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Liquid Droplet of Protein-Polyelectrolyte Complex for High-Concentration Formulations

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ABSTRACT

The formulation of high-concentration protein solutions is a challenging issue for achieving subcutaneous administration. Previously, we developed a method of precipitation-redissolution using polyelectrolyte as a precipitant to produce protein solutions at high concentrations. However, the redissolution yield of proteins was insufficient. This study aims to optimize the solution conditions for practical applications by combining IgG and poly-L-(glutamic acid) (polyE). A systematic analysis of solution pH and polyE size conditions revealed that an acidic condition favors precipitation, whereas neutral pH values are more effective for the redissolution. We find that the optimal size for polyE ranged from 15,000 to 50,000. This slight modification in the procedure in comparison with previous studies increased the precipitation and redissolution yields to nearly 100%, without irreversible protein denaturation. The fully reversible IgG-polyE complex formed as a droplet structure, which is similar to a condensate of liquid-liquid phase separation. The droplet structure plays an indispensable role in the salt-induced, redissolved state, which is pertinent to the new application that takes advantage of the methods to produce highly concentrated protein solutions.

Introduction

Proteins have been developed as new drugs for a number of indications such as autoimmune diseases and cancers,[1-4] and in the last 20 years, the development of these protein drugs has increased significantly. Currently, the U.S. Food and Drug Administration has approved approximately 200 types of therapeutic antibodies, enzymes, and peptides.[5,6] Therapeutic proteins are usually injected intravenously, but subcutaneous injections have grown in popularity due to patient convenience. Accordingly, a high-concentration protein formulation must be prepared to inject large doses of a therapeutic protein.[7-9] Ultrafiltration is the most widely used industrial method to prepare high-concentration protein formulations.[8,10] Other techniques, such as gelation,[11] crystallization,[12] nanoparticle formation,[13] liquid-liquid separation,[14] and a spray-drying technique have been explored.[15,16] However, these methods have several disadvantages, such as high cost, time-consuming procedures, and possible effects of protein denaturation and aggregation.

We propose a precipitation-redissolution method to prepare high-concentration protein solutions. The mechanisms behind this method are that we produce a protein precipitate using salting-out reagents, such as ammonium sulfate and poly(ethylene glycol). We then increase the concentration of the dispersed protein by solvent exchange using a small volume of the solution.[17] In developing this precipitation method, we were able to prepare therapeutic proteins from high-concentration protein solutions without denaturing the protein.[18] One of the most important results of this study is that poly(amino acids) are a versatile precipitant for dozens of antibodies, therapeutic enzymes, and hormones.[18] Only small quantities of poly (amino acids) are needed to precipitate proteins, compared to ammonium sulfate or poly(ethylene glycol) where large amounts are needed.[18,19] Furthermore, a protein precipitated with a poly(amino acid) is highly tolerant to physicochemical stressors, such as heat, agitation, and oxidation.[18,20,21] Thus, a precipitation

Abbreviations used: CD, circular dichroism; GdnHCl, guanidine hydrochloride; pI, isoelectric point; polyE, poly-L-glutamic acid; polyE1, polyE of 1.5–5.5 kDa; polyE2, polyE of 3–15 kDa; polyE3, polyE of 15–50 kDa; polyE4, polyE of 50–100 kDa; PPC, protein–polyelectrolyte complex.

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method using poly(amino acids) holds great potential as a method for proteins in aqueous solution as well as providing method to increase protein concentration.[22,23]

Figure 1 shows the molecular mechanism of precipitation-redissolution method using poly(glutamic acid).[24-30] Briefly, a protein solution is mixed with a polyelectrolyte solution with a specific pH and ionic strength, producing a cloudy solution. The cloudy solution precipitates easily during either light centrifugation or simply by allowing the mixture to stand.[18,19] The precipitated protein-polyelectrolyte complex (PPC) redissolves easily using a physiological concentration of ions[28,29] because of electrostatic shielding between the protein and polyelectrolyte.[30] The PPC formulation did not affect the therapeutic protein's bioavailability.[19] The driving forces to form the complex, between the protein and the poly(amino acid), depend on the size of the complex, solvent's pH, ionic strength, and temperature. Thus, solution conditions play an important role in the yields of precipitation and redissolution. In a previous study, we investigated precipitation-redissolution under the following conditions: (1) we used poly(glutamic acid) and poly(lysine) to precipitate-redissolve various types of proteins, including monoclonal antibodies and tetrameric enzymes, (2) precipitation conditions included a pH solution value of either 2 units higher or lower than the isoelectric point (p*I*) of the protein, (3) we induced redissolution with a solution of 150-mM NaCl. These conditions resulted in superior performance, as confirmed in a preliminary experiment. However, we cannot confirm the condition for 100% precipitation and redissolution condition for all kinds of proteins.[18]

For practical applications, this study reports the optimal experimental conditions, including the determination of the optimal pH value and poly(amino acid) molecular weight (MW). We used the IgG protein, one of the most important antibodies used in synthesizing pharmaceutical proteins. We used the nontoxic polyelectrolyte poly(glutamic acid) (polyE). A systematic analysis revealed that acidic conditions at nondenaturing pH values were more favorable for precipitation and that at neutral pH, near the IgG isoelectric point, redissolution was far more effective, and finally, polyE, with a MW of 3000-50,000 was an effective precipitant. Furthermore, phase-contrast microscopy revealed that electrostatic interactions stabilize droplet forms but are reversible by salts. On the other hand, irreversible PPC has an amorphous state that is stabilized by both electrostatic and hydrophobic interactions. These results indicate that protein, in the reversible PPC form, keeps its native droplet form, similar to membrane-less organelles or droplets observed in living cells and formed by liquid-liquid phase separation.[31-35] We demonstrate that PPC droplet can be used in new medical applications to stabilize proteins in a concentrated state.

Experimental Section

Materials

Citrate, NaCl, guanidine hydrochloride (GdnHCl), and urea were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Poly-L-glutamic acids (mean MWs: 1500-5500 [polyE1], 3000-15,000 [polyE2], 15,000-50,000 [polyE3], and 50,000-100,000 [polyE4]) were from Sigma Chemical Co. (St. Louis, MO). These chemicals were of high quality analytical grade and were used as received. Human IgG protein was from Equitech-Bio, Inc. (Kernville, TX), dialyzed it to remove the co-solutes, and used it without further purification. IgG used here is a plasma derived protein with p*I* 7.3.

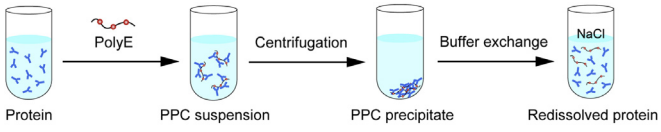

Figure 1. A schematic illustration of the precipitation-redissolution method using poly(glytamic acid) (polyE) as a model.

Preparation of the IgG–polyE Complex

The antibody stock solution was prepared with 6.0-mg/mL IgG in a 10-mM citrate buffer (pH = 4.0-6.0). Aliquots of 100 μL of various precipitant solutions containing 0-3.0 mg/mL polyE (polyE1, polyE2, polyE3, or polyE4) in a 10 mM citrate buffer (pH = 4.0-6.0) were mixed with 50 μL of the antibody stock solution in the corresponding buffer at pH. Samples of the IgG–polyE complex were centrifuged at 9000 × *g* for 5 min at 25°C. Initial and final IgG concentrations were determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Redissolution of the IgG-polyE Complex With NaCl

Aliquots of 100 μL of various precipitant solutions containing 2.25 mg/mL polyE (polyE1, polyE2, polyE3, or polyE4) in a 10-mM citrate buffer (pH = 4.0) or 0.75-mg/mL polyE (polyE1, polyE2, polyE3, or polyE4) in a 10-mM citrate buffer (pH = 5.0) were mixed with 50 μL of the antibody stock solution in the same buffer. Samples of the IgG-polyE complex were centrifuged at 9000 × *g* for 5 min at 25°C. Then, 135 μL of the supernatant was removed, and the same volume of 0 to 333-mM NaCl in a 10-mM citrate buffer (pH = 4.0-5.0) was added to the sample. The final IgG concentrations were determined using the ND-1000 spectrophotometer.

Redissolution of the IgG–polyE Complex With GdnHCl and Urea

Aliquots of 100 μL of precipitant solutions containing 3.0 mg/mL polyE (polyE1, polyE2, polyE3, or polyE4) in a 10-mM citrate buffer (pH = 4.0-5.0) were mixed with 50 μL of the antibody stock solution in the same buffer. Samples of the IgG-polyE complex were centrifuged at 9000 × *g* for 5 min at 25°C. Then, 135 μL of the supernatant was removed and 135 μL of 555 mM GdnHCl, NaCl, or urea was added to the sample in a 10-mM citrate buffer (pH = 4.0-5.0). The final IgG concentrations were determined using the ND-1000 spectrophotometer.

Redissolution of the IgG-polyE Complex by Changing pH

Aliquots of 100 μL of precipitant solutions containing 3.0 mg/mL polyE (polyE2 or polyE4) in a 10-mM citrate buffer (pH = 4.0) were mixed with 50 μL of the antibody stock solution in the same buffer. Samples of the IgG-polyE complex were centrifuged at 9000 × *g* for 5 min at 25°C. Then, 135 μL of the supernatant was removed, and 135 μL of 167-mM NaCl and 1 M NaOH/HCl ([NaOH]/[HCl] = 1-1.9) in a 10-mM citrate buffer (final pH of 4.0) was added to the sample. The final IgG concentrations were determined using the ND-1000 spectrophotometer.

Phase Contrast Microscopy

We imaged PPC morphology formed by different conditions using a phase contrast microscope (Primo Vert; Carl Zeiss, Germany) equipped with an AxioCam ERc5s camera (Carl Zeiss) and Axio Vision software (Carl Zeiss). A 15-μL sample was taken at several pH and polyE MW values from the IgG/polyE dispersion and then placed between glass slides, at room temperature, just before image capture.