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Research Paper

Role of a Novel Excipient Poly(Ethylene Glycol)-*b*-Poly(L-Histidine) in Retention of Physical Stability of Insulin in Aqueous Solutions

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Purpose. This study is to investigate whether poly(ethylene glycol) (PEG)-*b*-poly(L-histidine) [PEG-polyHis] can reduce aggregation of insulin in aqueous solutions on agitation by forming ionic complexes.
Materials and Methods. Insulin aggregation on agitation was monitored spectrophotometrically and by fibrillation studies with a dye Thioflavin T. Pluronic F-127 as a control and PEG-polyHis as a novel multifunctional excipient were added to prevent destabilization of insulin. Conformation of insulin was evaluated in a circular dichroism (CD) study.
Results. Ionic interactions between insulin and PEG-polyHis were induced in the pH range: 5.5–6.5. pH 5.5 was selected for further evaluation based on particle size/zeta potential studies. Ionic complexation with PEG-polyHis is more effective at pH 5.5 in stabilizing insulin (75% of insulin retained versus 0% with no excipient) than Pluronic F-127 (42% retained). PEG-polyHis guards against insulin aggregation in non-complexing pH conditions (pH 7.4), 64% insulin retained versus 58% with F-127 and 0% with no excipient) pointing to the potential role played by PEG in modulation of insulin surface adsorption. Rate of fibrillation was higher for plain insulin compared with addition of PEG-polyHis and Pluronic F-127 at both pH.
Conclusions. Understanding and manipulation of such polyelectrolyte-protein complexation will likely play a role in protein stabilization.

KEY WORDS: aqueous insulin stability; insulin aggregation; ionic complexation; PEG-*b*-poly(L-histidine); polymeric stabilizers.

INTRODUCTION

The most challenging task in the development of any protein formulation is the stabilization of a protein to achieve an acceptable shelf-life. Protein aggregation is arguably the most common and troubling manifestation of protein instability, encountered in almost all stages of protein drug development. Along with other physical and/or chemical instabilities of proteins, protein aggregation remains a major hindrance in rapid commercialization of potential protein drug candidates. A typical example is a therapeutic protein—insulin. Formulated human insulin aggregates on the surface of glass storage vials with relatively hydrophilic surfaces on standing demonstrating the challenges faced in insulin formulation. Contacts between formulated insulin and hydrophobic surfaces results in alteration of administered insulin, leading to formation of amyloid-like fibrils, which poses a problem in the purification, storage, and delivery of insulin solutions (1–4). Several other therapeutic proteins have shown similar problems on interaction with unfavorable surfaces. Administration of low concentrations of interleukin (IL)-1, via a syringe-pump system results in adsorptive losses of 20–80% (5). Similarly, reductions of 80–90% of the biological activity of a Food and Drug Administration-approved IL-2 formulation have been reported during delivery via polyethylene catheter tubing (6). Tzannis *et al.* have reported that the biological activity of the delivered protein was dramatically reduced by approximately 90% after a 24-h infusion program. Examination of the secondary and tertiary structure of both adsorbed and delivered protein indicated that transient surface association of IL-2 with the catheter tubing resulted in profound, irreversible structural changes that were responsible for the bioactivity losses (7).

Due to complication of structural modifications, proteins are commonly stabilized by addition of excipients. The often-used protein stabilizers include sugars, polyols, surfactants, salts, [poly(ethyelene glycol); PEGs], metal ions, and amino acids (8,9). There is no single pathway to follow in selection of a suitable stabilizer(s). Addition of human serum albumin or surfactants to the reconstitution medium can significantly decrease adsorptive losses and subsequent protein denaturation (5,6,10). However, the use of serum albumins is associated with problems including self-aggregation. Moreover, the addition of another protein complicates characterization of the formulation by the use of common analytical methods such as UV spectrophotometry, BCA assay and RP-HPLC methods. Nonionic surfactants such as Pluronics,

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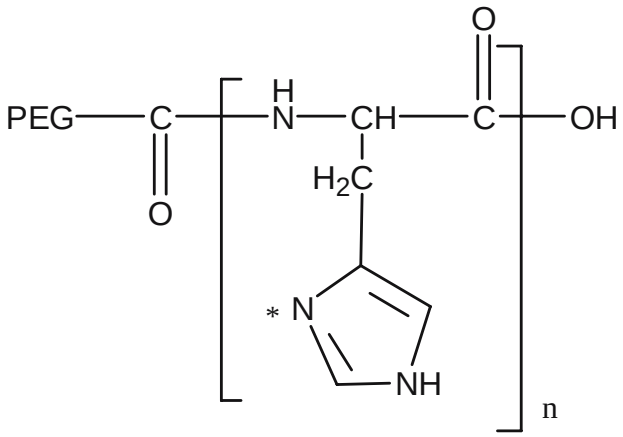
Tweens, Brijs have been known to reduce interfacially induced aggregation of insulin (11–14).

The present study is aimed at investigating the potential of poly(ethylene glycol)-b-poly(L-histidine) (PEG-polyHis: a cationic polyelectrolyte) in reduction of aggregation of insulin at the solid/aqueous interface. Recombinant human insulin was chosen as an unstable therapeutic protein in this study. There is a clinical requirement to prevent aggregation and degradation of insulin especially for long-tem use in infusion pumps for better management of hyperglycemia. Insulin also serves as an inexpensive biopharmaceutical drug to test the efficacy of novel protein stabilizers. The hypothesis proposed in this study aims to utilize smart macromolecules as a novel 'temporal and reversible molecular shield' to achieve reduction/elimination of insulin destabilization by inducing intermolecular interactions between insulin and PEG-polyHis. The imidazole ring in histidine has an electron pair on the unsaturated nitrogen that endows polyHis with an amphoteric nature by rapid and reversible protonation-deprotonation (Fig. 1). The pK_a value of PEG-polyHis lies between pH 6.5 and 7.0 (15, 16). Therefore, PEG-polyHis may form ionic complexes with an anionic protein such as insulin within a narrow pH range based on attraction between opposite charges on insulin and PEG-polyHis as depicted in Fig. 2. In a previous study from our group, PEG-polyHis significantly improved the stability of a non-therapeutic protein; BSA in aqueous solutions (17). This study is a preliminary attempt for understanding the potential of intermolecular interactions of insulin and PEG-polyHis in reducing aggregation of a therapeutic protein insulin. Pluronic F-127 was used as a positive control as used in other studies involving urease, rhIL-2, and recombinant human growth hormone (18,19).



PEG-Poly(L-Histidine) diblock copolymer

Fig. 1. Chemical structure of the diblock copolymer PEG-poly (L-Histidine). * indicates the lone pair of electrons available for reversible protonation.

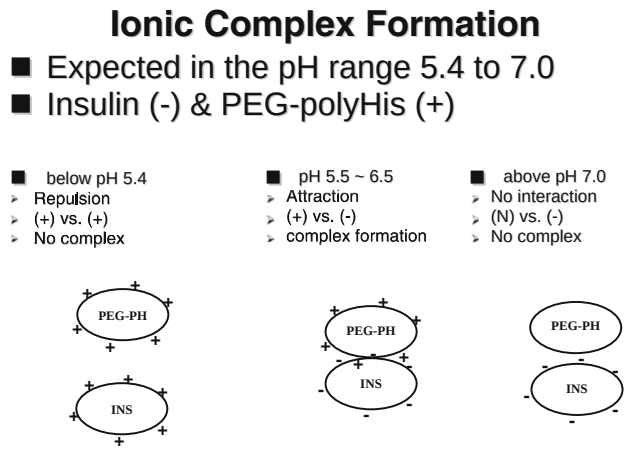


Fig. 2. Ionic Interactions of insulin (protein) and PEG-polyHis (polyelectrolyte, denoted as PEG-PH) as a function of pH.

MATERIALS AND METHODS

Insulin, recombinant human, USP (potency ≥ 27.5 U/mg, zinc content ≤ 1.08%), was purchased from Sigma Chemical Co., (St. Louis, MO). Pluronic F-127 (abbreviated as F-127 hereafter) was purchased from BASF Group (New Jersey). F-127 defines a block copolymer that is a solid, has a poly(propylene oxide) 'PO' block of 3,600 Da, and 70% weight of poly(ethylene oxide) 'EO'. DMSO-d_6, thioflavin T (ThT), 1,8-anilino naphthalene sulfonate (ANS) were purchased from Sigma-Aldrich, (St. Louis, MO). All buffer salts used in studies were of analytical reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO). Buffer solutions were prepared according to the standard pharmacopoeial procedures. Radioimmunoassay (RIA) kit was sourced from MP Biomedicals, Orangeburg, NY.

PEG-polyHis Synthesis and Characterization

PEG-polyHis was synthesized and characterized as previously reported (15,20). In brief, PEG-polyHis was synthesized by a coupling reaction between the monocarboxyl poly(ethylene glycol) (PEG-COOH) and poly(N^{im}-dinitrophenyl (DNP) L-His), followed by deprotection of poly((N^{im}-DNP L-His) block. The synthesis method includes selecting a blocking group for the primary amino group and imidazole ring of L-His and the synthesis of monomeric N^{im}-DNP-L-His N-carboxyanhydride NCA. Poly(N^{im}-DNP L-His) was synthesized by ring-opening polymerization of N^{im}-DNP L-His (NCA) in the presence of an amine initiator. The total molecular weight of PEG-polyHis used in this study was 7.5 kDa consisting of two blocks; PEG 2 kDa and polyHis 5.5 kDa (average degree of polymerization: 36) as determined by ^{1}H-NMR. Polydispersity of the diblock copolymer was 1.34 as determined by MALDI Time of Flight spectrometry. To independently confirm the apparent pK_a, acid–base titration was carried out by following the protocol as detailed in a previous study (9,12). The apparent pK_a range was determined to be 6.5–7.0. Therefore, the diblock copolymer may form ionic complexes with an anionic protein/peptide such as insulin with an isoelectric point (pI)—5.4 in a suitable and a narrow pH range.

Preparation of Insulin Solutions

Insulin solutions were made from a stock solution in 0.025 M hydrochloric acid (HCl) by adding appropriate pH buffers (ionic strength 0.15 M) to make desired insulin concentrations (21). The pH of insulin solutions was adjusted with addition of either 1 N sodium hydroxide (NaOH) or 1 N HCl. All insulin solutions were filtered through a 0.22-μm Acrodisc™ low protein-binding syringe filter (Pall Corporation, Ann Arbor, MI, USA).

Method of Analysis of Insulin in Aqueous Samples

For all samples, the concentration of insulin was checked by using an extinction coefficient of 1.0 for 1.0 mg/ml at 276 nm by UV spectrophotometry (21). Controls included aqueous buffers (pH 5.5 and 7.4) incorporating F-127 and PEG-polyHis, and blanks included aqueous buffers (pH 5.5 and 7.4).

Particle Size and Zeta Potential Analyses: pH-dependent Complexation

Zeta potential studies aided in determining the working pH range for complexation of insulin and PEG-polyHis (17). The aim was to optimize pH for inducing molecular-level interactions between insulin and PEG-polyHis such that insulin complexes could be tested against stresses encountered during agitation. PEG-polyHis concentration was fixed at 0.5 mg/ml. Insulin concentration was varied from 0.17 to 1.5 mg/ml. Therefore, the weight ratio of insulin to PEG-polyHis ranged from 1:3 to 3:1. Seven pH solutions were selected to evaluate complex formation: 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.4 (0.15 M ionic strength; the physiological ionic strength). The zeta potential of uncomplexed PEG-polyHis and insulin was measured within the experimental pH range as controls. Zeta potential as a function of weight ratio of insulin to PEG-polyHis and pH was obtained using a zetasizer (Malvern system) at 25°C.

Based on preliminary zeta potential results, particle size studies were designed to confirm the working pH range for complexation of insulin and PEG-polyHis. PEG-polyHis concentration was fixed at 0.5 mg/ml as earlier. Insulin concentration was varied from 0.17 to 1.0 mg/ml. Therefore, the weight ratio of insulin to PEG-polyHis ranged from 1:3 to 2:1. Six pH (buffer solutions of 0.15 M ionic strength) were selected to evaluate complex formation: 4.5, 5.0, 5.5, 6.0, 6.5, and 7.4. Dynamic light scattering (DLS) used a He–Ne laser, with a Bl-200SM Research Goniometer System from Brookhaven Instruments Limited. The apparatus consisted of digital correlator and a signal processor, which incorporated a computer. Measurements were made at 633 nm, at a 90° angle for the detector (photomultiplier tube) This angle is particularly useful for observing Rayleigh scatterers (i.e. particles much smaller than wavelength λ). Nonaggregated insulin and PEG-polyHis molecules behave as Rayleigh scatterers. For each sample at 25°C, light-scattering measurements were accumulated during a 10-min interval to reduce random signal noise and ensure a stable baseline. The experimentally determined autocorrelation function g(t) was used to obtain the size-distribution function G(τ) using CONTIN.

Effect of Ionic Strength on Intermolecular Interactions: Possibility of Hydrophobic Interactions

In a separate set of experiments, binding of insulin to the diblock copolymer PEG-polyHis was studied in two buffers: pH = 5.0 (buffer 1) and pH 8.0 (buffer 2) at optimized weight ratio(s) as determined in zeta potential/particle size studies. The pH of the buffers was selected to facilitate the study of the effect of PEG-polyHis' ionic transition on potential hydrophobic interactions with insulin. Two different ionic strengths: 0.05 and 0.15 M were chosen. A higher ionic strength may be expected to suppress electrostatic repulsion that especially occurs at pH 5.0 which could complicate the measurement of significant hydrophobic interactions.

Changes in pH affect the charges on PEG-polyHis and insulin. Therefore, comparison of binding behavior at different pH involves considering changes in both ionic and hydrophobic interactions. At a lower pH of 5.0, the net positive charges on insulin and PEG-polyHis may not only lead to increased electrostatic repulsion, which tends to decrease the extent of binding, but also to increased repulsion among adjacent bound proteins. However, any presence of interactions at this pH would suggest the possibility of significant hydrophobic interactions.

ANS Binding Characteristics of Insulin

1,8-Anilinonapthalene sulfonate (ANS: hydrophobic fluorescent dye) shows strong fluorescence on interaction with hydrophobic surfaces of a protein and/or polymers (22–24). Insulin and PEG-polyHis were dissolved at appropriate concentrations and various weight ratios as in particle size and zeta potential studies. Mixed solutions were incubated at room temperature for 4 h with a 15 μl of 10 mM ANS solution (as determined from optimization studies, more than optimal volumes used led to turbidity in the insulin solutions). Fluorescence emission was set at 460 nm with excitation at 380 nm. Microplate reader was used with average of five wells taken to estimate the fluorescence intensity for each experimental condition.

UV Absorbance Studies for Loss of Soluble Insulin

The aim was to estimate the loss of soluble insulin as a function of time by UV spectrophotometry. Aliquots of insulin stock solution in 0.15 M phosphate buffer were mixed to attain an initial concentration of ~1.0 mg/ml for insulin. PEG-polyHis concentration was fixed to provide an optimal weight ratio as determined in previous complexation studies. Similarly, in another sample set, F-127 was added to insulin solutions at a final concentration of 2.0 mg/ml. These polymeric excipients were co-dissolved with insulin in buffer solutions. Insulin solutions were incubated in cylindrical polyethylene tubes (12 × 75 mm) at 37°C with agitation in a water bath at 50 oscillations per minute. Care was taken to avoid the presence of air in the tubes that could complicate the evaluation of results. Air/water interface is hydrophobic in nature and leads to denaturation of proteins in solutions. Sampling was done at days 1, 4, 7 and then weekly intervals for a period of 4 weeks. At each sampling point, the contents from each tube were transferred to 1.5 ml eppendorf tubes and centrifuged at

10,000 × g for 10 min. The supernatant was carefully removed, and the absorbance of supernatant insulin solution was measured at 276 nm.

Kinetics of Fibrillation: Aggregation and Precipitation

Comparison experiments using PEG-polyHis and F-127 to investigate their ability to reduce/eliminate insulin aggregation were carried in the second phase of experiments. In order to correlate the propensity of insulin to fibrillate on interaction with the surfaces, the progress of fibrillation was studied for all the conditions listed in the previous experiment by using a dye thioflavin T (ThT) (25). ThT fluorescence was monitored for each sample in a 96-well plate using a Spectra Max M2 fluorescence plate reader (Molecular Devices). The excitation wavelength was set at 444 nm and emission was measured at 485 nm. Five replicates for each sample were used to minimize well-to-well variation. Results from >5 similar profiles were averaged to obtain the final results. Control experiments were run on the diblock copolymer PEG-polyHis, F-127 and plain insulin solutions incubated under similar conditions.

Conformational Evaluation of Insulin and Insulin/PEG-polyHis Complexes

Retention of secondary structure was monitored in a circular dichroism (CD) study. The CD spectra were recorded on AVIV 62DS spectropolarimeter equipped with a water bath operated at ~ 25°C. A quartz cuvette with 0.1 cm path length was used for far-UV measurements. Insulin solutions were scanned between 200 and 250 nm with 0.5 nm resolution and ten scans were taken. Insulin stability was estimated by using spectra and calculating the % α-helix content at each weekly interval. A plot of the percentage of α-helix vs. week incubated at 37°C for insulin at different pH was made.

Radioimmunoassay (RIA) of Recovered Soluble Insulin

Insulin amounts in recovered soluble samples after incubation in an aqueous medium with or without the polymeric excipients were determined using a commercially available human insulin RIA kit (ImmuChem™; MP Biomedicals, Orangeburg, NY) whose output was given in international insulin activity microunits per milliliter (μIU/ml). The detection range of this kit was 5.5–310 μIU/ml. Insulin samples from week 1, 2, 3 and 4 were analyzed. All samples were run in duplicate. Appropriate controls included freshly prepared insulin standards incubated with polymeric excipients at similar concentrations used in the real study. Recovered soluble insulin tested in RIA analyses was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC). The chromatograph was equipped with a HPLC LC pump; an injection valve (Rheodyne), a UV-Vis detector set at the wavelength of 215 nm and an integrator combined in an Agilent 1100 series HPLC system. An Extend™ 5-μm C_{18} (4.6 × 250 mm) (Agilent, USA) was employed. Insulin samples were analyzed at room temperature using a binary gradient consisting of (A) water/TFA (99.9/0.1) and (B) Acetonitrile/TFA (99.9/0.1). The gradient consisted of 15–65% B in 10 min, followed by equilibration at 15% B for 5 min. Flow rate was set at 1 ml/min.

RESULTS

Insulin/PEG-polyHis Complexation: pH-dependent Interactions

Zeta Potential Measurements

Towards validating the proposed concept, we selected PEG-polyHis as a cationic polyelectrolyte and insulin (isoelectric point (pI) = 5.4) as a model anionic protein. Variations of solution pH can significantly alter the electrostatic interactions by altering the charge present on amino acid residues with ionizable side chains. The zeta potential results provide evidence for intermolecular interactions between PEG-polyHis and insulin. Zeta potential results coincide well with pH-dependent changes in the net charges. Both insulin and PEG-polyHis bear a net positive charge below pH 5.4 and tend to repel each other. As an example, at pH 5.0, PEG-polyHis and insulin have zeta potential values of +18 mV and +7.4 mV. Using a weight ratio of 1:2 for insulin to PEG-polyHis, overall charges add up to 24.2 mV. Similarly, complexation is not significant above pH 7.0, because PEG-polyHis becomes neutral. However, insulin and PEG-polyHis could form ionic complexes in the pH range 5.5 to 6.5 due to opposite net charges. At pH 6.0, PEG-polyHis carries a positive charge of +12.4 mV compared to a negative potential of −11.6 mV for insulin. Interactions of these two at 1:2 weight ratio had an overall zeta potential of system as −0.5 mV. If insulin and PEG-polyHis interact to form optimal complexes, the value of the zeta potential should be near zero. This means that the surface charge of the complex is neutral because of charge neutralization between insulin and PEG-polyHis. Fig. 3 shows zeta potential as a function of insulin to PEG-polyHis weight ratio and pH. At pH 5.5 the zeta potential for insulin and PEG-polyHis mixture at weight ratio 1:2 and 1:1 had values close to zero. Therefore, it was decided to fix the weight ratio of insulin to PEG-polyHis as 1:2 for use at pH 5.5.

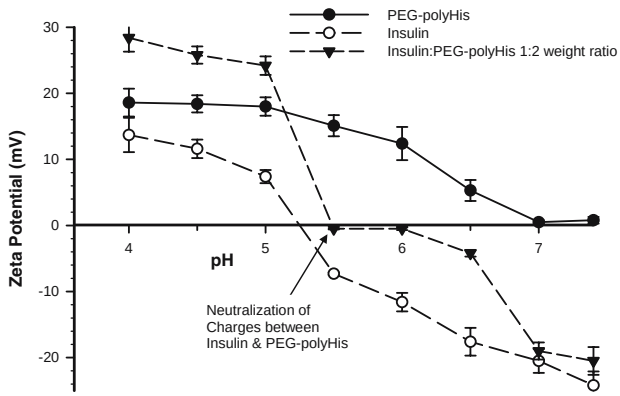


Fig. 3. Zeta potential of insulin, PEG-polyHis and intermolecular complexes (weight ratio 1:2 of insulin to PEG-polyHis, $n = 3$) as a function of pH at room temperature. PEG-polyHis concentration fixed at 0.5 mg/ml.

Particle Size Measurements

In the tested pH range 4.5–7.4, the average sizes of insulin were significantly influenced by varying the pH. At pH 4.5, the mixtures of insulin and PEG-polyHis solutions at differing mixing ratios resulted in particles smaller than 4 nm in size indicating that no significant ionic interaction is taking place based on similarity of positive charges on both species. Similarly, complexation is not significant at pH 7.4, because insulin bears a net negative charge and PEG-polyHis becomes neutral. Insulin and PEG-polyHis could form complexes in the pH range 5.5 to ~ 6.5 resulting in increased particle sizes of 8–15 nm, reflecting the primary role played by ionic interactions in the complexation. The size of the complexes increased with higher PEG-polyHis content. As an example, the insulin and PEG-polyHis complex particle were about 8 nm in diameter for a 2:1 ratio and 12 nm for a 1:2 weight ratio at pH 5.5. Use of higher PEG-polyHis amounts at pH 5.5 led to insignificant changes in particle sizes of complexes with insulin (see Fig. 4). It may be inferred that insulin and PEG-polyHis have completely complexed at 1:2 weight ratio (insulin : PEG-polyHis) and pH 5.5 and support zeta potential results.

Presence of Hydrophobic Interactions between Insulin and PEG-polyHis

To probe the role of hydrophobic interactions in assisting ion-pairing (HIP); a second set of experiment was designed in which pH were chosen to prevent any potential electrostatic interactions between PEG-polyHis and insulin. For buffer 1 at pH 5.0 and ionic strength 0.15 M, salts are likely to suppress electrostatic repulsions between the positively charged insulin and PEG-polyHis. No significant change in particle size was seen when lower a molarity buffer (0.05 M) was used for dissolving insulin and PEG-polyHis. Similarly, for buffer 2 at pH 8.0 and a lower ionic strength (0.05 M), no significant change in particle size was seen over controls (0.15 M buffer) ruling out any significant hydrophobic interactions. The use of buffers with different ionic strengths facilitated the study of potential hydrophobic interactions between the two components. It is likely that the major property of polyelectrolyte PEG-polyHis in these experiments that dictated binding is charge density. A decisive role for HIP can be ruled out in the present system.

In summary, particle size studies support zeta potential studies in determining suitable conditions for intermolecular interactions between insulin and PEG-polyHis. The weight ratio of insulin and PEG-polyHis was fixed at 1 to 2 for insulin aggregation and fibrillation studies at pH 5.5.

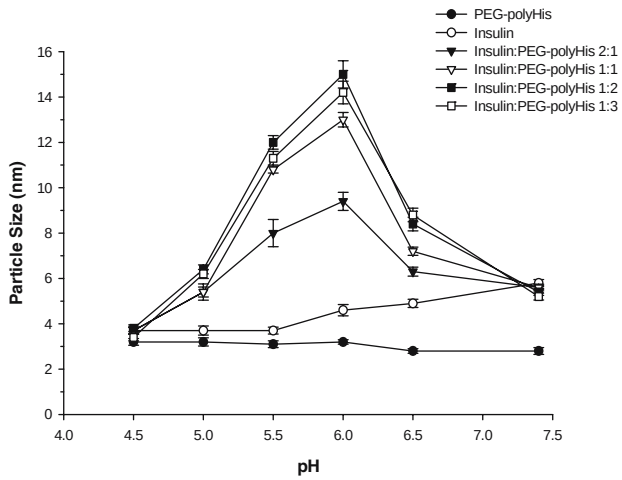


Fig. 4. Particle size of insulin, PEG-polyHis and intermolecular complexes (various weight ratio of insulin to PEG-polyHis, $n = 3$) as a function of pH at room temperature. PEG-polyHis concentration fixed at 0.5 mg/ml.

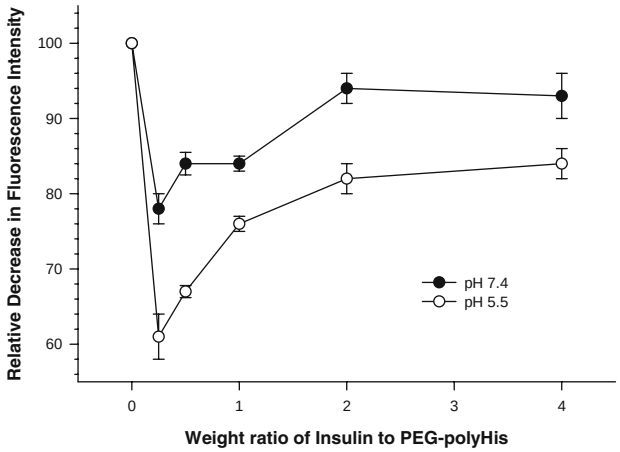


Fig. 5. Binding of ANS to Insulin and its complexes with PEG-polyHis. pH 7.4 (blend b system) and 5.5 (complex c system) were used for incubation at room temperature for 4 h.

ANS Binding Characteristics of Insulin

Insulin showed high fluorescence intensity on incubation with ANS in 0.1–1.0 mg/ml concentration range (the desired concentration in the studies). Regression coefficients values of 0.95 and higher seem to be satisfactory for this fluorescence study. PEG-polyHis showed very low fluorescence in the concentration range 0.25–2.0 mg/ml when compared to insulin at similar pH (results not shown here). Two pH were selected based on zeta potential and particle size studies: 5.5 (PEG-polyHis is ionized and able to ionically complex with insulin) and 7.4 (blend system of insulin and PEG-polyHis). Incubation of insulin and PEG-polyHis at 7.4 and 5.5 led to reduction in fluorescence intensity on binding to ANS to a variable degree. Varying the PEG-polyHis amount keeping the insulin amount fixed (0.25 mg/ml) showed a fall and rise pattern in fluorescence intensity as shown in Fig. 5. Increasing the amount of PEG-polyHis incubated with insulin led to decrease in fluorescence intensity; this suggests increased intermolecular interactions between insulin and PEG-polyHis limiting the access of ANS to the insulin backbone. More significantly, loss of fluorescence intensity is higher at pH 5.5 than at pH 7.4 suggesting that PEG-polyHis interacts with insulin more strongly by ionic interactions.

In subsequent results, the notation "b" refers to the physical admixture of insulin and PEG-polyHis at pH 7.4 in which no significant ionic complexation was found to occur in

zeta potential and particle size studies. Similarly, the notation "c" refers to the formulation of insulin and PEG-polyHis at pH 5.5 in which ionic interactions were induced and complexes optimized in terms of weights of insulin and PEG-polyHis necessary as determined in zeta potential and particle size studies.

Retention of Physical Stability of Insulin

Aggregation of Insulin

pH-dependent complexation studies aided in designing a suitable pH range for aqueous stability analyses of insulin on long-term incubation (4 weeks). The role of complexation of PEG-polyHis with insulin on stabilization is evident at pH 5.5. At this pH on incubation with PEG-polyHis [complex 'c' system], almost 75% of the soluble insulin content was retained at week 4 compared to about 42% insulin retention in presence of F-127 and near zero retention for insulin controls with no polymeric excipients. The poly(L-histidine) component of PEG-polyHis may be physically attached to the surface of insulin through ionic interactions with minimal disturbance of the protein structure and hydrophilic PEG component covered the surface of the protein potentially acting as the corona. This feature may prevent insulin surface adsorption, reduce unfolding, and reduce the extent of aggregation. At pH 7.4 [blend 'b' system], almost 64% of the original soluble insulin content was retained at week 4 compared to about 58% retention in presence of F-127 and zero retention for insulin controls. PEG-polyHis and F-127 slowed down the rate of destabilization suggesting the initial role of PEG component in preventing intermolecular interactions and surface denaturation of insulin. This is clearly demonstrated in insulin controls, where loss of soluble insulin is rapid. The kinetics of loss of soluble insulin in the presence and the absence of the polymeric excipients is presented in Fig. 6. To test whether the observed insulin aggregation is caused by mechanical shear stress and/or continual creation of an air/water interface, 1.0 mg/ml of insulin solution was incubated under similar conditions without agitation. Without agitation, insulin solution showed only a 5% drop in soluble insulin content up to 4 weeks (results not shown here). Experimental results demonstrate that agitation-associated effects (e.g., shear and continual creation of new solid/air interface) cause significant insulin instability.

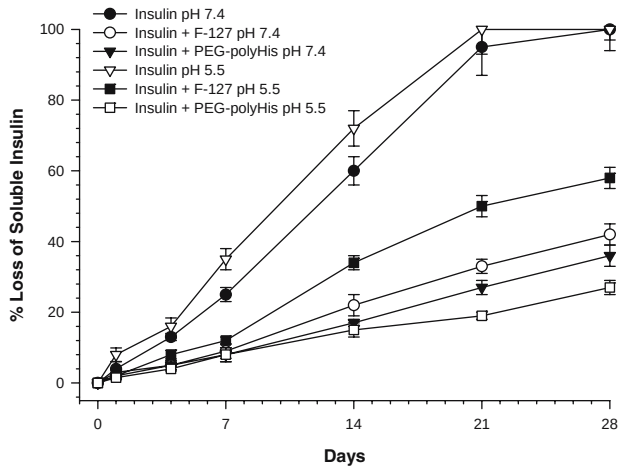


Fig. 6. Kinetics of loss of soluble insulin (Initial concentration: ~1.0 mg/ml, $n = 3$) under different pH conditions and/or on addition of PEG-polyHis (2.0 mg/ml, b and c systems) or F-127 (2.0 mg/ml). Ins refers to the native human insulin.

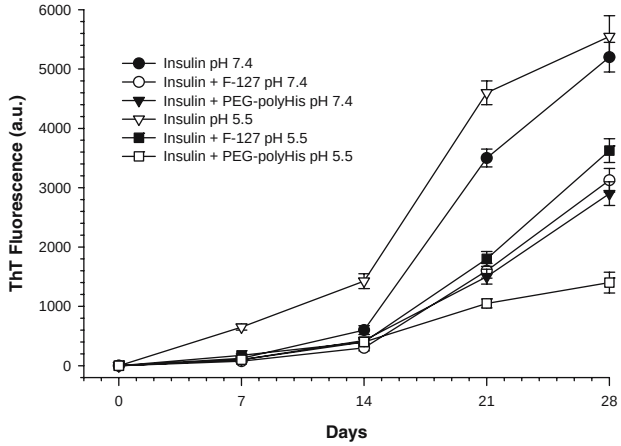


Fig. 7. Kinetics of fibrillation of insulin (Initial concentration: ~1.0 mg/ml, $n = 3$) under different incubation conditions and/or on addition of PEG-polyHis (2.0 mg/ml, b and c systems) or F-127 (2.0 mg/ml).

Kinetics of Fibrillation of Insulin

Reduction in insulin fibrillation upon the addition of the diblock copolymer PEG-polyHis and F-127 to insulin solutions were estimated through a change in ThT fluorescence. A higher fluorescence correlates to higher fibrillation of insulin (26). Measurement of rate of fibrillation of insulin in aqueous solutions was carried out with addition of F-127 (pH 7.4 and 5.5), and addition of PEG-polyHis ('b' system and 'c' system) in fluorescence studies. The amount of fibrils formed as measured by ThT signal increased as a function of time for all samples. The difference lay in the rate of fibrillation. Native insulin (pH 7.4 and 5.5 solution) was used as a control. At pH 7.4 ['b' system], higher ThT fluorescence (2,900 arbitrary units, a.u.) was seen for insulin with PEG-polyHis compared to the pH 5.5 complex system (1,400 a.u.) at week 4. However, the rate of fibrillation was slower compared to control plain insulin pointing to the initial role of PEG in slowing down the denaturation of insulin on interaction with surfaces and agitation. The results are summarized in a kinetic plot in Fig. 7. Conformational rigidity by complexation with PEG-polyHis at pH 5.5 may play a role in preventing fibrillation of insulin demonstrated by a lower ThT fluorescence. On comparison with insulin solutions with F-127, PEG-polyHis showed better preservation of insulin native structure at pH 5.5. For example, insulin incubated with PEG-polyHis showed lower fluorescence compared to insulin incubated with F-127 at pH 5.5 (1,400 a.u. with PEG-polyHis versus 3,625 a.u. with F-127). Plain insulin showed ThT fluorescence of 5,550 a.u. at pH 5.5. This can be attributed to lower stability of plain insulin at pH 5.5. This can also be seen when compared with plain insulin at pH 7.4 PEG-polyHis and F-127 slowed down the rate of fibril formation, possibly by different mechanisms.

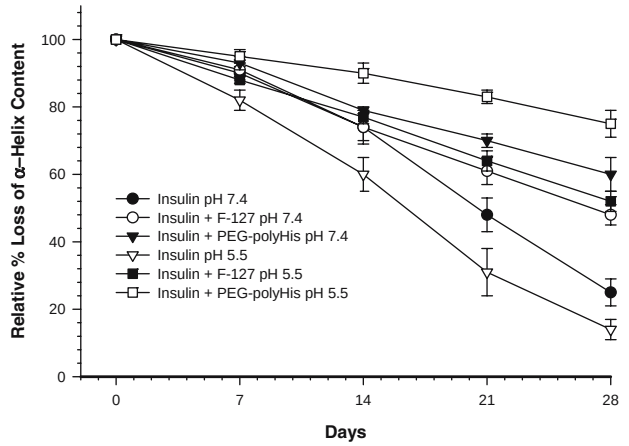


Fig. 8. Kinetics of loss of α-helix content of insulin (Initial concentration: ~ 1.0 mg/ml, $n = 3$) under different incubation conditions and/or on addition of PEG-polyHis (2.0 mg/ml, b and c systems) or F-127 (2.0 mg/ml).

Secondary Structure

Extensive losses in tertiary structure can lead to subsequent loss of secondary structure. The structural characterization of insulin depends upon the remarkable sensitivity of far-UV CD to the backbone conformation of insulin; the far-UV CD of a protein generally reflects the secondary structure content of the protein. Usually the magnitudes of two negative minima at 208 and 222 nm (α-helix) are measured. In the stability test, the α-helix percentage of insulin was obtained from circular dichroism (CD) spectra.

To estimate the retention of secondary structure of insulin in the presence of the diblock copolymer in aqueous solutions at a non-complexing pH: 7.4 and one favorable pH: 5.5 for complexation, the pH- and time-dependent decline in content of α-helix structure were monitored and the results are presented in Fig. 8. To minimize the contribution of buffer and PEG-polyHis in far-UV CD signal, the signal from the buffer and PEG-polyHis, previously determined, was subtracted from total signal of insulin and PEG-polyHis. Decomplexation was carried out to avoid changes in signal from insulin because of ionic interactions with the copolymer. The role of complexation of PEG-polyHis with insulin evident at pH 5.5 is remarkable in terms of minimum loss of alpha-helical structure. At this pH ['c' system], almost 75% of the original α-helix content was retained at week 4 compared to about 52% retention in presence of F-127 (43–45% original α-helix content at pH 7.4). Possibly, conformational rigidity imparted by complexation at pH 5.5 plays a role in preventing destabilization of insulin. At pH 7.4 ['b' system], almost 60% of the original α-helix content was retained at week 4 compared to about 48% retention in presence of F-127 and 25% retention for insulin with no additives. The diblock copolymer at pH 7.4 having PEG component may be preventing surface interactions between insulin and the tubes leading to reduced unfolding of insulin. PEG-polyHis and F-127 slowed down the rate of destabilization suggesting the initial role of PEG component in preventing agitation-induced effects of intermolecular interactions. The results seem to support the results of fibrillation analyses of insulin on a similar timescale.

Radioimmunoassay

Analyses of released insulin by RIA and RP-HPLC revealed an excellent correlation between these two methods. However, RIA was more accurate in estimating the insulin content in aqueous solutions especially for the samples from weeks 3 and 4. No interference was seen from polymeric excipients. Comparative results from RP-HPLC and RIA for studies at pH 5.5 are presented in Fig. 9 (a for insulin control, b for insulin + F-127, weight ratio 1:2, c for insulin + PEG-polyHis, weight ratio 1:2). Similar results were obtained for insulin incubation at pH 7.4 (results not shown here). It is interesting to note that insulin content estimations by RIA in presence of polymeric excipients were closer to those from RP-HPLC than for insulin controls. This may be explained on the basis of better preservation of secondary and tertiary structure of insulin in the presence of F-127 and PEG-polyHis as shown in Fig. 8.

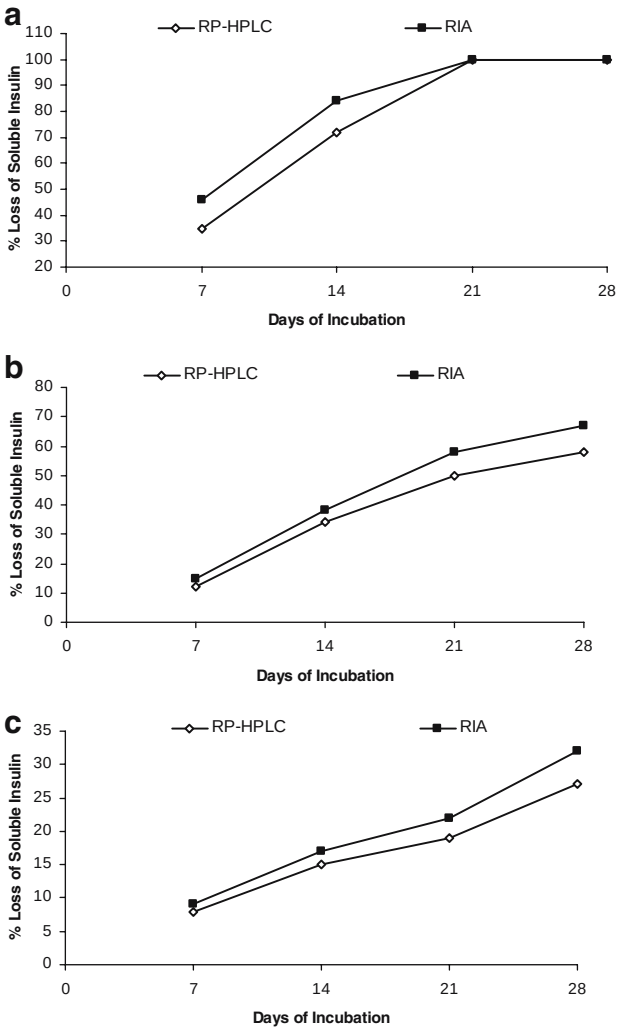


Fig. 9. Comparison of recovered soluble insulin by radioimmunoassay (RIA) and reversed-phase HPLC, pH 5.5, insulin controls without any polymeric excipient (**a**), pH 5.5, insulin + F-127, weight ratio 1:2 (**b**), pH 5.5, insulin + PEG-polyHis, weight ratio 1:2, 'c' system (**c**).

DISCUSSION

Maintaining the native and biologically active, three-dimensional structure of insulin is critical to preserving the biopotency of an insulin formulation. The use of infusion delivery devices places a high degree of thermo-mechanical stress on the protein formulation to be delivered. This thermo-mechanical energy is imparted to insulin and can result in denaturation and subsequent aggregation of the protein, as well as protein precipitation. Sluzky *et al.* investigated the fundamental nature of the aggregation mechanism (27). Mechanical stresses and a presence of a hydrophobic surface mimicked the destabilizing conditions present in infusion pumps. Experimental observations were consistent with their model of monomer denaturation at hydrophobic surfaces followed by the formation of stable intermediate species which facilitated subsequent macro-aggregation. To assist in maintaining the biologically active structure of insulin, various excipients are generally added as stabilizers. It is hypothesized that a surfactant-stabilized insulin formulation gains greater physical stability against aggregation or fibril formation from interactions of an appropriate surfactant with a partially unfolded insulin monomer. Further, it is hypothesized that an appropriate surfactant prevents non-specific aggregation of insulin and correct refolding of the denatured portions of the molecule, thus further stabilizing the formulation against aggregation and fibril formation (19,28,29). Pluronics have been investigated as surfactants/additives in formulating aqueous solutions of proteins. It has been demonstrated that nonionic surfactants Pluronics can bind to exposed hydrophobic domains of lipid bilayers facilitating the repair of disrupted cell membranes (30,31). There is no general consensus in the literature about the use of a specific stabilizer as a gold standard. Many different Pluronic-kind stabilizers with different hydrophile-lipophile (HLB) values have been reported to exert variable stabilizing/destabilizing effects (11–14). Some stabilizers that have been investigated for insulin stabilization include Pluronic F-68 (PEO-PPO-PEO, solid, molecular weight 8,400), Pluronic(s) L-31, L-81, L-101, L-121 (PEO-PPO-PEO, molecular weights 1,060, 2,500, 3,610, 4,450 respectively) Pluronic-17R8 (PPO-PEO-PPO, solid, molecular weight 7,000), Pluronic-25R5 (PPO-PEO-PPO, paste, molecular weight 4,250), Genapol™ PF-10, PF-20, PF-40, PF-80 (PEO-PPO-PEO, molecular weights 1,950, 2,190, 2,920, 8,750 respectively). In absence of a general unifying theory, it is difficult to objectively select one stabilizer. Basically, Pluronics can function as artificial chaperones of hydrophobic surface denatured insulin. PPO segments of F-127 may interact with exposed hydrophobic domains while the PEO segments provide added steric protection against self-association of unfolded insulin.

Based on reports in the literature, a rational stabilization strategy for insulin should either prevent contacts between the protein and the solid surface, or prevent unfolding of the protein. Numerous studies reported on proteins-polyelectrolytes complex formation have been motivated by interest in enzyme stabilization and immobilization. Polyelectrolyte-enzyme interactions aim at decreasing the denaturation process of a native enzyme, protecting the enzyme from external stresses while at the same time protecting the catalytic activity of the active center. Several investigations have reported that a variety of water-soluble polymers stabilize the enzymatic activities of glucose dehydrogenase, alcohol dehydrogenase, β-galactosidase, and trypsin (32–35). It has been suggested that electrostatic interactions between the enzymes and the polymers play a dominant role in stabilization of enzyme activities and nonenzymatic proteins (36,37). Our hypothesis that reversible molecular shielding of insulin with PEG-polyHis by ionic complexation will protect insulin from deleterious conditions has been proved correct. PEG conjugated to the polyelectrolyte may help prevent insulin aggregation, interfacial interactions between solid container and the aqueous phase (38,39). PEG-polyHis reduces insulin aggregation in a non-complexing pH 7.4 ('b' system) pointing to the role played by PEG in modulating surface adsorption. PEG-polyHis/insulin formulations are superior in physical stability to the same insulin formulation with F-127. PEG-polyHis-stabilized insulin formulations at pH 5.5 possess superior physical stability to that of an F-127-stabilized insulin formulation, and therefore, can be characterized as providing an even more dramatic effect on insulin stabilization. Complexing insulin with PEG-polyHis at pH 5.5 ('c' system) reduced the aggregation of insulin most effectively compared to the blend system (pH 7.4). Complexation may induce the formation of PEG-polyHis cover over the insulin surface and prevent direct exposure of insulin to the surfaces. Results indicate the requirement for complexation for most effective protection against aggregation of insulin.

It can safely be suggested that recovered soluble insulin as estimated by RP-HPLC retained its immunoreactivity in a RIA assay. Retention of immunoreactivity of insulin is a good indicator or bioactivity. Similar results have been reported in very few studies comparing the RIA assay with UV spectrophotometry and RP-HPLC (11,40). Overall, the use of polymeric excipients proved effective in retention of higher structures of insulin.

Electrostatic and hydrophobic interactions are common factors in protein-polyelectrolyte complexation. Complexation is dependant on the charge properties of the protein and the polyelectrolyte. The complex formation is known mainly, but not exclusively to be caused by electrostatic interactions between the proteins and the polyelectrolytes. Hydrophobic interactions play a minimal role in this particular system. Protein surface charge is known to influence the complexation behavior. Mozhaev *et al.* have used concept to artificially introduce negative charges into α-chymotrypsin and showed additional activation and stabilization on complexation with polybrene (41). The present study is a proof-of-principle investigation into the utility of a novel protein stabilizer with possibly a different mechanism of stabilization. It must be mentioned that an absolute control over the molecular weight of polyHistidine component is a very tough task. Changing the molecular weight of PEG is a relatively simpler modification. A higher concentration of positive charges on polyHis may provide multipoint electrostatic interactions with a protein providing additional conformational rigidity and provide supplementary stabilization against stresses. The multipoint electrostatic interactions may become stronger on increasing polyHis' molecular weight. Moreover, addition of lysine into PEG-polyHis chain to form modified copolymer may have stronger binding than PEG-polyHis alone. Binding between insulin and the polyelectrolyte may

be enhanced by hydrophobic interactions (42). Bromberg synthesized a special class of hydrophobically modified polyelectrolytes wherein poly(acrylic acid) was conjugated with Pluronic F-127 surfactant. The presence of Pluronic-PAA did not induce any significant structural change in insulin (43). It is interesting to note that both Pluronic-PAA and insulin had a negative net charge. Thus the formation of polyelectrolyte complexes can be ruled out, but hydrophobic interactions must be present. This study points to the role of hydrophobic interactions between synthetic polymers and insulin. Therefore, the electrostatic interactions between insulin and the modified copolymer can be strengthened through hydrophobic interactions.

CONCLUSION

A lower amount of insulin aggregates on complexation with PEG-polyHis. These aggregates are primarily caused by the presence of the agitation. To retain insulin stability by forming ionic complexes between oppositely charged protein and the copolymer, PEG-polyHis was synthesized. The complexation of anionic insulin was tested by examining the size and surface charge of complexes and by investigating retention of insulin's conformational stability. The maintenance of physical stability of insulin by ionic complexation is strong evidence supporting the hypothesis that addition of a multifunctional PEG-polyHis can be a beneficial strategy.

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