Non-covalent PEGylation of L-asparaginase using PEGylated polyelectrolyte

Takaaki Kurinomaru and Kentaro Shiraki *

Faculty of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan

To whom correspondence should be addressed. Tel.: +81-29-8535306. Fax: +81-29-8535215. E-mail: shiraki@bk.tsukuba.ac.jp

Abstract

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

Keywords

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

Abbreviations

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

Introduction

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

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

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

nitrilotriacetic acid[20] have also been designed. However, there have been few studies regarding non-covalent PEGylation of therapeutic proteins.

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

Experimental Section

Materials

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

Protein concentrations

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

Dynamic light scattering

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

MOPS solution (η = 0.87 cP). All results are presented as the averages of three independent experiments.

Circular dichroism

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

Enzyme assay

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

Enzyme Degradation of Trypsin

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

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

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

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

Shaking Treatment

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

Heat Treatment

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

Results

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

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

Figure Legends

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

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.

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.

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

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

Tables

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

[a] Z-average size.

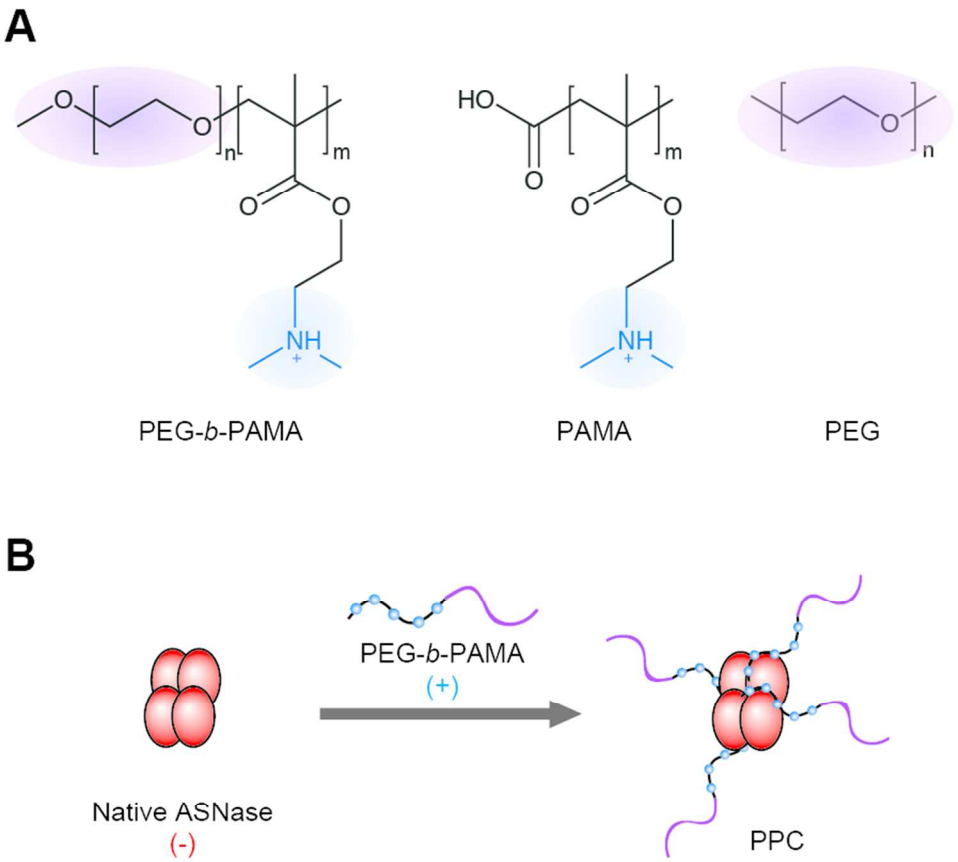
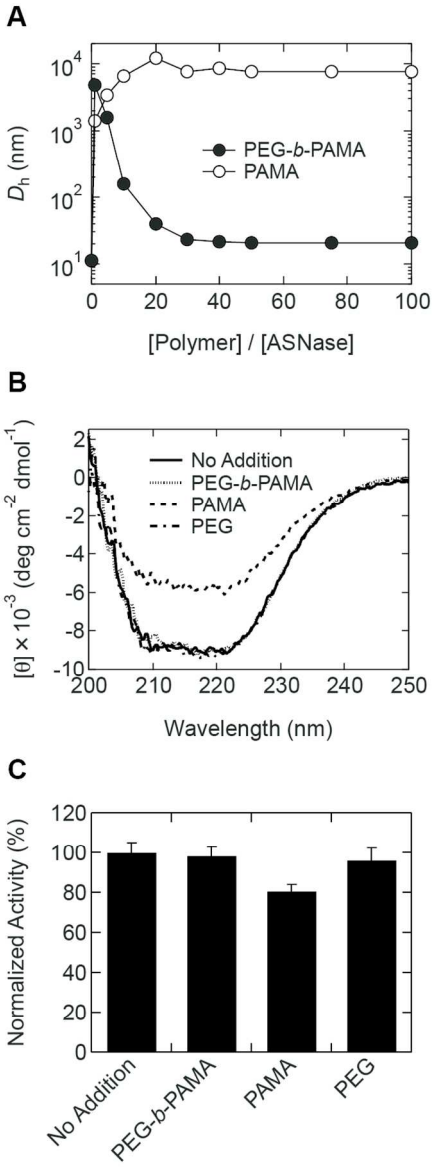


Figure 1. (A) Chemical structures of polymers. **(B)** Schematic illustration of PPC with PEG-*b*-PAMA. 79x72mm (300 x 300 DPI)



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79x152mm (300 x 300 DPI)

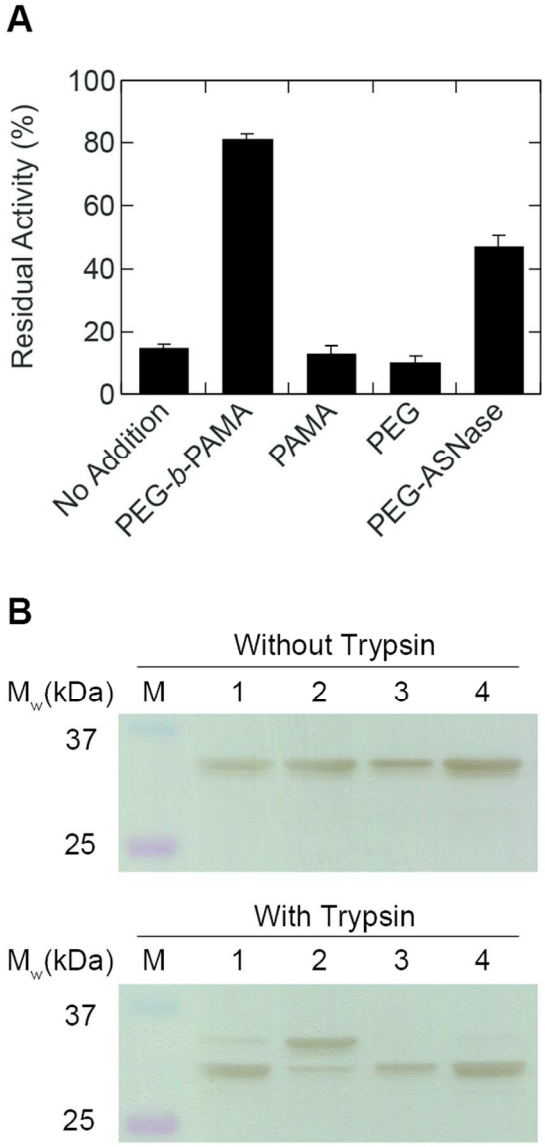

Figure 3. Proteolytic digestion of ASNase with polymer. **(A)** Residual activity of ASNase with polymers and PEG-ASNase after trypsin treatment. **(B)** SDS-PAGE of ASNase/polymer preincubated with or without trypsin treatment. Lane 1, native ASNase; Lane 2, ASNase/PEG-*b*-PAMA; Lane 3, ASNase/PAMA;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

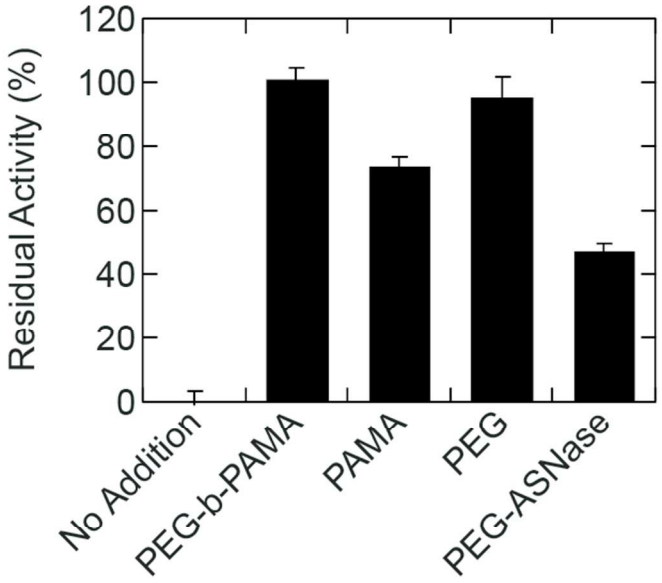

Figure 4. Residual activities of ASNase with polymers and PEG-ASNase after the shaking at 500 rpm for 6 hours.
79x54mm (300 x 300 DPI)

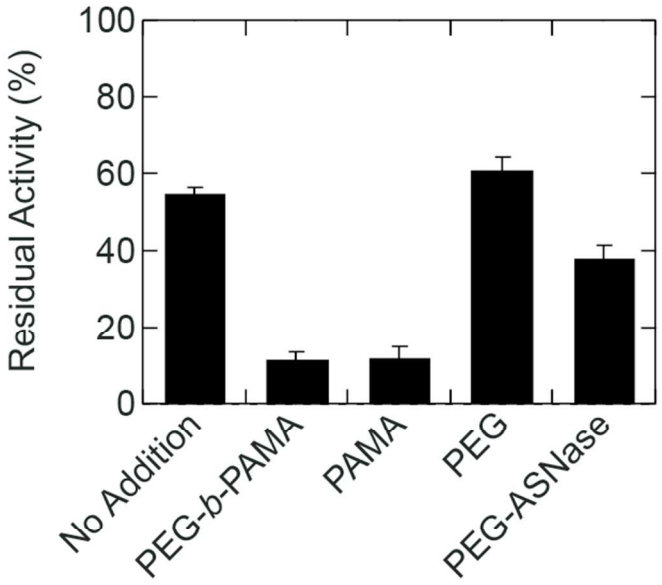

Figure 5. Residual activity of ASNase with polymers and PEG-ASNase after heat treatment at 60°C for 2 hours.
79x54mm (300 x 300 DPI)