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Impact of gold nanorod functionalization on biocorona formation and their biological implication

Sruthi Ann Alex, N. Chandrasekaran, Amitava Mukherjee *

Centre for Nanobiotechnology, VIT University, Vellore, India

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ABSTRACT

Gold nanorods (AuNRs) have distinctive opto-electronic properties, which facilitate their exploitation in various fields, especially biology and medicine. However, their interaction with the biomolecules in the physiological system can cause the formation of a protein corona, composing of a soft and hard layer, which ultimately alters the physiochemical properties, toxicity, and fate of the nanoparticles. Thus, the formation and composition of protein corona on three differently functionalized AuNRs [CTAB-AuNRs (positive), PEG-AuNRs (neutral), PSS-AuNRs (negative)] were studied for the three most abundant proteins in the blood serum namely, human serum albumin, immunoglobulin tG, and transferrin. The variation in protein adsorption, mean hydrodynamic size, and zeta potential were studied for different protein incubation periods (0, 0.5, 1, 4, 8, 12, and 24 h). The soft and hard corona layers were estimated for the individual proteins and mixture of the three proteins at the time point of maximum protein adsorption (1 h). Finally, in order to study the biological consequence of the coronation of AuNRs, the cell viability and uptake of AuNRs before and after protein corona formation were studied. The formation of IgG corona helped to reduce cellular toxicity, and at the same time, showed improved cell uptake when compared to the respective as-prepared AuNRs, highlighting their application potential for drug delivery applications.

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1. Introduction

Nanoparticle utilization for in vivo applications will inevitably result in their interaction with various biomolecules present in the surrounding physiological system before they reach the targeted area of interest. This interaction will result in the binding of a protein layer from the plasma known as the 'protein corona' layer [1]. The composition of this layer will determine the fate of the nanoparticles, and the nanoparticles, in turn, can also affect the protein function due to alterations in the protein structure. The morphology, type, and functionalization of nanoparticles will play a major role in determining the nature of proteins adsorbed [2,3]. Among the adsorbed layer, soft corona shows lower affinity and shorter residence time than the hard corona, which has stronger affinity and longer residence times. However, proteins with high concentration in the plasma will instantly occupy the nanoparticle surface and could later be replaced by proteins with lower concentrations and higher affinity [4,5]. Though the protein corona is composed of 3700 different proteins [6], nanoparticles get instantly covered by proteins of higher concentrations in the biological fluid; hence, we have studied the adsorption of the three most abundant proteins in the plasma, which are the most frequently studied [7,8], namely human serum albumin (HSA), immunoglobulin (IgG), and transferrin, and their effect on gold nanorods (AuNRs) of different functionalization.

Gold nanorods possess attractive physicochemical properties, which make them a promising tool for biological applications. They have two plasmonic peaks, one around 520 nm (transverse surface plasmon resonance) and the second >600 nm (longitudinal surface plasmon resonance), of which the latter can be tuned to the NIR region [9,10]. This characteristic will aid to augment photothermal therapy for cancer treatment as normal cells absorb NIR to a far lesser extent [11,12]. These features make them an attractive drug delivery vehicle also due to their enhanced uptake into cells [13,14]. They have immense potential for opto-electron microscopic imaging due to their photo-stability, resistance to photodegradation, facile preparation, and two-photon enhanced luminescence [15].

For the formation of a bio-nano interface, the most significant role is played by the protein corona, which will in turn finally define the NP destiny, thereby, necessitating the understanding of the formation and composition of protein corona and their thermodynamics, kinetics, toxicity, or uptake. Several studies have looked into the composition of corona layer formed on silver and gold nanoparticles when surrounded by serum proteins or individual proteins [8,16–18]. In general, though corona layer formation could reduce the application efficacy in biological system and be considered as an obstacle for in vivo applications, they can be used to alter the performance/efficiency or cellular uptake and toxicity. Casals et al. have compared citrate-capped AuNPs (size range:

* Corresponding author at: Centre for Nanobiotechnology, VIT University, Vellore 632014, India.
E-mail address: amitav@vit.ac.in (A. Mukherjee).

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4–40 nm) after modification with mercaptoundecanoic acid (MUA; negative) and aminoundecanethiol (positive) and observed that MUA-capped AuNPs were unable to form hard corona despite extended time periods [19]. Luby et al. have studied the protein coronation for EGF-capped 10-nm AuNPs under dynamic and lateral flow conditions [17]. In order to avoid the potential toxicity of the CTAB capping over AuNRs, PEGylation is done to improve biocompatibility; however, this involves a possible trade-off between the cellular uptake percentage and the longevity of the nanorods in the circulatory system. Protein coronation studies with citrate, cysteine, thioglycolic acid, and PEG (2 and 5 kDa) on AuNPs indicated that 5-kDa PEG-AuNPs were unable to form corona, and 2-kDa PEG-AuNPs could not form corona with fibrinogen or transferrin, but with BSA alone [20]. Similar works also suggested that the chain length of PEG can determine the extent of protein coronation (higher the PEG molecular weight, the protein corona formation will become lower) [18,21]. Dawson and co-workers were able to identify the spatial location of coronated proteins, their binding sites, and functional motifs with the help of antibody-labelled gold nanoparticles, differential centrifugal sedimentation, and several imaging techniques [22]. Fluorescent reporters have also been bound to nanoparticles to determine the accessible proteins present on the nanoparticle interface as these protein fragments determine their recognition by cellular receptors and triggering of biological pathways [23]. The time-dependent adsorption kinetics and individual protein coronation of HSA, IgG, and fibrinogen over citrate- and lipoic acid-coated 40-nm AuNPs and AgNPs suggested that fibrinogen coating causes irreversible aggregation, and thereby, showed reduced uptake by the cells [8]. A similar study was performed by Monteiro-Riviere et al., wherein the impact of protein coronation of HSA, IgG, and transferrin over citrate- and silica-coated AgNPs of different sizes on their uptake by HEK cells was studied [16]. However, the effect of protein corona on application efficacy, toxicity, and behavior of gold nanorods has not been studied till date despite their numerous biomedical applications.

In the current study, the protein corona formation of individual proteins (HSA, IgG, and transferrin) and their mixture on gold nanorods and their impact on cells were explored. To the best of our knowledge, this is the first-ever work that investigates the coronation of three biologically relevant proteins over gold nanorods of different functionalizations (CTAB, PEG, and PSS) and their impact on cellular uptake and viability. The adsorption of proteins was studied for various time points, and the mean hydrodynamic diameter and zeta potential analyses were done for the protein-adsorbed AuNRs. The effect of variation in protein corona percentage (for both individual and protein mixture) on the toxicity and cellular uptake of AuNRs with three different surface functionalization was analyzed to provide a deeper understanding on their effect when nanoparticles are used for biological applications.

2. Materials and methods

2.1. Chemicals

Hydrogen tetrachloroaurate hydrate ($HAuCl_4.2H_2O$) was procured from SRL Pvt. Ltd. (India), and silver nitrate ($AgNO_3$) was from Merck Specialties Pvt. Ltd. (India). Hydrochloric acid (HCl) and ascorbic acid were obtained from SD Fine Chemicals Ltd. (India). CTAB, sodium borohydride ($NaBH_4$), poly(ethylene glycol)methylether thiol (average Mw $\approx$ 2000), and poly(4-styrenesulfonic acid) solution (average Mw $\approx$ 75,000), and the three proteins, human serum albumin, immunoglobulin G (from human serum), and transferrin, were procured from Sigma-Aldrich (India). Analytical-grade chemicals have been used without further purification. Milli-Q water of 18.2 MΩ·cm from Cascada BioWater filtration unit (Pall Corporation, USA) was used for the work. All the glassware (Borosil or Corning) was immersed in a cleaning solution for a few hours. Then, the glassware was washed thoroughly with water, followed by detergent solution, and finally rinsed with distilled water and dried thoroughly. For the in vitro studies, Dulbecco's modified Eagle's Medium (DMEM), fetal bovine serum (FBS), antibiotic and antimycotic solution, 10× phosphate buffered saline, trypsin, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and dimethyl sulphoxide were purchased from Himedia laboratories, India.

2.2. Preparation of AuNRs with different functionalizations

CTAB-coated AuNRs were prepared using our previous protocol. In brief, the seed solution preparation (brown in color) involved the addition of ice-cold (0.3 mL, 0.01 M) $NaBH_4$ to a mixture of (2.5 mL, 0.5 mM) $HAuCl_4$ and (2.5 mL, 0.2 M) CTAB. For the growth solution, (2.8 mL, 0.079 M) ascorbic acid was added to a mixture containing (200 mL, 0.75 mM) $HAuCl_4$, (200 mL, 0.2 M) CTAB, and (12 mL, 0.004 M) $AgNO_3$. The 3-h incubated seed solution (0.48 mL) was added to the above solution and incubated for 24 h to give CTAB-capped AuNRs (violet in color). The excess CTAB was removed by centrifugation at 9000 rpm for 30 min twice, and the resultant solution can be stored for a minimum of three months at room temperature (28 °C) [24].

For the functionalization of AuNRs with PEG or PSS, the AuNRs were concentrated to $1/10^{th}$ of the original volume. Then, 1 mL of CTAB-AuNRs was stirred mildly with NaCl solution (8 mL, 1 mM), and 1 mL of thiolated PEG (0.2 mg/mL (100 μM); 30 min) or PSS (10 mg/mL (133 μM); 2 h), respectively, at room temperature. The varied concentrations and incubation times of PEG/PSS used was based on the availability of thiol groups on PEG, which aids the covalent binding of PEG to AuNRs. The unbound polymer was removed by centrifugation at 9000 rpm for 30 min, and the PEG- or PSS-functionalized AuNRs were resuspended in dd H_2O and can be stored at 4 °C [24,25]. The concentration of the three different AuNRs was estimated using inductively coupled plasma–optical emission spectroscopy (ICP-OES), and 10 mg/L of AuNRs was used for the experiments.

2.3. Estimation of soft/hard corona bound on AuNRs of different capping

The protein solutions of HSA, IgG, and transferrin were prepared in phosphate buffer (10 mM, pH 7.0). 200 μL AuNRs (10 mg/L) was added to 200 μL protein (with a fixed final concentrations of HSA (40 mg/mL), IgG (12 mg/mL), and/or transferrin (3 mg/mL)). The concentration of AuNRs was lower than the physiological concentrations of proteins studied in order to simulate the conditions similar to when AuNRs are utilized for bio-applications. After incubation for various time intervals, the solution was centrifuged twice at 7000 rpm, for 10 min, and resuspended with phosphate buffer. 50 μL of 2× Laemmli sample buffer was added to 200 μL of sample, incubated for 5 min at 90 °C, and then centrifuged down [26]. At each step, the supernatant was taken, and the protein concentration was measured using Bradford reagent by reading the absorbance at 595 nm. The measured protein concentrations were used for the estimation of soft and hard protein corona layers.

2.4. Cellular uptake of protein-bound nanorods

The hcT 116 cell line (colorectal carcinoma cell line; ATCC® CCL-247 ™) was obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in 25 cm^2 culture flask till they reached confluency in 5% carbon dioxide (CO_2) incubator at 37 °C. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% of antibiotic and antimycotic solution. The viable cells in the growth phase were trypsinized and counted using tryphan blue dye exclusion method with the help of a hemocytometer and seeded in 6-well plates at a cell concentration of 4×10^5 cells per well [27]. The cells were allowed to reach confluence, after which, CTAB-, PEG-, and PSS-capped AuNRs, each in presence and absence of different protein coronas (HSA, IgG, transferrin, and all three protein mixture) were added to the cells. After 24 h, the cells were washed with PBS buffer to remove any surface-bound particles and trypsinized. The cells from each well and the

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