

Research Article

Biolabeling and Binding Evaluation of Amphiphilic Nanocrystallopolymers

Kwang-Suk Jang

Department of Chemical Engineering and Research Center of Chemical Technology, Hankyong National University, Anseong 17579, Republic of Korea

Correspondence should be addressed to Kwang-Suk Jang; jang@hknu.ac.kr

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Surfactant-like inorganic-organic hybrid molecules named as nanocrystallopolymers were designed by conjugation of the hydrophilic synthetic poly(amino acid), poly- α,β -(N-(2-hydroxyethyl)L-aspartamide), with hydrophobic inorganic nanoparticles. In aqueous media, amphiphilic nanocrystallopolymers form self-aggregates with unique morphologies. Here, a simple biolabeling method of nanocrystallopolymers was developed. Biotin was selected as a model biomolecule. The specific binding of biotin-labeled nanocrystallopolymers to the targeted surface was evaluated with a surface plasmon resonance sensor.

1. Introduction

Inorganic nanoparticles have been extensively studied for biomedical applications such as cellular imaging, cancer diagnosis and therapy, cell and protein separation, and biosensors [1–11]. Enhancing the stabilities of inorganic nanoparticles in aqueous media is one of the most challenging aspects in the field of biomedical engineering. Recently, amphiphilic polymers have been widely used for fabrication of inorganic nanoparticle-loaded capsules [8–11]. A dispersion of inorganic nanoparticles with hydrophobic ligands in an organic solvent can be loaded in polymeric shells forming emulsions. After evaporation of the organic solvent with a low boiling point, nanoparticle-loaded polymeric micelles can be obtained.

Hydrophilic polymers chemically bound to hydrophobic nanoparticles forming micelle-like aggregates have been reported [12–14]. Surfactant-like inorganic-organic hybrid molecules named as nanocrystallopolymers were designed by conjugating the hydrophilic synthetic poly(amino acid), poly- α,β -(N-(2-hydroxyethyl)L-aspartamide) (PHEA) with hydrophobic Au nanoparticles (PHEA-g-Au NC). Because dodecanethiolate-protected Au nanocrystals have hydrophobic surfaces, the conjugated Au nanoparticles act as the hydrophobic part of the amphiphilic nanocrystallopolymers. In aqueous media, amphiphilic nanocrystallopolymers form

spherical aggregates, core-shell unimolecular micelles, and cylindrical aggregates according to their hydrophilic/hydrophobic compositions. The self-aggregates are composed of a core part of inorganic nanoparticles and a shell part of water-soluble biocompatible polymers; therefore, they are expected to be advantageous for use in biological systems. Au nanoparticles exhibit unique optoelectric properties and can be readily synthesized by several different methods and easily tagged to diverse biomolecules or chemicals. Therefore, Au nanoparticles are among the most widely studied inorganic nanoparticles, together with magnetic nanoparticles and quantum dots, in biological detection, cancer treatment, and so forth.

In this study, nanocrystallopolymers were labeled with biotin as a model biomolecule. Self-aggregates of the nanocrystallopolymers have unique morphologies. Biolabeling of nanocrystallopolymers is expected to expand the potentials for biomedical applications such as cellular imaging, cancer diagnosis and therapy, cell and protein separation, and biosensors. Synthesis of poly(amino acid) and biotin-functionalization were verified by ^1H -nuclear magnetic resonance (NMR) spectra, and the morphologies of self-aggregates of the nanocrystallopolymers were confirmed by transmission electron microscopy (TEM). The specific binding of biotin-labeled nanocrystallopolymers to the targeted

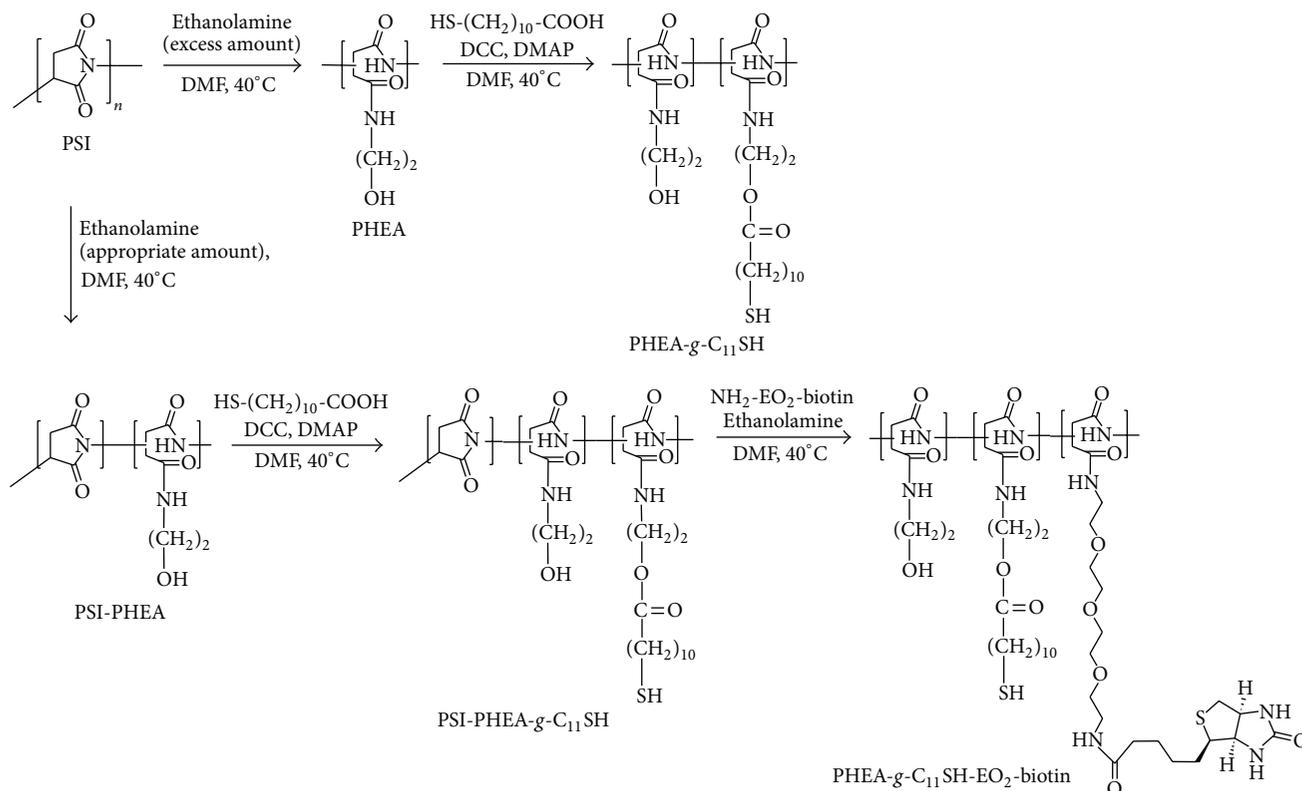


FIGURE 1: Synthetic routes of PHEA-*g*-C₁₁SH and PHEA-*g*-C₁₁SH-EO₂-biotin.

surface was evaluated with a surface plasmon resonance (SPR) sensor.

2. Experimental

Dodecanethiolate-protected Au nanocrystals were synthesized according to the Brust-Schiffrin method as previously reported, and their average core diameter was confirmed to be 1.5 nm average core diameter by TEM [12, 15–17]. PHEA conjugated with the undecanethiols, PHEA-*g*-C₁₁SH (P-*g*-C₁₁SH), was prepared by the simple esterification reaction [12]. The biotin-conjugated backbone, PHEA-*g*-C₁₁SH-EO₂-biotin (P-*g*-C₁₁SH-biot), was prepared by the aminolysis of the partially converted backbone poly(succinimide)- (PSI)-PHEA with amine-terminated biotin having a short ethylene oxide (EO₂) spacer. All synthetic routes are shown in Figure 1. The synthesis of both backbone polymers was verified by ¹H NMR spectra.

Au nanocrystallopolymeric aggregates in aqueous media were prepared as previously reported [12]. The Au nanocrystal-THF solution was added dropwise to the polymer-water solution under vigorous stirring, and the mixture was stirred continuously for 1 day to achieve ligand place-exchange. By removing the THF and unused Au nanocrystals, the Au nanocrystallopolymer forming the Au nanocrystalloaggregates, P-*g*-Au NC and P-*g*-Au NC-biot, was obtained in the aqueous solution. The solution was filtered through a 0.45 μm polyvinylidene fluoride (PVDF) filter to remove impurities for further characterizations. The formation of

micelles was visualized by TEM. For the TEM analysis of negatively stained nanocrystallopolymers, the solution containing 0.1% (w/v) phosphotungstic acid was placed on a copper grid covered with a formvar carbon membrane. Then, the grid was exposed for removing the solvent. Hydrodynamic diameter was measured by dynamic light scattering (DLS) and calculated with nonnegative least squares algorithms. ¹H-NMR spectra of P-*g*-Au NC and P-*g*-Au NC-biot were obtained with D₂O as a solvent.

Specific binding of biotin-labeled Au nanocrystallopolymers to avidin was verified by SPR measurement. P-*g*-Au NC and P-*g*-Au NC-biot were dissolved in phosphate buffered saline (PBS; 0.01 M, pH 7.4) solution at a concentration of 100 μg/mL and flowed into the channels of a streptavidin-immobilized gold sensor chip at a rate of 10 μL/min. For the P-*g*-Au NC-biot experiment, 1 μM solution of bovine serum albumin (BSA) and avidin in PBS solution were flowed at the same rate.

3. Results and Discussion

PHEA, a water-soluble biocompatible polymer with a poly(amino acid) structure, can be prepared from PSI and can be easily modified to form graft structures [18–20]. The molecular weight (*M_n*) of PHEA determined by gel permeation chromatography was 19,800 (PDI = 1.32) [21]. Undecanethiols (C₁₁SH) can be grafted onto the PHEA backbone by forming an ester bond between 11-mercaptoundecanoic acid and PHEA. The grafted C₁₁SH acts as a linker between

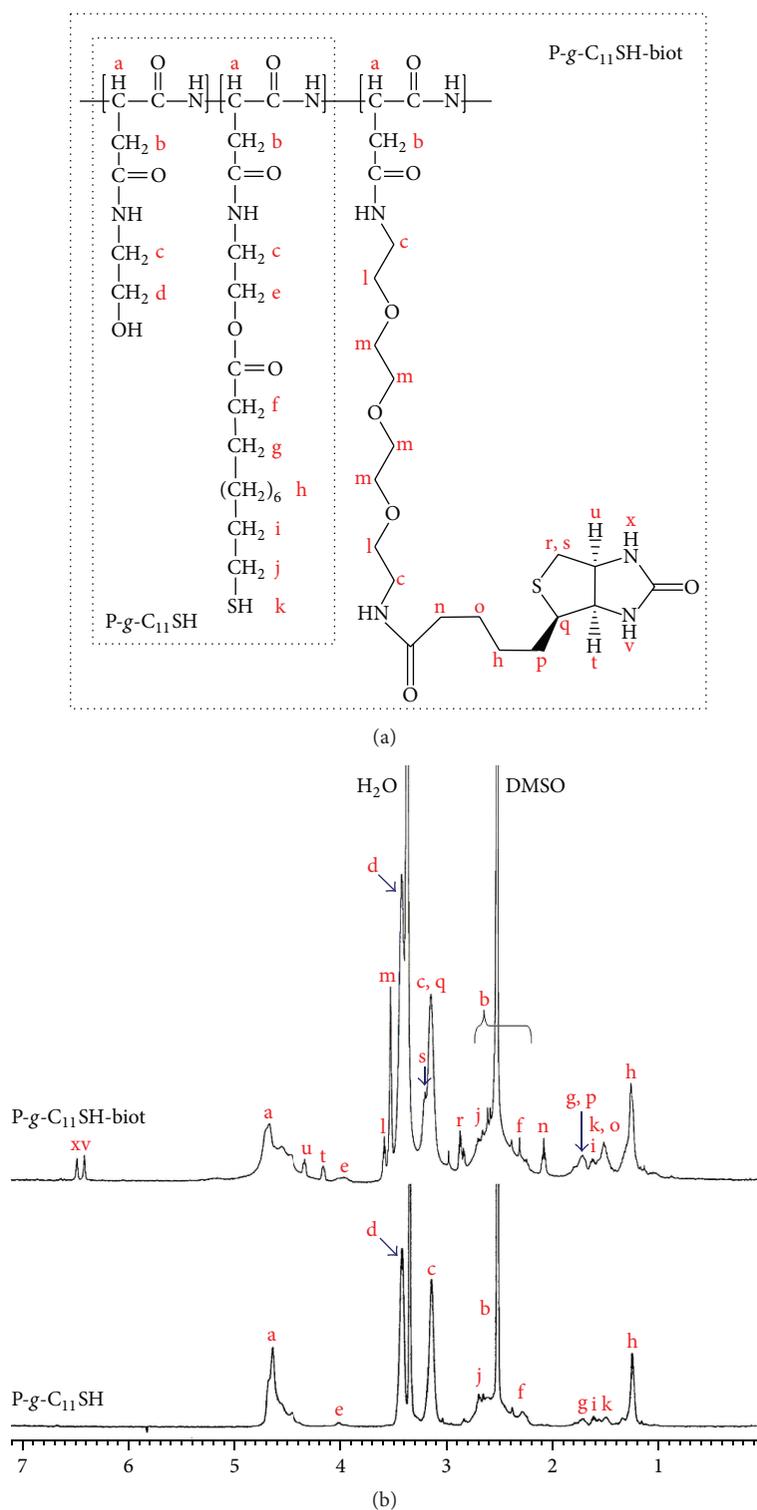


FIGURE 2: (a) Molecular formulas and (b) ¹H-NMR spectra of PHEA-g-C₁₁SH and PHEA-g-C₁₁SH-EO₂-biotin in DMSO-*d*₆.

the polymer and the Au nanocrystals. Thus, the conjugated amount of C₁₁SH is strongly correlated with the conjugated amounts of Au nanocrystals. Synthesis of P-g-C₁₁SH and P-g-C₁₁SH-biot was confirmed by ¹H-NMR spectra as shown in Figure 2. All peaks from the biotin with the EO₂ spacer

are shown at the right position. From ¹H-NMR spectra, the grafted mole percent (degree of substitution, DS) of C₁₁SH was determined as 2.92 and 3.20 mol% for P-g-C₁₁SH and P-g-C₁₁SH-biot, respectively. There was no significant difference between the conjugated amounts of C₁₁SH in

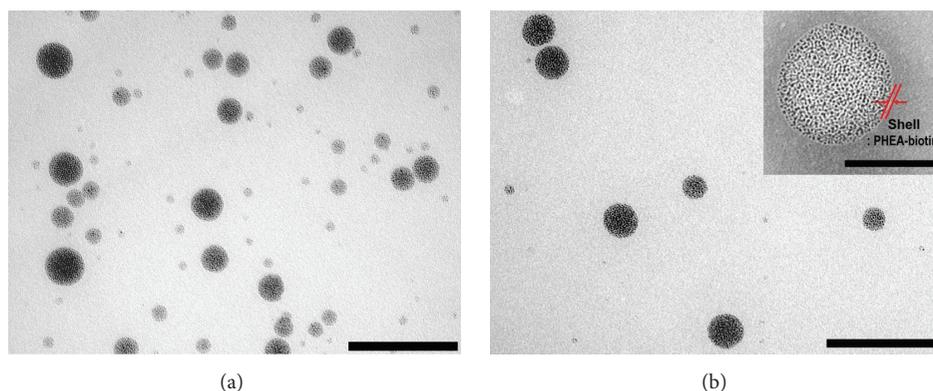


FIGURE 3: TEM images of (a) P-g-Au NC and (b) P-g-Au NC-biot (inset: magnified image of the P-g-Au NC-biotin aggregate negatively stained with PTA). Scale bars: (a) 200 nm; (b) 200 nm (inset: 100 nm).

both polymers. Thus, conjugation of similar amount of Au nanocrystals was expected. The DS of biotin in P-g-C₁₁SH-biot was calculated as 1.79 mol%. Biotin was successfully grafted onto the polymer backbone.

The Au nanocrystallopolymers P-g-Au NC and P-g-Au NC-biot were fabricated by ligand place-exchange reaction. Undecanethiols conjugated onto backbone polymers can participate in the ligand place-exchange to form PHEA or PHEA-biotin grafted with alkanethiolate-protected Au nanocrystals. For both polymers, it was determined that, on an average, 2 polymer chains were conjugated to 1 Au nanocrystal from the following analysis results. The weight% of Au in dodecanethiol-protected Au nanocrystals measured by thermal gravimetric analysis was 75%. The atomic weight% of Au in Au nanocrystallopolymers determined by inductively coupled plasma atomic emission spectrometry was 18.8% for P-g-Au NC and 17.1% for P-g-Au NC-biot. The prepared nanocrystallopolymers are composed of hydrophilic polymer backbones and grafted hydrophobic Au nanocrystals. Since conjugated nanocrystals are the hydrophobic component of amphiphiles, nanocrystals aggregate in the core part by dynamic self-assembly and surface-active properties [12]. Surfactant-like properties of the nanocrystallopolymers and micelle-like morphologies of their self-aggregates were confirmed by surface tension measurement and small-angle neutron scattering analysis [12]. The critical aggregation concentration of the nanocrystallopolymers was measured to be 0.075 g/L [12]. From the SANS analysis, the average diameter and polymer shell thickness of the self-like aggregates were found to be 71.4 nm and 3.85 nm, respectively [12].

Figures 3(a) and 3(b) show the TEM images of self-aggregates formed from P-g-Au NC and P-g-Au NC-biot, respectively. In aqueous medium, both nanocrystallopolymers form micelle-like aggregates by self-assembly. Hydrophobic Au nanocrystals are tightly packed inside a self-aggregate to form a core, and hydrophilic PHEA should be located at the surface of the core part to form a corona layer. Only the core parts filled with closely packed Au nanocrystals were observed due to the very high electron density of metals, while the organic corona layer was not visible by TEM.

To confirm the existence of polymer shells, samples were negatively stained with phosphotungstic acid. The staining revealed the presence of biotinylated PHEA shells (inset of Figure 3(b)). Figure 4 shows hydrodynamic diameters of P-g-Au NC and P-g-Au NC-biot measured by DLS. The mean hydrodynamic diameters obtained were 89.5 nm for P-g-Au NC and 94.9 nm for P-g-Au NC-biot. The slightly larger size and broader size distribution of P-g-Au NC-biot can be explained as follows: the size of the shell layer would increase by the conjugation of biotins with short hydrophilic EO₂ spacers (2.04 nm). As shown in Figure 5, the original Au nanocrystals are soluble in hydrophobic solvents such as hexane, but the self-aggregates of P-g-Au NC and P-g-Au NC-biot are stable in water and do not proceed to the oil phase. The effective external exposure of biotins to the aqueous media was verified by the ¹H-NMR spectra of both Au nanocrystallopolymers in D₂O as shown in Figure 6. Peaks from the PHEA backbone and linked biotins with EO₂ spacer are clearly shown, while the peaks from methylenes of hydrocarbons in both nanocrystallopolymers are drastically reduced and unclear due to their location at the core of the self-aggregates.

The binding affinity of P-g-Au NC-biot to streptavidin or avidin by the avidin-biotin specific interaction was monitored with the SPR biosensor. In this SPR study, the solution concentration of P-g-Au NC and P-g-Au NC-biot each was 100 μg/mL. Because the concentrations are over the critical aggregation concentration of Au nanocrystallopolymers, 75 μg/mL [12], they are expected to form micelle-like aggregates in the SPR flow channels. Measurement was performed with the Au sensor chip covalently attached with streptavidin; overall results are shown in Figure 7. At first, P-g-Au NC and P-g-Au NC-biot were injected to the streptavidin-coated surfaces. In both cases, SPR responses increased immediately after the injection. After the following injection of buffer solution (0.01 M PBS), unbound nanocrystallopolymers were washed out and SPR responses decreased. According to the SPR response differences, P-g-Au NC rarely bound to the streptavidin-coated surface, while P-g-Au NC-biot bound to the surface by the specific interaction of streptavidin and

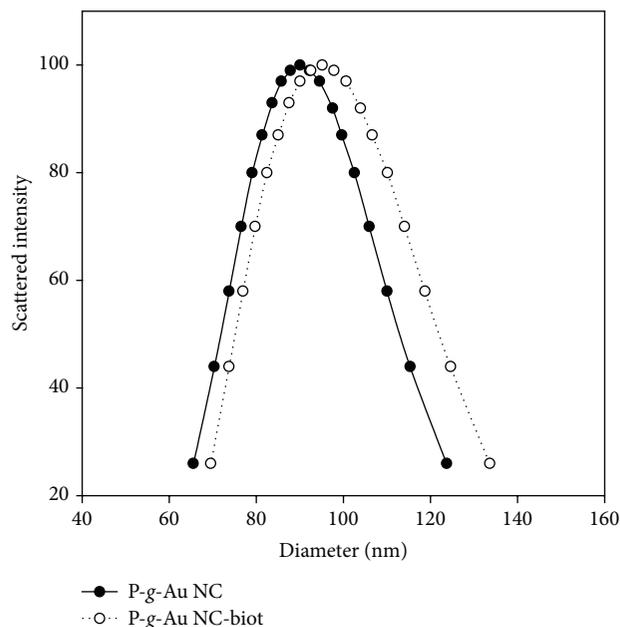


FIGURE 4: Size distributions of P-g-Au NC and P-g-Au NC-biot in DDI water by DLS measurements.

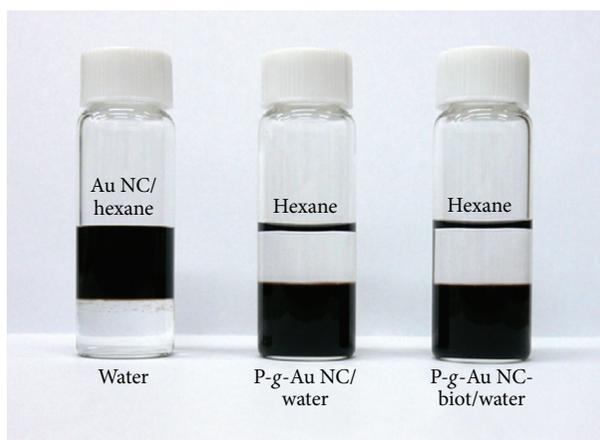


FIGURE 5: Phase separations of hexane solution of Au nanocrystals with DDI water, and water solutions of both Au nanocrystallopolymers with hexane.

biotin. To ensure the streptavidin-biotin binding, solutions of BSA and avidin were additionally injected to the channel of the P-g-Au NC-biotin covered surface. No binding of BSA and some binding of avidin to the biotin-exposed surface were observed. The high binding specificity of P-g-Au NC-biot to streptavidin/avidin was confirmed. From the SPR study, we can conclude that biotins conjugated to nanocrystallopolymers are effectively exposed and that the polymer backbone, PHEA, does not demonstrate nonspecific binding characteristics. Results of this study indicate that the nanocrystallopolymers can be labeled with biomolecules such as biotin. The biotin grafted into the nanocrystallopolymers was successfully exposed to the surface of the nanocrystallopolymers, and it had a specific binding property.

We anticipate that biolabeling of nanocrystallopolymers can expand their potential for biomedical applications.

4. Conclusions

An amphiphilic nanocrystallopolymer, P-g-Au NC, was successfully labeled with biotin (P-g-Au NC-biot) by simple modification of the synthetic scheme of the backbone polymer. This method can be applied to any molecule of interest that bears the amine group, and additive biomolecular labeling through avidin-biotin chemistry is possible. The specific interaction with streptavidin/avidin was evaluated with SPR measurement. From the SPR study, it is concluded that biotins are effectively exposed to the surface of micelle-like

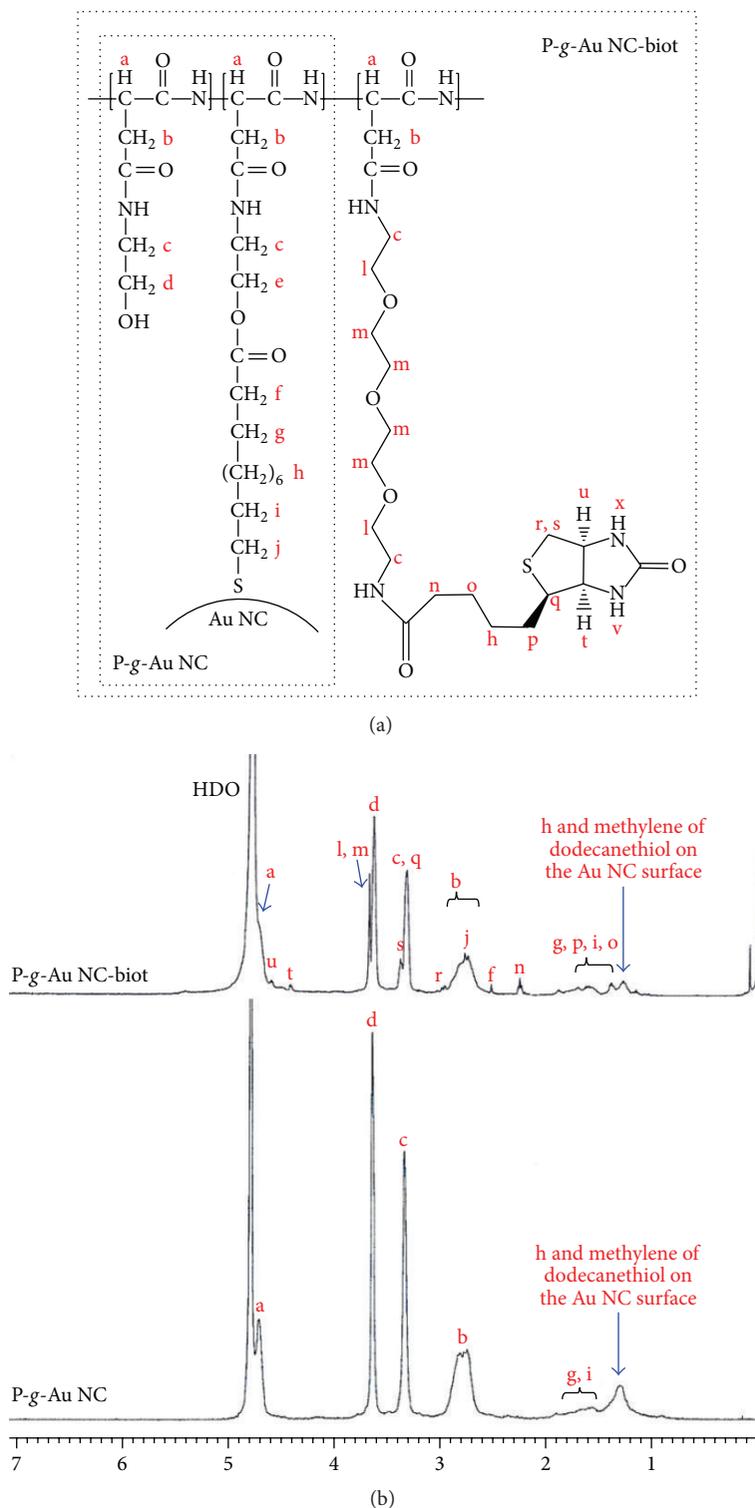


FIGURE 6: (a) Molecular formulas and (b) $^1\text{H-NMR}$ spectra in D_2O of P-g-Au NC and P-g-Au NC-biotin.

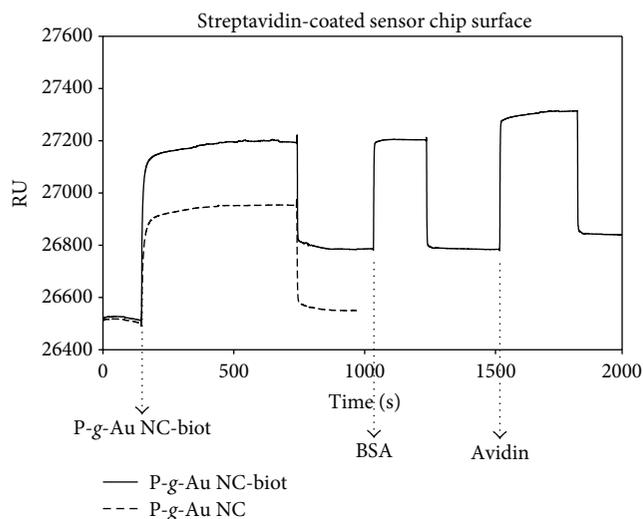


FIGURE 7: SPR responses for the binding of P-g-Au NC and P-g-Au NC-biot to the streptavidin/avidin.

aggregates. We anticipate that such nanocrystallopolymers will be useful in many biomedical applications such as cellular imaging, cancer diagnosis and therapy, cell and protein separation, and biosensors.

Competing Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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