

Noncovalent PEGylation of L-Asparaginase Using PEGylated Polyelectrolyte

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1 Non-covalent PEGylation of L-asparaginase using
2 PEGylated polyelectrolyte

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10

11 **Abstract**

12 Non-covalent PEGylation has great potential for stabilization of therapeutic proteins. Here,
13 we demonstrated that the non-covalent PEGylation with a PEGylated polyelectrolyte stabilized a
14 therapeutic protein, L-asparaginase (ASNase). Anionic ASNase and cationic poly(ethylene glycol)-
15 *block*-poly(*N,N*-dimethylaminoethyl methacrylate) (PEG-*b*-PAMA) formed a water-soluble protein–
16 polyelectrolyte complex (PPC) without loss of secondary structure and enzyme activity. PPC with
17 PEG-*b*-PAMA successfully inhibited the shaking-induced inactivation and aggregation of ASNase
18 as well as protease digestion, corresponding to the behaviors of covalently PEGylated ASNase. Thus,
19 non-covalent PEGylation by PEGylated polyelectrolytes is a new candidate for handling of
20 therapeutic proteins.

21

22

23 **Keywords**

24 Complexation, Pegylation, Polyelectrolytes, Protein aggregation, Protein formulation, Stabilization

25

26 **Abbreviations**

27 L-Asn, L-asparagine; ASNase, L-asparaginase; CD, circular dichroism; DLS, dynamic light
28 scattering; MOPS, 3-(*N*-morpholino)propanesulfonic acid; M_w , molecular weight; PAGE,
29 polyacrylamide gel electrophoresis; PAMA, poly(*N,N*-dimethylaminoethyl methacrylate); PEG,
30 poly(ethylene glycol); PEG-ASNase, poly(ethylene glycol)-L-asparaginase; PEG-*b*-PAMA,
31 poly(ethylene glycol)-*block*-poly(*N,N*-dimethylaminoethyl methacrylate); PMSF,
32 phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris,
33 tris(hydroxymethyl)aminomethane

34

35 **Introduction**

36 Progress in the fields of recombinant technology and biotechnology have markedly increased
37 the numbers of therapeutic proteins.¹ Many therapeutic proteins have large and multi-domain
38 structures consisting of homo- or heteropolypeptide chains. The formation of quaternary structures is
39 required for bioactivity of multi-domain enzymes, such as tetrameric L-asparaginase (ASNase),
40 tetrameric uricase, and trimeric arginase.² In comparison with single-domain proteins, multi-domain
41 proteins are prone to aggregation and are inactivated by common stresses, such as changes in
42 temperature, pH shift, and mechanical stress.³ Aggregation is the most serious problem for
43 therapeutic proteins causing loss of their bioactivities, and therefore such aggregates in therapeutic
44 proteins are generally unacceptable.⁴ Accordingly, a method for stabilization of multi-domain
45 proteins is important for pharmaceuticals.

46 Covalent attachment of polymers to the protein surface is one strategy for stabilization of
47 proteins.^{3, 5, 6} Especially, conjugation of poly(ethylene glycol) (PEGylation) is the most promising
48 method for protein therapy.⁷⁻¹² PEG is a hydrophilic, non-ionic, and non-toxic polymer that provides
49 a steric shield for proteins, resulting in improvement of the pharmacological properties of the
50 proteins. In addition, PEGylation also protects proteins against aggregation and protease digestion *in*
51 *vitro*.¹³⁻¹⁵ At present, 12 types of PEGylated therapeutic protein have been approved by the US Food
52 and Drug Administration,¹² and are used for several diseases, such as severe combined
53 immunodeficiency disease, acute lymphoblastic leukemia, and refractory chronic gout.¹¹ However,
54 covalent PEGylation requires chemical reaction for conjugation of PEG to proteins, which is both
55 time-consuming and costly.

56 Non-covalent PEGylation has been suggested as an alternative method for stabilizing
57 proteins using PEG.¹⁶⁻²¹ A common strategy for non-covalent PEGylation involves designing
58 functional PEG derivatives that bind to proteins. For example, Mueller et al. synthesized several
59 PEG derivatives conjugated with hydrophobic ligands, which reduced the aggregation of salmon
60 calcitonin and lysozyme.^{17, 18, 21} Similarly, PEG derivatives conjugated with sugars,¹⁹ biotin,¹⁶ and

61 nitrilotriacetic acid²⁰ have also been designed. However, there have been few studies regarding non-
62 covalent PEGylation of therapeutic proteins.

63 PEGylated polyelectrolytes are also PEG derivatives. The polyelectrolyte interacts strongly
64 with complementary charged proteins through multiple electrostatic interactions, resulting in the
65 formation of various types of protein–polyelectrolyte complex (PPC).^{22–28} We have recently shown
66 that PEGylated polyelectrolyte could form a water-soluble PPC with α -amylase and β -galactosidase
67 due to the PEG segment of the polyelectrolyte.²⁵ Here, we investigated stabilization of therapeutic
68 protein through non-covalent PEGylation using PEGylated polyelectrolytes. We selected ASNase as
69 a model therapeutic protein which used in acute lymphoblastic leukemia. Anionic ASNase and
70 cationic poly(ethylene glycol)-*block*-poly(*N,N*-dimethylaminoethyl methacrylate) (PEG-*b*-PAMA)
71 formed a water-soluble PPC with maintenance of the original secondary structure and enzyme
72 activity of ASNase. As expected, PEG-*b*-PAMA protected ASNase against protease digestion and
73 shaking-induced inactivation. It is important to note that these protective effects of PEG-*b*-PAMA
74 were comparable to those of commercial PEGylated ASNase (PEG-ASNase).

75

76 **Experimental Section**

77 **Materials**

78 L-Asparaginase (ASNase) from *Escherichia coli* was from Kyowa Hakko Kirin Co. Ltd.
79 (Tokyo, Japan). Nessler's reagent, 3-(*N*-morpholino)propanesulfonic acid (MOPS), poly(ethylene
80 glycol)-L-asparaginase (PEG-ASNase) from *Escherichia coli*, and trypsin from bovine pancreas
81 were from Sigma Chemical Co. (St. Louis, MO). Poly(*N,N*-dimethylaminoethyl methacrylate)
82 (PAMA) with average molecular weight (M_w) 4.2 kDa, poly(ethylene glycol)-*block*-poly(*N,N*-
83 dimethylaminoethyl methacrylate) (PEG-*b*-PAMA) with average M_w (PEG) 5.0 kDa and M_w
84 (PAMA) 5.5 kDa were from Polymer Source Inc. (Dorval, QC, Canada). Ammonium sulfate, L-
85 asparagine (L-Asn), poly(ethylene glycol) with average M_w 7.5 kDa, and trichloroacetic acid (TCA)
86 were from Wako Pure Chemical. Ind., Ltd. (Osaka, Japan). Phenylmethylsulfonyl fluoride (PMSF)
87 was from Nacalai Tesque (Kyoto, Japan). These chemicals were of high-quality analytical grade and
88 were used as received.

89

90

91 **Protein concentrations**

92 The concentrations of proteins were determined from the absorbance at 280 nm using a
93 spectrophotometer (V-630; Japan Spectroscopic Co., Ltd., Tokyo, Japan) with extinction coefficients
94 of $94020 \text{ M}^{-1} \text{ cm}^{-1}$ (ASNase and PEG-ASNase) or $37650 \text{ M}^{-1} \text{ cm}^{-1}$ (trypsin).²⁹

95

96 **Dynamic light scattering**

97 Dynamic light scattering (DLS) experiments were performed using a Zetasizer Nano ZS light
98 scattering photometer (Malvern Instruments, Worcestershire, UK) equipped with a 4 mW He-Ne ion
99 laser ($\lambda = 633 \text{ nm}$). The sizes of the protein with polymer were determined as follows. Solutions
100 containing $1.0 \text{ }\mu\text{M}$ ASNase, $0 - 100 \text{ }\mu\text{M}$ polymers, and 10 mM MOPS buffer (pH 7.0) were placed
101 in a 1-cm path length disposable cuvette, and DLS measurements were performed at 25°C at a
102 detection angle of 173° . The viscosity of the solutions was approximated by the value of the 10 mM

103 MOPS solution ($\eta = 0.87$ cP). All results are presented as the averages of three independent
104 experiments.

105

106 **Circular dichroism**

107 Circular dichroism (CD) experiments were performed in a 1-mm path length quartz cuvette
108 using a J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). Solutions
109 containing 1.0 μ M ASNase, 0 – 40 μ M polymers, and 10 mM MOPS buffer (pH 7.0) were prepared,
110 and the spectra were measured at 25°C. The CD spectra of the samples were corrected by
111 subtracting the corresponding spectra of the buffers in the absence of proteins.

112

113 **Enzyme assay**

114 The enzyme activities of ASNase and PEG-ASNase were measured as follows. An aliquot of
115 50 μ L of the protein solution was incubated with 950 μ L of the substrate solution containing 22 mM
116 L-Asn, 10 mM MOPS (pH 7.0) at 37°C for 2.0 min (ASNase) or 10 min (PEG-ASNase). The
117 reaction was stopped by the addition of 250 μ L of 3.0 M TCA in the assay mixture. Subsequently,
118 the sample was mixed with Nessler's reagent to measure the ammonia released after L-Asn
119 hydrolysis. The absorbance was monitored spectrophotometrically at 450 nm. The concentration of
120 ammonia produced by the enzymatic reaction was determined from a reference curve using
121 ammonium sulfate as a standard. One unit of enzyme activity was defined as the amount of enzyme
122 required to produce 1.0 μ mol ammonia per min at 37°C.

123

124 **Enzyme Degradation of Trypsin**

125 Trypsin solution of 1.0 μ M was prepared by dissolving the lyophilized trypsin in cold 1.0
126 mM HCl. An aliquot of 5.0 μ L of trypsin solution was added to 500 μ L protein solutions (ASNase:
127 1.0 μ M ASNase, 0 – 40 μ M polymers, 10 mM MOPS, pH 7.0; PEG-ASNase: 1.0 μ M PEG-ASNase,

128 10 mM MOPS, pH 7.0) at 37°C. After incubation for respective periods, enzyme activities of
129 ASNase were measured by above-mentioned assay.

130

131 **Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

132 An aliquot of 5.0 μ L of trypsin solution was added to 500 μ L protein solutions (ASNase: 1.0
133 μ M ASNase, 0 – 40 μ M polymer, 10 mM MOPS, pH 7.0; PEG-ASNase: 1.0 μ M PEG-ASNase, 10
134 mM MOPS, pH 7.0) at 37°C. After incubation for 2.0 h, 5.0 μ L of 100 mM PMSF in ethanol was
135 added to stop the trypsin digestion reaction. The samples were then mixed with an equal volume of
136 loading buffer containing 4.0% (w/v) SDS, 20% sucrose, 0.010% (w/v) bromophenol blue, and 125
137 mM Tris-HCl (pH 6.8). The samples were boiled for 15 min followed by loading on a 14%
138 polyacrylamide gel with a standard ladder marker, which was obtained from Bio-Rad Laboratories
139 (Hercules, CA). After gel electrophoresis, the gels were stained with silver nitrate.

140

141 **Shaking Treatment**

142 Aliquots of 1.2 mL of protein solutions (ASNase: 1.0 μ M ASNase, 0 – 40 μ M polymers, 10
143 mM MOPS, pH 7.0; PEG-ASNase: 1.0 μ M PEG-ASNase, 10 mM MOPS, pH 7.0) were added to
144 2.0-mL microcentrifuge tubes (Thermo Scientific, Waltham, MA). The samples were then shaken at
145 500 rpm for 0 – 6 hours. The enzyme activities and sizes of the samples were measured by the assay
146 described above.

147

148 **Heat Treatment**

149 Protein solutions (ASNase: 1.0 μ M ASNase, 0 – 40 μ M polymers, 10 mM MOPS, pH 7.0;
150 PEG-ASNase: 1.0 μ M PEG-ASNase, 10 mM MOPS, pH 7.0) were heated at 60°C for 0 – 2 h. The
151 enzyme activities and sizes of the samples were measured by the assay described above.

152

153 **Results**

154 **Preparation and Characterization of ASNase/PEG-*b*-PAMA Complexes.**

155 Figure 1A shows the chemical structures of polymers used in this study. Cationic
156 poly(ethylene glycol)-*block*-poly(*N,N*-dimethylaminoethyl methacrylate) (PEG-*b*-PAMA) had PEG
157 and PAMA regions with average M_w of 5.0 kDa and 5.5 kDa, respectively. Two types of
158 homopolymer were also used for control experiments: cationic PAMA with an average M_w of 4.2
159 kDa and non-ionic PEG with an average M_w of 7.5 kDa, which were similar to PEG-*b*-PAMA.
160 Anionic L-asparaginase (ASNase), which is used in treatment of acute lymphoblastic leukemia, was
161 selected as a multi-domain therapeutic protein.

162 We demonstrated previously that cationic PEGylated polyelectrolytes bind to anionic
163 proteins, resulting in the formation of water-dispersed protein–polyelectrolyte complexes (PPC).²⁵
164 Therefore, we first prepared a PPC between cationic PEG-*b*-PAMA and anionic ASNase, as
165 illustrated in Figure 1B. Figure 2A shows the hydrodynamic diameter (D_h) of ASNase in the absence
166 or presence of polyelectrolytes. The D_h of ASNase alone was 11.1 nm, but increased to > 1000 nm
167 with the addition of 1.0 μ M PEG-*b*-PAMA. When further PEG-*b*-PAMA was added to ASNase
168 solution, the D_h decreased with increasing concentration of PEG-*b*-PAMA, and then reached a
169 plateau of 22 nm at around 40 μ M PEG-*b*-PAMA. In contrast, the D_h of ASNase increased sharply
170 with increasing concentration of cationic PAMA with a plateau of > 1000 nm at around 30 μ M
171 PAMA. The D_h of PEG-*b*-PAMA and PAMA alone could not be detected by DLS. These results
172 indicated that ASNase formed a soluble PPC with PEG-*b*-PAMA, while ASNase formed an
173 aggregative PPC with PAMA.

174 We next characterized the structure and enzyme activity of ASNase in the presence of
175 polymers. Far-UV circular dichroism (CD) spectra of ASNase in the presence of PEG-*b*-PAMA and
176 PEG were identical to those of the native ASNase, whereas those of ASNase in the presence of
177 PAMA decreased (Figure 2B). These results indicated that PAMA denatured the ASNase in
178 aggregate form, as shown in Figure 2A. Furthermore, PEG-*b*-PAMA as well as PEG did not affect
179 the enzyme activity of ASNase, whereas it decreased in the presence of PAMA (Figure 2C). These

180 results indicated that ASNase retained the original properties of secondary structure and enzyme
181 activity after the formation of a PPC with PEG-*b*-PAMA.

182

183 **Stress Tolerance of ASNase Formed PPC with PEG-*b*-PAMA.**

184 PEGylation is one of the major strategies to stabilize pharmaceutical proteins *in vivo* due to
185 the steric hindrance of PEG segments on the protein surface.⁷⁻¹² It is of interest to determine whether
186 non-covalent interaction between ASNase and PEG-*b*-PAMA stabilizes the proteins to the same
187 extent as in covalently conjugated PEGylated proteins toward various stresses, including proteolytic
188 degradation, and agitation. Therefore, we prepared commercially available PEG-conjugated L-
189 asparaginase (PEG-ASNase) as a model PEGylated protein.

190 Figure 3A shows the enzyme activities of ASNase in the absence or presence of polymers
191 preincubated with trypsin, which hydrolyzes peptide bonds at the carboxyl end of basic amino acids
192 in the proteins. The residual activity of native ASNase was 15%, indicating that ASNase was
193 inactivated by trypsin proteolysis. Similarly, ASNase in the presence of PAMA and PEG was
194 inactivated by trypsin proteolysis. In contrast, the addition of PEG-*b*-PAMA showed a protective
195 effect against trypsin proteolysis. The residual activity of ASNase/PEG-*b*-PAMA was 81%, which
196 was higher than that of PEG-ASNase (47%).

197 The proteolytic digestion of ASNase by trypsin was further evaluated by SDS-PAGE (Figure
198 3B). Without trypsin treatment, only a band of monomeric size (35 kDa) was observed under all
199 conditions. After trypsin digestion, the band of 35 kDa disappeared in the samples of ASNase alone,
200 ASNase with PAMA, and ASNase with PEG. In contrast, the band of 35 kDa remained in the
201 presence of PEG-*b*-PAMA after trypsin treatment, corresponding with the residual activity (Figure
202 3A). These results indicated that the PPC with PEG-*b*-PAMA had a protective effect against
203 proteolysis by trypsin.

204 We then evaluated the protective effects of PEGylated polyelectrolyte against shaking. A
205 solution of native ASNase shaken at 500 rpm for 6 h showed visible suspension and the enzyme
206 activity disappeared completely (Figure 4). DLS measurements indicated that D_h of native ASNase

207 after shaking was > 1000 nm (Table 1), suggesting that the native ASNase was inactivated due to
208 aggregation induced by shaking. In contrast, the residual activity of ASNase with polymers remained
209 above 75%, and that of PEG-ASNase remained at 47%. It is interesting to note that D_h of
210 ASNase/PEG-*b*-PAMA after shaking remained constant, whereas those of other samples were above
211 1000 nm (Table 1). These results indicated that the PEGylated polyelectrolytes inhibited shaking-
212 induced protein aggregation.

213 We finally confirmed the effects of PEGylated polyelectrolyte against heat-induced
214 inactivation of ASNase. Heating temperature was chosen 60 °C because of the ASNase did not
215 inactivated below 40 °C (data not shown). The residual activities of native ASNase and PEG-
216 ASNase were 55% and 38%, respectively (Figure 5). With the addition of non-ionic PEG, there was
217 a slight change in the residual activity to 60%. In contrast, the addition of cationic polyelectrolytes,
218 PEG-*b*-PAMA and PAMA, the enzyme activities decreased about 10%. The ASNase and
219 polyelectrolyte solutions after heating showed visible aggregates with D_h values > 330 nm (Table 1).
220 These results suggested that polyelectrolytes accelerated the heat-induced inactivation of ASNase.

221

222 **Discussion**

223 This study showed stabilization of therapeutic proteins using PEGylated polyelectrolytes, as
224 summarized below. PEGylated cationic PEG-*b*-PAMA and anionic ASNase formed soluble
225 complexes without conformational changes, whereas non-PEGylated PAMA formed aggregative
226 complexes with conformational changes (Figure 2A,B), corresponding to the results reported
227 previously.²⁵ This is because the electrostatic interactions between proteins and polyelectrolytes are
228 driving forces that stabilize PPC, which was also supported by the control data in which non-charged
229 PEG alone did not affect the size of ASNase (Table 1). It is interesting to note that the D_h of
230 ASNase with PEG-*b*-PAMA was identical to that of PEG-ASNase (Table 1), suggesting that the
231 conformation of the PPC with PEG-*b*-PAMA may be similar to that of PEGylated proteins.

232 Several authors, including our group, reported previously that cationic polyelectrolytes can
233 bind to anionic enzymes, resulting in inhibition of enzyme activities.^{23, 25, 30, 31} However, the results
234 of the present study showed that the enzyme activity of ASNase did not change despite PPC
235 formation (Figure 2C). This unexpected difference in inhibition of polyelectrolytes may be due to
236 several factors, such as the types of compounds, pH of the solution, and the method used for enzyme
237 assay. Although it is difficult to determine whether polyelectrolytes inhibit the enzyme activity, PPC
238 is more favorable for enzyme activity of ASNase than PEG-ASNase because the covalent
239 PEGylation produces a decrease in activity of proteins.⁷⁻¹² Under the present experimental condition,
240 the enzyme activities of native ASNase and PEG-ASNase were about 35 U/mL and 10 U/mL,
241 respectively.

242 PEG-conjugated proteins are protected from proteolytic digestion by proteases *in vitro* and *in*
243 *vivo*. In fact, the residual activity of PEG-ASNase toward trypsin was higher than that of native
244 ASNase (Figure 3). In this study, PEG-*b*-PAMA protected ASNase from proteolytic digestion to a
245 comparable extent to PEG-ASNase, whereas the PAMA and PEG did not (Figure 3). These results
246 indicated that binding of PEG-*b*-PAMA to the surface of ASNase provided a shield. We concluded
247 that the protective mechanism of PPC with PEG-*b*-PAMA is similar to that of PEGylated proteins,
248 i.e., steric hindrance of PEG on ASNase/PEG-*b*-PAMA complex inhibits access to protease.

249 Shaking is one of the major causes of protein aggregation.^{3, 4} Aggregation by shaking is
250 primarily attributable to the contact of proteins and air–water interfaces.⁴ Our results indicated that
251 native ASNase also formed visible aggregates on shaking stress (Table 1), resulting in inactivation
252 of the enzyme (Figure 4). In contrast, the solutions of ASNase with polymer and PEG-ASNase were
253 clear and retained enzyme activity. It is emphasized that the D_h of ASNase/PEG-*b*-PAMA after
254 shaking remained constant, even though DLS measurement is sensitive to small amounts of
255 aggregates. It is possible that the steric hindrance of PEG on the ASNase/PEG-*b*-PAMA complex
256 conferred protection from the contact of air–water interfaces for the protease. Thus, PPC with
257 PEGylated polyelectrolyte is better protected against shaking stress as well as proteolytic digestion
258 than PEGylated proteins.

259 The data for heat treatment on ASNase are complex, and it is difficult to understand the
260 mechanism compared to other types of stress. Briefly, PEG-*b*-PAMA and PAMA accelerated
261 irreversible inactivation of ASNase against heat treatment, whereas the others did not (Figure 5).
262 This result was inconsistent with a previous report in which PEGylated polyelectrolyte stabilized hen
263 egg white lysozyme against heat-induced inactivation.²² We concluded that the inconsistency
264 resulted from monomeric or oligomeric forms of the proteins as follows. The tetrameric ASNase
265 loses enzyme activity due to dissociation of subunits during heat treatment at 60°C, and then the
266 enzyme activity is restored by decreasing temperature due to the re-association of subunits.^{13, 32}
267 Furthermore, the denaturation temperature of ASNase is 62°C, which was independent even in the
268 presence of polymers. Accordingly, the polyelectrolytes are thought to inhibit the re-association of
269 ASNase through electrostatic interaction, resulting in irreversible inactivation of ASNase.

270 In summary, we showed that PEGylated polyelectrolytes stabilize the therapeutic protein,
271 ASNase. PEG-*b*-PAMA successfully protected ASNase against trypsin digestion and shaking-
272 induced aggregation due to PPC formation. The stabilizing effects of PPC with PEG-*b*-PAMA were
273 similar to those of covalent PEGylated ASNase, suggesting that non-covalent PEGylation occurred
274 with PEGylated polyelectrolytes. Other therapeutic proteins with multi-domain structures would be
275 stabilized by PEGylated polyelectrolytes. We believe that complex formation between PEGylated

276 polyelectrolytes and proteins will expand the applications of therapeutic proteins, such as
277 formulation and drug delivery systems.
278

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282

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360 **Table Legends**

361 **Table 1.** Hydrodynamic diameters (D_h) of ASNase in the absence or presence of polymers.

362 Parentheses show polydispersity index (PDI).

363

364 **Figure Legends**

365 **Figure 1.** (A) Chemical structures of polymers. (B) Schematic illustration of PPC with PEG-*b*-
366 PAMA.

367

368 **Figure 2.** Characterization of ASNase/PEG-*b*-PAMA complexes. (A) Hydrodynamic diameter (D_h)
369 variations of the ASNase in the presence of polymers at various [polymer]/[ASNase] ratios. (B) Far-
370 UV CD spectra of ASNase in the absence or presence of polymers at [ASNase]/[polymer] = 1:40.
371 (C) Normalized enzyme activity of the ASNase in the absence or presence of polymers at
372 [ASNase]/[polymer] = 1:40.

373

374 **Figure 3.** Proteolytic digestion of ASNase with polymer. (A) Residual activity of ASNase with
375 polymers and PEG-ASNase after trypsin treatment. (B) SDS-PAGE of ASNase/polymer
376 preincubated with or without trypsin treatment. Lane 1, native ASNase; Lane 2, ASNase/PEG-*b*-
377 PAMA; Lane 3, ASNase/PAMA; Lane 4, ASNase/PEG; M, standard ladder marker.

378

379 **Figure 4.** Residual activities of ASNase with polymers and PEG-ASNase after the shaking at 500
380 rpm for 6 hours.

381

382 **Figure 5.** Residual activity of ASNase with polymers and PEG-ASNase after heat treatment at 60°C
383 for 2 hours.

1 **Tables**2 **Table 1.** Hydrodynamic diameters (D_h) of ASNase in the absence or presence of polymers.

3 Parentheses show polydispersity index (PDI).

	D_h (nm) ^a		
	No Stress	Shaking	Heating
No Addition	10.8 ± 0.06 (0.20)	> 1000 (0.92)	10.1 ± 0.13 (0.29)
PEG- <i>b</i> -PAMA	26.2 ± 0.28 (0.46)	28.9 ± 0.38 (0.67)	330 ± 2.61 (0.18)
PAMA	> 1000 (1.00)	> 1000 (1.00)	> 1000 (0.88)
PEG	11.3 ± 0.02 (0.22)	> 1000 (0.84)	13.3 ± 0.40 (0.47)
PEG-ASNase	24.5 ± 0.18 (0.25)	> 1000 (1.00)	28.0 ± 3.01 (0.59)

4 ^a Z-average size.

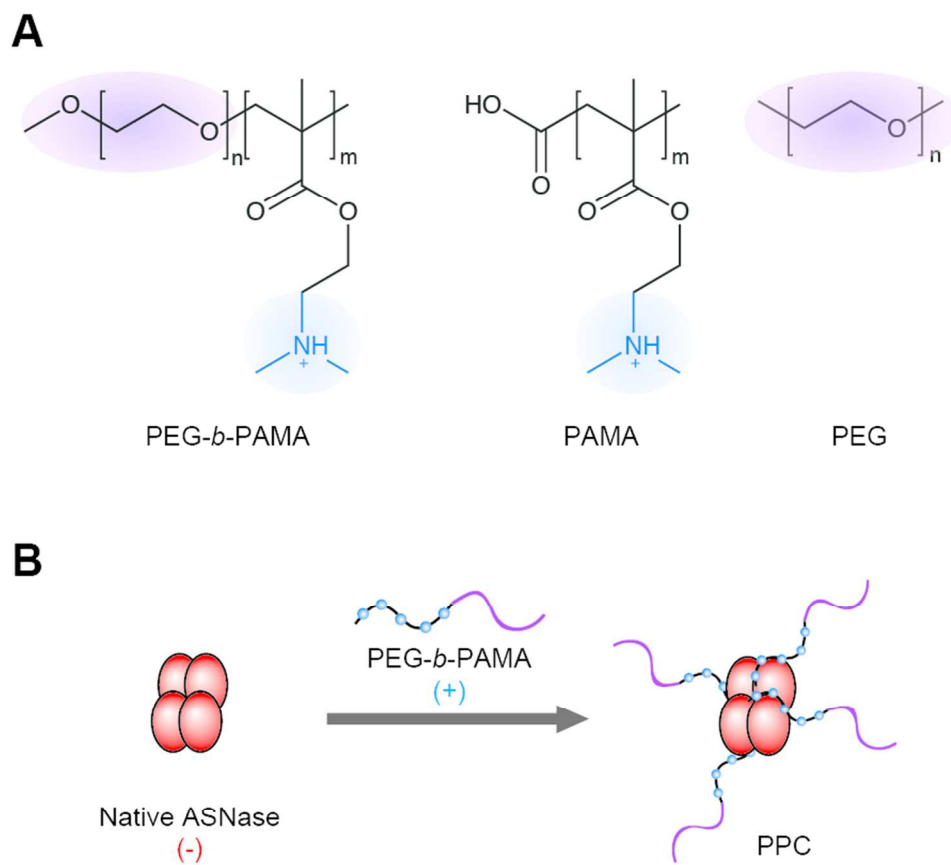


Figure 1. (A) Chemical structures of polymers. **(B)** Schematic illustration of PPC with PEG-*b*-PAMA.
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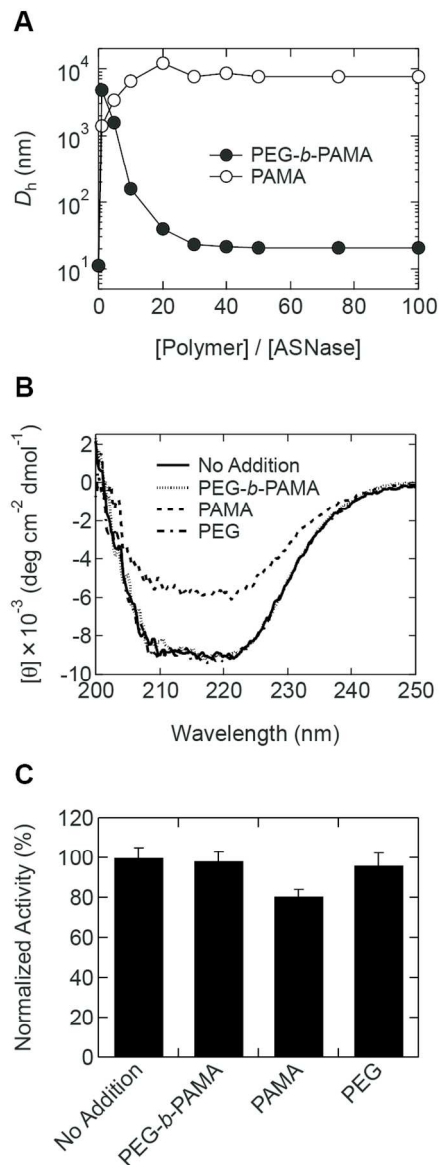


Figure 2. Characterization of ASNase/PEG-*b*-PAMA complexes. **(A)** Hydrodynamic diameter (D_h) variations of the ASNase in the presence of polymers at various [polymer]/[ASNase] ratios. **(B)** Far-UV CD spectra of ASNase in the absence or presence of polymers at [ASNase]/[polymer] = 1:40. **(C)** Normalized enzyme activity of the ASNase in the absence or presence of polymers at [ASNase]/[polymer] = 1:40.
79x152mm (300 x 300 DPI)

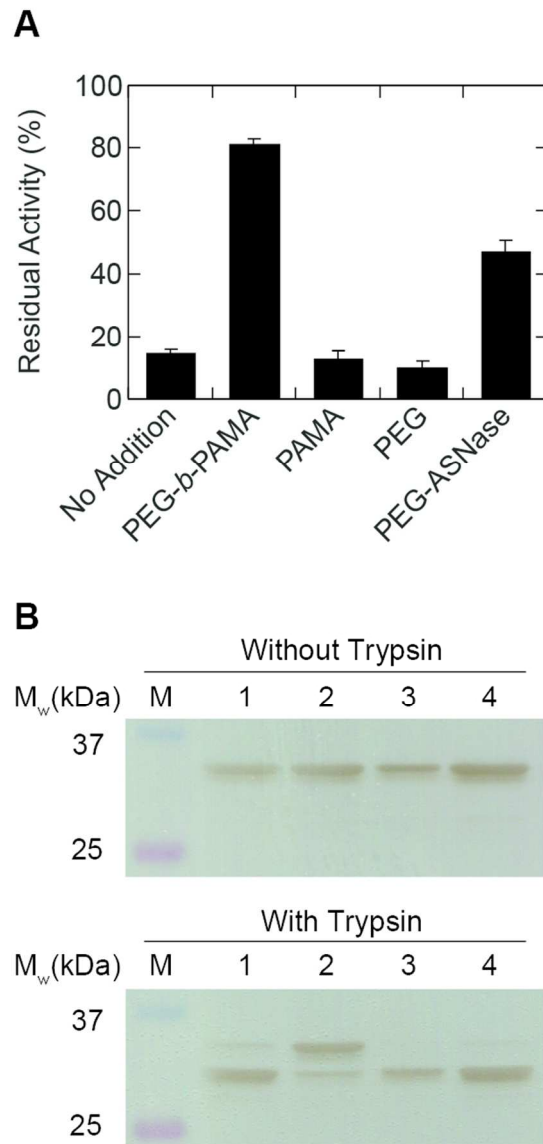


Figure 3. Proteolytic digestion of ASNase with polymer. **(A)** Residual activity of ASNase with polymers and PEG-ASNase after trypsin treatment. **(B)** SDS-PAGE of ASNase/polymer preincubated with or without trypsin treatment. Lane 1, native ASNase; Lane 2, ASNase/PEG-*b*-PAMA; Lane 3, ASNase/PAMA; Lane 4, ASNase/PEG; M, standard ladder marker.
79x116mm (300 x 300 DPI)

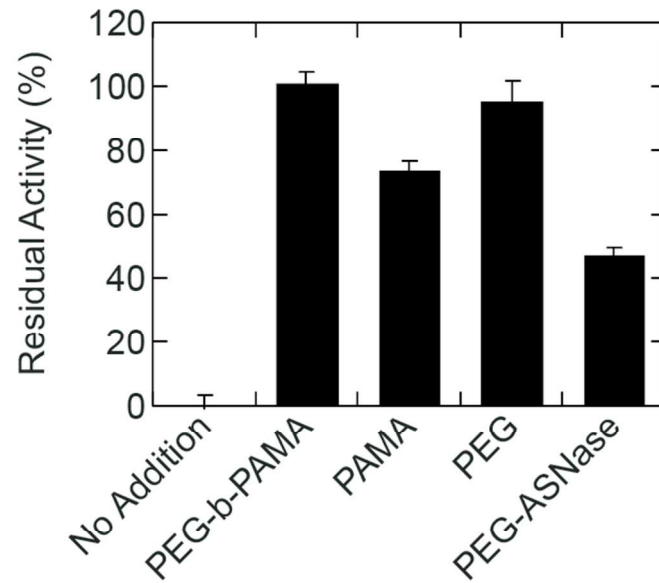


Figure 4. Residual activities of ASNase with polymers and PEG-ASNase after the shaking at 500 rpm for 6 hours.

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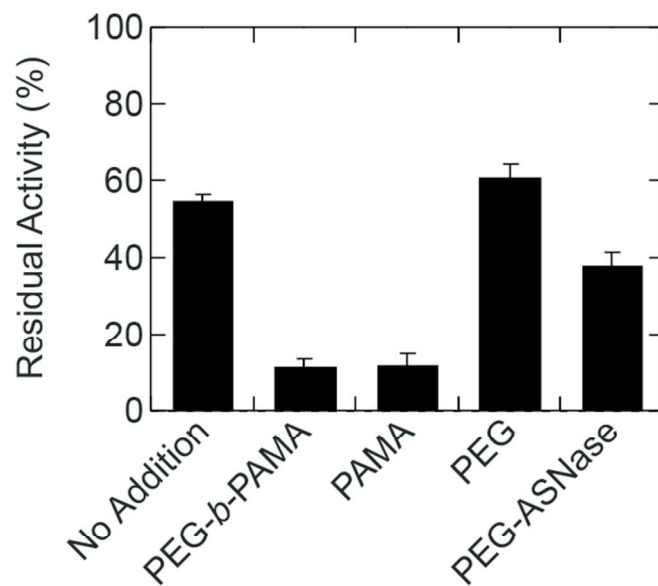


Figure 5. Residual activity of ASNase with polymers and PEG-ASNase after heat treatment at 60°C for 2 hours.

79x54mm (300 x 300 DPI)