New Method of Preparing a Stable and Functional Antibody-Polymer Nanoparticle Based on Deflocculation *via* Ultra-Sonication

Yo Han Choi, Sanghee Kim, and Min Su Han†,*

BT Research Group, Electronics and Telecommunications Research Institute, Daejeon 305-700, Korea
*†Department of Chemistry, Chung-Ang University, Seoul 156-756, Korea. *E-mail: mshan@cau.ac.kr*
Received August 21, 2009, Accepted September 27, 2009

Key Words: Polystyrene nanoparticle, Antibody, Deflocculation, Flocculation, Ultra-sonication

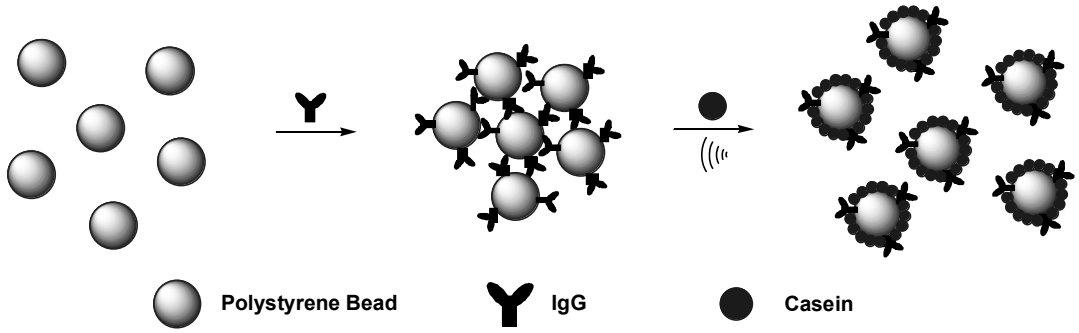
Polystyrene (PS) beads are a new kind of spherical and functional polymer material developed in recent years.[1] Due to their properties such as biocompatibility, non-toxicity, high surface area, strong adsorption ability, and chemical inertness, PS beads have been widely used in various academic and practical fields.[2] PS nanoparticles with bioconjugates are especially being increasingly used as tools in various applications, including drug delivery systems, medical diagnostic tests, and cellular imaging.[3] The particles can be prepared by immobilizing proteins on solid surfaces through entrapment, adsorption, and covalent bonding.[4] Among these methods, physical adsorption is the simplest, and it requires the mildest experimental conditions for obtaining a protein-nanoparticle complex.[5] The stability of PS nanoparticle-proteins in the immobilization process is, however, sensitive to various factors such as the concentration of the electrolyte, the pH of the buffer, the PsI of the proteins, and the coverage of the protein on the nanoparticles.[6] Due to these sensitivities, it is very tricky to prepare a stable protein adsorbent PS nanoparticle without flocculation and the PS nanoparticles are generally prepared using a flocculation test.[7]

Generally, when the part of PS nanoparticle surface is covered with proteins after the addition of limited amount of protein, the PS nanoparticles will flocculate surgically. The flocculators are induced through mixing and high collision frequency between the proteins on PS nanoparticles and the surface of other PS nanoparticles, and therefore, through rapid particle growth.[8] However, the entire surface of PS nanoparticles will be covered with protein after the excess addition of the adsorbent proteins in preparing PS conjugated with proteins, which makes the nanoparticles disperse well without a flocculation test.[9] This method will be ineffective, however, if expensive proteins such as monoclonal antibodies will be used. One challenge, using the limited amount of expensive protein, is to discover a simple method of preparing PS nanoparticle proteins that resists flocculation while maintaining suspension stability and functionality.

Herein, we describe that a rare amount of proteins, such as monoclonal antibodies, can be easily conjugated on the nanoparticles with the successive addition of excess mock proteins such as casein and sonication. A flocculated PS nanoparticles functionalized with limited amount of protein were dispersed *via* sonication, but they flocculated again after several minutes.[10] By the way, we found that the flocculate was dispersed clearly *via* sonication with prior addition of excess bovine serum albumin (BSA) and the dispersed PS nanoparticle was stable for more than two weeks. This inspired us to solve the limitation of the previous tricky problems in preparing high-cost protein-PS nanoparticles. A rare amount of proteins, such as monoclonal antibodies, can be easily conjugated on the nanoparticles with the successive addition of excess proteins such as BSA or casein and sonication. BSA and casein are selected as the excess proteins because they are the most frequently and commonly used proteins for the blocking purpose in immunoassays. In immunoassays such as Western blotting and ELISA, the blocking of vacant area (nitrocellulose in Western blotting, and polystyrene in ELISA) after the adsorption of the preceding protein is one of the major steps. BSA and casein are generally used for this purpose. The preceding proteins span any kinds of proteins from hormones to antibodies. Therefore, BSA and casein would be the most acceptable second proteins which can be applied to our method. The new method is described in Scheme 1.

The method has possibilities that antibodies conjugated on nanoparticle were detached and deactivated in preparing processes. Firstly, to prove the concept and eliminate the possibility of the detachment, FITC (fluorescein isothiocyanate)-labeled BSA was prepared as the model for high-cost proteins such as monoclonal antibodies.[11] One mg of PS nanoparticles was flocculated with the addition of 50 μg of FITC-labeled BSA in 1 mL of PBS. After total flocculation, another 2 mg of the excess unlabeled BSA was added to every 150 μL of the flocculated mixture. The control group was washed with repeated centrifugation and re-suspension with 1 mL of PBS after simple vortex mixing. The mixture of this group was still turbid, though, as shown in Fig. 1 (a)-i. The experimental group was sonicated before the washing step, which made the mixture transparent [Fig. 1 (a)-ii]. The relative fluorescence unit (RFU) of each group was measured with a fluorometer after the final washing. Compared with the control group, which was not sonicated at all, the experimental group showed an RFU with a decrease of only 2.19% [Fig. 1 (b)]. The results showed that the FITC-labeled BSA in the flocculate was not detached with the addition of the excess unlabeled BSA and the succeeding sonication. The results mean that a rare amount of proteins, such as monoclonal antibodies, can be easily conjugated on the nanoparticles with the successive addition of excess proteins such as casein and sonication without detachment as depicted in Scheme 1.

However, it is possible that the excess amount of additional



Scheme 1. Schematic model for the method of preparing PS nanoparticles with conjugated monoclonal antibodies *via* excess proteins such as casein and sonication.

protein and suceeding sonication in this method could induce notable deactivation of the adsorbent protein on the PS nanoparticle. Using the antigen-antibody interaction as a model, the retainment of the antibody function was examined to elucidate this possibility. A stable PS nanoparticle conjugated with anti-human serum albumin (HSA) polyclonal antibodies from rabbits was prepared through flocculation *via* the addition of the antibodies and through deflocculation *via* the successive addition of casein and sonication as described above. The BSA was substituted with casein in this experiment to prevent any cross-binding between the BSA and the HSA. Also, PS nanoparticles attached with a limited amount of only casein or a non-specific-antibody (rabbit total IgG) were prepared for comparisons. Each PS nanoparticle group was mixed with FITC-conjugated HSA and incubated for two hours, after which it was harvested *via* centrifugation by 28,500× g for one hour at 4 °C. The pellet was re-suspended with PBST (PBS with 0.05% Tween 20) and centrifuged again. This washing step was repeated three times to remove the unbound FITC-conjugated HSA. The fluorescence of each PS nanoparticle group was measured at 518 nm, and the results are summarized in Fig. 2. Compared with the fluorescence of the PS nanoparticles without IgG or with non-specific total rabbit IgG, that of the PS nanoparticle with anti-HSA rabbit antibodies had an almost 10 times and an almost 26 times higher intensity, respectively, which shows that the deflocculation process does not affect the functionality of anti-HSA antibodies. So, we could prepare the PS nanoparticle conjugated with antibody without the detachment and the disfuctionalities of the antibody.

This method can be expanded to prepare the PS nanoparticle conjugated with a other proteins. To test the generality of our method, we prepared a IgG-conjugated PS nanoparticles from commercially available nanoparticles. Anti-PSA (prostate-specific antigen) monoclonal antibody (Biodesign) was conjugated on amine-reactive PS nanoparticles (Molecular Probes) according to the manufacturer's instructions. We introduced smaller amount of IgG than suggested by instruc-

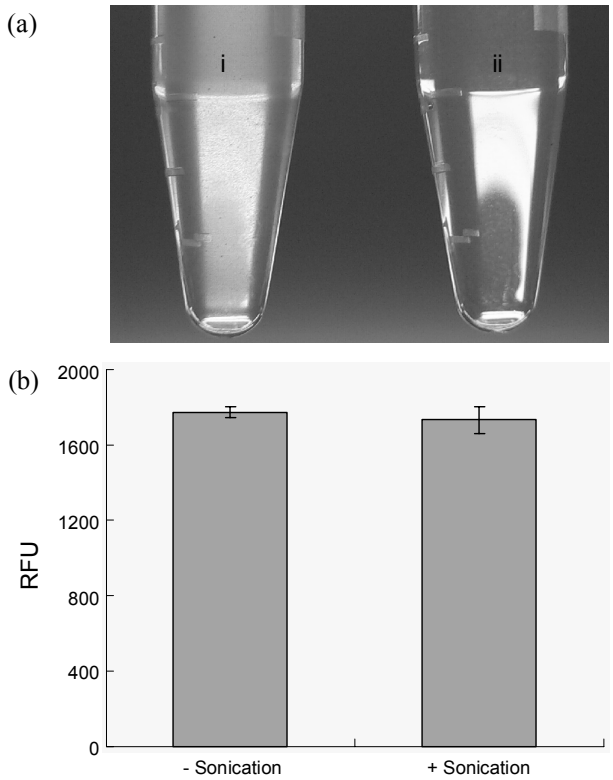


Figure 1. (a) PS nanoparticles were flocculated with FITC-labeled BSA. After the addition of the excess amount of unlabeled BSA, the mixture was vortex-mixed (i) or sonicated (ii). (b) The maintenance of the fluorescence of the PS nanoparticles was measured after they were washed to remove unbound proteins.

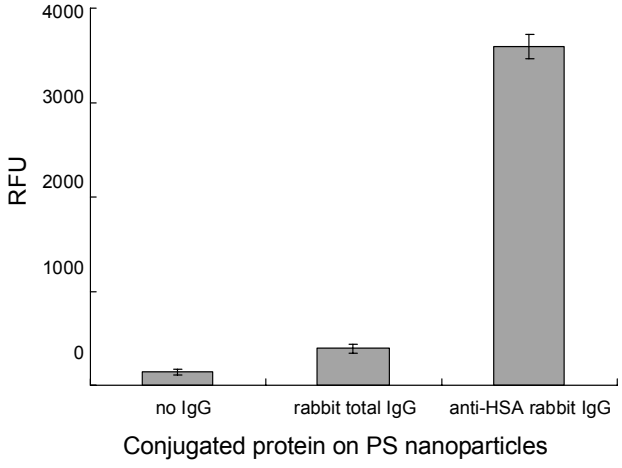


Figure 2. Fluorescence intensity of FITC-labeled HSA bound to PS nanoparticles functionalized with only casein, non-specific antibody pool, or anti-HSA antibody.

tions, and there happened visible flocculation as predicted from the instruction. The flocculation was successfully dissolved through excess addition of casein and sonication, and we confirmed that the PS nanoparticle congugated with antibody was stable for several days and could specifically bind to PSA. Therefore, an IgG-attached PS nanoparticle can be prepared very easily with this process of flocculation followed by deflocculation, without using a huge amount of IgG to obtain dispersed nanoparticles. In spite of using PS nanoparticle bearing different surface functionalities and size and target proteins, our method provides a general scheme for preparing stable bio-conjugated PS nanoparticles, which is very important because previous method must carefully control the experimetal conditions such as salt condition, pH, and concentration of proteins to prepare bio-conjugated PS nanoparticles without flocculation.

In summary, we prepared stable bio-conjugated PS nanoparticles using the successive addition of excess mock proteins such as casein and sonication. This method can offer a general scheme for preparing a stable and dispersed PS nanoparticle conjugated with expensive rare proteins and dramatically improving the simplicity than with the conventional method using a flocculation test. This simplicity is important in using PS nanoparticle-protein conjugates for drug delivery systems, medical diagnostic tests, separation media, and cellular imaging, among others. In addition, this method might be applicable to the preparation not only of other polymer nanoparticle bio-conjugates but also of silica and metal nanoparticle bio-conjugates.

Experiments

The synthesis of PS nanoparticles. PS nanoparticles were synthesized by emulsion polymerization.[12] 20 g of styrene monomer (Sigma Aldrich), 0.1 g of divinylbenzene (Sigma Aldrich), and 360 mg of sodium dodecyl sulfate (Sigma Aldrich) were added to 250 mL of distilled water and stirred at 250 rpm for 2 hr at room temperature. The following polymerization process was performed at 70 °C overnight in the presence of 50 mg of potassium peroxosulfate (Sigma Aldrich) with nitrogen purging. The average diameter of PS nanoparticle was measured to be 110 nm by dynamic light scattering (DLS-700, Otsuka Electronics Co. Ltd., Kyoto, Japan) which generates the hydrodynamic diameters (D_h) of PS nanoparticles with polydispersity. The morphology of synthesized PS nanoparticle was observed with Sirion scanning electron microscope (FEI Company). PS nanoparticle emulsion was placed on a piece of silicon wafer and allowed to be air-dried at room temperature before Au sputtering.

The determination of dispersity of synthesized PS nanoparticles. The monodispersity of synthesized PS nanoparticles were determined by calculating the degree of polydispersity (P_d) coefficient of variations. The area mean ($(\bar{d}^2)^{1/2}$, $\bar{d}_{NA}$), length mean diameters ($\bar{d}$, $\bar{d}_{NL}$) of nanoparticles, and standard deviation (σ) were used to the calculation of degree of polydispersity and coefficient of variation by following equations:

$$P_d = \bar{d}_{NA} / \bar{d}_{NL} = \{1 + \frac{\sigma^2}{\bar{d}^2}\}^{1/2} \quad (1)$$

The polydispersity (P_d) value of the PS nanoparticles was determined as 1.005.

Preparation of bio-conjugate polystyrene beads. Small amount of target protein was conjugated on PS nanoparticles as follows. One mg of PS nanoparticles was flocculated with the addition of 50 μg of target protein (FITC-labeled BSA, HSA, IgG, rabit total IgG respectively) in 1 mL of PBS or distilled water. After total floc σlation, another 20 mg of BSA or casein was added to the flocculated mixture. The mixture was still turbid at this step. This mixture was sonicated for five seconds with 0.5 sec intervals at 1/10 of total output (7 W, 20 kHz) by a tip-sonicator (Sonopuls HD2070, Bandelin GmbH & Co., KG). The cleared mixture was harvested *via* centrifugation by 28,500× g for one hour at 4 °C. The pellet was re-suspended with PBST (PBS with 0.05% Tween 20) and centrifuged again. This washing step was repeated three to five times to remove any unbound protein. PBS was used for the final resuspension.

Acknowledgments. This work was supported by the Ministry of Knowledge Economy in Korea [2009-F-008-01, Programmable Bio-CMOS Field Effect Transistors].

References

1. a) Zhang, G.; Niu, A.; Peng, S.; Jing, M.; Tu, Y.; Li, M.; Chi, U. *Acc. Chem. Res*. **2001**, *34*, 249; b) Moffitt, M.; Khougaz, K.; Eisenberg, A. *Acc. Chem. Res.* **1996**, *29*, 95; c) Klapper, M.; Nenov, S.; Haschick, R.; Müller, K.; Müllen, K. *Acc. Chem. Res.* **2008**, *41*, 1190.
2. a) Kawaguchi, H. *Prog. Polym. Sci.* **2000**, *25*, 1171; b) Arshady, R. *Biomaterials* **1993**, *14*, 5.
3. a) Vonarbourg, A.; Passirani, C.; Saulnier, P.; Benoit, J.-P. *Biomaterials* **2006**, *27*, 4356; b) Sassolas, A.; Leca-Bouvier, B. D.; Blum, L. J. *Chem. Rev.* **2008**, *108*, 109; c) Ha, C.-S.; Gardella, J. A. *Chem. Rev.* **2005**, *105*, 4205; d) Seydack, M. *Biosens. Bioelectron.* **2005**, *20*, 2454.
4. a) Janes, K. A.; Calvo, P.; Alonso, M. J. *Adv. Drug Deliv. Rev.* **2001**, *47*, 83; b) Malmsten, M.; Van Alstine, J. M. *J. Colloid Interface Sci.* **1996**, *177*, 502; c) Beeskow, T.; Kroner, K. H.; Anspach, F. B. *J. Colloid Interface Sci.* **1997**, *196*, 278; d) Malmsten, M.; Emoto, K.; Van Alstine, J. M. *J. Colloid Interface Sci.* **1998**, *202*, 507; e) Sofia, S. J.; Premnath, V.; Merrill, E. W. *Macromolecules* **1999**, *31*, 5059.
5. Wu, X.; Narsimhan, G. *Langmuir* **2008**, *24*, 4989.
6. a) Song, D.; Forciniti, D. *J. Colloid Interface Sci.* **2000**, *221*, 25; b) Peek, L. J.; Roberts, L.; Berkland, C. *Langmuir* **2008**, *24*, 9775; c) Tamai, H.; Fujii, A.; Suzawa, T. *J. Colloid Interface Sci.* **1987**, *118*, 176.
7. a) Tirado-Miranda, M.; Schmitt, A.; Callejas-Fernández, J.; Ferná ndez-Barbero, A. *Eur. Biophys. J.* **2003**, *32*, 128; b) Lin, W.; Garnett, M. C.; Davies, M. C.; Bignotti, F.; Ferrut, P.; Davis, S. S.; Illum, L. *Biomoterials* **1997**, *18*, 559; c) Stolnik, S.; Dunn, S. E.; Garnett, M. C.; Davies, M. C.; Coombes, A. G. A.; Taylor, D. C.; Irving, M. P.; Purkiss, S. C.; Tadros, T. F.; Davis, S. S.; Illum, L. *Pharma. Res.* **1994**, *11*, 1800.
8. Spicer, P. T.; Pratsinis, S. E. *AIChE J.* **1996**, *42*, 1612.
9. Peula, J. M.; Hidalgo-Alvarex, R.; de las Nieves, F. J. *J. Biomater. Sci. Polym. Ed*. **1995**, *7*, 241.
10. Yan, Y. D.; Glover, S. M.; Jameson, G. J.; Biggs, S. *Int. J. Miner. Process.* **2004**, *73*, 161.
11. Harlow, E.; Lane, D. *Antibodies, A Laboratory Manual*; Cold Spring Harbor Laboratory, 1988.
12. Kim, S.; Kim, C. A.; Choi, Y. H.; Jung, M. Y. *Polym. Bull.* **2009**, *62*, 23.