Stabilization and Release Effects of Pluronic Polyols in Protein Drug Delivery

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Abstract

This paper explores qualifications of the surfactant hydrogel Pluronic F127 as a protein drug delivery mechanism. Computer modeling techniques were used to predict the behavior of the surfactant in a hydrous environment and to predict the hydrophobicity of the surface residues of the test enzyme, urease. These theoretical calculations were tested using fluorescent probing and derivatization techniques on the surfactant and test protein. Surfactant preservation of the bioactivity of urease was assayed, and the hydrogel's ability to time-release the protein from its matrix was examined.

1 Introduction

Protein drug synthesis is a field of burgeoning significance in the pharmaceutics community [1], and delivery of polypeptide agents to biological targets has become a task of great importance. Proteins face a variety of denaturant influences and while attempts can be made to control a protein's environment as much as possible, a polypeptide in an ideal environment will still have some proclivity for final states that are not bioactive (see Appendix). It has thus become the task of many pharmaceutical researchers to preserve the bioactivity of specific protein drugs and find methods for their delivery.

Non-ionic surfactants are a strong solution to the protein druggist's dilemma. The polypeptide stabilizing abilities of surfactants have been verified by past experimentation; the addition of surfactants such as Tween has been shown to increase the ability of polypeptides to remain bioactive [2].

Pluronic F127, an amphipathic polymer of polyoxyethylene (PEO) and polyoxypropylene (PPO), is of particular interest. It is a polymer with two 96 unit PEO chains surrounding one 69 unit PPO chain [9]. This surfactant has an intriguing property in aqueous solution: at room temperature, a solution of F127 will relinquish its liquid state for that of a non-fluid hydrogel (See Table 6). The following study examines the usefulness of the surfactant Pluronic F127 as a protein drug delivery and protection device.

There are many theories as to how non-ionic surfactants are able to assist the maintenance of a protein's native state. One hypothesis is that the surfactant, as an amphipathic molecule, can compete with enzyme for the denaturing surface, for they have been shown to specifically help prevent surface-induced denaturation [3]. Other investigations suggest

that the surfactant can help decrease the potential energy of a surfactant-adsorbed protein through weak hydrophobic interactions, thus lowering the ΔG of the system and increasing the native conformation's stability [2].

To ascertain the various properties of F127 with respect to protein drug preservation and delivery, it was necessary to explore the interaction between the surfactant and a given polypeptide. The protein chosen was urease, an enzyme which carries out the simple conversion of urea into carbon dioxide and ammonia, an easily quantifiable process. The bioactivity of the test protein, which serves as a "drug" for the the intents of these trials, was assessed in such a manner. Meanwhile, studies were also conducted on the time-releasing abilities of the surfactant hydrogel to help measure its suitability as a drug delivery mechanism.

Pluronic F127 shows great promise as a drug delivery device, and merits examination. This study provides a detailed look at the various aspects of the surfactant's qualifications as an agent for protecting and releasing a polypeptide drug.

2 Materials and Methods

Pluronic F127 was obtained as a generous donation from the BASF Group (Mount Olive, NJ). Urease was procured in powder and 230 μM unit tablet forms from Sigma Chemical Co. (St. Louis, MO). All aqueous solutions were created using de-ionized distilled water. Fluorescent spectrophotometry was performed in in a Perkin-Elmer (Norfolk, CT) LS 503 Fluorescent Spectrophotometer. UV spectrophotometry was performed in a Shimadzuu UV spectrophotometer. Fluorescein isothiocyanate (FITC) and 1,8 anilinonapthalene sulfonate (ANS) were obtained from Molecular Probes, Inc. Phosphate buffer solution (PBS, pH 7.4)

were prepared according to standard procedure.

2.1 Computer Modeling

Using Quanta97 v. 97.0711, (Molecular Simulations Inc. 1986-1998 All Rights Reserved), and obtaining a urease profile from the Brookhaven Protein Data Bank at http://www.pdb.bnl.gov, it was possible to simulate the interactions of the polymers in a computer environment. Pluronic F127 is a polymer of the formula: $HO-[CH_2CH_2O]_x-[CH_2CH(CH_3)O]_y-[CH_2CH_2O]_x-H$ [11]. The program's molecule builder was first used to create the polymer, which was then simulated in a hydrous environment. A conjugate gradients method was used to predict aspects of its folding structure in water [10]. An $8\mathring{A}$ thick surface hydration cloud was simulated surrounding the polymer, and the model was allowed to progress towards energetic equilibrium, with the root mean square force on the molecule diminishing as the molecule's conformation neared the base of the potential well.

To verify the hydrophobicity assessment made by Quanta, an alternate method of hydrophobicity testing was employed. Water was used as a probe molecule to test the potential energy of the molecular system when it interacted with the reportedly hydrophobic urease residues to observe the program's energy predictions. A hydrophobic probe derived from the hydrophobic monomer of F127 was also applied to purportedly hydrophobic regions on the molecule's surface, and the the energy interaction maps of water and the the phobic probe were compared.

2.2 FITC-urease Tagging and Polarization Fluorescent Spectrophotometry

9.8 mL of sodium bicarbonate/sodium carbonate buffer at 8.5 pH was mixed with 460 μM units of urease and .2 mL of FITC at room temperature for 60 minutes. The solution was placed in a dialysis tubing of 6,000-8,000 Da permeability. The bag was stirred in a PBS bath for 36 hours, assuring diffusion of all non-reacted FITC from the bag. The resulting solution was then taken from the bath and lyophilized.

Solutions of F127 in PBS were prepared at concentrations of .9 mM, .09 mM, .009 mM, and .9 μM. Each sample of F127 solution received 5 mg of FITC-urease and was mixed until total enzyme dissolution. The samples were then each scanned from 490 nm to 620 nm. A blank sample without any surfactant was also prepared. The readings were taken using polarization crystals, such that p-vales were measured, where $P = \frac{I_V - I_H G}{I_V + I_H G}$ while I_V and I_H are the intensities of two orthogonal polarization states and G is a 'grating' factor specific to the incident wavelength which depends on the parameters of the measuring system [8].

2.3 1,8 ANS and Urease Fluorescent Spectrophotometry

PBS solutions of urease at 1, 2, 3, 4, and 0 unit/mL concentrations were prepared. A 4 mL sample of each solution was placed in a corresponding test tube and each tube received 20 μL of stock 1,8 ANS solution. Fluorescent spectrophotometry was performed on samples from each tube on a range of 400 nm to 560 nm.

A second experiment was performed which included ANS, urease, and surfactant. 20 mL PBS F127 solutions of 5 mM, .5 mM, .05 mM, 5 μM, were prepared, and each one received

20 μL of standard ANS solution and 5 mg of urease powder in 10 mL of PBS. The resulting samples were scanned on range of wavelengths corresponding to these ANS trials. A standard curve for fluorescence of an ANS-F127 mixture was created using surfactant concentrations of 1 mM, .1 mM, .01 mM, and 1 μM.

2.4 Hydrogel Releasing Study

An aqueous solution of Pluronic F127 (20% surfactant by mass) was prepared. The surfactant required approximately 36 hours to dissolve at 4°C. One 230 μM unit tablet of urease was added in 20 mL surfactant solution and mixed until full dissolution. Of eight 50 mL beakers, four were filled with 5 mL of the surfactant-urease solution, and four received 5 mL unadulterated surfactant solution as controls. Each beaker was placed in a 37°C bath, to allow for the hydrogel's formation. 50 mL PBS was poured over the gels, and UV spectrophotometric measurements were taken at a wavelength of 280 nm every hour for 8 hours, using a PBS blank as a background for the readings. Each reading was in fact the mean of 5 readings taken over the course of one minute. Buffer solutions at the end of this experiment were retained for the bioactivity assay.

2.5 Urease Bioactivity Study

This assay was performed using the end products of the previously described release study. 500 μL of urease solution was taken from each of the four beakers containing urease in the release study and distributed among four test tubes. Average concentrations of urease were calculated based on an absorption standard curve for varying concentrations of urease in PBS.

500 μL urease-buffer solution without surfactant was prepared at this average concentration. The standard test for urea nitrogen was performed on the samples using phenol nitroprusside and sodium hypochlorite solutions (See Sigma Co. Catalog)

3 Results and Data

3.1 Modeling

The first task accomplished with Quanta was an examination of the mutual-potential minimization theory. A hydrophobic surface residue of the protein was allowed to interact with a water molecule in a computer environment, and potential energy for the system at each interaction coordinate was plotted in Figure 1. A similar interaction was plotted for a hydrophobe and the residue, and the potential energy of the system was lowered in comparison to the water-urease map.

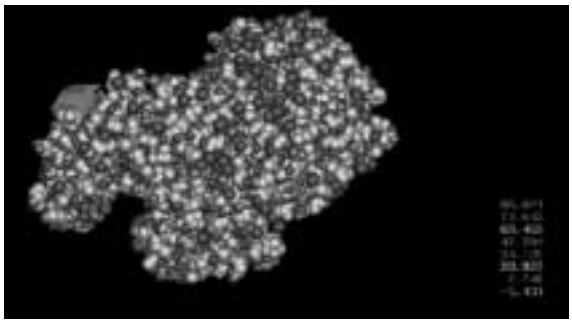

Figure 1: An energy contour map (top left) for the interaction of water and the B-100 proline residue on urease. Notice the darker region (of increased potential) surrounding a hydrophobic surface region.

The original expectation in the enlisting of Quanta as a resource for this experiment was to predict the exact binding stoichiometry of F127 to urease. This aim was not achievable:

Quanta has too many limitations to create a complex folding structure for an F127 polymer. It was feasible, however, to view the initial stages of the hydrophobic interactions which are theorized to cause bundling of the the polypropylene region of F127. Figure 2 shows a helical, but predominantly linear piece of an unfolded F127 with no energy consideration taken into account and no solvent to speak of.

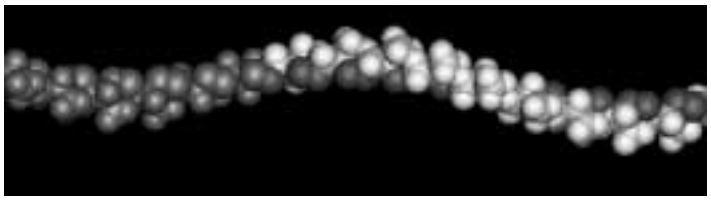

Figure 2: A polymer pluronic approximately half the size of F127, shaped in a regular, helical conformation in no solvent. Shaded region on the left is hydrophilic.

Correspondingly, Figure 3 exhibits the initial behavior of F127 in a hydrated medium using a conjugate gradients method for energy minimization.

Figure 3: Folding behavior is visible in the same Pluronic polymer as in Table 2. Here the chain is dissolved in water, and has been allowed to twist down a potential gradient such that the rms force is diminished almost down to nothing. rmsf=.384163 μdynes. Shaded region on the left is hydrophilic.

3.2 FITC-Urease Polarization Study

FITC-urease was examined using a fluorescent polarization measuring technique. It's relative mobility at different F127 concentrations was probed, and the results are shown in Figure 4.

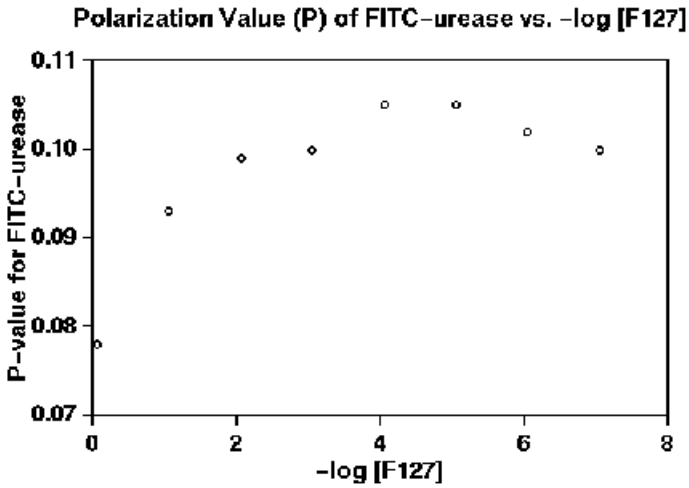

Figure 4: Viewing the breaking up of aggregates by Pluronic F127 in PBS solution of FITC-urease and

3.3 1,8 ANS Studies

For ANS studies, a standard background of ANS-F127 interaction was acquired, the results of which are quoted in Table 1.

-log [F127]	Flrscnt. Em. of ANS-F127 soln. at 480 nm
0	95.82
1	72.00
2	48.02
3	32.66

Table 1: Standard Curve for 1,8 ANS Fluorescence in Variant [F127]

As a test of the presence of hydrophobic regions on the surface of urease accessible to

ANS, a fluorescent spectrophotometry was run on an ANS-urease solution. The results are quoted in Table 2.

[urease] (μM units/mL)	Flrscnt. Em. at 463 nm
10	242.3
20	252.3
30	289.2
40	317.0
50	39.26

Table 2: Demonstration of the hydrophobicity of the surface of urease: ANS fluorescence

Having ascertained the natures of the interactions between ANS and F127 and ANS and urease, it then became useful to explore the interaction of all three compounds. The results of this displacement study are shown in Table 3.

-log 2[F127]	Flrscnt. Em. of urease-ANS-F127 soln. at 480 nm
-1	441.2
0	159.0
1	126.6
2	124.7
3	132.1
∞ (blank)	122.1

Table 3: Displacement of ANS by surfactant: difficult to read because of noise from the hydrophobic regions on F127

3.4 Bioactivity Assay

At the end of each release study, assays were made of protein's bio-catalytic abilities. The results of these studies are shown in Table 4.

prprd. urease-PBS	rels. F127-urease	pure rels. F127	net urease	rel. bioact. of F127-urease (%)
.059	.075	.049	.026	44.1
–	–	–	–	–
std. dev.	.010	–	–	–

Table 4: Urease Bioactivity Test Using Urea Nitrogen Probing at 570 nm

3.5 Release Data

Data were obtained from many trials of the release study. Table 5 represents a condensation of average values for the studies.

	control hydrogel	std. dev.	urease-F127 hydrogel	std. dev.	net abs. due to urease
Hr. 1	.053	.0034	.047	.001	.006
Hr. 2	.066	.0025	.053	.003	.013
Hr. 3	.075	.003	.056	.002	.019
Hr. 4	.086	.011	.065	.005	.021
Hr. 5	.098	.010	.070	.001	.028
Hr. 6	.116	.013	.075	.004	.041
Hr. 7	.129	.016	.079	.005	.050
Hr. 8	.142	.015	.086	.006	.056

Table 5: Mean Absorptions at 280 nm for Samples from Release Study PBS's

As an exploration of the behavior of the diffusion of urease from the hydrogel in comparison to the classical Fickian diffusion, Figure 5 shows the square-root of absorption (which is directly proportional to urease concentration) linear fitted curve.

3.6 Viscosity Study

As an additional assessment of the hydrogel's properties, a viscosity assay was done of an aqueous F127 solution to observe its gelation. The results, obtained by an experimenter at NEU Pharmacy Labs, are quoted in Table 6.

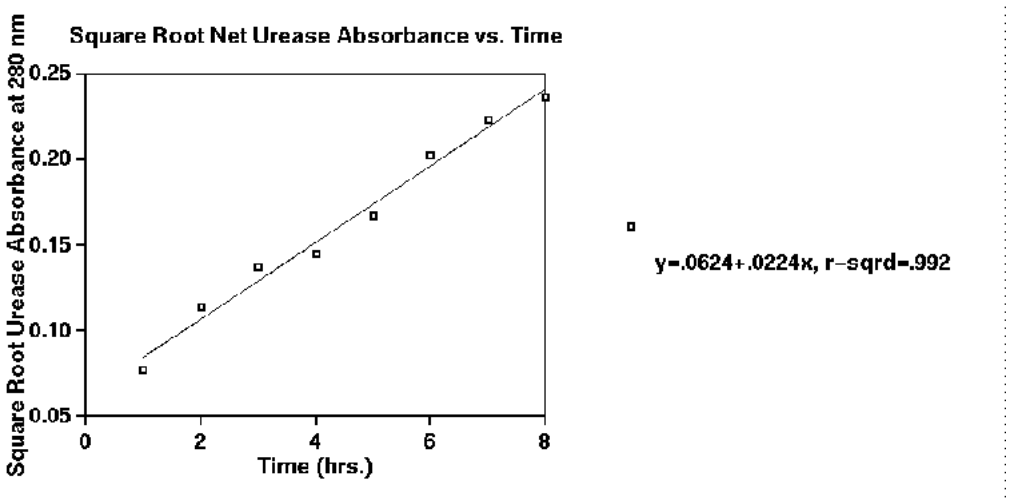

Figure 5: Sqrt. Urease Absorption at 280 nm During Diffusion from F127 Hydrogel: Appears to be Fickian diffusion

4 Discussion

The trials have produced data which indicate strongly that Pluronic F127 would indeed be an effective drug delivery device in the capacities which were tested here.

The computer modeling performed on Quanta gave a useful assessment of what kind of behavior was expected from urease and F127 in their interplay. First of all, the surfactant molecule, when submerged in water, tended to minimize its energy by folding, compacting the polyoxypropylene units exposed to water and coiling them together into a smaller hydrophobic unit with less surface area exposed to the hydrous environment. Given the computing power available and the nature of the modeling program, a fully-folded model was not possible, but the differences between Figures 2 and 3 are apparent. If the modeling capabilities were greater, an even more compacted hydrophobic core would be expected (there is a several Å loss of length from the unfolded to folded state).

Temp. ($°C$)	Viscosity (cps)
5	34
10	36
15	40
20	142
15	1144
30	1145
35	1144
40	1145
45	1145
50	1145

Table 6: Measuring the viscosity of 70% by mass hydrous solution of F127 as a function of temperature. Gelation occurs at 15°C

The FITC-urease study was definitively enlightening. The expected result for the study was to have polarization increase with the concentration of F127 and level off at a saturation point where most if not all of the FITC-urease was weakly bound with F127 polymers. The P values obtained were a measure of the polarization of the fluorophore (urease) particles in the sample PBS solution. As surfactant bound to urease, its tumbling rate would decrease, decreasing the rotational change it can go through during an excited state, and thus highly polarizing the emission spectrum [8].

The result of the trial was exactly to what was expected. In Figure 4, the polarization values for a logarithmic scale of surfactant concentrations are shown. P-values are found to *decrease* as the surfactant concentration is increased, almost in a mirror image of the expected behavior. This result can be explained using knowledge of protein aggregation (See Appendix). This has provided evidence that the assumption that most of the urease in solution is in a solitary state until interacting with F127 is a dubious one. If there were a significant contingent of urease aggregates present in solution before the addition of surfactant, then the polarization values for the blank would be high: protein aggregates are

high volume molecular complexes which therefore exhibit a slowed tumbling and thus emit highly polarized light [8]. Surfactants, if helping to minimize the potential of the *native* state, would decrease the equilibrium concentration of the aggregate state, thus lowering the polarization of the emission. Thus, Figure 4 provides evidence for the argument that surfactants help break up protein aggregates and corroborates the notion that F127 can help in the stabilization of proteins.

Given the contractile nature of the hydrophobic region, it is feasible to connect the unit with the surfactant's stabilizing ability. A less unweildy hydrophobic unit might make a mutual exclusion of water by surfactant and hydrophobic residue a much more favorable and practicable arrangement. We can see clearly from the evidence provided from the ANS probing that there is definitely a significant presence of hydrophobic residues on the surface of urease (see Table 2). Because the probe only fluoresces in a hydrophobic environment, we can assume that the observed increase in emission intensity as the urease concentration increases holds promise of many hydrophobic surface regions on urease [2]. Granted, F127's hydrophobic region will be larger than an ANS probe, but the character of the protein's surface has nevertheless been somewhat illuminated.

ANS probing of the urease-F127 interaction gave some insight into the interplay between the two molecules. When the experiment was originally designed, the hope was that less surfactant would begin to allow more ANS to bind to hydrophobic regions on urease, thus increasing the fluorescent intensity of the sample and indicating less binding of F127 to urease as the concentration of F127 went down. As can be observed in Table 3, the fluorescent intensity of the samples decrease for a certain range of concentrations along with the F127

concentration, and then increase again once the F127 concentration is low enough. This result is curious, and merits further investigation. It would seem that, for most of the F127 concentration range, the noise from the binding of ANS to F127 hydrophobic regions is too loud; Table 1 shows that such background is significant. Yet, as F127 concentration approaches zero, the fluorescent intensity stops decreasing. This result is explainable by a process whereby the surfactant, now in very low concentration, has yielded many free hydrophobic residues to ANS. 1,8 ANS is abnormally fluorescent when bound to proteins [4]. Thus, the surfactant has been giving off ANS signals *and* garnering most urease hydrophobic regions, but once at a competitive concentration relative to the surfactant, the ANS now has the opportunity to signal from the urease surface and thus the fluorescence increases slightly.

Pluronic F127 gel turned out to be quite successful in its preservation of the protein's bioactivity. The samples taken from the end result of the release study were certainly somewhat denatured by comparison to the prepared urease solution, yet they still had retained 44.1% of their bioactivity after 8 hours in a thermal environment different from that of the jack beans form which the urease was extracted. The catalysis of urea in ammonia and carbon dioxide was found to decrease over time, but nevertheless was to a substantial degree maintained by the presence of the surfactant. Such results support other studies documenting the stabilizing properties of surfactants, and also in particular demonstrate that F127 is not distinct from Tweens in this respect.

The release studies gave promising results with respect to the possibility of F127 becoming a drug delivery device. While proteins are large macromolecules, the properties of the hydrogel were ones that permitted the diffusion of urease up into the buffer solution above

the layer of gel. Figure 4 gives strong evidence for a claim that diffusion of the protein from the gel behaves in a classical, Fickian way, its solute-diffused directly proportional to the square root of the time allowed to pass [7]. Thus, this hydrogel gives us a material which retains a protein within its matrix, but allows for gradual, quantifiable diffusion of the drug into the surrounding medium in significant concentrations.

Finally, the viscosity data regarding the aqueous solution of surfactant are a useful way of viewing the delivery power of the hydrogel. Because its viscosity is so high at body temperature, a protein-drug containing gel could conceivably be injected into a patient and remain concentrated, without flowing throughout the body, for hours at a time. Granted, F127 is water soluble, and would eventually dissolve, but the amount of it that would remain potent is significantly greater than that of a simple protein drug solution. The gelation is one of many positive characteristics for Pluronic F127 as a drug delivery device.

5 Conclusion

Pluronic F127 is a surfactant molecule with some highly beneficial characteristics that make it a strong candidate for a protein drug delivery device. Its interaction with polypeptides is one of surface adsorption which minimizes potential energy by hiding hydrophobic residues from the hydrous medium, as was predicted by computer modeling and verified by experiment. It has helpful abilities with regard to bioactivity maintenance and native state stabilization, and also harbors a hydrogel character which would assist in time-release drug delivery. Given its positive capabilities, Pluronic F127 most certainly deserves further study as a polypeptide delivery mechanism.

6 Acknowledgments

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A Protein Stability

Proteins *do* require significant protection from the elements. Their structure is one subtly defined by ionic, covalent, hydrogen-bonding, and hydrophobic interactions of the residue chains of their amino acids, and a slight change in conditions is often all that it takes to cause physical denaturation, or unfolding, of the polypeptide. Such fragility does not lend itself to the variant environments that a protein drug must experience. The simple change of conditions from a test tube to the human body is enough to cause total physical denaturation of the protein. Lyophilization, or freeze-drying, is another common procedure applied to protein drugs which is a large source of physical degradation for the protein's bioactivity [3].

In addition, a protein in a completely *controlled* environment can nevertheless can fold out of this active, or native state. Such deactivation can result from an improper temperature, pH, or solvent [5]. Also, a polypeptide in an ideal medium still has the potential to leave its native state. Processes such as aggregation, surface-adsorbing denaturation, and precipitation all can cause an enzyme to leave its native state, even though it may be in a perfectly hospitable environment.

The reason for the precarious nature of a protein's native state is a direct result of the fact that the active state is only attained when that conformation represents the thermodynamic equilibrium of the protein and its solvent. In other words, the Gibbs free energy for the conversion from a completely unfolded polypeptide (primary structure) to a native state (tertiary structure) must be negative. Since basic thermodynamics tells us that $\Delta G = \Delta H - T\Delta S$, we can see immediately why a change in temperature can have a drastic effect

on the conformation of the protein. Given that the heat and entropy of the folding reaction remain relatively constant, a change in temperature can easily alter ΔG to the point where the native state is no longer the thermodynamically favorable conformation.

The minimization of potential energy, however, is another important factor in the stability of proteins which is more pertinent to this study. The force on a folding polymer must be, from physics, $\mathbf{F} = -\nabla U$. A molecular system reaches equilibrium when it hits a minimum of total potential energy for the system (analogously, a ball will not stand still on a hillside, but rather roll down to the bottom of the hill). The protein will continue to fold until all of the 'nearby' conformations require it to increase it's potential energy, and its gradient thus always points in such a way that it will not leave its equilibrium unless perturbed [10]. The potential energy of a folding protein is highly dependent on a variety of factors including the bonds that the protein forms with itself, solvent atoms, and the affinities that the amino acid chain residues of the protein have for water.

Hydrophobic interactions especially especially cause the protein to denature. Normally, a protein will fold to keep the number of exposed hydrophobic residues as low as possible, thus minimizing the potential energy of the system. In the process of converting from a primary chain to a complex tertiary shape with a predominantly hydrophobic core and hydrophilic surface, and polypeptide will go through intermediate conformations which, left to their own devices, will progress to the native state in a clement medium.

While partially unfolded, however, polypeptides may often take advantage of certain resources available to them for energy minimization. Large groupings of intermediate states called aggregates can form a mutually beneficially clumping up polypeptide where each

chain exposes more hydrophilic residues to the water and hides more hydrophobic ones than in the native state [2, 6]. Aggregates are completely non-bioactive states which remain in an equilibrium concentration in any protein solution. Proteins can also adsorb along container surfaces, sacrificing their bioactivity to attain a lower potential energy by offering hydrophobic residues to an anhydrous container surface instead of the aqueous medium. Surface adsorption is one of most definitively halted denaturations when surfactants are added to a protein solution [5].

Proteins are indeed fickle creatures, delicate structures which are only slightly more prone to bioactivity than to total physical denaturation. It is for this reason that the search for methods of protein stabilization has become a wide-ranging and significant aspect to protein drug delivery research.