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Citation	Macromolecular Bioscience, 18(6): 1800016
Issue Date	2018-04-24
Type	Journal Article
Text version	author
URL	http://hdl.handle.net/10119/17045
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Description	

DOI: 10.1002/((please add manuscript number))
Article type: Communication

Zwitterionic Polymer Design that Inhibits Aggregation and Facilitates Insulin Refolding: Mechanistic Insights and Importance of Hydrophobicity

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Keywords: zwitterionic polymers, RAFT polymerization, protein aggregation, refolding, hydrophobicity

We describe the synthesis of a zwitterionic polymer, poly-sulfobetaine (poly-SPB), which shows remarkable efficiency in the suppression of insulin aggregation. Hydrophobic modification of the polymer resulted in almost complete inhibition at very low polymer concentrations. Further studies revealed that these polymers facilitate the complete retention of insulin's secondary structure, which is otherwise lost after incubation. Refolding studies showed that addition of polymers to pre-aggregated insulin sample lead to the refolding of denatured insulin, indicating their potential to facilitate the refolding of the denatured proteins. In addition, 2D NMR studies showed that the presence of hydrophobic poly-SPB alters the hydrophobic environment of insulin, which may suppress hydrophobic interactions that lead to aggregation. These results indicate the enormous potential of these polymers for suppressing insulin aggregation and provide significant insight into the complex mechanism of insulin protection by zwitterionic polymers.

Between 1980 and 2014, the global prevalence of diabetes doubled from 4.7% to 8.5%, and the number of people affected has quadrupled from 108 million to 422 million.[1] The protein insulin regulates sugar in the blood stream and is widely used to treat diabetes. Insulin is usually injected into the body, although this method is not suitable for all diabetes patients. Furthermore, insulin amyloidosis has frequently been found at the site of repeated insulin injections in diabetic patients.[2] Insulin pumps eliminate the need for daily injections and assist in the delivery of precise amounts of insulin.[3] However, the application of insulin pumps as a prospective method for administering insulin is hampered by protein denaturation and aggregation, which results in decreased protein activity over time, thereby causing under-dosing.[4] Moreover, protein aggregation triggers potentially treacherous immune responses. Therefore, there is a serious need for compounds that are capable of suppressing insulin aggregation.

Protein denaturation and aggregation are among the most serious concerns in the field of protein biopharmaceutics. Much research has been conducted to develop biopharmaceutical proteins such as recombinant proteins, monoclonal antibodies, etc.[5] Over the years, a number of compounds including arginine, proline, cyclodextrin, and others, have been reported to suppress aggregation.[6] However, satisfactory efficiencies have not yet been achieved with these compounds. Furthermore, few compounds have been reported to facilitate the refolding of denatured proteins.

Polyampholytes and zwitterionic polymers are particularly interesting in this regard. They have been reported to exhibit unique properties and have been applied in cryopreservation,[7,8] antibiofouling,[9] and many other fields.[10,11] In our previous reports, we developed zwitterionic polymeric systems that showed favorable efficiency against the thermal aggregation of lysozymes.[12,13] However, very high

polymer concentrations (in the range of 10–15%) were required to achieve high efficiency. Furthermore, the interactions between such polymers and proteins were not investigated.

Therefore, we used reversible addition fragmentation chain transfer (RAFT) polymerization to develop homopolymers and various copolymers of SPB with a hydrophobic monomer (butyl methacrylate; BuMA; **Scheme 1**). The polymers were characterized using ^{1}H and ^{13}C NMR (**Figures S1–S10**). The presence of BuMA in the copolymer was verified by ^{1}H-NMR (**Figure S1**1), ^{13}C-NMR (**Figure S12**), and contact angle measurements (**Table 1**). The amount of BuMA in each copolymer was quantified by ^{1}H-NMR and is reported in **Table 1**.

The kinetic study by ^{1}H NMR, in which polymerization was monitored by observing the loss of vinyl protons, indicated that polymerization was completed in 6 h (**Figures S13 and S14**). The conversion of the monomer and the relationship between $\ln([M]_0/[M])$ and time was also monitored by ^{1}H NMR (**Figures S15 and S16**). The linear curve thus observed indicated that the polymerization followed first-order reaction kinetics.[14] GPC curves of the polymers suggested unimodal distribution. The molecular weights of the polymers were consistent with the theoretical molecular weight for the corresponding feed ratio, and the polydispersity index (M_w/M_n) was well within the range of living polymerization (**Table 1**).

Scheme 1. Synthesis of a) poly-SPB and b) poly-(SPB-r-BuMA) by RAFT polymerization.

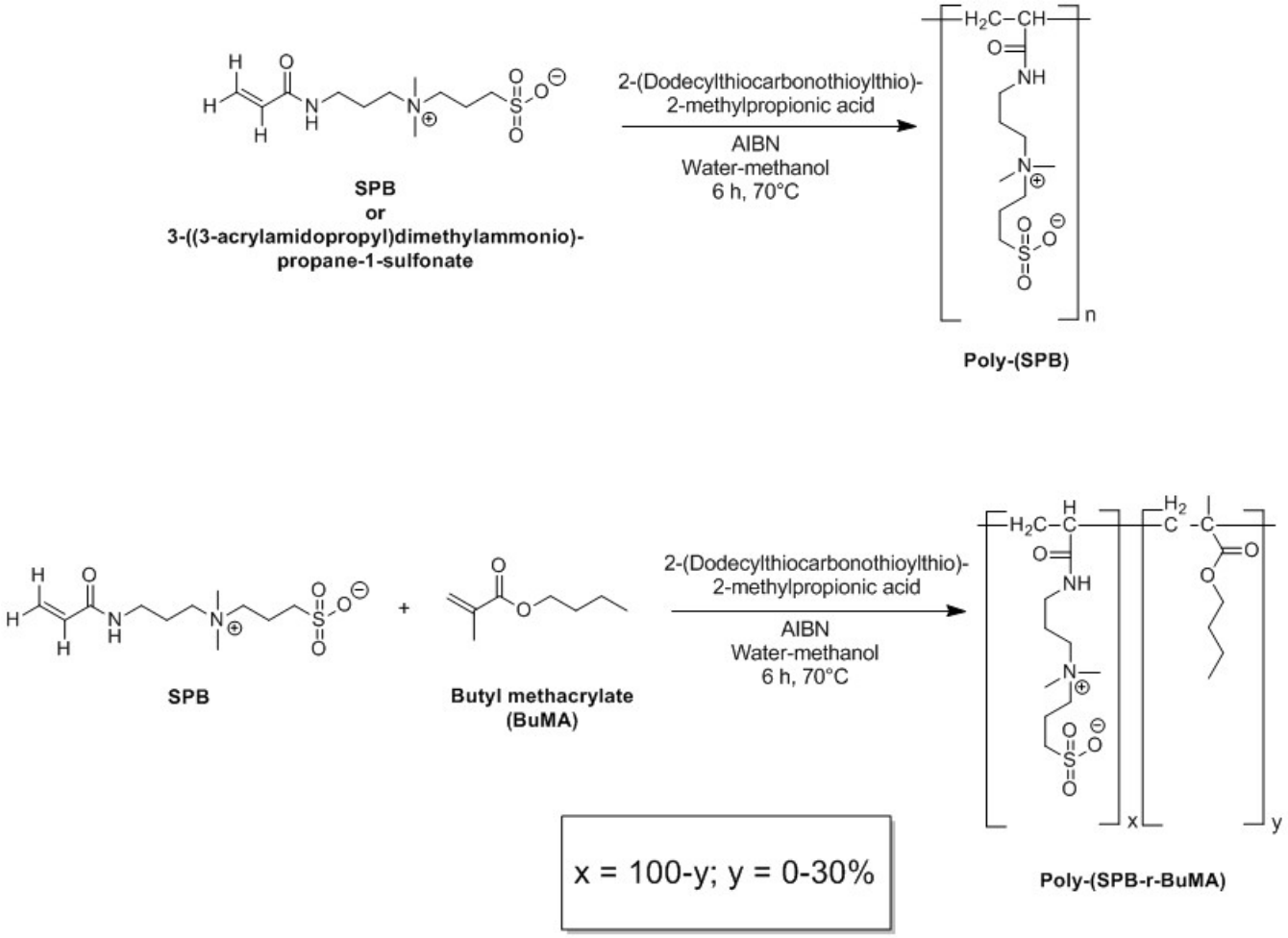

Table 1. Characteristics of various polymers prepared via RAFT polymerization.

Polymers		SPB	BuMA	Molar ratio[b]	M_n x 10^{-3},[c]	M_w/M_n[c]	Contact Angle[d]
***Poly*-SPB**	In feed	100	0	100:0.5:2.5	10.9	1.32	43.5 ±2.1
	In polymer[a]	100	0				
Poly-SPB With 10% BuMA	In feed	90	10	110:0.5:2.5	11.4	1.28	46.5 ±1.3
	In polymer[a]	89.7	10.3				
Poly-SPB With 20% BuMA	In feed	80	20	120:0.5:2.5	12.0	1.49	50.0 ±0.65
	In polymer[a]	85.4	14.6				
Poly-SPB With 25% BuMA	In feed	75	25	125:0.5:2.5	12.6	1.40	52.5 ±0.45
	In polymer[a]	78.2	21.8				
Poly-SPB With 30% BuMA	In feed	70	30	130:0.5:2.5	13.2	1.36	53.8 ±1.2
	In polymer[a]	72.1	27.9				

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

Insulin aggregation and suppression by polymers was observed by incubating insulin with and without additives at 37 °C for 12 h. When no additive was used, extensive aggregation took place (**Figure S17A**), however, on addition of polymers, especially hydrophobic derivatives suppressed the aggregation of insulin (**Figure 17B-F**). This was quantitively established UV-Vis spectroscopy by analyzing aggregation at 500 nm as a function of the increased absorbance of the solution. When only insulin was incubated at 37 °C, almost no change in absorbance was observed for the first 3 h. Subsequently, increased absorbance was observed, indicating the onset of aggregation, and the increase reached a maximum at approximately 10 h. In contrast, when the polymer was added prior to incubation, aggregation was largely suppressed, and the addition of hydrophobic poly-SPB almost completely suppressed aggregation (**Figure S18**). These results clearly demonstrate the exemplary ability of hydrophobic poly-SPB to suppress insulin aggregation. Transmission electron microscopy (TEM)

reveals that hydrophobic poly-SPB shows self-assembly (**Figure S19**), which may be related to the high aggregation inhibition behavior. Previous studies also indicate that self-assembled polymers nanogels[15] and polymer coated metallic nanoparticles[16] show significantly higher efficiency than its linear counterparts.

Protein aggregation is usually accompanied by the formation of amyloid-like fibrils, which cause many neurodegenerative diseases,[17] and therefore the prevention of protein aggregation is of paramount importance for the development of new protein biopharmaceuticals. Toward this objective, we investigated the effect of these polymers on the fibrillation of insulin, using TEM and Thioflavin T (ThT) assays. The fibrillation of insulin over time was obtained by incubating insulin with and without polymers at pH 2.0 and 37°C. When insulin was incubated in the absence of any additive, a characteristic sigmoidal curve consisting of an initial lag phase, a subsequent elongation phase, and a final saturation phase was obtained, and almost 100% fibrillation was observed at approximately 10 h. The sigmoidal curve was fitted by applying the following equation to the acquired data:[18]

$$Y = y_i + m_i x + \frac{y_f + m_f x}{1 + e^{-\left[\frac{x - x_0}{\tau}\right]}}$$

Here, Y refers to the fluorescence intensity; x is time; x_0 is the time required to attain 50% of maximal fluorescence; and lag time is given by $x_0 - 2\tau$, where $1/\tau$ is the apparent rate constant for the growth of fibrils, calculated by the slope of the curve (**Figure S20**).

The lag time without any additive was calculated to be approximately 6.8 h. When poly-SPB was added, ThT intensity dropped significantly, and the intensity further decreased with increasing concentrations of poly-SPB (**Figure 1A**), indicating poly-SPB's ability to suppress fibrillation (lag time with the highest concentration was 14.1 h). When using hydrophobic poly-SPB, ThT fluorescence was markedly reduced, and the effect became more pronounced as the amount of BuMA was increased (**Figure 1B–1E**). Higher amounts of BuMA, i.e. 25% and 30%, led to almost complete inhibition of insulin fibrillation. When insulin was incubated without any additive, almost 100% fibrillation occurred within 12 h, whereas when poly-SPB with 30% BuMA was added, less than 0.5% fibrillation took place at the

very low polymer concentration of 1.5% (**Figure 1F**), indicating the tremendous ability of this polymer to inhibit fibrillation. Even after extensive incubation (24 h), less than 2.5% fibrillation was observed. Lag times (calculated for the 1.5% polymer concentration) also increased with increasing hydrophobicity: lag times were 18.6 h, 20.9 h, 22.8 h, and 24.8 h for poly-SPB with 10%, 20%, 25% and 30% BuMA, respectively. These results confirm that hydrophobic derivatives of poly-SPB arrest the growth and formation of toxic fibrils. They exhibit higher efficiencies than reported in previous studies using quinones,[19] and osmolytes[20]. Compared to our previous report, where 15% polymer concentration was used for aggregation inhibition,[12] these polymers yield significantly higher efficiency at both 1% and 1.5% concentration, indicating ten-fold increase in the efficiency with these hydrophobic sulfobetaines. TEM analysis confirmed this behavior and demonstrated that when only insulin was incubated in PBS (pH 2), extensive fibrillation took place, however fibrillation was completely suppressed by addition of poly-SPB with 25% BuMA **(Figure S19)**.

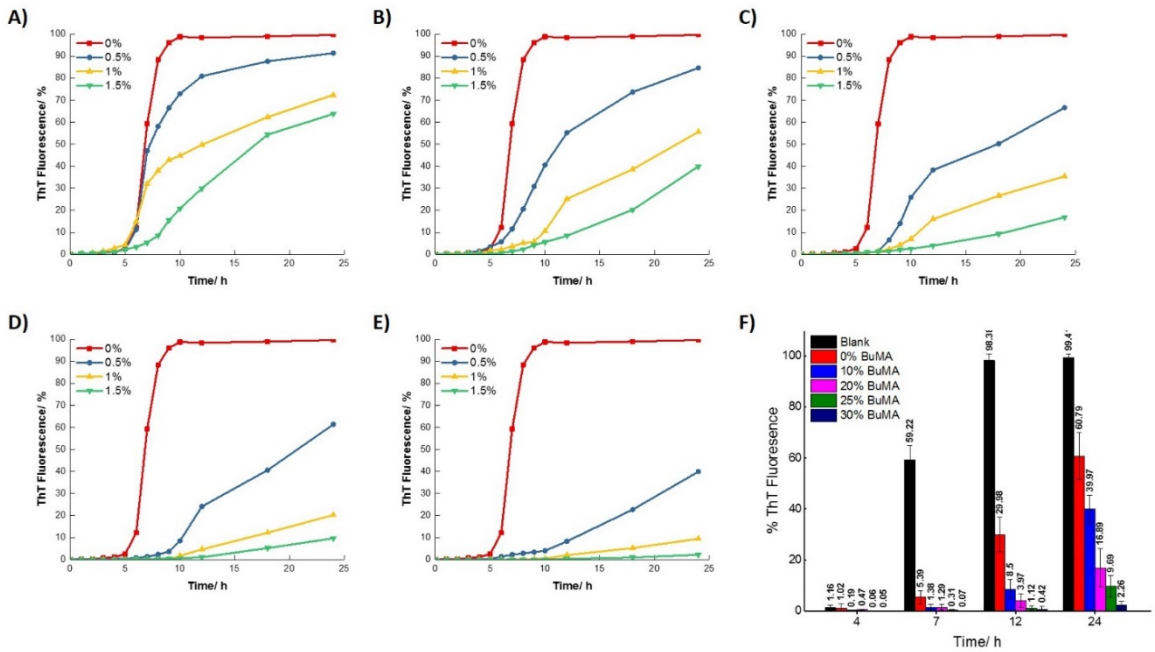

Figure 1. ThT fluorescence of insulin when incubated at 37°C in the presence of poly-SPB with A) 0% BuMA, B) 10% BuMA, C) 20% BuMA, D) 25% BuMA, and E) 30% BuMA. F) Comparison of ThT fluorescence of different polymers at 1.5% concentration, data are expressed as the mean ± SD of 3 independent experiments.

Next, the effects of poly-SPBs and their hydrophobic derivatives on the higher-order structures of insulin were studied by circular dichroism (CD) spectroscopy. As can be seen from **Figure 2A**, in the absence of any additive, the intensity of CD bands decreased significantly, especially around 225 nm corresponding to α-helix, indicating unfolding and loss of secondary structure. However, with the addition of poly-SPB, CD bands continued to appear, although at lower intensities. Upon adding hydrophobic poly-SPB to insulin, the CD bands retained almost all of their intensity: the CD bands of insulin incubated with poly-SPB with 25% and 30% BuMA were exactly identical to that of the original insulin, indicating that these polymers help to preserve insulin's secondary structure. For further confirmation, the contents of secondary structure elements (SSE) were estimated by deconvoluting the CD spectra using the CDSSTR algorithm with reference set 7 of the DichroWeb server.[21,22] **Figure 2B** clearly shows that when insulin was incubated without any polymer, a significant change in its secondary structure was observed, as represented by the decreased SSE content, especially *α*-helix, and the adoption of unordered conformation by the protein. However, the addition of polymers, especially poly-SPB with 25% and 30% BuMA, yielded almost identical SSE contents, indicating that the polymers prevent changes in insulin's secondary structure, which in turn inhibits aggregation and the formation of amyloid-like fibrils. A detailed analysis of the contents in the presence of different polymers and at different times is provided in **Table S1**.

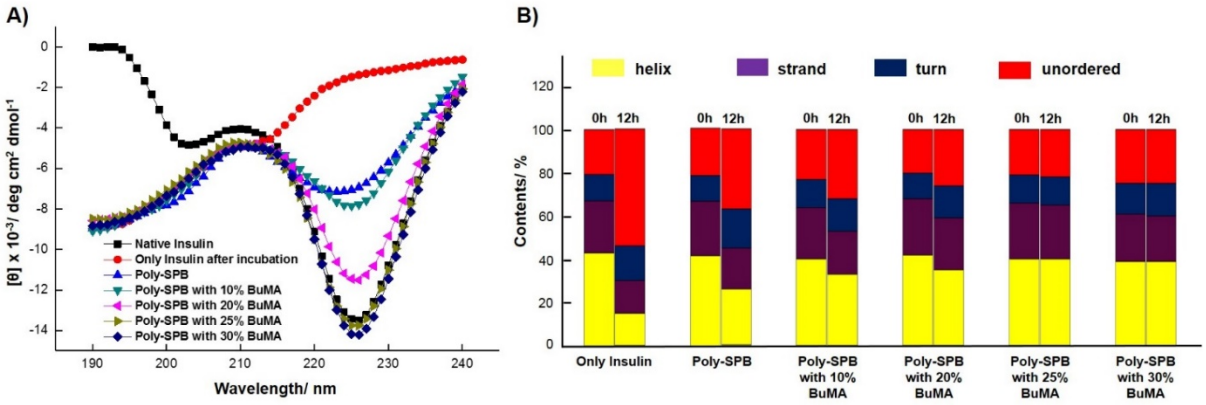

Figure 2. A) Representative far-UV CD spectra of insulin in the presence of various polymers (1.5% polymer concentration) after incubation at 37°C for 12 h, and B) percentages of secondary structure contents. Note: 0h represents the data collected immediately after mixing the insulin and polymer solution.

To ascertain the propensity of these polymers to facilitate resolubilization and refolding of denatured insulin, ThT assays and CD spectroscopy were conducted. Polymers were added to the preincubated insulin solution (at 37°C for 12 h), and the mixture was further incubated at 25°C for 5 h. **Figure 3A** unambiguously illustrates that poly-SPB facilitates the refolding of denatured insulin, with poly-SPB with 30% BuMA yielding approximately 40% recovery, as calculated by the decrease in ThT intensity after incubation of the denatured insulin with the polymer. CD spectroscopy (**Figure 3B**) also indicates a similar behavior. After preincubation, the intensities of the CD bands significantly decreased, whereas incubation with polymers enabled signal recovery, and the intensities of the CD bands were higher than in the absence of polymers. Although the recovery yield was not very high, it was nonetheless comparable to the results obtained in a recent study using polymer nanoparticles.[23] Compared to the previously reported synthesis polymer nanoparticles, which involved cross-linkers and surfactants, the zwitterionic polymers reported here were synthesized in a very facile manner. In addition, their ability to facilitate refolding suggests that these polymers have the potential to achieve higher efficiency with slight modifications.

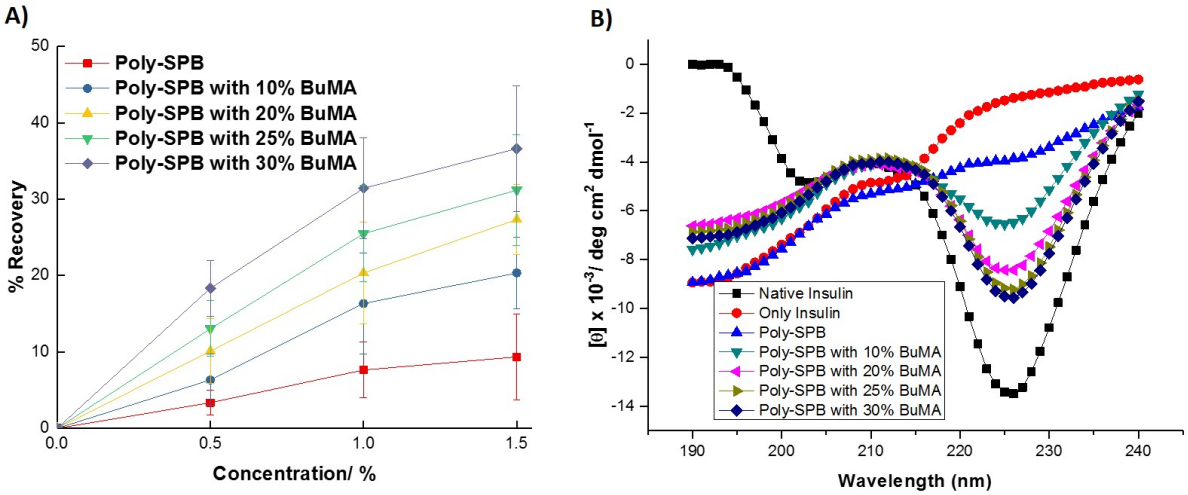

Figure 3. Refolding efficiency obtained after adding polymers to preincubated insulin solution: A) recovery yield calculated from ThT assays and B) representative far-UV CD spectra of insulin in the presence of various polymers.

Previous reports have firmly established that when proteins are exposed to extreme conditions, they tend to misfold (either partially or completely), and as a result, the hydrophobic domains of the proteins are exposed. Intermolecular interactions between such hydrophobic domains in the protein chain induce aggregation.[24] To elucidate the protective activity of the polymers reported here, we investigated the interaction between insulin and poly-SPB with 30% BuMA using 2D NMR. ^{1}H-^{1}H TOCSY and ^{1}H-^{1}H NOESY spectra were recorded with WATERGATE for solvent suppression. The observed ^{1}H signals were assigned by referring the chemical shifts registered in Protein Data Bank (PDB ID: 5MIZ). No significant change was observed in the chemical shift or the intensity of insulin peaks upon adding the polymer, indicating that insulin retained its original conformation in the presence of the polymer. **Figure 4** clearly shows that adding the polymer shifted Valine-3 (V3(A)) and Leucine-13 (L13(A)) of the A chain and Leucine-17 of the B chain (L17(B)) residues. As shown in **Figure 4B,** V3(A) is located in the α-helix and L13(A) and L17(B) are present in the 3_{10} helix. Changes in their chemical shifts indicate the binding of the polymer to the secondary structure elements, suggesting that these interactions assist in the inhibition of aggregation and the retention of higher order structures. More importantly, V and L are well-known hydrophobic amino acids (**Figure 4C**), and shifts in these residues clearly suggest that the hydrophobic environment of insulin was altered in the presence of poly-SPB with 25% BuMA. These results indicate that these polymers interact with the hydrophobic domains of insulin, preventing aggregation and facilitating refolding. This conclusion corresponds well with those of previous studies, which suggested that in order for any compound to facilitate refolding, it must interact effectively with specific residues, especially the hydrophobic domains of the denatured proteins.[23,25]

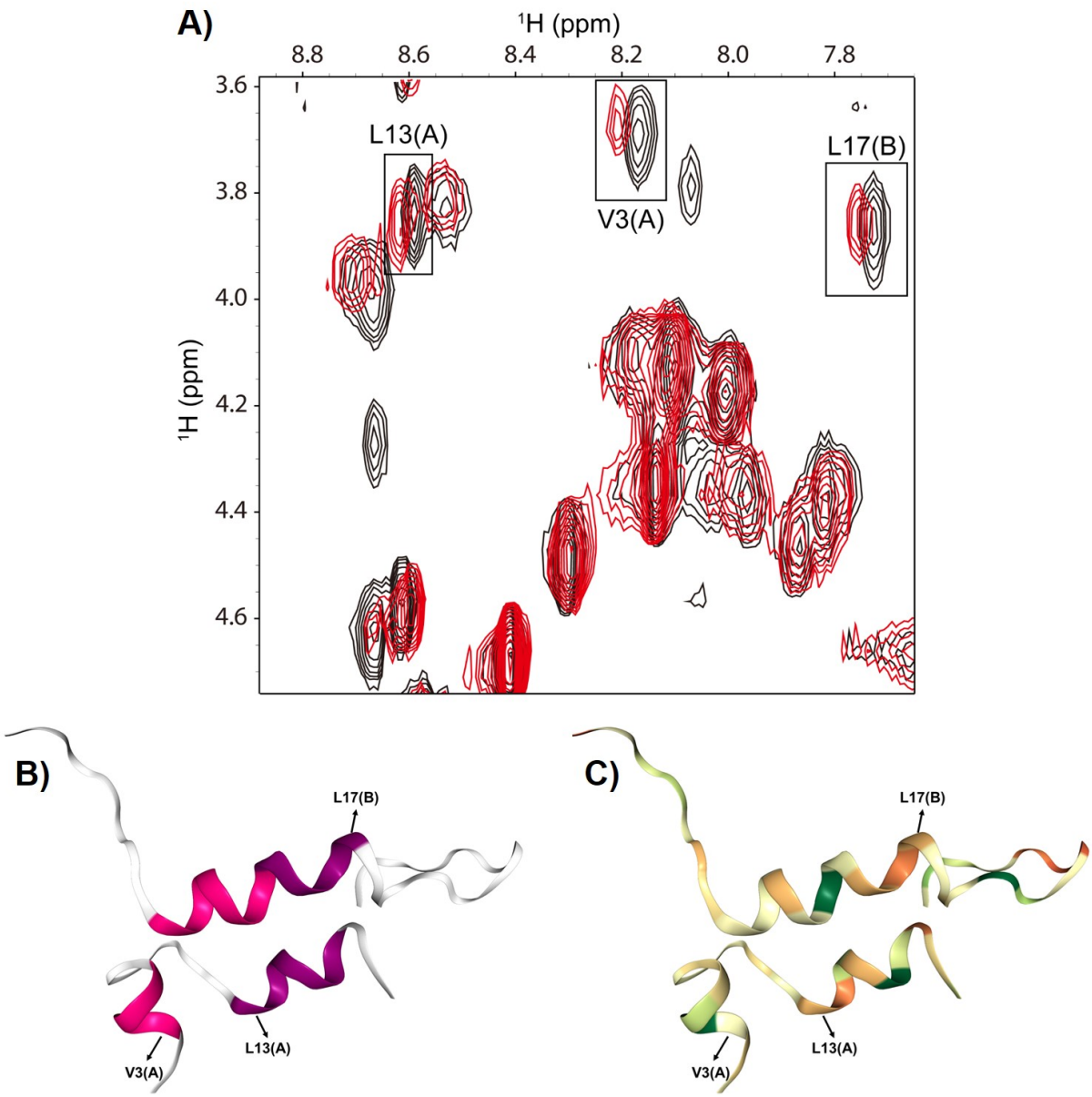

Figure 4. A) Hα/HN region of overlaid ^{1}H-^{1}H TOCSY spectra for only insulin (black) and insulin and poly-SPB with 25% BuMA (red). MD ensemble of bovine insulin by B) secondary structure and C) hydrophobicity (PDB ID: 5MIZ).

In summary, we have demonstrated that hydrophobic poly-SPB is highly efficient at suppressing insulin aggregation. Even at very low polymer concentrations and over long incubation periods (24 h), the polymers significantly inhibited aggregation and fibrillation of insulin. Upon incubation with these polymers, insulin retained its secondary structure, inhibiting aggregation and the formation of fibrils. Further investigations indicated the ability of these polymers to facilitate refolding, thus potentially providing a new avenue for the

development of polymeric systems as efficient refolding agents, which can be used to treat numerous disorders. Mechanistic investigations revealed that hydrophobic polymers alter the hydrophobic environment of insulin and suppress aggregation-inducing collisions. Further studies are underway to detail the operative mechanism based on computational simulations.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Conflict of Interest
The authors declare no conflict of interest.

Received: ((will be filled in by the editorial staff))
Revised: ((will be filled in by the editorial staff))
Published online: ((will be filled in by the editorial staff))

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A highly efficient hydrophobic zwitterionic polymers for the inhibition of insulin aggregation was developed by RAFT polymerization. Additionally, these polymers help in refolding of the denatured insulin, thus providing a potential method to cure numerous aggregation-induced neurodegenerative diseases. Mechanistic investigations revealed that they facilitate the preservation of secondary structure of insulin and prevent the aggregation-induced collisions by altering the hydrophobic environment of insulin.

Protein aggregation

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