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Synchronous delivery systems composed of Au nanoparticles and stimuli-sensitive diblock terpolymer

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A method to construct synchronous delivery systems via direct self-assembly of Au nanoparticles on the poly[(*N*-isopropylacrylamide-*r*-acrylamide)-*b*-L-lactic acid] (PNAL) nanospheres has been presented in this paper. To achieve amphiphilic diblock terpolymer, hydrophobic poly (L-lactic acid) (PLLA) block was added to poly(*N*-isopropylacrylamide-*r*-acrylamide) (PNA) block via Michel-type addition reaction. Lower critical solubility temperature (LCST) was modulated at 35.6 °C which is close to the body temperature, but higher than poly(*N*-isopropylacrylamide) (PNIPAAm) homopolymer by controlling the ratio between isopropylacrylamide (IPAAm) monomers and acrylamide (AAm) monomers. Using this amphiphilic diblock terpolymer, PNAL nanospheres were fabricated by emulsion/evaporation technique followed by direct self-assembly of Au nanoparticles on the PNAL nanospheres due to the high affinity of amino groups donated from PNA block. The 'core' site of Au@PNAL nanospheres can load various lyphophilic drugs. Moreover, Au nanoparticles in the 'shell' domain of PNAL nanospheres give optimal environment to conjugate various biomolecules. Therefore, it is expected that Au@PNAL hybrid nanospheres can be utilized in synchronous delivery of both biomolecules in the 'shell' domain and various therapeutic drugs in the 'core' domain.

1. Introduction

Recently, immobilization of biomolecules onto solid surfaces is attracting considerable attention in the field of biological engineering and biotechnology [1–5]. Especially, Au has been widely applied as a promising material because amine groups and cysteine residues in proteins can bind to, for instance, Au nanoparticles in a stable connection such as electrostatic stabilization [1a, 2]. Parallel to this application, a number of studies have been focused on the fabrication of Au nanoparticles-organic/inorganic hybrid structures using polyurethane (PU) [1a], silica [6, 7, 8e], polystyrene (PS) [8b], and stimuli-sensitive polymers (SSPs) [9]. It is mainly because this approach can endow Au nanoparticles with additional functions by adequately selecting either a planar or a spherical substrate.

Poly(*N*-isopropylacrylamide) (PNIPAAm) is one of the SSPs referred to as a thermosensitive polymer due to its distinct phase transition at a specific lower critical solubility temperature (LCST) [10–13]. PNIPAAm has a LCST at 32 °C in water. PNIPAAm is hydrophilic below its LCST; however, it turns to be hydrophobic when it is heated up above the LCST. Owing to this 'smart' character, PNIPAAm has been consistently studied for biomedical applications in forms of micelle [10c], tablet [13], and hydrogel [14b].

We previously reported a technique to construct thermosensitive 'shell-in-shell' structures with PNIPAAm, poly(L-lactic acid) (PLLA), poly(D-lactic acid) and poly(ethylene glycol) via a modified-double-emulsion method [15]. In this study, a more advanced but simpler technique has been presented to construct multifunctional hybrid nanospheres via direct self-assembly of Au nanoparticles on poly[(*N*-isopropylacrylamide-*r*-acrylamide)-*b*-L-lactic acid] (PNAL) nanospheres; hereafter abbreviated as Au@PNAL. This synthetic strategy aims at providing several advantages over other core-shell structures composed of polymeric materials and Au nanoparticles. Primarily, instead of introducing any coupling agent or surface modification, Au nanoparticles can be directly assembled on

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the surface of PNAL nanospheres by virtue of primary amino groups donated from acrylamide (AAm) molecules of PNAL diblock terpolymer, because primary amino groups have an excellent binding affinity to noble metals such as gold or silver. Therefore, the 'shell' domain of Au@PNAL becomes a good environment to conjugate biomolecules. Furthermore, the LCST of poly(*N*-isopropylacrylamide-*r*-acrylamide) (PNA) has been modulated from 32 °C up to around 36 °C through the manipulation of the ratio between *N*-isopropylacrylamide (NIPAAm) and AAm units. In this manner, the LCST of Au@PNAL was elevated closer to the body temperature and the thermal response could render Au@PNAL a triggerable function by the thermal stimuli from the body in order to release out lyphophilic drug loaded in Au@PNAL. This novel structure is expected to be utilizable for the synchronous delivery of therapeutic drugs and biomolecules. Whilst most of the reported studies are based on methods to bind Au nanoparticles on planar substrates, or solid micro-sized particles, it is therefore noteworthy to suggest a pathway for the direct self-assembly of Au nanoparticles on the spherical, nano-sized, and thermosensitive substrates.

2. Materials and methods

2.1. Materials

L,L-lactide (99.5%) was kindly donated by PURAC corporation. *N*-isopropylacrylamide (NIPAAm, 98%), benzoyl peroxide (BPO, 40 wt.-% blended in dibutyl phthalate), β-mercaptoethanol (99%), stannous 2-ethylhexanoate (98%), $HAuCl_4{\cdot}3H_2O$ (99%), $C_6H_5Na_3O_7{\cdot}2H_2O$ (TSC, 99%), $NaBH_4$ (99%), and poly(vinyl alcohol) (PVA, Mw of 15 kDa) were purchased from Sigma-Aldrich chemicals. Ammonium molybdate (99%) was purchased from KEBO chemicals. All organic solvents were reagent grade. Water was purified with a Milli-Q system. After finishing the reaction, all kinds of polymers were precipitated by diethyl ether three times and dried under vacuum for 24 hrs unless stated otherwise.

2.2. Methods

2.2.1. Characterization and analysis

The ^{1}H NMR spectra were recorded on a Bruker Avance-600 MHz spectrometer with $CDCl_3$ as a solvent: ^{1}H NMR (600 MHz, $CDCl_3$, 25 °C): δ = 1.13 (*d*, NH_2 on AAm), 1.14 (*d*, CH_3 on PNIPAAm), 1.50 (*broad s*, CH_2 on PNIPAAm and AAm), 1.58 (*d*, CH_3 on PLA), 2.05 (*broad s*, CH on PNIPAAm and AAm), 3.98 (*broad s*, NH-CH on PNIPAAm), and 5.16 (*q*, CH on PLA).

Molecular weight and polydispersity were examined on a gel-permeation chromatograph system (Model 1100 series, Hewlett Packard) with THF as a solvent and PMMA as a standard. Transmission electron microscopy (TEM) images were taken by using JEOL TEM 2000EX at 200 kV. Particle size was measured by quasi-elastic light scattering (QELS) spectrometer (Brookhaven BI-90) equipped with He-Ne laser beam (λ = 633 nm) at 20 °C. Varian Cary 300 UV-Vis spectrophotometer was used for the LCST determination and thermal response examination.

2.2.2. PNA random copolymer synthesis

NIPAAm (4.51 g), AAm (0.5 g) and β-mercaptoethanol (0.075 g) were dissolved in 15 mL of THF. The reaction was carried out at 60 °C for 12 hrs under N_2 gas.

2.2.3. PNAL diblock terpolymer synthesis

PNA (1 g), L-lactide (5 g), and 2-ethyl hexanoate (0.18 g) were dissolved in 30 mL of anhydrous toluene and reacted at 110 °C by supplying N_2 gas for 10 hrs.

2.2.4. Au nanoparticles preparation

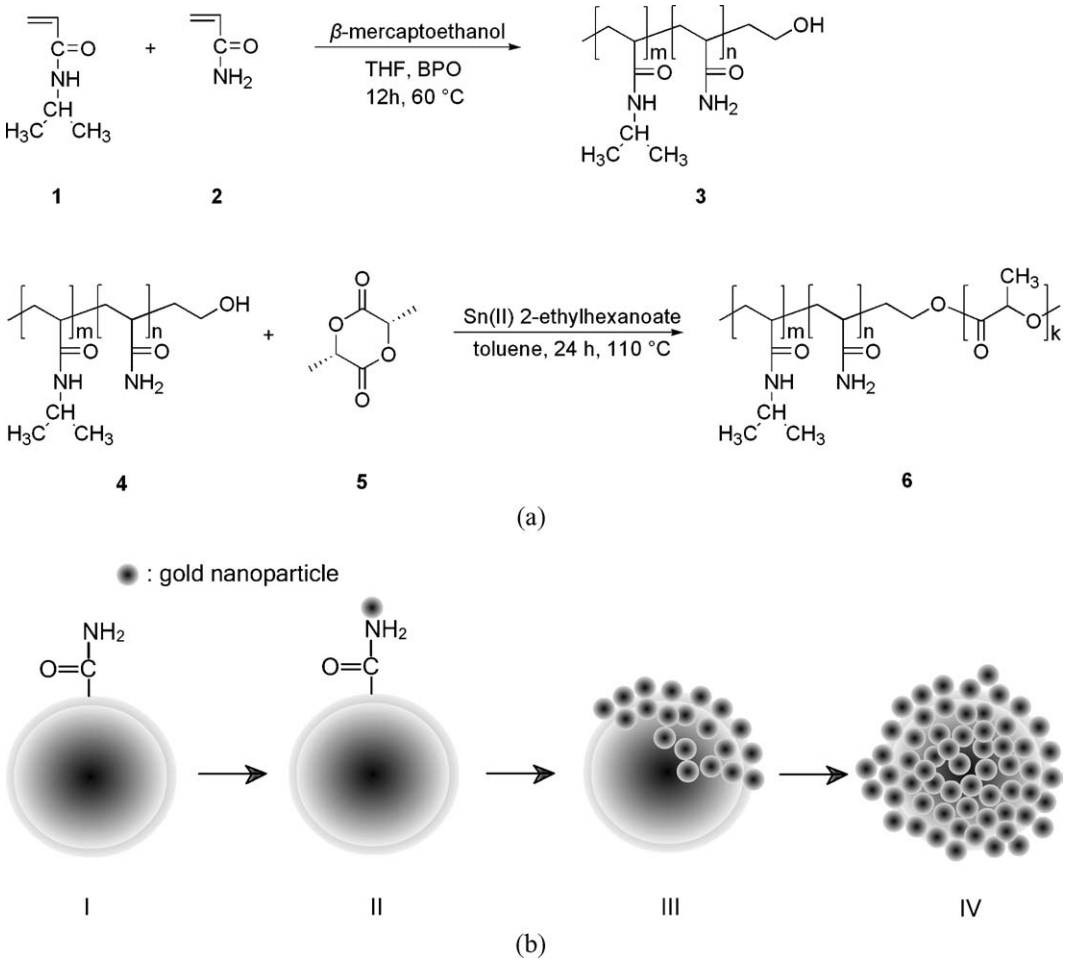
1 mL of $HAuCl_4{\cdot}3H_2O$ was added to 10 mL of 1 wt.-% TSC aqueous solution (solution I). The mixture was vigorously agitated for 10 mins. Separately, 1 g of $NaBH_4$ was dissolved in 100 mL of 1 wt.-% TSC aqueous solution (solution II). Then, 100 μL of solution II was added to solution I and maintained for stabilization for 1 min. This step was repeated tem times until dark-red colored solution was obtained (solution III).

2.2.5. Au@PNAL fabrication

μL of 4 wt.-% PNAL solution in methylene chloride was added to the mixture of 3 mL of 0.6 wt.-% of PVA aqueous solution and 3 mL of solution III (solution IV). Solution IV was sonicated in an ice bath for 1 min with 10 units of output power and left in an ice bath for 1 min more to be cooled down. This step was repeated 4 times until translucent violet 'oil-in-water' emulsion could be obtained. The residual methylene chloride in the Au@PNAL spheres was evaporated by agitating the emulsion under ambient conditions for 24 hrs.

3. Results and discussion

Scheme 1(a) shows the pathway to synthesize PNAL amphiphilic diblock terpolymer. PNA random copolymer was synthesized from NIPAAm and AAm monomers via free-radical polymerization. The weight ratio of two monomers was fixed at 95:5 (NIPAAm:AAm). Michael-type addition of thiol to vinyl monomer/oligomer is a main concept to produce hydroxyl-terminated homo/copolymer before another block of PLLA is added to the PNA random copolymer. This is a useful technique to crosslink vinyl monomers or introduce heterogeneous composition in polymer synthesis [10, 15, 16]. In our scheme, β-mercaptoethanol was used as a chain transfer agent. After accomplishing the synthesis of hydroxyl-terminated PNA random copolymer, PLLA block was subsequently added to the PNA copolymer through the ring-opening polymerization (ROP) initiated at hydroxyl groups of PNA copolymer. Molecular weight of PNAL diblock terpolymer was targeted at 40 kDa, which has been confirmed by gel-permeation chromatography (GPC) results of PNA with 7.89 kDa ($\bar{M}_w$, $\bar{M}_w/\bar{M}_n$: 1.14) and PNAL with 38.4 kDa ($\bar{M}_w$, $\bar{M}_w/\bar{M}_n$: 1.20). The molecular weight ratio between PNA and PLLA blocks was fixed at



Scheme 1 Strategy to fabricate Au@PNAL spheres: (a) Synthesis pathway to PNAL diblock terpolymer (1. NIPAAm. 2. AAm. 3. PNA. 4. Hydroxyl-terminated PNA. 5. L-lactide. 6. PNAL). (b) Schematic illustration of direct self-assembly of Au nanoparticles on the PNAL nanospheres; Step I. PNAL nanospheres. Step II. Direct self-assembly of Au nanoparticles to the primary amide groups of AAm. Steps III.–IV. Completion of the self-assembly of Au nanoparticles.

1:4 (PNA:PLLA). Scheme 1(b) schematically depicts the direct self-assembly of Au nanoparticles on the PNAL nanospheres. Many of the published articles describe the binding character of colloidal Au onto secondary amine [1a], amino acid containing primary amine such as valine [17a], primary fatty amine [17b] or electronegative nitrogen (tertiary amine) in sp^2 hybridized ring such as pyridine [18] for various purposes. In the present work, primary amide groups are the targeted site for Au nanoparticles' deposition. Using PNAL diblock terpolymer, PNAL nanospheres were fabricated by a typical 'oil-in-water' emulsion/evaporation technique in a similar way as mentioned elsewhere [19]. The data from quasi-elastic light scattering (QELS) particle-size measurement evidence that PNAL spheres with 104 nm of mean diameter were obtained while maintaining a monomodal size distribution (polydispersity index: 0.145). Also, no significant change was observed in sphere size even after the self-assembly of Au nanoparticles (115 nm of mean diameter and 0.139 of polydispersity index).

Fig. 1(a) and (b) are TEM images of a series of bare and Au nanoparticle-deposited PNAL nanospheres. Shown in Fig. 1(a) is photograph to prove that PNAL nanospheres were homogeneously well-defined before the self-assembly. The outer shells of PNAL nanospheres are clearly seen as designated by arrows in the inset of Fig. 1(a). Before taking TEM images of bare PNAL nanospheres, PNAL nanospheres on copper grids were stained by a negative staining agent, ammonium molybdate, because PNAL spheres tend to highly transmit the electron beam so as to cause too weak contrast at the interfaces between PNAL nanospheres and the surface of copper grid, making it too difficult to obtain clear TEM image of PNAL spheres without such a treatment.[1] In Fig. 1(b), Au nanoparticles (dark spots) bound on the PNAL nanospheres are clearly visible. Au nanoparticles which cover the PNAL spheres were obtained by reducing $HAuCl_4 \cdot 3H_2O$ by $NaBH_4$ in the presence of a capping agent, trisodium citrate (TSC). Au particle size of 4 to 10 nm was obtained in the similar procedure as suggested in our previous studies [15, 20].

The LCST of SSP can be determined by scanning the change of the optical transparency as a function of temperature. The cloud point of SSP indicates the critical point for the phase transition from hydrophilic phase to hydrophobic phase whose evaluation is normally carried out at 500 nm of wavelength [10, 11, 15]. PNA

[1]After a drop of PNAL emulsion was placed on the copper grid, the upper surface of the copper grid was immediately faced on a drop of 2 wt.-% ammonium molybdate solution on the paraffin film. It was left for 2 mins. Then, copper grid was dried in a desiccator for 12 hrs before TEM images were taken.

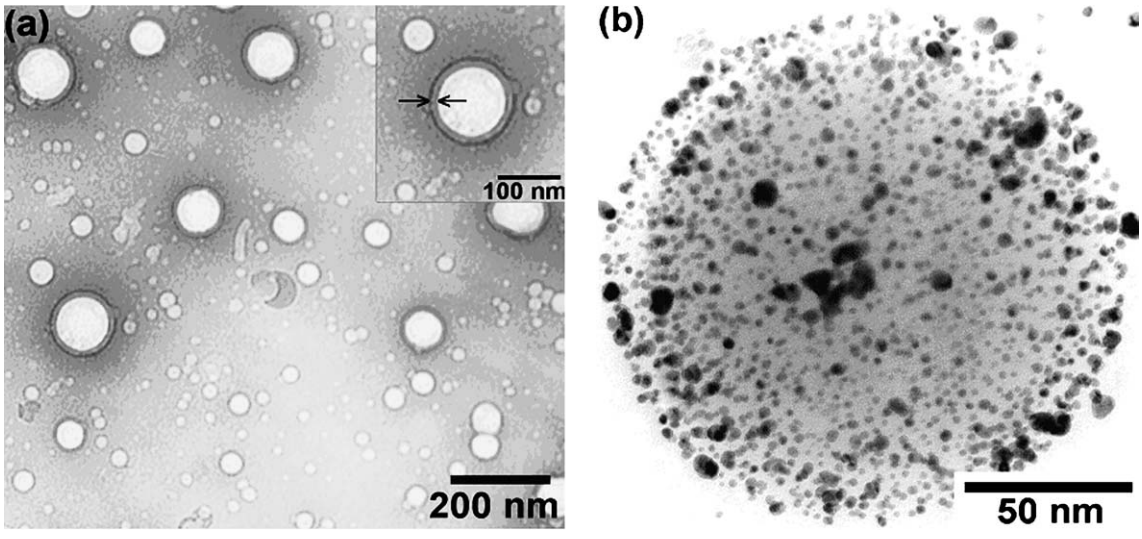


Figure 1 TEM images of (a), (b) PNAL nanospheres (taken after negative staining) and (c), (d) Au@PNAL (without staining).

aqueous solution is transparent at ambient temperature due to its high solubility to water. However, as temperature increases, PNA solution starts to be cloudy and turned to be turbid completely at around 41 °C as demonstrated in Fig. 2(a). This phenomenon mainly occurs as a result of the decrease of transmittance rate. The graph in Fig. 2(c) is numerically obtained by differentiating Fig. 2(a) to evaluate the minimum point of the curve in Fig. 2(a). This corresponds to the LCST of PNA of 35.6 °C elevated higher than the LCST of pure PNIPAAm homopolymer. In a similar way, the optical behavior of the emulsion containing PNA spheres was also examined. As presented in Fig. 2(b), the visible light absorption curve as a function of temperature is in a similar sigmoid shape with Fig. 1(a), which implies that PNAL spheres also exhibit an identical thermal response to heat regardless of the PLLA block added, although the amplitude of the change is not as severe as PNA solution.

Chung *et al.* reported that PNIPAAm micelles loading adriamycin showed a reversible thermoresponsible on/off switching behavior for drug release through the LCST [10d]. In this case, the inner core site of PNAL nanospheres is composed of self-aggregates of PLLA segments which can be also loaded with lyphophilic drug. In this respect, the interaction parameter between the lyphophilic drug and the core segment should be optimized to achieve high transport efficiency of the entrapped drug [21]. The outer PNA chains can play several important roles in the system as a whole. PNA chains stabilize the nanospheres in the emulsion and allow Au nanoparticles to be deposited. In addition, the thermosensitive PNAL nanospheres are capable of initiating the drug release by deformation of PNA triggered by the heat absorbed, for instance with a local hyperthermia [22].

In Fig. 3, the absorption spectrum of Au@PNAL are presented and compared to that of PNAL nanospheres. The inset displays the absorption spectrum of colloidal Au nanoparticles, which reveals a plasmon band maximum (λ_{max}) at 484 nm with transparent dark-red color. PNAL emulsion shows broad absorption band over the whole range; from 400 nm to 800 nm wavelength (Fig. 3(a)). In contrast, as Au nanoparticles starts to be self-assembled on the PNAL nanospheres, λ_{max} of the spectrum appears at almost the same point with Au colloid (Fig. 3(b)). As more particles are deposited on the PNAL nanospheres, the absorption band grows sharper

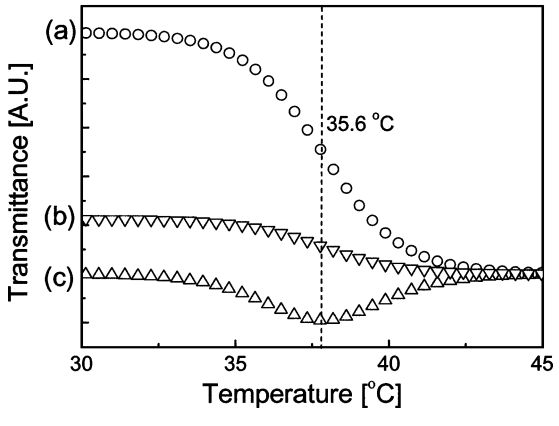


Figure 2 UV-Vis spectra to determine the LCST of (a) PNA solution and (b) PNAL nanospheres. (c) Differentiated curve of spectrum given in (a).

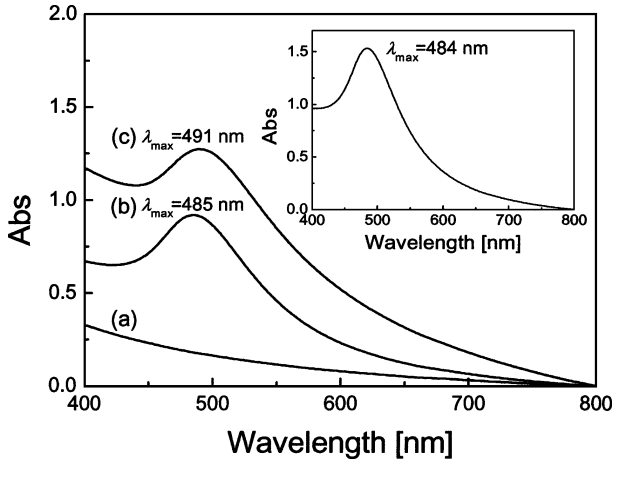


Figure 3 UV-Vis spectra of (a) PNAL emulsion, (b) PNAL nanospheres partly-covered by Au nanoparticles, and (c) PNAL nanospheres with a full coverage by Au nanoparticles. The inset graph represents the absorption spectrum of colloidal Au nanoparticles.

and shifts toward the long wavelength region.[2] The color of Au@PNAL emulsion was dark and translucent violet. The red-shift in λ_{max} is attributed to the coupling of plasmon resonances of neighboring Au nanoparticles deposited on the PNAL substrate [8]. Therefore, the position gap of λ_{max} in Fig. 3(b) and Fig. 3(c) stems from the red-shift caused by the difference in the progress of Au nanoparticles' self-assembly on the PNAL nanospheres. These observations indicate that the Au nanoparticles behave as discrete but coupled particles and not as a thin, continuous metal shell [8 g]. It is also possible to predict the extent of self-assembly of Au nanoparticles by observing the shift of λ_{max} from UV-Vis spectrum.

4. Conclusion

In summary, we have presented a novel technique to fabricate Au@PNAL hybrid nanospheres supported by characterization and analysis data. No coupling agent is required between Au nanoparticles and PNAL nanospheres due to the existence of amino groups from diblock terpolymer itself. The LCST of PNAL nanosphere was 35.6 °C as revealed by UV-Vis spectroscopy, which is very close to the body temperature. From all the results obtained in this work, we believe that this novel hybrid nanosphere possesses multifunction and high potential in a wide variety of applications such as synchronized delivery of therapeutic drugs and biomolecules in a controlled manner.

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[2]This phenomenon is called the red-shift.