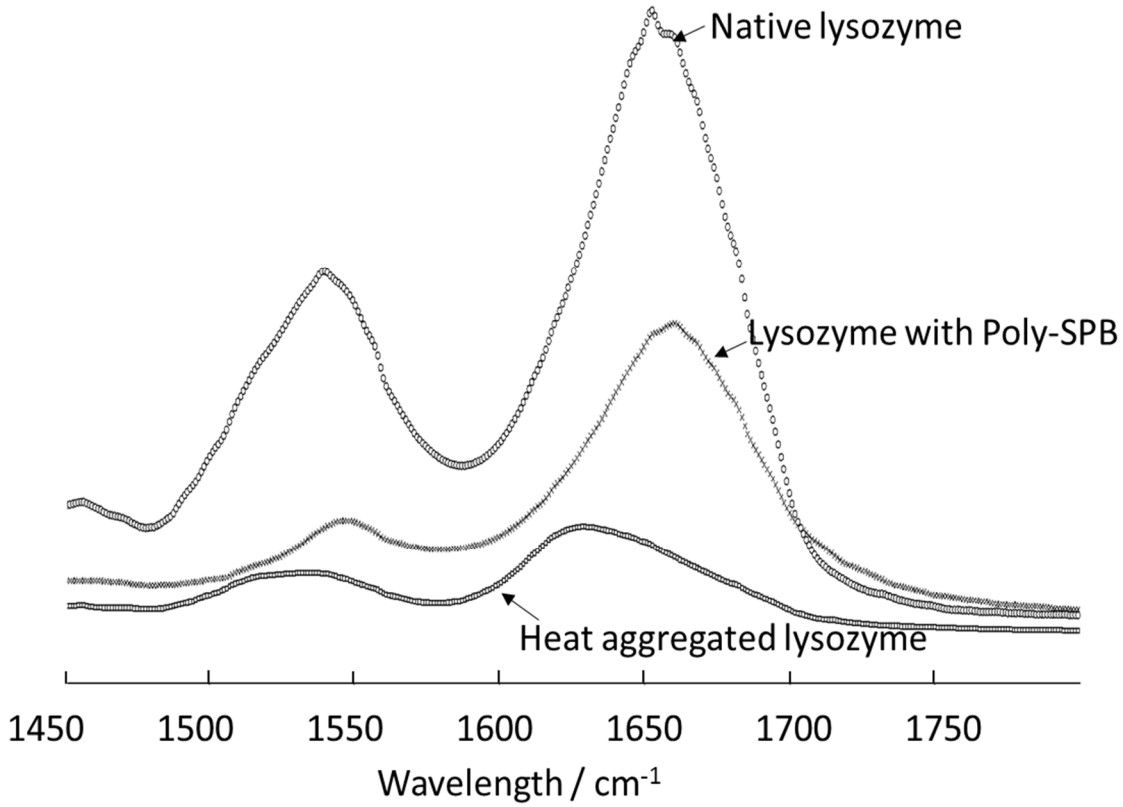
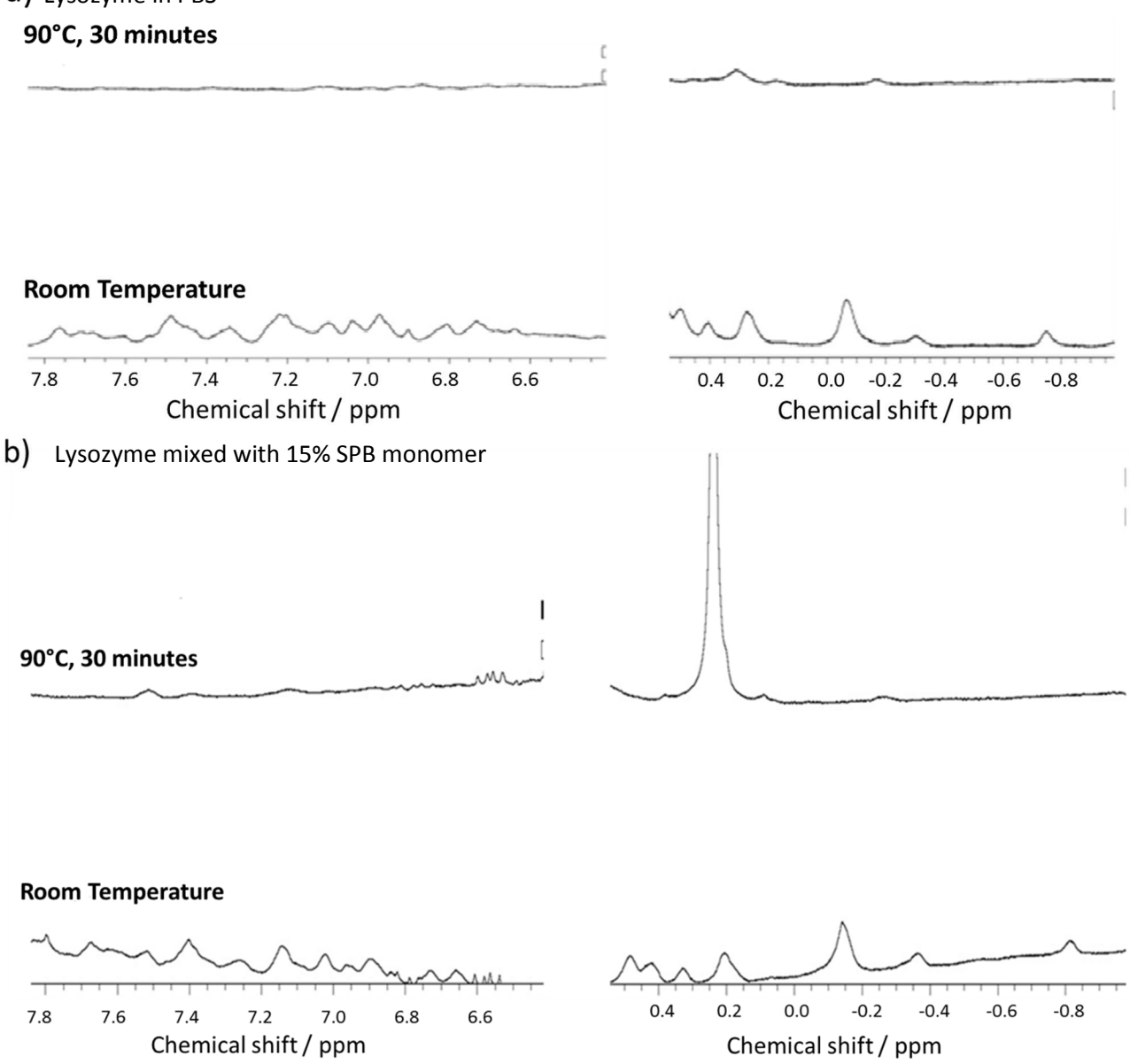


Figure 7. ATR-FTIR of native lysozyme in the presence of poly-SPB and lysozyme (aggregated) after heating at 90°C for 30 min.



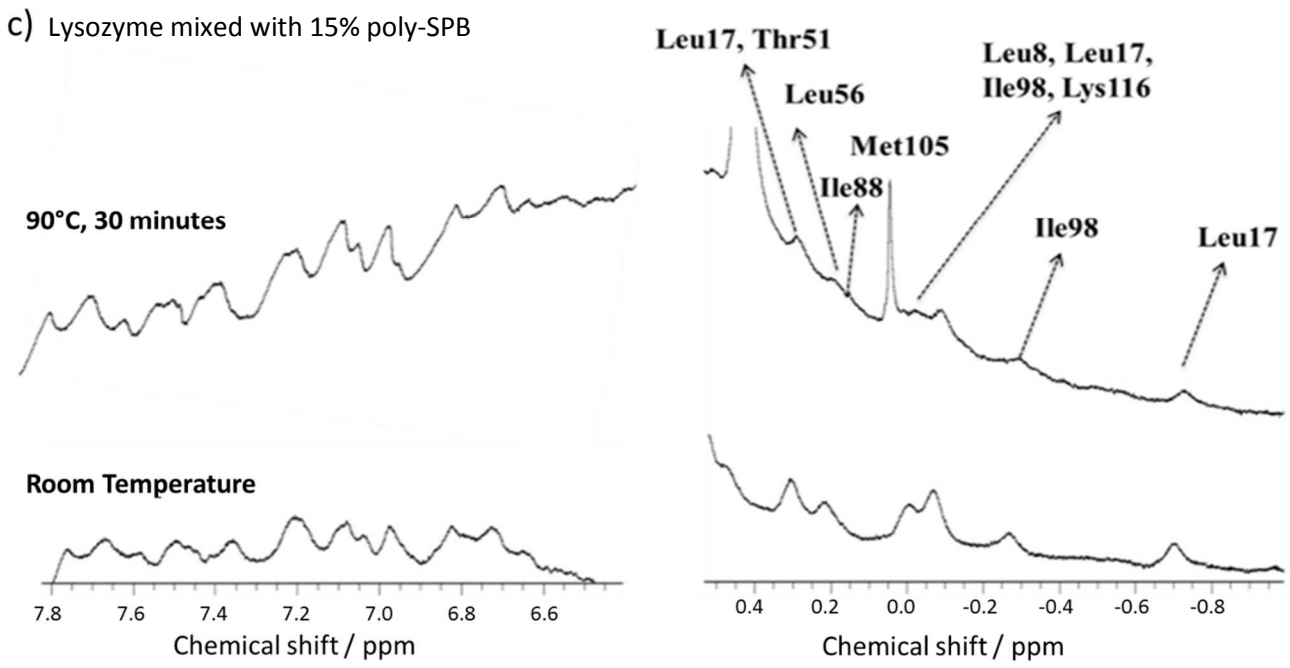


Figure 8. a) 1H NMR spectra of a mixture of a) lysozyme in PBS, b) lysozyme mixed with monomer SPB, and c) lysozyme mixed with poly-SPB at room temperature and after heating to 90°C for 30 min.

Graphical abstract

Zwitterionic polymer was synthesized via RAFT polymerization. This polymer prevented heat induced protein aggregation, by preventing hydrophobic interactions between protein chains in solution; thus acting as a molecular shield.