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ARTICLE

Design of Polysulfobetaine Derivatives for Enhanced Inhibition of Protein Aggregation†

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Protein aggregation and misfolding are implicated in neurodegerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease. The inhibition of these processes by polymers is a promising therapeutic approach but currently an underexplored area of research. In this study, we synthesised and evaluated new polysulfobetaine (PSPB) derivatives for protein stabilisation under thermal stress. These PSPB derivatives effectively stabilised various proteins, including lysozyme, insulin, and lactate dehydrogenase, indicating their broad-spectrum utility. The enhanced stabilisation was due to the introduction of a hydrophobic moiety into the PSPB structure, suggesting the vital role of hydrophobic interactions in preventing protein aggregation. The incorporation of trehalose also improved protein stability, likely due to a synergistic effect. Additionally, transforming the PSPB structure into a star-shaped architecture increased the surface area and functional sites. This enhanced its interaction with proteins, which effectively hindered aggregation. These findings underscore the potential of PSPB derivatives as biopharmaceutical stabilisers for the treatment of protein-aggregation-related neurodegenerative diseases.

Introduction

Neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's disease, are a significant area of concern in modern medical science.[1] These diseases are deeply intertwined with protein misfolding and aggregation, which cause cellular damage and progressive degeneration of neuronal structures.[2,3] Therefore, understanding and strategically intervening in these processes are not only crucial for comprehending neurodegenerative diseases but also serve as a potential therapeutic avenue. Protein aggregates are also cytotoxic and capable of eliciting immune responses, including allergic reactions and autoimmune disorders, potentially leading to severe adverse effects and fatalities.

In protein therapeutics, protein aggregation poses substantial difficulties. Protein aggregates, which are formed during the production and storage phases, compromise the biological activity and therapeutic efficacy of protein-based drugs. Their presence significantly influences the pharmacokinetics and pharmacodynamics of such drugs, altering their distribution, metabolism, and elimination profiles, as well as their interactions with biological systems.[4] This necessitates the implementation of additional, often costly purification processes, which, in turn, diminish the overall yield.[5,6]

Conventional approaches for mitigating protein aggregation rely primarily on small molecules. Compounds such as polyamines,[7,8] polyphenols,[9] nanoparticles,[10,11] polypeptides,[12–14] and nondetergent sulfobetaines,[15,16] although effective to a certain extent, have limitations in their application. These include the necessity for high doses and molecular architecture that hinders functional modifications. This gap in the therapeutic landscape highlights the need for more adaptable and multifaceted agents capable of addressing the complexities of protein aggregation with greater efficiency and specificity.

Polymers, particularly zwitterionic polymers, have emerged as promising candidates owing to their inherent modifiability.[1] These polymers can be tailored in terms of molecular weight, functionality, and structure to meet specific therapeutic needs. The potential of polymers to combat protein aggregation, although immense, remains underexplored in contemporary research, presenting an opportunity for groundbreaking advancement.

Our previous research laid the foundation in this field by introducing a zwitterionic polymer, polysulfobetaine (PSPB), with considerable activity against protein aggregation.[17–21] However, the quest for enhanced efficacy led us to further explore PSPB derivatives. In this study, we expanded the variety of PSPB structures by constructing a star-shaped architecture, using hydrophobic comonomers such as butyl methacrylate (BuMA) and styrene, and introducing trehalose. These derivatives were engineered to target specific mechanisms of protein aggregation, thereby enhancing the protective capability of PSPB.

In addition to the synthesis of these diverse PSPB derivatives, we investigated their structure–activity relationships. Structure–activity relationship studies are crucial for elucidating the effect of structural changes on biological activity, particularly the inhibition of protein aggregation. Systematic analysis of this effect enabled the identification of key factors that enhance the anti-aggregation efficacy of PSPB. This approach is pivotal to the development of polymers as an effective and targeted therapeutic solution for

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protein-aggregation-related neurodegenerative diseases, thereby broadening the scope of polymer science in medical applications.

Materials and methods

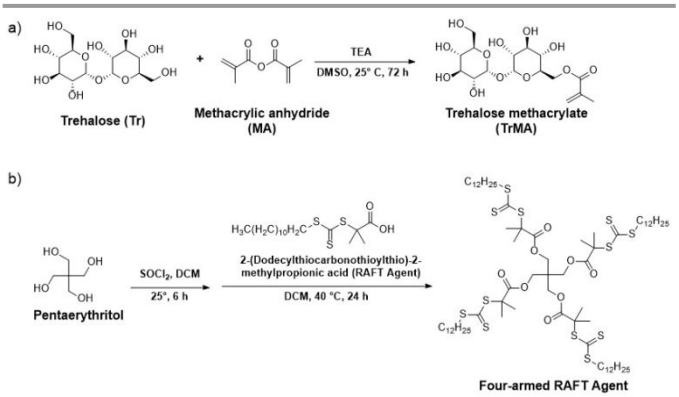
Materials

Sulfobetaine (SPB) was donated by Osaka Organic Chemical Industry, Ltd. (Osaka, Japan) and used without further purification. BuMA, *N-tert*-butylacrylamide (NTBAm), and methacrylic anhydride were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). BuMA was purified by passing it through an inhibitor removal column (prepacked column, Sigma-Aldrich Corp., St. Louis, MO, USA). 2-(Dodecylthiocarbonothioylthio)-2-methylpropionic acid (reversible addition–fragmentation chain transfer (RAFT) agent), thioflavin T (ThT), triethylamine, *N*-isopropylacrylamide (NIPAm), *Micrococcus lysodeikticus* ATCC No. 4698, styrene, lysozyme from chicken egg, and insulin from bovine pancreas were purchased from Sigma-Aldrich. Trehalose dihydrate and methanol were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Synthesis of polymers

Synthesis of trehalose methacrylate (TrMA) and four-armed RAFT agent. TrMA was synthesised by first drying trehalose dihydrate at 110 °C under vacuum for 48 h. Equimolar amounts of dried trehalose and methacrylic anhydride were mixed with anhydrous dimethylsulfoxide. Anhydrous triethylamine was then added, and the reaction mixture was stirred under nitrogen atmosphere for 72 h at 25 °C. The product was purified by successive precipitation with diethyl ether, followed by washing with hexane (three times) and drying under vacuum (Scheme 1a).

The four-armed RAFT agent was synthesised by adding a dry thionyl chloride/dichloromethane solution (2 M) to pentaerythritol under an inert atmosphere and then allowing the reaction to proceed at 25 °C for 6 h (Scheme 1b). Subsequently, the solvent was removed using a rotary evaporator and dried under vacuum for 2 h. The product was then dissolved in anhydrous dichloromethane. The RAFT agent was added, and the reaction was continued at 45 °C for 24 h. The reaction mixture was then washed thrice with water and saturated $NaHCO_3$, and dried over dry $MgSO_4$. The product was purified by column chromatography using an ethyl acetate/hexane mixture.



Scheme 1 Schematic representation of precursor synthesis. (a) Trehalose methacrylate and (b) four-armed reversible addition–fragmentation chain transfer (RAFT) agent.

Synthesis of PSPB. The SPB monomer, RAFT agent, and azobisisobutyronitrile were added to a round-bottom flask and dissolved in a methanol/water mixture (3:1 v/v). The solution was then purged with nitrogen gas for 1 h and stirred at 70 °C for 24 h. The reaction mixture was then dialysed for 3 days (12 water changes). Water was removed under reduced pressure, and the residue was dried under vacuum to obtain the final polymer.

On the other hand, the four-armed PSPB was synthesised using the four-armed RAFT agent as the chain transfer agent and a 1,4-dioxane/water mixture (2:1 v/v).

Incorporation of the second monomer. The PSPB derivatives were synthesised by adding the desired monomer unit to the reaction mixture and then proceeding with the polymerisation reaction in a methanol/water mixture (3:1 v/v) using the procedure described above (Fig. 1).

Gel permeation chromatography (GPC)

The molecular weights and polydispersity indices of the polymers were determined by GPC using a BioSep SEC-s2000 column (Phenomenex, Inc., CA, USA) and e2695 separation module equipped with a 2414 refractive index detector (Waters, MA, USA) . The mobile phase was a 0.1 M NaBr solution with a flow rate of 1 mL/min. Pullulan was used as the calibration standard.

Protein aggregation inhibition

Residual enzyme activity. A lysozyme solution in phosphate-buffered saline (PBS; pH 7.4) was mixed with the polymer solution of different concentrations to achieve a final lysozyme concentration of 0.1 mg/mL. The solution was heated at 90 °C for 30 min. *M. lysodeikticus* (2 mL; 0.25 mg/mL in PBS) and 100 µL of the lysozyme/polymer solution were placed in a quartz cuvette and mixed well. Turbidity was measured by ultraviolet–visible spectrophotometry (UV-1600PC, Shimadzu Corp., Kyoto, Japan) at 600 nm for 0–6 min with constant stirring at room temperature.

ThT assay. A ThT stock solution was prepared by adding 8 mg of ThT to 10 mL of PBS (pH 7.4), and the solution was then filtered through a 0.22-µm filter (Millex-GP filter unit). The working solution was prepared by adding 2 mL of the stock solution to 98 mL of PBS (pH 7.4). An insulin solution (100 µM) in PBS (pH 2) or lysozyme solution (56 µM) in PBS (pH 7.4) was then mixed with the polymer, and the mixture was incubated at 37 °C for 12 h with stirring at 300 rpm (for insulin) or 90 °C for 30 min (for lysozyme). At certain time intervals, 100 µL of the solution was removed and mixed with 2 mL of ThT solution. The fluorescence intensity was observed at excitation and emission wavelengths of 450 and 484 nm, respectively (FP-8600, JASCO, Japan). The increased intensity corresponds to amyloid formation because ThT binds to amyloid fibrils.[22]

Lactate dehydrogenase (LDH) activity assay (incubation). The residual LDH activity after denaturation was evaluated by first mixing the polymer and LDH (20.7 mU/mL in PBS) and then incubating the mixture at 37 °C for 1 h using a Bioshaker BR-40LFA (Taitec Corp., Japan). After incubation, 5 µL of the sample was dispensed into a well of a 96-well plate. Sodium pyruvate and reduced

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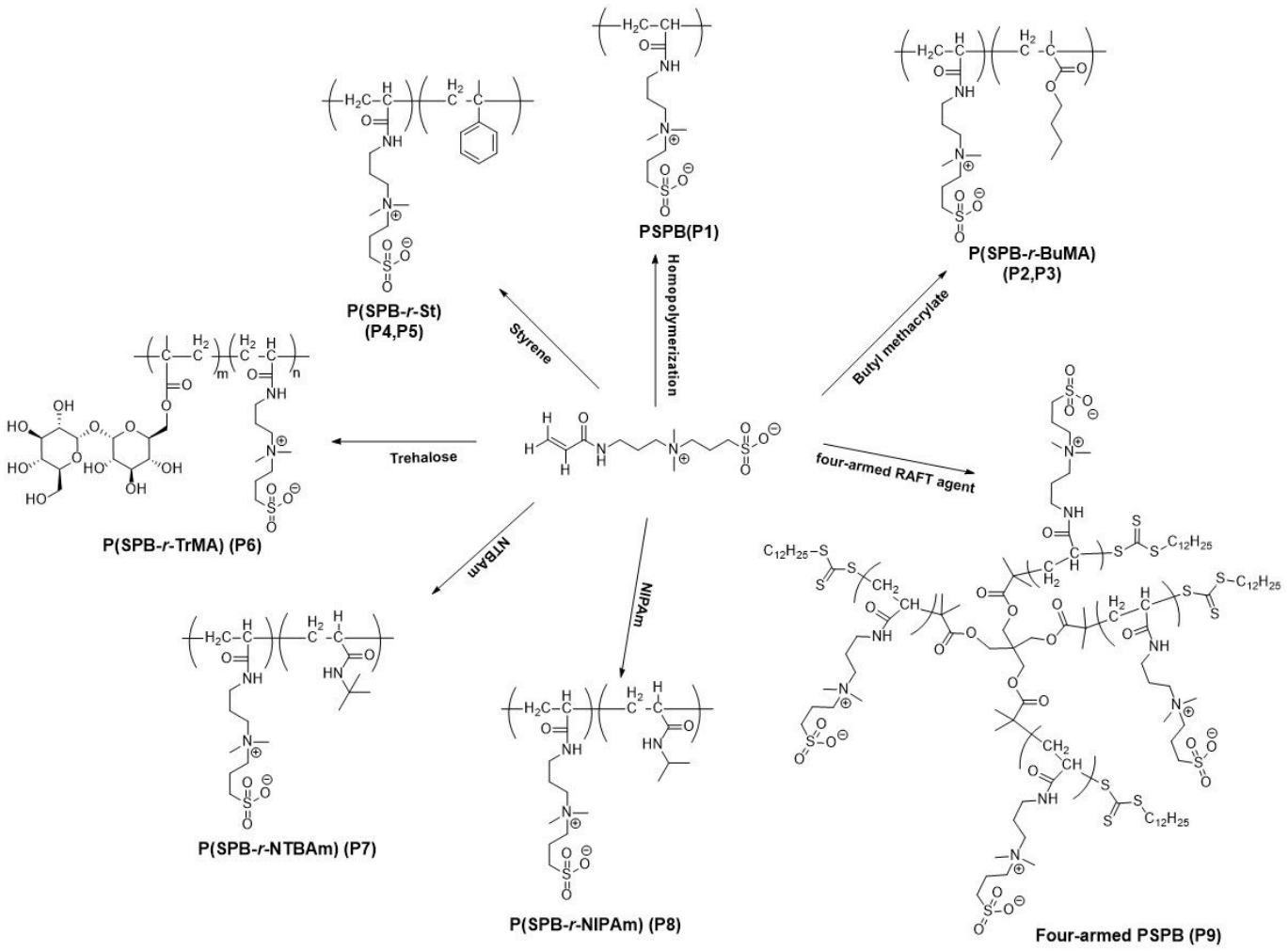

Fig. 1 Schematic illustration of the syntheses of various polysulfobetaine (PSPB) derivatives (BuMA: butyl methacrylate; NIPAm: *N*-isopropylacrylamide; NTBAm: *N*-*tert*-butylacrylamide; TrMA: trehalose methacrylate; St: styrene; RAFT: reversible addition–fragmentation chain transfer).

nicotinamide adenine dinucleotide were prepared in PBS at concentrations of 10 and 63 mM, respectively. A master mix was freshly prepared by combining 200 µL of the reduced nicotinamide adenine dinucleotide solution and 500 µL of the sodium pyruvate solution with 49.3 mL of chilled PBS. Subsequently, 195 µL of this master mix was added to each well, ensuring thorough mixing. The absorbance was measured at 340 nm at various time points using an Infinite 200 PRO M Nano+ microplate reader (Tecan). The LDH activity was determined by monitoring the rate of absorbance reduction compared to that of an unincubated control LDH sample over a 10-minute duration.

Refolding of insulin. Initially, insulin (100 µM in PBS, pH 2) was denatured by heating at 37 °C for 12 h with constant stirring. Upon cooling to room temperature, the solution was combined with an equal volume of the polymer solution at varying concentrations. The resulting mixture was incubated at 25 °C for 5 h. The yield of recovered insulin was assessed using a ThT assay.

Results and discussion

Synthesis and characterisation of polymers

All PSPB derivatives were synthesised in such a way as to obtain random copolymers. To obtain the copolymers, SPB was retained as the first component and different monomers were added prior to the reaction. RAFT polymerisation was used to control the size of the polymer and ratio of the comonomer. The polymers were characterised by ^{1}H nuclear magnetic resonance spectroscopy (Figs. S1–S11) and GPC. The ratio of the comonomer was determined by comparing their characteristic proton with the methylene protons of SPB. A summary of the characteristics of the polymers is presented in Table 1. The ratio of each monomer in the polymers was well controlled and in accordance with the initial feed ratio. Furthermore, the GPC curves indicated that all polymers had a unimodal molecular weight distribution with polydispersity indices (M_w/M_n) ranging from 1.0 to 1.4 and the observed M_n values were consistent with the theoretical molecular weights for the corresponding feed ratios.

Table 1 Characteristics of various polysulfobetaine (PSPB) derivatives prepared via reversible addition–fragmentation chain transfer (RAFT) polymerisation.

Entry	Polymer	Comonomer	Molar ratio[b]	% composition of 2nd Monomer		M_n ($\times 10^{-3}$)[c]	M_w/M_n[c]
				In feed	In polymer[a]		
1	PSPB	-	40:1:5	-	-	10.9	1.32
2	P(SPB-*r*-BuMA)	*N*-butyl methacrylate	40:1:5	10	10.3	11.4	1.28
3			40:1:5	30	27.9	13.2	1.36
4	P(SPB-*r*-St)	Styrene	40:1:5	5	3.6	8.4	1.30
5			40:1:5	10	7.7	8.9	1.03
6	P(SPB-*r*-TrMA)	Trehalose	40:1:5	50	43.3	14.7	1.05
7	P(SPB-*r*-NTBAm)	*N*-*tert*-butylacrylamide	40:1:5	30	33.8	12.8	1.07
8	P(SPB-*r*-NIPAm)	*N*-isopropylacrylamide	40:1:5	30	34.6	12.1	1.11
9	Four-armed PSPB	-	160:1:5	-	-	57.8	1.19

[a]Determined by ^{1}H NMR. [b][monomer]:[initiator]:[RAFT agent]. [c]Determined by GPC.

Stabilisation of proteins by the polymers

To assess the ability of the synthesised polymers to inhibit the thermal aggregation of lysozyme, the residual enzymatic activity was measured after heat treatment, both in the presence and absence of the polymers, using *M. lysodeikticus* cells. Enzymatic degradation of the bacterial cell wall by lysozyme was monitored through the decrease in turbidity of the cell suspension, quantified by the reduction in absorbance at 600 nm.[7] The rate of absorbance decrease, derived from the slope of the absorbance–time plot, correlates directly with the residual enzymatic activity.

Fig. 2a shows that the polymers exhibited varying degrees of efficacy. PSPB (P1) demonstrated moderate ability to prevent protein aggregation, leading to an activity retention of approximately 34% at 10% (w/v). However, its efficacy was significantly enhanced by the integration of a hydrophobic monomer. The incorporation of a small percentage of BuMA (10% BuMA; P2) notably increased the activity retention to approximately 59%. This enhancement was more pronounced at a higher hydrophobic content (30% BuMA; P3), resulting in nearly 70% activity retention. A further increase in efficacy was observed using the more hydrophobic monomer styrene (P4 and P5). In particular, the PSPB derivative with 10% styrene (P5) achieved impressive activity retention exceeding 85%, underscoring the crucial role of hydrophobic groups in amplifying efficacy. This corresponds well with previous reports, suggesting that hydrophobic polymers may interact with the hydrophobic domains of proteins, thereby altering their environment and reducing aggregation-inducing interactions.[17,20]

The incorporation of trehalose into PSPB (P6) also substantially improved the thermal stability of lysozyme. This improvement is likely attributable to the ability of trehalose to replace water molecules around the protein, thus preserving the protein structure under stress conditions.[23,24] This mechanism complements the

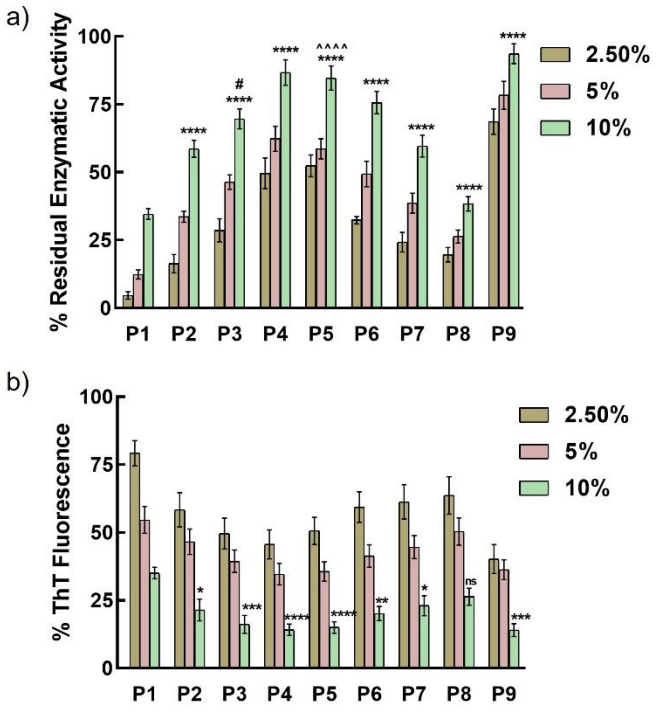

Fig. 2 Lysozyme stabilisation by polysulfobetaine (PSPB) derivatives. A) Residual enzymatic activity in the presence and absence of PSPB derivatives at different polymer concentrations. b) Thioflavin T (ThT) fluorescence assays in the presence and absence of PSPB derivatives at different polymer concentrations. Experiments were conducted in triplicate, and errors bars indicate the standard deviation of the mean. The significances, calculated against polymers P1, P2, and P3, are marked as *, #, and ^ respectively, and ****$p < 0.0001$ (ns: not statistically different).

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hydration shell of PSPB, which mitigates protein–protein interactions and aggregation. This dual-action mechanism indicates a synergistic effect in which molecular stabilisation by trehalose is coupled with the protective hydration barrier of PSPB, forming a robust defence against thermally induced protein aggregation.

Modifications with the comonomers NTBAm (P7) and NIPAm (P8) also resulted in enhanced efficacy compared to PSPB, although the effect was slightly less than that of the more hydrophobic comonomers, BuMA and styrene. This modest increase may be due to the balance between hydrophilic (with PSPB) and hydrophobic (with polyNTBAm or polyNIPAm) interactions, which are crucial for maintaining protein stability and preventing aggregation. The improved solubility and dispersion properties of the copolymers may also have contributed to this effect, particularly under conditions conducive to protein aggregation. Notably, the polymer with a more hydrophobic comonomer (NTBAm) exhibited higher efficacy than that with a less hydrophobic one (NIPAm).

The preparation of a four-armed poly-SPB has demonstrated remarkable efficacy in preventing protein aggregation, primarily due to its unique architecture that facilitates efficient protein encapsulation. This efficacy is attributed not just to the multiple functional sites and expanded,[25–27] provided by its branched design but significantly to how the polymer physically folds around the protein. This interaction minimizes aggregation-inducing interactions, acting as a protective embrace rather than solely stabilizing through a hydration shell. The enhanced solubility and dispersion characteristics of the four-armed poly-SPB, along with its spatial arrangement, efficiently encase proteins, providing a robust barrier against environmental stressors known to induce aggregation. The inherent molecular flexibility of this architecture aligns seamlessly with protein surfaces, offering augmented protection. This elucidates the superiority of the four-armed poly-SPB in maintaining protein stability under severe thermal stress, showcasing its innovative potential for protein stabilization.

Given the critical association of amyloid fibrillation with numerous neurodegenerative diseases and its significance in the development of protein biopharmaceuticals,[28] we quantitatively assessed the impact of the polymers on this process using a ThT assay. In this context, an elevated ThT fluorescence intensity is indicative of increased amyloid-like fibril formation, which correlates with increased protein denaturation. Mirroring the trends observed in the residual enzymatic activity measurements, PSPB (P1) effectively hindered the formation of amyloid-like fibrils (Fig. 2b). This effect was amplified by the introduction of a hydrophobic comonomer into the polymer matrix. Notably, only 15% ThT fluorescence was observed in the presence of P5 (10% polymer concentration), conclusively demonstrating the potent ability of hydrophobic derivatives not only in preserving enzymatic activity but also in averting the formation of deleterious amyloid fibrils. P6, which contained trehalose, also displayed significant efficacy, and this trend persisted with P7 and P8. Critically, the star-shaped polymer P9 afforded the greatest protection against amyloid fibril formation, thereby substantially inhibiting lysozyme denaturation.

This comprehensive analysis underscores the pivotal role of the polymer structure and composition in inhibiting lysozyme aggregation. The results clearly highlight that while PSPB provides a foundational level of protection, the incorporation of hydrophobic components and trehalose and adoption of a star-shaped architecture significantly enhance the ability of the polymer to prevent both enzyme inactivation and amyloid fibril formation in lysozyme, thereby offering promising avenues for the development of effective biopharmaceutical stabilisers.

To extend the scope of our study to a broader range of proteins, we explored the protective efficacy of our synthesised polymers against the aggregation of insulin and LDH. Insulin, a crucial therapeutic agent in diabetes management, was initially used as a secondary model protein. Given that insulin suspensions are typically stored at low temperatures (4–8 °C) to avert denaturation, developing a stabilising agent conducive to ambient temperature storage is of paramount importance, especially for enhancing the utility of automated insulin pumps. The impact of the polymers on insulin fibrillation was also evaluated using a ThT assay. In the absence of polymers, insulin exhibited significant fibril formation, as evidenced by the high ThT fluorescence (Fig. 3a). However, the addition of PSPB (P1) resulted in a notable reduction in ThT fluorescence, with a further decrease upon the introduction of a hydrophobic comonomer, paralleling the findings of the lysozyme studies. Remarkably, the addition of PSPB containing 30% BuMA (P3) resulted in near-complete inhibition of insulin fibrillation (less than 0.5%) at a polymer concentration of only 1.5%. This trend was echoed by the styrene-containing PSPB (P4 and P5). P7 and P8 also demonstrated enhanced efficacy, which was consistent with the lysozyme results. Notably, the star-shaped PSPB derivative P9 exhibited extraordinary efficacy, with only 0.3% fibrillation observed at a polymer concentration of 1.5 %.

Subsequently, we examined LDH, an enzyme ubiquitous in living cells and integral to anaerobic respiration.[29] LDH is prone to

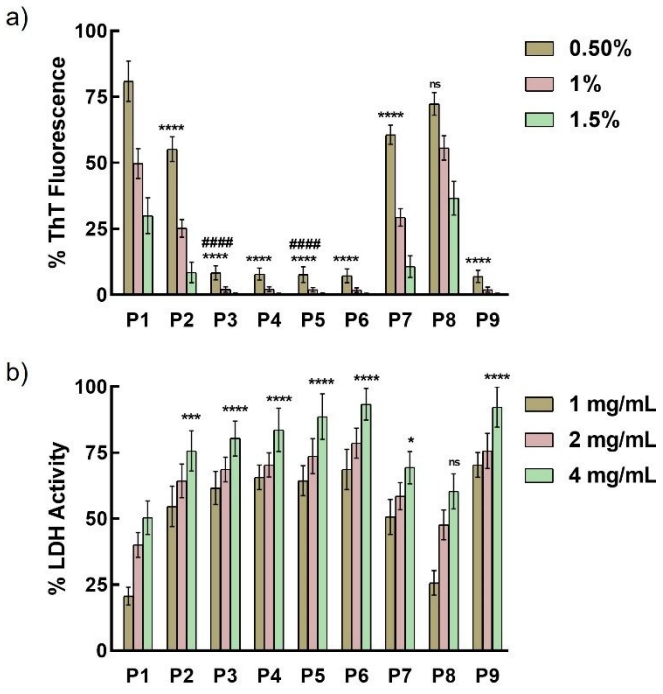

Fig. 3 Efficacy of polysulfobetaine (PSPB) derivatives against the aggregation of different proteins. a) Thioflavin T (ThT) fluorescence assays of bovine insulin incubated in the

presence and absence of PSPB derivatives at different polymer concentrations. b) Residual activity of lactate dehydrogenase (LDH) incubated in the presence and absence of PSPB derivatives at different polymer concentrations. Experiments were conducted in triplicate, and errors bars indicate the standard deviation of the mean. The significances, calculated against polymers P1 and P2, are marked as * and #, respectively, and ****p < 0.0001 (ns: not statistically different).

aggregation under various conditions, including thermal stress.[30] To assess the efficacy of PSPB derivatives in preserving LDH activity, we monitored the enzymatic function of LDH, specifically its ability to catalyse the reversible reduction of pyruvate to L-lactate, via changes in the absorbance at 340 nm.[31] Consistent with the insulin results, PSPB-based polymers markedly shielded LDH from thermal aggregation (Fig. 3b). Enhancement in polymer hydrophobicity, the addition of trehalose, and alteration of the polymer architecture (i.e., star-shaped P9) significantly bolstered this protective effect. When LDH was incubated with P9 at a concentration of 4 mg/mL, over 92% of its enzymatic activity was retained, demonstrating the potent stabilising ability of the polymer.

Overall, these findings substantiate the exceptional ability of PSPB-based polymers to protect diverse proteins from denaturation and aggregation, highlighting their potential applicability in a wide range of biopharmaceutical contexts.

Refolding of proteins facilitated by the polymers

Protein refolding or resolubilisation is crucial for protein stabilisation because it prevents protein misfolding and restores proteins to their native conformation. This mechanism is vital for the production of recombinant proteins and therapeutic management of neurodegenerative disorders such as Alzheimer's disease.[33–35] It involves the transformation of misfolded or solubilised proteins back into their functionally active form and is a key strategy for mitigating amyloid plaque aggregation. The ability of our synthesised polymers to assist in the resolubilisation and refolding of denatured insulin was evaluated using a ThT assay. Fig. 4 shows that PSPB (P1) significantly facilitated the refolding of denatured insulin, with approximately 38% recovery achieved using the derivative containing 30% BuMA (P3), as evidenced by the reduction in ThT fluorescence intensity after incubation. Other polymer derivatives mirrored this result, notably P9, which achieved a recovery yield exceeding 40%. While these recovery yields are moderate, they were on par with some of the nanoparticles reported in recent study employing polymer nanoparticles[36], and further studies need to be conducted to develop a system with higher efficacy for use in clinical applications. However, the zwitterionic polymers in our study have the advantage of a more straightforward synthetic approach, unlike the synthesis of these polymer nanoparticles, which necessitate the use of cross-linkers and surfactants. Furthermore, the demonstrated ability of these polymers to aid protein refolding indicates that with minor modifications, even higher efficacy in protein stabilisation applications can be attained.

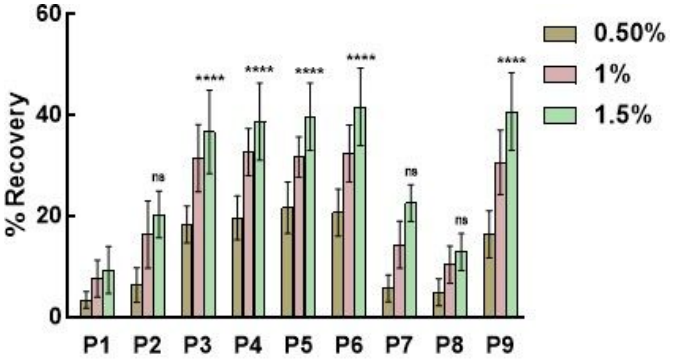

Fig. 4 Refolding efficiency (recovery yield) of insulin in the presence of polysulfobetaine derivatives at different polymer concentrations determined using a thioflavin T assay. Experiments were conducted in triplicate, and errors bars indicate the standard deviation of the mean. The significances, calculated against polymer P1, are marked as *, and ****p < 0.0001, (ns: not statistically different).

Conclusions

This study highlights the promising role of PSPB and its derivatives in addressing the complex issue of protein aggregation, particularly in protein therapeutics and neurodegenerative diseases. Our systematic investigation of PSPB derivatives not only demonstrated their high efficacy in stabilising key proteins, such as lysozyme, insulin, and LDH, but also underscored the remarkable versatility of the PSPB backbone. The incorporation of hydrophobic comonomers and trehalose and construction of a star-shaped architecture significantly augmented the protective ability of PSPB against protein denaturation and aggregation. The ease with which PSPB can be synthesised and its characteristics can be finely tuned unequivocally establishes the enormous potential of the PSPB backbone as a foundational platform for crafting a revolutionary new class of highly specialised and effective polymers. On a broader scale, this study offers promising new directions in biopharmaceutical stability and medical therapeutics for neurodegenerative diseases. Further studies are underway to elucidate the molecular mechanisms of action of these polymers in protecting proteins from aggregation.

Author contributions

Conceptualisation: R.R. and K.M., Methodology: R.R., Funding Acquisition: R.R. and K.M., Investigation: R.R., Formal Analysis: R.R., Writing—Original Draft: R.R., Writing—Review & Editing: R.R. and K.M.

Conflicts of interest

There are no conflicts to declare.

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