J. Microbiol. Biotechnol. (2010), **20**(10), 1424–1429
doi: 10.4014/jmb.1006.06004
First published online 7 August 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Synthesis of an Amphiphilic Poly(γ-Glutamic Acid)–Cholesterol Conjugate and Its Application as an Artificial Chaperone

Lee, Eun-Hye[1], Yoshiki Kamigaito[1], Takashi Tsujimoto[1], Hiroshi Uyama[1]*, and Moon-Hee Sung[2,3]*

[1]*Department of Applied Chemistry, Graduate School of Engineering, Osaka University, Osaka 565-0871, Japan*
[2]*Department of Bio & Nanochemistry, Kookmin University, Seoul 136-702, Korea*
[3]*BioLeaders Corp., Daejeon 305-500, Korea*

Received: June 3, 2010 / Revised: June 28, 2010 / Accepted: June 29, 2010

A poly(γ-glutamic acid) (γPGA)–cholesterol conjugate was synthesized and its properties were then evaluated. The conjugate exhibited an amphiphilic nature derived from the hydrophilic γPGA backbone and the hydrophobic cholesterol side chain. The conjugate spontaneously formed nanoparticles, becoming an aqueous solution when at low concentrations, and at high concentrations the result was the formation of a physical gel. By utilizing the self-aggregating properties of the conjugate in water, an artificial chaperone was developed. A complex of protein, with the nanoparticles of the conjugate, was formed and the protein was released upon the dissociation of the nanoparticles through the addition of β-cyclodextrin. For denatured carbonic anhydrase, the activity was recovered in the artificial chaperone of the nanoparticle conjugate.

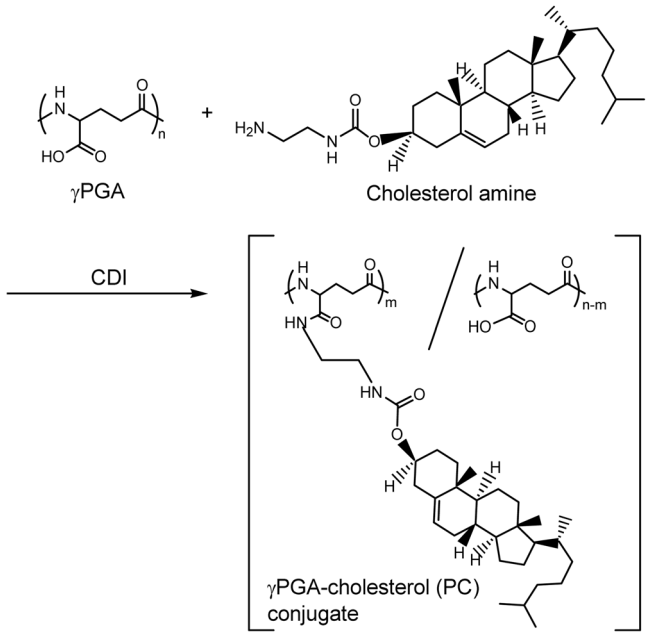
Keywords: Poly(γ-glutamic acid), cholesterol, amphiphilic polymer, artificial chaperone

Expression of cloned genes in bacteria is widely used for the production of pharmaceutical proteins both within industry and for biochemical studies of proteins in research. However, in many cases, recombinant proteins that are expressed in bacteria often form biologically inactive aggregates, called inclusion bodies (IB). In order to recover the biological activity of the proteins, the IB must be refolded into active aggregates [4]. In nature, a molecular chaperone, a giant molecular aggregate of protein, refolds proteins from denatured structures. It is often difficult to construct this system *in vitro*, and thus for the purposes of refolding of a variety of proteins in the field of biotechnology, artificial chaperoning systems have received much attention. In 1995, Rozema and Gellman [25] pioneered an artificial chaperoning system for efficient protein refolding. Many researchers have attempted to design an artificial chaperone system using ionic/nonionic detergents [28], cyclodextrins [25], polyols [7], poly(ethylene glycol) [5], and so forth. In most cases, however, it is difficult to completely remove the additives after treatment.

Amphiphilic polymers form stable nanoparticles in water, and consist of a hydrophilic outer part and a hydrophobic inner. The self-assembling properties of amphiphilic polymers provide for their high utility for many applications in industry; for example, as drug carriers [6], surface modifiers [8], colloidal dispersants [18], and as matrixes of artificial chaperones [1, 3].

Poly(γ-glutamic acid) (γPGA) is a bacterially synthesized biopolymer and has been extensively studied from industrial standpoints. The bacteria of the *Bacillus* species, such as *B. anthracis*, *B. licheniformis*, and *B. subtilis*, are often used for the production of γPGAs [11, 12, 23, 24, 26]. Unlike conventional natural peptides, γPGA, which consists exclusively of glutamic acid in a γ-amide linkage of the glutamate unit, is substantially biodegradable, nontoxic to humans, and even edible. Its multifunctionality has made it a promising biomaterial for a variety of uses, such as in health foods, moisturizers in cosmetics, chelating agents in waste water treatment, and hydrogels for environmental, agricultural, and biomedical product applications [11, 13–15, 20, 23, 26]. Recently, we found that γPGA strongly influenced enzymatic activity and stability, with the formation of a specific complex between enzyme proteins and γPGA. In addition, γPGA was efficient in the suppression of denaturation in enzymes during thermal treatment or freeze-thaw processes.

**Corresponding author*
H.U.
Phone: +81-6-6879-7364; Fax: +81-6-6879-7367;
E-mail: uyama@chem.eng.osaka-u.ac.jp
M.-H.S.
Phone: +82-2-910-4808; Fax: +82-2-910 8550;
E-mail: smoonhee@kookmin.ac.kr, smoonhee@bioleaders.co.kr



Scheme 1. A simplified scheme for the synthesis of the γPGA–cholesterol (PC) conjugate.

Cholesterol groups are often used for supramolecular assembly, owing to their strong hydrophobic interactions [6, 16, 19]. It has been hypothesized that the combination of the characteristics of γPGA, and cholesterol, should afford the amphiphilic nanoparticles to conjugate in water, whereby proteins could be efficiently refolded. In this study, we have designed an artificial chaperone, consisting of the highly hydrophilic γPGA of the polymer backbone and a strong hydrophobic cholesterol group in the side chain. This amphiphilic polymer [γPGA–cholesterol (PC) conjugate], which was synthesized according to Scheme 1, formed nanoparticles in water and exhibited chaperone-like activity.

MATERIALS AND METHODS

Materials

γPGA (acid form, M_w=2×10^3 kDa) was acquired through BioLeaders Corp. (Korea). Carbonic anhydrase from bovine erythrocytes was purchased from Sigma-Aldrich (U.S.A.). All solvents and reagents were used without further purification.

Synthesis of γPGA–Cholesterol Conjugate

Cholesterol amine was synthesized in accordance with the literature [9]. A typical run of the conjugate synthesis was as follows (PC 3 in Table 1): To a mixture of γPGA (0.39 g, 3 mmol) in 10 ml of dimethyl sulfoxide (DMSO), and cholesterol amine (0.036 g, 0.075 mmol) in 10 ml of tetrahydrofuran (THF), 1,1'-carbonylbis-1*H*-imidazole (CDI) (0.049 g, 0.3 mmol) was added with gentle stirring and kept at 40°C for 12 h. After the reaction solution was cooled to room temperature, the solvents were evaporated under reduced pressure and the residue was poured into 200 ml of toluene. The precipitate was collected by filtration and dried *in vacuo*. The resulting product was dissolved in distilled water, and sodium hydrogen carbonate (0.25 g, 3 mmol) was added to the solution, which was then treated by Amberlite IR-120H for ion exchange. After the removal of Amberlite by filtration, the product was subjected to dialysis using a cellulose membrane (cutoff molecular weight of 2×10^3) in distilled water for 2 days. The solution was lyophilized to render the conjugate (70% yield). The structure was confirmed by ^{1}H NMR (Bruker DPX 400). [^{1}H NMR (in DMSO-d_6): δ 0.8–1.5 (br, CH_3, CH_2, and CH of choresterol), 1.7, 1.9 (br, β-CH_2 of γPGA), 2.2 (br, γ-CH_2 of γPGA), 3.0–3.1 (br, NHCH_2), 4.1–4.3 (br, α-CH of γPGA), 5.3 (d, CH=C), and 8.3 (br, NH of γPGA).] The introduced ratio of cholesterol was calculated from the ratio of the integrated area of the peaks attributed to the methine proton (α-CH of γPGA, δ 4.1–4.3) and olefinic cholesterol proton (CH=C of cholesterol amine, δ 5.3).

Determination of Critical Micelle Concentration

The critical micelle concentration (CMC) was determined *via* the fluorescence spectroscopic method, using pyrene as a hydrophobic fluorescence probe [2, 16]. Aliquots of pyrene solutions (1.5×10^{-5} M in acetone) were added to the containers, and the solvent was removed under reduced pressure. Aqueous PC conjugate solutions of different concentrations, from 5×10^{-3} to 5 mg/ml, were added to the containers containing pyrene. The mixtures were incubated for 24 h at 20°C, and the excitation spectra were recorded on an F-2500 fluorescence spectrophotometer (Hitachi, Japan). The excitation spectra were recorded from 300 to 360 nm with an emission wavelength of 390 nm. The ratio ($I_{337.5}/I_{323}$) of the fluorescent intensities, at 337.5 and 323 nm, was calculated to evaluate the hydrophobicity around the pyrene molecules, and the rising point of $I_{337.5}/I_{323}$ was defined as the CMC.

Measurements of Fluorescence

For the examination of the trap and release function of the conjugate nanoparticles, a fluorescence study was carried out. To the conjugate

Table 1. Properties of γPGA–cholesterol (PC) conjugate.

Sample	Feed of cholesterol amine to γPGA (mol%)	Yield (%)	Introduction ratio of cholesterol (mol%)[a]	Diameter (nm)[b]
PC 1	1.00	80	0.88	350±10
PC 2	1.25	85	1.20	320±50
PC 3	2.50	70	2.00	250±90

[a]Determined by ^{1}H NMR.
[b]Determined by DLS at 0.5 mg/ml.

solution, a concentrated stock of fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA, 30 mg/ml) in 0.1 M PBS (pH 7.4) was added to produce the solution with the final concentrations of FITC-BSA and the conjugate of 0.03 mg/ml and 5.0 mg/ml, respectively. After 24 h of incubation, 10 mM β-cyclodextrin (β-CD) was added to the mixture. All samples were incubated at 20°C and their fluorescence spectra were recorded.

Measurement of Dynamic Light Scattering

The size of micelles was measured by using the dynamic light scattering (DLS) apparatus of DLS-6006 (Otsuka Electronics, Japan) equipped with a He–Ne laser and a thermoregulated bath at 25°C. Before the measurement, the solution was filtered through a 5 μm syringe filter and the filtrate was sonicated using a probe-type sonifier at 0°C for 10 min.

Measurement of Viscosity

The conjugate was dissolved in water at various concentrations and kept at room temperature for 1 day. The viscosity of the conjugate was measured by a ThermoHaake Rheostress RS1 rheometer with a parallel plate of 20 mm diameter and a 1 mm gap.

Refolding of Carbonic Anhydrase

Carbonic anhydrase (CA, 30 mg/ml) was denatured by incubation at 25°C for 16 h in a 50 mM Tris-sulfate buffer (pH 7.5) containing 10 M urea. This solution was diluted a thousand times by the buffer containing 5 mg/ml of the conjugate and incubated at 37°C for 2 h. β-CD was added to the mixture, which was then incubated at 37°C for 24 h. The activity of CA was determined according to an assay of the *p*-nitrophenyl acetate (*p*NPA) hydrolysis [21]. The assay started by mixing 10 μl of 100 mM *p*NPA in acetonitrile and 1.0 ml of the above sample solution (mixture of CA and conjugate) for 20 s, and the amount of the resulting *p*-nitrophenol was monitored from the absorbance change at 400 nm as a function of time by using a U-2810 UV–Vis spectrophotometer (Hitachi, Japan). The percent recovery of enzymatic activity was calculated on the basis of the initial velocity of the activity of native CA [22].

Results and Discussion

Synthesis and Characterization of γPGA–Cholesterol Conjugate

Cholesterol has only secondary hydroxy groups of low reactivity; thus, it is difficult for it to directly react with γPGA. Therefore, we selected a primary amine derivative of cholesterol and allowed for the reaction with γPGA in the presence of CDI as a dehydrating agent, in order to produce the γPGA–cholesterol (PC) conjugate (Scheme 1). In the ^{1}H NMR spectrum of the conjugate, a peak at δ 2.8 ascribed to the terminal methylene proton of cholesterol amine (NH_2CH_2) was not found, and a new peak at around δ 3.0–3.1 ($NHCH_2$) attributed to the corresponding amide appeared, indicating the formation of the desired conjugate. In this study, three conjugate samples with different contents of the cholesterol moiety were synthesized and used for applications of the artificial chaperone (Table 1).

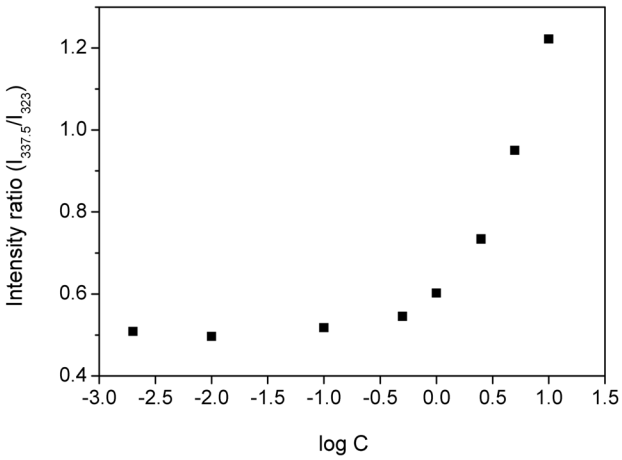


Fig. 1. Plot of the fluorescence intensity ratio ($I_{337.5}/I_{323}$) obtained from the excitation spectra of PC 1 as a function of the concentration of the conjugate.
The final concentration of pyrene was 6.2×10^{-7} M.

The content of the cholesterol group in the conjugate increased as a function of the feed ratio.

The CMC of the conjugate was determined by the fluorescence spectroscopic method, with use of pyrene as a probe [2, 16], which is sensitive to the polarity of the solubilizing medium. The CMC of the conjugate (PC 1) was approximately 0.5 mg/ml, and the CMC value scarcely depended on the cholesterol content of the conjugate (Fig. 1).

In the DLS measurement of the dilute concentration of the conjugate (0.5 mg/ml), nanoparticles of an average diameter of 250–350 nm were detected. The particle size decreased slightly with an increase in the content of the cholesterol group. For PC 3, the particle size of the conjugate was found to be almost constant for two weeks

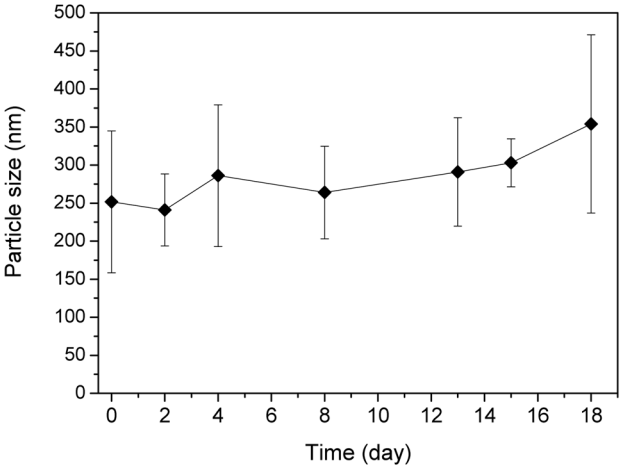


Fig. 2. Particle size of PC 3 as a function of time, measured by DLS in an aqueous solution.
The concentration of the conjugate was 0.5 mg/ml.

(Fig. 2), indicating a good stability of the nanoparticles of the conjugate.

Fig. 3A shows the effect of the concentration of the conjugates on the viscosity. For the conjugates with the lowest content of the cholesterol group (PC 1), the viscosity slightly increased as a function of the concentration. However, other conjugates (PC 2 and PC 3) showed a remarkable increase of viscosity with increasing concentrations. Furthermore, physical gels were formed in the high concentrations of these samples. This is likely due to a strong hydrophobic interaction in the cholesterol group for the conjugate bearing its high content.

Cyclodextrins include a variety of compounds in its cavity, and cholesterol is known to form an inclusion complex with β-CD [17, 27]. The effect of the addition of β-CD on the viscosity of the conjugate was examined (Fig. 3B). When fixing the concentration of PC 2 at 50 mg/ml, a sudden decrease in the viscosity was observed at a β-CD concentration of 8 mM, which is probably due to the formation of the inclusion complex of β-CD, and the cholesterol group of the conjugate, resulting in the dissociation of the aggregation of the conjugate.

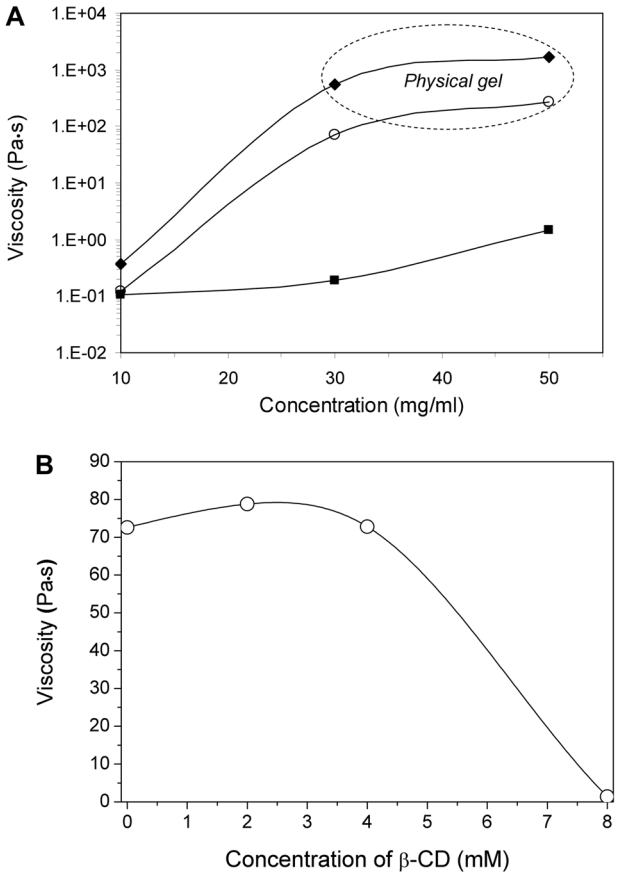


Fig. 3. Viscosities of the γPGA–cholesterol conjugate aqueous solutions of (**A**) PC 1 (■), PC 2 (○), and PC 3 (◆), and (**B**) 50 mg/ml of PC 2 in the presence of β-CD.
The measurement was carried out with a shear stress of 10 Pa at a 0.1 Hz frequency, at room temperature. The viscosity value represents the mean of three independent experiments.

Interaction Between the γPGA–Cholesterol Conjugate and Protein

FITC-labeled proteins are often used for mechanistic studies in order to elucidate the interaction of proteins with target molecules. In this study, the interaction between FITC-BSA and the PC conjugate was examined using fluorescence spectroscopy (Fig. 4). A remarkable increase of the largest intensity was found by the addition of the conjugate. In addition, a slight red shift from 519 to 522 nm of the maximum emission peak was observed. These data indicate that the surrounding environment of the FITC moiety becomes hydrophobic in the presence of the conjugate [10], and that when the complex between FITC-BSA and the conjugate is formed, the protein is included in the nanoparticles of the conjugate.

Through the addition of β-CD to the mixture of FITC-BSA and the PC conjugate, the peak intensity again decreased, suggesting the disappearance of the strong interaction between FITC-BSA and the PC conjugate. This result appears to be closely related to the rapid decrease of the viscosity of the conjugate by the addition of β-CD (Fig. 3B).

Chaperone-Like Activity of γPGA–Cholesterol Conjugate

CA was selected as a model protein for the evaluation of the chaperone-like activity of the PC conjugate. The CA was denatured by urea. Fig. 5 shows the recovery of the enzyme activity of CA by the addition of the PC conjugate, as a function of β-CD concentration. The complex protein

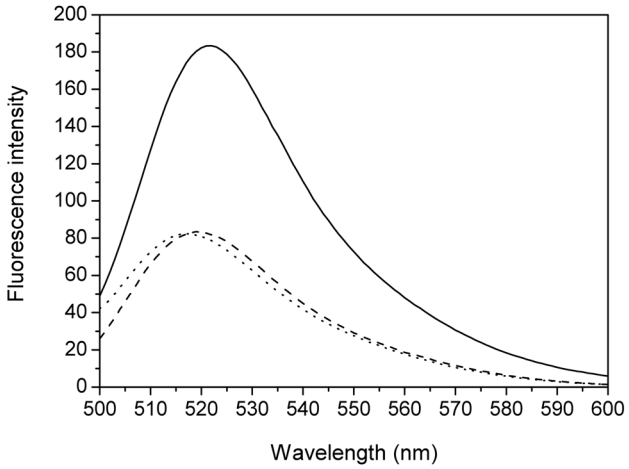


Fig. 4. Fluorescence spectra of FITC-BSA (dashed line), a mixture of FITC-BSA and PC 1 (solid line), and a mixture of the FITC-BSA–PC 1 complex and β-CD (dotted line), at 25°C.
The concentrations of FITC-BSA, PC 1, and β-CD were 0.03 mg/ml, 5 mg/ml, and 10 mM of β-CD, respectively.

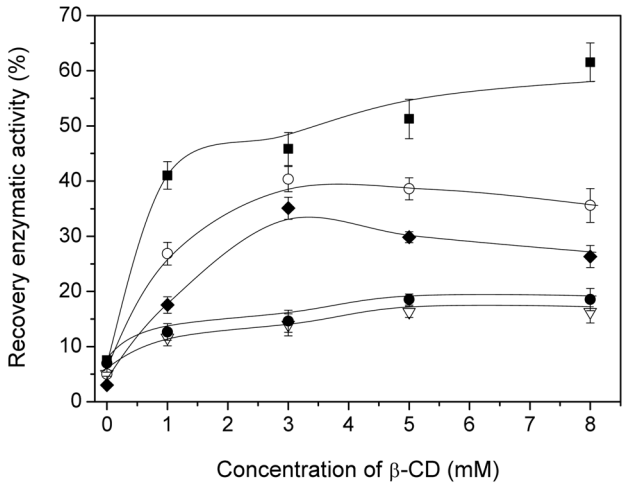


Fig. 5. Activity recovery of denatured carbonic anhydrase (CA) by the γPGA–cholesterol conjugate as a function of the concentration of β-CD (n=3).
(▽) Denatured CA, (●) γPGA, (■) PC 1, (○) PC 2, and (◆) PC 3. The final concentrations of CA and the conjugate were 0.03 mg/ml and 5 mg/ml, respectively.

was released in a refolded form upon the dissociation of the nanoparticles in the presence of β-CD. Where there were low concentrations of β-CD (less than 3 mM), the recovery activity of the CA was found to be low, whereas an increasing concentration of β-CD exhibited a higher recovery activity. At the same time, of the higher concentrations of β-CD, a slight decrease in the recovery activities of PC 2 and PC 3 was observed. Without the conjugate, the recovery activity was approximately 15%, whereas the presence of the conjugate resulted in an efficient recovery in the activity of CA. The recovery activity in the presence of γPGA and β-CD was found to be similar to that without the conjugate, indicating that amphiphilic properties are required for the recovery of activity. PC 1 showed the highest recovery in activity. PC 3 showed the lowest recovery activity than the other conjugates, which may be due to the precipitation formed when the denatured CA solution was diluted by the buffer containing the PC 3.

In the present study, an amphiphilic polymer consisting of a hydrophilic γPGA backbone, and a cholesterol group of the side chain, was synthesized and its self-aggregating properties in water were examined. Stable nanoparticles were formed in low concentrations of the γPGA–cholesterol conjugate, and gel formation was observed in high concentrations. The dissociation of the nanoparticles took place by the addition of β-CD. The complex was formed from the conjugate nanoparticles and proteins, and protein was included in the nanoparticles of the conjugate. The conjugate nanoparticles showed good chaperone-like activity, with the conjugate efficiently refolding the denatured carbonic anhydrase in the presence of β-CD. The combination of characteristics of γPGA and the self-aggregating properties of the present conjugate will provide for potential applications as an artificial chaperone for various biochemical fields. Further investigations, including on the refolding of other useful proteins, and examination of the mechanisms of refolding in the present conjugate nanoparticles, are currently under way in our laboratory.

Acknowledgments

The work was supported by a 2010 research fund of Kookmin University.

References

1. Akiyoshi, K., Y. Sasaki, and J. Sunamoto. 1999. Molecular chaperone-like activity of hydrogel nanoparticles of hydrophobized pullulan: Thermal stabilization with refolding of carbonic anhydrase B. *Bioconjugate Chem.* **10:** 321–324.
2. Astafieva, I., X. F. Zhong, and A. Eisenberg. 1993. Critical micellization phenomena in block polyelectrolyte solutions. *Macromolecules* **26:** 7339–7352.
3. Cavalieri, F., E. Chiessi, and G. Paradossi. 2007. Chaperone-like activity of nanoparticles of hydrophobized poly(vinyl alcohol). *Soft Mater.* **3:** 718–724.
4. Clark, E. D. B. 1998. Refolding of recombinant proteins. *Curr. Opin. Biotechnol.* **9:** 157–163.
5. Cleland, J. L., C. Hedgepeth, and D. I. C. Wang. 1992. Polyethylene glycol enhanced refolding of bovine carbonic anhydrase B. *J. Biol. Chem.* **267:** 13327–13334.
6. Dutta, P., S. Shrivastava, and J. Dey. 2009. Amphiphilic polymer nanoparticles: Characterization and assessment as new drug carriers. *Macromol. Biosci.* **9:** 1116–1126.
7. Gerlsma, S. Y. 1968. Reversible denaturation of ribonuclease in aqueous solutions as influenced by polyhydric alcohols and some other additives. *J. Biol. Chem.* **243:** 957–961.
8. Hou, A. and Y. Shi. 2009. Polymerization and surface active properties of water-soluble amphiphilic polysiloxane copolymers modified with quaternary ammonium salts and long-carbon chain groups. *Mater. Sci. Eng. B* **163:** 99–104.
9. Ishi-i, T., R. Iguchi, E. Snip, M. Ikeda, and S. Shinkai. 2001. Fullerene can reinforce the organogel structure of porphyrin-appended cholesterol derivatives: Novel odd–even effect of the $(CH_2)_n$ spacer on the organogel stability. *Langmuir* **17:** 5825–5833.
10. Kim, M. S., K. S. Seo, G. Khang, and H. B. Lee. 2005. Preparation of a gradient biotinylated polyethylene surface to bind streptavidin-FITC. *Bioconjugate Chem.* **16:** 245–249.
11. Kim, T. W., T. Y. Lee, H. C. Bae, J. H. Hahm, Y. H. Kim, C. Park, *et al*. 2007. Oral administration of high molecular mass poly-γ-glutamate induces NK cell-mediated antitumor immunity. *J. Immun.* **179:** 775–780.
12. Ko, Y. H. and R. A. Gross. 1998. Effects of glucose and glycerol on γ-poly(glutamic acid) formation by *Bacillus licheniformis* ATCC 9945a. *Biotechnol. Bioeng.* **57:** 430–437.
13. Lee, E.-H., H. Uyama, O. H. Kwon, and M. H. Sung. 2009. Fabrication of ultrafine fibers of poly(γ-glutamic acid) and its derivative by electrospinning. *Polym. Bull.* **63:** 735–742.

14. Lee, E.-H., Y. Kamigaito, T. Tsujimoto, H. Uyama, S. Seki, S. Tagawa, and M. H. Sung. 2010. Preparation of poly(γ-glutamic acid) hydrogel/apatite composites and their application for scaffold of cell proliferation. *J. Soc. Fiber Sci. Technol. Jap.* **66:** 104–111.
15. Lee, T. Y., Y. H. Kim, S. W. Yoon, J. C. Choi, J. M. Yang, C. J. Kim, J. T. Schiller, M. H. Sung, and H. Poo. 2009. Oral administration of poly-gamma-glutamate induces TLR4- and dendritic cell-dependent antitumor effect. *Cancer Immunol. Immunother.* **58:** 1781–1794.
16. Liu, X.-M., K. P. Pramoda, Y.-Y. Yang, S. Y. Chow, and C. He. 2004. Cholesteryl-grafted functional amphiphilic poly(*N*-isoproprylacrylamide-*co*-*N*-hydroxylmethylacrylamide): Synthesis, temperature-sensitivity, self-assembly and encapsulation of a hydrophobic agent. *Biomaterials* **25:** 2619–2628.
17. Ohtani, Y., T. Irie, K. Uekama, K. Fukunaga, and J. Pitha. 1989. Differential effects of α-, β- and γ-cyclodextrins on human erythrocytes. *Eur. J. Biochem.* **186:** 17–22.
18. Okoshi, K., N. Sano, T. Okumura, A. Tagaya, J. Magoshi, Y. Koike, M. Fujiki, and J. Watanabe. 2003. The Christiansen effect of brightly colored colloidal dispersion with an amphiphilic polymer. *J. Colloid Interface Sci.* **263:** 473–477.
19. Ooya, T., K. M. Huh, M. Saitoh, E. Tamiya, and K. Park. 2005. Self-assembly of cholesterol–hydrotropic dendrimer conjugates into micelle-like structure: Preparation and hydrotropic solubilization of paclitaxel. *Sci. Technol. Adv. Mat.* **6:** 452–456.
20. Park, C., Y. H. Choi, H. J. Shin, H. Poo, J. J. Song, C. J. Kim, and M. H. Sung. 2005. Effect of high-molecular-weight poly-γ-glutamic acid from *Bacillus subtilis* (*chungkookjang*) on Ca solubility and intestinal adsorption. *J. Microbiol. Biotechnol.* **15:** 855–858.
21. Pocker, Y. and D. R. Storm. 1968. The catalytic versatility of erythrocyte carbonic anhydrase. IV. Kinetic studies of enzyme-catalyzed hydrolyses of *p*-nitrophenyl esters. *Biochemistry* **7:** 1202–1214.
22. Pocker, Y. and L. J. Guilbelt. 1972. Catalytic versatility of erythrocyte carbonic anhydrase. Kinetic studies of the enzyme-catalyzed hydrolysis of methylpyridyl carbonates. *Biochemistry* **11:** 180–190.
23. Richard, A. and A. Margaritis. 2001. Poly(glutamic acid) for biomedical applications. *Crit. Rev. Biotechnol.* **21:** 219–232.
24. Roelants, G. E. and J. W. Goodman. 1968. Immunochemical studies on the poly-γ-D-glutamyl capsule of *Bacillus anthracis*. IV. The association with peritoneal exudate cell ribonucleic acid of the polypeptide in immunogenic and nonimmunogenic forms. *Biochemistry* **7:** 1432–1440.
25. Rozema, D. and S. H. Gellman. 1995. Artificial chaperones: Protein refolding *via* sequential use of detergent and cyclodextrin. *J. Am. Chem. Soc.* **117:** 2373–2374.
26. Sung, M. H., C. Park, C. J. Kim, H. Poo, K. Soda, and M. Ashiuchi. 2005. Natural and edible biopolymer poly-γ-glutamic acid: Synthesis, production, and applications. *Chem. Rec.* **5:** 352–366.
27. Van de Manakker, F., M. Van der Pot, T. Vermonden, C. F. Van Nostrum, and W. E. Hennink. 2008. Self-assembling hydrogels based on β-cyclodextrin/cholesterol inclusion complexes. *Macromolecules* **41:** 1766–1773.
28. Wetlaufer, D. B. and Y. Xie. 1995. Control of aggregation in protein refolding: A variety of surfactants promote renaturation of carbonic anhydrase II. *Protein Sci.* **4:** 1535–1543.