On the Structural Preservation of Recombinant Human Growth Hormone in a Dried Film of a Synthetic Biodegradable Polymer

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Abstract □ In this work we describe the structural investigation of the model protein recombinant human growth hormone (rhGH) under conditions relevant to polymeric sustained-delivery depots, including the dried protein entrapped in a film of poly(DL-lactic-*co*-glycolic)acid. At each step of the procedure, dehydration of rhGH by lyophilization, suspension in methylene chloride, and drying from that suspension, the structure of rhGH was probed noninvasively using Fourier transform infrared (FTIR) spectroscopy. We found that the structure of rhGH was significantly changed by the dehydration process as indicated by a marked drop in the α-helix content and increase in the β-sheet content. Subsequent suspension of this powder in methylene chloride, drying from that suspension, and drying from a methylene chloride/PLGA solution introduced only minor additional structural changes when using appropriate conditions. This result is likely due to the limited molecular mobility of proteins in nonprotein-dissolving organic solvents. Finally, when rhGH was co-lyophilized with the lyoprotectant trehalose, which preserves the secondary structure, the rhGH entrapped in the PLGA matrix also had a nativelike secondary structure.

Introduction

Recent advances in biotechnology have facilitated the development of numerous biopharmaceuticals (peptides and proteins). The dozens of biopharmaceuticals already approved by the Federal Drug Administration (FDA),[1] in addition to hundreds of others in clinical trials,[2] will substantially aid in the prevention and management of disease.[1,3] However, drug delivery has been a major stumbling block. Because proteins are rapidly degraded in the gastrointestinal tract,[4] the oral route is impractical. Even when delivered by direct injection (e.g., insulin),[5] frequent administrations are often required. A promising approach is the sustained delivery of proteins from polymer matrixes,[6−8] which offers advantages such as targeting specific tissues and increasing patient compliance (and comfort). Poly(lactic-*co*-glycolic)acid (PLGA) is a good candidate for a protein encapsulation matrix.[9] PLGA, an FDA-approved material, has been intensively studied for its biocompatibility, toxicology, and degradation kinetics.[10−13] For this investigation we chose the model protein recombinant human growth hormone (rhGH) under a variety of conditions relevant to its encapsulation in PLGA as the model polymer.

Protein encapsulation in polymers is challenging because proteins are susceptible to deleterious chemical and structural modifications,[8,14−17] which may be induced by typical encapsulation procedures (e.g., the double-emulsion-solvent-evaporation-technique).[8,16] During such encapsulation procedures, protein structural perturbations may occur due to exposure to organic solvents,[18,19] mechanical stress,[20] creation of hydrophobic interfaces,[21] and dehydration.[22−25] Structural alterations caused by these factors may reverse upon release of protein from the polymeric device into the aqueous environment of body fluids. For example, lyophilization-induced structural changes are frequently reversible for small proteins.[25]

Even so, structural alteration is often the first step toward formation of irreversible aggregates when dehydrated proteins are exposed to moist environments in vitro, simulating the slow rehydration process occurring in a sustained-delivery device in vivo.[26−29] Dehydration-induced structural changes in rhGH are known to promote the formation of insoluble aggregates under accelerated storage conditions.[30] Aggregates may have lower activity (decreasing the efficiency of the expensive pharmaceutical protein) and increased immunogenicity, and thus must be avoided.[13] The simplest procedure to avoid such detrimental reactions would be to avoid any protein structural changes when processing. For rhGH, the addition of sugar excipients, such as trehalose, efficiently prevented storage-induced solid-state aggregation and conserved the protein structure upon lyophilization.[30]

It may be difficult to avoid such structural alterations when following the most common protocol of protein encapsulation in hydrophobic polymers, the double-emulsion-solvent-evaporation technique (also called the water-in-oil-in-water or w/o/w, technique).[16] In this approach, an aqueous protein solution is added to an organic solvent (typically methylene chloride) to form the first emulsion after probe sonication. Next, a second emulsion is formed by adding the first one to an aqueous solution containing an emulsifier followed by homogenization. Finally, microspheres are obtained after polymer hardening by solvent evaporation and dehydration of the particles, typically by lyophilization. A major difficulty is the exposure of the protein to denaturing conditions in aqueous solution. Under such conditions, proteins are relatively unstable and easy to denature, for example by organic solvents.[19] Structural investigations on the consequences of encapsulating the model proteins bovine serum albumin and lysozyme in PLGA microspheres by the w/o/w technique demonstrated substantial structural changes of both proteins.[31] Some of these changes could be prevented by adding the excipient trehalose to the aqueous protein solutions, but the proteins still were very much structurally perturbed in the micro-

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spheres, as probed by Fourier transform infrared (FTIR) spectroscopy.[31]

Recently, Johnson et al.[13] reported the successful stabilization of a Zn-protein complex in PLGA for rhGH using a novel cryogenic, nonaqueous methodology (no emulsification). The encapsulated protein was delivered from PLGA with unaltered properties compared with rhGH before the procedure, although the protein structure in the polymer was not reported.[13] Herein, we employ a different (more general) nonaqueous approach, taking advantage of the increased rigidity of dehydrated proteins in organic solvents[19] and employing a sugar excipient to stabilize the protein structure. We have specifically focused on the protein secondary structure under various conditions related to processing in PLGA.

Recent developments of FTIR spectroscopy make this technique the method of choice to noninvasively probe the protein structure under all relevant conditions. Encapsulation in polymers involves steps during which the protein is in different physical states. All these conditions can be analyzed using FTIR spectroscopy; several recent works have utilized this technique to characterize the secondary structure of proteins in the amorphous solid state,[22,24,25,29] in powder suspension in organic solvents,[32,33] and also encapsulated in PLGA.[31] Utilizing FTIR spectroscopy as a noninvasive spectroscopic method, we demonstrate that rhGH can be encapsulated in PLGA with a nativelike secondary structure.

Materials and Methods

Protein—Recombinant human growth hormone (rhGH) was produced at Genentech, Inc. (South San Francisco, CA). The protein bulk containing 2 mg/mL of rhGH, 88 mM mannitol, and 5 mM sodium phosphate (pH 7.8) was buffer-exchanged into a 100 mM ammonium bicarbonate solution and lyophilized to obtain excipient-free protein.[34]

Chemicals—Trehalose and poly(DL-lactic-*co*-glycolic)acid (50:50; MW 40 000−75 000; lot no. 56H1176) were obtained from Sigma Chemical Company (St. Louis, MO) and used as supplied. All other chemicals were of analytical grade and from various suppliers.

Preparation of Lyophilized Excipient/rhGH Samples—Lyophilized excipient-free rhGH was reconstituted with deionized water to form a stock solution containing 2 mg/mL of protein, and trehalose was added at the required protein-to-trehalose ratio of 1:250 (mol:mol). Samples were lyophilized in a Labconco Freezone 6 unit at a chamber pressure of <100 μmHg and a shelf temperature of −42 °C for 48 h. The lyophilized material was used immediately or was sealed in glass vials and stored at 2−8 °C until use.

FTIR Spectroscopy—FTIR studies were conducted with a Nicolet Magna-IR System 560 optical bench as described previously.[19,25,30] A total of 256 scans at 2 cm^{-1} resolution using Happ-Ganzel apodization were averaged to obtain each spectrum. For all experiments involving aqueous solutions, a Spectra Tech liquid cell equipped with CaF_2 windows and 15-μm thick spacers was used. For all experiments involving suspensions in organic solvents, the same liquid cell was used with a 50-μm spacer. Samples of rhGH were dried in poly(lactic-*co*-glycolic)acid by casting (vide infra) and measured as thin films on a CaF_2 window.

Lyophilized protein powders were measured as KBr pellets (1 mg of protein per 200 mg of KBr).[19,25,33,35] It is important to note that the influence of the KBr pellet method on the vibrational spectrum of rhGH has been tested before.[35] In the current study, we have been careful to employ a KBr pellet method that has been shown previously not to induce any artifactual structural changes (i.e., same result as obtained with an IR microscope). It has also been reported that the KBr pellet method does not introduce spectral changes for various other proteins as well.[22] In addition, for recombinant human deoxyribonuclease, the sole protein reported to exhibit structural alterations due to IR sample preparation in KBr,[36] a recent study revealed that under judiciously mild conditions there were no structural changes that could be attributed to pressing the protein in a KBr pellet.[37]

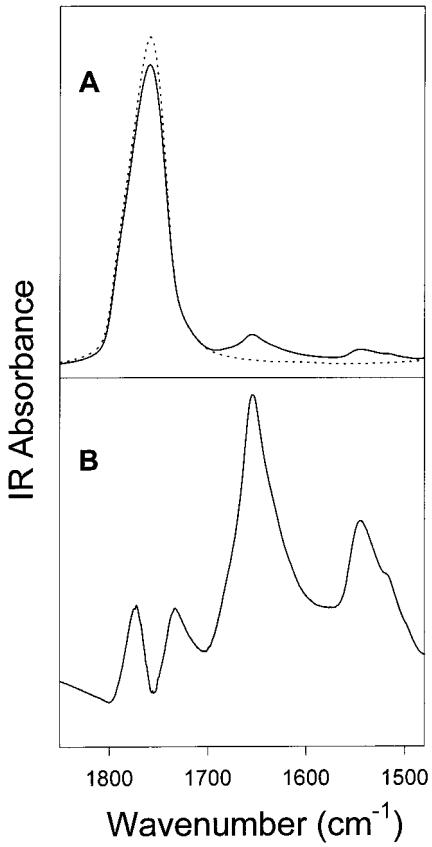

Figure 1—FTIR spectra of (A) rhGH encapsulated in PLGA (solid line) and of pure PLGA (dotted line) and (B) the rhGH spectrum after subtraction of the polymer background.

Each protein sample was measured at least four times. When necessary, spectra were corrected for the solvent background in an interactive manner using the Nicolet OMNIC 3.1 software[19,25,33] to obtain the protein vibrational spectra. We previously confirmed that this procedure is reliable for water background subtraction when using 15-μm thick spacers.[19]

RhGH was suspended in methylene chloride (25 mg protein/mL) using (1) a homogenizer (Virtishear Tempest 500W with an 18-mm shaft generator) for 2 min at the minimum speed setting of 1, (2) a probe sonicator (Branson Sonifier model 450) at 50 W at 50% duty cycle for 2 min, or (3) a sonication bath (Branson Sonication Bath model 2210) for 2 min. After suspension, the IR cell was immediately filled with the protein suspensions and the IR spectra were recorded. Encapsulation of lyophilized and co-lyophilized rhGH samples in poly(lactic-*co*-glycolic) acid (50:50) was carried out by suspending 10 mg of protein in a solution of 98 mg of PLGA in 1 mL of methylene chloride as already described. After suspension, samples were spread as thin films on a CaF_2 window and dried over a flow of dry N_2.

Spectra recorded for rhGH entrapped in PLGA were corrected for the polymer contribution. Although the polymer band with a maximum at ca. 1760 cm^{-1} is well separated from the amide I area (1700−1600 cm^{-1}), we were careful in excluding any artifactual influences of the subtraction procedure on the amide I spectrum. It can be seen from Figure 1A that the spectrum of PLGA was not identical in the absence and presence of the protein and could not be totally eliminated by the subtraction procedure. Thus, in principle, false subtraction could have led to the creation of some artifactual bands at ca. 1680−1700 cm^{-1}, where tailing of the peak into the amide I occurred. However, we were able to exclude such artifacts. To do so, the PLGA background was subtracted from lyophilized and co-lyophilized rhGH samples entrapped in PLGA employing different subtraction factors (e.g., 2.4133, 2.4737, and 2.5744 for the lyophilized protein and 0.9106, 0.9736, and 1.0456 for the co-lyophilizate), and no subtraction at all. For each of the spectra obtained, we calculated the secondary

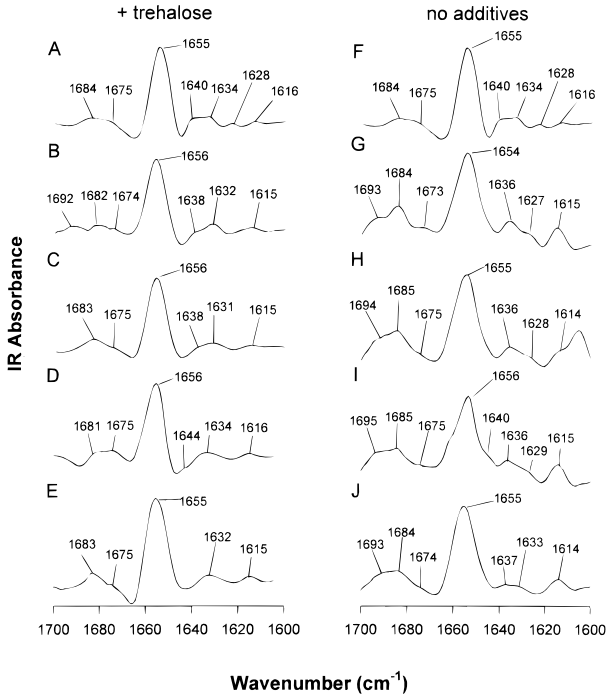

Figure 2—Second-derivative FTIR spectra of rhGH in the amide I region under various conditions. (A, F) rhGH in aqueous solution at pH 7.8; (B–E) rhGH co-lyophilized with trehalose at a 1:250 molar ratio from an aqueous solution at pH 7.8 under different conditions; [(B) co-lyophilized powder, (C) suspension in methylene chloride by homogenization, (D) dried powder after suspension in methylene chloride, and (E) entrapped in PLGA]; (G–J) rhGH powder lyophilized from pH 7.8 without additives under different conditions [(G) lyophilized powder, (H) suspension of the powder in methylene chloride by homogenization, (I) dried from suspension in methylene chloride, and (J) entrapped in PLGA].

structure by Gaussian curve-fitting. For the case of the lyophilized protein, the α-helix content determined was 39%, 35%, 37%, and 41% for four situations tested. For the case of the co-lyophilized protein, the α-helix content determined was 48%, 45%, 48%, and 46% for the four situations tested. The values were the same within the error of the method, excluding a significant influence of the PLGA background subtraction procedure on the amide I protein spectrum.

In addition, second-derivative spectra of rhGH entrapped in PLGA (Figure 2) were analyzed for any new components that could have been the result of the subtraction procedure. This analysis demonstrated no new components in these samples (Figures 2E and 2J) when compared with the spectrum of the lyophilized protein (Figures 2B and 2G). This result also excludes creation of artificial bands by the subtraction procedure.

FTIR Data Analysis—*Second Derivatization*—All spectra were analyzed by second derivatization in the amide I region for their component composition.[19,25,33,38,39] Second-derivative spectra were smoothed with an 11-point smoothing function (10.6 cm^{-1}).[19,25]

Fourier Self-Deconvolution (FSD)—FSD was applied to the unsmoothed spectra to enable quantification of the secondary structure in the amide I region by Gaussian curve-fitting[19,33,40,41] using the program OMNIC 3.1. The parameters chosen, a value of 24 for the full-width at half-maximum (fwhm) and $k = 2.4$ for the enhancement factor, are in the range of those published.[19,33,39,41–43] Note that FSD alters the band shapes but preserves the integrated band intensities when over-deconvolution is avoided.[38,42] The values chosen for FSD in our analyses were checked for the risk of such over-deconvolution (which could result in distorted band areas)[40,41] by the strategy outlined by Griebenow and Klibanov.[19]

Gaussian Curve-Fitting—The frequencies of the band centers found in the second-derivative spectra in the amide I region were used as starting parameters for the Gaussian curve-fitting (performed using the program GRAMS 386 from Galactic Industries, Inc.). The secondary structure contents were calculated from the areas of the individual assigned bands and their fraction of the total area in the amide I region.[19,33,39] Gaussian curve-fitting was performed in the amide I region after band-narrowing of the protein vibrational spectra by FSD.[39,41,43] In all cases, a linear baseline was fitted. The secondary structure content is reported as the averaged standard deviation of at least four measurements. To compare the structure of rhGH under different conditions, we performed a *t* test to determine within the confidence level of 95% whether the α-helix and β-sheet content were statistically significantly different. The null hypothesis used was that for both samples the values were the same within the error.

Band Assignments—The band assignment in the amide I region followed those in the literature[19,24,30,33,41] and is summarized for some typical samples in Table 1. For the aqueous solution, the main band at 1655 cm^{-1} was assigned to α-helices and a band at 1631 cm^{-1} to β-sheets. All other bands were assigned to other structural elements (β-turns, random coil, extended chains). The solution secondary structure content determined by Gaussian curve-fitting in the amide I region using these assignments was very similar to that determined previously [30,35] and to the X-ray crystal structure (60% α-helix).[44] The relatively high α-helix content of rhGH is advantageous when studying the influence of the encapsulation procedure on the structure of the protein under various conditions. For lyophilized or suspended proteins, the α-helix content is the most reliable criterion to describe their structural integrity when utilizing FTIR spectroscopy.[19,25,30]

Table 1—Infrared Band Positions, Areas, and Assignments for rhGH under Various Conditions[a]

	band position (cm^{-1})			
sample	SD[b]	Gaussian curve-fitting[c]	area (%)	assignment
aqueous solution (pH 7.8)	1684	1680	18 ± 3	other[d]
	1670	1670	7 ± 1	other
	1655	1655	57 ± 3	α-helix
	1641	1639	11 ± 3	other
	1628	1631	7 ± 3	β-sheet
co-lyophilized with trehalose[e]	1692	1693	5 ± 2	β-sheet
	1680	1681	9 ± 2	other
	1675	1671	15 ± 3	other
	1656	1656	52 ± 3	α-helix
	1639	1641	10 ± 1	other
	1632	1632	9 ± 2	β-sheet
co-lyophilizate entrapped in PLGA[e,f]	1683	1681	11 ± 1	other
	1674	1671	7 ± 1	other
	1656	1656	48 ± 1	α-helix
	1639	1642	23 ± 3	other
	1631	1630	11 ± 1	β-sheet

[a] Data are the average and standard deviation of four to five independent determinations. [b] Second derivative. [c] Gaussian curve-fitting was performed on Fourier self-deconvolved amide I spectra. [d] Other structures include random coil, extended chains, and turns. [e] rhGH co-lyophilized with 1:250 protein: trehalose (mol:mol) prior to encapsulation. [f] The rhGH:trehalose co-lyophilizate was homogenized in methylene chloride containing PLGA and the suspension was subsequently dried to entrap the protein.

Results and Discussion

Previously, we reported the use of FTIR spectroscopy as a tool to investigate the structural alteration of rhGH upon dehydration[35] and to develop strategies for solid-state stabilization.[30] Herein, we have further extended the use of FTIR spectroscopy to probe rhGH structure under a variety of conditions relevant to the encapsulation of the protein in sustained-release formulations.

The structural consequences of the different steps of a nonaqueous encapsulation procedure were probed. Lyophilization of rhGH in the absence of excipients induces significant structural changes, in particular a decrease in the α-helix and an increase in the β-sheet content.[30,35] Our FTIR spectroscopic data obtained for rhGH in aqueous solution at pH 7.8 (Figure 3A) and for the powder lyophilized from that pH value (Figure 3B) are in agreement

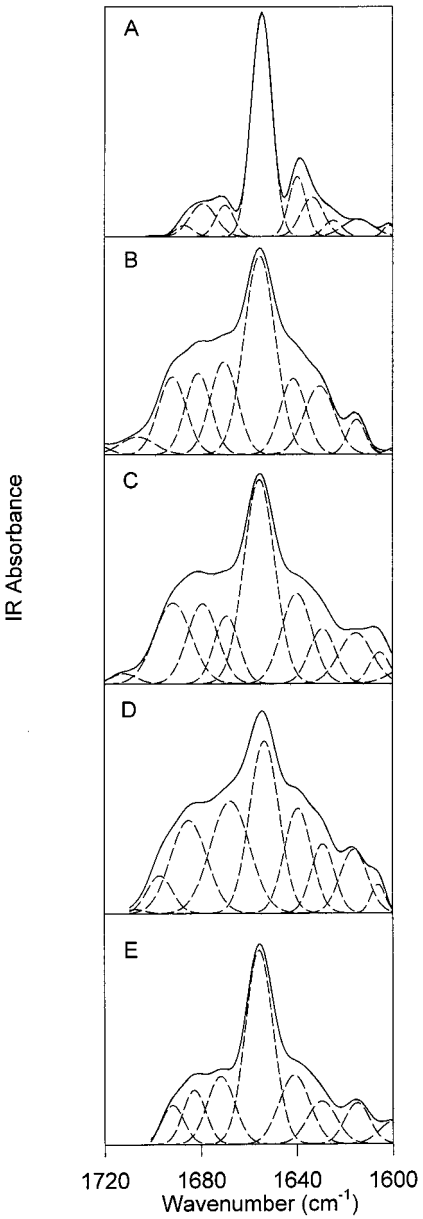

Figure 3—Fourier self-deconvolved FTIR spectra and Gaussian curve-fitting of rhGH without additives in the amide I spectral region under various conditions: (A) aqueous solution at pH 7.8, (B) lyophilized powder from that solution, (C) suspension of the powder in methylene chloride by homogenization, (D) dried from this suspension in methylene chloride, and (E) entrapped in PLGA. The solid lines represent the superimposed FSD and the curve-fit, and the dashed curves represent the individual Gaussian bands.

with these findings: the spectrum of rhGH in the amide I spectral region (1700−1600 cm^{-1}) showed pronounced changes. In particular, a broadening of the spectrum was observed for the lyophilized powder when compared with the aqueous spectrum. Such spectral changes are indicative of structural changes caused by the lyophilization process.[19,22,24,25,33,45] Quantitative analysis of the spectra by Gaussian curve-fitting (Table 2) revealed a statistically significant 20% decrease in the α-helix and 11% increase of the β-sheet content upon lyophilization, which is in agreement with results of previous investigations.[30,35]

When the excipient-free protein was suspended in methylene chloride using a homogenizer, the FTIR spectrum of this suspension (Figure 3C) was similar to the one of the lyophilized protein (Figure 3B). Quantitative analysis also revealed that there were no significant additional structural changes in the α-helix content and only a minor (but statistically significant) increase in the β-sheet content to 22% upon suspension of the protein in the organic solvent (Table 2).

Table 2—Secondary Structure of Excipient-Free rhGH under Various Conditions

sample	secondary structure content (%)[a] α-helix	β-sheet	other[b]
aqueous solution	57 ± 3	7 ± 3	36 ± 1
lyophilized powder	37 ± 4	18 ± 1	45 ± 3
powder suspension by probe sonication			
suspended in methylene chloride	23 ± 2	36 ± 2	41 ± 2
dried from methylene chloride	28 ± 3	33 ± 5	39 ± 4
powder suspension by homogenization			
suspended in methylene chloride	35 ± 2	22 ± 2	43 ± 2
dried from methylene chloride	29 ± 2	24 ± 1	47 ± 3
dried from methylene chloride/PLGA	39 ± 1	12 ± 3	49 ± 2

[a] The secondary structure of rhGH was calculated by Gaussian curve-fitting of the Fourier self-deconvolved amide I spectra. [b] Other structures include random coil, extended chains, and turns.

These data are in agreement with the hypothesis that dehydrated proteins have lower molecular mobility than in the aqueous state, which precludes them from being denatured by the organic solvent.[19,33] In contrast, another FTIR spectroscopic study found that suspension of the protein subtilisin in ethanol, hexane, and pyridine led to some structural rearrangements.[32] In that report, the exact details of the suspension conditions were not presented. Thus, to determine whether the suspension conditions used could affect the protein structure, we also suspended rhGH using probe sonication in methylene chloride. In this case, we found a significant additional reduction in the α-helix content to 23% and increase of the β-sheet content to 36% (Table 2). This additional structural instability may be related to the increased localized heating effect of suspensions achieved by probe sonication versus homogenization, which would promote increased molecular mobility.[46] Therefore, the means by which the suspension is obtained is important. FTIR spectroscopy may be useful as a tool to optimize the processing conditions with respect to structural preservation of pharmaceutical proteins.

We examined the effect of removing the organic solvent, which would be the next step in the preparation of an implantable or injectable depot. The spectra for rhGH suspended in methylene chloride with the homogenizer and of the dried powder obtained from that suspension (Figures 2C and 2D) were qualitatively quite similar. Quantitative analysis of the two spectra showed that some minor additional structural changes occurred upon drying of the suspension. There was a slight but statistically significant decrease in the α-helix content from 35% to 29% observed during this process, whereas the β-sheet content remained unchanged (Table 2).

A similar result was obtained when rhGH, suspended by probe sonication in methylene chloride, was dried from that solvent. The spectra were qualitatively very similar (data not shown) and the quantitative data (Table 2) were also similar. Thus, we conclude that removal of the organic solvent resulted in only minor additional structural alterations.

We also tested whether the presence of the polymer PLGA would have any influence on the structure of rhGH when following the same processing protocol. The protein was suspended in methylene chloride containing the polymer (98 mg/mL) using the homogenizer.[47] This suspension was directly applied to a FTIR window and a thin film produced by solvent evaporation under a stream of dry nitrogen gas. The structure of rhGH dried in the PLGA

film was essentially the same as in the lyophilized powder. The α-helix content was the same, but a small decrease in the β-sheet content was observed (Table 2). Thus, the structure of excipient-free rhGH dried in the presence of PLGA was not native. However, it was the freeze-drying process beforehand and not exposure to the organic solvent and/or PLGA that caused the structural perturbations.

Thus, to preserve the structure of rhGH in PLGA, the lyophilized formulation must first be optimized. It is well established from FTIR spectroscopic studies that lyophilization-induced structural changes can be prevented by addition of a co-lyophilized sugar or polyol.[22,24,25] For rhGH, several sugars proficient in stabilization against dehydration-induced structural changes (and also solid-state aggregation) have been identified; for example, trehalose.[30] Trehalose is an efficient lyoprotectant,[48] and has been shown to ameliorate the dehydration-induced structural alteration of various unrelated proteins, such as lysozyme[49,50] and bovine serum albumin.[51]

rhGH was co-lyophilized with trehalose at an excipient-to-protein ratio of 250:1 (mol:mol). At this ratio, the secondary structure of rhGH was largely preserved upon lyophilization. The second-derivative spectrum of the co-lyophilizate (Figure 2B) was very similar to that of the aqueous solution (Figure 2A). The main component was the band assigned to α-helices with a maximum at 1655 cm^{-1}. No significant changes in the frequencies or amplitudes of any component were noted in the second-derivative spectra. The quantitative analysis of the FTIR spectrum of the co-lyophilizate (Figure 4B) confirmed that the secondary structure was largely preserved by the lyoprotectant. The α-helix content for the sample was 52%, only slightly lower than in aqueous solution (57%, Table 3). An increase of 7% for the β-sheet was observed in the co-lyophilizate compared with the 11% increase for the excipient-free protein.

As the subsequent step, the rhGH:trehalose co-lyophilizate was suspended in methylene chloride using three different methods: probe sonication, homogenization, and a sonication bath. Probe sonication resulted in a statistically significant decrease in the α-helix content from 52% to 44%, indicating some pronounced structural changes by the method (Table 3). When we dried rhGH from this suspension, no further structural changes were noted (Table 3). These observations are in agreement with data for excipient-free protein as already discussed. Probe sonication in both cases caused some additional structural perturbation upon suspension in the organic solvent, and caution should be exercised when using this method.

A higher degree of structural preservation was achieved upon homogenization (Figures 2C and 4C) or suspension using the sonication bath (Table 3). The spectral changes upon suspension were very small in both cases. For example, the second-derivative spectrum of the rhGH:trehalose co-lyophilizate suspended by homogenization in methylene chloride (Figure 2C) was quite similar to that of the co-lyophilizate before suspension (Figure 2B). Quantitative analysis of the spectra by Gaussian curve-fitting did not indicate any statistically significant structural alterations upon suspension: the α-helix and β-sheet contents were, within the 95% confidence interval, the same as for the co-lyophilizate before suspension (Table 3).

The secondary structure was also determined for the protein in the co-lyophilizate dried from the organic solvent. Second-derivative spectra did not indicate any structural changes upon organic solvent removal. For the case of rhGH suspended by homogenization, the spectrum of the suspended protein and of the dried protein after suspension were very similar (Figures 2C and 2D). Furthermore, the second-derivative spectrum of the co-lyophilized protein

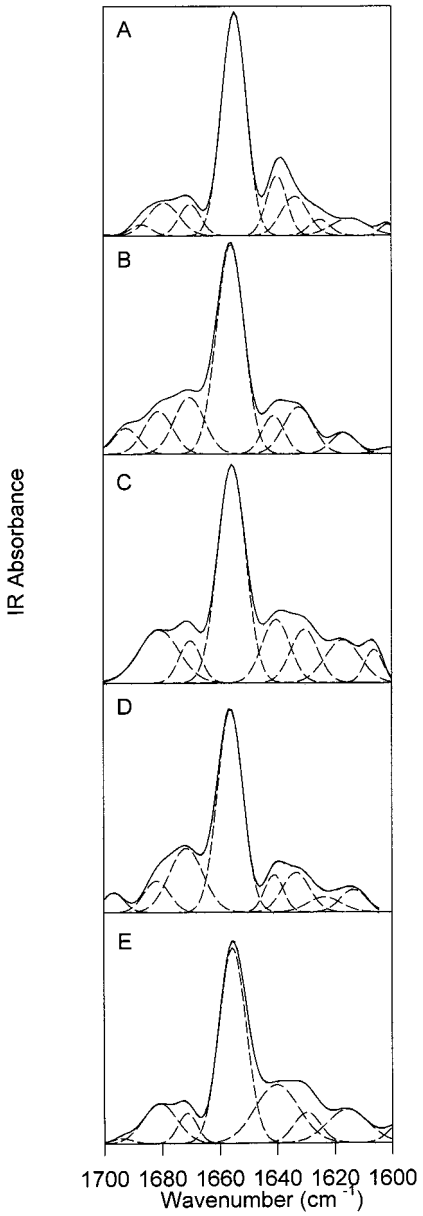

Figure 4—Fourier self-deconvolved FTIR spectra and Gaussian curve-fitting of rhGH in the amide I spectral region under various conditions: (A) aqueous solution at pH 7.8; (B) rhGH co-lyophilized with trehalose at pH 7.8; (C) suspension of the co-lyophilizate in methylene chloride by homogenization; (D) dried from this suspension in methylene chloride; and (E) entrapped in PLGA. The solid lines represent the superimposed FSD and the curve-fit, and the dashed curves represent the individual Gaussian bands.

dried after suspension in methylene chloride was comparable to that in aqueous solution.

Quantitative analysis of the spectra by Gaussian curve-fitting was in agreement with the qualitative observations (Figure 4D, Table 3). For the sample suspended by homogenization, the α-helix content (50%) and β-sheet content (13%) were the same, within the 95% confidence interval, as for the dried protein after suspension (48% and 14%, respectively). A slight but statistically significant decreases in α-helix (from 49% to 45%) and β-sheet content (from 16% to 13%) were noted for the sample suspended with the sonication bath upon drying (Table 3).

The trehalose-containing protein was then suspended in a solution of methylene chloride containing PLGA using the homogenizer and sonication bath because these methods were mild with respect to maintaining protein structure. The suspension was cast on a FTIR window and

Table 3—Secondary Structure of rhGH Co-Lyophilized with Trehalose under Various Conditions

sample	secondary structure content (%)[a]		
	α-helix	β-sheet	other[b]
aqueous solution[c]	57 ± 3	7 ± 3	36 ± 1
powder co-lyophilized with trehalose[d]	52 ± 3	14 ± 2	34 ± 1
powder suspension by probe sonication			
suspended in methylene chloride	44 ± 2	9 ± 2	47 ± 3
dried from methylene chloride	44 ± 1	12 ± 1	44 ± 3
powder suspension by homogenization			
suspended in methylene chloride	50 ± 2	13 ± 1	37 ± 1
dried from methylene chloride	48 ± 5	14 ± 3	38 ± 2
dried from methylene chloride/PLGA	48 ± 1	11 ± 1	41 ± 1
powder suspension by sonication bath			
suspended in methylene chloride	49 ± 2	16 ± 2	36 ± 3
dried from methylene chloride	45 ± 1	13 ± 2	42 ± 3
dried from methylene chloride/PLGA	51 ± 2	8 ± 3	41 ± 2

[a] The secondary structure of rhGH was calculated by Gaussian curve-fitting of the Fourier self-deconvolved amide I spectra. [b] Other structures include random coil, extended chains, and turns. [c] The aqueous solution did not contain trehalose. [d] The co-lyophilized powder was obtained using a protein-to-trehalose ratio of 1:250 (mol:mol).

evaporated under a stream of dry N_2 gas. The second-derivative spectra qualitatively revealed that the structure of rhGH was very similar to that in aqueous solution and to that of the co-lyophilizate. For example, for the sample obtained by homogenization, only minor differences were evident when comparing the spectrum of the aqueous solution (Figure 2A) and of the entrapped protein (Figure 2E). Quantitative data obtained by Gaussian curve-fitting were in agreement with this qualitative analysis. The spectra of rhGH co-lyophilized with trehalose dried in PLGA from a suspension that was homogenized (Figure 4E) or prepared using the sonication bath (data not shown) were similar to that of the aqueous solution (Figure 4A) and the co-lyophilizate (Figure 4B). Some minor differences were noted in both cases for bands representing unordered secondary structure (ca. 1640 cm^{-1}) and β-sheet structure (ca. 1631 cm^{-1}). Some changes in the area of these components indicate a minor structural rearrangement occurring under the different conditions.

For the protein co-lyophilized with trehalose and entrapped in PLGA, quantitative analysis of the spectra (Table 3) demonstrated that the processing conditions produced only very minor structural changes. The α-helix content of rhGH entrapped in PLGA by homogenization was 48% and thus only slightly lower than for the co-lyophilizate before encapsulation (52%). In addition, only small differences were found for the β-sheet content (11% and 14%, respectively). The same result was evident when employing the sonication bath. The α-helix content for rhGH entrapped in PLGA was 51%, the same (within the 95% confidence interval) as for the co-lyophilized powder (52%), and the β-sheet content was only slightly decreased (14% and 8%, respectively).

The data clearly demonstrate that a nonaqueous strategy can be employed to entrap rhGH in PLGA with a nativelike secondary structure. The key was to lyophilize the protein under conditions ensuring structural integrity and then employ processing conditions that are not detrimental to the structure. This finding is intriguing in light of the common wisdom that organic solvents, such as the one in our study, are known to denature proteins.[8] It should be noted that in the present study, following the lyophilization step, all processes were nonaqueous. A likely explanation is the proposed decreased molecular mobility of proteins in anhydrous organic milieu compared with the aqueous state.

Such increased rigidity of proteins in organic solvents was originally proposed as an explanation for kinetic observations when employing suspended dehydrated enzymes as catalysts in nonaqueous organic solvents. For instance, increased rigidity has been attributed to differences in the enzymatic activity in hydrophobic and hydrophilic organic solvents.[52−54] In addition, it has been hypothesized that the plunge in enzymatic activity when comparing aqueous and nonaqueous systems can in part be attributed to increased rigidity of enzymes in organic solvents.[54,55] Also, the phenomenon of so-called 'molecular imprinting' can only be explained when assuming an increased rigidity of enzymes in organic solvents.[56−58] Furthermore, proteins show an increased stability toward thermal denaturation in organic solvents.[59,60] A few spectroscopic investigations have directly addressed the issue of protein rigidity in organic solvents. Nuclear magnetic resonance experiments on the tyrosyl ring motion in α-lytic protease,[61] electron spin resonance spectroscopic studies,[62,63] time-resolved fluorescence anisotropy measurements,[64] and recently FTIR spectroscopic experiments[19] support the view that proteins have restricted mobility in organic solvents when compared with aqueous systems. Theoretical works support this picture,[65,66] and it has also been pointed out that water serves as a molecular lubricant and promotes extremely fast conformational fluctuations.[67]

In summary, this report illustrates that FTIR spectroscopy can be used as a tool to probe protein structure under various conditions relevant to encapsulation in PLGA. This approach should be generally applicable to the structural stabilization of proteins during encapsulation in other polymeric depots.

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