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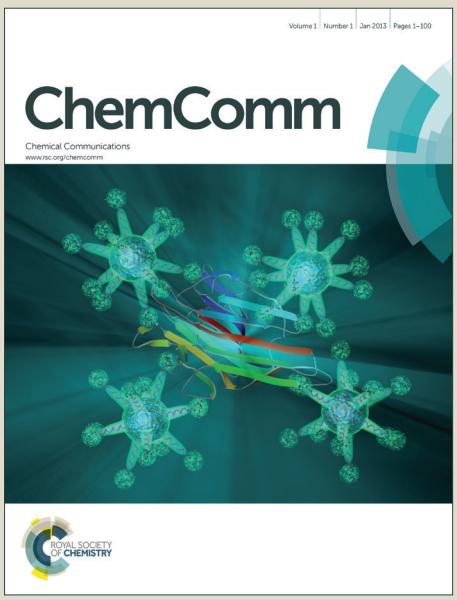

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Label-free detection of C-reactive protein using highly dispersible gold nanoparticles synthesized by reducible biomimetic block copolymers

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Biomimetic block copolymer-protected gold nanoparticles (AuNPs) were prepared by autoreduction. Excellent colloidal stability of the AuNPs was observed at a high salt concentration and over a wide pH range. Functional AuNPs are useful for label-free detection of an inflammation biomarker, C-reactive protein (CRP).

Gold nanoparticles (AuNPs) are promising candidates as nanoscopically assembled materials for numerous applications due to the unique electronic and optical properties that result from their nanometer size and narrow size distribution.[1] AuNPs are also used in applied biological applications such as imaging, labeling, sensing, etc.[2] Surface modification of AuNPs to improve colloidal stability in aqueous media, functionalization, and biocompatibility is quite important for extending their application in biomedical fields.[3] Thiol compounds have been widely used for the surface modification of gold surfaces with chemical species ranging from small molecules[4] to synthetic polymers.[5] Thiol-capped AuNPs are typically produced using conventional reducible agents such as $NaBH_4$ in the presence of thiol-functional molecules. Recently, it has been reported that amine-functional polymers can be utilized for the synthesis of highly stable AuNPs where the amine-functional polymers have double functionality as both reducing and protecting agents. Ishii et al. synthesized poly(ethylene glycol) (PEG)-*b*-poly(2-(*N*,*N*-dimethylamino)ethylmethacrylate) (DMAEMA)] for preparing PEGylated AuNPs by the autoreduction of tetrachloroauric acid ($HAuCl_4$).[6] Yuan and co-workers followed this autoreduction procedure and synthesized poly(2-metacryloyloxyethyl phosphorylcholine) (MPC)-*b*-DMAEMA and successfully prepared AuNPs.[7] Coated with hydrophilic PEG or PMPC chains, the AuNPs were well dispersed in aqueous media. As a more eco-friendly and biomimetic system, the preparation of AuNPs by reduction of $HAuCl_4$ with plasma proteins has been performed, playing an essential role in improving desirable optical properties as well as enabling conjugation of biofunctional groups. Xie et al. demonstrated the synthesis of highly fluorescent protein-stabilized Au nanoclusters (AuNCs) using a bovine-serum albumin (BSA)-templated method.[8] It has been suggested that tyrosine (Tyr) and cysteine (Cys) residues in BSA are important in the production of the protein-stabilized AuNCs. This is because Cys residues, similar to thiol-protected AuNPs, are able to stabilize AuNCs, and Tyr residues can reduce Au(III) into Au(0) or Au(I) under alkali conditions.[9] Although these methods are robust enough to obtain AuNPs**,** the colloidal stability of the prepared AuNPs at a high salt concentration and over a wide pH range has not been well researched.

In this study, we have newly synthesized a thiol-terminated biomimetic block copolymer, poly(2-methacryloyloxyethyl phosphorylcholine)-*b*-poly(*N*-methacryloyl-(*L*)-tyrosine methylester) (PMPC-*b*-PMAT; Figure 1A), which bears two unique functional groups at the side chain: MPC residues that mimic a biomembrane and Tyr residues as a reducing agent. The biomimetic PMPC-*b*-PMAT-protected AuNPs were prepared by autoreduction without the addition of any reducing agent, showing excellent colloidal stability at a high salt concentration and over a wide pH range because of the effective steric stabilization of MPC. Label-free detection of an inflammation marker, C-reactive protein (CRP), was demonstrated by using the aggregation of AuNPs via the binding of MPC to CRP.

To obtain PMPC-*b*-PMAT, PMPC was synthesized as a macro chain transfer agent (macro-CTA) via reversible addition fragmentation chain transfer (RAFT) polymerization. The degree of polymerization (DP_n) of macro-CTA was adjusted to nearly 100 by changing the [MPC]/[RAFT reagent] ratio; the DP_n of the synthesized macro-CTA was 98. Block copolymerization of MAT proceeded at a [MAT]/[macro-CTA] ratio of 20. Under our experimental conditions, DP_n of MAT was 6, which was much lower than that of the target DP_n. Although we tried to increase the feed concentration of MAT, the polymerization was significantly reduced (data not shown). The polydispersity (M_w/M_n) of PMPC-*b*-PMAT determined by gel-permeation chromatography (GPC) was 1.12. PMPC-*b*-PMAT showed an amphiphilic nature and the critical micelle concentration (cmc) of PMPC-*b*-PMAT in water was 4.0 mg/L (≈0.13 μM) in water.

For preparing AuNPs by autoreduction, $HAuCl_4$/phosphate buffer saline (PBS) (200 μL, 10 mM) was added to the polymer aqueous solution at different concentrations under vigorous stirring. NaOH aqueous solution (50 μL, 1 M) was introduced 2 min later, and the reaction was allowed to proceed under vigorous stirring at 60 °C for 15 h. The absorption band (λ_{LSPR}) from the localized surface plasmon resonance (LSPR) of AuNPs is described as a function of the PMPC-*b*-PMAT concentration in the feed (See ESI,† Figure S1). The λ_{LSPR} of AuNPs decreased with an increase in the polymer concentration, reaching a minimum at a concentration of 3 mM. The UV-Vis absorption spectrum of AuNPs prepared at 3 mM PMPC-*b*-PMAT shows clear LSPR absorption (λ_{LSPR} ~ 520 nm), as shown in Figure 1B. The TEM image reveals well-dispersed and spherical AuNPs with sizes of 3 - 13 nm (as bare AuNPs), as shown in Figure 1C. The dependence of λ_{LSPR} on PMPC-*b*-PMAT concentration suggests that the size of AuNPs was regulated by changes in the ratio of polymer and $HAuCl_4$ because the block copolymer could work as stabilizers to protect AuNPs generated by autoreduction. In contrast, the increase of λ_{LSPR} at a higher polymer concentration (>10 mM) was unexpected due to the stabilizer effect (See ESI,† Figure S1), but such increase may be attributed to the uncontrollable quick reduction of $HAuCl_4$. In the following study, the concentration of PMPC-*b*-PMAT was fixed at 3 mM to prepare the smallest possible, and well-dispersed AuNPs.

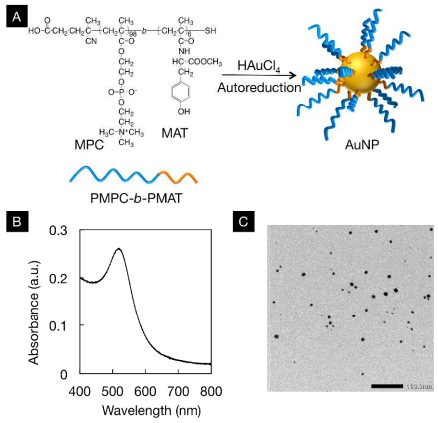

Figure 1 Synthesis of the PMPC-*b*-PMAT-protected AuNPs: (A) Synthetic scheme of the AuNPs; (B) UV-Vis absorption spectrum of PMPC-*b*-PMAT-protected AuNPs; (C) TEM image of PMPC-*b*-PMAT-protected AuNPs; for (B) and (C), AuNPs was prepared by 3 mM PMPC-*b*-PMAT and each analysis was conducted using AuNPs /153 mM NaCl aqueous solution.

Thermal gravimetric analysis (TGA) of PMPC-*b*-PMAT-protected AuNPs was performed to determine the surface density of PMPC-*b*-PMAT on the AuNPs, as shown in Figure S2. The result indicated that PMPC-*b*-PMAT decomposed in the temperature range of 230-450 °C. The residual mass of gold was calculated to be 55.1%. Thus, the surface density of the PMPC-*b*-PMAT chain can be calculated to be 0.45 chains/nm^2 (see ESI,†).

The colloidal stability of the AuNPs bearing PMPC-*b*-PMAT in various ionic strength media (0 mM ≤ [NaCl] ≤ 3825 mM) is represented in Figure 2A. Both the size and λ_{LSPR} of the AuNPs aqueous solution did not change with additional increments of ion strength up to ~ 3.8 M NaCl in the aqueous medium. These results suggest that surface-immobilized MPC block copolymers are quite effective for improving the colloidal stability of AuNPs. The contribution of MPC copolymers to stabilize AuNPs is in agreement with the earlier studies.[7,10]

Figure 2B shows the ζ-potentials of the AuNPs bearing PMPC-*b*-PMAT in aqueous media. They were almost neutral (-10 ~ 0 mV) and stable over the pH range of 4-11. The weak dependence of the ζ-potentials may originate in partially protonated phosphorylcholine (PC) groups at a low pH.[11] Jin et al. studied the stability of AuNPs covered with an 11-mercaptoundecyl phosphorylcholine (PC-SH) in various pH media.[11] At pH > 6, the ζ-potentials of PC-SH-protected AuNPs was almost neutral due to the anti-parallel orientation of the PC groups. However, the orientation was destroyed at the low pH because the phosphate groups in PC were partially protonated. The neutral ζ-potential is important for strong non-specific resistance to other particles. The surface of PMPC-*b*-PMAT-protected AuNPs has a thick hydration layer with a large number of PC groups. The hydration layer can preserve PC orientation which, in turn, provides very effective steric stabilization.

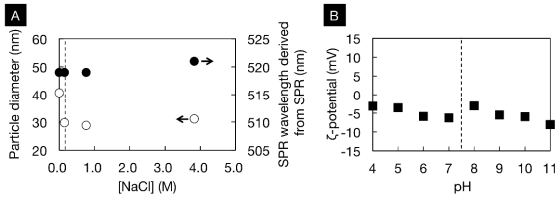

Figure 2 Colloidal stability of the PMPC-*b*-PMAT-protected AuNPs under (A) high salt concentration and (B) over a wide pH range. The dashed line represents physiological ionic condition. Size of AuNPs for (A) was estimated by dynamic light scattering measurement.

Finally, we applied the PMPC-*b*-PMAT-protected AuNPs for label-free detection of C-reactive protein (CRP). CRP is one of the most well-known human acute-phase proteins in relation to inflammation and infection.[12] The native form of CRP is "pentraxin" (115 kDa), which is composed of five identical subunits (23 kDa) with noncovalent protomers arranged as a symmetrical pentagon around a central pore. Each protomer has a PC binding site with the aid of two coordinated Ca^{2+} adjacent to a hydrophobic pocket. Phenylalanine (Phe)-66 and glutamic acid (Glu)-81 are the two key residues mediating the binding of PC to CRP.[13] In the analogy of PC to MPC, recently, the MPC polymer has shown to be a good indicator for detecting CRP,[14] since MPC has a PC group at the side chain and reduces non-specific interaction with plasma protein.[15] We then demonstrate a PMPC-*b*-PMAT-protected AuNPs-based LSPR sensor for label-free detection of CRP.

Figure 3A shows pH dependent aggregation of PMPC-*b*-PMAT-protected AuNPs in contact with 50 nM CRP with the existence of 1 mM Ca^{2+}. The colloidal stability of MPC-*b*-PMAT-protected AuNPs was preserved in the range of pH 5.5 to 7.4 when CRP was not in the medium. The CRP remarkably induced aggregation of the AuNPs and the particle diameter increased signigicantly at the lower pH condition. This pH dependence is due to affinities for interaction between CRP and Ca^{2+}.[14,16] Figure 3B shows the CRP-concentration dependence on the aggregate sizes formed by the AuNPs and CRP. When Ca^{2+} was not present in the medium, the size of PMPC-*b*-PMAT-protected AuNPs did not change regardless of CRP concentration. In contrast, the AuNPs formed remarkable aggregates in the presence of 1.0 mM Ca^{2+} and the size of the aggregates increased with an increase in the concentration of

CRP over the range of 0 to 80 nM. Limit of detection for CRP was found to be between 20 and 40 nM. The size of the AuNP could not be determined when the CRP concentration was greater than 100 nM because of the instrument detection limit. CRP-mediated aggregation of AuNPs in the presence of Ca^{2+} was also confirmed by a red shift of λ_{LSPR} and TEM imaging (Figures 3B and 3C).

Figure 3D shows the precipitation in the solution of PMPC-*b*-PMAT-protected AuNPs after being in contact with CRP for one night. Whereas the PMPC-*b*-PMAT-protected AuNPs were well dispersed, even in contact with CRP in the Ca^{2+}-free condition, the AuNPs underwent a remarkable precipitation when the CRP concentration was above 40 nM in the solution containing Ca^{2+}. Since the size of AuNPs (~8.6 nm) is equivalent to the dimension of disc-shaped CRP (~10 nm in diameter), we suggest that the CRP binding to the PC groups displayed on the AuNPs induces inter-particle cross-linking, leading to the macroscopic aggregation. Significantly, the dynamic range of the AuNPs corresponds to the clinically relevant upregulation of CRP from normal (< 10 nM) to acute-phase levels (> 100 nM), which is demonstrated by the biomimetic design of the PC-CRP interplay on the AuNPs.[14] Therefore, our system may be used for direct, colorimetric screening of inflammation and infection by the naked eye without requiring any apparatus.

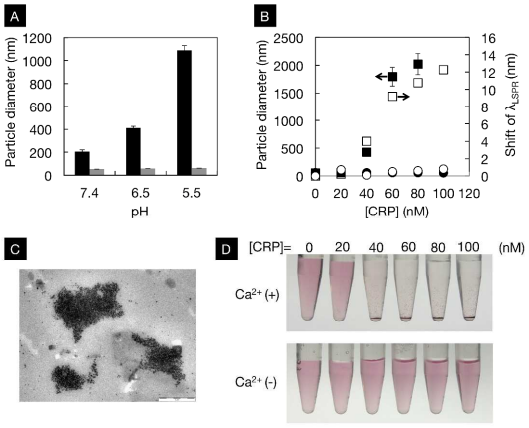

Figure 3 Detection of CRP by PMPC-*b*-PMAT-protected AuNPs: (A) pH dependent aggregation of PMPC-*b*-PMAT-protected AuNPs in contact with CRP for 30 min with the existence of 1mM Ca^{2+}. ■: [CRP]= 50 nM; ■ [CRP]= 0 nM (B) Change in the size and the red shift of λ_{LSPR} of AuNPs in contact with CRP for 30 min at pH 5.5. ■,□: [Ca^{2+}]= 1 mM; ●,○: [Ca^{2+}]= 0 mM (C) TEM image of PMPC-*b*-PMAT-protected AuNPs after contact with CRP in an acidic medium (pH=5.5) for 30 min. Scale bar represents 1 μm. (D) Photographs of CRP-contacted AuNPs after incubation for one night pH 5.5.

In conclusion, we newly synthesized a reducible MPC block copolymer. Autoreduction of gold ions with the reducible MPC block copolymers results in the generation of various, highly dispersible AuNPs, which can be used for biomedical applications. As proof of this concept, the label-free detection of CRP as an infection biomarker was successfully demonstrated by using the biomimetic block copolymer-protected AuNPs. Although there are limited reports of detecting CRP using AuNPs,[17] the current study is the first effort conducted with AuNPs bearing synthetic polymers.

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