Feasibility of Antibody–Poly(glutamic acid) Complexes: Preparation of High-concentration Antibody Formulations and Their Pharmaceutical Properties

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Abstract

Development of high-concentration antibody formulations for subcutaneous administration remains challenging. Recently, a precipitation–redissolution method was proposed to prepare suspensions or precipitates of salt-dissociable protein–poly(amino acid) complexes. To elucidate the utility of this method for protein therapy, we investigated the feasibility of a precipitation–redissolution method using poly(amino acid) for high-concentration antibody formulation. Omalizumab and adalimumab formulations of 150 mg/mL could be prepared using poly-L-glutamic acid (polyE) from low-concentration stock solutions. Enzyme-linked immunosorbent assay (ELISA), circular dichroism (CD), and size exclusion chromatography (SEC) revealed that the formation of antibody–polyE complex and precipitation–redissolution process did not significantly affect the immunoreactivity or secondary structure of the antibodies. The precipitation–redissolution method was less time-consuming and more effective than lyophilization–redissolution, evaporation–redissolution, and ultrafiltration from the viewpoint of final yield. Scalability was confirmed from 400 μL to 1.0 L. The general toxicity and pharmacokinetic profiles of the antibody–polyE complex formulations were similar to those of conventional antibody formulations. These results suggested that the precipitation–redissolution method using poly(amino acid) has great potential as a concentration method for antibody formulation and medicinal use.

Keywords

Complexation, Dissolution, Polyelectrolytes, Precipitation, Formulation, Suspensions

Abbreviations

CD, circular dichroism; ELISA, enzyme-linked immunosorbent assay; FcεRIα, high-affinity immunoglobulin ε receptor subunit α; HPLC, high-performance liquid chromatography; HRP,

43 horseradish peroxidase; IgE, immunoglobulin E; IgG, immunoglobulin G; MOPS, 3-(*N*-
44 morpholino)propanesulfonic acid; p*I*, Isoelectric point; polyE, poly-L-glutamic acid; PPC, protein–
45 polyelectrolyte complex; SEC, size exclusion chromatography; TMB, 3,3',5,5'-tetramethylbenzidine;
46 TNF-α, tumor necrosis factor α; Tris, Tris(hydroxymethyl)aminomethane

Introduction

Monoclonal antibodies have generated considerable interest as biopharmaceuticals over the past two decades because of their high target specificity and biocompatibility. At present, over 20 types of monoclonal antibody have been approved by the US Food and Drug Administration[1] and used for several types of disease, such as cancer and autoimmune diseases.[2–5] Despite advances in antibody drug development, the routes of administration for these antibodies remain challenging. The major route of delivery for antibodies has been intravenous administration due to the high bioavailability.[6] In contrast, subcutaneous administration has been in great demand as an alternative route that allows at-home administration by patients and improves compliance rates.[7–10] However, the desirable concentration of antibodies for subcutaneous administration is usually above 100 mg/mL with a volume limitation of 1.5 mL.[9, 10]

Several methods have been developed to obtain high-concentration protein solutions that would enable proteins to be used in formulations, for example with additives such as arginine or other amino acids,[11, 12] ultrafiltration,[13] gelation,[14] crystallization,[15] liquid–liquid phase separation,[16] and spray drying.[6, 17] However, these methods are still time-consuming and costly. Methods for the suspension or precipitation of protein have also been reported for use in concentrated protein formulations.[18, 19] If the precipitates of protein can be fully resolubilized by the simple method, such precipitates can be used as concentrated protein solutions. Recently, we have demonstrated the complex precipitation–redissolution method with poly(amino acid) as a precipitant.[20] Briefly, charged polyelectrolytes, including poly-L-lysine and poly-L-glutamic acid, interact strongly with complementary charged proteins through multiple electrostatic interactions, resulting in the formation of a protein–polyelectrolyte complex (PPC),[20–26] which can often be precipitated depending on the experimental conditions, such as pH, ionic strength, and stoichiometric ratio.[24, 27, 28] PPC precipitates are then redissolved by the addition of buffer with high ionic strength such that the final concentration reaches 150 mM, which corresponds to physiological conditions.[20, 28, 29] This simple system has been applied successfully for several types of therapeutic protein, including

enzymes, antibodies, and peptide hormones.[20] However, the utility of this precipitation–redissolution method for protein therapy is still unclear.

In this study, we demonstrated the feasibility of the complex precipitation–redissolution method using poly(amino acid) for high-concentration antibody formulation. As described below, antibody formulations with concentrations over 100 mg/mL of adalimumab for autoimmune disease and omalizumab for allergic asthma could be prepared by addition of poly-L-glutamic acid (polyE). The formation of antibody–polyE complex and precipitation–redissolution processes did not significantly change the immunoactivity or secondary structure of the antibodies, and did not result in undesirable aggregation. Comparison of time required, yield, and aggregate ratio indicated that the precipitation–redissolution method was more effective for application than the conventional concentration methods, including lyophilization–redissolution, evaporation–redissolution, and ultrafiltration. The precipitation–redissolution method was successfully performed from a scale of 400 μL to 1.0 L, indicating that this method could be scaled up to 2500-fold. Finally, the general toxicity and pharmacokinetic profiles of the antibody–polyE complex formulation were similar to those of conventional antibody formulations. These results suggested that this simple method represents a new strategy for preparing high-concentration antibody formulations, and we expect that this method and complex formulations would be applicable for medicinal use.

Experimental Section

Materials

Adalimumab was obtained from transfected Chinese hamster ovary (CHO) cell cultures and purified on a protein-A column. Omalizumab was purchased from Novartis Pharma KK (Tokyo, Japan) and purified on a protein-A column to remove the additives. Citrate, sodium chloride (NaCl), sodium phosphate, and potassium chloride (KCl) were from Kanto Chemical Co., Inc. (Tokyo, Japan). Polysorbate 20 was from Wako Pure Chemical. Ind., Ltd. (Osaka, Japan). 3-(*N*-morpholino)Propanesulfonic acid (MOPS) and Blocking One were from Nacalai Tesque, Inc. (Kyoto, Japan). Tris(hydroxymethyl)aminomethane (Tris) was from Bio-Rad Laboratories (Hercules, CA). Biotinylated anti-human IgG monoclonal antibody, rat IgG, and poly-L-glutamic acid sodium salt with average molecular weights of 3000 – 15000 Da (polyE1) and 50000 – 100000 Da (polyE2) were from Sigma Chemical Co. (St. Louis, MO). Human IgE was from Abcam (Cambridge, MA). Biotinylated anti-human IgE monoclonal antibody was from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Human tumor necrosis factor α (TNF-α) was from Gibco Life Technologies Ltd. (Grand Island, NY). Human high-affinity immunoglobulin epsilon receptor subunit alpha (FcεRIα) was from Sino Biological Inc. (Beijing, China). Streptavidin-labeled horseradish peroxidase (Avidin-HRP) was from Thermo Fisher Scientific (Waltham, MA). 3,3',5,5'-Tetramethylbenzidine (TMB) was from KPL (Gennep, the Netherlands). Human IgG1 Therapeutic EIA Kit was from Cayman Chemical Co. (Ann Arbor, MI). Sucrose was from Dai-Nippon Meiji Sugar Co., Ltd. (Tokyo, Japan). L-Histidine hydrochloride was from Mitsubishi Tanabe Pharma Co. (Osaka, Japan). L-Histidine was from Kyowa Hakko Bio Co., Ltd. (Tokyo, Japan). Glucose 50% injection was from Terumo Co. (Tokyo Japan). These chemicals were of high-quality analytical grade and were used as received.

Preparation of antibody–poly(amino acid) complex and redissolution

The procedure of precipitation and redissolution of antibody–polyE1 complex was described as follows. *Step 1:* The antibody stock solutions containing low (1.0 mg/mL) and high (30 mg/mL)

concentration antibodies in 10 mM buffer (MOPS at pH 6.5, for adalimumab; citrate at pH 5.5 for omalizumab) were prepared. *Step 2:* Aliquots of 200 μL of various concentrations of polyE1 in 10 mM buffer (MOPS at pH 6.5, for adalimumab; citrate at pH 5.5 for omalizumab) were mixed with aliquots of 200 μL antibody stock solution in the same buffer. *Step 3:* The samples of antibody–polyE mixture were centrifuged at 1000 × g for 3 minutes at 25°C. *Step 4:* The supernatant of 370 μL was removed and precipitates were resuspended by vortexing. The nominal value of the samples was 30 μL. *Step 5:* 10 μL of 600 mM NaCl in 10 mM buffer was added. The concentrations of antibodies for each step were determined from the absorbance at 280 nm using a spectrometer (SpectraMax Plus384; Molecular Devices Co., Ltd., Sunnyvale, CA). The immnoreactivity, secondary structure, and aggregation ratio were determined as described below.

In addition to the above original scale experiments (400 μL), both 50-fold scale up (20 mL; medium scale) and 2500-fold scale up (1.0 L; large scale) experiments were performed. Table 3 shows the volumes of these scales at Step 2. A concentration of 0.2 mg/mL polyE1 solution was mixed with 2.0 mg/mL adalimumab solution, and then precipitation–redissolution procedures were performed as described above. It is note that the final concentration of antibody at Step 5 is five times higher than that at Step 1. The containers and centrifuges used for each scale were 1.5 mL centrifuge tubes (MS-4215M; Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and Kubota 3740 for mini-scale, 50 mL centrifuge tubes (430829; Corning Inc., Corning, NY) and Kubota 5220 for medium-scale, and 1 L Nalgene Polycarbonate Centrifuge Bottles (3122-1010; Thermo Fisher Scientific Inc., Waltham, MA) and Kubota 9900 for large-scale. All centrifuges were purchased from Kubota Co. (Tokyo, Japan).

Concentration of antibody solutions by several methods

To compare the efficiencies of the concentration methods, 150 mg/mL omalizumab and adalimumab solutions were prepared by the following methods. *i) Precipitation–redissolution*: 250 μL of 0.15 mg/mL polyE1 in 10 mM buffer (MOPS, pH 6.5, for adalimumab; citrate, pH 5.5 for omalizumab) and 250 μL of 30 mg/mL antibodies in same buffer. The samples of antibody–polyE

mixture were centrifuged at 1000 × g for 3 minutes at 25°C. 462.5 μL of supernatant was removed and 12.5 μL of 600 mM NaCl in 10 mM buffer was added. The final volume of the concentrated antibodies was 50 μL. *ii) Lyophilization−redissolution*: 250 μL of 30 mg/mL antibodies in 10 mM buffer were added to vials and frozen onto a pre-cooled shelf (Drying Chamber DRC-1100; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at -40 °C for 2 hours. Subsequently, the frozen samples were primary dried at -20 °C for 12 hours, and were secondary dried at 25 °C for 4 hours under 0.67 Pa. After lyophylization, 50 μL of 10 mM buffer were added to the vials to redissolve the lyophilized cakes. *iii) Evaporation−redissolution*: 250 μL of 30 mg/mL antibodies in 10 mM buffer were added to tubes and evaporated in a rotatory evaporator (Centrifugal vaporizer CVE-100D; Tokyo Rikakikai). After evaporation, 50 μL of 10 mM buffer were added to the tube to redissolve the cakes. *iv) Ultrafiltration*: 250 μL of 30 mg/mL antibodies in 10 mM buffer were put into an ultrafiltration unit with a 50 kDa cut-off (UFC5050; Millipore Co., Ltd., Billerica, MA) and centrifuged at 14000 × *g* at 4°C until the volume was decreased to 50 μL. The time required, final concentration, and aggregation ratio were determined as described below.

Immunoassay

The immunoreactivities of omalizumab were measured by competitive enzyme-linked immunosorbent assay (ELISA) to confirm structural integrity of redissolved antibody. The wells of 96-well ELISA microplates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) were coated with human FcεRIα, and the plates were incubated at 4°C overnight. The wells were washed a total of five times by addition of washing buffer containing 24.8 mM Tris, 136.9 mM NaCl, 2.7 mM KCl, and 0.1% polysorbate 20 (pH 7.4). The wells were then blocked with Blocking One at room temperature for 1 hour. After washing of the wells, solutions containing human IgE at a constant concentration and omalizumab of different concentrations were added to the wells and incubated at room temperature for 1 hour. After washing of the wells, 100 μL of biotinylated anti-human IgE monoclonal antibody was added to each well and plates were incubated at room temperature for 1 hour. After washing of

the wells, 100 μL of streptavidin-labeled horseradish peroxidase was added to each well and plates were incubated at room temperature for 30 minutes. After washing of the wells, 100 μL of TMB was added to each well and plates were incubated at room temperature for more than 15 minutes. Finally, 100 μL of 0.1% HCl was added and the absorbance at 450 nm was determined using a microplate reader (SpectraMax Plus384; Molecular Devices).

The immunoreactivities of adalimumab were measured by ELISA. Two types of adalimumab with or without precipitation–redissolution method were used, as described above. The wells of polystyrene 96-well ELISA microplates were coated with human TNF-α, and the plates were incubated at 4°C overnight. After washing of the wells, Blocking One were added and plates were incubated at room temperature for 1 hour. After washing of the wells, 100 μL of adalimumab solutions with or without precipitation–redissolution process of different concentrations were added to the wells and plates were incubated at room temperature for 1 hour. After washing of the wells, 100 μL of biotinylated anti-human IgG monoclonal antibody was added to each well and plates were incubated at room temperature for 1 hour. Subsequent procedures were as described above.

Circular dichroism

Circular dichroism (CD) experiments were performed using a spectropolarimeter (J-720; Japan Spectroscopic Co., Ltd., Tokyo, Japan). Two types of antibodies with or without precipitation–redissolution method were used, as described above. The samples containing 1.0 mg/mL antibodies in 10 mM buffer were added to a 1-mm path length quartz cuvette, and then CD spectra of antibodies were measured at 25°C. Scans were taken from 195 to 250 nm at a rate of 100 nm/min with a sample interval of 0.2 nm, 0.25 s response, and 1.0 nm bandwidth. 50 scans were averaged. Obtained far-UV CD spectra were plotted in the wavelength range of 210 – 250 nm because of the large noise below 210 nm. The CD spectra of the samples were corrected by subtracting the corresponding spectra of the buffers in the absence of antibodies.

Size exclusion chromatography (SEC)

Two types of antibodies with or without precipitation–redissolution method were used, as described above. The samples containing 1.0 mg/mL antibodies in 10 mM buffer were centrifuged at 14000 × *g* for 10 minutes, and then the supernatant was analyzed by SE-HPLC. The analysis was performed on an SEC column (Yarra 3u SEC-3000; Phenomenex Inc., Torrance, CA) on the HPLC system (LC-20A; Shimadzu Corp., Kyoto, Japan) at a constant flow rate of 0.5 mL/min. The mobile phase consisted of 100 mM sodium phosphate buffer and 0.5 M NaCl (pH 6.8).

General toxicity test

We evaluated general toxicity tests using two types of samples, IgG–polyE2 complex formulation and conventional IgG formulation as a control. Each formulation was prepared as described below. *IgG–polyE2 complex formulation:* Aliquots of 4.5 mL of 0.24 mg/mL polyE2, 8.0% glucose, and 10 mM citrate (pH 5.0) were added to 4.5 mL of 2.0 mg/mL IgG and 10 mM citrate (pH 5.0) aseptically. The samples of antibody–polyE mixture were centrifuged at 1000 × g for 3 minutes at 25°C. 8.1 mL of supernatant was removed and precipitates were resuspended by vortexing. The nominal value of the samples was 900 μL containing 10 mg/mL IgG, 1.2 mg/mL polyE2, 4.0 % glucose, 10 mM citrate (pH 5.0). *Conventional IgG formulation:* Aliquots of 500 μL of 10 mg/mL IgG, 4.0 % glucose, 10 mM citrate (pH 5.0) was prepared.

Five-week-old Crl:CD (SD) rats were from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The samples were injected subcutaneously into the backs of six rats in two groups at 50 mg/kg (333 nmol/kg). The body weights of the rat were measured for 0 – 14 days. Weight checks of the organs (heart, lung, liver, spleen, and kidney), pathology assessment, and blood test were performed for 1 and 2 weeks after injection. In the pathology assessment, the above organs and administration site were assessed. In the blood test, the following items were measured: white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), activated partial thromboplastin time (APTT), prothrombin time (PT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine

aminotransferase (ALT), lactase dehydrogenase (LDH), total bilirubin (TB), total protein (TP), albumin (ALB), total cholesterol (TC), triglyceride (TG), phospholipid (PL), glucose (GLC), blood urea nitrogen (BUN), creatinine (CRE), inorganic phosphorus (Pi), calcium (Ca), sodium (Na), potassium (K), and chlorine (Cl).

Pharmacokinetic profile

We evaluated pharmacokinetic studies using two types of samples, omalizumab−polyE2 complex formulation and conventional omalizumab formulation as a control. Each formulation was prepared as described below. *Omalizumab−polyE2 complex formulation:* Aliquots of 2.5 mL of 0.24 mg/mL polyE2, 8.0% glucose, and 20 mM citrate (pH 5.5) were added to 2.5 mL of 2.0 mg/mL omalizumab and 20 mM citrate (pH 5.5) aseptically. The samples of antibody−polyE mixture were centrifuged at 1000 × g for 3 minutes at 25°C. 4.5 mL of supernatant was removed and precipitates were resuspended by vortexing. The nominal value of the samples was 500 μL containing 10 mg/mL omalizumab, 1.2 mg/mL polyE2, 4.0 % glucose, 20 mM citrate (pH 5.5). *Conventional omalizumab formulation:* Aliquots of 500 μL of 10 mg/mL omalizumab, 10.4% sucrose, 0.20% L-histidine hydrochloride, 0.13% L-histidine, and 0.04% polysorbate 20 was prepared. Sterile syringes were filled with the samples. Each sample, conventional, and complex formulation of omalizumab was injected subcutaneously into the backs of three rats at 10 mg/kg (66.7 nmol/kg), and the animals were kept in cages for 0 – 22 days. Blood was collected from the tail vein at 0 – 22 days after injection. Finally, blood concentration of omalizumab was measured using a Human IgG1 Therapeutic EIA Kit (Cayman Chemical Co., Ann Arbor, MI). All animal experiments were approved by the institutional animal care and use committee of Terumo Corp. before the experiments.

Results

Preparation of high-concentration antibody solution by the precipitation–redissolution method

Previously, we reported a precipitation–redissolution method using poly(amino acid), which enables the preparation of salt-dissociable precipitates of protein–poly(amino acid) complexes.[20] To evaluate the efficiency of antibody concentration by the precipitation–redissolution method, we selected two antibody drugs: adalimumab (p*I* = 8.7; for autoimmune disease) and omalizumab (*p*I =7.6; for allergic asthma). Anionic poly-L-glutamic acid with average molecular weight of 3000 – 15000 Da (polyE1) was selected for cationic antibodies at pH 5.5 – 6.5, because protein–poly(amino acid) complexes were formed only when poly(amino acid) had a charge complementary to that of the protein at experimental pH. Two types of stock solution containing low (1.0 mg/mL) or high (30 mg/mL) concentrations of antibodies were mixed with the polyE1 solutions. The yield of antibody under the milder condition (1,000 g for 3 minutes at 25°C) was identical to that under the harder condition (15,000 g for 20 minutes at 25°C). Thus, the centrifugation condition was selected at 1,000 g for 3 minutes at 25°C in this study. Figure 1 shows the final concentration of antibodies at Step 5. In Figure 1A, final concentration of antibodies increased sharply with increasing concentration of polyE1, and reached a plateau at around 0.05 eq., which was consistent with the previous report.[20] Similar profiles were observed even at high concentration of stock solution (Figure 1B); final concentration values of omalizumab and adalimumab reached about 150 mg/mL at 0.05 eq. The yields of these antibodies were also about 100% even at high protein concentration (150 mg/mL) as well as low concentration (5 mg/mL).

We next characterized the activity and structure of the redissolved antibodies. As shown in Figures 2A and 2B, the immunoreactivities of redissolved omalizumab and adalimumab were identical to those of native antibodies; the 50% inhibitory concentration values (IC_{50}) of omalizumab and the 50% effective concentration values (EC_{50}) of adalimumab were equivalent to those without polyE1 (Table 1, Figure 2A,B). Thus, the activities of the antibodies were not altered by the concentration and redissolution process. In addition, far-UV circular dichroism (CD) spectra of redissolved antibodies were identical to those of the native antibodies (Figure 2C,D), although

slight changes were observed in the far-UV CD spectra of adalimumab at 218 nm. These results suggested that the secondary structure of the antibodies were significantly maintained even in the redissolved state. Furthermore, SEC analysis indicated that the soluble aggregates were not increased in the redissolved antibody solutions (Figure 2E,F). These results suggested that redissolved antibodies retained the original activity, structure, and aggregation properties.

Feasibility studies of precipitation–redissolution method

We performed feasibility studies of the precipitation–redissolution method using poly(amino acid) to confirm its utility for pharmaceutical applications. First, the efficiency of the precipitation–redissolution method was compared to three conventional concentration methods, i.e., lyophilization–redissolution, evaporation–redissolution, and ultrafiltration. As expected, we obtained 150 mg/mL antibody solutions by the precipitation–redissolution method (Table 2). On the other hand, the final concentrations of antibody solutions obtained by the conventional methods were 72.7 – 136 mg/mL, suggesting that some antibody was lost in the concentration process in these methods. The time required for the precipitation–redissolution method was about 2 hours, which was shorter than those of lyophilization and evaporation (Table 2). Furthermore, SEC analysis revealed that soluble aggregates did not increase in the concentrated antibody solutions prepared by the precipitation–redissolution method and ultrafiltration, but increased in those prepared by lyophilization and evaporation (Table 2). These results suggested that the precipitation–redissolution method is a good alternative to the well-known concentration methods.

To examine whether the precipitation–redissolution method could be scaled up, we prepared 10 mg/mL adalimumab from 2.0 mg/mL adalimumab by the addition of 0.1 mg/mL polyE1 at three different scales−original-scale (400 μL), 50-fold scale up (20 mL; medium-scale), and 2500-fold scale up (1.0 L; large-scale). Similar to the original-scale, the adalimumab–polyE mixed solution formed a complex and was suspended immediately at the medium- (data not shown) and large-scales (Figure 3 Step 2) At the large scale, the complex was spontaneously precipitated almost completely in 2 hours at Step 2. After centrifugation (Step 3), the supernatant was removed and resuspended

by mild agitation (Step 4). Finally, the suspension was fully dissolved by the addition of NaCl in the same buffer (Step 5). ELISA and SEC analysis revealed that the immunoreactivities and the amounts of soluble aggregates of redissolved adalimumab at medium and large scales were similar to those at the original-scale (Table 3), suggesting that the properties of redissolved antibody were independent of the scale. Thus, we confirmed that the precipitation–redissolution method could be scaled up by 2500-fold.

Finally, to test the potential of antibody–poly(amino acid) complexes for medicinal use, general toxicity tests of the antibody–poly(amino acid) complex suspension (Step 4) were performed. Anionic poly-L-glutamic acid with average molecular weight of 50000 – 100000 Da (polyE2) was selected because we speculated that poly(amino acids) with larger molecular weight would have longer blood half-lives and stronger influence.[30] In addition, we selected rat IgG (pI ≈ 7.0) to avoid immune reaction in rats. Suspensions of the complex containing 10 mg/mL rat IgG and 1.2 mg/mL polyE2 (Step 4) were prepared by the precipitation–redissolution method, and then injected subcutaneously into rats at 50 mg/kg (333 nmol/kg). Comparison of administration of conventional IgG formulation and IgG–polyE2 complex formulation indicated no significant difference in rat body weight or the weights of several organs (heart, lung, liver, spleen, and kidney) between the two groups (Figure 4A,B). In addition, no significant differences in pathological findings or blood composition were observed between the two groups (data not shown). These results suggested that antibody–polyE complexes did not have general toxicity in these experimental animals. Furthermore, we performed pharmacokinetic analysis to compare the behaviors of the conventional omalizumab and omalizumab–polyE2 complex formulation in blood. Figure 4C shows the plasma concentration of omalizumab–time curve in rats after subcutaneous administration. The curve of omalizumab–polyE2 complex (Step 4) corresponded to that of the conventional omalizumab. These results indicated that the antibody–polyE complex formulation did not affect the bioavailability of the therapeutic antibody.

Discussion

High-concentration (> 100 mg/mL) antibody formulations are indispensable for subcutaneous administration because of the limitation of injection volume (typically 1.5 mL).[9, 10] As demonstrated above, 150 mg/mL antibody formulations were successfully prepared from 30 mg/mL antibody solutions using the precipitation–redissolution method with poly(amino acid) (Figure 1). It is emphasized that the procedures involved in the precipitation–redissolution method are quite simple: (i) polyE is added to the antibody solution, resulting in precipitation of the antibody–polyE complex; (ii) the antibody–polyE precipitates can be suspended easily; (iii) the antibody–polyE complexes can be redissolved with saline solution. Note that the protein concentration was changed from 1.0 mg/mL to 30 mg/mL, indicating that the precipitation–redissolution method could be used for preparation of desirable concentrations of antibody formulations. In addition, the precipitation–redissolution method would be applicable for several types of therapeutic protein, including enzymes, antibodies, and peptide hormones.[20]

It is possible that quality of redissolved antibody formulation is comparable to the conventional antibody formulation. ELISA revealed that the immunoreactivity of redissolved antibodies was similar to that of native antibodies (Table 1, Figure 2A and 2B). Far-UV CD spectra suggested that the secondary structure of antibodies did not significantly change by the precipitation–redissolution process (Figure 2C and 2D). In addition, SEC analysis showed that soluble aggregates of redissolved antibodies were similar to those of the native antibodies (Figure 2E and 2F). Furthermore, pharmacokinetic profile of omalizumab/polyE complex formulation was similar to that of the conventional omalizumab formulation (Figure 4C). Although further investigation of structural and colloidal properties remains unclear, these *in vitro* and *in vivo* evaluations supported that the redissolved antibodies are likely to be applicable for protein therapy.

Comparison with conventional concentration methods indicated significant advantages of the precipitation–redissolution method with regard to time required and yield (Table 2). The precipitation–redissolution method successfully prepared antibody solutions of about 150 mg/mL within 2 hours, whereas lyophilization–redissolution, evaporation–redissolution, and ultrafiltration

failed to reach this concentration (Table 2). Although visible aggregates were not observed in the adalimumab concentrated by the conventional methods, the final concentrations were lower than 150 mg/mL. The loss of adalimumab in these methods were due to the unfavorable influences of these processes, such as adsorption to the container or ultrafiltration membrane[31–34] or the generation of water-ice crystals at the periphery of the container.[35] In contrast, the final concentrations of omalizumab obtained by the conventional methods were significantly lower than that achieved by the precipitation–redissolution method (Table 2), Visible aggregates were observed in the omalizumab solution during the concentration process, suggesting that the aggregates decreased the yield of concentrated omalizumab. Thus, the precipitation–redissolution method enables prompt and successful concentration of antibodies without soluble aggregates.

The precipitation–redissolution method has another significant advantage with regard to scalability. The adalimumab solutions with different volume scale were successfully concentrated 5-fold without loss of quality (Table 3). Interestingly, centrifugation is not always necessary to prepare highly concentrated antibody formulations on a large scale. As shown in Figure 3, the adalimumab–polyE complex was formed and the solution was suspended immediately. The suspension began to precipitate spontaneously a few minutes later and precipitated almost completely within 2 hours (Step 2). The supernatant of the precipitation solution without centrifugation did not contain any antibody (data not shown). The volume of supernatant collected was 0.98 L, meaning that the theoretical maximum concentration of redissolved adalimumab was >5-fold even without centrifugation. This is important for the application of the precipitation–redissolution method for industrialization, e.g., to reduce the costs associated with centrifuges.

Poly(amino acids) are not listed on the Inactive Ingredient Search for Approved Drug Products of the US Food and Drug Administration, although poly(amino acids) have been used as biocompatible materials.[36–39] Nevertheless, *in vivo* tests revealed that the general toxicity and pharmacokinetic profile of the antibody–polyE complex formulations were identical to those of the conventional antibody formulation (Figure 4). The antibody–polyE complex could be easily dissociated by saline solution; the salts at physiological ionic concentration (150 mM) shielded

the electrostatic interaction between antibody and polyE and then dissociated the precipitable complexes.[28, 29] Accordingly, it was suggested that the suspension of antibody–polyE complex injected into rats was immediately dissolved to antibody and polyE at physiological ionic strength in the subdermal space. Therefore, we concluded that the high-concentration antibody–poly(amino acid) complex has biocompatibility for application in protein therapy.

In summary, this study indicated the feasibility of the precipitation–redissolution method for preparation of antibody–poly(amino acid) complex formulations. This simple, quick, and scalable method allows the formation of high-concentration protein solutions without loss of structure or function. More importantly, antibody–polyE complex formulations have the same level of biocompatibility as conventional antibody formulations. Due to its several advantages, the precipitation–redissolution method could be beneficial in medical applications. We expect that protein–poly(amino acid) complex formulations prepared by this method could be used in protein therapy.

Acknowledgment

This study was supported financially by University of Tsukuba and Terumo Corp.

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Table Legends

Table 1. Immunoreactivities of redissolved antibodies

Table 2. Comparison of the precipitation–redissolution method with conventional concentration methods

Table 3. Scaling up of the precipitation–redissolution method for preparation of 10 mg/mL adalimumab from 2.0 mg/mL stock solution

Figure Legends

Figure 1. Precipitation–redissolution profiles of antibodies by polyE1. Various concentrations of polyE1 were added to stock solutions containing 1.0 mg/mL (low) (A) or 30 mg/mL (high) (B) antibodyes in 10 mM buffer. The final concentrations of antibodies at Step 5 in the presence of polyE1 were measured. The final concentration of antibodies in the absence of polyE1 was lower than that of stock solution due to the dilution of precipitation–redissolution process. Closed circles, omalizumab; open circles, adalimumab.

Figure 2. Characterization of omalizumab (A,C,E) and adalimumab (B,D,F) with or witout precipitation−redissolution method. (A,B) Binding curve of antibodies. dashed lines; native antibodies, open circles; low-concentration redissolved antibodies and closed circles; high-concentration redissolved antibodies. (C,D) Far-UV CD spectra of antibodies. solid lines; native antibodies and dushed lines; high-concentration redissolved antibodies. (E,F) SEC chromatograms of antibodies, solid lines; native antibodies, dotted lines; low-concentration redissolved antibodies ans dushed lines; high-concentration redissolved antibodies, arrowhead; monomer peak and arrow; aggregate peak.

Figure 3. Outline of precipitation–redissolution method for adalimumab at the scale of 1.0 L. Aliquots of 0.5 L of 0.2 mg/mL polyE1 solution in 10 mM MOPS (pH 6.5) was added to 0.5 L of stock solution containing 2.0 mg/mL adalimumab in the same buffer (Step 1), and antibody–polyE complex suspension was formed immediately (Step 2). After centrifugation (Step 3), 0.91 L of supernatant was removed and the precipitate was resuspended by mild agitation (Step 4) followed by addition of 0.01 L buffer containing NaCl (Step 5).

519 **Figure 4.** (A,B) General toxicity tests in rats; body weight of rats (A) and weight of organs (B).
520 Conventional IgG formulation, closed symbols; IgG-polyE2 complex fomulation, open symbols. (C)
521 Plasma concentration of omalizumab–time curve in rats after subcutaneous administration.
522 Conventional omalizumab formulation, open circles; omalizumab–polyE2 complex formulation,
523 closed circles.

Tables

Table 1. Immunoreactivities of redissolved antibodies

Antibody	Concentration	$Activity_{control}$	$Activity_{Step5}$
Omalizumab	Low	3.99 (nM) [a]	3.92 (nM) [a]
	High		3.77 (nM) [a]
Adalimumab	Low	327 (pM) [b]	321 (nM) [b]
	High		309 (pM) [b]

[a] 50% inhibitory concentration.

[b] 50% effective concentration.

Table 2. Comparison of the precipitation–redissolution method with conventional concentration methods

		Adalimumab		Omalizumab	
Concentration method	Time Require (hours)	Final concentration (mg/mL)	Aggregation peak ratio (%)	Final concentration (mg/mL)	Aggregation peak ratio (%)
Precipitation–redissolution	2	151	9.67	154	3.98
Lyophilization-redissolution	18	136	12.7	86.0	7.35
Evapolation-redissolution	5	133	19.4	96.1	8.61
Ultrafiltration	2	131	7.29	72.7	3.58

6 **Table 3.** Scaling up of the precipitation–redissolution method for preparation of 10 mg/mL adalimumab from 2.0 mg/mL stock solution

Scale	Final concentration (mg/mL)	Immunoreactivity (pM)	Aggregate peak ratio (%)
Mini (400 μL)	10.2	302	8.09
Medium (20 mL)	10.1	317	8.42
Large (1.0 L)	10.4	289	8.52

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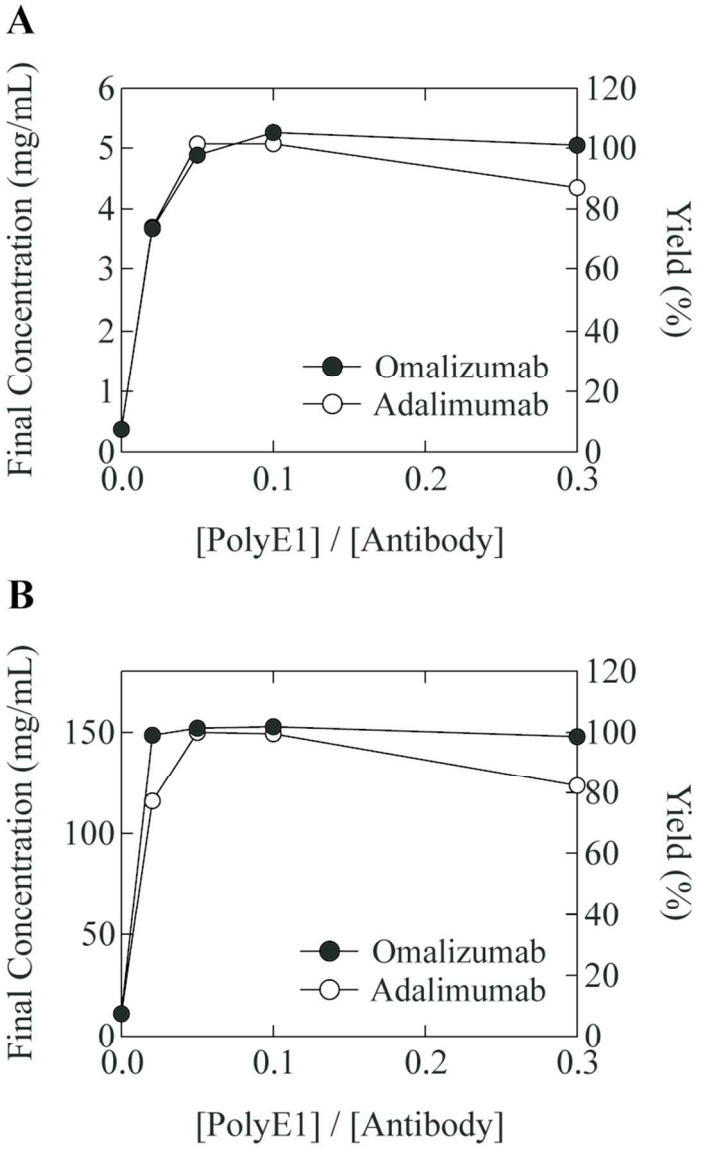

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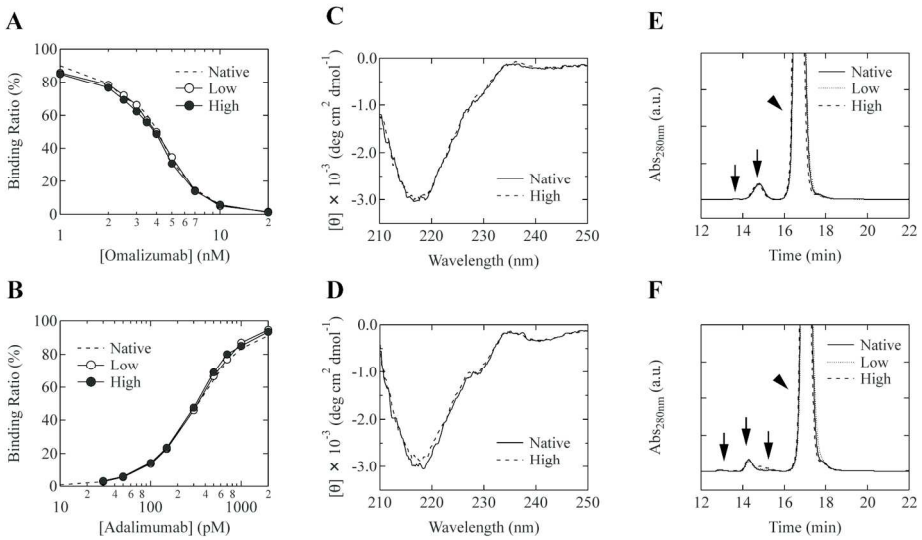

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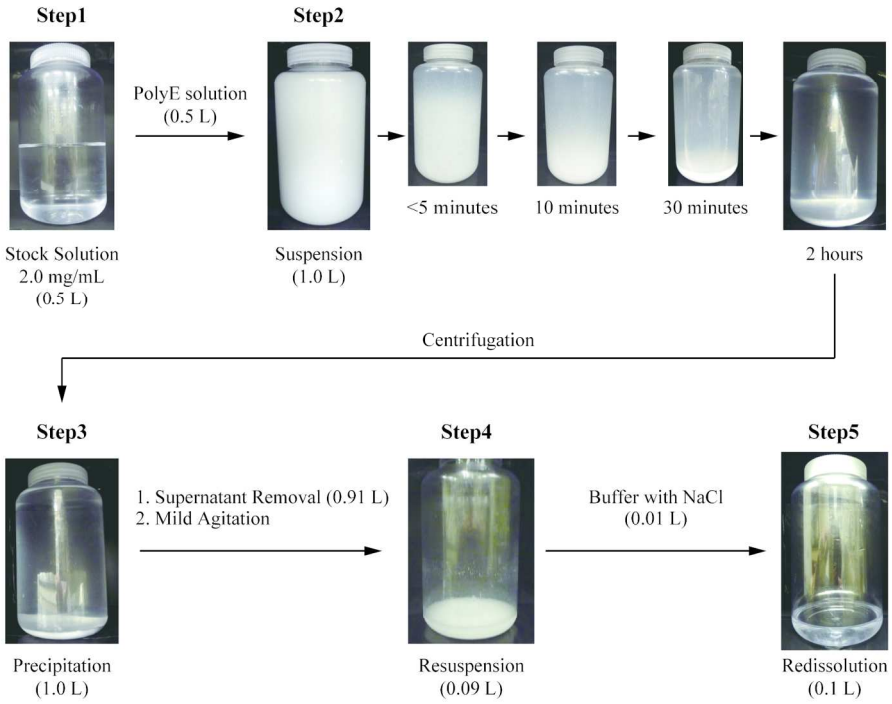

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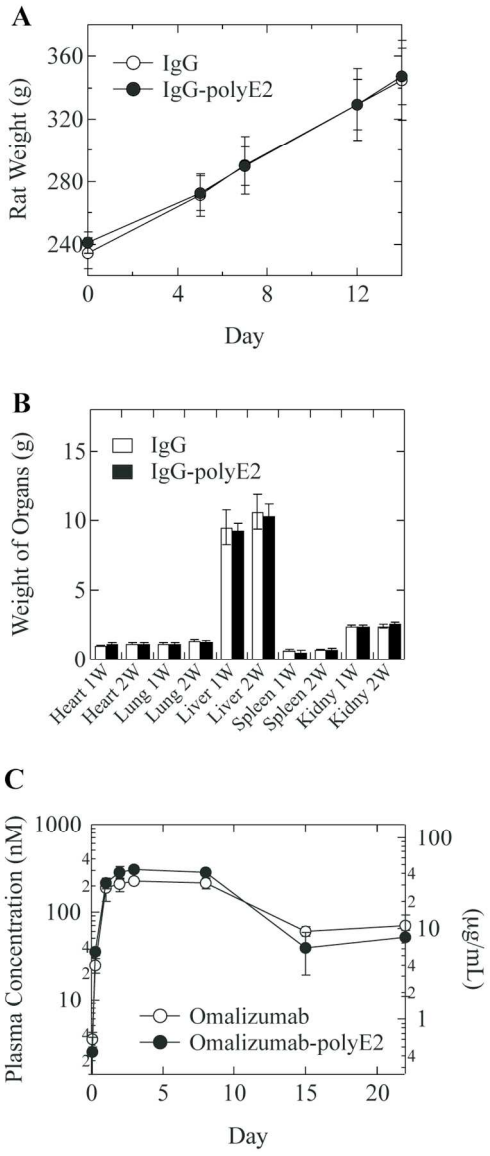

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