

Feasibility of Antibody Poly(Glutamic Acid) Complexes: Preparation of High-Concentration Antibody Formulations and Their Pharmaceutical Properties

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17 **Abstract**

18 Development of high-concentration antibody formulations for subcutaneous administration
19 remains challenging. Recently, a precipitation–redissolution method was proposed to prepare
20 suspensions or precipitates of salt-dissociable protein–poly(amino acid) complexes. To elucidate the
21 utility of this method for protein therapy, we investigated the feasibility of a precipitation–
22 redissolution method using poly(amino acid) for high-concentration antibody formulation.
23 Omalizumab and adalimumab formulations of 150 mg/mL could be prepared using poly-L-glutamic
24 acid (polyE) from low-concentration stock solutions. Enzyme-linked immunosorbent assay (ELISA),
25 circular dichroism (CD), and size exclusion chromatography (SEC) revealed that the formation of
26 antibody–polyE complex and precipitation–redissolution process did not significantly affect the
27 immunoreactivity or secondary structure of the antibodies. The precipitation–redissolution method
28 was less time-consuming and more effective than lyophilization–redissolution, evaporation–
29 redissolution, and ultrafiltration from the viewpoint of final yield. Scalability was confirmed from
30 400 μ L to 1.0 L. The general toxicity and pharmacokinetic profiles of the antibody–polyE complex
31 formulations were similar to those of conventional antibody formulations. These results suggested
32 that the precipitation–redissolution method using poly(amino acid) has great potential as a
33 concentration method for antibody formulation and medicinal use.

34

35

36 **Keywords**

37 Complexation, Dissolution, Polyelectrolytes, Precipitation, Formulation, Suspensions

38

39

40 **Abbreviations**

41 CD, circular dichroism; ELISA, enzyme-linked immunosorbent assay; Fc ϵ RI α , high-affinity
42 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; *pI*, 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

47 **Introduction**

48 Monoclonal antibodies have generated considerable interest as biopharmaceuticals over the
49 past two decades because of their high target specificity and biocompatibility. At present, over 20
50 types of monoclonal antibody have been approved by the US Food and Drug Administration¹ and
51 used for several types of disease, such as cancer and autoimmune diseases.²⁻⁵ Despite advances in
52 antibody drug development, the routes of administration for these antibodies remain challenging.
53 The major route of delivery for antibodies has been intravenous administration due to the high
54 bioavailability.⁶ In contrast, subcutaneous administration has been in great demand as an alternative
55 route that allows at-home administration by patients and improves compliance rates.⁷⁻¹⁰ However,
56 the desirable concentration of antibodies for subcutaneous administration is usually above 100
57 mg/mL with a volume limitation of 1.5 mL.^{9, 10}

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

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

75 In this study, we demonstrated the feasibility of the complex precipitation–redissolution
76 method using poly(amino acid) for high-concentration antibody formulation. As described below,
77 antibody formulations with concentrations over 100 mg/mL of adalimumab for autoimmune disease
78 and omalizumab for allergic asthma could be prepared by addition of poly-L-glutamic acid (polyE).
79 The formation of antibody–polyE complex and precipitation–redissolution processes did not
80 significantly change the immunoactivity or secondary structure of the antibodies, and did not result
81 in undesirable aggregation. Comparison of time required, yield, and aggregate ratio indicated that
82 the precipitation–redissolution method was more effective for application than the conventional
83 concentration methods, including lyophilization–redissolution, evaporation–redissolution, and
84 ultrafiltration. The precipitation–redissolution method was successfully performed from a scale of
85 400 μ L to 1.0 L, indicating that this method could be scaled up to 2500-fold. Finally, the general
86 toxicity and pharmacokinetic profiles of the antibody–polyE complex formulation were similar to
87 those of conventional antibody formulations. These results suggested that this simple method
88 represents a new strategy for preparing high-concentration antibody formulations, and we expect that
89 this method and complex formulations would be applicable for medicinal use.

90 **Experimental Section**

91 **Materials**

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

113

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

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

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

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

138

139 **Concentration of antibody solutions by several methods**

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

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

158

159 **Immunoassay**

160 The immunoreactivities of omalizumab were measured by competitive enzyme-linked
161 immunosorbent assay (ELISA) to confirm structural integrity of redissolved antibody. The wells of
162 96-well ELISA microplates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) were coated with human
163 $\text{Fc}\epsilon\text{RI}\alpha$, and the plates were incubated at 4°C overnight. The wells were washed a total of five times
164 by addition of washing buffer containing 24.8 mM Tris , 136.9 mM NaCl , 2.7 mM KCl , and 0.1%
165 polysorbate 20 (pH 7.4). The wells were then blocked with Blocking One at room temperature for 1
166 hour. After washing of the wells, solutions containing human IgE at a constant concentration and
167 omalizumab of different concentrations were added to the wells and incubated at room temperature
168 for 1 hour. After washing of the wells, $100 \mu\text{L}$ of biotinylated anti-human IgE monoclonal antibody
169 was added to each well and plates were incubated at room temperature for 1 hour. After washing of

170 the wells, 100 μ L of streptavidin-labeled horseradish peroxidase was added to each well and plates
171 were incubated at room temperature for 30 minutes. After washing of the wells, 100 μ L of TMB was
172 added to each well and plates were incubated at room temperature for more than 15 minutes. Finally,
173 100 μ L of 0.1% HCl was added and the absorbance at 450 nm was determined using a microplate
174 reader (SpectraMax Plus384; Molecular Devices).

175 The immunoreactivities of adalimumab were measured by ELISA. Two types of adalimumab
176 with or without precipitation–redissolution method were used, as described above. The wells of
177 polystyrene 96-well ELISA microplates were coated with human TNF- α , and the plates were
178 incubated at 4°C overnight. After washing of the wells, Blocking One were added and plates were
179 incubated at room temperature for 1 hour. After washing of the wells, 100 μ L of adalimumab
180 solutions with or without precipitation–redissolution process of different concentrations were added
181 to the wells and plates were incubated at room temperature for 1 hour. After washing of the wells,
182 100 μ L of biotinylated anti-human IgG monoclonal antibody was added to each well and plates were
183 incubated at room temperature for 1 hour. Subsequent procedures were as described above.

184

185 **Circular dichroism**

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

195

196 **Size exclusion chromatography (SEC)**

197 Two types of antibodies with or without precipitation–redissolution method were used, as
198 described above. The samples containing 1.0 mg/mL antibodies in 10 mM buffer were centrifuged at
199 $14000 \times g$ for 10 minutes, and then the supernatant was analyzed by SE-HPLC. The analysis was
200 performed on an SEC column (Yarra 3u SEC-3000; Phenomenex Inc., Torrance, CA) on the HPLC
201 system (LC-20A; Shimadzu Corp., Kyoto, Japan) at a constant flow rate of 0.5 mL/min. The mobile
202 phase consisted of 100 mM sodium phosphate buffer and 0.5 M NaCl (pH 6.8).

203

204 **General toxicity test**

205 We evaluated general toxicity tests using two types of samples, IgG–polyE2 complex
206 formulation and conventional IgG formulation as a control. Each formulation was prepared as
207 described below. *IgG–polyE2 complex formulation:* Aliquots of 4.5 mL of 0.24 mg/mL polyE2,
208 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
209 citrate (pH 5.0) aseptically. The samples of antibody–polyE mixture were centrifuged at $1000 \times g$
210 for 3 minutes at 25°C. 8.1 mL of supernatant was removed and precipitates were resuspended by
211 vortexing. The nominal value of the samples was 900 μ L containing 10 mg/mL IgG, 1.2 mg/mL
212 polyE2, 4.0 % glucose, 10 mM citrate (pH 5.0). *Conventional IgG formulation:* Aliquots of 500 μ L
213 of 10 mg/mL IgG, 4.0 % glucose, 10 mM citrate (pH 5.0) was prepared.

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

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

228

229 **Pharmacokinetic profile**

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

247 **Results**

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

249 Previously, we reported a precipitation–redissolution method using poly(amino acid), which
250 enables the preparation of salt-dissociable precipitates of protein–poly(amino acid) complexes.²⁰ To
251 evaluate the efficiency of antibody concentration by the precipitation–redissolution method, we
252 selected two antibody drugs: adalimumab ($pI = 8.7$; for autoimmune disease) and omalizumab (pI
253 $=7.6$; for allergic asthma). Anionic poly-L-glutamic acid with average molecular weight of 3000 –
254 15000 Da (polyE1) was selected for cationic antibodies at pH 5.5 – 6.5, because protein–poly(amino
255 acid) complexes were formed only when poly(amino acid) had a charge complementary to that of
256 the protein at experimental pH. Two types of stock solution containing low (1.0 mg/mL) or high (30
257 mg/mL) concentrations of antibodies were mixed with the polyE1 solutions. The yield of antibody
258 under the milder condition (1,000 g for 3 minutes at 25°C) was identical to that under the harder
259 condition (15,000 g for 20 minutes at 25°C). Thus, the centrifugation condition was selected at 1,000
260 g for 3 minutes at 25°C in this study. Figure 1 shows the final concentration of antibodies at Step 5.
261 In Figure 1A, final concentration of antibodies increased sharply with increasing concentration of
262 polyE1, and reached a plateau at around 0.05 eq., which was consistent with the previous report.²⁰
263 Similar profiles were observed even at high concentration of stock solution (Figure 1B); final
264 concentration values of omalizumab and adalimumab reached about 150 mg/mL at 0.05 eq. The
265 yields of these antibodies were also about 100% even at high protein concentration (150 mg/mL) as
266 well as low concentration (5 mg/mL).

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

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

279

280 **Feasibility studies of precipitation–redissolution method**

281 We performed feasibility studies of the precipitation–redissolution method using poly(amino
282 acid) to confirm its utility for pharmaceutical applications. First, the efficiency of the precipitation–
283 redissolution method was compared to three conventional concentration methods, i.e.,
284 lyophilization–redissolution, evaporation–redissolution, and ultrafiltration. As expected, we obtained
285 150 mg/mL antibody solutions by the precipitation–redissolution method (Table 2). On the other
286 hand, the final concentrations of antibody solutions obtained by the conventional methods were 72.7
287 – 136 mg/mL, suggesting that some antibody was lost in the concentration process in these methods.
288 The time required for the precipitation–redissolution method was about 2 hours, which was shorter
289 than those of lyophilization and evaporation (Table 2). Furthermore, SEC analysis revealed that
290 soluble aggregates did not increase in the concentrated antibody solutions prepared by the
291 precipitation–redissolution method and ultrafiltration, but increased in those prepared by
292 lyophilization and evaporation (Table 2). These results suggested that the precipitation–redissolution
293 method is a good alternative to the well-known concentration methods.

294 To examine whether the precipitation–redissolution method could be scaled up, we prepared
295 10 mg/mL adalimumab from 2.0 mg/mL adalimumab by the addition of 0.1 mg/mL polyE1 at three
296 different scales—original-scale (400 μ L), 50-fold scale up (20 mL; medium-scale), and 2500-fold
297 scale up (1.0 L; large-scale). Similar to the original-scale, the adalimumab–polyE mixed solution
298 formed a complex and was suspended immediately at the medium- (data not shown) and large-scales
299 (Figure 3 Step 2) At the large scale, the complex was spontaneously precipitated almost completely
300 in 2 hours at Step 2. After centrifugation (Step 3), the supernatant was removed and resuspended

301 by mild agitation (Step 4). Finally, the suspension was fully dissolved by the addition of NaCl in the
302 same buffer (Step 5). ELISA and SEC analysis revealed that the immunoreactivities and the amounts
303 of soluble aggregates of redissolved adalimumab at medium and large scales were similar to those at
304 the original-scale (Table 3), suggesting that the properties of redissolved antibody were independent
305 of the scale. Thus, we confirmed that the precipitation–redissolution method could be scaled up by
306 2500-fold.

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

326 **Discussion**

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

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

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

353 failed to reach this concentration (Table 2). Although visible aggregates were not observed in the
354 adalimumab concentrated by the conventional methods, the final concentrations were lower than 150
355 mg/mL. The loss of adalimumab in these methods were due to the unfavorable influences of these
356 processes, such as adsorption to the container or ultrafiltration membrane³¹⁻³⁴ or the generation of
357 water-ice crystals at the periphery of the container.³⁵ In contrast, the final concentrations of
358 omalizumab obtained by the conventional methods were significantly lower than that achieved by
359 the precipitation–redissolution method (Table 2), Visible aggregates were observed in the
360 omalizumab solution during the concentration process, suggesting that the aggregates decreased the
361 yield of concentrated omalizumab. Thus, the precipitation–redissolution method enables prompt and
362 successful concentration of antibodies without soluble aggregates.

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

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

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

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

393

394

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487 **Table Legends**

488 **Table 1.** Immunoreactivities of redissolved antibodies

489

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

492

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

495 **Figure Legends**

496

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

503

504 **Figure 2.** Characterization of omalizumab (A,C,E) and adalimumab (B,D,F) with or without
505 precipitation–redissolution method. (A,B) Binding curve of antibodies. dashed lines; native
506 antibodies, open circles; low-concentration redissolved antibodies and closed circles; high-
507 concentration redissolved antibodies. (C,D) Far-UV CD spectra of antibodies. solid lines; native
508 antibodies and dashed lines; high-concentration redissolved antibodies. (E,F) SEC chromatograms
509 of antibodies, solid lines; native antibodies, dotted lines; low-concentration redissolved antibodies
510 and dashed lines; high-concentration redissolved antibodies, arrowhead; monomer peak and arrow;
511 aggregate peak.

512

513 **Figure 3.** Outline of precipitation–redissolution method for adalimumab at the scale of 1.0 L.
514 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
515 stock solution containing 2.0 mg/mL adalimumab in the same buffer (Step 1), and antibody–polyE
516 complex suspension was formed immediately (Step 2). After centrifugation (Step 3), 0.91 L of
517 supernatant was removed and the precipitate was resuspended by mild agitation (Step 4) followed by
518 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.

1 **Tables**2 **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

3 ^a 50% inhibitory concentration.4 ^b 50% effective concentration.

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

Concentration method	Time Require (hours)	Adalimumab		Omalizumab	
		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
Evaporation-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 μ L)	10.2	302	8.09
Medium (20 mL)	10.1	317	8.42
Large (1.0 L)	10.4	289	8.52

7

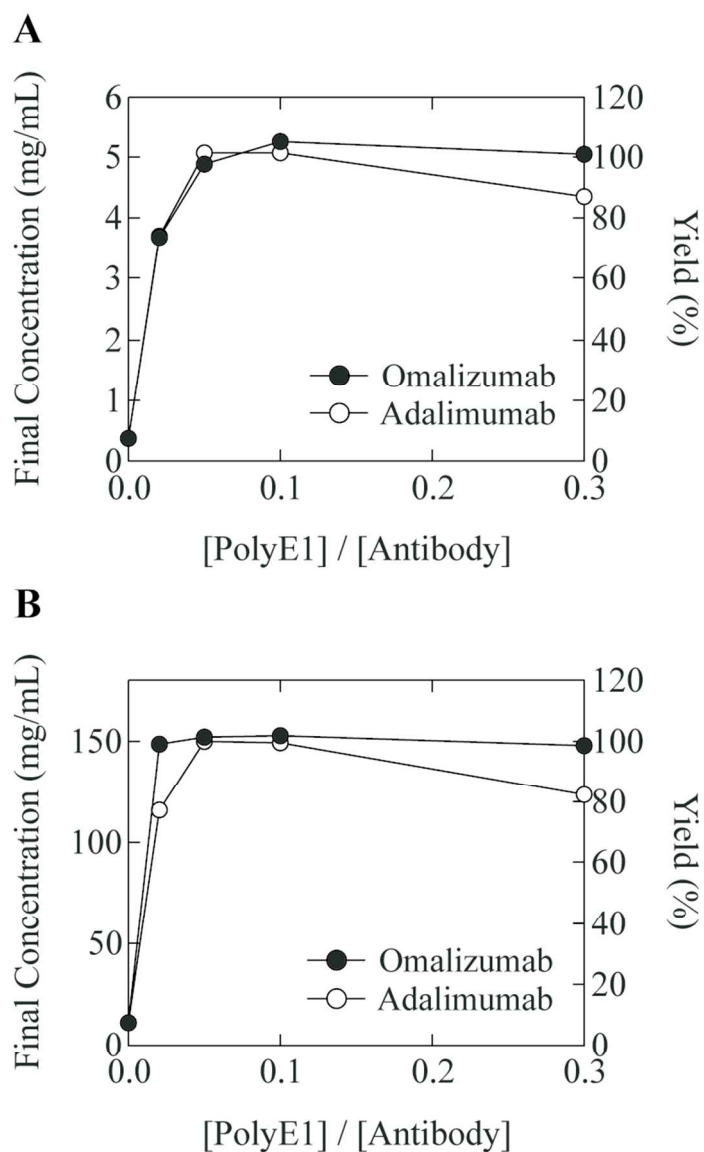


Figure 1. Precipitation–redissolution profiles of antibodies by polyE1. Various concentrations of polyE1 were added to stock solutions containing 1.0 mg/mL (low) (A) or 30 mg/mL (high) (B) antibodies in 10 mM buffer. The final concentrations of antibodies at Step 5 in the presence of polyE1 were measured. 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.
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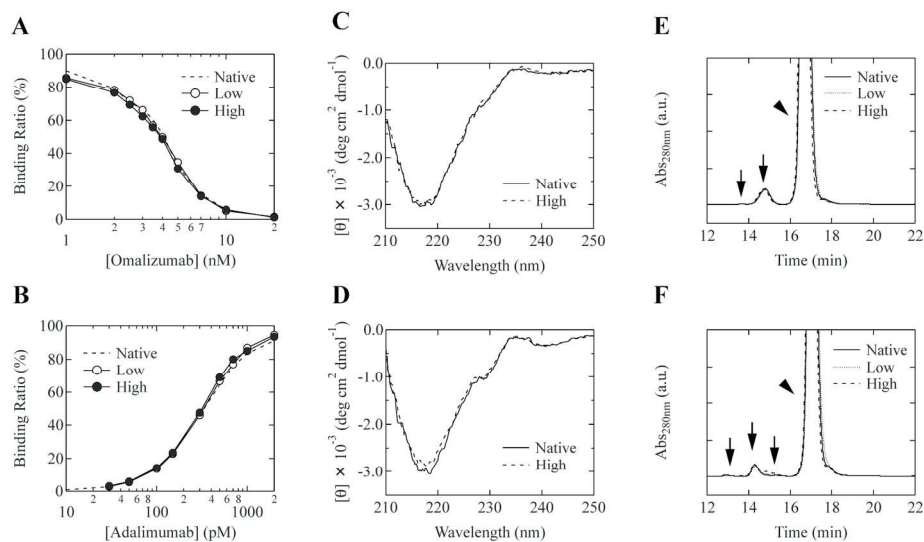


Figure 2. Characterization of omalizumab (A,C,E) and adalimumab (B,D,F) with or without precipitation–redissolution method. (A,B) Binding curve of antibodies. dashed lines; native antibodies, open circles; low-concentration redissolved antibodies and closed circles; high-concentration redissolved antibodies. (C,D) Far-UV CD spectra of antibodies. solid lines; native antibodies and dashed lines; high-concentration redissolved antibodies. (E,F) SEC chromatograms of antibodies, solid lines; native antibodies, dotted lines; low-concentration redissolved antibodies and dashed lines; high-concentration redissolved antibodies, arrowhead; monomer peak and arrow; aggregate peak.
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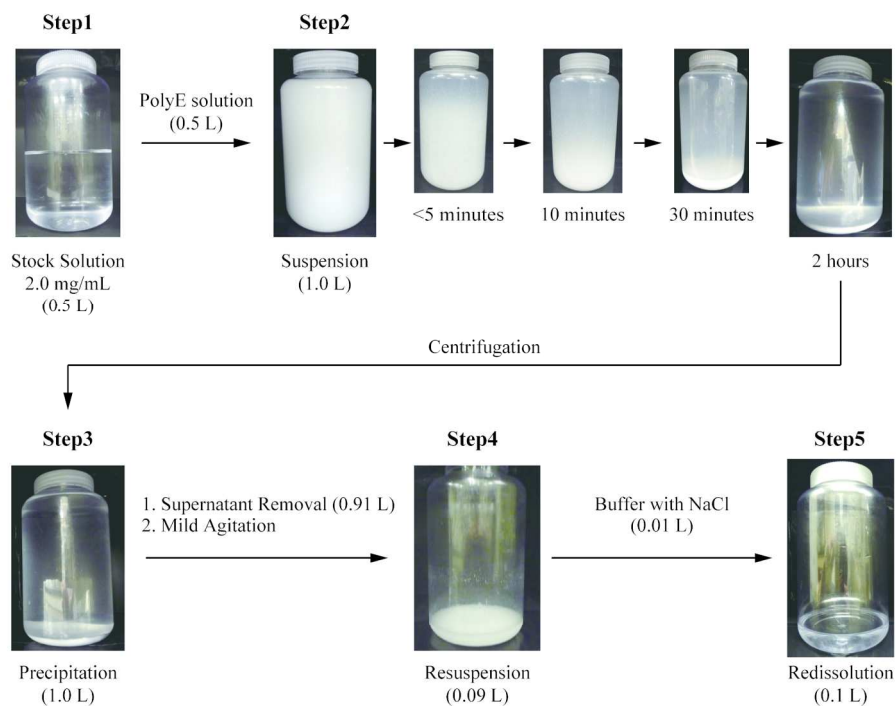


Figure 3. Outline of precipitation-redissolution method for adalimumab at the scale of 1.0 L. Aliquots of 0.5 L of 0.2 mg/mL polyE1 solution in 10 mM MOPS (pH 6.5) was added to 0.5 L of stock solution containing 2.0 mg/mL adalimumab in the same buffer (Step 1), and antibody-polyE complex suspension was 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).
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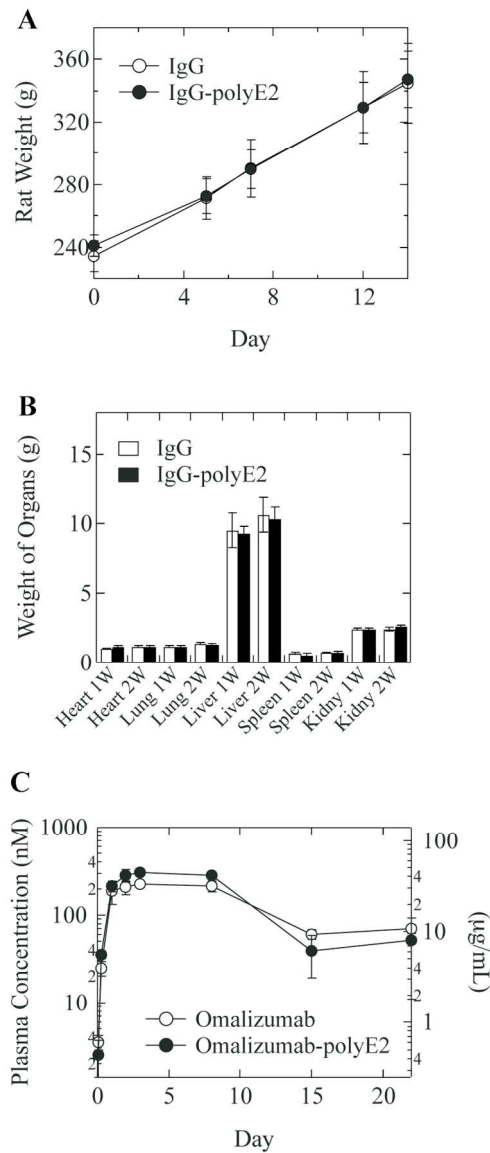


Figure 4. (A,B) General toxicity tests in rats; body weight of rats (A) and weight of organs (B). Conventional IgG formulation, closed symbols; IgG-polyE2 complex fomulation, open symbols. (C) Plasma concentration of omalizumab-time curve in rats after subcutaneous administration. Conventional omalizumab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