# Effect of Gelation on the Chemical Stability and Conformation of Leuprolide

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***Purpose.*** The purpose of this study was to characterize the conformation, aggregation, and stability of leuprolide on gelation.

***Methods.*** Infrared spectra (FTIR) of leuprolide solutions and gels were collected in water, propylene glycol (PG), dimethyl sulfoxide (DMSO), and trifluoroethanol (TFE). Leuprolide solution and gel stability data were obtained by SEC and RP-HPLC.

***Results.*** Leuprolide was induced to gel with increasing peptide concentration, introduction of salts, and gentle agitation. Leuprolide dissolved in water (400 mg/ml) demonstrated FTIR spectra consisting of two major bands of equal intensity at 1615 $cm^{-1}$ and 1630 $cm^{-1}$, similar to inter- and intra-molecular β-sheet structure in proteins. When samples were gently agitated for 24 hours at 25°C, the formulation was observed to change from a viscous liquid to an opaque gel with a concomitant shift in infrared spectra from the equal intensity bands to mostly 1630 $cm^{-1}$, indicating a shift to a preferred β-sheet structure. Incubation of leuprolide with 20–200 mM salts at 25°C and 37°C also produced gels ranging from clear to cloudy and stringy white precipitates. The gel and precipitate were marked by a shift of the predominant β-sheet band to 1630 $cm^{-1}$ and 1615 $cm^{-1}$, respectively. Leuprolide was also observed to gel and/or precipitate in mixtures of water, PG or TFE, but not in DMSO.

***Conclusions.*** Birefringence was noted in many of the firmer gels. Both solutions and gels demonstrated minimal dimer or trimer formation, with no larger order aggregates detected. The chemical stability profile of gelled leuprolide was similar to that of the non-gelled water formulation by RP-HPLC.

**KEY WORDS:** leuprolide acetate; gelation; aggregation; FTIR; SEC; RP-HPLC.

## INTRODUCTION

Leuprolide (pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt) is a potent luteinizing hormone-releasing hormone (LHRH) agonist that stimulates the release of luteinizing hormone. The active conformer of LHRH analogs has been proposed from energy minima calculations to consist of two β-turns: a type II' β-turn centered at $Tyr^5$-$Arg^8$, and a second type III β-turn perpendicular to the first centered at $pGlu^1$-$Ser^4$ (1). The N-terminal residues, pGlu-His-Trp, appear to be the region responsible for activity (2). Furthermore, circular dichroism studies of leuprolide solutions (0.1–20 mg/ml) dissolved in secondary structure-inducing solvent, TFE, showed increased β-bend conformation and aggregation at higher peptide concentration (3). Aqueous formulations of deterelix (10–20 mg/ml) have also been observed to aggregate, gel, and crystallize (4,5). Powell and coworkers noted that the addition of salts, specifically anions, affected the liquid crystal stability, as well as the temperature at which birefringent liquid crystals formed (4). In contrast, little work has been presented on the physical stability of leuprolide in non-aqueous solvents (6).

Leuprolide has been used successfully for the palliative treatment of prostate cancer and endometriosis by saturating and down regulating pituitary receptors, resulting in the suppression of tumor growth and endometrial hyperplasia, respectively (7). Therefore, sustained release formulations of leuprolide in DUROS™ osmotically driven implants were of interest (8). Miniature implants require highly concentrated formulations, and LHRH analogs have been documented to gel; therefore, novel formulation strategies for extended in vivo stability were required. The objectives of this study were threefold: to structurally characterize the effect of leuprolide concentration, salt content, and agitation on gelation; to determine the extent of leuprolide gelation and its effect on peptide conformation in varying solvent systems by FTIR; to determine the stability of highly concentrated gelled and non-gelled peptide solutions by SEC and RP-HPLC.

## MATERIALS AND METHODS

### Materials

Leuprolide acetate was obtained from Mallinckrodt (St. Louis, MO) and aqueous solutions ranged between pH 5.0–5.6. PG, TFE, NaCl, $CaCl_2$, $Na_2CO_3$, $NaH_2PO_4$ and $Na_2SO_4$ were obtained from Sigma (St. Louis, MO). DMSO was obtained from Fisher (Pittsburgh, PA).

### Gelation Studies

Leuprolide solutions were prepared in water, TFE/$H_2O$, neat PG, and neat DMSO at 400 mg/ml. Samples were stored in glass or polypropylene vials at 25°C and 37°C for up to 6 weeks. Agitation induced gelled samples were prepared by slow inversion on a rotating wheel for 72 hours. Salt induced gelation studies were performed by first preparing aqueous salt solutions containing 20 mM, 100 mM, or 200 mM NaCl, $CaCl_2$, $Na_2CO_3$, $NaH_2PO_4$ and $Na_2SO_4$, then reconstituting leuprolide into the salt solutions, and storing at 25°C and 37°C for up to 4 weeks.

Leuprolide stability samples were stored in titanium tubes (4 mm × 45 mm) capped with C-flex plugs and sealed into polyfoil bags. Gelation was induced in solution samples by gentle agitation prior to storage at 37°C.

### Fourier Transform Infrared Spectroscopy

Leuprolide solutions were prepared at 50, 100, 200, and 400 mg/ml. All FTIR spectra were collected using a Nicolet 550 FTIR system (Madison, WI). Spectra were collected by placing leuprolide solutions on a single-bounce, zinc-selenide attenuated total reflectance (ATR) crystal (Spectra-Tech, Shelton, CT). 1024 interferograms were averaged with spectral resolution of 4 $cm^{-1}$. Second derivative plots were calculated using Nicolet Omnic software and smoothed with a 9-point smoothing function as described elsewhere (9).

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### Size Exclusion Chromatography

Leuprolide aggregation was assayed using a Pharmacia HR 10/30 10 mm × 100 mm column at a flow rate of 0.5 ml/min. The mobile phase was 100 mM ammonium phosphate, pH 2.0, 200 mM sodium chloride, 30% acetonitrile, and leuprolide was detected at 210 nm. Peptide standards (Sigma, St. Louis, MO) were chosen for having pI's relatively close to leuprolide (pI 10.5), and included cytochrome C, growth releasing factor, angiotensin, Trp-Leu-Arg-Phe, and bursin.

### Reverse Phase HPLC

Leuprolide degradation products from stability samples were separated using a binary gradient HPLC system (Waters, Milford, MA) equipped with a HaiSil C-18 4.6 mm × 250 mm column (Higgins Analytical, Mountain View, CA) and UV photodiode detection at 210 nm. Mobile phase A consisted of 100 mM sodium phosphate, pH 3.0 and mobile phase B was acetonitrile/water (90/10). The column was equilibrated at 10% B with a flow rate of 0.8 ml/min. A step gradient was established that consisted of an isocratic hold at 10% B for 5 minutes, 10–21% B in 5 minutes, isocratic at 21% B for 20 minutes, 21–60% B in 15 minutes, 60–80% B in 1 minute and an isocratic hold at 80% B for 5 minutes.

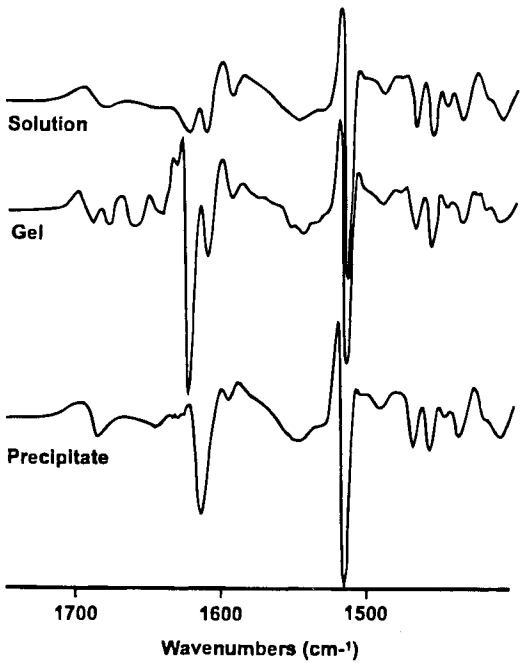


**Fig. 1.** FTIR spectra of aqueous leuprolide (400 mg/ml) (a) in solution, (b) agitation induced gel, and (c) salt induced precipitate. The sharp tyrosine bending mode at 1510 $cm^{-1}$ can be used as an internal standard for intensity.

## RESULTS

### Aqueous Gel Conformation

The FTIR spectra for aqueous leuprolide solutions (50 mg/ml) showed little distinct structure, with broad bands at 1615 $cm^{-1}$, 1630 $cm^{-1}$ and 1683 $cm^{-1}$, representative of a highly flexible, rapidly changing structure, where the time averaged conformation appeared to be β-sheet and β-turn based on literature assignments (10). Spectra collected from 50 mg/ml leuprolide in 90% TFE showed a shift to β-sheet structure at 1630 $cm^{-1}$, while retaining a small β-turn band at 1683 $cm^{-1}$ (data not shown), inconsistent with circular dichroism data indicating a β-turn conformation at lower concentrations (3). In addition, as the concentration of leuprolide in water was increased (100–400 mg/ml), two distinct bands at 1615 $cm^{-1}$ and 1630 $cm^{-1}$ were observed, indicative of aggregate and β-sheet, respectively, instead of β-turn structure (10–14) (Figure 1a). More specifically, the 1615 $cm^{-1}$ band was always associated with aggregation and precipitation, while the 1630 $cm^{-1}$ band was associated with gelation and indicative of inter-molecular β-sheet structure. The effect of increased leuprolide concentration, above 100 mg/ml, did not alter the ratio of aggregate and intra-/inter-molecular β-sheet bands by FTIR. However, increasing peptide concentration increased the rate of leuprolide gelation, in agreement with the findings of other workers (4,5).

Aqueous leuprolide solutions agitated gently overnight formed translucent soft gels characterized by a shift in FTIR spectra to the 1630 $cm^{-1}$ band, further characterized in the literature as an extended inter-molecular anti-parallel β-sheet structure (Figure 1b) (11,12,15). These soft gels were also faintly birefringent. The leuprolide solutions were observed to gel after 4 hours, and FTIR spectra at 24 and 72 hours appeared virtually identical to the 4 hour spectra. Increasing the temperature of the gels from 37°C to 50°C, or diluting in water, disrupted the gel and reformed the original solution. Agitation induced gels were produced with and without a headspace, in metallic, polypropylene, and glass vials, indicating that interfacial denaturation was not a major factor for initiation of gelation. Instead, the gel structure may be thermodynamically more stable than the solution conformation. The shift in secondary structure also suggests that the highly concentrated leuprolide solution is at a metastable equilibrium that can be easily driven toward an alternative structural energy minima with time and agitation.

### Effect of Salt on Gelation

Rapid addition of NaCl to a final concentration of 200 mM caused 400 mg/ml aqueous leuprolide solutions to precipitate into a fibrilar suspension. In some cases, long fibrils could be stretched several feet. FTIR spectra of the leuprolide salt precipitates shifted predominantly to the 1615 $cm^{-1}$ band, suggestive of precipitated aggregate β-sheet structure (11,12) (Figure 1c).

Addition of leuprolide (400 mg/ml) to $CaCl_2$ solutions resulted in soft and hard gels ranging from clear to opaque when stored at 37°C for 2 weeks. For example, 20 mM $CaCl_2$ gelled more slowly and formed a softer, stringy gel than did 200 mM $CaCl_2$ (Figure 2). Increasing the salt concentration to 200 mM $CaCl_2$ also produced harder gels that were opaque with small white spots of precipitate. The resulting FTIR spectra were similar to those of agitation induced aqueous gels, with the characteristic intense band at 1630–1633 $cm^{-1}$ and a weaker band at 1615 $cm^{-1}$ (Figure 1b,2b). Interestingly, a third β-sheet band was resolved at 1624 $cm^{-1}$, and weak β-turn and α-helix bands also appeared at 1697 $cm^{-1}$ and 1660 $cm^{-1}$, respectively (10,15). The 1697 $cm^{-1}$ band has been assigned to both β-turn and anti-parallel β-sheet structures, where precise assignment was difficult (11). However, the combination of 1630 $cm^{-1}$ and

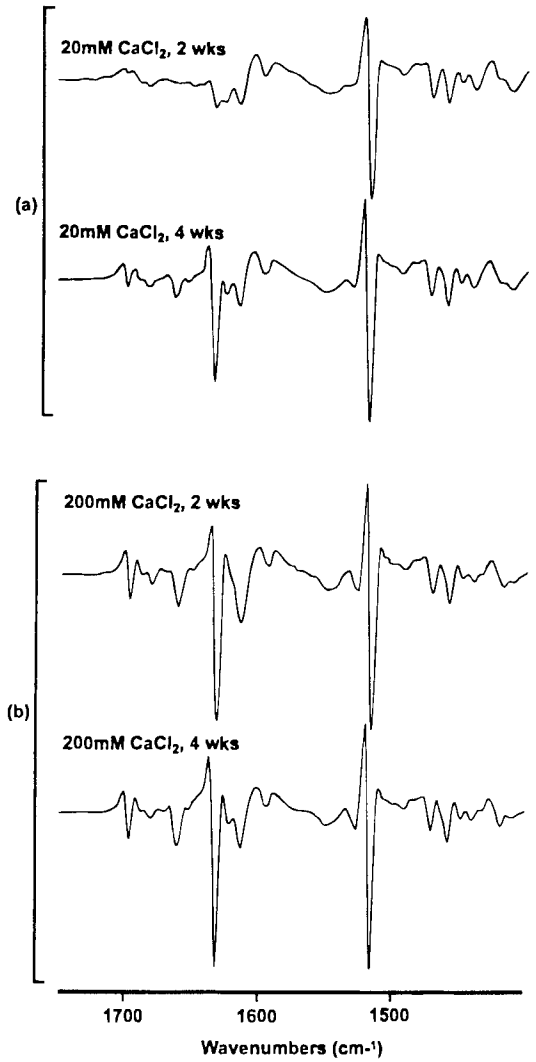


Fig. 2. Salt induced aqueous leuprolide gels (400 mg/ml) in (a) 20 mM $CaCl_2$ at 2 and 4 weeks, and (b) 200 mM $CaCl_2$ at 2 and 4 weeks.

1670–1697 $cm^{-1}$ bands is usually indicative of anti-parallel β-sheet structure (13).

Similarly, the addition of divalent anions instead of monovalent anions produced firmer gels generally consistent with the Hofmeister series (5,16). Overall, birefringent gels formed faster, clearer, and harder with $SO_4^{-2} > H_2PO_4^- > HCO_3 > Cl^-$. Within the subset of divalent anions, the addition of 20 mM $Na_2SO_4$ produced a firmer gel quicker than either $Na_2CO_3$ or $NaH_2PO_4$ at 2 weeks (Figure 3).

## Effect of Solvent on Conformation

Using a structure inducing solvent, such as TFE, was explored as an alternative method for inducing leuprolide gelation. TFE is considered a structure inducing solvent because of its relatively poor hydrogen bonding potential with respect to that of water. This is similar to work by Powers and colleagues, in which the addition of TFE to relatively dilute solutions of leuprolide induced a β-turn structure (3). Solubilizing leuprolide (400 mg/ml) in 30% TFE/70% $H_2O$ did not appreciably change the structure from the two equally intense bands at 1615 $cm^{-1}$ and 1630 $cm^{-1}$. However, 60% TFE produced a viscous soft gel solution with a slight increase in the 1630 $cm^{-1}$ band (Figure 4a). Leuprolide solubilized in 90% TFE produced a clear stiff gel, with spectra comprised predominantly of the 1630 $cm^{-1}$ band (Figure 4a). Therefore, increasing TFE concentrations shifted the hydrogen bonding motif from β-turn to intermolecular β-sheet, similar to that seen previously for increasing leuprolide concentration.

As a result, we were interested in the effect of hydrogen bonding potential in other classes of non-aqueous solvents on secondary structure and the extent of gelation. Leuprolide (400 mg/ml) dissolved in PG, a less polar protic solvent, exhibited slightly different FTIR spectra: a β-turn band at 1690 $cm^{-1}$ and β-sheet bands at 1642 $cm^{-1}$ and 1630 $cm^{-1}$ (Figure 4b). Studies on melittin in lipids suggested that the combination of the 1630 $cm^{-1}$ and 1680 $cm^{-1}$ β-type bands was indicative of an anti-parallel β-sheet structure (13). However, anti-parallel β-sheet bands from tachyplesin and myoglobin have been assigned as high as 1685 $cm^{-1}$ and 1695 $cm^{-1}$, respectively (11). Similar to aqueous leuprolide solutions, the PG solutions were seen to gel with agitation and time, with a concomitant increase in the 1690 $cm^{-1}$ and 1642 $cm^{-1}$ bands (Figure 4b). The PG gels were very firm in texture and displayed highly birefringent liquid crystal formation.

Solubilization of leuprolide (400 mg/ml) in neat DMSO produced a very different structure, as expected from an aprotic, polar solvent (Figure 5). The FTIR spectra displayed wide, unresolved bands indicative of a rapidly changing structure, comprised of an α-helix band at 1658 $cm^{-1}$, random coil at 1648 $cm^{-1}$, with minor β-sheet and aggregate bands at 1632 $cm^{-1}$ and 1615 $cm^{-1}$, respectively. Leuprolide (400 mg/ml) in 100% DMSO was not observed to gel with agitation, nor on stability for 2 years at 37°C. Titration of moisture into the leuprolide solution (5% $H_2O$/95% DMSO and 10% $H_2O$/90% DMSO) did not appreciably change the peptide conformation.

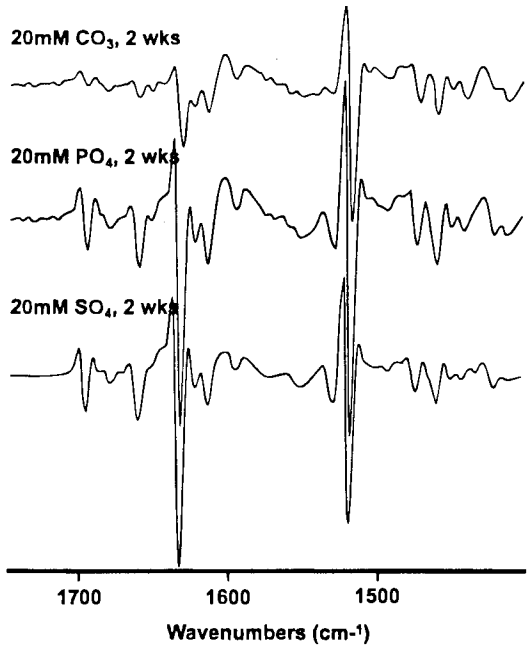


Fig. 3. Salt induced aqueous gels (400 mg/ml) in (a) 20 mM $Na_2CO_3$, (b) 20 mM $NaH_2PO_4$, and (c) 20 mM $Na_2SO_4$ at 2 weeks.

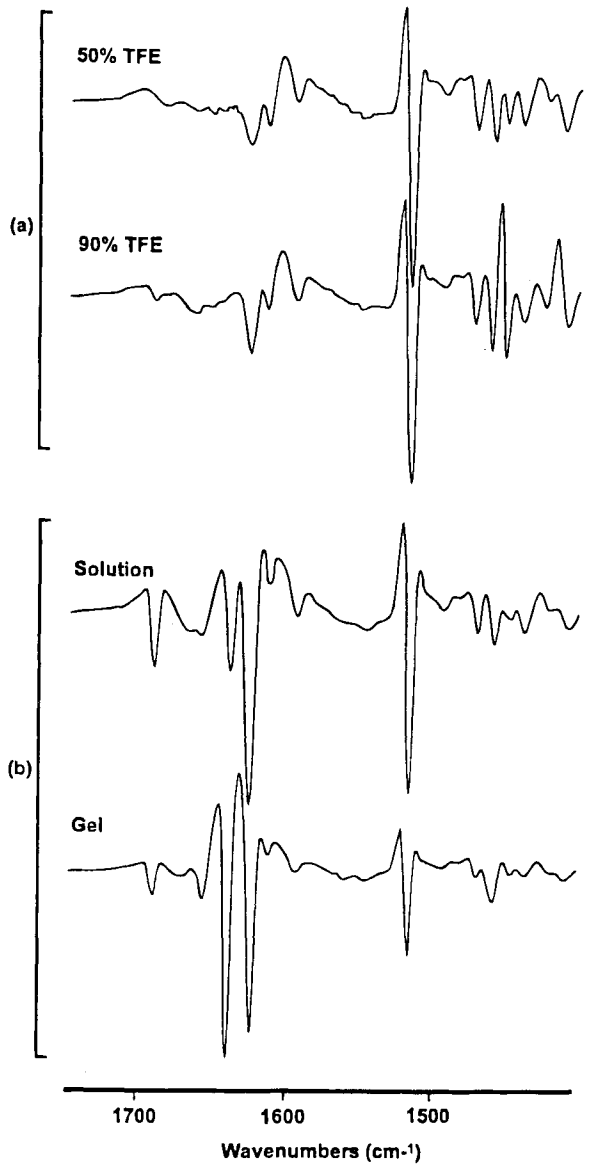


**Fig. 4.** FTIR spectra of leuprolide (400 mg/ml) in (a) 60% TFE/40% $H_2O$ and 90% TFE/10% $H_2O$, and (b) solution and gel in PG.

However, 30% $H_2O$/70% DMSO FTIR spectra showed the emergence of the 1615 $cm^{-1}$ and 1630 $cm^{-1}$ bands, similar to the aqueous spectra (data not shown). This may indicate preferential hydration of water around leuprolide to stabilize the structure, similar to that observed in studies with cytochrome C and lysozyme in non-aqueous solvent mixtures (17). Leuprolide was not observed to gel in solvents that did not sustain a β-sheet structure, such as DMSO, indicating that the β-sheet/gelation process was no longer energetically favorable. This is consistent with previous gelation work, which indicated that gelation occurs with an overall increase in β-sheet structure (14,18).

### Gel Stability

To examine the effects of gelation on chemical stability, leuprolide solutions and agitation induced gels in water and PG were incubated at 37°C for up to 6 months. The samples were dissolved in water and analyzed by RP-HPLC and SEC to determine if gelation altered chemical degradation or aggregation rates due to a more ordered structure facilitating degradation. Leuprolide solution stability at 37°C indicated a $t_{90}$ = 10.5 months and $t_{90}$ = 13.3 months for water and PG, respectively, from 2 year data (data not shown). Chemical and physical degradation products were similar between both formulations and those observed by other workers (2,3,19,20). Stability data on PG gels and solutions at 37°C for 1.5 months revealed 98.5% and 97.4% leuprolide remaining, respectively. Furthermore, agitation induced aqueous leuprolide gel and solution formulations showed 92.6% and 93.5% stability at 37°C for 4.5 months, respectively. Six month stability data for aqueous solution and gel formulations showed no statistical difference in leuprolide stability by RP-HPLC (data not shown). SEC data revealed no oligomers larger than trimers, and the extent of aggregation agreed well with RP-HPLC data.

## DISCUSSION

### Aqueous Gel Conformation

Proteins such as BSA, β-lactoglobulin, ovalbumin, and α-lactalbumin have been well documented to gel on variation of temperature, pH, and salt concentration (21–25). FTIR has been used extensively to characterize protein structures as well as membrane penetrating peptides such as mellitin, alamethicin, tachyplesin, and magainins (11–13).

Leuprolide gels in a manner consistent with other peptides, including β-amyloid peptide and insulinotropin, which have been documented to form gel-like aggregates with increased peptide concentration (14,26–29). In some cases nucleation has been observed, as well as a shift from α-helix to β-sheet structure upon gelation for proteins such as BSA, β amyloid peptides, calcitonin, and insulinotropin (18,26,29,30). Insulinotropin structure was observed to shift from α-helix to anti-parallel β-sheet aggregates in order to energy minimize the hydrophobic regions upon gelation, similar to leuprolide (14,18). Protein

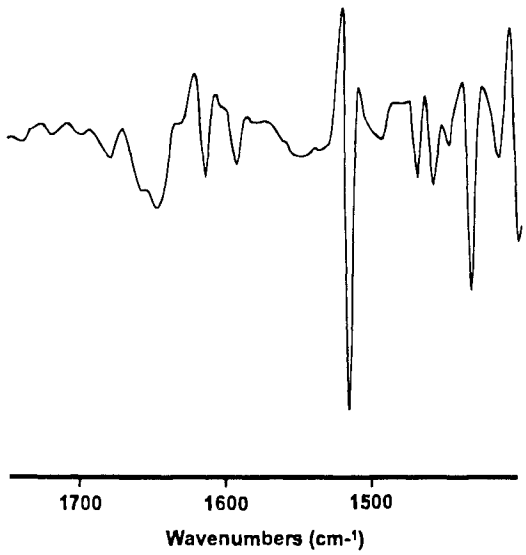


**Fig. 5.** FTIR spectra of leuprolide (400 mg/ml) in neat DMSO.

gelation as a function of concentration, temperature, temperature cycling, time, pH and salt is well documented. However, little data on agitation induced gelation of peptides and proteins could be found.

Previous circular dichroism data on leuprolide solutions between 0.1–20 mg/ml report a β-turn structure (3). With increasing leuprolide concentration (50–400 mg/ml), the peptide may transition from a β-turn structure, through an unordered conformation to a β-sheet structure. This would fulfill the peptide hydrogen bonding requirements, and allow a more ordered, solvated packing structure.

### Effect of Salt on Gelation

The solvent and salt induced gel data both indicated an anti-parallel inter-molecular β-sheet structure. Ordering of the β-sheet structure into a gel is possible considering the nature of the peptide. Aqueous gels were prepared at pH 5, where $His^2$ and $Arg^8$ are protonated. The charged residues appear on the same face of the leuprolide β-sheet structure and can be rotated to the solvent exposed sides, allowing dimers to form along the hydrophobic faces. Staggering of the dimers in a brick-like fashion and ring stacking of the hydrophobic interiors could allow the initiation of gelation. For example, $Ca^{+2}$ ions have been shown to bind to amyloid fragment $\beta A(31–35)NH_2$ by carboxy side chains and backbone carbonyls in a similar fashion (31).

Furthermore, at low salt concentrations, anions can form weak salt bridges between the charged residues in the β-sheet dimer to further stabilize the gel matrix (Figure 6). If ionic interactions between anions and protonated Arg and His residues occur, then divalent anions would be more effective than monovalent anions. In fact, the clearest, firmest birefringent gels prepared in these studies were generated with divalent anions.

Addition of salt to leuprolide caused gelation, consistent with Powell's findings in which deterelix liquid crystal stability increased with increasing anion hydrophobicity from the Hofmeister series (5). In addition, gels were birefringent, consistent with liquid crystal formation observed for other LHRH analogs (5). As the salt concentration increases and approaches charge neutralization (200 mM $CaCl_2$ or $Na_2SO_4$:300 mM leuprolide), the gel progressed from clear to cloudy, consistent with gel studies on β-lactoglobulin (22,23). β-lactoglobulin gels became opaque with increasing salt, and the protein matrix was observed to collapse in the gel. Therefore, charge neutralization decreased the ability of leuprolide to solubilize in the gel matrix, and pockets of precipitate appeared. Other workers have found that gelation of ovalbumin was strongest when the pH is far from the pI, concluding that gelation is governed by electrostatic as well as hydrophobic interactions (25).

### Effect of Solvent on Conformation

The addition of non-aqueous solvents affects the hydrogen bonding potential of leuprolide. Classically, this results in a secondary structure energetically minimized to bond with itself rather than with the non-aqueous solvent (TFE). In this study, gelation occurred more rapidly in TFE or PG than in water, consistent with the assumption that leuprolide would prefer to aggregate in β-sheet structures rather than bond with the solvent. FTIR spectra also indicated that the PG induced gel was a

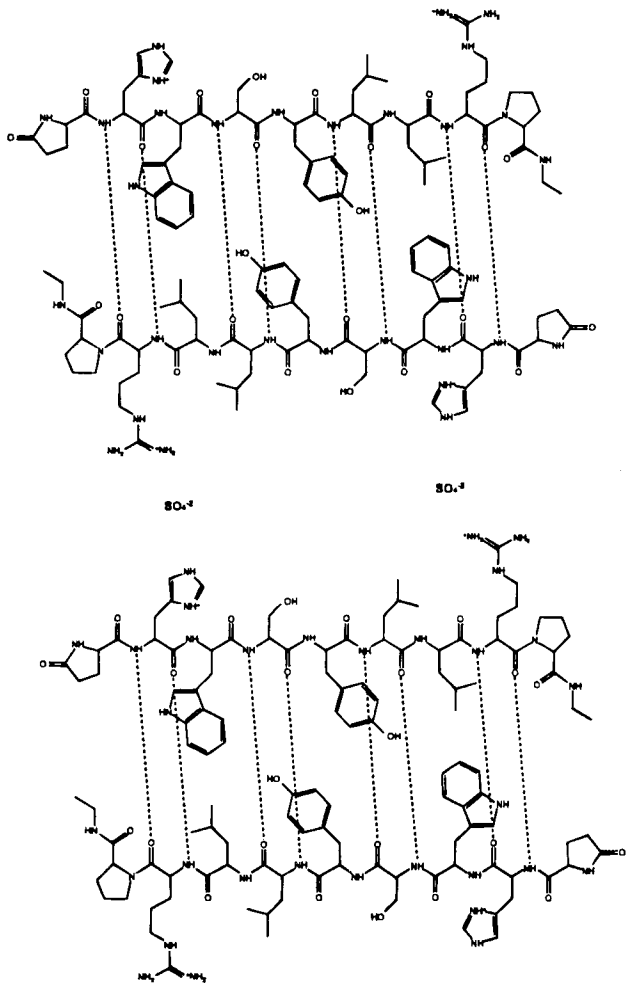

**Fig. 6.** Proposed structure of gelled leuprolide in the presence of divalent anions.

β-sheet structure, however the bands were shifted from those observed in the aqueous and TFE leuprolide gels. The shift to 1642 $cm^{-1}$ in PG, as compared to 1630 $cm^{-1}$ in water, may indicate a more extended, random β-sheet structure in PG than in water. Theoretically, the β-sheet structure in the water gel contains 100 $H_2O$ molecules per leuprolide molecule and may have a better solvated and more ordered structure than the PG gel, which contains only 24 PG molecules per leuprolide molecule.

In contrast, the total lack of hydrogen bonding in an aprotic solvent such as DMSO radically altered the secondary structure. The stabilization of β-sheet structure observed under aqueous conditions indicated that hydrogen bonding ability was of more importance than solvent polarity. This secondary structure was observed to change in DMSO to a relatively unstable conformation consisting of α-helix and random coil. Further, the DMSO formulation was not observed to gel, indicating that β-sheet structure may be required for leuprolide gelation.

Other workers have addressed the relationship between β-sheet structure and gelation by generating peptide analogs with varying ionic and hydrophobic forces within the molecule. For example, when uncharged Gly or Gln residues were substituted for charged Glu residues, β amyloid analogs were pro-

duced with an increased random coil conformation, decreased β-sheet structure, and decreased ability to form amyloid plaques (29). Further studies have shown that the binding of nicotine to the His residue in the 1-28 helical region of β(1-42) amyloid peptide retards the α-helix to β-sheet transformation important for plaque formation (32).

### Gel Stability

All leuprolide gels were completely reversible upon dissolution in water. Stability studies also indicated that gelation did not alter leuprolide chemical or physical stability. Therefore, ordering of a peptide solution into a liquid crystal did not align chemically reactive functional groups and accelerate degradation or aggregation processes. Alternatively, the decreased flexibility in the gelled state did not retard chemical degradation due to limited molecular motion or oxygen diffusion. Therefore, chemical and physical stability was more affected by peptide concentration than by the onset of gelation.

## CONCLUSIONS

Leuprolide gelation was observed with water, PG, and TFE, but not with DMSO. This indicates that the polarity of the solvent does not affect gelation, but the hydrogen bonding potential does. Specifically, leuprolide solubilized in a polar aprotic solvent must adapt to a random coil conformation not susceptible to β-sheet formation and gelation. Furthermore, leuprolide solution structure in water or PG appeared to be in a metastable state that could be driven to a gel or to a precipitated structural energy minima. The addition of divalent anions also induced leuprolide gelation, indicating that gelation is affected by electrostatic as well as hydrophobic forces.

Gelled and non-gelled leuprolide solutions stored at 37°C showed very similar chemical and physical stability, indicating that chemical stability and dimerization processes at high peptide concentration were independent of gelation.

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