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Polymer Nanoparticle−Protein Interface. Evaluation of the Contribution of Positively Charged Functional Groups to Protein Affinity

Yusuke Yonamine,[†] Keiichi Yoshimatsu,[†] Shih-Hui Lee,[†] Yu Hoshino,[‡] Yoshio Okahata,[§] and Kenneth J. Shea*,[†]

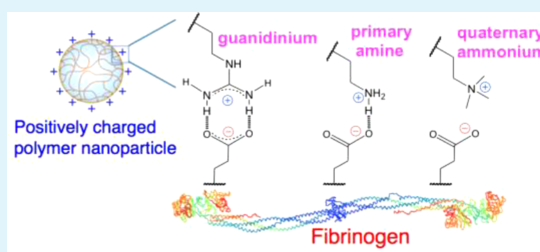
[†]Department of Chemistry, University of California Irvine, Irvine, California 92697, United States

[‡]Department of Chemical Engineering, Kyushu University, 744 Motooka, Fukuoka 819-0395, Japan

[§]Department of Biomolecular Engineering, Tokyo Institute of Technology, Yokohama 226-8501, Japan

Supporting Information

ABSTRACT: Cationic-functionalized polymer nanoparticles (NPs) show strikingly distinct affinities to proteins depending on the nature of the cationic functional group. *N*-Isopropylacrylamide (NIPAm) polymer NPs incorporating three types of positively charged functional groups (guanidinium, primary amino, and quaternary ammonium groups) were prepared by precipitation polymerization. The affinities to fibrinogen, a protein with an isoelectric point (pI) of 5.5, were compared using UV−vis spectrometry and a quartz crystal microbalance (QCM). Guanidinium-containing NPs showed the highest affinity to fibrinogen. The observation is attributed to strong, specific interactions with carboxylate groups on the protein surface. The affinity of the positively charged NPs to proteins with a range of pIs revealed that protein-NP affinity is due to a combination of ionic, hydrogen bonding, and hydrophobic interactions. Protein affinity can be modulated by varying the composition of these functional monomers in the acrylamide NPs. Engineered NPs containing the guanidinium group with hydrophobic and hydrogen bonding functional groups were used in an affinity precipitation for the selective separation of fibrinogen from a plasma protein mixture. Circular dichroism (CD) revealed that the protein was not denatured in the process of binding or release.



KEYWORDS: *polymer nanoparticles, plastic antibodies, protein interaction, guanidinium, fibrinogen, affinity precipitation*

■ INTRODUCTION

Proteins play a central role in biology: enzymes in biochemical reactions, antibodies in the immune system, and receptors and channels in cell signaling.[1,2] Proteins utilize characteristic properties of 20 amino acids for substrate recognition. The side chains can be categorized as electrically charged, polar uncharged, and hydrophobic. The positively charged amino acids include arginine, lysine, and histidine. Interestingly, their function in proteins is often distinct.[3−6] For example, in conjunction with hydrophobic residues, arginine frequently contributes to stabilize protein−protein interactions at the interface of homocomplexes[3−5] and heterocomplexes.[6] This has been attributed to the guanidinium group in arginine.[7] The guanidinium−oxyanion affinity has been exploited in synthetic binders[8] and in polymers[9−12] with high affinity to target proteins.

Synthetic nanoparticles (NPs) that can selectively capture target proteins in biological fluids are a novel class of materials that hold great promise in nanomedicine for drug delivery, imaging, diagnostics, and as protein separation media.[13−20] Fundamental insight into the interaction of NPs with proteins would provide direction in controlling NP- protein interactions.[13−18] We showed previously that synthetic polymer NPs composed of negatively charged and hydrophobic monomers can be formulated to interact specifically with target biomolecules in vitro and in vivo ("plastic antibody").[21−24] However, to target a broad range of biomolecules, expansion of the pool of positively charged functional monomers is required. To date, there have been a number of studies of interactions of positively charged polymers with a broad range of biogenic targets,[25−43] including DNA,[29−33] proteins,[34,35] viruses,[36] bacteria[37−43] for visual sensing,[29−31,34,35,38,39] imaging,[39,40] disinfection,[36,37,40−42] and several therapeutic applications.[32,41,42] However, relatively few studies have systematically investigated the effects of the positively charged functional groups on biomacromolecule affinity. In this report, we describe synthesis of three types of positively charged synthetic polymer NPs that incorporate different sources of positive charge. Evaluation of NP interactions with selected proteins revealed striking differences in the NP affinities to the proteins.

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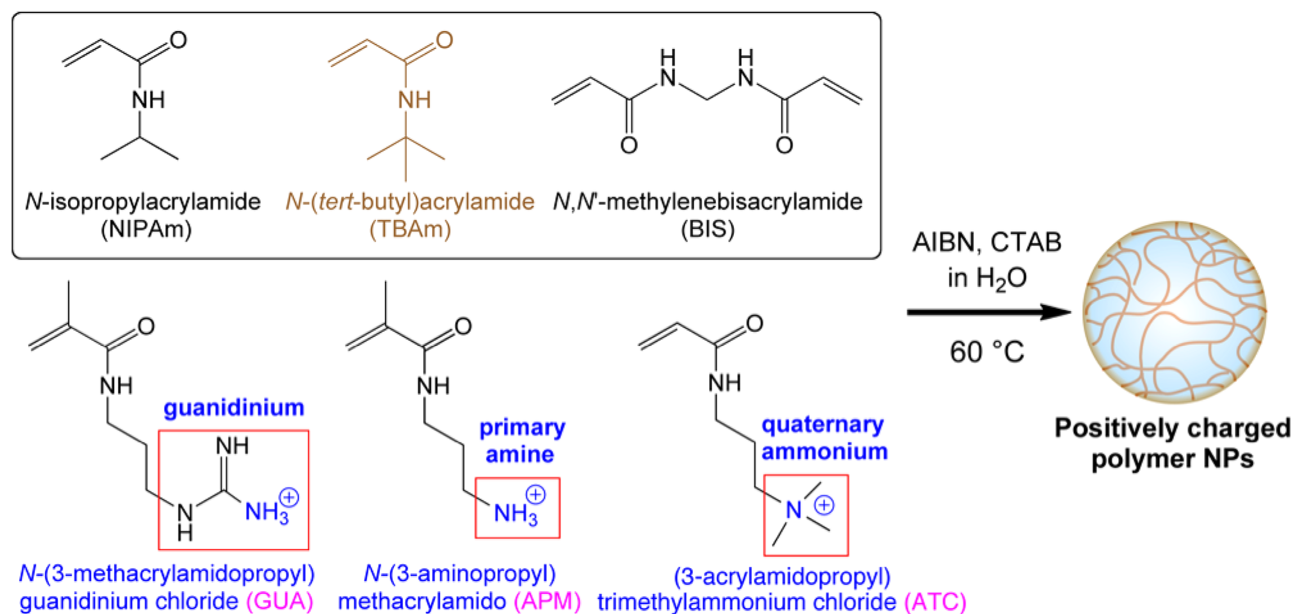

Figure 1. Synthesis of polymer NPs. Three types of positively charged monomers (GUA, APM, or ATC) were used as the source of positive charge. AIBN (azobisisobutyronitrile) and CTAB (cetyltrimethylammonium bromide), were used as initiator and surfactant.

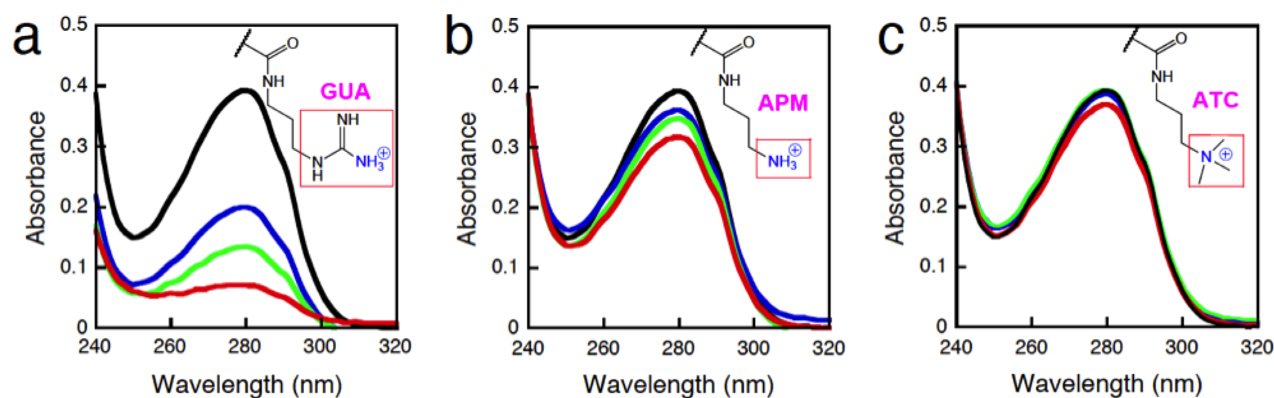

Figure 2. Interaction of positively charged NPs and fibrinogen evaluated by UV−visible spectrometry. UV spectrum of (a) GUA, (b) APM, and (c) ATC NPs. Black line indicates the condition without NP. The feed ratios of positively charged monomers are 5% (blue), 10% (green), and 20% (red), respectively. The actual incorporation ratios were evaluated by ^{1}H−NMR spectroscopy and elemental analysis (see Figures S1−4 in the Supporting Information). The final concentrations of proteins and NPs were 500 and 1000 μg/mL, respectively.

As in the protein−protein interface, NPs incorporating a guanidinium group were shown to have a higher protein affinity compared with other positively charged groups.

■ RESULTS AND DISSCUSSION

The three types of positively charged functional monomers were included in this study, a guanidinium containing monomer (*N*-(3-methacrylamidopropyl) guanidinium chloride; GUA) and a primary amine containing monomer (*N*-(3-aminopropyl) methacrylamide hydrochloride ; APM). These mimic the positively charged amino acids, arginine and lysine, respectively (see the Supporting Information (SI) for synthesis of GUA). A quaternary ammonium monomer ((3-acrylamidopropyl)trimethylammonium chloride; ATC) was used as the third source of positive charge. The *N*-isopropylacrylamide (NIPAm) based NPs were prepared with various monomer feed ratios of GUA, APM or ATC (5, 10, and 20 mol %, each; Table S1 in the Supporting Information). On the basis of previous studies,[3−6,21−24,44,45] hydrophobicity is essential to stabilize the NP association with proteins, hence a hydrophobic monomer, *N*-t-butylacrylamide (TBAm), was also incorporated (40 mol %). *N,N*′-methylenebisacrylamide (BIS, 2 mol %) was used as cross-linker. The NPs were formed by precipitation polymerization in water using small amounts of a cationic surfactant (cetyltrimethylammonium bromide; CTAB) (Figure 1). Following dialysis of the NP solutions to remove surfactant and oligomers, dynamic light scattering (DLS) revealed the NPs were monodisperse with hydrodynamic diameters ranging from 24 to 62 nm (see Table S1 in the Supporting Information). The composition of the polymer NPs was established by ^{1}H−NMR spectroscopy and elemental analysis (see Figures S1−S4 in the Supporting Information). For the GUA, APM, and ATC NPs (20%), the actual molar ratios were 12.3, 14.3, and 12.2 mol % (^{1}H−NMR spectroscopy). These values were consistent with the results from elemental analysis (Cl and C), 15.8, 15.3, and 12.2 mol %, respectively (see Table S3 in the Supporting Information).

Fibrinogen was selected as the target protein for this work. The protein is relatively hydrophobic[46,47] and negatively charged at physiological pH (pI = 5.5).[48] Fibrinogen has an

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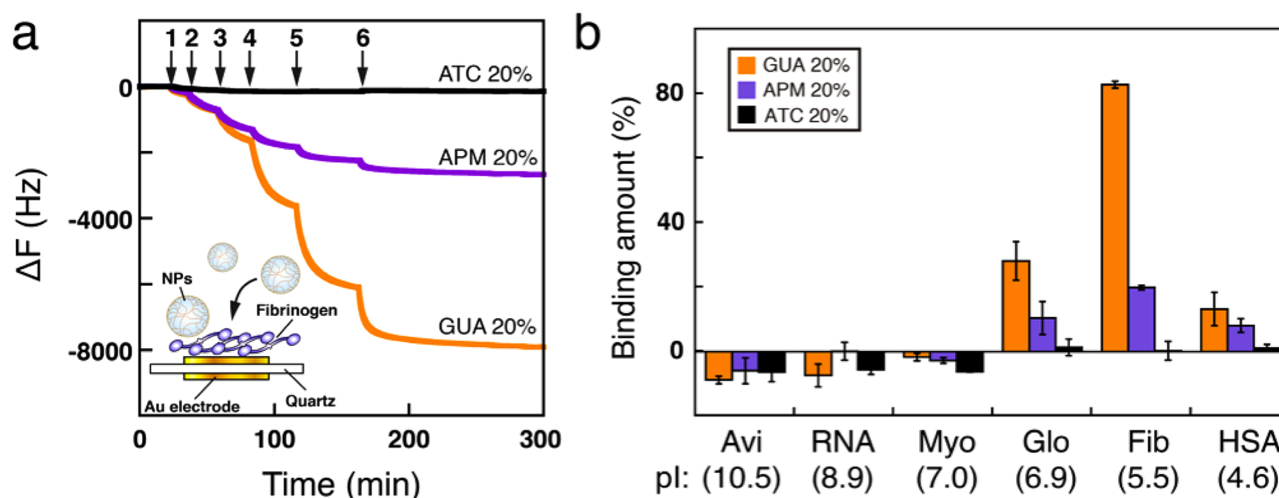

Figure 3. Interactions between NPs containing 20% positively charged monomers and various proteins (orange, GUA 20%; purple, APM 20%; black, ATC 20%.). (a) QCM analysis of interactions between fibrinogen and positively charged NPs. NP solutions were injected into the 27-MHz QCM cell at the time points indicated by the arrows. Final concentrations after each arrow are (1) 1.0, (2) 3.0, (3) 6.9, (4) 15, (5) 29, and (6) 56 μg/mL. (b) Interaction of positively charged NPs with various proteins evaluated by UV−visible spectrometry. Binding amount ratio of the protein to the NP was calculated by the absorbance at 280 nm. The three-letter codes represent: Avi, avidin; RNA, ribonuclease A; Myo, myoglobin; Glo, γ-globulin; Fib, fibrinogen; and HSA, human serum albumin, respectively. Isoelectric points (pI) of each protein[48,52,53] are indicated in brackets. The data represent the average of three measurements and the error bars are standard deviations.

elongated structure (45 nm) that consists of two outer D domains, each connected by a coiled-coil segment to its central E domain[49] forming a "dumbbell-shaped" structure. The E domain is capped with two sets of negatively charged fibrinopeptides A and B (Figure S5 in the Supporting Information), cleavage of which by thrombin exposes positively charged polymerization sites (termed E_A and E_B, respectively) and initiates end-to-middle intermolecular D to E associations, resulting in homocomplex assembly (fibril).[49] This protein−protein association can be assisted by both hydrophobic and electrostatic interactions between positively charged E_A and E_B surfaces and the negatively charged D domain. We investigated whether the positively charged NPs can present a protein-like interface that mimics the E_A and E_B surfaces and are complementary to the negatively charged and hydrophobic D domain on fibrinogen. This designed NP could be used to "capture" fibrinogen.

Interactions of the positively charged NPs with fibrinogen were evaluated by UV−visible spectrometry (Figure 2). Fibrinogen was dissolved in phosphate-buffered saline (PBS; 35 mM phosphate and 150 mM NaCl, pH 7.3) and mixed with positively charged NP (GUA, APM or ATC) solutions (the final concentrations of proteins and NPs were 500 μg/mL and 1000 μg/mL, respectively), and the NP-protein solution was incubated at 37 °C for 30 min. Aggregation was observed in the cases of the GUA NPs and all the samples were centrifuged. The supernatant was filtrated through 0.22 μm filter to remove the NPs and bound fibrinogen. The removal of NPs by filtration was confirmed by monitoring fluorescent dye labeled NPs. The result revealed that more than 99% of NPs were removed in all cases (Figure S6 in the Supporting Information). The interaction between fibrinogen and the NPs was evaluated by measuring the decrease of UV absorbance at 280 nm of the filtrate (Figure 2). In the case of the GUA NPs, a significant decrease in absorbance was observed. The amount of bound fibrinogen to GUA NPs increased as the mol % of GUA monomer in the NP increased. These results imply a specific interaction between the guanidinium groups on the NP and the protein. On the other hand, APM NPs showed only a slight decrease in absorbance, and ATC NPs did not interact with fibrinogen at all. Following these preliminary findings, a detailed analysis of NP-fibrinogen binding was undertaken using a quartz crystal microbalance (QCM). NPs containing 20% positively charged monomers were selected for the analysis. A monolayer of fibrinogen was covalently immobilized on the QCM electrode (ca. −3000 Hz; see Figure S7 in the Supporting Information), and NP solutions were injected into the QCM cells intermittently. The time courses of the frequency change (ΔF) are shown in Figure 3a. The largest increase of mass on the QCM electrode was observed with an increase in concentration of GUA NP (up to −8000 Hz). In the case of APM NPs, the frequency changes were lower (up to −2500 Hz), indicating weaker affinity to fibrinogen, and the ATC NP did not show any interaction. The trend was consistent with the results of UV−visible spectrometry and show that there is a significant difference in the NP-fibrinogen interaction depending on the nature of the positively charged functional group. A guanidinium group in GUA NP can form two hydrogen bonds and interact electrostatically with a carboxylate group[7] on fibrinogen, resulting in specific interactions at the interface. Meanwhile, the primary amine group in the APM NP forms only one hydrogen bond with electrostatic interaction, and a quaternary ammonium group in ATC NP does not form a hydrogen bond, resulting in a relatively weak interaction with fibrinogen.

We next examined the interactions of the positively charged NPs with proteins with various isoelectric points (pI) by UV−visible spectrometry (Figure 3b). The percentage of protein bound to the NP was calculated from the absorbance of the protein samples with and without NPs. Compared to fibrinogen, the NPs only weakly bound to human serum albumin (HSA) even though HSA has a lower pI value. This result may be attributed to the absence of a combination of negative charges and hydrophobicity that is important for protein-NP association. Although HSA has hydrophobic binding pockets available to small-molecule ligands, the cavities

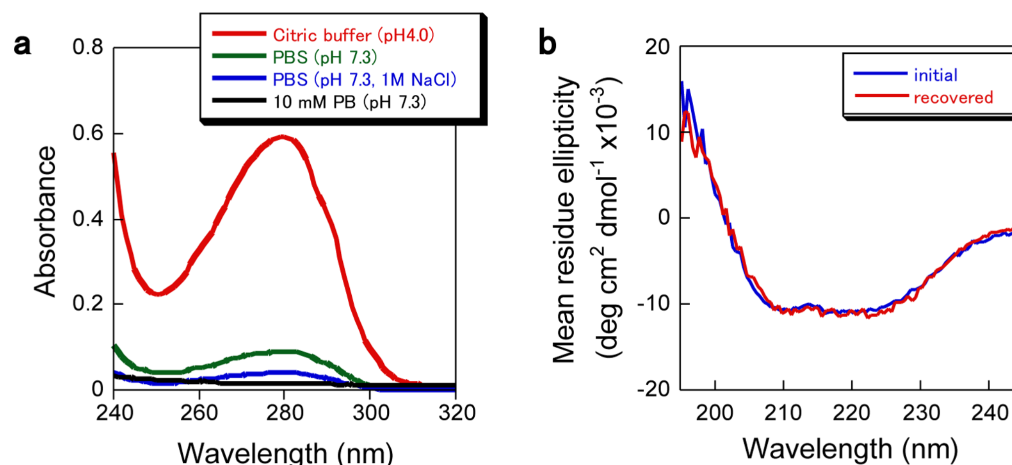

Figure 4. Optimization of elution conditions and examination of the conformation of fibrinogen. (a) UV spectra of fibrinogen-eluted solutions at various conditions. Red: 20 mM citric buffer (pH 4.0), recovery 50%. Green: 35 mM phosphate and 150 mM NaCl buffer (pH 7.3), recovery 7.7%. Blue: 35 mM phosphate and 1 M NaCl buffer (pH 7.3), recovery 3.4%. Black: 10 mM phosphate buffer (pH 7.3), recovery 1.3%. (b) Circular dichroism spectra of the initial (blue line) and recovered (red line) fibrinogen solution.

are buried in the protein[50,51] and are independent of the negatively charged outer surfaces. The lack of a cooperative effect of negative charge and hydrophobicity can lower the affinity of HSA to the NPs. On the other hand, the neutral protein γ-globulin interacted more strongly with the positively charged NPs, indicating that some domains of γ-globulin associated with antigen protein interactions can bind tightly to the NPs. On the other hand, myoglobin (pI = 7.0), an oxygen-carrying protein, did not show any interaction. In the case of positively charged proteins (avidin and ribonuclease A), no interaction was observed, presumably because of charge repulsion. The binding amount of the proteins in this case showed slightly negative values. We attribute this to facilitation of protein filtration by charge repulsion between the trapped positively charged NPs on the filter and the proteins. These results suggest that positively charged NPs, more specifically those formulated with guandinium groups (GUA NP), bind selectively to certain proteins due to a combination of electrostatic, hydrogen bonding, and hydrophobic interactions. These same interactions are common to protein−protein binding. Because of the distinctive "personalities" of each protein and NP, these results suggest the possibility to develop "matched pairs" of proteins and synthetic polymer NPs with high mutual affinity.

When fibrinogen and GUA 20% NP solutions were mixed and incubated, NP−fibrinogen aggregation was observed. This result encouraged exploration of the capability of the GUA containing NP as fibrinogen separation media. Conditions were optimized to recover fibrinogen from the aggregates (Figure 4). The solution was centrifuged and the supernatant was removed. To the resultant pellet, various solutions were added to elute the fibrinogen and the recovery ratio was calculated using the absorbance at 280 nm of the eluted solutions. The recovery ratio was highest (50%) with an acidic buffer (20 mM citric buffer, pH 4.0; Figure 4a). This result is attributed to protonation of the carboxyl groups on fibrinogen weakening the robust guanidinium−carboxylate binding. Because the circular dichroism (CD) of recovered fibrinogen was identical to native fibrinogen (Figure 4b), the protein was not denatured in the process of binding or release.

Finally, we have taken advantage of the selective interaction of the GUA NPs to a human plasma protein by demonstrating the immunoprecipitation of fibrinogen with a synthetic polymer NP that mimics a fibrinogen antibody (Figure 5a). Solutions

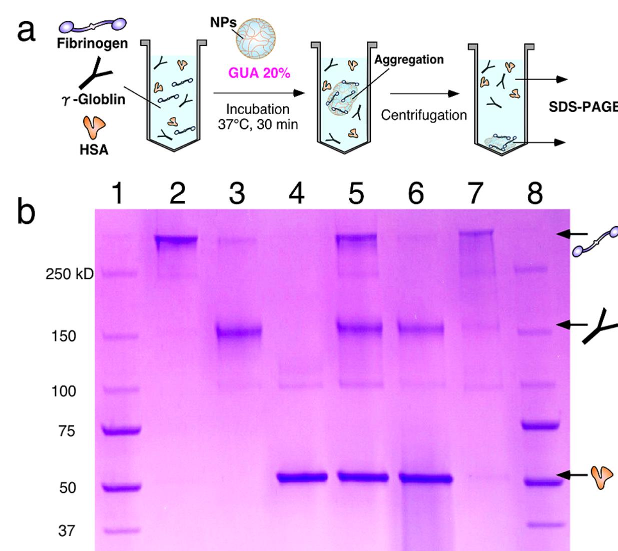

Figure 5. Immunoprecipitation of fibrinogen with GUA 20% NP. (a) Schematic diagram of the procedure. (b) SDS-PAGE analysis. Lanes 1 and 8: molecular weight marker. Lane 2: fibrinogen. Lane 3: γ-globulin. Lane 4: HSA. Lane 5: protein mixture. Lane 6: supernatant of the centrifuged solution. Lane 7: the elution from precipitation. The SDS-PAGE gel was stained with Coomassie Brilliant Blue R-250.

containing fibrinogen, γ-globulin and HSA were prepared in PBS and mixed with GUA 20% NP solution (the final concentrations of each protein and NP were 250 μg/mL and 1000 μg/mL, respectively). Aggregation was observed after the incubation. The supernatant and the precipitate were separated by centrifugation. After elution of the proteins from the pellet with citrate buffer (pH 4.0), the proteins in the solution were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). The result confirmed that most of the fibrinogen was selectively captured from solution by the GUA NPs (Figure 5b).

■ CONCLUSIONS

In conclusion, we prepared positively charged polymer NPs incorporating guanidinium, primary amino and quaternary ammonium groups and compared their interactions with fibrinogen. The GUA NP showed substantially higher affinity to fibrinogen, which was attributed in part to specific interactions with surface carboxylate groups on fibrinogen. The affinities of the positively charged NPs to various proteins were also investigated. The study reveals that there is no simple relationship between affinity and pI. The affinity of GUA NPs to proteins is not only due to the negatively charged surface but also to additional protein−protein-like interactions that include hydrogen bonding and hydrophobic interactions. Taking advantage of the selectivity of the GUA NP, we undertook an immunoprecipitation and separated fibrinogen from a plasma protein mixture. These results draw attention to the ability of guanidinium-functionalized synthetic polymer NPs to form a complementary protein-like interface with selected proteins to achieve highly specific interactions and strengthen parallels between synthetic polymer NP−protein and protein−protein interactions.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures for monomer and nanoparticle synthesis, characterization data (NMR, DLS) and procedures for evaluating nanoparticle−protein binding, capture, and release. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: kjshea@uci.edu.

Notes

The authors declare no competing financial interest.

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