J. Microbiol. Biotechnol. (2009), **19**(9), 960–965
doi: 10.4014/jmb.0812.688
First published online 18 April 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Seed-Conjugated Polymer Bead for β2-Microglobulin Removal at Neutral pH

Kim, Mira, Sungsoo Kang, Eun-Kyung Myung, Minkoo Ahn, Jeong-Hyun Choi, Seung R. Paik, and Yoon-Sik Lee*

School of Chemical and Biological Engineering, Seoul National University, Seoul 151-744, Korea

Received: December 22, 2008 / Revised: February 18, 2009 / Accepted: March 12, 2009

β2-Microglobulin (β2m) is known to be a major factor for dialysis-related amyloidosis. We have studied β2m removal through an aggregation process, which was initiated by a ligand that could catch the β2m monomer and promote its aggregation into fibril. As a ligand, we have prepared β2m fibril fragments and used them as a seed. The seed was coupled to PEGylated-PS beads to remove the monomeric β2m from solution. The β2m seed-conjugated resin effectively adsorbed the β2m monomers with a capacity of 3.6 mg/ml *via* promoting their aggregation into fibrils on the resin at pH 7.4.

Keywords: β2-Microglobulin, dialysis-related amyloidosis, amyloid seed, polymer bead, protein aggregation

Dialysis-related amyloidosis (DRA) is common in patients with end-stage kidney disease [11, 14, 15, 33]. β2-Microglobulin (β2m) is the major protein constituent of this amyloid [14]. The concentration of β2m in patients undergoing hemodialysis for more than 5 years is 30–50 times higher than that of a normal individual. β2m noncovalently binds to the light chain of the major histocompatibility complex class I (MHC-I), is released to the serum and is then carried to the kidney [4, 6]. Renal failure disrupts the process of catabolic β2m degradation in the kidney, which results in increased levels of β2m in the patients. Hemodialysis is not sufficient to get rid of the β2m. Elimination of β2m from blood serum, therefore, has gained much attention [12, 20].

Removal of β2m using a conventional adsorption column based on hydrophobic adsorption with additional size-exclusion property is insufficient in terms of its specificity and affinity toward β2m. [1, 10, 13, 31, 32] Antibodies could be employed for the removal, but this method is not cost-effective and their storage stability might be also problematic [2, 16, 23, 28].

Native β2m contains 99 amino acid residues and has a β-sandwich structure stabilized by a single disulfide bond [17, 30]. Incubation of β2m under acidic conditions results in the formation of amyloid fibrils. Under physiologically relevant, neutral pH, however, β2m is hardly converted into amyloid fibrils [26, 35, 36]. A few regulators that accelerate fibrillation at neutral pH have been examined, including the β2m amyloid seeds [26, 35, 36].

Fibril growth of β2m at neutral pH was shown to be directed by the self-seeding effect [8, 18]. In the present study, we focused on localization of the seeds on the polymer beads (resin) and removal of β2m from solution by inducing fibrillization on the resin surface.

Materials and Methods

Materials

N-Hydroxysuccinimide (NHS, 97%), 4-dimethylaminopyridine (DMAP), and succinic anhydride (SA, 99%) were supplied from Fluka. Acetic anhydride, *N,N'*-diisopropyl carbodiimide (DIC), diisopropylethylamine (DIEA), and polyoxyethylene-20-sorbitan monolaurate (Tween 20) were purchased from Aldrich. *N*-Methylpyrrolidone (NMP) and dimethyl formamide (DMF) were purchased from Junsei Chemical Co. 9-Fluorenylmethyl carbamate (Fmoc)-methionine and aminomethyl polystyrene (AM PS) resin were supplied from BeadTech, Korea. The fluorescein isothiocyanate (FITC) Protein Labeling Kit was obtained from Molecular Probes, Inc. The diethylaminoethyl (DEAE) Sepharose FF column was bought from GE Healthcare, U.S.A. Filter membranes were purchased from Millex.

Purification of β2m and Preparation of β2m Seeds

The DNA sequence encoding human β2m was amplified from pCMV-SPORT6 (21C Frontier Human Gene Bank, KUGI, Korea) and inserted into the NdeI/HindIII sites of pRSET A vector (Invitrogen) [9]. Wild-type β2m was overexpressed in *E. coli* BL21 (DE3). *E. coli* containing the β2m expression plasmid was incubated in Luria–Bertani (LB) medium with 0.1 mg/ml ampicillin at 37°C. Harvested β2m was subjected to air-oxidation in 8 M urea and

*Corresponding author
Phone: +82-2-880-7073; Fax: +82-2-876-9625;
E-mail: yslee@snu.ac.kr

refolded through dialysis against 20 mM Tris-Cl, pH 8.0 [29]. β2m was purified using a DEAE–Sepharose FF column (0–500 mM NaCl concentration gradient) and size-exclusion chromatography (Hiprep 26/60 Sephacryl S-100). This β2m solution was dialyzed against deionized water and lyophilized to β2m powder.

β2m (1 mg/ml in 0.15 M NaCl, 50 mM sodium citrate buffer, pH 2.5) was incubated at 37°C for 3 days [21, 24]. Aggregation kinetics was monitored with thioflavin-T binding fluorescence and the mature fibrils were confirmed with a transmission electron microscope (TEM). The seeds were obtained by fragmentation of amyloid fibrils using 15 cycles of sonications with 80 sec for a cycle (20 sec, on; 60 sec, off) [25, 34]. Then, the fibrillar fragments were filtered through a 0.22-μm pore-sized membrane filter.

FITC Labeling to β2m Monomer

β2m monomer solution (200 μl, 1.7 mg/ml in 20 mM PBS, pH 7.4) and 1 M sodium bicarbonate (20 μl) were combined in a reaction tube with a stirring bar. While stirring the protein solution in a tube, FITC stock solution (6.6 μl, 10 mg/ml in DMSO) was added. The reaction mixture was stirred for 1 h at room temperature. After the reaction mixture was loaded onto the gel bed of the spin column (30,000 MW size exclusion resin in PBS with 2 mM sodium azide), it was then centrifuged for 3 min at 5,000 rpm to collect the labeled protein. α-Synuclein monomer was labeled with FITC using the same method.

Preparation of β2m Seed-Conjugated Resin and Control Resin

HiCore resin (core-shell type PEGylated-PS bead) was prepared from AM PS bead using a previously developed method [19]. The loading amount of the amino group was analyzed quantitatively by Fmoc-titration [7, 22] and sulfur elementary analysis after Fmoc-Met coupling.

HiCore resin (1 g, 0.35 mmol NH_2/g) was mixed with SA (3 eq. 105 mg) and DIEA (6 eq. 366 μl) in NMP (10 ml) (Fig. 1). The resin mixture was agitated in a 15-ml polypropylene syringe reaction vessel with a frit and a polytetrafluoroethylene (PTFE) valve (Libra tube) for 24 h at 25°C. The product, carboxy functionalized resin, was filtered and washed with NMP, MeOH, and DCM. The conversion of the amine group to the carboxylic group was confirmed by negative color change of Kaiser's ninhydrin test [17].

NHS solution (121 mg in 3 ml of DMF) was added to the carboxy functionalized resin in a Libra tube and cooled to 0°C using an ice bath. DIC solution (134 μl in 4 ml of DCM) and DMAP solution (1.3 mg in 1 ml of DMF) were added to the resin mixture while keeping the temperature to 0°C. After 5 min, the resin mixture was shaken for 18 h at 25°C, filtered, and washed with DMF and DCM.

The pH of the β2m seed solution was adjusted to above pH 9 with Na_2CO_3. NHS-activated resin (100 mg) was reacted with the β2m amyloid fibrillar fragments seeds (1 ml, 0.5 mg/ml in pH 9.5 Tris buffer) in the 5-ml Libra tube for 3 h at 25°C. The seed-conjugated resin was filtered and washed with PBS (pH 7.4). Since the weight change of the resin was negligible upon seed immobilization, quantification of the seeds on the resin was difficult to monitor. Instead, we checked for coupling of the seeds to the beads with a scanning electron microscope (SEM) and fluorescence microscope.

As a control, the amino group of HiCore resin (100 mg, 0.35 mmol/g) was acetylated with acetic anhydride (3 eq. 10 μl) and DIEA (6 eq. 37 μl) in NMP (3 ml) for 1 h. Completion of acetylation was confirmed by Kaiser's ninhydrin test.

Removal of β2m Using the Seed-Conjugated Resin and Its Quantitative Analysis

The β2m seed-conjugated resin (10 mg) and the acetylated resin (10 mg) were treated with BSA (0.5% in pH 7.4, 20 mM PBS) for 1 h at 25°C, which was filtered and washed with 0.1% Tween 20 solution in 20 mM PBS, pH 7.4, 5 times within 30 min. Each resin was incubated and shaken in FITC–β2m monomer solution (50 μl, 0.6 mg/ml) in a 1-ml microtube in the dark at 37°C. The reactions were carried out for either 1 or 3 days. The resin was washed with PBS containing 0.1% Tween 20 solution. FITC-labeled α-synuclein (50 μl, 0.6 mg/ml) was also reacted with this resin (10 mg) using the same process.

For obtaining quantitative data, 10 mg of β2m seed-conjugated resin was incubated with native β2m solution (1 ml, 0.39 mg/ml) for 24 h at 37°C. For quantitative analysis, the decrease in concentration of β2m was monitored with UV–Vis absorbance at 595 nm using the Bradford assay [5]. The β2m concentrations were measured before and after incubation of the monomeric protein with the seed-conjugated resins.

RESULTS

Purification of β2m and Preparation of β2m Seed

β2m was purified according to a previously reported procedure [25, 26]. The purity of β2m was greater than

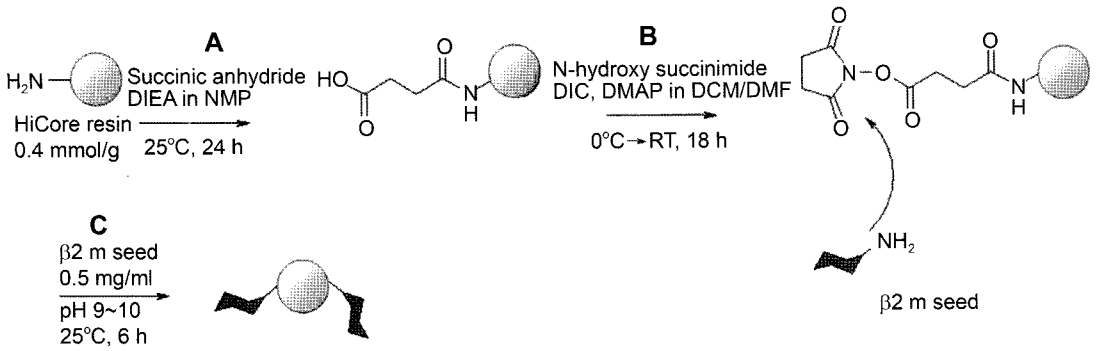

Fig. 1. Preparation of β2m seed-conjugated resin: **A**. Conversion of amine group to carboxyl group, **B**. Activation to NHS active ester; **C**. Coupling of β2m seed to the resin.

Fig. 2. TEM image of β2m: **A**. Monomer; **B**. Amyloid fibril; **C**. Fibril fragment.

95% according to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) mass analysis. For β2m fibril formation, β2m monomers were incubated at pH 2.5 with sodium citrate buffer. Thioflavin-T binding fluorescence reached a maximum after 20 h of incubation. The incubation time was extended for 3 days to obtain mature fibrils. The images of mature fibrils showed straight needle-like morphology, which was confirmed by TEM. The mature fibrils were chopped down to shorter fibrillar fragments by sonication. In order to obtain more homogenized fibrillar fragments, the resulting fibrillar products were filtered through a 0.22-μm pore-sized membrane. The filtered fibrillar fragments exhibited regular lengths (30–50 nm) and diameters (5–10 nm), which were confirmed by TEM (Fig. 2).

FITC Labeling of β2m Monomer

After the FITC labeling reaction was completed, FITC-labeled β2m was separated from the light green-colored FITC solution using a spin column with centrifugation. The labeled product revealed that one or two FITC molecules were coupled to β2m monomer, which was confirmed by MALDI–TOF. The small peak of 11.8 kDa corresponds to unlabeled β2m monomer, the main peak of 12.2 kDa to one FITC-labeled β2m monomer, and the 12.6 kDa to two FITC-labeled β2m monomers.

Preparation of β2m Seed-Conjugated Resin

For β2m seed coupling, we prepared HiCore resin (0.35 mmol NH_2/g), and converted the amino group to carboxyl group quantitatively by reacting with SA. Then, the carboxylated resin was activated using NHS for coupling with amino groups of the β2m fibrillar fragments as seeds. To confirm the coupling of β2m amyloid seeds to the resin, SEM analysis was performed (Fig. 3). Compared with the image of the bare resin, we could detect small, branch-like spots attached on the smooth surface of the resin. In order to reconfirm the results of β2m seed coupling, the same resin was monitored by fluorescence microscopy after JC-1 treatment. JC-1 is a fluorescent dye that interacts with the fibrillation-state protein structure [37].

β2m Adsorption to the β2m Seed-Coupled Polymer Bead

After incubation with FITC–β2m solution for 1 or 3 days, the acetylated resin and the seed-conjugated resin were analyzed by confocal laser scanning microscopy. Bare HiCore resin showed a slight fluorescence background, which was used to adjust a base level for the confocal laser fluorescence detection. Acetylated resin, used as a control, exerted no positive charge, while possessing PEG chains on the surface of the resin to interrupt protein adhesion. The control and seed-conjugated resins were treated with BSA to prevent nonspecific binding.

Seed-conjugated polymer beads showed obvious fluorescence signals after incubation with FITC–β2m solution at neutral pH for 1 day (Fig. 4). When the duration of incubation was increased to 3 days, the fluorescent signal became stronger compared with the 1-day incubation period. On the other hand, the control resin only gave very weak fluorescent signals even after 3 days of incubation

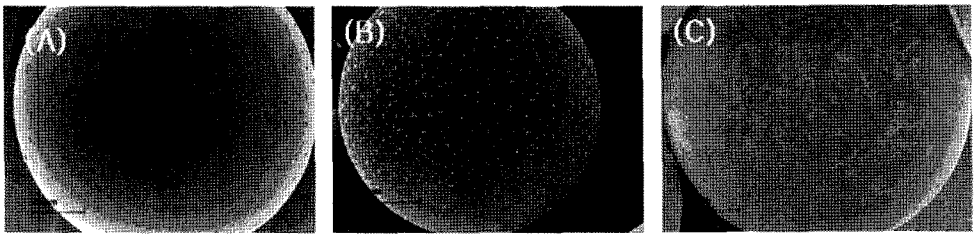

Fig. 3. SEM image of resin: **A**. Bare resin; **B**. Seed-conjugated resin; **C**. Seed-conjugated resin after β2m incubation for 24 h.

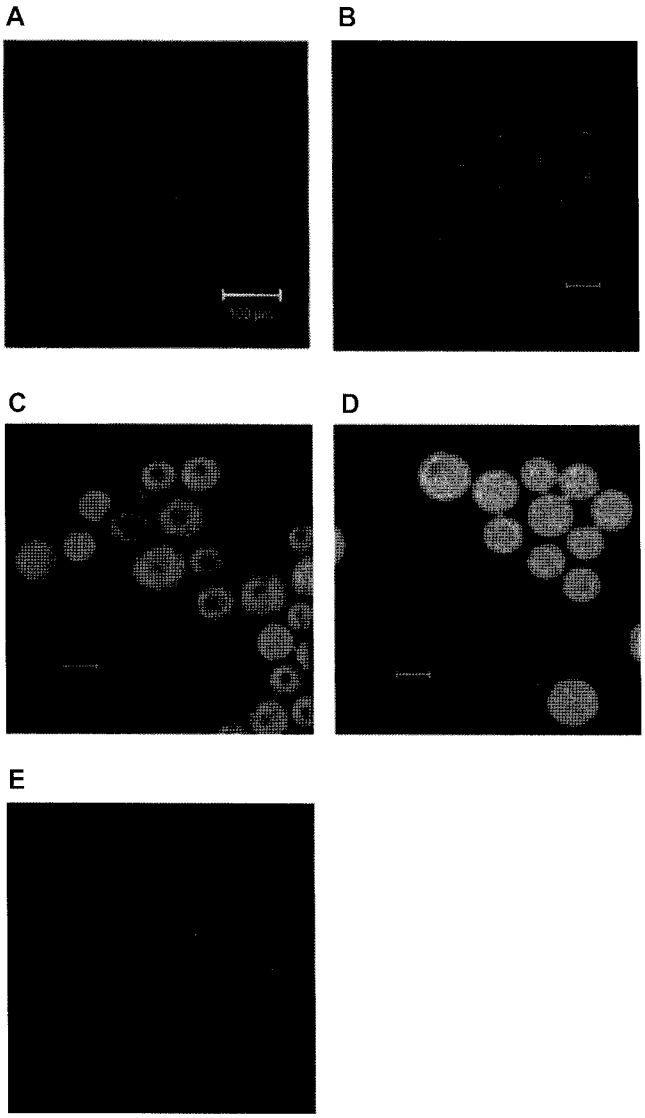

Fig. 4. Confocal microscope image of resin: **A**. Bare resin; **B**. Acetylated resin after FITC-β2m (0.6 mg/ml) incubation for 3 days; **C**. Seed-conjugated resin after FITC–β2m (0.6 mg/ml) incubation for 1 day; **D**. Seed-conjugated resin after FITC–β2m (0.6 mg/ml) incubation for 3 days; **E**. Seed-conjugated resin after FITC–α-synuclein (0.6 mg/ml) incubation for 1 day.

with FITC–β2m solution at neutral pH. We thus proved that the seed on the resin was very effective in adsorbing β2m from its monomeric solution.

For quantitative assay purposes, the seed-conjugated resins and the acetylated resins were reacted in β2m monomer solution at neutral pH. The initial concentration of β2m was 0.39 mg/ml, which was reduced to 0.30 mg/ml after treating the β2m solution (1 ml) with the seed-coupled polymer bead (10 mg, 0.025 ml) for one day, which corresponds to 9 mg/g polymer bead (Fig. 5). On the other hand, the decreased concentration of β2m in the control experiment was 0.03 mg/ml. The adsorption capacity of β2m per resin volume was about 3.6 mg/ml (mg β2m/l

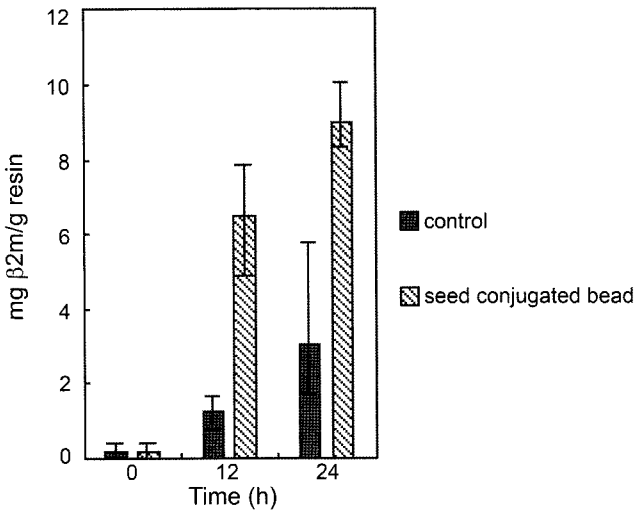

Fig. 5. Decreased amounts of β2m monomer per polymer bead (mg/g) from solution: the solid bar represents the control, the bar with stripes represent seed-conjugated resin[a]. The error bar was obtained after triple experiments.
[a]The β2m solution (1 ml, 0.4 mg/ml) was incubated with resin (10 mg) at 37°C under agitation.

settled resin). This is comparable to the previously reported results showing that the β2m adsorption site density was 2–4 mg/ml when using immunoadsorbent chromatography [2, 16, 23, 28] and 1 mg/ml when using a hydrophobic adsorbent column [1], even though their systems are different from ours.

We observed the surface morphology change of the seed-coupled resin after β2m adsorption for 24 h with SEM, and confirmed that fibril-like shapes of β2m aggregates had grown on the surface of the resin (Fig. 4).

DISCUSSION

In the present study, we demonstrated that β2m seeds that were coupled on a resin successfully captured β2m. The seeds on the resin removed β2m under neutral pH. The untreated resin revealed nonspecific adhesion of β2m. β2m fibril fractions were used as seeds to allow for binding of the β2m monomer to the resin and thereby initiate fibrillation on the resin. Removing β2m through the use of such a specific ligand is a more effective method than the one based on simple hydrophobic interactions. β2m fibril fractions can be easily obtained and have great ability as seeds to continuously promote β2m amyloid formation on the polymer bead surface.

For the purpose of designing a more advanced system, we are currently searching other types of resins to use as a solid support. We are also involved in investigations into the detailed mechanisms and fundamental theories of β2m fibril formation under neutral conditions.

Acknowledgments

This research was supported by Seoul R & BD program (10538) and a grant (F104AB010005-07A0201-00510) from Korea Biotech R&D Group of Next-Generation Growth Engine Project of the Ministry of Education, Science and Technology, Republic of Korea.

References

1. Abe, T., K. Uchita, H. Orita, M. Kamimura, M. Oda, H. Hasegawa, *et al.* 2003. Effect of β2-microglobulin adsorption column on dialysis-related amyloidosis. *Kidney Int.* **64:** 1522–1528.
2. Ameer, G. A., E. A. Grovender, H. Ploegh, D. Ting, W. F. Owen, M. Rupnick, and R. Langer. 2001. A novel immunoadsorption device for removing β2-microglobulin from whole blood. *Kidney Int.* **59:** 1544–1550.
3. Bethea, M. and D. T. Forman. 1990. Beta 2-microglobulin: Its significance and clinical usefulness. *Ann. Clin. Lab. Sci.* **20:** 163–168.
4. Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley. 1987. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* **329:** 506–512.
5. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
6. Bui, H. H., A. J. Schiewe, H. Grafenstein, and I. S. Haworth. 2006. Structural prediction of peptides binding to MHC class I molecules. *Proteins* **63:** 43–52.
7. Chan, W. C. and P. W. White. 2000. Fmoc solid phase peptide synthesis: A practical approach. pp. 41–76. Oxford University Press, New York.
8. Chatani, E., H. Naiki, and Y. Goto. 2006. Seeding-dependent propagation and maturation of β2-microglobulin amyloid fibrils under high pressure. *J. Mol. Biol.* **359:** 1086–1096.
9. Chiba, T., Y. Hagihara, T. Higurashi, K. Hasegawa, H. Naiki, and Y. Goto. 2003. Amyloid fibril formation in the context of full-length protein: Effects of proline mutations on the amyloid fibril formation of β2-microglobulin. *J. Biol. Chem.* **278:** 47016–47024.
10. Davankov, V., L. Pavlovaa, M. Tsyurupa, J. Bradyb, M. Balsamo, and E. Yousha. 2000. Polymeric adsorbent for removing toxic proteins from blood of patients with kidney failure. *J. Chromatogr. B Biomed. Sci. Appl.* **739:** 73–80.
11. Deppermann, D., K. Andrassy, H. Seelig, F. Ritz, and D. Post. 1980. Evidence for dependence of beta-thromboglobulin levels on renal function (The Society of Nephrology, German speaking 1979). *Kidney Int.* **17:** 403–404.
12. Drueke, T. B. 2000. β2-Microglobulin and amyloidosis. *Nephrol. Dial. Transplant.* **15:** 17–24.
13. Furuyoshi, S., M. Nakatani, J. Taman, H. Kutsuki, S. Takata, and N. Tani. 1998. New adsorption column (Lixelle) to eliminate beta2-microglobulin for direct hemoperfusion. *Ther. Apher. Dial.* **2:** 13–17.
14. Franco, A., A. Caballero, J. Chisvert, J. Luflo, R. Perez,and M. Martinez. 1988. Beta2-microglobulin (β2m) levels in hemodialysis (HD) patients. Its variation during RD with different membranes. *Kidney Int.* **34:** 288–302.
15. Gejyo, F., T. Yamada, S. Odani, Y. Nakagawa, M. Arakawa, T. Kunitomo, *et al.* 1985. A new form of amyloid protein associated with chronic hemodialysis was identified as β2-microglobulin. *Biochem. Biophys. Res Commun.* **129:** 701–706.
16. Grovender, E. A., B. Kellogg, J. Singh, D. Blom, H. Ploegh, K. D. Wittrup, R. Langer, and G. A. Ameer. 2004. Single-chain antibody fragment-based adsorbent for the extracorporeal removal of β2-microglobulin. *Kidney Int.* **65:** 310–322.
17. Kaiser, E. T., R. L. Colescott, C. D. Blossinger, and P. I. Cook. 1970. Color test for detection of free terminal amino groups in solid-phase synthesis of peptides. *Anal. Biochem.* **34:** 595–598.
18. Kihara, M., E. Chatani, M. Sakai, K. Hasegawa, H. Naiki, and Y. Goto. 2005. Seeding-dependent maturation of β2-microglobulin amyloid fibrils at neutral pH. *J. Biol. Chem.* **280:** 12012–12018.
19. Kim, H., J. K. Cho, W. J. Chung, and Y. S. Lee. 2004. Core-shell-type resins for solid-phase peptide synthesis: Comparison with gel-type resins in solid-phase photolytic cleavage reaction. *Organic Lett.* **6:** 3273–3276.
20. Lornoy, W., I. Becaus, J. M. Billiouw, L. Sierens, P. V. Malderen, and P. D. Haenens. 2000. Hepatitis C and management of end-stage renal disease. *Nephrol. Dial. Transplant.* **15:** 49–54.
21. McParland, V. J., N. M. Kad, A. P. Kalverda, A. Brown, P. Kirwin-Jones, M. G. Hunter, M. Sunde, and S. E. Radford. 2000. Partially unfolded states of β_2-microglobulin and amyloid formation *in vitro*. *Biochemistry* **39:** 8735–8746.
22. Meinhofer, J., M. Waki, E. P. Heimer, T. J. Lambross, R. C. Makofske, and C. D. Chang. 1979. Solid phase synthesis without repetitive acidolysis: Preparation of leucyl-alanyl-glycyl-valine using 9-fluorenyl-methyloxy-carbonyl-amino acids. *Int. J. Peptide Protein Res.* **13:** 35–42.
23. Mogi, M., M. Harada, T. Adachi, K. Kojima, and T. Nagatsu. 1989. Selective removal of β2-microglobulin from human plasma by high-performance immunoaffinity chromatography. *J. Chromatogr.* **496:** 194–200.
24. Naiki, H., N. Hashimoto, S. Suzuki, H. Kimura, K. Nakakuki, and F. Gejyo. 1997. Establishment of a kinetic model of dialysis-related amyloid fibril extension *in vitro*. *Amyloid* **4:** 223–232.
25. Ohhashi, Y., M. Kihara, H. Naiki, and Y. Goto. 2005. Ultrasonication-induced amyloid fibril formation of β2-microglobulin. *J. Biol. Chem.* **280:** 32843–32848.
26. Ohhashi, Y., K. Hasegawa, H. Naiki, and Y. Goto. 2004. Optimum amyloid fibril formation of a peptide fragment suggests the amyloidogenic preference of β2-microglobulin under physiological conditions. *J. Biol. Chem.* **279:** 10814–10821.
27. Radford, S. E., W. S. Gosal, and G. W. Platt. 2005. Towards an understanding of the structural molecular mechanism of β2-microglobulin amyloid formation *in vitro*. *Biochim. et Biophys. Acta* **1753:** 51–63.
28. Shabunina, I. V., O. I. Afanas'eva, and S. N. Pokrovskii. 2001. Immunosorbent for removal of β2-microglobulin from human blood plasma. *Bull. Exp. Biol. Med. Immunol. Microbiol.* **4:** 984–986.

29. Singh, S. M. and A. K. Panda. 2005. Solubilization and refolding of bacterial inclusion body proteins. *J. Biosci. Bioeng.* **99:** 303–310.
30. Sunde, M., L. C. Serpell, M. Bartlam, P. E. Fraser, M. B. Pepys, and C. C. F. Blake. 1997. Common core structure of amyloid fibrils by synchrotron X-ray diffraction. *J. Mol. Biol.* **273:** 729–739.
31. Tsuchida, K., Y. Takemoto, and T. Nakamura. 1998. Lixelle adsorbent to remove inflammatory cytokines. *Artif. Organs* **22:** 1064–1067.
32. Tsuchida, K., Y. Takemoto, and K. Sugimura. 2002. Adsorption of endotoxin by beta(2)-microglobulin adsorbent column (Lixelle): The new approach for endotoxinemia. *Ther. Apher.* **6:** 116–118.
33. Winchester, J. F. 2005. Novel changes in β2-microglobulin in dialysis patients. *Clin. Chem.* **51:** 1089–1090.
34. Yamamoto, K., H. Yagi, D. Ozawa, K. Sasahara, H. Naiki, and Y. Goto. 2008. Thiol compounds inhibit the formation of amyloid fibrils by β2-microglobulin at neutral pH. *J. Mol. Biol.* **376:** 258–268.
35. Yamamoto, S., K. Hasegawa, I. Yamaguchi, S. Tsutsumi, J. Kardos, Y. Goto, F. Gejyo, and H. Naiki. 2004. Low concentrations of sodium dodecyl sulfate induce the extension of β2-microglobulin-related amyloid fibrils at a neutral pH. *Biochemistry* **43:** 11075–11082.
36. Yamamoto, S., I. Yamaguchi, K. Hasegawa, S. Tsutsumi, Y. Goto, F. Gejyo, and H. Naiki. 2004. Glycosaminoglycans enhance the trifluoroethanol-induced extension of β2-microglobulin–related amyloid fibrils at a neutral pH. *J. Am. Soc. Nephrol.* **15:** 126–133.
37. Lee, J. H., I. H. Lee, Y. J. Choe, S. Kang, H. Y. Kim, W. P. Gai, J. S. Hahn, and S. R. Paik. 2009. Real-time analysis of amyloid fibril formation of α-synuclein with a fibrillation state-specific fluorescent probe of JC-1. *Biochem. J.* **418:** 311–323.