

Drug Delivery Matrix Containing Native Protein Precipitates Suspended in a Poloxamer Gel

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Abstract □ Sustained delivery systems can achieve more constant blood levels of protein therapeutics than those obtained with bolus doses, leading to improved drug efficacy and fewer adverse side effects. Several different polymeric delivery systems have been studied, including poloxamers, which are unique because they can be prepared in aqueous buffers that are compatible with proteins. Poloxamers are nontoxic block copolymers of poly(ethylene oxide) and poly(propylene oxide). Certain poloxamers exhibit reversible thermal gelation. Thus, a solution of protein and poloxamer prepared at low temperatures and injected extravascularly will form a gel as it warms to body temperature. Subsequently, the protein is released slowly from the gel. To date, however, poloxamer gel delivery systems have been limited to relatively low protein concentrations (i.e., ≤ 0.4 mg/mL) that produce a completely soluble protein and an optically clear gel. Much higher concentrations of other protein drugs might be needed to obtain an efficacious sustained dose. In the current *in vitro* study we found that a poloxamer 407 (22% wt/wt) matrix could be prepared containing tens of milligrams/milliliter of the model proteins α -chymotrypsin and lactate dehydrogenase. Under these conditions the protein forms a homogeneous suspension. Warming through the poloxamer 407 transition temperature (ca. 18 °C) results in a gel that retains a homogenous distribution of protein precipitates for several days at 37 °C. Infrared spectroscopy documented that the precipitated proteins in the suspension have native secondary structure. Furthermore, the fully active protein can be recovered completely when the gel is dissolved in excess buffer. Finally, at the higher protein concentrations used to form the suspensions in poloxamer 407, protein stability during incubation at 37 °C was greatly improved over that seen at lower protein concentrations.

Introduction

Recombinant protein pharmaceuticals have already provided unique therapies for several previously untreated diseases, and numerous new protein drugs are being developed. Because of the barriers to delivery by oral, transmucosal, and transdermal routes, most proteins are administered parenterally, which can lead to rapid elimination from the circulation.¹ Hence, in order to maintain therapeutically effective blood levels, it is often necessary to administer large or frequent doses. The inconvenience and potential adverse side effects of this approach might be circumvented by employing systems that provide sustained delivery. Relatively constant blood levels can improve the efficacy of the drug and the patient's quality of life, while minimizing side effects. Most notably, several different polymeric matrices have been developed to provide sustained drug delivery.^{1,2} However, with many of these systems (e.g., polylactides or -glycolides) the polymers are only soluble in organic solvents, which can cause denaturation of the protein and limit protein solubility.^{1,2} Alternative approaches in which a dried protein powder is suspended

in the organic solvent necessitate development of an appropriate formulation and freeze-drying processing to prevent protein denaturation during lyophilization.^{2,3} Furthermore, suspension of even a native dried protein in organic solvent can lead to extensive denaturation.⁴

In contrast, proteins are compatible with aqueous gels such as those formed by poloxamers. Poloxamers are nontoxic block copolymers of poly(ethylene oxide) and poly(propylene oxide).⁵⁻⁸ An important characteristic of some poloxamers is that they exhibit reversible thermal gelation.⁵⁻⁸ For example, with high enough concentration (ca. $\geq 20\%$ wt/wt), poloxamer 407 (molecular weight 11 500) is liquid at relatively low temperatures, e.g., 4 °C, but rapidly forms a highly viscous gel upon warming above a characteristic transition temperature, e.g., 15–18 °C for a 22% solution. Thus, a solution of protein and poloxamer prepared at low temperatures and injected extravascularly will form a gel as it warms to body temperature.⁵⁻⁸ Subsequently, the protein should be released slowly either by diffusion from the gel or as the gel slowly dissolves. With this approach sustained delivery (on the time scale of hours) of urease and interleukin-2 has been obtained in rodents, after intraperitoneal and intramuscular injections, without any systemic or local toxic effects of the poloxamer 407 gel or protein.⁵⁻⁸

To date, however, poloxamer gel delivery systems have been limited to relatively low protein concentrations that produce a completely soluble protein and an optically clear gel. For example, the protein concentrations of urease and interleukin-2 used in previous studies were 400 and 6.3 μ g/mL, respectively.⁶⁻⁸ Obviously, much higher concentrations of other protein drugs might be needed to obtain an efficacious sustained dose. The purpose of the current *in vitro* study was to investigate the possibility of developing a gel matrix of poloxamer 407 that contains protein at concentrations of tens of milligrams/milliliter. We discovered that this goal could be achieved in a surprising fashion: at high concentrations in poloxamer 407, proteins form homogeneous suspensions with full retention of native secondary structure. Furthermore, the protein can be recovered completely when the gel is dissolved in excess buffer.

Material and Methods

Materials—Porcine heart lactate dehydrogenase (Type XVIII) and bovine pancreatic α -chymotrypsin (Type II) were purchased from Sigma Chemical Co. and used without further purification. Poloxamer 407 was obtained from BASF Corp. Buffer salts and enzyme substrates were purchased from Sigma Chemical Co.

Preparation of Protein Suspensions in Poloxamer 407—Poloxamer 407 solutions were prepared by adding the dry powder to the appropriate type and amount of ice cold buffer in a capped test tube and mixing the tube contents overnight by repeated inversion on a rotating mixer at 4 °C. For experiments on enzyme recovery from the gel and infrared spectroscopic analysis of secondary structure, α -chymotrypsin powder was dissolved in 30 mM sodium phosphate buffer (pH 7.4 at 23 °C) to a concentration of 100 mg/mL. The protein

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solution was mixed 1:4 with an ice-cold solution (27.5%, w/w) of poloxamer 407 in the same buffer to give final protein concentration of 20 mg/mL and a final poloxamer concentration of 22% (w/w). For experiments testing protein stability, the protein and poloxamer were prepared in 50 mM Tris/HCl (pH 7.4 at 23 °C) containing 0.1% (w/v) sodium azide.

With lactate dehydrogenase, the commercial ammonium sulfate suspension was pelleted by centrifugation for 15 min at 4000g at 4 °C. The supernatant was discarded and the pellet was dissolved in 50 mM Tris/HCl buffer (pH 7.4 at 23 °C) with 0.1% (wt/vol) sodium azide, against which the enzyme was dialyzed overnight at 4 °C. The protein solution was then mixed 1:3 with 29.4% poloxamer 407 in the same buffer. To concentrate the protein for infrared spectroscopic analysis of protein secondary structure, the ice-cold enzyme suspension was centrifuged for 15 min at 4000g (4 °C) to pellet the enzyme precipitate. The supernatant containing enzyme dissolved in poloxamer 407 was removed. The concentration of the protein in the pellet was 63 mg/mL.

Enzyme Activity Assays—To a 50 μ L aliquot of the poloxamer gel containing the protein suspension was added 950 μ L of the appropriate buffer (see above), which was at room temperature. Both poloxamer 407 and protein dissolved completely within a few seconds. Catalytic activity was measured in aliquots removed from this solution or from a control solution prepared in buffer without poloxamer. Catalytic activity of α -chymotrypsin was measured according to the method of Del Mar et al.⁹ Lactate dehydrogenase activity was measured as described by Anchordoquy and Carpenter.¹⁰

Determination of Protein Solubility in Poloxamer 407—Suspensions of proteins in 22% poloxamer 407 were prepared as described above. The samples were chilled on ice to keep poloxamer 407 in the liquid state and centrifuged for 15 min at 4000g (4 °C) to pellet the precipitated enzyme. The catalytic activities were determined in the supernatant and in the original suspension. On the basis of the initial protein concentration in the original suspension, the fractional activity recovered in the supernatant was used to calculate the concentration of soluble protein in the supernatant.

Stability Studies—Suspensions of α -chymotrypsin were prepared in 20% (w/w) poloxamer 407 in 50 mM Tris/HCl buffer (pH 7.4 at 23 °C) and 0.1% (w/v) sodium azide. Lactate dehydrogenase was prepared in the same buffer with 22% (w/w) poloxamer 407. Initial ice-cold suspensions containing 10 mg/mL α -chymotrypsin or 12 mg/mL lactate dehydrogenase in poloxamer 407 were diluted as needed with ice-cold poloxamer 407 solution to obtain the desired final protein concentrations. Aliquots (50 μ L) were pipetted into 1.5 mL Eppendorf tubes, which were capped and placed in a 37 °C incubator. After incubation, the samples were dissolved in buffer and assayed for residual catalytic activity as described above. Samples containing the same protein concentrations dissolved in buffer without poloxamer 407 were also tested as controls. The results are presented as the percentage of the initial catalytic activity that was recovered after incubation at 37 °C.

Infrared Spectroscopic Analysis of Protein Secondary Structure and Poloxamer 407 Phase Behavior—Samples were prepared for infrared measurement in a CaF₂ cell (Beckman FH-01) with a 6 μ m spacer. To load samples containing poloxamer 407, the cell and the solution were chilled on ice and the ice-cold solution was injected into the cell filling port. The cell was mounted in a custom-made Peltier device, which allowed precise control of sample temperature (± 0.3 °C). The temperature of the sample was monitored with a fine wire thermocouple, which was placed directly into a small depression in one of the CaF₂ windows, and a digital thermometer. Infrared spectra were recorded using a Nicolet Magna 550 spectrometer equipped with a dTGS detector. For each spectrum a 512-scan interferogram was collected in single beam mode with a 4 cm^{-1} resolution. For the purposes of generating the protein amide I spectra, reference spectra were recorded under identical scan conditions with only the corresponding buffer (with or without poloxamer 407) in the cell. The spectra of liquid and gaseous water were subtracted from the observed protein spectra according to previously established criteria, with a double subtraction procedure.^{4,11} Second-derivative amide I spectra were obtained with the derivative function of Omnic software (Nicolet). The final protein spectrum was smoothed with a seven-point function to remove white noise.

The phase behavior of the poloxamer 407 was determined by monitoring the frequency of CH₃ stretching and CH₂ scissoring vibrational modes of the poloxamer. At each temperature tested, a

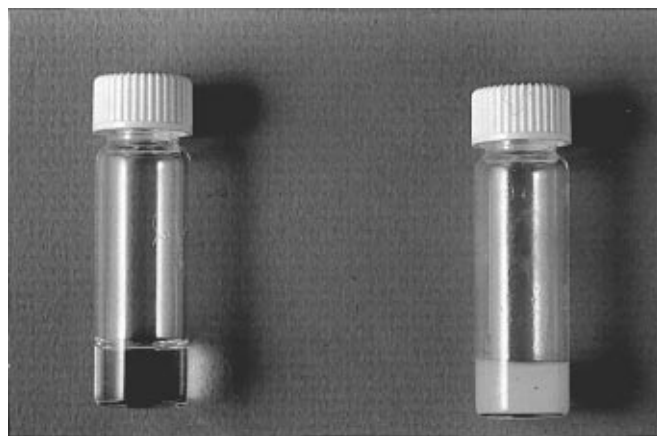


Figure 1—Appearance of 22% (w/w) poloxamer 407 gels prepared without (left) and with 20 mg/mL α -chymotrypsin (right). Samples were prepared as described in the Experimental Section and photographed at room temperature (ca. 23 °C).

spectrum was acquired and the midpoint of the CH bands determined using Omnic (Nicolet) software. The frequency of the midpoints was plotted as a function of sample temperature.

Effect of Protein Stabilizers on Poloxamer Phase Transition Temperature—Solutions (10 mL) containing various concentrations of poloxamer 407 and either sucrose or polyethylene glycol (MW 8000) in 30 mM sodium phosphate buffer (pH 7.4 at 23 °C) were prepared in 15 mL conical-bottom cell culture tubes. The samples were placed into a water/ethylene glycol bath set at -5 °C. The bath temperature was increased in small increments (2–5 °C). After allowing approximately 5 min for temperature equilibration, a sample tube was removed briefly from the bath and inverted. Samples that had undergone the transition from the liquid to the gel state would not flow down in the inverted tube. The lowest temperature at which such gelling occurred was designated the transition temperature for a sample.

Results and Discussion

In order to be able to assess accurately the effects of incorporation into and removal from poloxamer 407 gels on protein function, we chose two enzymes, monomeric α -chymotrypsin and tetrameric lactate dehydrogenase, as model systems. In the first experiment, ice-cold buffer (30 mM sodium phosphate, pH 7.4 at 23 °C) containing 100 mg/mL α -chymotrypsin was mixed 1:4 with the same buffer containing 27.5% (w/w) poloxamer 407, resulting in 20 mg/mL protein and 22% poloxamer 407. Immediately upon mixing, the protein formed a homogeneous, white suspension of precipitated protein. The mixture formed a gel at temperatures above 18 °C, similar to 22% poloxamer 407 alone. The gel without protein was clear and colorless, whereas the sample containing α -chymotrypsin had a uniform milky, white appearance (Figure 1). Similar results were obtained with lactate dehydrogenase, subtilisin, bovine serum albumin, and insulin (data not shown). Examination of the gel by light microscopy revealed that the protein precipitates were homogeneously distributed with a size ranging from about 5–10 μ m. The solubility of the model enzymes in 22% poloxamer 407 was 0.16 ± 0.07 and 7.40 ± 1.24 mg/mL (mean \pm SD, $n = 3$) for α -chymotrypsin and lactate dehydrogenase, respectively.

Even after 18 days at 37 °C (the longest duration tested), homogenous distribution of the protein precipitates was maintained in the gel (data not shown); i.e., no settling occurred. This physical property would be important for drug delivery systems in which it is desirable to maintain a relatively constant release rate of protein as the gel matrix dissolves. In contrast, at low temperatures (e.g., 4 °C), where

the system is a liquid, the protein precipitates settle toward the bottom of the container, a process that can be hastened with centrifugation. This manipulation can be used to increase the concentration of the protein in the matrix by at least about 10-fold (data not shown). Thus, for proteins with limited solubility in aqueous solution, a high protein concentration in the poloxamer could be obtained by forming a suspension and then concentrating the precipitates by centrifugation at low temperature. Alternatively, we have obtained, and concentrated further by low temperature centrifugation, essentially identical protein suspensions by simply dissolving the appropriate mass of dried poloxamer powder in a protein solution (data not shown).

Although the physical properties of the poloxamer gel containing the protein suspension are highly suitable for drug delivery, the system will be of little benefit, unless a functional protein can be recovered from the gel. At first glance, this would appear unlikely, because when solution conditions (e.g., denaturants or high temperature) induce protein precipitation, the protein is often irreversibly denatured and aggregated. In order to ascertain the effects of incorporation of a protein into a suspension in a poloxamer 407 gel, we employed infrared spectroscopy to monitor alterations in the protein secondary structure. Infrared spectroscopy can be used with any type of sample (e.g., solution, suspensions, dried solids).^{3,4} The second-derivative infrared spectra, in the conformationally sensitive amide I region,^{3,4,11} for α -chymotrypsin and lactate dehydrogenase suspensions in poloxamer are only slightly altered relative to the respective spectra for the native protein dissolved in buffer (Figure 2). Relative to those for control samples in buffer, the spectra for lactate dehydrogenase prepared in poloxamer 407 have a slight decrease in the absorbance of the band representing α -helix at 1656 cm^{-1} , which is compensated by a minor absorbance increase in the region assigned to turns at $1670\text{--}1690\text{ cm}^{-1}$ (Figure 2A).⁹ With α -chymotrypsin, relative to samples in buffer, the spectra for the sample prepared in poloxamer 407 have minor alterations in the β -sheet band at 1637 cm^{-1} , the random coil band at $1648\text{--}1651\text{ cm}^{-1}$, and the bands in the region assigned to turns at $1660\text{--}1690\text{ cm}^{-1}$ (Figure 2B).⁹ These spectral alterations are trivial compared to results obtained when proteins are precipitated by chemical or thermal denaturation.^{4,12} The infrared spectra for such samples are dominated by large bands around 1620 and 1695 cm^{-1} , which are due to the formation of an extensive intermolecular β sheet.^{4,12} Between these bands there is usually a featureless contour composed of broadened and fused bands.

Since less than 1% of a 20 mg/mL sample of α -chymotrypsin is soluble in 22% (w/w) poloxamer 407, the infrared spectra reflect essentially the structure of only precipitated protein. With lactate dehydrogenase, which has a much higher solubility in poloxamer, the initial suspension was concentrated by low-temperature centrifugation. The final sample contained approximately 88% precipitated protein. On the basis of the similarity of the infrared spectra between the protein solutions in buffer and suspensions in poloxamer, it can be concluded that the precipitated proteins have native secondary structures. Similar infrared spectroscopic results were obtained with insulin, bovine serum albumin, and subtilisin (data not shown).

Thus, instead of fostering protein denaturation, poloxamer appears to decrease protein solubility, which results in a "salting out" of the native protein molecules. Such phase separation of proteins by additives (e.g., ammonium sulfate) is routinely used to fractionate and concentrate proteins.¹³ Commonly, soluble and fully functional protein molecules can be recovered from the salted-out sample, if the concentration of the salting-out agent(s) is(are) reduced sufficiently.¹¹ Since the native secondary structures of α -chymotrypsin and lactate

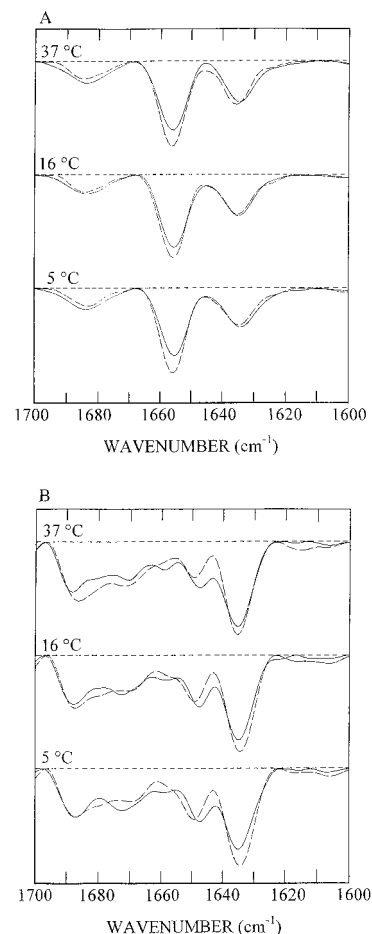


Figure 2—Second-derivative infrared spectra for proteins in the amide I region. Samples in buffer are shown by dashed lines and those in 22% (w/w) poloxamer 407 by the solid lines. The sample temperatures at which the spectra were acquired are noted in the figure. (A) Lactate dehydrogenase prepared at 23 mg/mL in buffer and 63 mg/mL in poloxamer 407. (B) Chymotrypsin prepared at 20 mg/mL in both buffer and poloxamer 407.

dehydrogenase were retained in precipitates in poloxamer, it is not surprising that fully functional enzymes could be recovered if the poloxamer were diluted. When 22% poloxamer 407 gels containing the suspended proteins were diluted (at least 100-fold) in buffer (at 23 °C), the poloxamer and enzymes dissolved completely and $104.9 \pm 7.3\%$ (mean \pm SD, $n = 3$) and $101.7 \pm 16.9\%$ (mean \pm SD, $n = 3$), respectively, of the catalytic activities originally placed into the poloxamer were recovered for lactate dehydrogenase and α -chymotrypsin.

Another advantage of infrared spectroscopy is that it can be used to monitor simultaneously the phase behavior of the poloxamer, by measuring the frequency of the poloxamer CH vibrations, as well as the protein secondary structure as a function of sample temperature. Increasing the temperature through the liquid to gel transition of poloxamer led to a decrease in the frequency of both the CH_2 scissoring (Figure 3A) and CH_3 stretching modes (Figure 3B). Plots of frequency versus temperature show a sharp reduction in frequency at the transition temperature, followed by a plateau at higher temperatures (Figure 3). The presence of a 20 mg/mL α -chymotrypsin suspension has minimal effect on the apparent transition temperature (Figure 3). Also, the secondary structure of the incorporated protein is essentially the same in any state of the poloxamer. The second-derivative amide I spectra of α -chymotrypsin and lactate dehydrogenase in the poloxamer are very similar to those for the proteins in buffer,

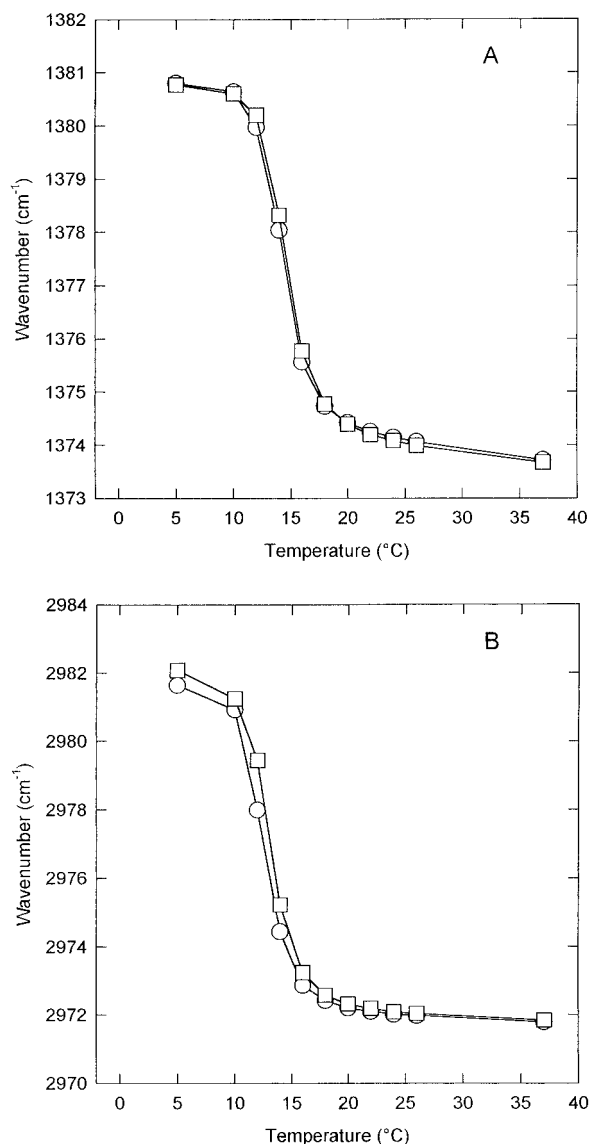


Figure 3—The effect of temperature on frequency of the infrared vibrational modes for CH groups in poloxamer 407. Samples contained 22% (w/w) poloxamer 407 alone (squares) and 22% poloxamer 407 with 20 mg/mL α -chymotrypsin (circles). (A) CH_2 scissoring mode frequency. (B) CH_3 stretching mode frequency.

at temperatures below, above, and in the middle of the poloxamer phase transition zone (Figures 2 and 3).

The thermotropic changes in the poloxamer's CH infrared spectral bands are similar to those seen with the liquid crystalline to gel phase transition during cooling of phospholipids.^{14–16} The decreased frequency of CH vibrations in the gel phase of poloxamer, as is the case with phospholipids, is most likely due to enhanced intermolecular interactions—hydrophobic, in the case of poloxamer—between hydrocarbon chains. This conclusion is consistent with earlier light scattering, ultrasonic velocity, and NMR spectroscopic studies on the poloxamer gelling process.^{17–19}

Another critical property required for a sustained delivery formulation is maintenance of protein stability at body temperature (i.e., 37 °C). Stabilities of lactate dehydrogenase in buffer and in poloxamer gels at 37 °C for 18 days were compared at high (12 mg/mL) and low (0.6 mg/mL) protein concentrations. About 60% of the initial activity was recovered in either preparation at low protein concentration, whereas essentially complete recovery of activity was observed with the high protein concentration samples (Figure 4A).

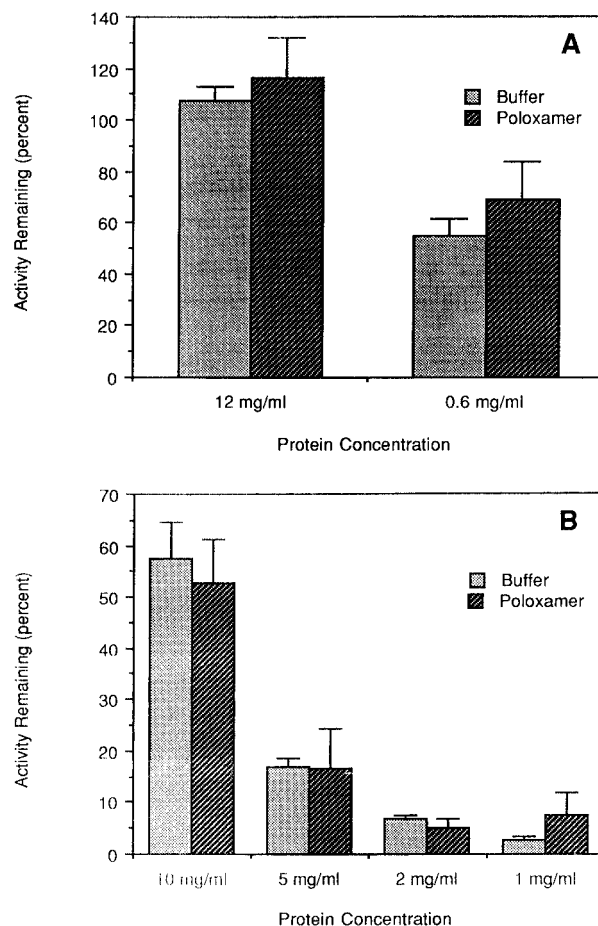


Figure 4—Effect of protein concentration and poloxamer 407 on stability of proteins at 37 °C. (A) Lactate dehydrogenase incubated for 18 days. (B) Chymotrypsin incubated for 4 days. Values given are the means \pm SD for three samples. See Experimental section for sample preparation and incubation protocols.

Similarly, equivalent stability was observed with α -chymotrypsin in buffer and poloxamer gels, and reduction in protein concentration greatly reduced stability (Figure 4B). Thus, even though formation of a precipitate of native protein in poloxamer may not itself increase long-term stability at 37 °C, preparing products at high protein concentrations provides an extra benefit of increasing protein stability.

Any given protein drug formulation would have to be optimized further for long-term stability in poloxamer gels, as each protein has unique physicochemical properties that dictate its relative chemical and physical stabilities.²⁰ Chemical degradation is usually minimized by specific adjustments in solution conditions. For example, adjusting the pH can substantially retard the rate of deamidation.²⁰ In contrast, increasing physical stability, which in itself may be linked with chemical stability, often requires the use of nonspecific additives which increase overall protein thermodynamic stability (e.g., sucrose) and/or inhibit aggregation between protein molecules (e.g., nonionic surfactants).²¹ Thus, it is important that a poloxamer-based protein drug delivery system be amenable to the inclusion of protein stabilizers. To address this issue we tested the effects of two common protein stabilizers, sucrose and polyethylene glycol (PEG), on the phase transition temperature of various concentrations of poloxamer 407 (Figure 5). Poloxamer 407 alone did not form a gel at concentrations below 20% (w/w). Conversely, in the presence of either PEG or sucrose, gels could be formed at lower poloxamer concentrations. Moreover, the poloxamer transition temperature decreased in the presence of sucrose

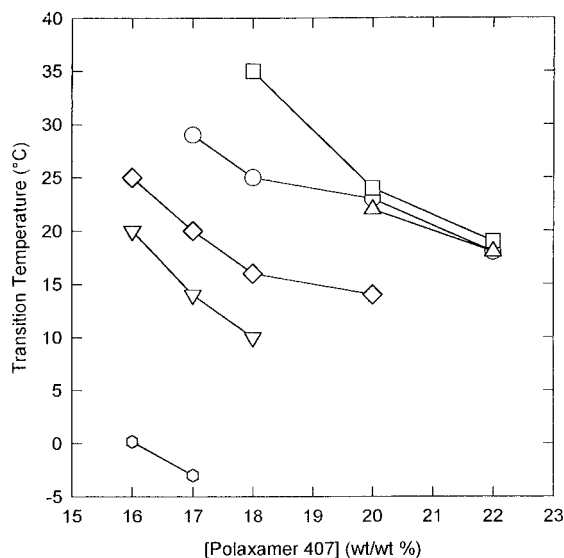


Figure 5—Effect of protein stabilizers on liquid-gel transition temperature of poloxamer 407. Samples were prepared in 30 mM sodium phosphate buffer (pH 7.4 at 23 °C). Absence of symbol at poloxamer 407 concentrations greater than that shown in a given plot (except for poloxamer in buffer alone) is due to insolubility of protein stabilizer and/or poloxamer 407 at that given combination. Absence of symbol at poloxamer concentration less than that shown in a given plot indicates that poloxamer gelling did not occur at that given combination, when tested up to 55 °C. Samples were prepared in buffer alone (triangles), in polyethylene glycol (molecular weight 8000) at 0.1 (circles) and 1.0% (w/v) (squares), or in sucrose at 0.5 M (diamonds), 1.0 M (inverted triangles) and 1.5 M (octagons).

and increased in the presence of PEG. With 0.5 and 1.0 M sucrose a poloxamer system could be obtained with a gel transition temperature convenient for parenteral administration (i.e., gelling between 10 and 25 °C). However, with 1.5 M sucrose the poloxamer transition temperature was at or below 0 °C. This property could be useful if it were necessary to prepare poloxamer gel systems (e.g., contained in an implantable device) in a manufacturing facility and ship to the end user at refrigerated temperatures. The retention of the gel phase would assure homogeneous distribution of the protein precipitates in the gel matrix during shipping. Alternatively, for systems to be mixed at the site of use, the poloxamer-stabilizer combinations with higher transition temperatures would be more convenient.

Obviously, *in vivo* testing of protein suspensions in poloxamer gels will be needed to assess the full utility of this sustained release approach. However, the current *in vitro* studies have documented that this drug delivery system has

many desirable attributes: (1) a high concentration of native protein can be obtained in a system that forms a gel at body temperature and maintains a homogeneous suspension of the protein precipitates; (2) dissolution of the poloxamer gel leads to complete recovery of fully functional protein molecules; (3) preparing the protein at the high concentration desired for sustained delivery leads to an increase in the long-term stability of the protein at 37 °C; and (4) known protein stabilizers can be incorporated into the poloxamer gel and used, as needed, to manipulate the phase transition behavior of the gel.

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