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Polyethylene-glycol-modified Zwitterionic Polymers assisted Protein Aggregation Arrest and Refolding

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Protein aggregation limits the development of protein-based drugs, and leads to neurological disorders. In this study, the zwitterionic polymer, poly-sulfobetaine (p-SPB), was modified using the polyethylene glycol (PEG), and exhibited the remarkable suppression of heat-induced lysozyme aggregation. The study revealed that the modified polymers prevented the formation of amyloid fibrils, and retained the enzymatic activity of the lysozyme, which is lost after heating. An increase in the molecular weight of the polymers afforded higher efficacy to perpetuate enzymatic activity. Along with the effectiveness of these polymers for lysozyme aggregation arrest, further evaluations revealed that these polymers facilitated the refolding of protein because of their tendency to dissolve the already formed fibrils, and regained the lost lysozyme activity. These findings suggest that the amalgamation of PEG and p-SPB has the potential to protect proteins from aggregation via altering the hydrophobic environment of the lysozyme, creating a molecular shield around the protein molecules, and thus, providing scope for the development of protein-based biopharmaceuticals.

Design, System, Application

This study addresses the modification of the zwitterionic polymer, p-SPB, using PEG to enhance the polymer's efficiency to restrain protein aggregation. We synthesized different polymers through the addition of PEG via reversible addition-fragmentation chain transfer polymerization. The newly synthesized PEG-based polymers were effective in protecting the lysozyme from thermal aggregation, even at higher temperatures. The formation of amyloid fibril in the protein after heating was suppressed in the presence of the polymers with higher molecular weights, which are also capable of retaining the enzymatic activity of the lysozyme even after prolonged heating. Along with their ability to inhibit lysozyme aggregation, the prepared polymers eased the refolding of lysozyme by dissolving the already formed fibrils, affording a high recovery rate. In addition, the lost activity of the lysozyme was regained when it was incubated with the PEG-based polymers. This approach widens the scope for using polymeric materials in the study of proteins, and the polymers can be used for improving the shelf-life of proteins, which can help grow the protein-based drugs industry.

Introduction

Having earlier played a small role in medical treatments, the application of protein therapeutics has increased exponentially. Protein drugs are promising biotherapeutic agents for the treatment of critical health problems via overcoming the limitations of conventional drugs, and provide high selectivity, specificity, and potency.[1] For instance, insulin is a protein drug used to regulate the level of sugar in the bloodstream, and is also widely applied for the treatment of diabetes. Insulin pumps were introduced to eliminate the usage of regular injections for insulin dosages, assisting in the delivery of accurate volumes of insulin.[2] However, the instability of protein and its tendency to aggregate obstruct the application of insulin pumps for insulin administration, eventually leading to insulin underdosing.[3]

Protein aggregation is of serious concern to the development of protein-based drugs, and leads to neurological disorders such as Alzheimer's, Parkinson's, and Huntington's disease.[4,5] More than 10 million people worldwide suffer from Parkinson's disease, and approximately five million people in the United States of America (USA) suffer from Alzheimer's disease.[6]
Proteins under optimal conditions attain specific conformations through self-assembling. However, external factors such as physical stress and changes in temperature or pH result in the protein undergoing misfolding, unfolding, and aggregation.[7] Factors such as thermodynamic behavior influence the robustness of the proteins.[8] Proteins lose their functionality under external stress, further leading to their misfolding, which results in the changing of protein conformations into non-native structures. Proteins such as Aβ1-40,[9] lysozyme,[10] and insulin[11] are known for their formations of amyloid fibrils that cause

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incurable neurodegenerative diseases. Removal of the amyloid fibrils formed because of protein aggregation or refolding of already misfolded proteins is a potential strategy for treating these neurodegenerative diseases and increasing the shelf life of therapeutic proteins.

Prevention of protein aggregation has been approached via various techniques. Tirrell et al. showed that modifying the insulin protein by replacing the proline residue present at position 28 with another group prevented the protein from undergoing denaturation.[12,13] However, such a strategy is highly protein-specific, and thus, cannot be applied for different types of proteins.[14] Another easier method to protect the protein from denaturation is the addition of excipients such as the small molecules of proline,[15] polyamines,[16] and guanidine[17], or various polymeric materials, which alter the external environment of the protein and allow it to retain its native structure even after exposure to physical or chemical stress.
The application of polymeric materials as additives has recently been explored for the protection of proteins from aggregation.[1] These polymeric inhibitors provide the scope for alterations with varying molecular weights and surface charges in accordance with our requirements, and their interactions with biological molecules can be affected via the incorporation of groups with different functionalities. The class of polymers known as zwitterionic polymers exhibit structures similar to those of the proteins (owing to the presence of positive as well as negative charges on the same repeating unit), and have been widely applied in the biomedical field.[18] Zwitterionic polymers exhibit excellent anti-bio-fouling properties[19] because of their ability to retain the water structure at the interface of the polymer and material,[20,21] and have also been used to improve the stability of proteins.[22] Rajan et al. reported the effectiveness of a zwitterionic polymer, poly-sulfobetaine (p-SPB) in inhibiting heat-induced protein aggregation.[23–25] It was found that the p-SPB can retain the secondary structure of the lysozyme, which is lost after prolonged heating, and facilitate the retention of enzymatic activity. This is because of the anti-biofouling property of the poly-zwitterions, i.e., p-SPB acts as a molecular shield and prevents the aggregation-prone collision (between the exposed hydrophobic domains in the misfolded chains) from occurring between the hydrophobic parts of the protein, protecting the protein from aggregation. However, a higher concentration of the polymer was required to prevent the aggregation of lysozyme. Other polymeric systems made of zwitterionic polymers such as microneedles[26] and graft polymers[27,28] also exhibited high efficiency in stabilizing proteins.
In this study, we modify the p-SPB through the addition of a side group via living radical polymerization. We aim to incorporate the polyethylene glycol group (PEG) into the p-SPB as the side group. PEG-based compounds have been applied extensively in the fields of biomaterial and biomedical because of their ability to increase biocompatibility, solubility, and shield the molecule against deactivation.[29–31] PEGylation has been reported in the study of proteins, wherein the PEG covalently conjugates with the protein, protecting it from denaturation. This strategy has been widely applied for the treatment of various diseases in protein-based therapeutics.[32–34] In 2013, Muraoka et al. synthesized triangular PEG via a multi-step reaction using pentaerythritol and substituted oligoethylene glycol.[35] The non-linear PEG molecule was observed to become dehydrated and change its conformation, resulting in an increase in hydrophobicity, which resulted in a strong interaction with the unfolded protein, thus leading to protection of the lysozyme from aggregation.

Herein, we study the amalgamation of p-SPB and PEG. We synthesize the different random and block co-polymers through the addition of different PEG groups to the SPB via reversible-addition fragmentation chain transfer (RAFT) polymerization to investigate the effectiveness of the different polymers in inhibiting protein aggregation. We investigate the effects of PEG introduction, chain length, and hydrophobicity on suppressing the thermal aggregation of lysozyme. We also introduced a hydrophobic monomer into the copolymers using butyl methacrylate (BuMA). Hydrophobicity was introduced because a previous report demonstrated that an increase in the hydrophobicity of the polymer may help in the interaction of polymers with the hydrophobic domains of proteins, thus suppressing the aggregation of proteins.[36] Furthermore, we investigate the propensity of the PEG-based zwitterionic polymers to facilitate the refolding of denatured lysozyme

Experimental

Materials

The SPB monomer was donated by Osaka Organic Chemical Ind. Ltd. (Osaka, Japan), and used in the condition it was received. 2-(Dodecylthiocarbonothioylthio)-2-methyl propionic acid (reversible addition-fragmentation chain transfer (RAFT) agent), polyethylene glycol methacrylate (PGMA), Thioflavin T (ThT), *Micrococcus lysodeikticus*, and hen egg-white lysozyme were purchased from Sigma Aldrich (Burlington, Massachusetts, USA), and used without further purification. Azobisisobutyronitrile (AIBN, initiator, used after recrystallization) and 2-methoxyethyl (E)-4-cyano-4-((2-cyano-5-methoxy-5-oxopentan-2-yl)diazenyl)pentanoate (VPE-0201, azo based initiator with PEG unit) were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan).

Synthesis of zwitterionic polymers

Synthesis of random SPB copolymer using PGMA. The SPB monomer (7 mmol), PGMA (0.78 mmol), RAFT agent (0.116 mmol), and AIBN (0.023 mmol) were dissolved in the mixture of methanol and water (3:1 v/v%). The solution was purged with nitrogen gas for 1 h, and the sealed solution flask was subsequently placed into the oil bath at 70 °C for 12 h (Scheme 1A). Twenty microliters was removed from the sample periodically and quenched in liquid nitrogen to observe its ^{1}H-NMR spectrum (in deuterium oxide) (Fig. S1) for the rate of conversion calculation. Upon completion of the reaction, the mixture was dialyzed against distilled water for three days with

the change of water four-to-five times a day, and the dry product was obtained after lyophilization. The same procedure was followed to prepare the p-SPB with 15% PGMA. In addition, a similar procedure was followed to prepare the polymers with chain lengths of p-SPB.

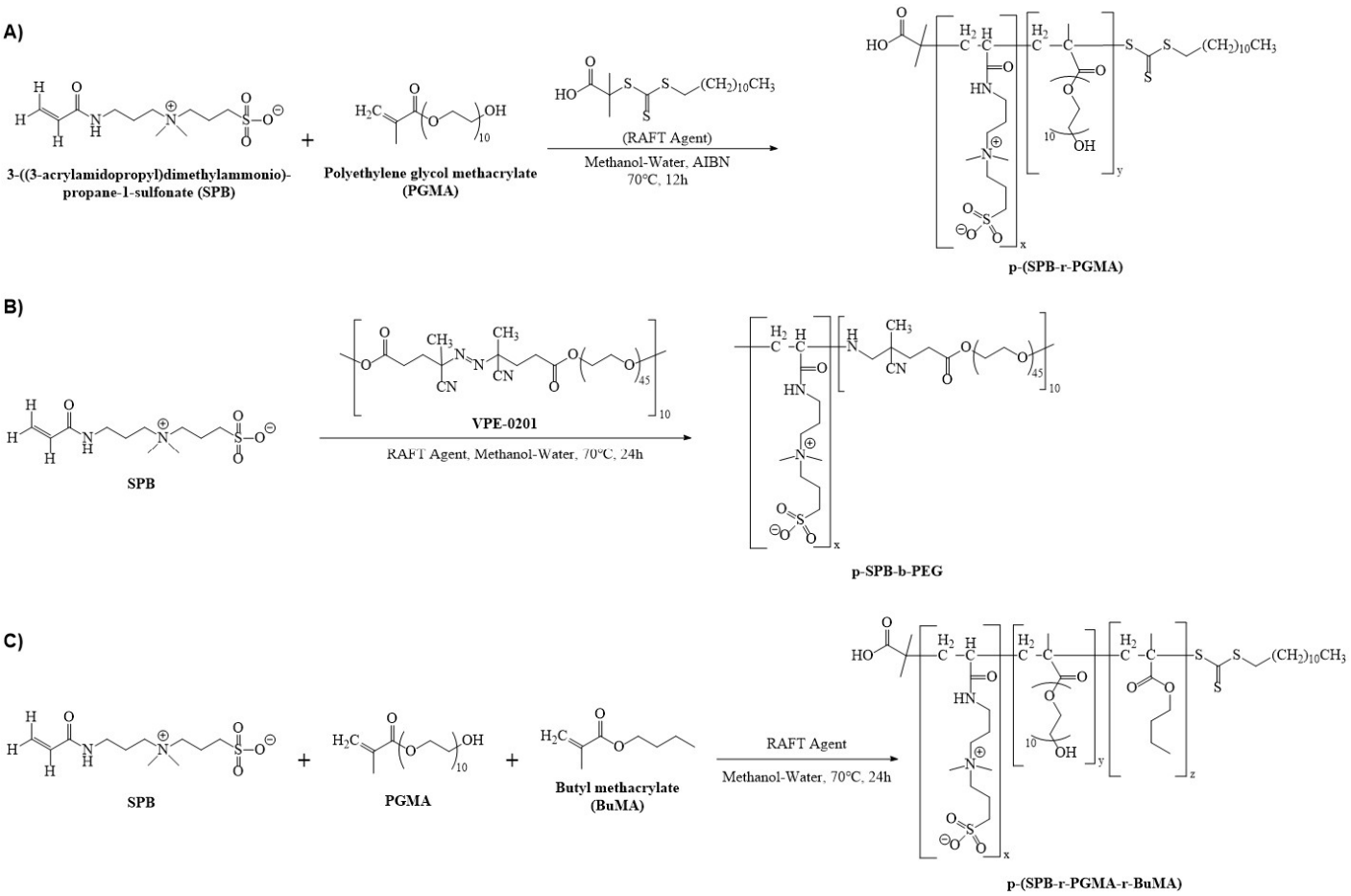
Synthesis of SPB and PEG block copolymer. The SPB monomer (7 mmol), RAFT agent (0.116 mmol), and VPE-0201 (0.023 mmol) were dissolved in the mixture of methanol and water (3:1 v/v%). The solution was purged with nitrogen gas for 1 h, and the sealed solution flask was subsequently placed in the oil bath at 70 °C for 24 h (Scheme 1B). Twenty microliters was removed from the sample periodically and quenched in liquid nitrogen to observe its ^{1}H-NMR spectrum (in deuterium oxide) (Fig. S2) for the rate of conversion calculation. Upon completion of the reaction, the mixture was dialyzed against distilled water for three days with the change of water four-to-five times a day, and the dry product was obtained after lyophilization. The same procedure was followed to prepare the p-SPB with 15% PGMA. In addition, a similar procedure was followed to prepare the, polymers with chain lengths of p-SPB.

Synthesis of random SPB tri-copolymer using PGMA and BuMA. The SPB monomer (7 mmol), PGMA (0.544 mmol), BuMA (0.11 mmol), RAFT agent (0.116 mmol), and AIBN (0.023 mmol) were dissolved in the mixture of methanol and water (3:1 v/v%). The solution was purged with nitrogen gas for 1 h, and the sealed solution flask was subsequently placed in the oil bath at 70 °C for 12 h (Scheme 1C). Upon completion of the reaction, the mixture was dialyzed against distilled water for three days with the change of water four-to-five times a day, and the dry product was obtained after lyophilization.

Molecular Weight Determination. Gel permeation chromatography (GPC, BioSeps2000 column; Phenomenex, Inc., CA, USA) with a high-performance liquid chromatography data system (Shimadzu) integrating a refractive index detector was used to measure the molecular weights and distributions (polydispersity index, PDI) of the polymers. Pullulan (Shodex Group, Tokyo, Japan) and a NaBr solution (pH 7.4, 0.1 M) were used as the mobile phase and standard, respectively.

Protein aggregation arrest

Lysozyme from hen egg-white was employed as the model protein to evaluate the efficiency of the PEG-based zwitterionic polymers in inhibiting the formation of aggregates. Lysozyme is a commonly studied protein widely applied for the study of aggregation because of its known primary and three-dimensional structure.[37,38] The lysozyme solution (20 µM) was prepared in the PBS solution (pH 7.4) and exposed to thermal



Scheme 1 Synthesis of poly-SPB with A) PGMA, B) PEG, and C) PGMA and BuMA.

treatment (90 °C for 15 min) with and without polymers. The formation of aggregates was further characterized using various techniques.

ThT assay

The formation of fibrils in the lysozyme after heating at 90 °C for 15 min was determined using the ThT (2-[4-(dimethylamino)phenyl]-3,6-dimethyl-1,3-benzothiazol-3-ium chloride) assay. ThT dye binds with β-amyloid fibrils, resulting in high fluorescence intensity.[39] The stock solution was prepared via the addition of 4 mg ThT to 5 mL PBS solution (pH 7.4), followed by filtration using a 0.22 µm filter. Two milliliters of the stock solution prepared was diluted in 98 mL of the PBS solution to prepare the working solution. The lysozyme-polymer solution was prepared by mixing equal volumes of the lysozyme and polymer solutions. The final lysozyme concentrations in the lysozyme-polymer solutions were 10 µM, and those of the polymers were 2.5, 5, and 10 w/v%. The lysozyme-polymer solution was heated for 15 min. Following this, 2 mL of the ThT working solution was mixed with 100 µL of the lysozyme-polymer solution, and the fluorescence intensity of ThT was measured using the JASCO FP-8600 spectrofluorometer (Tokyo, Japan) with the excitation and emission wavelengths of 450 and 485 nm, respectively.

Residual enzymatic activity

The lysozyme solution (20 µM) was mixed with the polymer solution of varying concentration in the PBS solution (pH 7.4). The prepared lysozyme-polymer solution was subsequently heated at 90 °C for 15 min.

Table 1. Overview of polymers prepared via RAFT polymerization.

Entry	Polymer		SPB	PGMA	BuMA	Molar Ratio [b]	M_n x10^{-3}[c]	M_w/M_n[c]
R1	p-(SPB-10%PGMA) *DP 60	In Feed In Polymer[a]	90 90.5	10 9.5	0 0	60:1:0.2	10.3	1.37
R2	p-(SPB-10%PGMA) *DP 100	In Feed In Polymer[a]	90 88.5	10 11.5	0 0	100:1:0.2	17.7	1.40
R3	p-(SPB-15%PGMA) *DP 60	In Feed In Polymer[a]	85 83.5	15 16.5	0 0	60:1:0.2	10.1	1.39
R4	p-(SPB-15%PGMA) *DP 100	In Feed In Polymer[a]	85 85.8	15 14.2	0 0	100:1:0.2	19.3	1.48
B1	p-SPB-b-PEG *DP 60	In Feed In Polymer[a]	60 34	0 0	0 0	60:1:0.2	10.9	1.63
B2	p-SPB-b-PEG *DP 100	In Feed In Polymer[a]	100 94	0 0	0 0	100:1:0.2	19.3	1.50
T1	p-(SPB-15%PGMA-10%BuMA) *DP 60	In Feed In Polymer[a]	75 **	15 **	10 **	60:1:0.2	12.6	1.40
T2	p-(SPB-15%PGMA-10%BuMA) *DP 100	In Feed In Polymer[a]	75 **	15 **	10 **	100:1:0.2	21.1	1.35

*DP represents the total degree of polymerization of all the monomers. **Compositions that were difficult to determine because of the merging of peaks in the NMR spectra. Determined using (a) 1H-NMR; (b) [SPB]:[RAFT]:[Initiator]; (c) GPC.

Two milliliters of the *Micrococcus Lysodeikticus* (0.25 mg/mL) was mixed with 100 µL of the lysozyme-polymer solution. The absorbance of the resultant mixture was measured using UV-vis spectrophotometry (UV-1800, Shimadzu) at 600 nm from 0 to 360 s with constant stirring. The continuous downward slope indicates the residual enzymatic activity of lysozyme.

Refolding of lysozyme

The lysozyme (20 µM) in PBS (pH 7.4) solution was heated at 75 °C for 45 min to evaluate the efficiency of PEG-based zwitterionic polymers in facilitating the refolding of lysozyme. After cooling the solution at room temperature for 10 min, an equal volume of the polymer solution with a different concentration was added to the heated lysozyme solution, and the resultant lysozyme-polymer mixture was incubated at 25 °C for 6 h. Further characterizations were conducted via ThT assay and residual enzymatic activity analyses following the procedures mentioned above.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). All experiments were conducted in triplicate. An ordinary one-way analysis of variance with Dunnett's multiple comparison test was used to compare the data. Differences were considered statistically significant at $P < 0.05$.

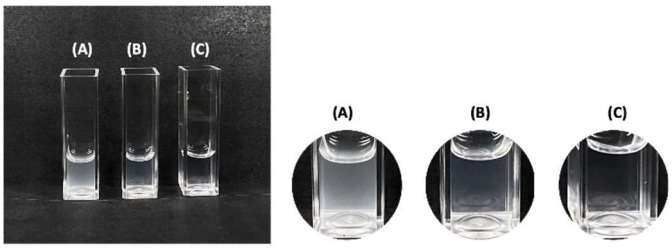


Fig. **1** Photograph of lysozyme in PBS solution after heating at 90 °C for 15 min with and without additives. Lysozyme A) only, B) in the presence of p-SPB-r-PGMA, and C) in the presence of p-SPB-b-PEG. Image of lysozyme solutions dissolved in cuvettes (left), and clear view of the occurrence of turbidity after heat treatment (right).

Results and discussion

Polymer characterization

RAFT polymerization of the SPB monomer with different groups such as the PGMA, BuMA, and PEG-based-macro-initiator (VPE-020) was conducted to obtain the random and block SPB-based copolymers (Scheme 1). The p-SPB polymers with different side groups of PEG and BuMA and degrees of polymerization (DP) were synthesized from the 3-((3-acrylamidopropyl)-dimethylammonio)-propane-1-sulfonate monomer via RAFT polymerization. Polymer formation was investigated via ^{1}H-NMR analysis (Figs. S1, S2), by observing the loss of vinyl protons at approximately 5.7 and 6.2 ppm during polymerization.[40] From the ^{1}H-NMR analysis (Fig. S3), the new peak observed at approximately 3.6 ppm suggested that the PEG group was successfully incorporated into the SPB via RAFT polymerization with the PGMA or in the presence of the VPE-0201 initiator.

Both the PGMA and BuMA were incorporated into the p-SPB, and the resultant structure was characterized via ^{1}H-NMR analysis (Fig. S4) to further evaluate the effect of the increase in hydrophobicity of the p-SPB and PEG co-polymer. The PEG-based zwitterionic polymers prepared were characterized via ^{1}H-NMR and ^{13}C-NMR analyses (Figs. S5-S20), using deuterium oxide as the solvent.

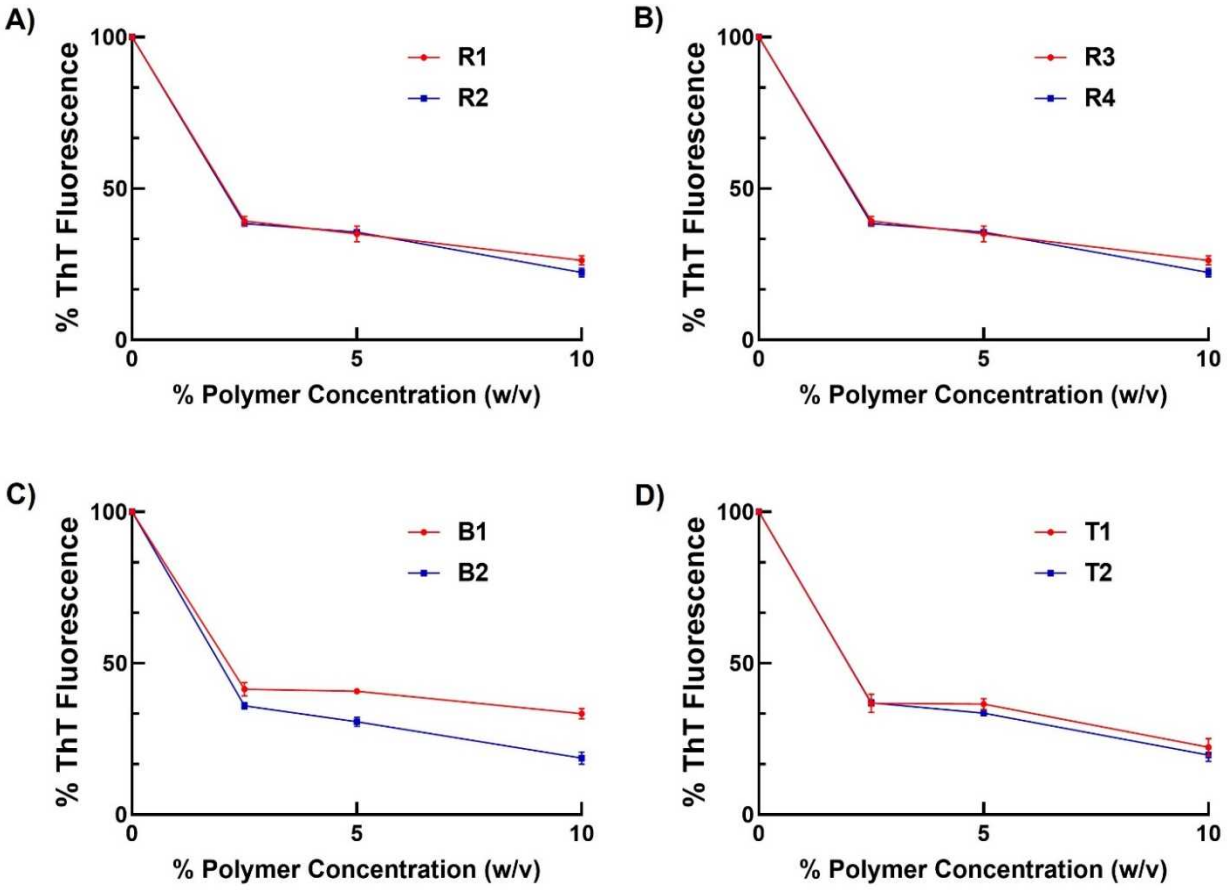


Fig. 2 ThT fluorescence intensity of lysozyme when heated in the presence of polymers: A) R1 & R2; B) R3 & R4; C) B1 & B2; and D) T1 & T2. The intensity signals are with respect to lysozyme heated without any additive. Error bars indicate standard deviation of the mean.

From the ^{1}H-NMR analysis, the monomeric ratio of all the polymers was evaluated from the integration value of the PEG peak (Table 1), and the composition of the monomers in the polymer exhibited similarity to that of the in-feed ratio. The molecular weights of the polymers prepared were determined using gel permeation chromatography (GPC). The results showed that the GPC curves of the polymers had unimodal distribution (Fig. S21). The molecular weights of the polymers were comparable to those of their theoretical values (in-feed), and their polydispersity indices (M_w/M_n) were found to be within the range of living polymerization (Table 1).

Protein aggregation inhibition

In this study, the lysozyme (20 µM) in PBS (pH 7.4) solution was exposed to thermal treatment with and without the PEG-based p-SPB. The polymer and lysozyme solutions were mixed in equal volumes and heated at 90 °C for 15 min. The solution only containing lysozyme was observed to become highly turbid owing to the formation of aggregates. However, the turbidity significantly decreased when the lysozyme mixed with p-(SPB-r-PGMA) was heated, showing that the polymer inhibited the formation of aggregates. In the presence of the p-SPB-b-PEG, the lysozyme-polymer solution was clear even after heating, indicating that the polymer suppressed lysozyme aggregation to a greater extent (Fig. 1).

Amyloid fibril formation

Proteins undergoing aggregation form amyloid fibrils, which further lead to several diseases such as Alzheimer's and Parkinson's.[10] Hence, it is essential that the formation of such fibrils is suppressed. ThT dye has the tendency to bind with amyloid fibrils, imparting high fluorescence intensity. It has been hypothesized that the ThT molecule becomes intercalated between the depressions of the amyloid fibrils.[41–43]

When the lysozyme was heated at 90 °C for 15 min in the presence of the polymers with 2.5, 5, and 10 w/v% concentration, the ThT assay results showed that the ThT fluorescence intensity significantly decreased compared with that of the lysozyme heated without an additive (Fig. 2), thus showing that the prepared polymers appreciably suppressed the formation of amyloid fibrils. As the polymer concentration increased, the fluorescence intensity decreased, indicating that the polymers inhibited amyloid fibril formation at the higher concentrations. In Fig. 2A–D, the polymers with higher molecular weights (DP 100), i.e., the R2, R4, B2, and T2 polymers, have lower fluorescence intensities than their lower-molecular-weight (DP 60) counterparts. As the chain length of SPB increased, the polymers showed improved properties for inhibiting the amyloid fibrils owing to the anti-biofouling property of SPB.[36]

Comparing the inhibitions of heat-induced lysozyme aggregation in the presence of different p-SPB-based polymers with similar degrees of polymerization (Fig. 3A and 3B) showed that the random (R1, R2, R3, and R4), block (B1 & B2), and

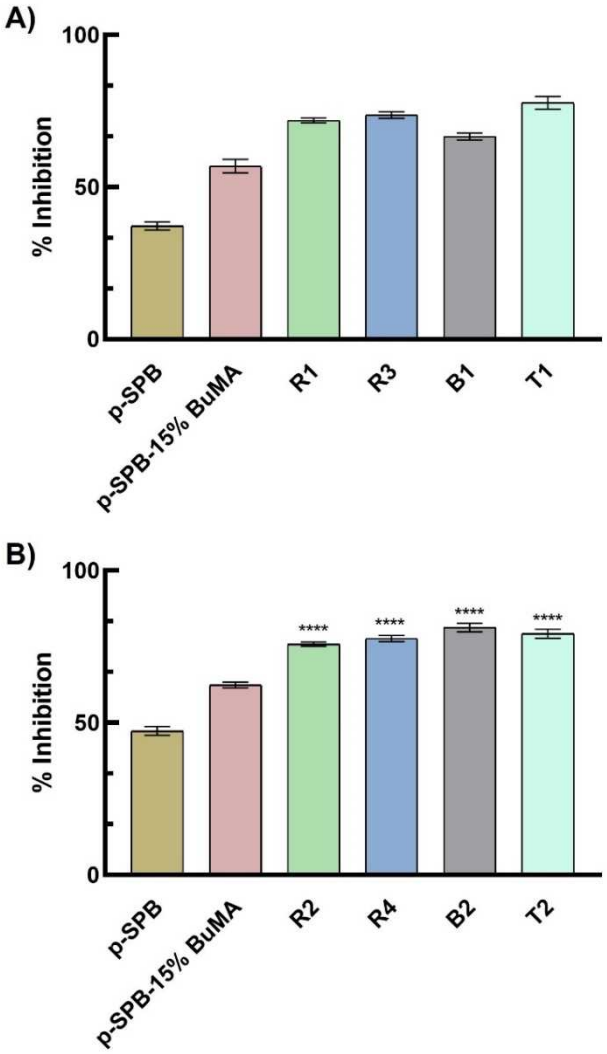


Fig. 3 Inhibition % of lysozyme aggregation in the presence of different poly-SPB-based polymers (10 w/v%): Polymers with DP A) 60; and B) 100. Error bars indicate standard deviation of the mean. **** indicate that the data sets are significant against p-SPB and pSPB-15% BuMA. ****$p < 0.0001$.

random-tri (T1 & T2) co-polymers prepared exhibited higher aggregation inhibitions than the previously reported poly-SPB[23] and poly-(SPB-r-BuMA)[36] at 10 w/v% concentration, with up to 80% aggregation inhibition. This was attributed to the incorporation of the PEG group, i.e., better efficiency in inhibiting the amyloid fibrils. PEG has the tendency to undergo hydrophobic interactions with protein, masking the hydrophobic domains of the protein, thus preventing aggregation-prone collisions.[44] It is speculated that the p-SPB component prevents the collision between two protein molecules because of its anti-biofouling property, whereas the PEG component is speculated to prevent the protein chain from undergoing aggregation, thus resulting in the effective combination of PEG and SPB for protein aggregation arrest. In Fig. 3B, the R2, R4, B2, and T2 polymers exhibit higher inhibition % than the R1, R3, B1, and T1 polymers. The B2 polymer, i.e., p-SPB-b-PEG with DP100, exhibited the highest inhibition % among all the polymers prepared (also seen in Fig. 1B), which may be attributed to its longer PEG chain length (n = 45). A similar result was not observed for the B1 polymer, i.e.,

p-SPB-b-PEG with DP 60 (Fig. 3A), because of its shorter p-SPB chain length compared with that in the feed ratio (Table 1). This result also supports the previous finding that higher molecular weight polymers exhibit superior inhibition efficacy because of the anti-biofouling property of SPB.

Residual enzymatic activity

The efficiency of the prepared polymers for inhibiting the protein aggregation was also evaluated using the enzymatic assay of lysozyme in the presence of the *Micrococcus Lysodeikticus* bacteria. Lysozyme is an enzyme that breaks down the cell walls of gram-positive bacteria via hydrolyzation. The bacterial suspension was highly turbid; however, turbidity began to decrease with the addition of lysozyme to the suspension because it disrupted the cell walls of the bacteria. The term residual enzymatic activity refers to the remaining efficiency of lysozyme to perform cell lysis after being treated at high temperatures. The reduction in the turbidity via the addition of lysozyme to the cell suspension was observed using UV-vis spectroscopy, where absorbance was observed against time. The residual enzymatic activity % was calculated from the slope of the graph plotted between absorbance and time (s).

The enzymatic activity of lysozyme was lost when lysozyme was heated at a high temperature. However, Fig 4A and 4B show that lysozyme heated at 90 °C in the presence of polymers

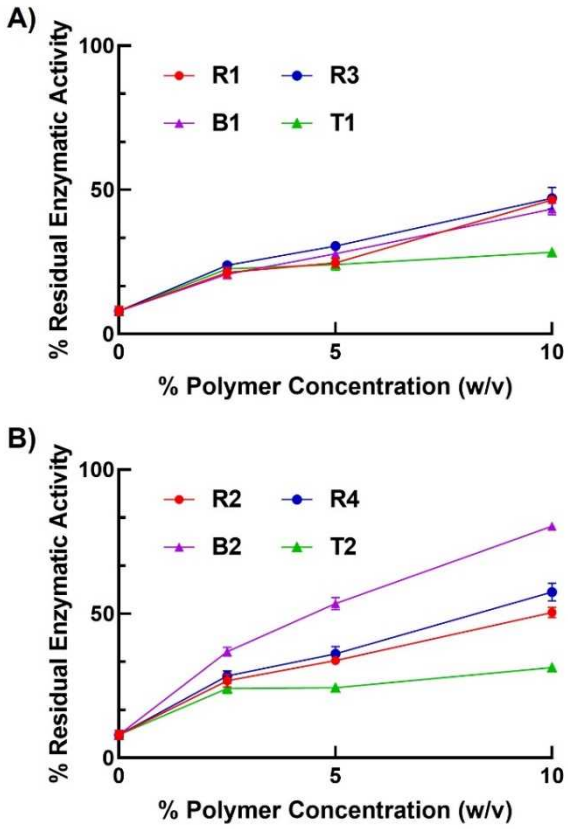


Fig. 4 Residual Enzymatic Activity of lysozyme heated in the presence of polymers with different concentrations: Polymers with DP A) 60; and B) 100. Error bars indicate standard deviation of the mean.

retained up to 50% of its enzymatic activity. Using Fig. 4B, it can be concluded that the R2, R4, B2, and T2 polymers are more capable of suppressing the amyloid fibrils as well as retaining the enzymatic activity of lysozyme even after being heated at elevated temperatures, because of their higher molecular weights. As the concentration of the polymers increased, the residual enzymatic activity of the B2 polymer reached up to 80%; however, the T1 & T2 polymer, i.e., p-(SPB-15%PGMA-10%BuMA) did not exhibit significant retention of the enzymatic activity of lysozyme even at 10% w/v polymer concentration. This could be attributed to the shorter SPB chain present in the T1 & T2 polymer because it consists of the two side groups, PGMA and BuMA.

Comparing the efficiency of the PEG-based poly-SPB in retaining the enzymatic activity of lysozyme (Fig. 5) showed that the polymers exhibited superior properties to the earlier reported additives p-SPB and PEG. In addition, evaluating the enzymatic activity of the lysozyme heated in the presence of only PEG with different molecular weights revealed that PEG with higher molecular weight, i.e., PEG2000, retains more enzymatic

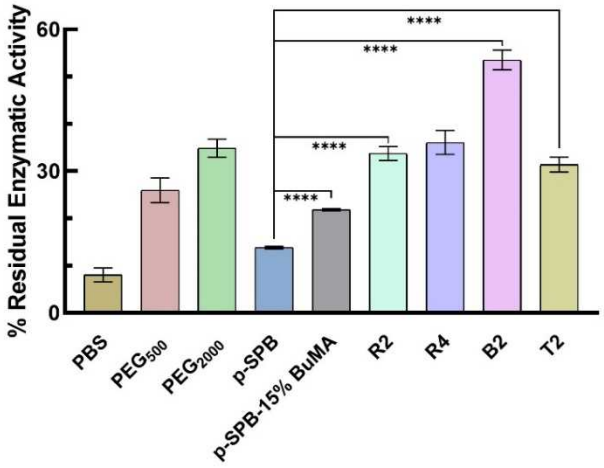


Fig. 5 Residual enzymatic activity % of lysozyme heated in the presence of different additives (10 w/v%). Error bars indicate standard deviation of the mean. ****P < 0.0001.

activity than PEG with lower molecular weight. This result also supports the finding that the B2 polymer, i.e., p-SPB-b-PEG (DP100), exhibits superior properties for protecting the lysozyme from thermal-induced aggregation because of its higher molecular weight and longer PEG chain length.

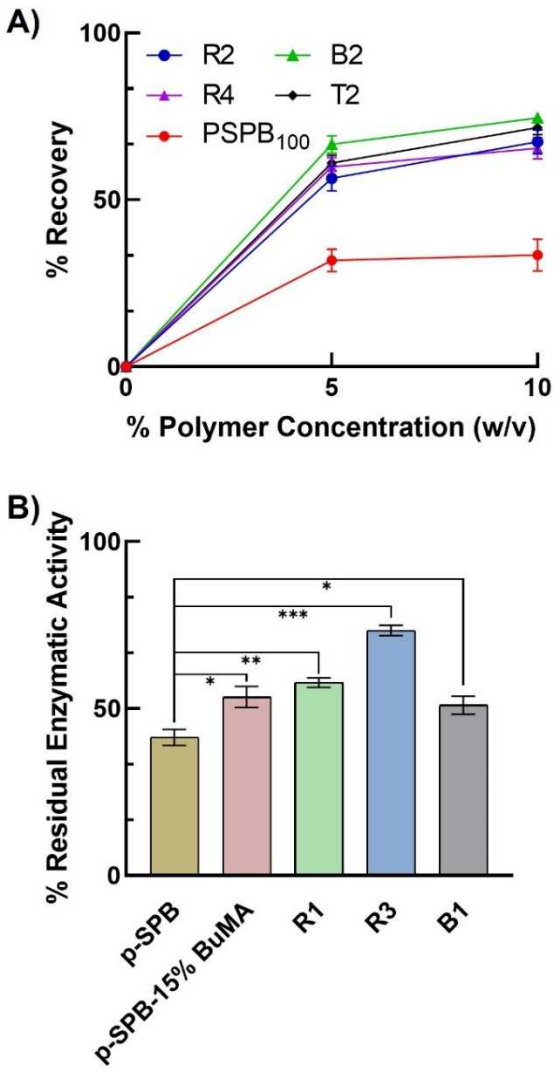


Fig. 6 Refolding efficiency obtained via the addition of polymers to the denatured lysozyme solution: A) Recovery yield obtained from ThT fluorescence intensity; B) Increase in residual enzymatic activity at 10 w/v% polymer concentration. Error bars indicate standard deviation of the mean. ***P < 0.001.

Refolding of Protein

Apart from the efficiency of the prepared zwitterionic polymers in protecting lysozyme from undergoing thermal aggregation, the robustness of these polymers in facilitating the re-solubilization and refolding of denatured lysozyme was evaluated via ThT assay and residual enzymatic activity analyses. Proteins can attain their native structure through refolding, rendering this process equally as essential as protecting the protein from aggregation because it is a vital step in recombinant proteins' production[45], and can be used to cure neurodegenerative diseases. Preventing the stabilization of misfolded proteins is crucial for achieving refolding.

Fig. 6A shows that the PEG-based p-SPB polymers facilitate the refolding of denatured lysozyme. The recovery yield % was calculated by the decrease in the ThT fluorescence intensity after incubating the heated lysozyme with polymers. The recovery yield for the B2 polymer was as high as 75%. Upon evaluating the residual enzymatic activity of lysozyme, the addition of polymers to the denatured lysozyme solution was observed to increase the enzymatic activity by approximately 30% (Fig. 6B).

Together with inhibiting protein aggregation, the results suggest that these polymers are highly capable of solubilizing the already formed amyloid fibrils and regaining the lost lysozyme enzymatic activity. It can be theorized that the PEG forms hydrophobic interactions with the aggregates formed, destabilizing the monomeric intermediates. In addition, the shielding capability of the p-SPB stabilizes the protein. Thus, the amalgamation of p-SPB and PEG stabilizes the protein and assists in the restoration of the misfolded protein to its native state.

Conclusions

Protein aggregation limits the development of protein-based biopharmaceutical products. In this study, we demonstrated that along with the anti-biofouling property of SPB, the incorporation of PEG yielded excellent efficiency in inhibiting the heat-induced aggregation of lysozyme. PEG incorporated into the p-SPB significantly reduced the formation of amyloid fibrils, even at lower concentrations. The chain length of PEG also played a crucial role in masking the hydrophobic domains of the protein, thus preventing the collision between aggregation-prone species. The effect of the PEG chain length resulted in the high efficiency of the p-SPB-b-PEG to suppress the formation of amyloid fibrils and retain the enzymatic activity of lysozyme by up to 80% even after prolonged heating at elevated temperatures. Along with the robustness of the polymers in protecting lysozyme from aggregation, the zwitterionic polymers demonstrated excellent effectiveness in easing the re-solubilization and refolding of denatured lysozyme, providing the recovery yield of approximately 75%. The ability of these polymers to facilitate refolding suggests their high potential to achieve higher efficiency following few alterations. This study widens the scope for utilizing polymers to inhibit protein aggregation as well as facilitate protein refolding, which pave the way for promising developments in protein-based therapeutics. Further studies are underway to investigate the exact mechanism for protein aggregation arrest using the amalgamation of PEG and p-SPB.

Author Contributions

Conceptualization, R.R. and K.M.; Methodology, A.D., R.R., and K.M.; Funding Acquisition, R.R. and K.M.; Investigation, A.D.; Formal Analysis, A.D.; Writing—Original Draft, A.D. and R.R.; Writing—Reviewing and Editing, A.D., R.R., and K.M.

Conflicts of interest

The authors have no conflicts to declare.

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