Conformational Stability of Lyophilized PEGylated Proteins in a Phase-Separating System

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Abstract □ PEGylation of proteins is of great interest to the pharmaceutical industry as covalent attachment of poly(ethylene glycol) (PEG) molecules can increase protein sera half-lives and reduce antigenicity. Not surprisingly, PEGylation significantly alters the surface characteristics of a protein, and consequently, its conformational stability during freezing and drying. Freeze concentration-induced phase separation between excipients has been previously shown to cause degradation of the secondary structure in lyophilized hemoglobin. In this report we show how PEGylation of two proteins, hemoglobin- and brain-derived neurotrophic factor (BDNF), influences partitioning and protein secondary structure as determined by FTIR spectroscopy in a system prone to freezing-induced phase separation. PEGylation of hemoglobin reduces the loss of structure induced by lyophilization in a PEG/dextran system that phase separates during freezing, perhaps due to altered partitioning. The partition coefficient for native hemoglobin favors the dextran-rich phase (PEG/dextran partition coefficient = 0.3), while PEGylated hemoglobin favors the PEG phase (partition coefficient = 3.1). In addition, we demonstrate that PEGylation alters hemoglobin's stability during lyophilization in the absence of other excipients. In contrast, because native BDNF already partitions into the PEG-rich phase, PEGylation of BDNF has a less dramatic effect on both partition coefficients and conformational stability during lyophilization. This is the first report on the effects of PEGylation on protein structural stability during lyophilization and points out the need to consider modification of formulations in response to changing protein surface characteristics.

Introduction

The covalent attachment of poly(ethylene glycol) (PEG) to enzymes and proteins,[1] a process known as PEGylation, has received recent increased attention. PEGylation has created enzyme derivatives with improved solubility in organic solutes, making the process of interest to the field of nonaqueous enzymology.[2,3] Attachment to PEG has been used as a method of immobilizing enzymes.[4] Of greater interest to the pharmaceutical biotechnology arena, PEGylated proteins have demonstrated dramatically increased plasma half-lives and very low immunogenicity.[5] It is thought that PEGylation provides a biocompatible "protective coating" to proteins, reducing immune response. It also increases the effective molecular weight of the protein and thus reduces the rate of clearance through the kidney. These are both extremely attractive properties for proteins administered as therapeutic agents since clearance of such drugs from the bloodstream is often too rapid for a therapeutic response. In addition, PEGylated proteins generally remain biologically active. Reports of numerous PEGylated proteins exist in the literature, including superoxide dismutase,[6] horse cytochrome *c*,[7] hemoglobin,[8−10] recombinant human granulocyte-colony stimulating factor,[11] human growth hormone and growth hormone antagonist,[12] and brain-derived neurotrophic factor,[13] just to name a few. With the original patent covering PEGylation[1] expiring, many biotechnology companies are now considering PEGylated versions of protein products.

PEGylation affects a protein's immunogenicity and circulation half-life in part by altering the protein's surface characteristics. Clearly, PEGylation should also significantly alter a protein's partitioning in a PEG/dextran two-phase system. Similarly, we can expect that PEGylation may change the protein's storage stability and behavior in a given formulation. Considering PEG's incompatibility with a number of polymers and salts in aqueous solutions, phase separation of a PEGylated protein from other formulation components may be a potential instability pathway in lyophilization formulations.

Partitioning of proteins in an aqueous two-phase system depends on specific surface features of the protein, along with system conditions such as total composition, pH, ionic strength, etc. Numerous approaches to altering the partitioning behavior of a protein have been considered for improving selectivity of two-phase separation methods. These include immunoaffinity partitioning[14] and metal affinity partitioning,[15,16] to name a few. Metal affinity partitioning involves partially chelating a transition metal ion to a linear polymer such as poly(ethylene glycol) (PEG). Transition metals demonstrate strong affinity for electron-rich amino acid residues such as histidine and cysteine. Thus, the use of PEG-bound metal chelates in PEG/dextran two-phase systems can dramatically increase the partitioning of proteins with accessible surface histidines or cysteines to the PEG phase.

In this paper, we investigate the stability of PEGylated hemoglobin and BDNF lyophilized in PEG/dextran formulations. By altering the partitioning of protein in a PEG/dextran two-phase system through PEGylation, we provide additional information on the mechanisms responsible for protein damage during lyophilization. To our knowledge, this is also the first report on the conformational stability of PEGylated therapeutic proteins during freeze-drying and as such offers an introduction to possible concerns in formulating such products. We also consider a noncovalent attachment of PEG to hemoglobin via chelation using a PEG-copper complex.

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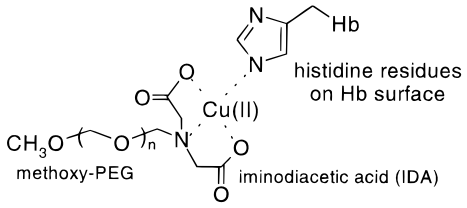


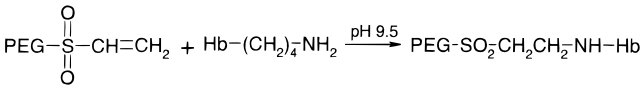
Figure 1—Structural representation of Cu(II)IDA-PEG. Copper partially coordinated with the IDA-modified PEG has a strong affinity for electron-rich surface amino acids. Adapted from Guinn.[16]

Materials and Methods

Materials—Recombinant human hemoglobin (Hb) was provided by Somatogen, Inc. (Boulder, CO). Poly(ethylene glycol) 3350 (PEG), average molecular weight 3350, was obtained from Sigma Chemical Co. and dextran T500, weight average molecular weight 519000, was obtained from Pharmacia Biotech. Polymers were dissolved in a stock solution at 20% (w/w) and combined to obtain solutions of 4% (w/w) PEG, 4% (w/w) dextran, and 10 mg/mL Hb (or PEG-Hb) with 5 mM potasssium phosphate buffered at pH 7.4 and 150 mM NaCl (or KCl). Cu(II)IDA-PEG 5000, a copper-chelated PEG derivative (average PEG molecular weight = 5000), was synthesized by Martin Guinn (lot no. MRG 5-105).[16] A structural representation of Cu(II)IDA-PEG is shown in Figure 1. For closer comparison to the 5000 molecular weight copper-chelated PEG, hemoglobin was also lyophilized in a solution of PEG 4600 (Aldrich) and dextran T500.

Human brain-derived neurotrophic factor (BDNF) and PEG-BDNF were supplied by AMGEN, Inc. (Thousand Oaks, CA). BDNF is a noncovalent homodimer with an approximate molecular weight of 27.8 kDa.[17] PEG-BDNF contains a PEG with average molecular weight of 20 kDa covalently attached to the N-terminal methionine of each BDNF monomer unit.[18] BDNF was received in 10 mM sodium phosphate/150 mM NaCl (pH 7.0). PEG-BDNF was received in 10 mM histidine/150 mM NaCl (pH 7.5), and transferred by dialysis to 10 mM sodium phosphate/150 mM NaCl (pH 7.0). All BDNF lyophilization solutions were made in this same sodium phosphate/NaCl buffer at a protein concentration of 1 mg/mL. It should be noted that, as defined in this report, mass concentrations of PEGylated proteins represent only the protein portion of the molecule. For example, a 1 mg/mL solution of BDNF and PEG-BDNF contains the same amount of protein.

PEGylation of Hemoglobin—Hb was PEGylated using a PEG–vinyl sulfone (molecular weight 5000)-activated PEG from Shearwater Polymers, Inc. (Huntsville, AL). At high pH (9–9.5), PEG–vinyl sulfone (PEG-VS) will react slowly with lysine groups:[19]



While more selective coupling with sulfhydryl groups occurs at lower pH (7–9), reaction with hemoglobin at lower pH was unsuccessful.

Hemoglobin in 5 mM potassium phosphate/150 mM NaCl was adjusted to pH 9.5 by careful addition of NaOH solution and a 2.4× molar excess of dry PEG-VS was dissolved in the solution. The reaction mixture was allowed to sit at 4 °C overnight. Half of the solution was dialyzed against 5 mM potassium phosphate/150 mM NaCl (pH 7.4) while the other half was dialyzed against 5 mM potassium phosphate/150 mM KCl (pH 7.4). Confirmation of PEGylation and fractionation of PEGylated and non-PEGylated hemoglobin was performed by FPLC size exclusion chromatography on a HiPrep 16/60 column containing Sephacryl S-200 resin (Pharmacia) with an elution buffer of 5 mM phosphate (pH 7.4)/150 mM NaCl at a flow rate of 0.7 mL/min. UV absorbance at 280 nm was used for detection. Hb and PEG-Hb concentrations were measured with a BCA total protein assay (Pierce).

Two-Phase Partitioning—The partitioning coefficient of each protein (concentration in the PEG phase divided by concentration in the dextran phase) was measured in a system of 7% PEG 3350/7% dextran/1 mg/mL protein. This system separates at room temperature. Samples were allowed to come to equilibrium over several hours, and the two phases were physically separated. Protein concentration in each phase was determined by UV absorbance at 280 nm for BDNF and PEG-BDNF and with a BCA total protein assay for Hb and PEG-Hb.

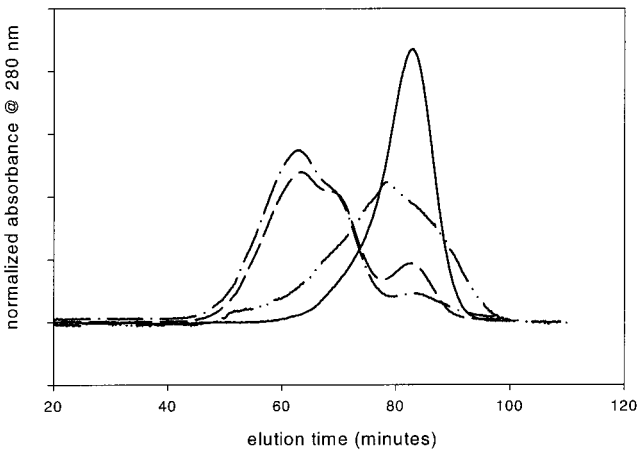


Figure 2—Normalized size exclusion chromatogram demonstrating PEGylation of hemoglobin. Solid trace: Hb; dashed trace: after PEGylation reaction; dash–dot trace: PEG-rich phase of 7% PEG/7% dextran/10 mg/mL PEG-Hb; dash–dot–dot trace: dextran-rich phase of 7% PEG/7% dextran/10 mg/mL PEG-Hb. All curves are normalized to the total area under peaks.

Lyophilization—Lyophilization was conducted in an FTS Systems microprocessor controlled tray dryer (Dura-Stop, Dura-Dry-MP). Samples were freeze-dried by the method previously described.[20] In short, samples for annealing experiments were quench frozen in a dry ice/acetone slurry and placed on the freeze-dryer shelf precooled to −7 °C. Samples were allowed to anneal at −7 °C for different lengths of time prior to drying. At "time zero", the samples were cooled to below −30 °C and held for 120 min. Primary drying proceeded at 60 mT vacuum with the shelf temperature set at −20 °C for 1000 min. Secondary drying consisted of 120 min at 0, 10, and 25 °C, while maintaining vacuum of 60 mT.

Infrared Spectroscopy—Protein secondary structure was monitored using Fourier transform infrared spectroscopy (FTIR). Spectral collection and processing were as previously described.[20,21]

IR Curve Fitting—Curve fitting of the second derivative IR spectra was used to determine the relative secondary structural content of BDNF in solution.[22,23] Normalized second derivative spectra were inverted by multiplying by −1 and then fitted with Gaussian band profiles using the Grams/386 (Galactic Enterprises) fitting routine. Component bands were assigned to secondary structural elements based on wavenumber according to the method of Dong and Caughey.[24]

Scanning Electron Microscopy—Lyophilized cake structures were examined using an ISI–SX-30 scanning electron microscope operating at an acceleration voltage of 30 kV. Dried samples were adhered with a graphite adhesive to SEM stubs and gold sputter coated.

Results and Discussion

Characterization of PEGylated rHb—PEGylation obviously increases the size and molecular weight of a protein; thus, size exclusion chromatography (SEC) can be used to evaluate the PEGylation reaction and offer some information on the extent of PEGylation. Figure 2 shows representative chromatograms of Hb before and after PEGylation. Clearly, PEGylation causes a gross shift to reduced elution times (and hence larger molecular weights), though it is also apparent that the entire Hb population was not PEGylated. Fractionation of PEG-Hb from non-PEGylated Hb was made between peaks at an elution time of approximately 75 min in Figure 2. A minimum of two peaks is identifiable in the PEGylated population, indicating that different degrees of PEGylation exist in the sample. Since PEG molecules (and therefore PEGylated

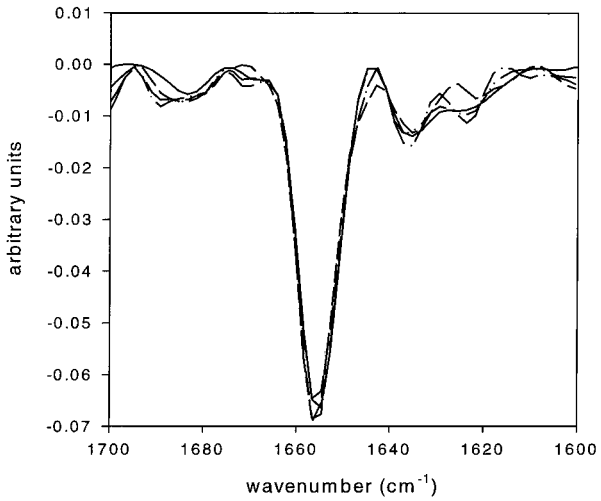


Figure 3—Second derivative IR spectra of hemoglobin controls in solution. Solid trace: Hb in buffer (pH 7.4); dashed trace: Hb in top phase of a 7% Cu(II)IDA-PEG/7% dextran system; dash–dot trace: Hb at PEGylation reaction conditions (pH 9.5); dash–dot–dot trace: PEG-Hb in buffer (pH 7.4).

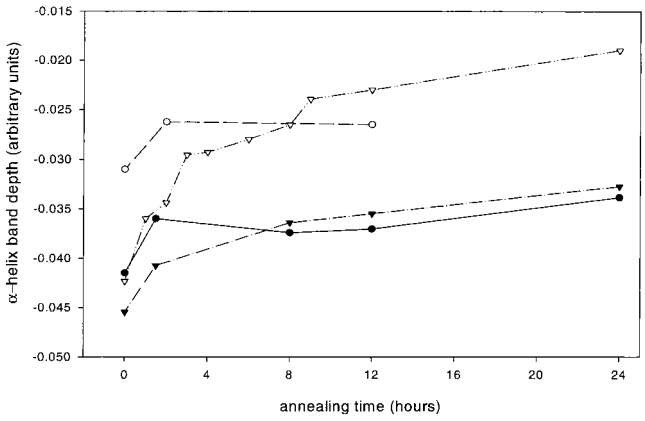


Figure 4—Effect of PEGylation on hemoglobin structure annealed in the PEG/dextran system. Depth of the α-helix band (ca. 1656 cm^{-1}) from second derivative infrared spectra of lyophilized Hb (open symbols) and PEG-Hb (filled symbols) as a function of sample annealing time at −7 °C prior to drying. Circles are for 10 mg/mL protein in 5 mM potassium phosphate/150 mM NaCl (pH 7.4). Triangles also contained 4% PEG 3350/4% dextran T500. Open triangles presented previously.[20,21]

proteins) "act" much larger in gel chromatography (as well as gel electrophoresis) than do proteins used for column calibration, it is difficult to assign quantitative information to the elution peaks corresponding to PEGylated proteins in Figure 2. However, based on the initial PEG-VS:HB stoichiometry and on SEC performed at different reaction times, the three main peaks observed by SEC can be tentatively assigned to un-PEGylated-, mono-PEGylated-, and di-PEGylated-Hb. PEGylation has no effect on the secondary structure of hemoglobin as seen by FTIR spectroscopy (Figure 3). However, partitioning of hemoglobin between the two phases of a system of 7% PEG 3350 and 7% dextran T500 is drastically effected by PEGylation. Hemoglobin partitions preferentially to the dextran phase with a partition coefficient (PEG phase/dextran phase) of about 0.3. PEGylated hemoglobin on the other hand has a partition coefficient of 3.1. In other words, while only about 30% of Hb is in the PEG phase, over 80% of the PEG-Hb sample is in the PEG phase.

Lyophilization of PEG-Hb—The formation of pure ice during freezing results in concentration of solutes. In protein formulations containing excipients, such concentration can cause solute−solute phase separation to become thermodynamically favored, albeit often with slow kinetics.[20,21,25] Annealing frozen solutions at subzero temperatures allows phase separations to proceed toward equilibrium and serves to magnify effects of such separations on protein stability. Annealing experiments that mimic those in the previous report[20] were conducted on PEG-Hb samples. In brief, samples were quench frozen and allowed to anneal for various lengths of time at −7 °C before freeze drying. After freeze-drying, hemoglobin structure in the dried solid was analyzed by IR spectroscopy as a function of annealing time. Figure 4 shows the depth of the α-helix band (ca. 1656 cm^{-1}) from second derivative IR spectra as a function of annealing time. The depth of the α-helix band is the primary indicator of "nativelike" structure in these hemoglobin studies (more negative values correspond to greater retention of nativelike structure; native hemoglobin has a α-helix band depth of ∼ −0.07 as can be seen in Figure 3). The first thing apparent in the data in Figure 4 is that PEG-Hb lyophilized in buffer alone retains more α-helix in the dried state than does Hb. This raises important questions regarding the possibility of covalently attached PEG acting as a stabilizer during freeze-drying. PEG is known to be an excellent protein stabilizer in frozen solutions.[26] However, studies in the literature have demonstrated the failure of PEG as a desiccoprotectant.[27] This is attributed to the tendency of PEG to crystallize during freeze-drying, making it unavailable to hydrogen bond with the protein[27] or form a "single phase glass".[25,28] It is likely, however, that covalent attachment to a protein surface will prevent, or at least greatly decrease, PEG crystallization. Whether one or two PEG molecules per protein would be sufficient to replace hydrogen bonds or form a protective glass is unknown.

Hb and PEG-Hb freeze-dried in 4% PEG/4% dextran also differ significantly in their native structural retention as seen in Figure 4. PEGylation appears to reduce the loss of nativelike structure with annealing. A number of explanations are possible. It may be that the added protection provided by PEGylation is also effective in reducing the damage in the phase-separating system. PEGylation also alters the surface properties of the protein, perhaps making it less active at the liquid−liquid interface created by the PEG/dextran phase separation. Since concentration and denaturation at this interface is a probable mechanism of damage,[20] removal of the protein from the interface by PEGylation could prevent this damage. Finally, if the loss of native structure of Hb with annealing is due to partitioning away from a stabilizer (PEG in this case), then reversing this partitioning would dampen the effect. Although dextran can form protective glasses and potentially replace some hydrogen bonds, its bulky size could make it less effective than PEG as a dessicoprotectant. This explanation seems unlikely, however, since Hb lyophilized in either 8% PEG or 8% dextran shows essentially equivalent levels of structural retention (data not shown).

PEG-Hb was also lyophilized in a 4% PEG/4% dextran solution with NaCl replaced with KCl. The results with PEG-Hb are the same as those seen with Hb: essentially no change in α-helix content occurs with annealing (Figure 5). We have previously shown[20] that switching to KCl prevents the phase separation from occurring. This is thought to be due to kinetic prevention of the PEG/dextran phase separation by the formation of a strong glass. Figure 5 also contains an anomalous result where an abrupt loss in structure occurs after 8 h of annealing. It is thought that this is the result of the thermodynamically unstable yet kinetically hindered phase separation relaxing sporadically. A corresponding radical change in cake structure is also seen in scanning electron microscopy images (not shown). The initiation of this relaxation is unknown. This

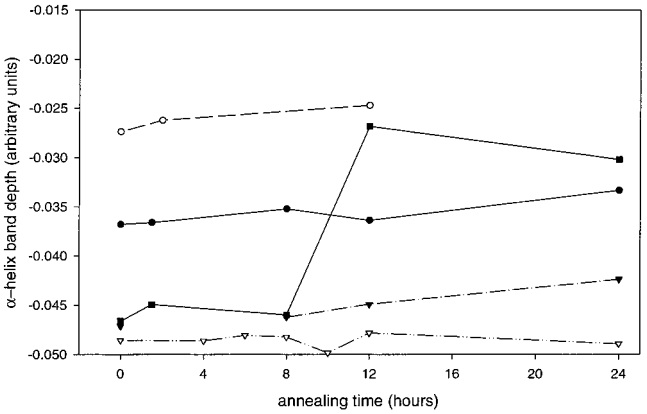


Figure 5—PEG-Hb lyophilized in KCl buffer. Depth of the α-helix band (ca. 1656 cm^{-1}) from second derivative infrared spectra of lyophilized Hb (open symbols) and PEG-Hb (filled symbols). Circles are for 10 mg/mL protein in 5 mM potassium phosphate/150 mM KCl (pH 7.4). Triangles also contain 4% PEG 3350/4% dextran T500. The square data points represent an anomalous example of a "relaxation" of the kinetically hindered phase separation (solutions same a triangles). Annealing is at –7 °C prior to drying. Open triangles presented previously.[20,21]

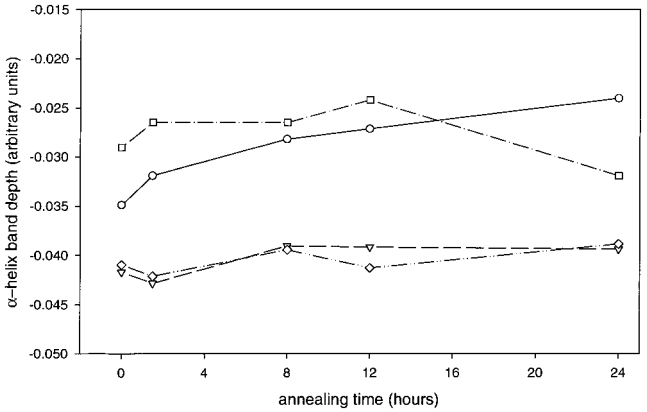


Figure 6—Hb lyophilized in Cu(II)IDA-PEG solutions. Circles, Hb in 3% PEG 4600/3% dextran T500; squares, Hb in 3% Cu(II)IDA-PEG; triangles, Hb in 3% Cu(II)IDA-PEG/3% dextran. All solutions contain 5 mM potassium phosphate/150 mM NaCl (pH 7.4).

is the only observed example of such sporadic behavior in kinetically hindered samples.

Lyophilization of Hb with Cu(II)IDA-PEG—Solutions of 4% Cu(II)IDA-PEG and 4% dextran T500 containing 10 mg/mL Hb form two phases at 0 °C. Thus, polymer concentrations were reduced to 3% Cu(II)IDA-PEG/3% dextran in freeze-drying studies to ensure single-phase solutions prior to freezing. Hb in a solution of 7% Cu(II)-IDA-PEG/7% dextran is entirely (within limits of detection) in the PEG-rich phase. While considerable copper-catalyzed oxidation of hemoglobin occurs in solutions of Cu-(II)IDA-PEG,[16] this is not a concern for the present study. Hb secondary structure is not affected by the presence of even high concentrations of Cu(II)IDA-PEG, as seen in Figure 3.

IR spectroscopy of Hb lyophilized in Cu(II)IDA-PEG-containing samples is summarized in Figure 6. There is not a significant change in α-helix content with annealing time in the system of 3% Cu(II)IDA-PEG/3% dextran. As in the case with PEG-Hb, the use of a metal affinity partitioning agent greatly increases the partitioning of hemoglobin to the PEG phase. The increased partitioning is even greater with the use of the metal-chelating ligand: essentially all of the hemoglobin is moved to the PEG phase at equilibrium. This increase in partitioning is larger than that caused by the covalent attachment of PEG-VS. This

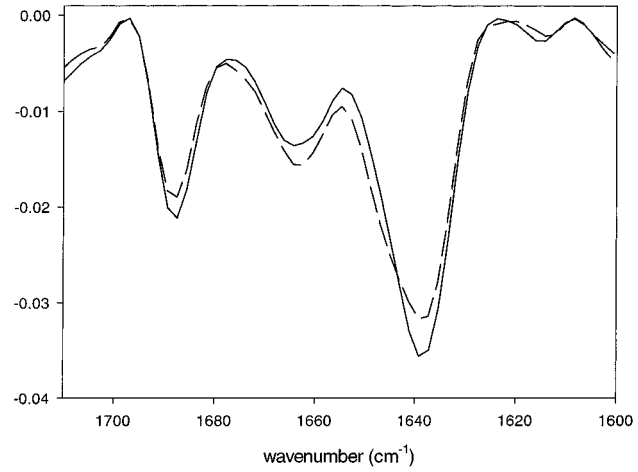


Figure 7—Amide I region of second derivative IR spectra of BDNF (solid trace) and PEG-BDNF (dashed trace) in solution (10 mM sodium phosphate (pH 7.0)/150 mM NaCl). Protein concentrations were >20 mg/mL.

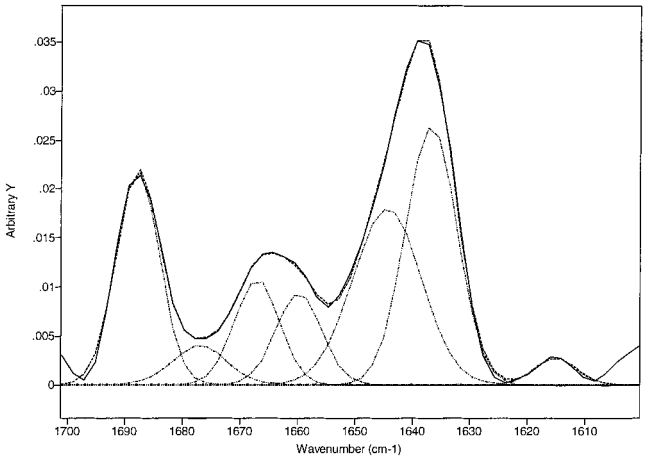


Figure 8—Curve-fitted inverted second derivative spectrum of BDNF in solution. Areas of fitted peaks are summarized in Table 1.

can be understood in terms of relative PEG attachment densities. Hb has 16 sites available for PEG attachment via Cu(II)IDA-PEG.[16] Although it is unlikely for steric reasons that all of the potential sites could ever be filled, there is almost certainly more PEG attached to Hb via Cu-(II)IDA-PEG chelation than by covalent attachment with PEG-VS. By increasing the affinity of the protein for the PEG phase, we are depleting the dextran phase of hemoglobin, but there is likely also a decreased concentration of protein at the liquid–liquid interface, due to the large difference in protein affinity of the two phases.

BDNF Solution Structure—FTIR spectroscopy of BDNF and PEG-BDNF in solution was first performed to gain information on the secondary structural content. Figure 7 shows the amide I region of second derivative IR spectra of BDNF and PEG-BDNF in 10 mM sodium phosphate/ 150 mM NaCl (pH 7.0). Quantitative analysis of BDNF secondary structure was obtained by curve fitting the second derivative spectra as seen in the example in Figure 8 and summarized in Table 1. This quantification is in fair agreement with reported structural assignments for BDNF (47% β-structure, 31% random coil, 22% reverse turns, no α-helix).[29] Table 1 shows that there is a slight increase in bands assignable to random structure and turns, and decreases in β sheet bands for the PEGylated protein, possibly indicating a less ordered structure.

Lyophilization of BDNF and PEG-BDNF—BDNF and PEG-BDNF were freeze-dried in the presence of 4%

Table 1—Band Positions, Relative Intensities, and Secondary Structural Assignment for the Amide I Region of Infrared Spectra of BDNF and PEG-BDNF

frequency (cm^{-1})	assignment	% area	
		BDNF	PEG-BDNF
1688	β-sheet	20.7	17.5
1677	turn	4.4	3.1
1667	turn	10.6	10.7
1660	turn	9.3	10.3
1644	random	26.9	35.1
1637	β-sheet	29.9	23.3

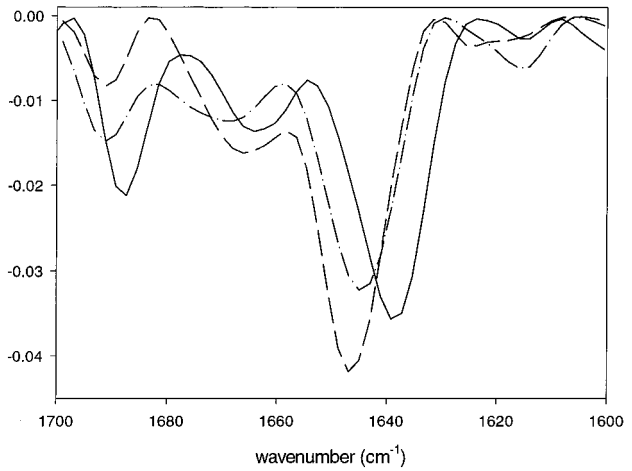


Figure 9—Second derivative IR spectra of BDNF (dashed trace) and PEG-BDNF (dash–dot trace) in the dried state, freeze-dried from buffer alone. The BDNF solution spectrum (solid trace) is shown for reference.

PEG/4% dextran, as well as in solutions containing only PEG or only dextran. Figure 9 shows the IR spectral changes seen with both BDNF and PEG-BDNF freeze-dried from buffer alone. In the dried state spectra, there is a considerable shift to higher wavenumbers for all of the structural bands. Frequency shifts in structural bands upon dehydration have been reported previously for β-sheet proteins.[23,30,31] Yet, it remains unresolved whether such a shift in band frequency reflects gross conformational changes in the protein upon dehydration or is simply due to a change in stretching vibration (and thus IR absorbance) in secondary structures due to the removal of hydrogen-bonded water. Allison et al.[30] report that shifts seen in the β-sheet bands upon lyophilization of chymotrypsinogen return to the frequencies of the solution state spectrum (before freeze drying) when the protein is freeze-dried in the presence of sucrose. This could be due to hydrogen bond replacement by the sugars.[32] In our studies with BDNF, all dried state spectra, regardless of excipient, have demonstrated the same shifting in resolved band frequencies. However, if the band frequencies and structural assignments for the solution spectra are fitted to the dried state spectra of BDNF, the result is a dramatic change in band area from the β-sheet band at 1637 cm^{-1} to the random structure band at 1644 cm^{-1} while maintaining the β-sheet band at 1688 cm^{-1}. This is an unlikely interpretation of the data: it seems unreasonable that one β-sheet vibrational mode would be nearly eliminated but not the other. Thus, for the sake of this study, we will interpret the shifting in band frequencies as a dehydration effect and not necessarily a secondary structural perturbation. In other words, the bands ca. 1645 and 1691 cm^{-1} in the dried solid will be considered to coincide with the 1637 and 1688 cm^{-1} β-sheet solution bands, respectively. The relative intensities of these bands, however, can be considered structural perturbations.

Table 2—Structural Retention of BDNF Freeze Dried in Various Excipient Combinations

excipient(s)	depth of band ca. 1690 cm^{-1} (arbitrary units)	β-sheet ratio[b]
solution (reference)	–0.0211	0.593
100 mM sucrose	–0.0174	0.544
8% PEG, 100 mM sucrose	–0.0149	0.682
8% PEG	–0.0145	0.442
8% PEG, 8 h[a]	–0.0132	0.411
8% PEG, 500 mM sucrose	–0.0118	0.304
4% PEG/4% dextran	–0.0101	0.285
500 mM sucrose	–0.0097	0.436
4% PEG/4% dextran, 8 h[a]	–0.0087	0.251
buffer alone	–0.0083	0.198
8% dextran, 500 mM sucrose	–0.0071	0.201
8% dextran, 100 mM sucrose	–0.0063	0.176
8% dextran, 8 h[a]	–0.0062	0.189
8% dextran	–0.0055	0.222

[a] Samples annealed for 8 h at –7 °C prior to drying (see Materials and Methods). [b] β-sheet ratio = (depth of band ca. 1690 cm^{-1})/(depth of band ca. 1640 cm^{-1}).

Table 3—Structural Retention of PEG-BDNF Freeze Dried in Various Excipient Combinations

excipient(s)	depth of band ca. 1690 cm^{-1} (arbitrary units)	β-sheet ratio[b]
solution (reference)	–0.0190	0.596
buffer alone	–0.0148	0.459
8% PEG	–0.0130	0.379
8% PEG, 100 mM sucrose	–0.0124	0.334
8% PEG, 8 h[a]	–0.0121	0.363
8% PEG, 500 mM sucrose	–0.0113	0.290
4% PEG/4% dextran, 8 h[a]	–0.0094	0.257
8% dextran, 8 h[a]	–0.0083	0.230
8% dextran, 500 mM sucrose	–0.0083	0.198
4% PEG/4% dextran	–0.0073	0.226
8% dextran, 100 mM sucrose	–0.0070	0.190
8% dextran	–0.0048	0.174

[a] Samples annealed for 8 h at –7 °C prior to drying (see materials and methods). [b] β-Sheet ratio = (depth of band ca. 1690 cm^{-1})/(depth of band ca. 1640 cm^{-1}).

The two β-sheet bands in BDNF presumably correspond to different types of β-sheet configurations. Monitoring the depth of the two β-sheet bands (as an indicator of band intensity) may be useful in resolving structural differences seen in different freeze-drying formulations. Table 2 reports the depth of the β-sheet band ca. 1690 cm^{-1} as well as the ratio of the band depth at 1640 cm^{-1} to that at 1690 cm^{-1} from second derivative IR spectra of BDNF in solution and freeze-dried in a number of formulations. Deviations in band depth from the solution spectrum are considered structural perturbations from the native structure. The formulations in Table 2 are ordered by the band depth at 1640 cm^{-1}. By this ordering, there is a clear segregation between samples containing PEG and those containing only dextran, with the PEG-containing samples retaining more nativelike structure. Unlike the hemoglobin results, little change in BDNF structure is seen with 8 h of annealing at −7 °C for the 4% PEG/4% dextran sample.

PEG-BDNF freeze-drying results are summarized in Table 3. While the order dictated by the β-sheet band at 1640 cm^{-1} is slightly different with PEG-BDNF than with BDNF, the general segregation between PEG and dextran samples remains. By this quantification, structural retention is better for PEG-BDNF lyophilized in buffer alone than in the presence of polymer. There may also be some

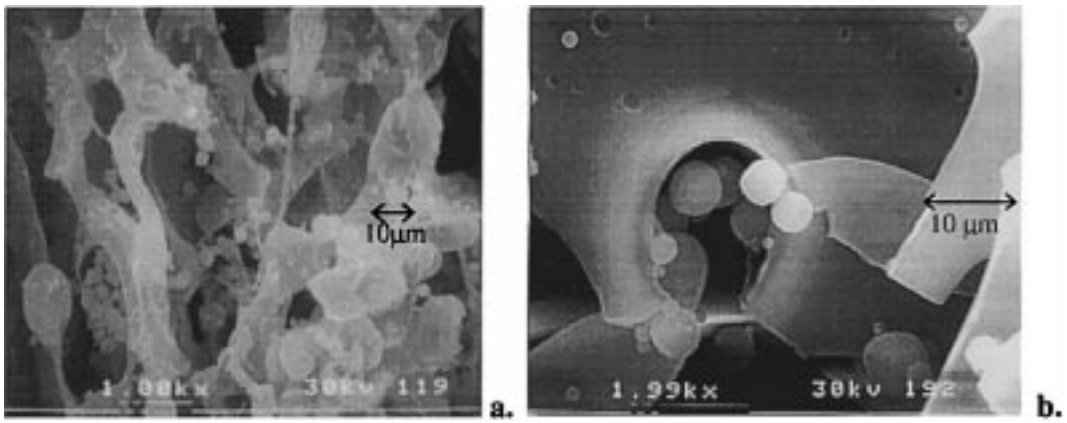


Figure 10—Scanning electron microscopy images showing potential polymer/protein phase separation. (a) BDNF freeze-dried in 8% dextran (1000× magnification) and (b) PEG-BDNF freeze-dried in 8% dextran (2000× magnification).

improvement in retention of secondary structure after annealing for PEGylated BDNF in the PEG/dextran system.

The detrimental effects of phase separation seen in hemoglobin studies (Figure 4) is not as apparent with BDNF or PEG-BDNF over the annealing times examined here, despite the fact that the PEG/dextran phase separation in the dried solid as seen by scanning electron microscopy looks similar (SEM not shown). The equilibrium partitioning of BDNF in a PEG/dextran two-phase system may be partly responsible for this result. While hemoglobin has a partition coefficient (concentration in the PEG phase divided by concentration in the dextran phase) of around 0.3 in a PEG/dextran two-phase system, BDNF's partition coefficient has been measured to be 2.4. This means that about 70% of hemoglobin partitions to the *dextran*-rich phase while about 78% of BDNF is in the *PEG*-rich phase. PEGylating hemoglobin causes the amount of protein in the PEG-rich phase to increase to over 80% while PEGylating BDNF causes the partition coefficient to increase to around 4.2 which means around 86% of the protein is in the PEG-rich phase. Thus, the change in partitioning as a result of PEGylation is considerably less for BDNF compared to hemoglobin. Therefore, unmodified BDNF may already receive the protective effect of a partitioning that favors the PEG-rich phase, namely removal from an interface and/or increased availability of PEG during drying. Alternatively, the differences in behavior between hemoglobin and BDNF may be intrinsic to the structural motifs of the two proteins. During lyophilization, secondary structural damage to hemoglobin is seen primarily as loss of α-helix. BDNF does not contain α-helical structure and therefore may not be sensitive to the same stresses.

Protein/Polymer Phase Separation—SEM images of the freeze-dried cake of BDNF and PEG-BDNF in dextran suggest a possible protein–polymer phase separation during lyophilization (Figure 10). A dispersed phase is present that is not typical of lyophilized dextran. Given BDNF's partitioning behavior in a PEG/dextran system, it may be reasonable to say that BDNF is more "PEG-like", which could lead to incompatibilities with dextran that result in a phase separation. Samples of PEG-BDNF-lyophilized in dextran show a similar separated phase. Phase separation in solutions of dextran and PEG-BDNF has been observed at room temperatures. Closer examination of BDNF structure when freeze-dried in dextran (Figure 11) suggests a structural change with annealing. The broad, flattened bands in the spectra of both BDNF and PEG-BDNF in dextran are typical of denatured proteins, representing a more disperse conformational population. These bands

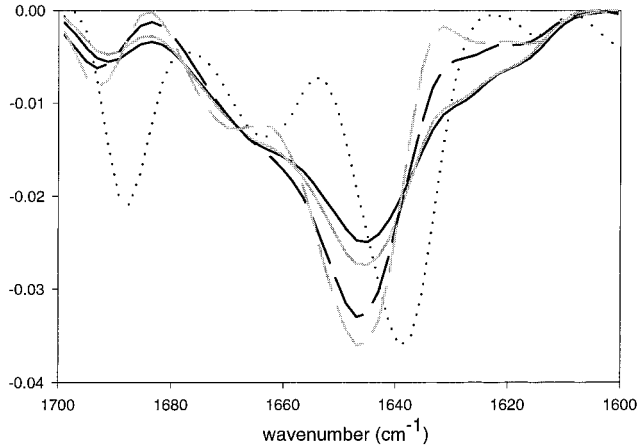


Figure 11—Second derivative IR spectra from BDNF (black lines) and PEG-BDNF (gray lines) lyophilized in 8% dextran. Broken lines were annealed at –7 °C for 8 h prior to drying while solid lines were not annealed. BDNF solution spectrum (dotted trace) is shown for reference.

narrow and deepen with annealing prior to drying, which could indicate added conformational stability. If a BDNF/dextran phase separation is occurring, more of the protein would be contained in a nondextran, high concentration protein phase with annealing. Protein self-association in a phase out of contact with dextran appears to provide BDNF with added stability in the dried state. This effect is greater with PEG-BDNF, emphasizing the greater separation driving force due to greater incompatibility with dextran.

Conclusions

Alteration of hemoglobin partitioning by PEGylation as well as by metal affinity ligands increases the structural integrity of the protein when lyophilized from a PEG/dextran system as well as from buffer alone. In contrast, both BDNF and PEGylated BDNF exhibit similar levels of damage during freeze-drying. This can in part be explained by the relative affinities of the various proteins and protein derivatives for PEG. Although PEG can be a potent stabilizing excipient, hemoglobin partitions away from a PEG-rich phase in PEG/dextran two-phase systems; this behavior is reversed upon PEGylation. On the other hand, unmodified BDNF favors the PEG-rich phase, and PEGylation only increases this tendency.

PEGylation of a protein can be expected to alter the protein's surface characteristics, and thus its interaction with other "surfaces", including interfaces and cosolutes.

Current theories of stabilizing mechanisms during lyophilization[27,28,33] all allude to the importance of compatibilities between the protein and a formulation's glass forming excipients. Addition of PEG to a protein surface changes the interactions between excipients and the protein. In this study, PEGylation actually improves hemoglobin structural retention in the dried state, and reduces BDNF structural loss during annealing in frozen solutions. However, caution is appropriate: therapeutic protein formulations optimized for the nonmodified protein may not generally provide adequate protection when formulating PEGylated derivatives.

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