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Effect of Surface Chemistry and Morphology of Gold Nanoparticle on the Structure and Activity of Common Blood Proteins

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Abhishek Chaudhary, Syamantak Khan, Abhishek Gupta and Chayan Kanti Nandi*

Keeping the native structure and activity of protein, while adsorbs onto the nanoparticle surface is one of the pre-requisite for the real biological application of nanoparticle. However, this phenomenon has poorly been understood. This is because of the lack of in-depth knowledge on the structural orientation of the adsorbed protein, complex surface chemistry and morphology of the nanoparticle. In this report, we present quantitative information on the structure and the activity of few major blood proteins when adsorbed onto different morphological and surface functionalized gold nanoparticles (GNPs). A profound effect of both the particle anisotropy and the surface ligands on the secondary structural change and consequently the activity of the proteins were observed. Further, a prominent effect on the cell viability assay was also observed, when performed the MTT assay by using MDA-MB 231 cell lines.

Nanoparticle morphology, in recent years, has been found to be very useful for cellular uptake, tumor ablation, toxic metal ion and small biomolecule sensing.[1-7] The main advantage of such anisotropic nanoparticle is the presence of different surface energies that arises due to different corners, edges, vertices, facets and further the presence of surface plasmon resonance (SPR) band at near IR region.[8,9] For example, because of the SPR band in the near infrared region, Triangular Gold Nano Plate (TGNP) and Gold Nano Rod (GNR) have effectively been used for photo thermal therapy in tissues.[10,11] However the conformational change and consequently the activity of the protein, while adsorbed onto such anisotropic nanoparticle has been poorly understood.[12] The native structural change in protein can alter the biodistribution and exposes new epitopes.[13,14] Further the formation of protein corona around nanoparticle when adsorbed onto the nanomaterial surface immensely hampers the nanoparticle targeting capability, efficiency of specific delivery, causing pro-inflammatory effect, blood coagulation and cardiovascular disease.[15-18] As a result, the application of nanoparticles in biology has become a challenging problem and proper care should be taken on the rational design of the nanoparticle.

*School of Basic Sciences, Indian Institute of Technology Mandi, Himachal Pradesh, 175001, India

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Recently, we have demonstrated that surface ligand chemistry and the size of the nanoparticles extensively affect the kinetics of protein adsorption and further the secondary structure of protein.[19-21] In this report we present the effect of GNPs morphology and surface chemistry on the structure and consequently the activity of few common blood proteins. Human serum albumin (HSA), bovine serum albumin (BSA) and hemoglobin (HB) were chosen as model systems (Fig. S1) for the study. HSA is the most abundant in human serum and plays an important role as a shuttle for broad range of endogenous and exogenous ligands. [22] BSA resembles almost 76 % structural similarity to HSA. Both BSA and HSA possess a single polypeptide chain with 583 amino acid residue and molecular weight of approximately 67 kDa. HB resembles the size and molecular weight of HSA and BSA, but has a completely different structure.[23] It carries oxygen in our body, making up about 96% of the red blood cells' dry content (by weight), and around 35% of the total content (including water). Four different types of closely similar size GNPs such as CTAB coated TGNP, GNR, CTAB coated spherical GNPs and citrate coated spherical GNPs were synthesized for a quantitative comparison of both the anisotropy and surface ligand effects (supporting information). All the GNPs were further surface functionalized using less toxic poly (3, 4 ethylene dioxythiophene) polystyrene sulfonate PEDOT: PSS.[24] Our data showed profound effect of both the particle anisotropy and the surface ligands on the secondary structural change which consequently affect the activity of the proteins. Further, a prominent effect of particle anisotropy on the cell viability assay was also observed.

All the particles were characterized by transmission electron microscope (TEM), dynamic light scattering (DLS) and their corresponding SPR bands (Fig. 1a-b and Fig. S2). The stability of the nanoparticles was further confirmed by measuring the zeta potential (Fig. S3 and Table S1).[25] The intensity correlation and the corresponding fit of the raw DLS data supports the monodispersity of the nanoparticles **(**Fig. S4). Concentration of the nanoparticles was calculated by assuming that all the $HAuCl_4$ have been converted to nanoparticles.[26] The surface functionalization of the GNPs by PEDOT: PSS was confirmed by measuring the change in the zeta potential and from TEM and DLS data (Fig. S3 and Table S1). The zeta potential of only PEDOT: PSS is -68 mV. The as synthesized

CTAB coated TGNP, GNR and spherical GNPs have the zeta potential of +70mV, +60 mV and +38 mV, respectively. After replacement by PEDOT: PSS the zeta potential for the TGNP, GNR and spherical GNPs was -8mV, -11 mV and -16 mV respectively, suggesting the complete exchange of CTAB by PEDOT: PSS. The particle size and their optical properties remains almost similar to that of initial synthesized CTAB coated GNPs, as were confirmed by the measured TEM image, hydrodynamic diameter and the corresponding SPR bands (Fig. 1 and Fig. S2).

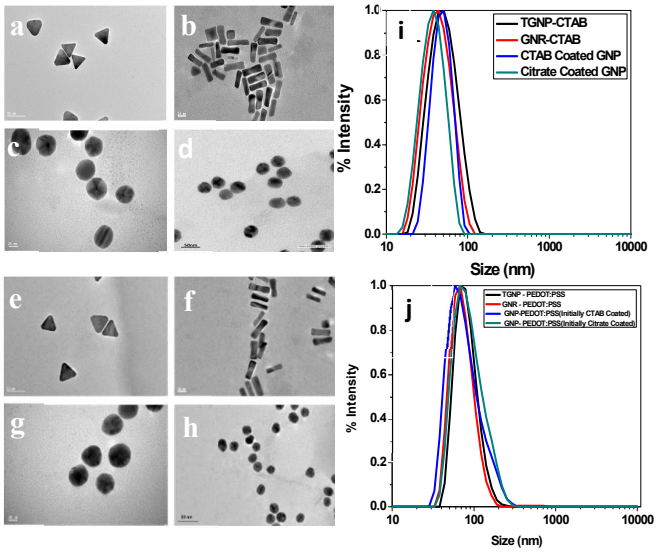

Fig. 1 Characterization of different GNPs. TEM images of (a) TGNP (b) GNR (c) CTAB coated spherical GNP (d) citrate spherical coated GNP (i) hydrodynamic diameter of as synthesized CTAB coated TGNP, GNR, spherical GNPs and citrate coated spherical GNPs .TEM images of (e) TGNP (f) GNR (g) CTAB coated spherical GNP (h) Citrate coated spherical GNP after surface functionalized with PEDOT:PSS and (j) hydrodynamic diameter when all the GNPs were surface functionalized with PEDOT:PSS.

Small increase in the hydrodynamic diameter, little red shift and decrease in intensity of the corresponding SPR bands confirms the successful surface functionalization by PEDOT: PSS. Circular dichroism (CD) spectra were measured to get information about the secondary structural change of the proteins in presence of different GNPs. HSA and BSA have two negative bands of almost similar magnitude at 222 and 208 nm (Fig. 2a, b). The band at 222 nm is related to the strong hydrogen-bonding environment of α helices and is relatively independent of their length. The spectrum for an all β sheet protein has, in general, a negative band at around 208 nm and a positive band between 195 - 200 nm.[27-28] On the other hand, the far UV CD of HB also appeared at 208 and 222 nm but with a little difference in the spectrum (Fig. 2c). This is may be due to the different number of alpha helices.[29] The percentage of α-helicity was calculated from the mean molar residual ellipticity ($^{\theta}$MRE) (Supporting Information), which gives a direct quantitative measurement of loss of α-helical structure of protein.[30] Citrate coated spherical GNPs showed minimum effect on the CD bands of HSA/BSA and hence the % helicity change (Table 1), whereas CTAB coated spherical GNPs showed a complete loss of the band at 208 at the same concentration (Fig. 2a,b), suggesting the major role of the surface ligands.[21] It is noteworthy to mention that in practice, most of the proteins have an isoelectric point below seven (HSA 4.7, BSA 4.7, Hb 6.9), and as a result at the experimental pH (pH 7.4), they will be slightly negatively charged. Consequently, the electrostatic repulsion between negatively charged citrate coated spherical GNPs and negatively charged proteins should hinder the protein adsorption. On the other hand, the positively charged CTAB coated GNPs could favor strong electrostatic interactions with the negatively charged proteins. As a result it could be concluded that, the little change in the CD spectrum in case of citrate coated spherical GNPs could be an effect of lesser number of proteins adsorbed, while the extensive change in the spectrum for CTAB coated spherical GNPs could be a result of more number of adsorbed proteins onto the GNPs surface. On the contrary, we and others have shown that HSA/BSA strongly interacts with negatively charged citrate coated GNPs, while protein adsorption on a positively charged CTAB coated GNPs is not that strong.[21, 31] The strong binding of BSA to citrate coated spherical GNPs could occur by the formation of salt bridges of the carboxylate ammonium type, between the citrate and the lysine on the protein surface.[19,31] The lesser extent of binding of proteins on CTAB coated GNPs is due to other decisive factors like the structure and properties of the surface ligands and the mechanical strain of the hydrophobic chains of the CTAB molecule. So the observed CD spectral change should be reversed that what have been observed. From the above results, it could be concluded that, not only the charge but protein orientation and attachment of the specific amino acids of the proteins onto the GNPs surface might play important role. Recently, using the theoretical simulation developed by our group, we have shown that in the case of citrate coated spherical GNPs, HSA protein is attached to the GNPs surface only with fewer numbers of amino acids of the III A domain with higher binding constant and with perpendicular orientation whereas in CTAB coated spherical GNPs, several amino acids from the entire protein are attached with a parallel orientation causing internal strain in the protein.[21] This observation explains the observed the CD spectral change (Fig. S5). As more number of amino acids from the entire protein in case CTAB coated GNPs is attached, the secondary structural change will be more than citrate coated GNPs. Although BSA uses IA domain when attached to citrate coated spherical GNPs, but again with fewer numbers of amino acids than on a CTAB coated GNPs,[12] which explains the similarity in CD spectral change. Interestingly HB, being structurally different, showed the similar type of spectral change, suggesting the important role of the surface ligand effect (Fig. 2c). HB also used large number of amino acids to interact with the CTAB coated GNPs while fewer numbers of amino acids were used on a citrate surface with a stable and higher binding constant (Fig. S5). Both the anisotropic GNPs (GNR and TGNP) showed large amount of helicity change than the citrate coated spherical GNPs but is less than the CTAB coated spherical GNPs at the similar concentration. Although the local concentration of the incoming proteins in TGNP and GNR will be higher in the edges, corners and facets because of lower in energy, [32-33] however the calculated

overall surface area for CTAB coated spherical GNPs is almost double than that of TGNP and GNR (Table S2). As a result, the number of proteins adsorbed on CTAB coated spherical GNPs is much higher than GNR and TGNP and therefore the total structural loss also will be higher. To get

more quantitative information of the anisotropic effect, the concentration of the CTAB coated spherical GNPs was reduced one order of magnitude to get an undistorted CD spectrum (only intensity changes) and to calculate % helicity change. Further all the GNPs were surface functionalized with PEDOT: PSS (it is hard to get citrate coated TGNP and GNR), which is very less toxic and less prone to any secondary structural change of the proteins mentioned here (Fig. S6). Interestingly, both the PEDOT: PSS surface functionalized spherical GNPs (earlier named as CTAB and citrate coated spherical GNPs) showed the minimum structural loss and the % helicity changes in this case was also close to the citrate coated spherical GNPs (Fig. 2d-f).

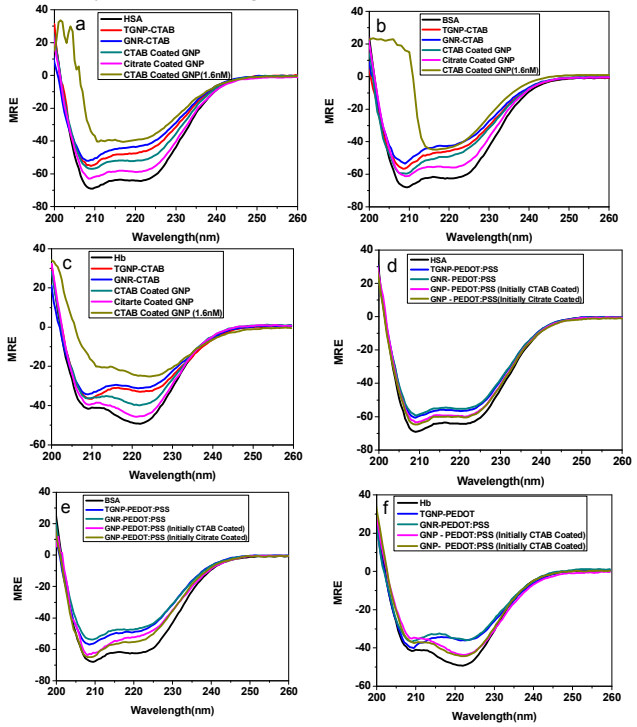

Fig. 2 (a) CD spectra of (a) HSA (b) BSA and (c) HB in presence of different as synthesized GNPs. CD spectra of (d) HSA (e) BSA and (f) HB in presence of PEDOT: PSS functionalized different GNPs.

On the other hand, for the anisotropic TGNP and GNR, when functionalized with PEDOT: PSS, although the change in helicity is reduced but was much higher than the PEDOT; PSS functionalized spherical GNPs (Table 1). This data exclusively suggest that anisotropy nature of the GNPs has significant effect on the secondary structure of the protein. The retention of the protein activity is of particular importance for any biological application involving nanoparticles. The evaluation of the protein activity was performed by using the well-established esterase activity test of HSA using para-nitrophenol reduction.

Table 1: The secondary structural changes in terms of % helicity change for HSA, BSA and HB proteins in presence of different GNPs (as synthesized and with PEDOT: PSS modified). The concentration for CTAB coated spherical GNPs was used one order of magnitude less than the other GNPs.

GNPs	As synthesized GNPs			PEDOT: PSS modified GNPs		
	HSA	BSA	HB	HSA	BSA	HB
TGNP	29	28	35.2	15.5	20.4	26.4
GNR	34	32.7	37.1	16.6	21.6	27
CTAB Coated spherical GNPs	22	24.4	20.4	7.6	12.5	11.4
CTAB Coated spherical GNPs	11.1	10.2	8	6.9	11.3	10.3

It is observed that HSA retains 86% of its original activity in the presence of citrate coated spherical GNPs, however, the activity is drastically reduced for the CTAB coated spherical GNPs (only 18 %). On the other hand, TGNP and GNR retain 61% and 56% of HSA activity, respectively (Fig. 3a). To get a quantitative idea, whether the surface coating agent and/or the anisotropy plays the role, the same activity check was performed with PEDOT: PSS modified GNPs. Interestingly, the CTAB coated spherical GNPs when modified with PEDOT: PSS, increases the protein activity to 80 % (close to the citrate coated GNPs,) whereas the anisotropic PEDOT: PSS modified GNPs (TGNP and GNR), the retention of activity increases a little (72 % and 65 % for TGNP and GNR respectively) (Fig. 3b).

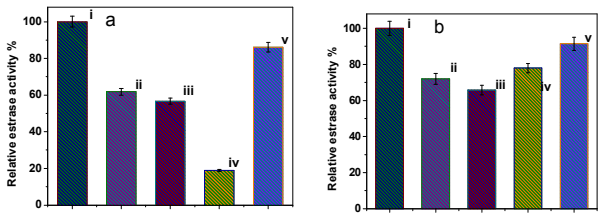

Fig. 3 Effect of Different GNPs on the activity of HSA (a) Relative esterase activity (%) of free HSA and HSA bound to CTAB coated GNPs (i- only HSA, HSA in presence of ii- TGNP, iii- GNR, iv- CTAB coated spherical GNPs, v- citrate coated spherical GNPs) and (b) Relative esterase activity (%) of free HSA and HSA bound to PEDOT:PSS modified GNPs (i- free HSA, HSA in presence of ii- TGNP, iii- GNR, iv- spherical GNPs (initially CTAB coated), v- spherical GNPs (initially citrate coated).

This data suggest that both the surface ligand (CTAB) and the anisotropy affect the protein activity profoundly. Finally, the viability assays, which is the overall dose-dependent toxicity of nanoparticles was performed by using an in vitro MTT assay on MDA-MB 231 cell line. The citrate coated GNPs showed no toxic effect on the MDA-MB 231 cell line. CTAB coated spherical GNPs showed the highest toxicity whereas minimum toxic effect was observed in the case of TGNP, among all the CTAB coated GNPs (Fig. 4a). On the other hand, the CTAB coated spherical GNPs, when modified with PEDOT: PSS, the cell viability increase drastically to 84 %. Interestingly, the anisotropic GNPs modified with PEDOT: PSS

showed a little increased in cell viability and is much less than the PEDOT: PSS modified or citrate coated spherical GNPs (Fig. 4b).

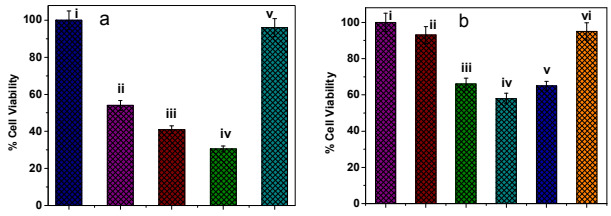

Fig. 4 (a) Cytotoxicity assay for different as synthesized GNPs (i-control, ii-TGNP, iii-GNR, iv-CTAB coated spherical GNPs, v-citrate coated spherical GNPs) on MDA-MB 231 cell lines measured by MTT assay after 24 hours of incubation. (b) Cytotoxicity assay for PEDOT: PSS functionalized GNPs (i-Control, ii-PEDOT:PSS, iii-TGNP, iv-GNR, v-spherical GNPs (initially CTAB coated), vi- spherical GNPs (initially citrate coated).

In Conclusion, we presented quantitative information on the structure and their activities of few major blood proteins when adsorbed onto different morphological anisotropic gold nanoparticles (GNPs). Human serum albumin (HSA), bovine serum albumin (BSA) and hemoglobin (HB) were chosen as model systems for the study. Four different types of closely similar size GNPs such as CTAB coated TGNP, GNR, spherical GNPs and citrate coated spherical GNPs were synthesized for a quantitative comparison of both the anisotropy and surface ligand effects. All the GNPs were further surface functionalized by PEDOT: PSS. Our data showed profound effect of both the particle anisotropy and the surface ligands on the secondary structural change which consequently affects the activity of the proteins. Further, a prominent effect of particle anisotropy on the cell viability assay was also observed.

Experimental: All four GNPs were synthesized using established methods (Supporting Information). The surface modification was done by using PEDOT:PSS. In the first step, extra CTAB was removed by carrying out centrifugation at 10000 rpm for 10 minutes followed by discarding the supernatant and the pellet was redispersed in double distilled water. This process was repeated one more time. To ensure the complete removal of CTAB from the above solution, 5 mL of the GNPs was equilibrated with 5 mL of chloroform. The solution was gently mixed and allowed to stand for 20 minutes. Finally, the chloroform layer was discarded. In the second step of the surface functionalization, 400 mL of the PEDOT:PSS solution (1.3 wt% dispersion in water) was added 5 to 10 mL of double distilled water. 1 mL of the resulting PEDOT:PSS solution was added to 2 mL of the GNP solution and allowed to stand for 10 minutes.

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TOC Graphical abstract

In-vitro cell cytotoxicity and conformational study of serum protein with anisotropic gold nanoparticles