

Variation of Protein Corona Composition of Gold Nanoparticles Following Plasmonic Heating

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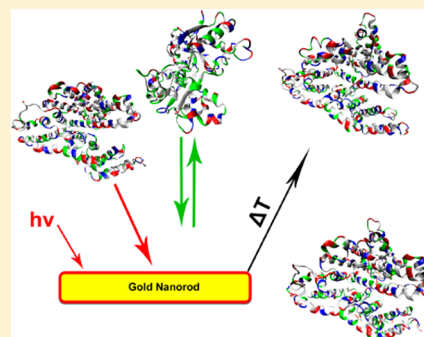
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S Supporting Information

ABSTRACT: It is well recognized that the primary interaction of most biological environments with nanoparticles (NPs) is strongly influenced by a long-lived (“hard”) protein corona that surrounds the NP and remains strongly adsorbed to its surface. The amount and composition of associated proteins in the corona adsorbed onto the NPs is related to several important factors, including the physicochemical properties of the NPs and the composition of the protein solution. Here, for the first time, it is shown that plasmonic heat induction (by laser activation) leads to significant changes in the composition of the hard protein corona adsorbed on low aspect ratio gold nanorods. Using mass spectrometry, several proteins in the corona were identified whose concentrations change most substantially as a result of photoinduced (plasmonic) heating versus simple thermal heating. Molecular modeling suggests that the origin of these changes in protein adsorption may be the result of protein conformational changes in response to much higher local temperatures that occur near the gold nanorods during photoinduced, plasmonic heating. These results may define new applications in vivo for NPs with hyperthermia capability and better define the likely interactions of cells with NPs after plasmonic heating. Potential changes in the protein corona following hyperthermia treatment may influence the final biological fate of plasmonic NPs in clinical applications and help elucidate safety considerations for hyperthermia applications.



KEYWORDS: Protein corona, nanoparticles, gold nanorods, hyperthermia, laser, photoinduced

Nanoparticles (NPs) are promising materials for a variety of new applications in many fields of science and technology, spanning physics, chemistry, coatings, electronics, information technology, and (most recently) biomedical and life sciences.^{1–7} Upon contact with biological environments, it is well-known that the surfaces of NPs are immediately covered by a corona of adsorbed proteins.⁸ Therefore, biological responses to NPs are strongly dependent to the composition and concentrations of associated proteins in this protein corona.^{8–10} Recent reports have confirmed that the physicochemical properties of NPs (size,¹¹ surface charge,¹¹ and surface smoothness/roughness¹²) have considerable effects on the composition of the protein corona. In addition to the NP properties, the concentration of proteins in the biological medium play a crucial role in the formation of the protein corona.¹³ For instance, increasing the concentration of plasma proteins (for example, from 10 to 55%) incubated with NPs caused a significant decrease in the relative amount of bound fibrinogen (one the most abundant proteins in plasma) at the

surface of 200 nm SiO₂ NPs due to increased competition from the other plasma proteins.¹³

A particularly promising biomedical application of some specific NPs (for example, gold and iron oxide NPs) is the hyperthermia treatment of tumors.^{14–17} Although there have been several studies on the composition of the protein corona at the surface of hyperthermic NPs,^{12,18} there is little available information regarding the effects of heating during hyperthermia on the protein corona composition. One recent example shows that even slight changes in the incubation temperature (in the physiological range of 35–39 °C) of NP–protein complexes can considerably change the composition of the protein corona and hence change the cellular uptake of NPs.¹⁹ It also is possible that hyperthermia may change the composition in the NP protein corona in a different way than thermal heating, because during plasmonic heating significantly

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higher temperatures will occur at the NP surface compared to the average solution temperature.^{20,21} Therefore, understanding the effect of local heat (for example, during laser activation of gold nanorods for hyperthermia) on the composition of the protein corona at the surface of gold NPs is critical to our understanding of biological fate and transport of hyperthermic NPs.

Among the many classes of functionalized NPs that have been used as biomedical therapeutic or diagnostic agents, functionalized gold nanorods (AuNRs) have become one of the most widely studied. In addition to being composed of a relatively bioinert core material, gold nanorods possess strong absorbance and light scattering properties that can be precisely controlled through the shape of the metal core.^{22,23} For instance, AuNRs with an aspect ratio of 3.5 or greater possess a strong longitudinal surface plasmon resonance in the near-IR region of the spectrum where light can easily penetrate biological tissue to depths of up to 1.0 cm.^{22,24} In addition, gold nanorods are potentially appealing hyperthermia agents, due to the variety of functionalization strategies that exist to control the surface chemistry of these AuNRs. Such surface functionalization can enhance both the biocompatibility and targeting opportunities of AuNRs both in vivo and in vitro.²² All of these properties make functionalized AuNRs appealing theranostic materials, combining in one structure the ability to simultaneously image biological targets with therapeutic properties.¹² Indeed, functionalized AuNRs have shown great promise in biomedical applications ranging from molecular (drug) delivery to hyperthermia cancer treatments.^{20,25,26}

In order to study the effect of plasmonic heating on the hard protein corona composition of AuNRs, we immersed cetyltrimethylammonium bromide (CTAB)-stabilized gold nanorods in fetal bovine serum (FBS) solutions and probed the protein corona composition at the surface of nanorods before and after plasmonic heating induced by continuous laser irradiation for various activation times. We studied the effect of plasmonic heating on AuNR–protein complexes formed at two different overall protein concentrations: 10 and 100% FBS. These fetal bovine serum concentrations were chosen because they simulate two different types of biological media: 10% FBS simulates an in vitro milieu and 100% FBS simulates an in vivo milieu. FBS solutions, which consist of a mixture of approximately 3700 proteins,⁸ are also suitable models of in vivo complexity. The AuNR–protein complexes were characterized by UV–vis absorbance spectroscopy, transmission electron microscopy, ζ -potential, and LC-MS/MS analysis, in order to determine changes in the protein corona composition after photoinduced heating.

The protein–AuNR complexes used in our study were prepared by incubating CTAB-AuNRs (twice purified and concentrated by centrifugation to a concentration of 3.2 nM) for 20 min in FBS solution. The AuNRs used in this study were prepared by a silver-assisted seeded growth procedure and had an aspect ratio of approximately 3.5 with a longitudinal plasmon band λ_{max} of 756 nm (Figure 1 and Figure S1, S2 of the Supporting Information).²² Following purification, AuNR–protein complexes were then prepared for heating studies by incubating the AuNRs in either 10 or 100% FBS solutions. After incubation in the FBS solutions, the AuNR longitudinal SPR λ_{max} blue shifted to 736 nm. This SPR blue shift indicated a change in the dielectric environment immediately surrounding the AuNRs,²⁷ consistent with protein corona formation. Our absorbance data indicated no significant AuNR aggregation

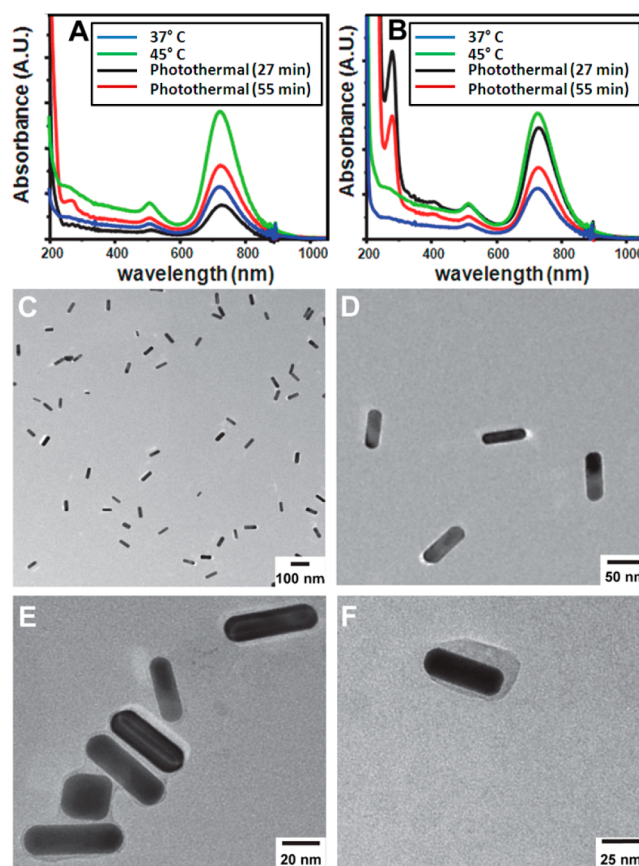


Figure 1. UV–vis absorption spectra and transmission electron micrographs of AuNR–protein complexes before and after laser irradiation or thermal treatment at 45 °C for 55 min. (A) UV–vis spectra for AuNRs after 10% FBS exposure, followed by hyperthermia treatment. (B) UV–vis spectra for AuNRs after 100% FBS exposure, followed by hyperthermia treatment. (C,D) TEM images of CTAB-AuNRs. (E) TEM image of a protein–AuNR complex (10% FBS). (F) TEM image of protein–AuNR complex (100% FBS).

during protein corona formation at either FBS concentration (AuNP aggregation has been reported for some previous studies of protein corona formation).^{18,28} Then 500 μL of the protein–AuNR complex solutions was then irradiated with a 48 mW laser ($\lambda = 785 \text{ nm}$) with a spot size of $\sim 1.5 \text{ mm}^2$ without stirring. Laser exposure continued for either 27.5 or 55 min, which should increase the temperature at the surface of the AuNRs up to $\sim 45 \text{ }^\circ\text{C}$ at least for short times.²⁵ A 55 min irradiation time was chosen because we have previously shown that under similar irradiation conditions, this period of time is sufficient to achieve an overall solution temperature of approximately 39 °C.²⁵ The average temperature of the irradiated solution was monitored during heating by placing a thermocouple in the solution at 27.5 min and again at 55 min (Supporting Information). For the photoinduced heated samples, the solution temperature after 27.5 min was approximately 36 °C, while after 55 min, the temperature was approximately 39 °C. This corresponds to an increase in the ambient temperature of nearly 7 °C during the course of these trials.

Following irradiation, the hard corona protein–AuNR complexes were separated from excess serum by centrifugation, and changes in the hard corona composition were examined by LC-MS/MS and ζ -potential analysis (Table 1; see Supporting

Table 1. ζ -Potential Analysis of AuNR-Protein Complexes before and after Laser Irradiation As a Function of Treatments and Protein Concentration

sample	ζ -potential (mV)
FBS control (no Au nanorods)	-24.5 ± 2.1
CTAB-AuNR control	$+23.7 \pm 3.2$
100% FBS-AuNR, 37 °C	-18.9 ± 1.4
100% FBS-AuNR, 45 °C	-13.5 ± 3.1
100% FBS-AuNR, 27 min laser	-12.7 ± 0.5
100% FBS-AuNR, 55 min laser	-12.8 ± 2.1
10% FBS-AuNR, 37 °C	-19.8 ± 1.6
10% FBS-AuNR, 45 °C	-16.9 ± 2.4
10% FBS-AuNR, 27 min laser	-17.1 ± 1.3
10% FBS-AuNR, 55 min laser	-16.3 ± 2.1

Information for preparation and procedures). Possible changes to the AuNR core following irradiation and heating were examined by UV–vis absorbance spectroscopy and transmission electron microscopy (TEM). The composition of the hard protein corona around the AuNRs was determined for four different treatment options: incubation at 37 °C, thermal heating at 45 °C, and photoinduced heating induced by continuous laser exposures (27.5 and 55 min, see Materials and Methods section of Supporting Information for details). It is important to note that although the solution temperature is uniform over the course of either thermal or photoinduced heating experiments, we expect the process of heating to be different in the two situations, because heating during plasmonic activation begins at the AuNR surface (heating from the “inside out”), while thermal heating occurs from the “outside in”. We found that CTAB-stabilized AuNRs that had not been exposed to FBS solution showed no significant changes in shape or surface chemistry over the course of the treatment time either during the purely thermal heating or photoinduced heating (see Supporting Information for details).

ζ -Potential analysis of the AuNR–protein complexes (Table 1) indicates a minor change in the overall AuNR–protein corona surface charge following either plasmonic heating or thermal incubation. The modest changes in ζ -potential may be consistent with some compositional change in the hard protein corona following heating. We found that the decrease in the overall surface charge was slightly greater for the 100% FBS treated AuNR than the 10% FBS sample (Table 1), regardless of the type of heating the AuNR–protein complexes experienced. Although again, we note that the overall change in the ζ -potential is relatively modest following heating and is not a statistically significant change for the 10% FBS samples. Furthermore, the length of the irradiation period (27.5 versus 55 min.) did not appear to have a significant effect on the overall charge of the protein–AuNP complexes. UV–vis spectra of the AuNR–protein complexes following heating revealed no significant changes in the shape or size of the AuNRs following irradiation (Figure 1, Supporting Information Table S6 and Figure S2). Interestingly, there is a peak in the ultraviolet region of the spectrum that appeared post-treatment for the laser-irradiated samples at ~ 260 nm (regardless of exposure time); this suggests that proteins with a higher concentration of aromatic residues (e.g., tryptophan, tyrosine, or phenylalanine) may be present in the corona of AuNR–protein complexes following photoinduced heating, perhaps due to changes in bound apolipoproteins.^{29–32}

To measure the protein adsorption profile of the hard protein corona at the surface of AuNRs following the thermal or photoinduced heating treatments, liquid chromatography mass spectrometry/mass spectrometry (LC MS/MS) techniques were employed. A semiquantitative assessment of the protein amounts present in the corona was conducted through application of a mass spectral counting (SpC) method¹³ (see Supporting Information for details). Normalized SpC (NSpC) values of the predominant corona proteins for different samples incubated with 10 and 100% protein solutions are presented in Figure 2 and Supporting Information Tables S1 and S2. Complete NSpC results for all detected proteins are presented in Supporting Information, Tables S3 and S4.

The LC-MS results indicate that the relative concentrations of several biologically significant proteins in the corona change as a result of the thermal and photoinduced treatments. Interestingly, the composition of the protein corona bound to

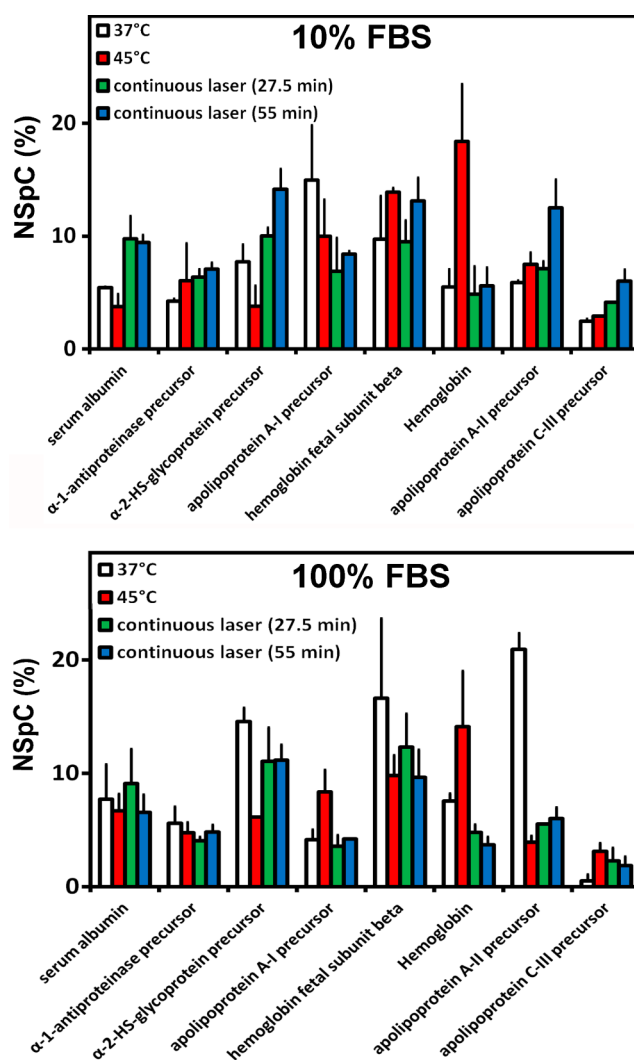


Figure 2. Graphs showing the variations of representative proteins (i.e., serum albumin α -1-antitrypsin precursor, α -2-HS-glycoprotein precursor, apolipoprotein A-I precursor, hemoglobin fetal subunit beta hemoglobin, apolipoprotein A-II precursor, and apolipoprotein C–III precursor) in the composition of protein corona around initially cationic gold nanorods after incubation with 10 and 100% FBS solutions after different treatments (i.e., incubation at 37 °C, 45 °C, and continuous laser irradiation) as determined by LC MS/MS.

the AuNR is different following photoinduced heating, compared either to thermal heating at 45 °C or to incubation under standard physiological temperatures (37 °C). As an example, note the changes in concentration of apolipoprotein A-II precursor in the AuNR sample incubated in 100% FBS. The apolipoprotein A-II precursor concentration was substantially decreased after plasmonic or thermal heating of the AuNRs (e.g., decreased by 68% following 55 min of irradiation). The final protein corona composition, as expected, also depends on the initial bulk FBS concentration (i.e., 10 vs 100%) during treatment. Indeed distinctly different changes in the protein corona composition occur in the 10% FBS AuNR sample, in which the content of apolipoprotein A II precursor increased slightly for the 10% FBS sample after heat treatments compared to the samples incubated at 37 °C with a substantial increase in the apolipoprotein A II precursor observed in the sample following photothermal irradiation for 55 min.

Several other biologically important proteins present in the hard corona also show significant changes in relative concentrations following laser-activated plasmonic heating. For the *in vitro* milieu model (i.e., 10% FBS solution), the amounts of several important proteins (e.g., serum albumin, α -2-HS-glycoprotein precursor, apolipoprotein A-II precursor, and apolipoprotein C-III precursor) in the protein corona were significantly increased, by continuous laser activation of AuNRs (versus incubation at 37 °C). In contrast, a few proteins (e.g., apolipoprotein A-I precursor) are decreased from the hard corona following irradiation. There are a few significant differences in the corona changes for an *in vivo* model (100% FBS) compared to the *in vitro* one; for example, the amount of α -1-antitrypsin precursor, hemoglobin fetal subunit beta, hemoglobin, and apolipoprotein A-II precursor in the corona composition after laser activation, were decreased.

We also compared the composition of the protein corona for both the 10% FBS and 100% FBS samples following heating as a function of molecular weight. According to the NSpC amounts in Figure 3 and Table S5 of Supporting Information, it appears that either plasmon-induced heating or purely thermal heating can decrease the relative amounts of low molecular weight proteins (<50 kDa) in the corona for an *in vivo* model (100% FBS). There are considerable differences in the proteins with molecular weight in range of <30 kDa, between 10 and 100% hard coronas. For instance, none of the heat treatments appear to significantly increase the amount of associated proteins (<30 kDa) in corona for the 10% FBS samples. In contrast, the amounts of these same proteins were significantly decreased in the protein corona in the 100% FBS samples following photoinduced heating but not thermal heating. Interestingly, in the 100% FBS samples no heat treatment appears to have a significant influence on the amount of proteins (>30 kDa) present in the protein corona. The variation in the observed protein corona at 10 versus 100% FBS concentrations must arise from both kinetic and thermodynamic factors: the high surface energy of NPs is lowered by the adsorption of proteins, and the essential irreversibility of adsorption by some proteins implies that the selection and exchange processes on short time scales will depend on the rates of diffusion of the multitude of proteins present in FBS that diffuse to the surface of AuNRs.¹⁵

Very recently, we showed that the degree of protein coverage on NP surfaces, as well as the protein corona composition, depend on the incubating temperature (for simple thermal heating) at which the protein corona is formed;¹⁹ in addition,

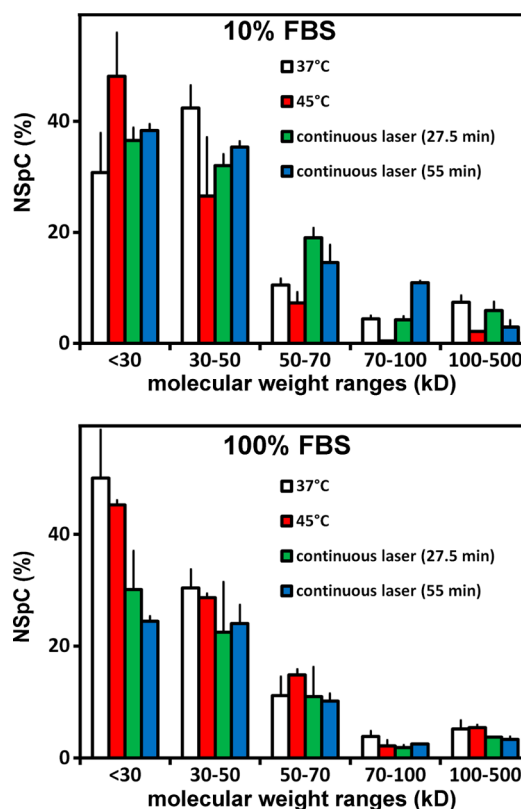


Figure 3. Normalized spectral counts (NSpC) of proteins of various molecular weights contained in the hard corona incubated in 10 and 100% FBS solutions after different treatments (incubation at 37 °C, 45 °C, and continuous laser irradiation) as determined by LC MS/MS.

we found that the cellular uptake of NPs is significantly affected by the temperature at which protein corona formation occurs.¹⁹ By extension, small variations in protein corona composition may have a significant difference on cellular uptake *in vitro*.¹⁹ Because the structures of small proteins are very sensitive to the incubation temperature, variations of only a few degrees in temperature may influence the protein folding (as we have previously shown both experimentally or computationally for amyloid beta proteins).³³ Contrary to conventional thermal heating of NP–protein complexes, however, plasmonic heating induces a substantial temperature gradient that starts at the AuNR surface and decays with distance. For instance, the temperature at the AuNR–solution interface has previously been shown to be as high as 45 °C during photoinduced activation (depending on [AuNR]), but decreases to ~36 °C over just a few millimeters.²¹

In order to model the gradient temperature decay from the surface of laser activated nanorods, we have employed an analytical simulation, as described in the Supporting Information. This simulation assumes a single laser-activated gold nanorod (40.0 nm × 7.0 nm) with a constant temperature at the AuNR–PBS interface of 45 °C. The PBS solution is initially assumed to have a constant temperature of 37 °C. Laser activation of the AuNR PBS for 10 ns results in a temperature gradient between 45 and 38 °C that extends up to 100 nm from the AuNR surface. After 540 ns, the same temperature gradient now extends up to 500 nm from the AuNR surface. The temperature gradient from the nanorod surface to the maximum affected PBS zone for these two cases has been illustrated in Figure 4 and Figure S4 of Supporting Information.

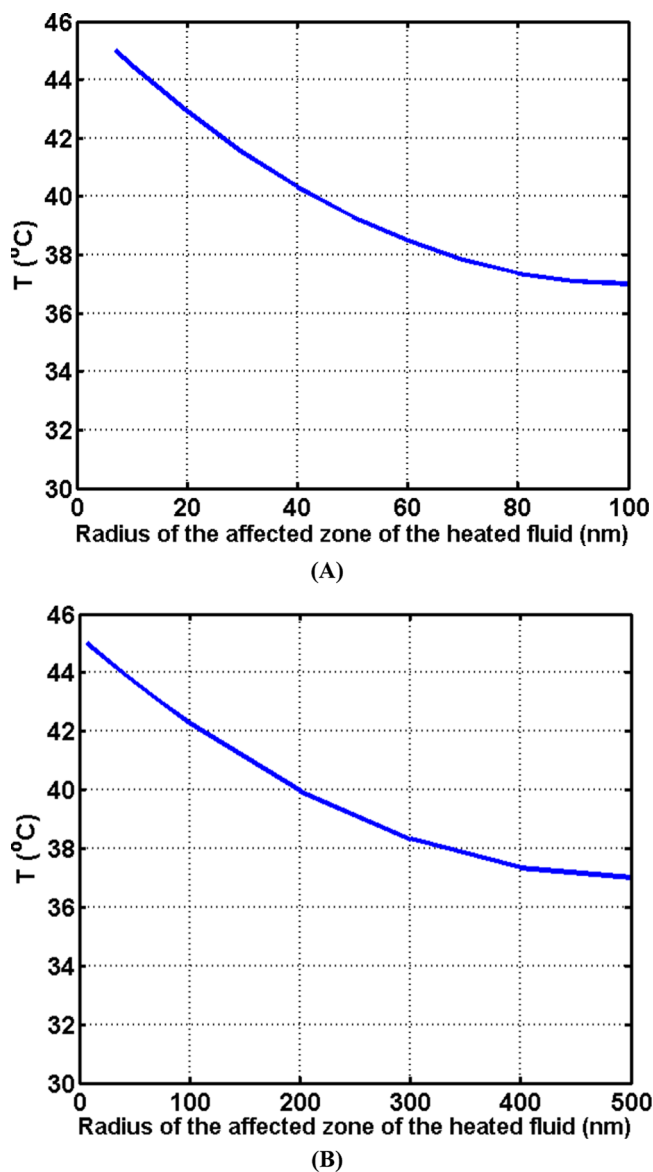


Figure 4. Temperature changes from the nanorod surface to the maximum radius of the heated fluid of (A) 100 nm in the case of 10 ns heating and (B) 500 nm for 540 ns heating.

These simulation results demonstrate that during photoinduced heating, unlike thermal heating, the local temperature at the surface of the laser-activated AuNRs can be substantially higher than the temperature of the remainder of the solution (>6 °C across the gradient). Previous studies have suggested that under similar experimental conditions, the global solution may rise by ~ 10 °C in the first 4 min of photoinduced heating, and the global solution temperature will be essentially homogeneous after ~ 20 min (which is roughly consistent with our own temperature measurements). Local heating zones with higher temperature near the AuNR will likely persist, however, over the full course of our photoinduced thermal activation period.^{20,21} Accordingly, even from very early heating times, the proteins adsorbed to the AuNR surface may experience very different solution temperatures than free proteins during photoinduced thermal treatments.

Because of the thermal gradient in solution that results from photoinduced heating of the AuNRs, any given protein (e.g., apolipoproteins) may have different conformations depending

on whether the proteins are adsorbed on the AuNR surface or are located a few hundred nanometers from the surface of laser activated nanorods. Such conformational differences as a function of position relative to the AuNR surface will change protein-particle adsorption-desorption rates that ultimately influence the composition of the hard corona; different proteins present in the protein corona may show very different conformational responses to photoinduced heating.

To demonstrate the effect of slight temperature variation on the protein conformations, molecular dynamic (MD) simulations were employed on apolipoprotein C III and apolipoprotein A I (see Supporting Information for detailed procedures used in the simulations). To quantitatively probe how much the structure of the apolipoprotein C III and apolipoprotein A I proteins are affected by the temperature, separate simulations were performed on both proteins at 5 different temperatures. The simulations for individual temperature/proteins (which were performed twice and the root-mean-square deviations (RMSD) and the radius of gyration (R_g) of the proteins by averaging over the simulations at each temperature) are shown in Figure 5.

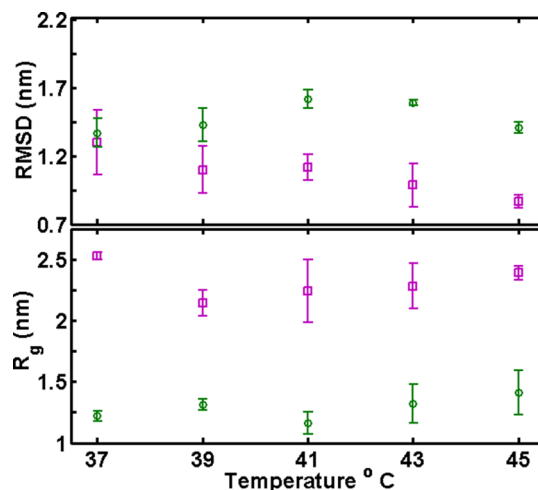


Figure 5. Results of the molecular dynamics simulation on apolipoprotein C-III (green circles) and apolipoprotein A-I (cyan squares). The RMSD and R_g and the corresponding error bars are calculated by performing two separate simulations at each temperature. The results show that the structures of these proteins are sensitive to the slight variation of temperature.

For both proteins, it can be seen that the RMSD and R_g are sensitive to the variations of the temperature. The differences in the RMSD and R_g values at different temperatures are in the order of few angstroms, which is sufficient to change the charge distribution of the protein and thus change their interaction with the NPs. Previously, it has been shown that some proteins (e.g., serum albumin) have a similar susceptibility to temperature variation, whereas other proteins (such as serotransferrin) showed no significant conformational variation over the same temperature range.¹⁹ Interestingly, our experimental data was in good agreement with the performed simulation results. The apolipoprotein C III, apolipoprotein A I, and serum albumin all showed large variations in the hard corona composition of the illuminated AuNRs compared to the conventional thermal heating control samples, while there was no difference in the relative serotransferrin content in the protein coronas of the illuminated AuNRs versus the thermally treated samples (see

Tables S3 and S4 of Supporting Information). It should be noted that the MD simulations indicate that certain protein conformations are susceptible to temperature changes; these conformational changes occur on a variety of time scales (from ps to ms). All the experimental characterization we have performed on the protein–AuNR complexes, however, require time delays of at least several minutes after photoinduced heating had been completed. As a result, fast local conformational changes must influence protein–protein or protein–AuNR interactions that lead to more permanent changes in the composition of the protein corona.

The changes observed in the protein corona composition may have implications for the biological fate and transport of AuNRs (or other hyperthermic NPs) following heating. One may speculate, for instance, that the observed increase of the apolipoproteins in the corona may promote prolonged circulation time in the bloodstream, based on prior literature of apolipoprotein effects on drug delivery.²⁷ One might also suspect that the changes plasmonic heating induced in the apolipoprotein A-II precursor concentration in the AuNR protein corona could make AuNRs less likely to cross specific biological barriers (e.g., blood brain barrier³¹) following hyperthermia treatment. As shown previously through MRI tests on a brain capillary endothelial cell in the presence of magnetic NPs with apolipoproteins in their corona composition,³¹ apolipoproteins are important in mediating NP transport through the blood brain barrier.

Previous studies of AuNRs in laser-activated hyperthermia and similar laser powers indicate that the overall solution temperature during AuNR hyperthermia is 37–45 °C (depending on AuNR concentration, time of heating, and initial temperature).²⁴ Interestingly, our results show that the type of heating the AuNRs undergo has a significant influence on the composition of the protein corona. As seen in Figures 2, 3, and Supporting Information Tables S1–S4, there are significant differences in the composition of the protein corona following photoinduced versus conventional thermal heating, under both in vitro and in vivo simulated conditions. The low molecular weight proteins (especially in the range of <30 kDa) appear to be present in the hard corona in higher proportions at 45 °C compared to the laser activated conditions. For proteins in the range of 30–70 kDa, the amounts of proteins present in the hard corona at 45 °C are lower but represent a higher proportion of the total protein profile compared to the laser activated conditions at 10 and 100% FBS, respectively; this may be related to the various corona thickness and composition at different protein concentrations, as shown before.¹³

In summary, we have examined the effect of laser-induced heating of gold nanorods on the composition of the hard protein corona associated with CTAB–AuNRs immersed in 10 and 100% solutions of FBS serum. Using experimental, analytical, and molecular dynamic simulation evaluations, we found that hyperthermia treatments had minimal effects on the overall surface charge of the protein corona associated with the gold nanorods following irradiation, but there were significant changes in the composition of the hard protein corona following both irradiation and thermal heating. More interestingly, plasmonic heating (i.e., photoinduced) and conventional thermal heating produced distinct changes on the composition of the AuNR protein corona. The compositional changes observed in the hard corona that are induced specifically by the laser irradiation may reflect relatively high localized temperature gradients right at the AuNR surface

during laser irradiation. The variation in the protein corona composition, following photoinduced local heating, may change the biological fate of NPs, and further understanding of the changing corona may have predictive value for NP therapy and for the study of nanotoxins.

■ ASSOCIATED CONTENT

📄 Supporting Information

Full experimental details, protein corona compositions, and characterization of gold nanorods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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