ACS OMEGA

Article

Cite This: ACS Omega 2019, 4, 12186−12193

http://pubs.acs.org/journal/acsodf

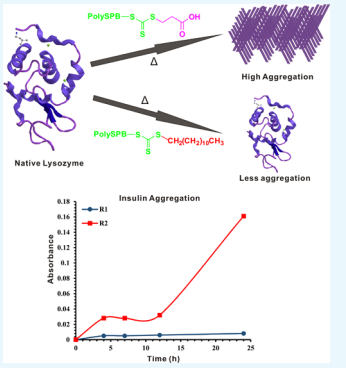
Comparative Study of Protein Aggregation Arrest by Zwitterionic Polysulfobetaines: Using Contrasting Raft Agents

Neha Sharma, Robin Rajan, Sparsh Makhaik, and Kazuaki Matsumura*

School of Materials Science, Japan Advanced Institute of Science and Technology, Asahidai, Nomi, Ishikawa 923-1292, Japan

Supporting Information

ABSTRACT: Protein aggregation has caused limitations in the study and development of protein-based biopharmaceuticals. We prepared different polysulfobetaine (poly-SPB) polymers via reversible addition fragmentation chain transfer (RAFT) polymerization. These polymers exhibited high efficiency in modulation of protein aggregation. We synthesized polysulfobetaines using two different RAFT agents, and analyzed the aggregation profile of lysozyme and insulin. In poly-SPBs, existence of a hydrophobic RAFT agent resulted in visible enhancement of the residual enzymatic activity of lysozyme, whereas it remained unaffected by the hydrophilic RAFT agent. In addition, these polymers resulted in significant suppression in the aggregation of insulin. Increase in the molecular weight of the polymer caused higher efficiency to perpetuate enzymatic activity of lysozyme upon thermal denaturation. The polymers arrested the formation of amyloid like fibrils of lysozyme and insulin, thus indicating their potential to inhibit aggregation. The results unambiguously demonstrate the importance of polysulfobetaine moiety and hydrophobicity in protein aggregation inhibition. This study gives insight into the protein aggregation inhibition by zwitterionic polymers, which have a potential to be developed as aggregation inhibitors in the future.



1. INTRODUCTION

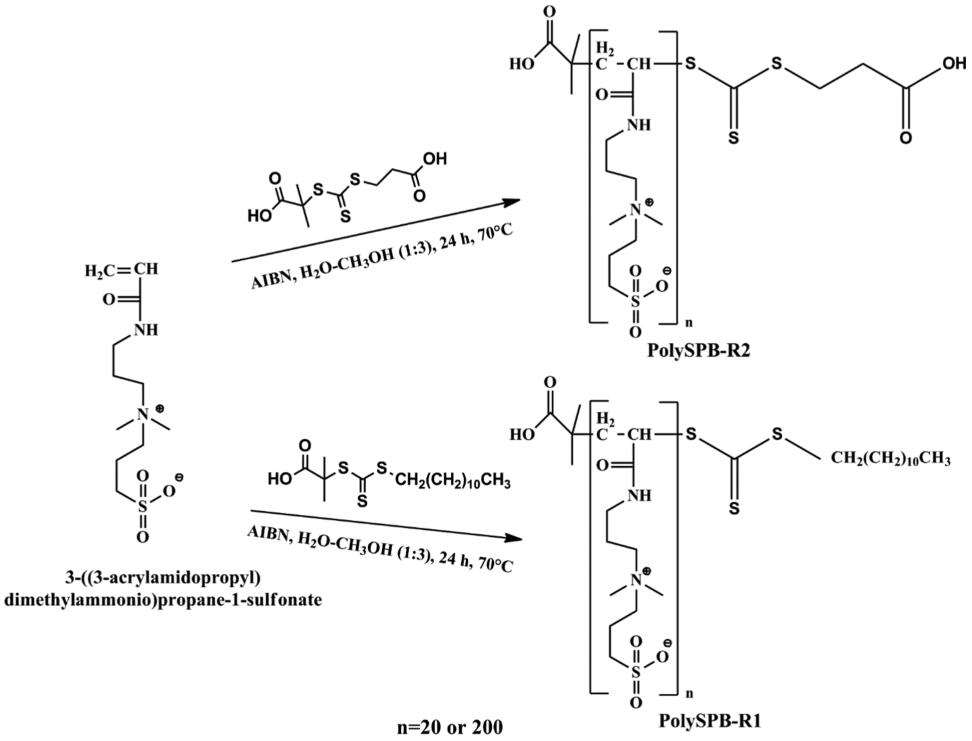
Polysulfobetaines (poly-SPBs) are zwitterionic polymers that have a cationic quaternary ammonium ion and an anionic sulfonate ion on the same monomer unit. Due to anti-polyelectrolytic effect, polysulfobetaines have been used in industrial applications, as they form open coiled chain structures in solution. The biocompatible properties of polysulfobetaines have been studied recently whose resemblance to phospholipids is responsible for such a behavior.[1−6] Sulfobetaine polymers show good hemocompatibility,[7] have antifouling properties,[8] suppress nonspecific binding of proteins and do not modify the structure of the hydrogen-bonded network of water molecules at the interface of the polymer and material.[9,10] They have been used for various applications including wound dressing[8] and cryopreservation,[11] as well as in separation science[12] and drug delivery.[13,14]

Protein aggregation is a biological phenomenon in which misfolded proteins cluster together intra- or extracellularly to form an aggregated mass of fibers with the β-sheet conformation called amyloids,[15] which ensue neurodegenerative diseases like Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis, etc.[16,17] Protein aggregation due to physical clumping involves changes in protein primary structure, and chemical aggregation involves changes in chemical structure, e.g., covalent bond formation.[18] Depending on the type of aggregation taking place, the aggregates may be soluble or insoluble.[19] Earlier, various compounds like arginine,[20] proline,[21] cyclodextrin,[22] and many other compounds[18] have been used to inhibit protein unfolding and aggregation. The success rates are not very high, so there is a scope and great need to develop new protein aggregation inhibitors. Certain amphiphilic proteins[23] and sulfobetaines with nondetergent properties[24] have shown protein aggregation inhibitor properties in solution by assisting in protein folding.

One of the common proteins used to study protein aggregation is hen egg-white lysozyme (HEWL) because of its easy availability and readily available information about its complete primary and higher-order structures.[25,26] Also, its folding and unfolding mechanisms have been studied in detail, and the unfolding intermediates have been identified.[27−30] Lysozyme is a small enzyme that acts as an antimicrobial protein by cleaving the cell walls of bacteria.[31] Lysozyme readily undergoes a thermally induced aggregation and forms amyloid-like fibrils.[32,33] The structure of the HEWL is similar to that of the human lysozyme, and certain mutations in this protein have been reported to induce an amyloid disease.[34,35] In addition, we used insulin protein, which regulates the sugar level in body and is secreted in the form of hormone from pancreas. Insulin is widely popular in the treatment of diabetes, which has affected millions of lives globally. It is commonly available in the form of injections and pumps where the aggregation of insulin decreases it activity over the time. Hence, it is a challenge to administer the protein in the treatment of diabetes that arouse the need for modulators.

Received: May 15, 2019
Accepted: June 14, 2019
Published: July 15, 2019

Scheme 1. Synthesis of Poly-SPB with Hydrophobic and Hydrophilic RAFT Agent



In our previous study, we showed that polysulfobetaine polymers can act as protein aggregation inhibitors against HEWL and insulin.[36−38] However, the structure−activity relationship, i.e., the importance of various polymer parameters such as hydrophobicity and molecular structure on the efficiency of inhibition had not been elucidated. Therefore, in this report, we have synthesized a library of poly-SPB polymers with different molecular weights, end groups, and functionality via reversible addition fragmentation chain transfer (RAFT) polymerization and then examined the aggregation inhibition of the hen egg lysozyme and bovine insulin. This was accomplished by measuring the residual enzymatic activity of lysozyme and aggregation profile of lysozyme and insulin in the poly-SPB solution by employing Thioflavin T (ThT) assay.

2. RESULTS AND DISCUSSION

2.1. Characterization. Polysulfobetaine polymers with different RAFT agents and degrees of polymerization (DP) were synthesized from the monomer 3-((3-acrylamidopropyl)-dimethylammonio)-propane-1-sulfonate by RAFT polymerization (Scheme 1).

The polymers were characterized using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy (Figures S1−S8). Polymer formation was analyzed by ^{1}H NMR by observing the loss of vinyl protons during the course of polymerization. Figure 1a,b clearly demonstrates that 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (R1) and 3-((((1-carboxyethyl)thio)carbonothioyl)thio)propanoic acid (R2) polymers with different molecular weights were obtained, indicated by the differences in the intensity of signals of the RAFT agent in polymers with different degrees of polymerization (DP). Since the relative amount of the RAFT agent end group in the polymer with DP 20 is higher than that in the polymer with DP 200, clear signals of the RAFT agent with higher intensity were obtained. Polymers with different levels of RAFT agents were also successfully synthesized (Figure 1c). The average molecular weights and polydispersity index (PDI) values of poly-SPB polymers were determined using gel permeation chromatography (GPC). Owing to the use of RAFT polymerization, the PDI was determined to be between 1.1 and 1.5, and the average molecular weights were similar to the theoretical values. A summary of the characteristics of all of the polymers synthesized is provided in Table 1. Poly-SPB of DP 20 and 200 with R1 will be denoted as R1-20 and R1-200, respectively. Similarly, with R2, polymers are indicated as R2-20 and R2-200.

2.2. Residual Enzymatic Activity. The protein aggregation inhibition property of polysulfobetaines was studied using an enzymatic assay of HEWL involving *Micrococcus lysodeikticus*. Lysozyme is an enzyme that can break down the cell walls of various bacteria by hydrolyzing them. Lysozyme clears the turbidity of the bacterial suspension, as it destroys the bacterial cell walls making bactericidal studies possible. The disappearance of turbidity in the poly-SPB solution with the addition of lysozyme was measured using spectrophotometric analysis where the absorbance was measured with time. The residual lysozyme activity of the solution at different concentrations was calculated from slope of the graph plotted between absorbance and time. The term residual lysozyme activity here refers to the remaining ability of the lysozyme to perform lysis of bacterial cells after it has been heated to 90 °C. Arginine hydrochloride was used as the control to assess the activity of polysulfobetaines (Figure S9). In addition, the stability of the dodecyl group of the RAFT agent was analyzed using mass spectrometry (MALDI) (Figure S10).

Residual activity of lysozyme was calculated both in the presence and in the absence of poly-SPBs to examine their capacity to prevent the thermal aggregation of lysozyme. Figure 2a,b unambiguously demonstrate that a polymer

DOI: 10.1021/acsomega.9b01409
ACS Omega 2019, 4, 12186−12193

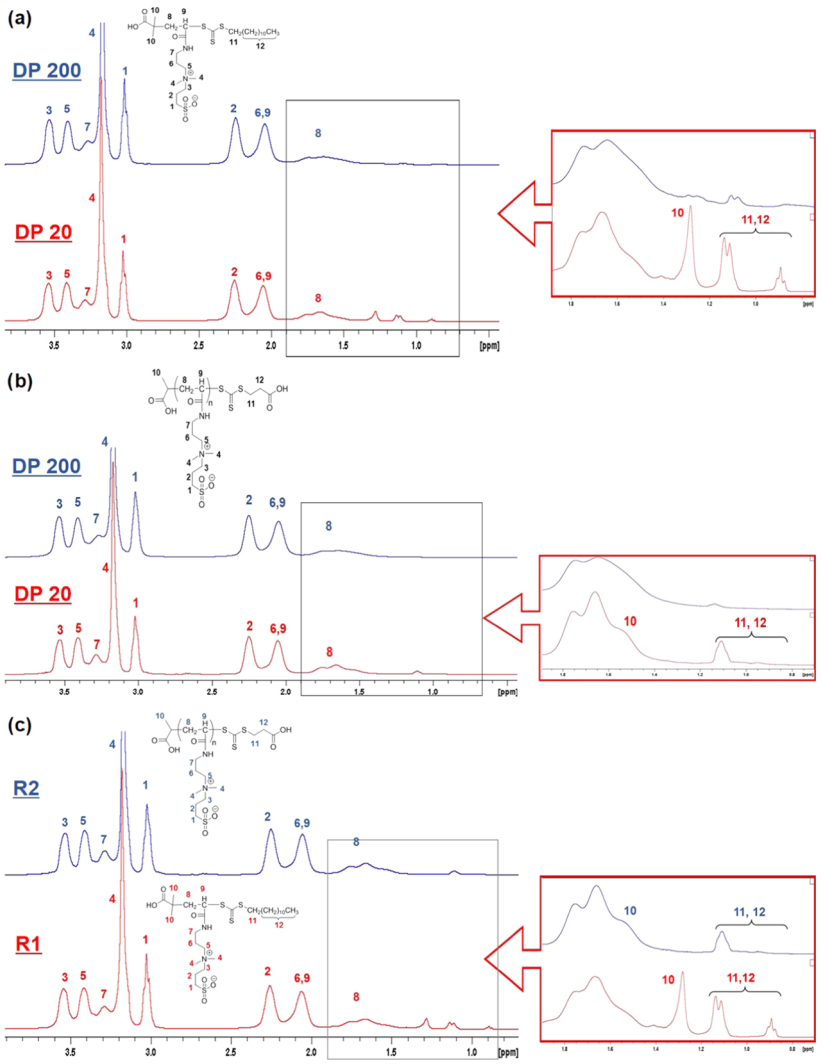


Figure 1. ^{1}H NMR of poly-SPB with (a) DP 20 and DP 200 using hydrophobic RAFT agent (R1), (b) with DP 20 and DP 200 using hydrophilic RAFT agent (R2), and (c) R2 and R1 with DP 20 in D_2O. M_n can be calculated from the NMR data, $M_n = nM_0 + M_e$ where n is DP (no. of repeating units), M_0 is the molecular weight of one monomer (repeating unit), and M_e is the molecular weight of the end-groups attached to the polymer.

Table 1. Characteristics of All of the Polymers Prepared via RAFT Polymerization

entry	polymer	RAFT agent	molar ratio[c]	$M_n \times 10^{-3}$[d]	M_w/M_n[d]
1	poly-SPB_{20}	hydrophobic (R1)[a]	100:1:5	5.5	1.13
2	poly-SPB_{20}	hydrophilic (R2)[b]	100:1:5	4.7	1.18
3	poly-SPB_{200}	hydrophobic (R1)[a]	1000:1:5	36.2	1.59
4	poly-SPB_{200}	hydrophilic (R2)[b]	1000:1:5	45.0	1.12

[a]2-(Dodecylthiocarbonothioylthio)-2-methylpropionic acid. [b]3-((((1-Carboxyethyl)thio)carbonothioyl)thio)-propanoic acid. [c][Monomer]/[initiator]/[RAFT agent]. [d]Determined by GPC.

synthesized with a hydrophobic RAFT agent (R1) shows better propensity to stabilize proteins during heating. The residual enzymatic activity obtained with R1 was markedly higher than that obtained with R2. A previous study had indicated that hydrophobic species are capable of masking the hydrophobic surfaces of proteins,[40] which, in turn, could prevent the aggregation-inducing collisions between the hydrophobic domains of the protein. Additionally, we can see that changing the RAFT agent in case of the higher-molecular-weight polymer did not yield a significant difference. This can be attributed to the relative amounts of RAFT agent in polymers with DP 20 and DP 200. In the case of R1-20 and R2-20, since the polymer chain is short, the end group has a significant effect on the overall properties of the polymer. Thus, the protein molecules are unable to interact due to the hindrance from the hydrophobic raft agent in the short-chain polymer. On the contrary, in R1-200 and R2-200, owing to the long polymer chain, the end group does not have any significant effect on the polymer properties. Similar results were obtained upon increasing the degree of polymerization, i.e., the molecular weight of the polymer. Increase in the DP from 20 to 200 resulted in an increase in residual enzymatic activity (Figure 2c,d), thus indicating that polymers with longer chains enable greater protection for proteins during

DOI: 10.1021/acsomega.9b01409
ACS Omega 2019, 4, 12186−12193

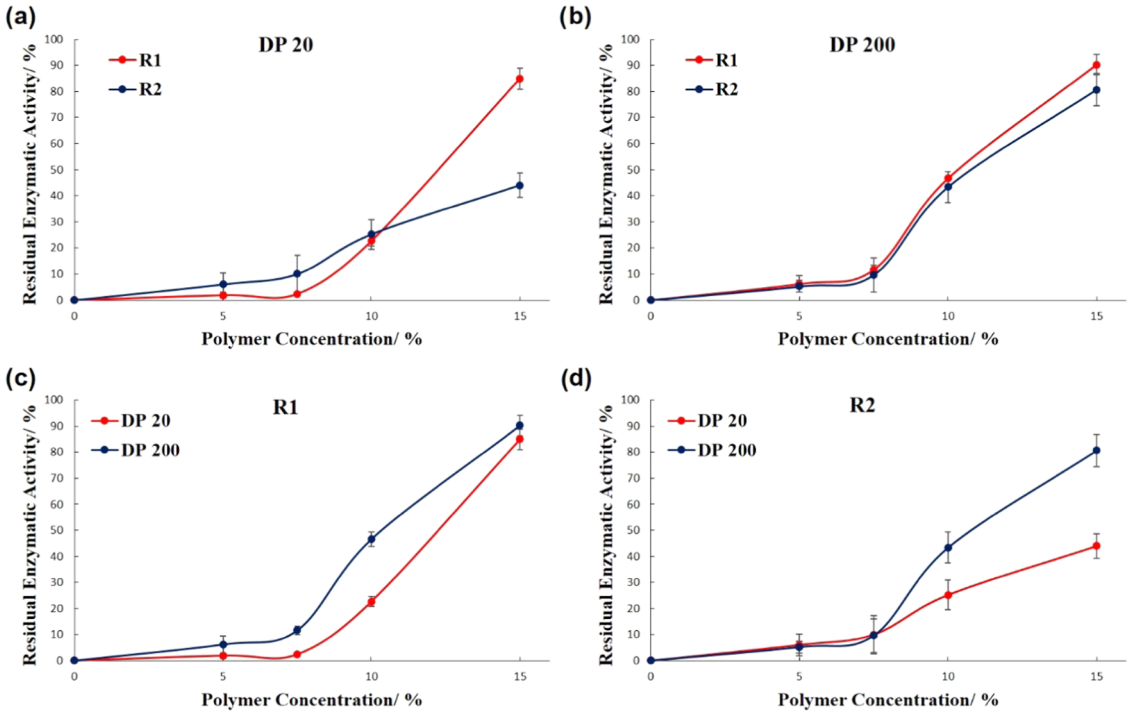


Figure 2. Enzymatic activity of lysozyme after treatment in the presence of poly-SPBs at 90 °C with (a) different RAFT agents with DP 20, (b) different RAFT agents with DP 200, (c) different DPs with R1, and (d) different DPs with R2.

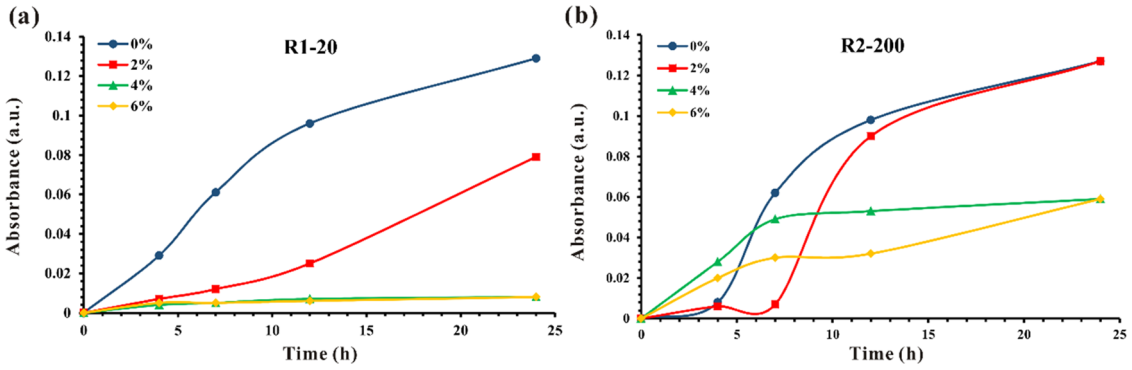


Figure 3. UV−vis analysis of insulin when incubated at 37 °C in the presence of (a) R1-20 and (b) R2-200 at 2% (red squares), 4% (green triangles), and 6% (yellow rhombus). Absorbance was measured at 500 nm over 24 h. Absorbance values indicated the extent of aggregation of insulin.

extreme stress. Since the inhibition of protein aggregation by poly-SPB is linked to their antibiofouling abilities, the increase in inhibition efficiency is due to the greater antibiofouling ability. As is well known, shorter polymer chains exhibit antibiofouling ability due to their hydrophilicity owing to the formation of the hydration layer. On the other hand, in polymer with longer chains, the effect due to the formation of the hydration layer is supplemented by the presence of steric repulsion (due to flexible polymer chains), which enables them to have a greater antibiofouling ability.[41,42]

2.3. Aggregation Profile of Insulin. Aggregation profile of insulin was studied in the polymer solution using spectrophotometric analysis, where an increase in absorbance was measured with respect to time. The analysis was carried out over 24 h, and absorbance was noted at time intervals such as 0, 4, 7, 12, and 24 h where insulin was incubated in polymer solution at 37 °C. Polymers were used at three different concentrations (2, 4, and 6%); it is noticeable that the concentration of the polymers to arrest the aggregation of insulin was much lower than that needed for lysozyme. It can be attributed to the fact that insulin is composed of 51 amino acids, which is less than that number of amino acids in lysozyme. Initially, we have done a simultaneous comparison of R1−R2 and DP 20−200. Figure 3a shows that in the presence of R1-20, insulin aggregation was reduced significantly. In addition, the decrease was linear with increase in concentration. This is due to the presence of the hydrophobic RAFT agent (R1) integrated with poly-SPB. Meanwhile, R2-200 did not show significant changes in the aggregation of insulin at 2% (Figure 3b). However, with rise in polymer concentration (4 and 6%), there was a decrease in the absorbance values, indicating the arrest of insulin aggregation in the presence of high-molecular-weight polymer with R2. Further, we discussed individual comparisons in the activity of poly-SPBs by utilizing Thioflavin T (ThT) assay, which is a widely acceptable method to study the aggregation of proteins.

DOI: 10.1021/acsomega.9b01409
ACS Omega 2019, 4, 12186−12193

2.4. Thioflavin T Assay. Proteins are known to form amyloid fibrils as a result of aggregation. These fibrils are reported to be responsible for a wide range of diseases such as Alzheimer's and prion diseases as well as several types of systemic amyloidosis.[43,44] Hence, suppression of the formation of the fibrils is very important for the development of the next generation of protein biopharmaceutics. Therefore, we used a ThT fluorescence assay to analyze the effect of sulfobetaine polymers on lysozyme and insulin fibrillation. ThT is a cationic benzothiazole salt that shows specific binding to amyloid fibrils,[45] which, in turn, leads to enhanced fluorescence.[46] Hence, a higher ThT fluorescence indicates an increased formation of amyloid fibrils. ThT assay demonstrated that a poly-SPB formed in the presence of a hydrophobic RAFT agent at DP 20 (R1-20) resulted in the greater suppression of fibril formation of lysozyme than a poly-SPB formed in the presence of R2 (R2-20), which is in accordance with the observation of enzymatic activity assay. Far UV-circular dichroism (CD) spectrum shows the preservation of the higher-order structure of lysozyme in the presence of R1-20 upon incubation at elevated temperature (Figure S12). In our previous report, attenuated total reflection Fourier transform infrared and NMR measurements exhibited that R1-20 prevents any changes in the secondary structure of lysozyme.[36] The same trend was observed for the increase in the molecular weight of the polymer, where a polymer with a longer chain length showed greater efficiency in suppressing the formation of fibrils (Figure 4). This result clearly suggests that

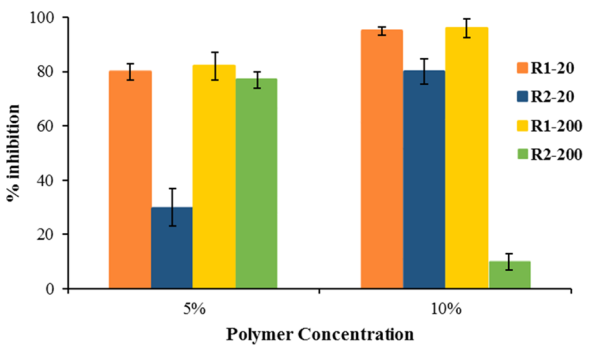


Figure 4. Aggregation of lysozyme at the concentration of 0.5 mg/mL, heated to 90 °C for 30 min in the presence of poly-SPB with DP 20 and 200 of R1 and R2 at 5 and 10% polymer concentrations. Data are expressed as the mean ± standard deviation (SD), where error bars are SD of three independent experiments (five samples each).

polysulfobetaines have excellent efficiency in inhibiting the formation of fibrils, and further changes in the polymer structure and functionality could render it more effective in stabilizing proteins under extreme stress.

In case of insulin, we analyzed insulin aggregation in all four polymers at 2, 4, and 6%. Figure 5a,b demonstrate the effect of the hydrophobic RAFT agent (R1) and the hydrophilic RAFT agent (R2) at 2, 4, and 6% polymer concentrations on the time-dependent aggregation profile of insulin. As shown in these figures, an increase in the concentration leads to significant increase in the inhibition property of these polymers. R1-20 exhibits higher efficiency (as can be seen in Figure S11) to arrest the aggregation of insulin even at 2%, whereas higher concentration of R2-20 was required to do the same. Micellar form of polymer caused hindrance in the aggregation by shielding the monomers/dimers of insulin. R1-20 also retains the higher-order structure of insulin upon incubating the protein at physiological temperature (Figure S13). Further, use of higher-molecular-weight polymers, i.e., R1-200 and R2-200, showed that R2-200 exhibits better inhibition than R1-200 (Figure 5c,d). These findings indicate that the hydrophobic RAFT agent (R1) with smaller DP (20) contributes significantly to arrest the aggregation of insulin (Figure 5e). In addition, activity was in the order of R1-20 > R2-20 > R2-200 > R1-200. The activity of R1-200 was lower than that of R2-200, since long chain of polymer tones down the effect of R1 in protein inhibition. Thus, we can conclude that R1-20 exhibits the best inhibitory effect in the aggregation of insulin. This is in complete accordance with the spectrophotometric analysis.

We propose a hypothetical model to explain the mechanism behind the activity of polymer, as presented as Figure 6. As polymers are zwitterionic in nature, we postulate that it will serve as a molecular shield between two protein molecules. The aggregation of protein will occur only if monomer molecules of protein comes in contact, forms oligomeric, and then fibrillary species. In addition, poly-SPBs may also act as molecular chaperons; in general, chaperons are ubiquitous class of compounds that are responsible for folding/refolding of the proteins. Recently, we have demonstrated that poly-SPB in conjugation with butyl methacrylate can cause the refolding of the fibrillary species of insulin protein.[38]

3. CONCLUSIONS

Through the study of the protein aggregation inhibition abilities of poly-SPBs with different chain lengths and RAFT agents, we were able to notice the change in the inhibition patterns of these polymers. The results unambiguously demonstrated that sulfobetaines have excellent efficiency in inhibiting lysozyme and insulin aggregation, and that a change in molecular weight and hydrophobicity of the polymers caused a greater stabilization of lysozyme and insulin. These investigations indicated the potential of these polymers to suppress the formation of amyloid fibrils. This study will further our ability to discern the myriad of properties associated with the protein aggregation inhibition capacity of polysulfobetaines, the polymers that could serve as an indispensable source to treat various problems associated with protein aggregation. Although differences in the aggregation inhibition tendencies of these polysulfobetaines have been examined and explained theoretically, further studies are underway to find out more about dependence of aggregation inhibition efficiencies of poly-SPBs on RAFT agents and different functional groups.

4. MATERIALS AND METHODS

4.1. Materials. Osaka Organic Chemical (Osaka, Japan) donated sulfobetaine monomer that was used without further purification. Insulin from bovine pancreas, lysozyme from chicken egg white, *M. lysodeikticus*, and 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (R1) were obtained from Sigma-Aldrich (Japan). Azobisisobutyronitrile (AIBN) was obtained from Wako Pure Chemical Industries (Osaka, Japan). 3-((((1-Carboxyethyl)thio)carbonothioyl)thio)-propanoic acid (R2) was purchased from Boron Molecular (North Carolina).

4.2. Synthesis of Poly-SPB. SPB polymer with hydrophobic and hydrophilic RAFT agents and DP were prepared.

DOI: 10.1021/acsomega.9b01409
ACS Omega 2019, 4, 12186−12193

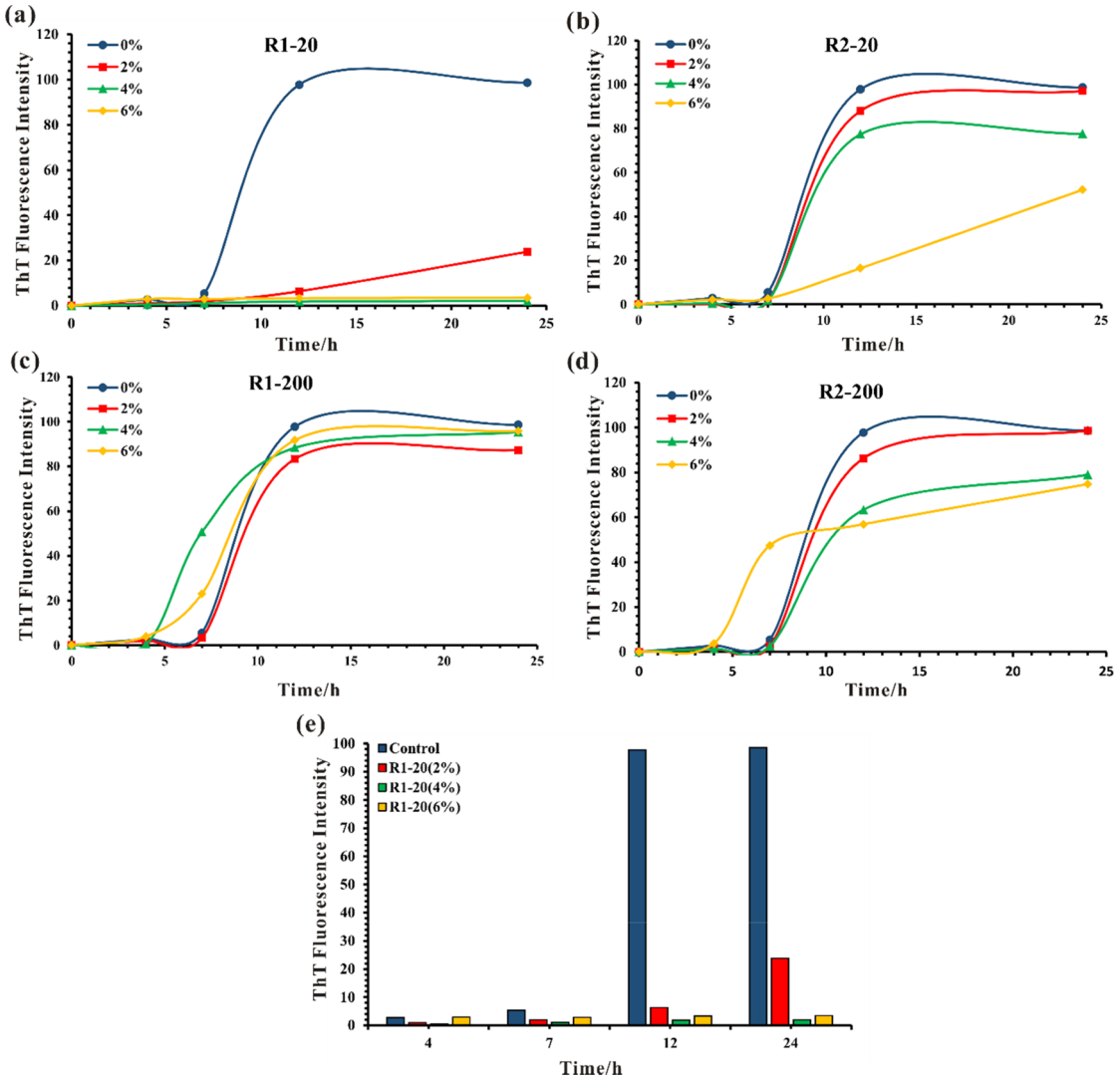


Figure 5. Thioflavin T fluorescence of insulin (0.5 mg/mL) when incubated at 37 °C for 24 h in the presence of different polymers. (a) R1-20, (b) R2-20, (c) R1-200, and (d) R2-200 at 0, 2, 4, and 6%, respectively. (e) Bar graph representation of the fluorescence intensity values of R1-20 and 2, 4, and 6%. Data are expressed as the mean ± SD of three independent experiments.

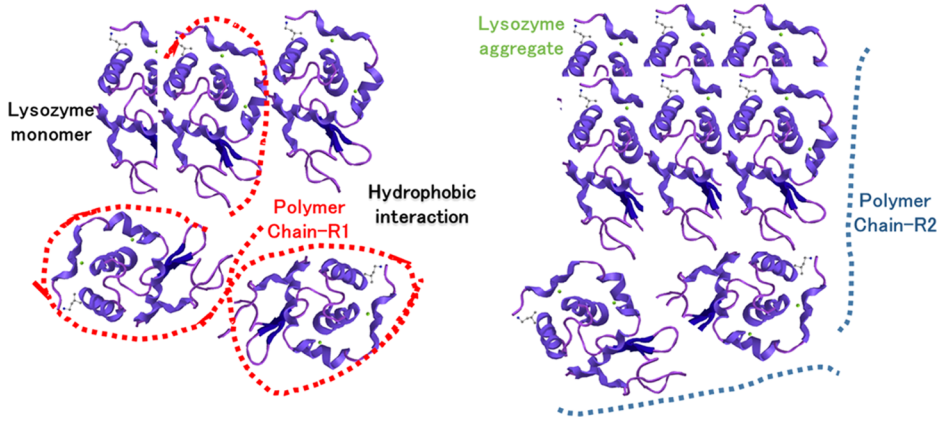


Figure 6. Schematic representation of the action mechanism of polymer chain to inhibit the aggregation of insulin monomer.

For instance, the methodology of polymer synthesis with DP 20 and R1 as RAFT agent is stated as follows: SPB monomer (8 mmol), R1 (0.4 mmol), and AIBN (0.08 mmol) were dissolved in a mixture of water and methanol (1:3), followed by purging with nitrogen gas for 1 h. Sealed solution flasks were then placed and stirred in an oil bath at 70 °C for 24 h. The samples (20−30 μL) were removed periodically, quenched in liquid nitrogen, and used for observing the ^{1}H NMR spectra (in D_2O) to calculate the conversion rate. For purification, the reaction mixture was dialyzed against water for

DOI: 10.1021/acsomega.9b01409
ACS Omega 2019, 4, 12186−12193

3 days with water changed five times, and the dry sample was obtained after lyophilization.

4.3. Nuclear Magnetic Resonance (NMR) Spectroscopy. ^{1}H and ^{13}C NMR spectra of the polymers were recorded using D_2O as the solvent using a 400 MHz Bruker Avance III HD spectrometer installed with the Topspin 3.5 software, which was used to process the spectra (Figures S1−S8).

4.4. Gel Permeation Chromatography (GPC). For the determination of molecular weight and polydispersity index (PDI) of the polymers, GPC (BioSeps2000 column; Phenomenex, Inc., CA) equipped with refractive index detector and a high-performance liquid chromatography data system (Shimadzu) was used. Mobile phase was NaBr solution (pH 7.4, 0.1 M) with the flow rate of 1 mL/min for the column, and pullulan was used as a standard with different molecular weights obtained from Shodex Group, Tokyo, Japan.

4.5. Evaluation of Protein Aggregation Inhibition by Poly-SPB. *4.5.1. Enzymatic Activity of Lysozyme after Heating in Poly-SPB.* To study the protein aggregation inhibition using poly-SPB, an enzymatic assay was done to determine the residual enzymatic activity of lysozyme after its thermal aggregation. The solutions of polymers at different concentrations (w/w %) were prepared in phosphate buffer solution (PBS). The solution of *M. lysodeikticus* (0.25 mg/mL) and lysozyme (0.1 mg/mL) was prepared in PBS as well. One hundred microliters of the polymer solution at different concentrations and 100 μL of the lysozyme solution were mixed and heated at 90 °C for 30 min. One hundred microliters of this solution was then taken and mixed with 2 mL of the solution of *M. lysodeikticus*. To check the turbidity, UV−vis measurements were made at 25 °C, and 600 nm for 6 min with constant stirring for each sample, using a V-670 spectrophotometer (JASCO Inc., Japan).[39]

4.5.2. Aggregation of Insulin in Poly-SPB. The UV−visible spectra were recorded using UV−vis spectrophotometer (UV-1800, Shimadzu Corp., Kyoto, Japan). The concentration of insulin was 0.5 mg/mL; and the solutions of polymers at different concentrations (w/w %) were prepared in PBS. Insulin in polymer solution was mixed and incubated at 37 °C for 24 h. The absorbance was measured at 0, 4, 7, 12, and 24 h to monitor the aggregation of insulin with and without polymers at 500 nm.

4.5.3. Amyloid Fibril Formation of Lysozyme and Insulin. Thioflavin T (ThT) was used to measure the amyloid fibrils, and stock solution was prepared by dissolving 8 mg ThT to 10 mL PBS (pH 7.4). The solution was then filtered through a 0.22 μm filter (Millex-GP Filter Unit). On diluting the stock solution, working solution was prepared by adding 49 mL PBS to 1 mL of stock. Lysozyme/insulin solution was mixed with the solution of polymer; using PBS as the solvent, at different concentrations and heated to 90 °C for 30 min (insulin was incubated at 37 °C for 24 h). One hundred microliters of this solution was added to 2 mL of the working solution of ThT and mixed well. Fluorescence intensity was measured at an excitation wavelength of 450 nm and emission wavelength of 484 nm using a JASCO FP-8600 spectrofluorometer.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01409.

Figures S1 and S2: ^{1}H NMR and ^{13}C NMR spectra of poly-SPB with a hydrophobic RAFT agent with DP 20, respectively, Figures S3 and S4: ^{1}H NMR and ^{13}C NMR spectra of poly-SPB with a hydrophobic RAFT agent with DP 200, respectively, Figures S5 and S6: ^{1}H NMR and ^{13}C NMR spectra of poly-SPB with a hydrophilic RAFT agent with DP 20, Figures S7 and S8: ^{1}H NMR and ^{13}C NMR spectra of poly-SPB with a hydrophilic RAFT agent with DP 200; Figure S9: enzymatic activity of lysozyme after treating at 90 °C in the presence of arginine hydrochloride and sulfobetaines, Figure S10; MALDI data of poly-SPB synthesized with a hydrophobic RAFT agent (R1) with DP 20 heated at 90 °C, Figure S11: TEM data of insulin in poly-SPB synthesized with a hydrophobic RAFT agent (R1) with DP 20, Figure S12: representing far UV-CD spectra of lysozyme in poly-SPB synthesized with a hydrophobic RAFT agent (R1) with DP 20 after 30 min heating at 90 °C, Figure S13: conformational data of insulin in poly-SPB synthesized with a hydrophobic RAFT agent (R1) with DP 20 after 24 h of incubation (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mkazuaki@jaist.ac.jp. Tel.: +81-761-51-1680.

ORCID

Robin Rajan: 0000-0002-6610-9661

Kazuaki Matsumura: 0000-0001-9484-3073

Author Contributions

N.S., R.R., and S.M. performed the experiments, analyzed the data, and wrote the paper; K.M. conceived and designed the experiments.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Nakabayashi, N.; Iwasaki, Y. Copolymers of 2-methacryloyloxyethyl phosphorylcholine (MPC) as biomaterials. *Bio-Med. Mater. Eng.* **2004**, *14*, 345−354.

(2) Matsuda, T.; Nagase, J.; Ghoda, A.; Hirano, Y.; Kidoaki, S.; Nakayama, Y. Phosphorylcholine-endcapped oligomer and block co-oligomer and surface biological reactivity. *Biomaterials* **2003**, *24*, 4517−4527.

(3) Ma, I. Y.; Lobb, E. J.; Billingham, N. C.; Armes, S. P.; Lewis, A. L.; Lloyd, A. W.; Salvage, J. Synthesis of Biocompatible Polymers. 1. Homopolymerization of 2-Methacryloyloxyethyl Phosphorylcholine via ATRP in Protic Solvents: An Optimization Study. *Macromolecules* **2002**, *35*, 9306−9314.

(4) Hirota, K.; Murakami, K.; Nemoto, K.; Miyake, Y. Coating of a surface with 2-methacryloyloxyethyl phosphorylcholine (MPC) copolymer significantly reduces retention of human pathogenic microorganisms. *FEMS Microbiol. Lett.* **2005**, *248*, 37−45.

(5) Feng, W.; Zhu, S.; Ishihara, K.; Brash, J. L. Adsorption of Fibrinogen and Lysozyme on Silicon Grafted with Poly(2-methacryloyloxyethyl Phosphorylcholine) via Surface-Initiated Atom Transfer Radical Polymerization. *Langmuir* **2005**, *21*, 5980−5987.

(6) Chung, Y. C.; Chiu, Y. H.; Wu, Y. W.; Tao, Y. T. Self-assembled biomimetic monolayers using phospholipid-containing disulfides. *Biomaterials* **2005**, *26*, 2313−2324.

(7) Yuan, Y.; Zang, X.; Ai, F.; Zhou, J.; Shen, J.; Lin, S. Grafting sulfobetaine monomer onto silicone surface to improve haemocompatibility. *Polym. Int.* **2004**, *53*, 121−126.

(8) Lalani, R.; Liu, L. Electrospun Zwitterionic Poly(Sulfobetaine Methacrylate) for Nonadherent, Superabsorbent, and Antimicrobial

DOI: 10.1021/acsomega.9b01409
ACS Omega 2019, 4, 12186−12193

Wound Dressing Applications. *Biomacromolecules* **2012**, *13*, 1853−1863.
(9) Kitano, H.; Mori, T.; Takeuchi, Y.; Tada, S.; Gemmei-Ide, M.; Yokoyama, Y.; Tanaka, M. Structure of Water Incorporated in Sulfobetaine Polymer Films as Studied by ATR-FTIR. *Macromol. Biosci.* **2005**, *5*, 314−321.
(10) Kondo, T.; Nomura, K.; Murou, M.; Gemmei-Ide, M.; Kitano, H.; Noguchi, H.; Uosaki, K.; Ohno, K.; Saruwatari, Y. Structure of water in the vicinity of a zwitterionic polymer brush as examined by sum frequency generation method. *Colloids Surf., B* **2012**, *100*, 126−132.
(11) Rajan, R.; Hayashi, F.; Nagashima, T.; Matsumura, K. Toward a Molecular Understanding of the Mechanism of Cryopreservation by Polyampholytes: Cell Membrane Interactions and Hydrophobicity. *Biomacromolecules* **2016**, *17*, 1882−1893.
(12) Cao, F.; Tan, L.; Xiang, L.; Liu, S.; Wang, Y. Application of the copolymers containing sulfobetaine methacrylate in protein separation by capillary electrophoresis. *J. Biomater. Sci., Polym. Ed.* **2013**, *24*, 2058−2070.
(13) Rajan, R.; Matsumura, K. Tunable Dual-Thermoresponsive Core-Shell Nanogels Exhibiting UCST and LCST Behavior. *Macromol. Rapid Commun.* **2017**, *38*, No. 1700478.
(14) Sun, H.; Chang, M. Y. Z.; Cheng, W.-I.; Wang, Q.; Commisso, A.; Capeling, M.; Wu, Y.; Cheng, C. Biodegradable zwitterionic sulfobetaine polymer and its conjugate with paclitaxel for sustained drug delivery. *Acta Biomater.* **2017**, *64*, 290−300.
(15) Ross, C.; Poirier, M. Protein aggregation and neurodegenerative disease. *Nat. Med.* **2004**, *10*, S10−17.
(16) Lansbury, P. T.; Lashuel, H. A. A century-old debate on protein aggregation and neurodegeneration enters the clinic. *Nature* **2006**, *443*, 774−779.
(17) Koo, E. H.; Lansbury, P. T. J.; Kelly, J. W. Amyloid diseases: abnormal protein aggregation in neurodegeneration. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9989−9990.
(18) Wang, W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int. J. Pharm.* **1999**, *185*, 129−188.
(19) Sluzky, V.; Tamada, J. A.; Klibanov, A. M.; Langer, R. Kinetics of insulin aggregation in aqueous solutions upon agitation in the presence of hydrophobic surfaces. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 9377−9381.
(20) Taneja, S.; Ahmad, F. Increased thermal stability of proteins in the presence of amino acids. *Biochem. J.* **1994**, *303*, 147−153.
(21) Samuel, D.; Kumar, T. K.; Ganesh, G.; Jayaraman, G.; Yang, P. W.; Chang, M. M.; Trivedi, V. D.; Wang, S. L.; Hwang, K. C.; Chang, D. K.; Yu, C. Proline inhibits aggregation during protein refolding. *Protein Sci.* **2000**, *9*, 344−352.
(22) Karuppiah, N.; Sharma, A. Cyclodextrins as protein folding aids. *Biochem. Biophys. Res. Commun.* **1995**, *211*, 60−66.
(23) Ow, S.-Y.; Bekard, I.; Blencowe, A.; Qiao, G. G.; Dunstan, D. E. A generic class of amyloid fibril inhibitors. *J. Mater. Chem. B* **2015**, *3*, 1350−1359.
(24) Expert-Bezançon, N.; Rabilloud, T.; Vuillard, L.; Goldberg, M. E. Physical−chemical features of non-detergent sulfobetaines active as protein-folding helpers. *Biophys. Chem.* **2002**, *100*, 469−479.
(25) Blake, C. C.; Koenig, D. F.; Mair, G. A.; North, A. C.; Phillips, D. C.; Sarma, V. R. Structure of hen egg-white lysozyme. A three-dimensional Fourier synthesis at 2 Angstrom resolution. *Nature* **1965**, *206*, 757−761.
(26) Canfield, R. E. The Amino Acid Sequence of Egg White Lysozyme. *J. Biol. Chem.* **1963**, *238*, 2698−2707.
(27) Redfield, C.; Dobson, C. M. Sequential proton NMR assignments and secondary structure of hen egg white lysozyme in solution. *Biochemistry* **1988**, *27*, 122−136.
(28) Dobson, C. M.; Evans, P. A.; Radford, S. E. Understanding how proteins fold: the lysozyme story so far. *Trends Biochem. Sci.* **1994**, *19*, 31−37.
(29) Itzhaki, L. S.; Evans, P. A.; Dobson, C. M.; Radford, S. E. Tertiary Interactions in the Folding Pathway of Hen Lysozyme: Kinetic Studies Using Fluorescent Probes. *Biochemistry* **1994**, *33*, 5212−5220.
(30) Radford, S. E.; Dobson, C. M.; Evans, P. A. The folding of hen lysozyme involves partially structured intermediates and multiple pathways. *Nature* **1992**, *358*, 302−307.
(31) Repaske, R. Lysis of gram-negative bacteria by lysozyme. *Biochim. Biophys. Acta* **1956**, *22*, 189−191.
(32) Arnaudov, L. N.; de Vries, R. Thermally Induced Fibrillar Aggregation of Hen Egg White Lysozyme. *Biophys. J.* **2005**, *88*, 515−526.
(33) Krebs, M. R. H.; Wilkins, D. K.; Chung, E. W.; Pitkeathly, M. C.; Chamberlain, A. K.; Zurdo, J.; Robinson, C. V.; Dobson, C. M. Formation and seeding of amyloid fibrils from wild-type hen lysozyme and a peptide fragment from the β-domain 11Edited by P. E. Wright. *J. Mol. Biol.* **2000**, *300*, 541−549.
(34) Pepys, M. B.; Hawkins, P. N.; Booth, D. R.; Vigushin, D. M.; Tennent, G. A.; Soutar, A. K.; Totty, N.; Nguyen, O.; Blake, C. C.; Terry, C. J.; et al. Human lysozyme gene mutations cause hereditary systemic amyloidosis. *Nature* **1993**, *362*, 553−557.
(35) Booth, D. R.; Sunde, M.; Bellotti, V.; Robinson, C. V.; Hutchinson, W. L.; Fraser, P. E.; Hawkins, P. N.; Dobson, C. M.; Radford, S. E.; Blake, C. C.; Pepys, M. B. Instability, unfolding and aggregation of human lysozyme variants underlying amyloid fibrillogenesis. *Nature* **1997**, *385*, 787−793.
(36) Rajan, R.; Matsumura, K. A zwitterionic polymer as a novel inhibitor of protein aggregation. *J. Mater. Chem. B* **2015**, 5683.
(37) Rajan, R.; Matsumura, K. Inhibition of protein aggregation by zwitterionic polymer-based core-shell nanogels. *Sci. Rep.* **2017**, *7*, No. 45777.
(38) Rajan, R.; Suzuki, Y.; Matsumura, K. Zwitterionic polymer design that inhibits aggregation and facilitates insulin refolding: mechanistic insights and importance of hydrophobicity. *Macromol. Biosci.* **2018**, *18*, No. 1800016.
(39) Kudou, M.; Shiraki, K.; Fujiwara, S.; Imanaka, T.; Takagi, M. Prevention of thermal inactivation and aggregation of lysozyme by polyamines. *Eur. J. Biochem.* **2003**, *270*, 4547−4554.
(40) Das, U.; Hariprasad, G.; Ethayathulla, A. S.; Manral, P.; Das, T. K.; Pasha, S.; Mann, A.; Ganguli, M.; Verma, A. K.; Bhat, R.; Chandrayan, S. K.; Ahmed, S.; Sharma, S.; Kaur, P.; Singh, T. P.; Srinivasan, A. Inhibition of Protein Aggregation: Supramolecular Assemblies of Arginine Hold the Key. *PLoS One* **2007**, *2*, No. e1176.
(41) Chen, S.; Li, L.; Zhao, C.; Zheng, J. Surface hydration: Principles and applications toward low-fouling/nonfouling biomaterials. *Polymer* **2010**, *51*, 5283−5293.
(42) Zhang, H.; Chiao, M. Anti-fouling Coatings of Poly-(dimethylsiloxane) Devices for Biological and Biomedical Applications. *J. Med. Biol. Eng.* **2015**, *35*, 143−155.
(43) Xing, Y.; Higuchi, K. Amyloid fibril proteins. *Mech. Ageing Dev.* **2002**, *123*, 1625−1636.
(44) Dobson, C. M. Protein folding and misfolding. *Nature* **2003**, *426*, 884−890.
(45) Khurana, R.; Coleman, C.; Ionescu-Zanetti, C.; Carter, S. A.; Krishna, V.; Grover, R. K.; Roy, R.; Singh, S. Mechanism of thioflavin T binding to amyloid fibrils. *J. Struct. Biol.* **2005**, *151*, 229−238.
(46) Groenning, M. Binding mode of Thioflavin T and other molecular probes in the context of amyloid fibrils-current status. *J. Chem. Biol.* **2010**, *3*, 1−18.

DOI: 10.1021/acsomega.9b01409
ACS Omega 2019, 4, 12186−12193