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Size-Dependent Protein-Nanoparticle Interactions in Citrate-Stabilized Gold Nanoparticles: The Emergence of the Protein Corona

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Abstract

Surface modifications of highly monodisperse citrate-stabilized gold nanoparticles (AuNPs) with sizes ranging from 3.5 to 150 nm after their exposure to cell culture media supplemented with fetal bovine serum were studied and characterized by the combined use of UV-vis spectroscopy, dynamic light scattering, and zeta potential measurements. In all the tested AuNPs, a dynamic process of protein adsorption was observed, evolving toward the formation of an irreversible hard protein coating known as the Protein Corona. Interestingly, the thickness and density of this protein coating were strongly dependent on the particle size, making it possible to identify different transition regimes as the size of the particles increased: (i) NP-protein complexes (or incomplete corona), (ii) the formation of a near-single dense protein corona layer, and (iii) the formation of a multilayer corona.

In addition, the different temporal patterns in the evolution of the protein coating come about more quickly for small particles than for the larger ones, further revealing the significant role that size plays in the kinetics of this process. Since the biological identity of the NPs is ultimately determined by the protein corona and different NP-biological interactions take place at different time scales, these results are relevant to biological and toxicological studies.
Introduction

Noble metal nanoparticles (NPs) are among the most widely used nanomaterials in biomedicine because of their outstanding optical, electromagnetic, and photothermal properties. These unique properties, together with the high reactivity of the NPs and their affinity for binding many different (bio)molecules, make them attractive candidates in a wide variety of uses and applications including delivery, diagnostics, and therapy.\textsuperscript{1,2} However, despite the rapid development of strategies for particle design and functionalization, relatively little is known about their \textit{in vitro} and \textit{in vivo} behavior in complex biological systems.\textsuperscript{3}

The ability to integrate NPs in biological systems ultimately relies on the fundamental understanding of the interaction of these inorganic NPs with biological fluids.\textsuperscript{4-6} A large number of biological fluids (plasma serum or otherwise) are complex aqueous media composed of electrolytes, proteins, lipids, and metabolites, able to adsorb (by electrostatic, hydrophobic, van der Waals, and dispersive forces) onto the surfaces of the NPs forming a protein dense coating known as Protein Corona (PC).\textsuperscript{7-11} A phenomena extensively studied previously in implants.\textsuperscript{12} The PC shields the original surface properties of the NPs acting as a “complex” surfactant, and alters their size and composition providing the NPs with a new biological identity.\textsuperscript{10,13,14} This corona is what is ultimately “seen” by cells, and critically determines the physiological response and interaction of the NPs with living systems, including cellular uptake, circulation lifetime, signaling, biodistribution, therapeutic effects, and toxicity.\textsuperscript{15-21}

The composition, structure and kinetics of the PC formation depends on (i) the specific characteristics of the biological environments in which NPs are dispersed, especially protein composition\textsuperscript{22} and concentration;\textsuperscript{16,23} (ii) the physicochemical features of the dispersed material, such as NP chemical composition\textsuperscript{24}, morphology,\textsuperscript{7,25,26} surface charge (functional groups),\textsuperscript{16,27} and hydrophobicity,\textsuperscript{7,28} and (iii) the exposure time, which directly correlates with the relative abundance of proteins and the different protein-NP binding constants (Vroman’s effect).\textsuperscript{24,27,29} Among these parameters, the morphology of the NPs relating to size and shape has been suggested to play an important role. Due to surface curvature effects,\textsuperscript{30} molecules tend to adopt different conformations on top of NPs as compared to flat surfaces indicating different protein-binding affinities, giving rise variation of the structure of PC according to the size of the NPs.\textsuperscript{31} Pioneering studies were performed by Cerdervall et al.\textsuperscript{7,32} who investigated the role of surface curvature in copolymer NPs of 70 and 200 nm after their dispersion in plasma, and further studies were later extended to other sizes and compositions including AuNPs,\textsuperscript{22,33-35} AgNPs,\textsuperscript{20} polystyrene NPs,\textsuperscript{36-38} SiO\textsubscript{2},\textsuperscript{14,15,25,36} metal oxide NPs,\textsuperscript{24,39,40} and polymer-coated FePt and CdSe/ZnS.\textsuperscript{41} Although all of the authors reported consistent results, it was often difficult to separate the effect of NP size from other physicochemical aspects, such as particle composition and surface chemistry. Some of them found that particle size and surface curvature influences the amount of bound protein and (to a lesser extent) the identity of these proteins.\textsuperscript{15,25,32}
contrast, other studies reported significant qualitative size-dependent changes in protein adsorption.\textsuperscript{40} So far, the underlying mechanism explaining size-dependent protein-particle specific interaction is not yet resolved. Moreover, in all these studies the diameter of the tested NPs was above that of most abundant serum proteins (6-12 nm). As a result, changes in the PC of NPs of a similar or even smaller size than proteins were not addressed. In this regard, only a few studies have provided data on this size regime, including AuNPs (2 nm,15 nm),\textsuperscript{16, 22} CdSe/ZnS (9-11 nm),\textsuperscript{42} and FePt (10 nm).\textsuperscript{41}

Little is known about the PC formation of NPs when they are smaller than most of the proteins themselves, with subsequent lack of knowledge of their biological consequences. Smaller NPs present some features that make them very attractive for biomedical applications: they are less prone to opsonization and have substantially longer lifetimes in the bloodstream, in such a way that a particle as small as 10 nm would not significantly activate the immune system.\textsuperscript{43, 44} They also have faster biodegradation (e.g., dissolution) together with excretion profiles which may avoid unwanted bioaccumulation.\textsuperscript{45} Moreover, the small size of the NP may affect the PC formation process. Thus, when NPs have sizes similar to (or even smaller than) those of proteins it may be difficult to form a compact corona since the number of proteins at the surface of the particle is too small for a crowding cooperative effect to be achieved.\textsuperscript{24, 46} Conversely, as the size of the particles becomes larger, it may be possible for a proper full density protein corona to develop.

Taking advantage of the recent improvements in the synthesis of sub-10 nm AuNPs,\textsuperscript{47, 48} we have studied the formation of PC on highly monodisperse citrate-stabilized AuNPs of different sizes (from 3.5 to 150 nm) after their exposure to cell culture media (CCM) supplemented with fetal bovine serum (FBS). By the combined use of UV-vis spectroscopy, dynamic light scattering, and zeta potential, a dynamic process of protein adsorption has been identified and characterized for all the tested particles, leading to the formation of an irreversible coating that mediates the colloidal stability of the AuNPs in the physiological medium. Interestingly, the thickness (and compactness) of this protein coating was strongly determined by the size of the NPs being possible to identify a transition regime from AuNP-complexes (or spare corona) in which the thickness of the corona was smaller than that expected for a single protein layer (AuNPs ≤ 12 nm), to the formation of a full corona composed by a dense protein monolayer for AuNPs sizes from 12 to 80 nm. Finally, for very large particles (> 80 nm), an additional regime could be identified in which a multilayered corona composed of 2-3 protein layers, similar to that reported in flat surfaces, seemed to be favored.\textsuperscript{49}

Results and Discussion

The protein adsorption process was studied by incubating citrate-stabilized AuNPs for 48 h at 37\textdegree C in complete cell culture media (cCCM); composed of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of fetal bovine serum (FBS). Highly monodisperse citrate-stabilized AuNPs with sizes ranging from 3.5 to 150 nm were synthesized following recently reported kinetically-
controlled seeded-growth approaches.\textsuperscript{47, 48} Citrate is a biocompatible reducer commonly used in the synthesis of AuNPs which provides a negative electrostatic stabilization once adsorbed on the NP surface. Besides, different from other surfactants that hinder the formation of the protein corona (PC), i.e., polyethylene glycol (PEG),\textsuperscript{26, 50, 51} the citrate layer is easily replaced, allowing the spontaneous adsorption of proteins under normal conditions of in vitro and in vivo exposure.\textsuperscript{50, 52} Moreover, FBS is a biological fluid that contains more than 3700 different proteins, with 12 orders of magnitude difference in their relative concentrations.\textsuperscript{7} Among all proteins in serum, albumin represents the most abundant fraction and accounts for $\sim$60\% in mass of the total proteins in solution (1 mM), with globulins the second most abundant ($\sim$40\%) and all the rest representing less than 1%.\textsuperscript{52} With this in mind, in Figure 1 the relative size of the particles used in this study is compared to proteins, using bovine serum albumin (BSA) and Immunoglobulin G (IgG) as model proteins.

For the study of the PC formation, it is important to previously select the proper exposure condition of the AuNPs in cCCM. cCCM is a high ionic strength medium in which the stability of citrate-coated AuNPs is strongly compromised due to the screening of the electrostatic repulsion between particles by the high concentration of charges in solution. This process is evidenced when performing exposure experiments in DMEM media (Fig. 2A), where the immediate aggregation of 10 nm AuNPs is clearly observed by UV-vis spectroscopy.\textsuperscript{53} Thus, in the absence of serum proteins, the localized surface plasmon resonance (LSPR) band of the particles rapidly vanishes, red-shifts and broadens, and a new band between 600 and 800 nm emerges, which can be univocally ascribed to the coupling of the plasmon modes of individual NPs when they come into contact.\textsuperscript{52, 54}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Representative TEM images of citrate-stabilized AuNPs of selected sizes used in this study. The relative difference in size between AuNPs and proteins has been compared by choosing BSA (65 kDa) and IgG (150 kDa), the two most abundant protein in serum, as model proteins. The inset spheres represent the size of the AuNPs scaled to the model proteins schematically drawn in the column on the left. The smallest AuNPs (3.6 nm) cannot accommodate more than 1-2 proteins while AuNPs larger than 30 nm can accommodate hundreds of them on their surfaces.}
\end{figure}
In the presence of proteins (fixed at the level of standard *in vitro* studies), particle concentration is the main driver for aggregation. Stability conditions of 10 nm AuNPs in cCCM were initially studied by exposing the same volume (1 mL) of particles of adjusted concentrations (from $10^{12}$ NP/mL to $10^{15}$ NP/mL) to an equal volume of cCCM (9 mL), thus keeping the concentration of proteins constant (Fig. 2B). The extent of aggregation was systematically quantified from UV-vis spectra, by calculating the aggregation parameter (AP) according to Lévy *et al.*\textsuperscript{54} after 1 h of exposure. AP values higher than 0.5 indicated significant aggregation. It is important to note that the LSPR peak and the calculation of this parameter were not affected by the presence of free protein in solution, since proteins absorb at wavelengths far lower than the characteristic AuNPs band. For the selected NP-protein exposure conditions, AuNPs were stable in cCCM at a low concentration of $2.5 \times 10^{12}$ NPs/mL while they formed aggregates when the concentration was increased above $2.5 \times 10^{13}$ NPs/mL. Since the concentration of proteins in the media does not represent a limiting factor (in all conditions the amount of proteins in solution is largely above that needed to fully cover all available NP surface), the aggregation is likely due to kinetic factors when mixing both solutions. That is, NPs destabilize and aggregate before proteins succeed in protecting their surfaces.

When the NP and the protein solutions are mixed, two different kinetic processes are competing at the same time: (i) the destabilization (and further aggregation) of the NPs promoted by the high ionic strength of the media in which they are dispersed and (ii) the stabilization of the NP surface against aggregation via protein adsorption. Thus, if the formation of a protective protein layer is faster than the characteristic time of destabilization (that is the time needed by two individual “bare” particles to bind to each other), then the resultant colloidal solution will remain stable. Conversely, the sample will destabilize and (partially or totally) agglomerate, forming aggregates immediately coated by proteins that will progressively sediment.\textsuperscript{55} Based on these results, it becomes clear that these two processes take place in the same time scale. For completeness, stability conditions were additionally evaluated for particles of different sizes (3.5 to 20 nm) and the results were normalized to: (1) the total number of NPs in solution (Fig. 2C), (2) the total surface area exposed by the NPs (Fig. 2D) and (3) the total gold mass in each sample (Fig. 2E), with it being possible to observe that curves hardly converge in any case. Only when the results were normalized to the total gold mass in each sample did they become comparable, which suggest that, in excess of free proteins, the aggregation of the particles is not exclusively determined by the probability of collisions of two individual particles but may be affected by additional parameters. Interestingly, small particles appear to be slightly more stable than large ones and thus are able to reach higher concentrations. This could be related to faster protein coating or a faster dispersion of the NP due to increased Brownian motion as size decreases. Consequently, stability conditions depend on both the characteristics of the AuNPs and the ratio between the NPs and protein concentration in the media.
Optimization of exposure conditions. (A) UV-vis spectra of AuNPs in different media. AuNPs aggregate in DMEM, even at low particle concentration, while they remain stable in cCCM. (B) Normalized UV-vis spectra of AuNPs in cCCM at different concentrations. The changes in the LSPR band indicate that AuNPs aggregate in cCCM when they were exposed to the medium at high concentration while their stability was not compromised at low concentration. (C-E) Experimentally measured aggregation parameter of AuNPs of different sizes after their exposure to cCCM at increasing NP concentrations. Results are normalized by particle number (NPs/mL) (C), total particle surface area (cm²/mL) (D) and total mass of Au atoms (µg/mL) (E). In all cases 1 mL of a solution of AuNPs 10x concentrated with respect to the desired concentration was added to 9 mL of the medium and rapidly mixed for a couple of minutes before being left undisturbed for 1 h under mild stirring.

Considering these results, we adjusted the concentration of AuNPs, from ~10^{13} NPs/mL for the smallest particles (3.5 nm) to ~10^9 NP/mL for the largest ones (150 nm), to be low enough to promote the formation of a protein layer before the characteristic time of destabilization. It is worth mentioning that these studies show how the exposure of NP to physiological media is concentration dependent and how dispersion of highly concentrated NP samples in these media (as i.v. injection or high NP dose toxicology studies) may be challenging.

Protein adsorption profile is a kinetic process in which a soft protein corona composed of loosely bound proteins in equilibrium with the free proteins in solution initially forms and evolves slowly toward an irreversible hard protein corona consisting of tightly bound proteins that do not readily desorb. In this process, the proteins adsorb/desorb and rearrange at the surface of the particle, leading to the hardening of the corona, a process that determines the final identity of the particle. In this stable (hard) conformation of the protein corona, crowding/cooperative effects between attached proteins are a key factor: once the protein layer is formed, the incorporation of a new protein implies
Figure 3. UV-vis characterization of purified AuNPs of selected sizes after their exposure to cCCM for different times: (A) 3.5 nm, (B) 5 nm, (C) 10 nm, (D) 20 nm, (E) 40 nm, (F) 100 nm. UV-Vis spectra before (dotted-line) and after their exposure at different times (blue t=30 min, green t=24 h, red t=48 h).

To obtain a PC hardening profile, the NP has to be purified from the exposure media to remove the unbound and loosely bound proteins, which is indeed a critical step. Purification of the AuNPs was performed by centrifugation followed by subsequent dispersion in protein-free aqueous solution after a proper optimization of the process. Additionally, the number of purification steps was determined to be at least 2. Otherwise the presence of unbounded free proteins was directly translated into bimodal size distributions in dynamic light scattering (DLS) measurements, in particular for the small nanoparticles (more details can be found in the SI).

The size-dependent evolution of the hardening of the protein corona was analyzed by UV-vis spectroscopy, DLS, and zeta potential measurements of purified samples. This combination of characterization techniques provides a remarkably robust analysis of the interaction between the proteins and the NPs in the colloidal state, bypassing complex postprocessing steps and/or avoiding chemical labeling strategies, such as luminescent or radioactive labelling, usually needed for protein detection. As observed in Table 1, important changes in the physicochemical properties of the purified AuNPs after exposure to cCCM are identified. The LSPR band of the AuNPs systematically red-shifts which can be ascribed to the change of the refractive index at the vicinity of the particles, and it is consistent with the spontaneous binding of media proteins. The shift is clearly observed within minutes and it takes 24-48 h to reach a stationary value, indicating a dynamic process of hardening of the protein corona, reaching a constant value faster for small particles than for the large
ones (Fig. 3). The extent of the red-shift also depends on particle size being \( \Delta \lambda \approx 9 \text{ nm} \) for the smallest AuNPs (3.5 nm) and \( \Delta \lambda \approx 6 \text{ nm} \) for the largest ones (150 nm). However, this cannot be directly correlated with the thickness and density of the protein layer since particles of different sizes present different sensitivity toward similar modifications of the surrounding environment (thickness, refractive index and chemical anchoring).

The absence of absorbance peaks at longer wavelengths is further evidence that under optimized conditions the AuNPs remain stable and well-dispersed in cCCM for all the sizes under study.

Table 1. Physicochemical Characterization of AuNPs before and after Exposure to cCCM for 48 h and Further Purified.

<table>
<thead>
<tr>
<th>Target Size (nm)</th>
<th>Citrate</th>
<th>TEM Diameter (nm)</th>
<th>DLS Diameter (nm)</th>
<th>( \zeta )-Potential (mV)</th>
<th>LSPR Maximum (nm)</th>
<th>\text{cCCM}</th>
<th>TEM Diameter (nm)</th>
<th>DLS Diameter (nm)</th>
<th>( \zeta )-Potential (mV)</th>
<th>LSPR Maximum (nm)</th>
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<tbody>
<tr>
<td>3.5</td>
<td>3.6 ± 0.4</td>
<td>5.5 ± 1.2</td>
<td>-36.8 ± 8.7</td>
<td>505.5</td>
<td>10.2 ± 3.9</td>
<td>-32.8 ± 5.1</td>
<td>515.0</td>
<td></td>
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<td></td>
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<tr>
<td>5</td>
<td>5.1 ± 0.8</td>
<td>6.6 ± 1.4</td>
<td>-37.2 ± 14.3</td>
<td>510.5</td>
<td>11.0 ± 3.6</td>
<td>-31.0 ± 7.8</td>
<td>519.5</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>6</td>
<td>6.2 ± 0.6</td>
<td>7.4 ± 1.6</td>
<td>-39.6 ± 11.1</td>
<td>513.0</td>
<td>13.3 ± 3.2</td>
<td>-26.1 ± 4.3</td>
<td>522.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>7.8 ± 0.8</td>
<td>9.1 ± 1.9</td>
<td>-42.6 ± 8.6</td>
<td>515.0</td>
<td>16.1 ± 4.3</td>
<td>-24.5 ± 6.2</td>
<td>523.5</td>
<td></td>
<td></td>
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<tr>
<td>9</td>
<td>9.3 ± 1.0</td>
<td>10.3 ± 2.2</td>
<td>-43.6 ± 19.8</td>
<td>516.0</td>
<td>22.6 ± 4.1</td>
<td>-22.5 ± 5.1</td>
<td>524.0</td>
<td></td>
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<tr>
<td>10</td>
<td>10.9 ± 1.0</td>
<td>10.9 ± 3.4</td>
<td>-45.8 ± 14.6</td>
<td>516.5</td>
<td>25.2 ± 5.3</td>
<td>-16.7 ± 5.3</td>
<td>525.5</td>
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<td></td>
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<tr>
<td>12</td>
<td>12.0 ± 1.1</td>
<td>12.9 ± 2.7</td>
<td>-42.9 ± 16.4</td>
<td>516.5</td>
<td>29.7 ± 6.9</td>
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<td>23.3 ± 4.8</td>
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<td>42.9 ± 9.3</td>
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<td>30</td>
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<td>32.0 ± 6.2</td>
<td>-48.3 ± 22.8</td>
<td>526.0</td>
<td>51.9 ± 11.9</td>
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<td>41.8 ± 8.2</td>
<td>-45.7 ± 23.4</td>
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<td>47.4 ± 11.8</td>
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<td>69.8 ± 15.2</td>
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<td>60</td>
<td>58.3 ± 4.6</td>
<td>55.8 ± 14.6</td>
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<td>536.5</td>
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<td>-50.2 ± 22.8</td>
<td>546.5</td>
<td>106.5 ± 29.1</td>
<td>-24.2 ± 6.6</td>
<td>553.0</td>
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<tr>
<td>100</td>
<td>110.6 ± 8.5</td>
<td>106.6 ± 34.1</td>
<td>-44.2 ± 14.5</td>
<td>556.5</td>
<td>137.6 ± 43.4</td>
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<td>561.3</td>
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<td>150</td>
<td>149.1 ± 11.9</td>
<td>139.3 ± 40.8</td>
<td>-44.1 ± 15.2</td>
<td>586.0</td>
<td>174.3 ± 57.5</td>
<td>-21.4 ± 7.4</td>
<td>597.5</td>
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DLS was used for an in-depth study of the PC and to determine variations in the hydrodynamic diameter (HD) of the samples. Obtained results are summarized in Table 1 and Figure 4, where it can be seen how the HD of the AuNPs systematically increases after their exposure to cCCM, which is associated with the direct adsorption of the proteins onto the surface of the NPs (Fig. 4-A, B). This increase strongly depends on the NP size and can be directly correlated with the thickness of the PC (thickness = 1/2 \( \Delta \text{HD} \) (Fig. 4-C, D). Thus, for small 3.5 nm AuNPs a protein layer of \(~2.5 \text{ nm}\) in thickness is observed \( \Delta \text{HD} \sim 5 \text{ nm} \). As the size of the particles progressively increases the protein coating becomes thicker, reaching a rather constant value of 8-10 nm \( \Delta \text{HD} \) from 16 to 20 nm) for AuNPs from 12 to 80 nm. Finally, for particles larger than 80 nm, the thickness of the corona seems to be even larger, obtaining an increase of 15-16 nm in thickness \( \Delta \text{HD} \) from 30 to 32 nm).

Considering the results obtained earlier and assuming that albumin (65 kDa, HD \sim 6-7 \text{ nm}) and other high molecular mass \((>65 \text{ kDa})\) proteins\(^{13}\) have been extensively found as the major constituents of the PC, it is reasonable to identify three different regimes: (i) small size regime, where the particles...
are smaller than mentioned proteins, and the thickness of the PC is thinner than that of a single layer of proteins, (ii) medium size regime, where the size of the particles starts to be significantly larger than of the proteins, and where the obtained thickness of the PC supports the overall formation of a single layer of proteins and (iii) large size regime, where the size of the particles is much larger than that of proteins, approaching a flat surface, and where the obtained PC thickness suggests the formation of a multilayer of proteins. It is worth noting that despite the PC not being completely developed in the small AuNP regime; it is robust and provides enough stability to prevent them from aggregation.

Figure 4. Hydrodynamic diameter (HD) measured by dynamic light scattering of purified AuNPs of different sizes after their exposure to cCCM for different times. (A) HD obtained by DLS for particles between 3.5 and 150 nm and (B) detailed for particles between 3.5 and 20 nm. (C, D) Respective increases in the HD showing doubling of the corona thickness. Straight lines indicate different tendencies in the resultant protein corona.

Similar evidence was obtained by zeta potential measurements used to assess the changes in particle surface charge after exposure to cCCM. Thus, concomitant with the observed red-shift of the LSPR position and the increase of the NP size measured by DLS, a drop in absolute surface charge of the particles toward the average value of the medium proteins was systematically observed. It accounts for the progressive screening of the negatively charged citrate-metal surface by the coating protein layer, which provides a long-term colloidal stability to the AuNPs in biological media. Interestingly, here again, zeta potential results depend on AuNP size (Fig. 5A), changing from -40 mV (citrate) to -30 mV for the smallest particles, and to -(15-20) mV for larger particles after protein coating. These results supports the previous idea that proteins are unable to form a dense layer on the surface of very
small AuNPs, while a proper protein corona is formed in the large ones, either by a single dense protein layer or a multilayered coating. Since the surface charge itself is not high enough to prevent NPs from electrostatic aggregation (an absolute value of 30 mV is usually considered a limit for particle stability), these results are further evidence that the stability of the NPs is mediated by steric repulsion provided by proteins (which stay in their native state) rather than via the former electrostatic repulsion provided by citrate.

As previously observed by UV-vis spectroscopy, time-dependent DLS and zeta potential results confirm the kinetic evolution of a hard protein corona and its dependence on particle size (Fig. 4 and Fig. 5), being faster (24 h) for smaller sizes than for larger ones (48 h). These results suggest that particle size plays a critical role in both the kinetic and the thickness of the protein layer: the smaller the particle the faster the kinetics evolution and the thinner (incomplete) the protein layer.

Figure 5. Zeta potential of purified AuNPs of different sizes after their exposure to cCCM for different times. (A) Absolute values of zeta potential measurements and (B) relative values in which 0 represents the surface charge of the citrate coated particles and 1 the charge of the proteins. The size-dependent protein corona evolution is clearly observed; where small particles present the most negative charge after exposure as a consequence of not forming a dense layer. For large particles, zeta potential is closer to that of the proteins indicating the formation of a denser layer.
Based on the obtained results, there seems to be a size limit below which a complete PC cannot fully
develop. Presumably, this can be related to geometric constraints or other effects (crowding,
cooperative effects) that could be a subject of further studies. As described by Nygren and
Alaeddine,\textsuperscript{57} and contrary to what might be expected, proteins do not distribute on surfaces randomly.
Instead, once the first proteins are attached they serve as nucleation centers for the deposition on new
ones, thereby stabilizing them, and this mechanism is repeated until the entire surface is filled. These
and other attempts to explain the irreversibility of this process seem to have in common that the initial
attachment of a protein to a surface is followed by a series of movements and/or rearrangements to
make the interaction more stable, ultimately resulting in an irreversible attachment. These processes
appear to be restricted when the particles become very small and their surface is limited to very few
proteins per NP (Fig. 6). On the other hand, the formation of a hard protein corona composed of more
than one layer of proteins is a plausible explanation for larger particles. In this case, when
approaching flat surfaces, proteins are subjected to larger conformational changes upon adsorption
leading to multiple protein adsorptions. Thus, the increase in the corona thickness of the larger
particles with respect to the smaller particles could be related to this effect along with the appearance
of defects on the first protein layer docking other proteins (Fig. 6). However, it remains unknown
whether particle size determines the nature of the PC only quantitatively (the number of proteins
attached) or also in a qualitatively way (the composition of proteins).\textsuperscript{15}

In order to see whether and how obtained results can be extrapolated to other systems we studied the
PC formation in the presence of pure albumin. Considering the high abundance of albumin in serum

Figure 6. Representative scheme of the formation process of a hard protein corona in citrate-stabilized
AuNPs of different sizes. (i) NP-protein complexes (or incomplete coating), (ii) the formation of a single dense
protein corona layer and (iii) the formation of a multilayer corona. Graphic on the left details the number of
proteins that can attach on the surface of the NP as a single monolayer. Calculations has been performed based
on the size of BSA chosen as model protein (see SI).
(represents ~60% of the total mass serum proteins), its high affinity for many different surfaces, and low specificity, albumin is known to be one of the main components of the protein corona when AuNPs, and other NPs, are exposed to serum or blood plasma.\textsuperscript{15, 25} Indeed, this high affinity of albumin for gold surfaces has been widely proven, resulting in the spontaneous formation of a dense and tightly bound albumin-rich coating in a process usually known as albuminization.\textsuperscript{41, 49, 51, 58, 59}

With the aim to elucidate whether the albuminization of the particles showed similar size dependent effects to those observed in the PC formation by serum proteins, analogous experiments were performed by exposing the particles to BSA in phosphate buffer solution. The obtained results are summarized in Table 2, confirming the formation of an irreversible BSA coating layer onto all the citrate-stabilized AuNPs after 48 h of exposure. BSA adsorption did not induce colloidal aggregation of the NPs, comparable to that given by FBS serum proteins in cCCM. This further indicates that the BSA preserves their native state and do not undergo denaturalization. The obtained results are in general agreement with those observed in the case of cCCM suggesting that BSA is unable to form a proper corona for the very small particles while a single layer or multilayer is formed on larger ones.

Slight differences observed between FBS and BSA may account for the presence of other proteins in combination with BSA. Briefly, the differences in the shift of the LSPR position (smaller in the case of BSA than FBS) along with the variations of the HD observed by DLS (smaller in the case of BSA than FBS) suggest that the presence of pure BSA may lead to the formation of a more structured, and consequently thinner, layer.

Table 2. Physicochemical Characterization of AuNPs before and after Exposure to BSA for 48 h and Further Purified.

<table>
<thead>
<tr>
<th>Target Size (nm)</th>
<th>Diameter BSA</th>
<th>ζ-Potential</th>
<th>LSPR maximum (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DLS (nm)</td>
<td>(mV)</td>
<td></td>
</tr>
<tr>
<td>3.5</td>
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<td>-30.7 ± 7.3</td>
<td>513.5</td>
</tr>
<tr>
<td>5</td>
<td>10.6 ± 1.7</td>
<td>-29.5 ± 6.8</td>
<td>518.0</td>
</tr>
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<td>6</td>
<td>12.6 ± 2.5</td>
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<td>520.0</td>
</tr>
<tr>
<td>7.5</td>
<td>14.9 ± 3.4</td>
<td>-23.5 ± 6.1</td>
<td>522.5</td>
</tr>
<tr>
<td>9</td>
<td>19.8 ± 4.4</td>
<td>-23.5 ± 4.7</td>
<td>522.5</td>
</tr>
<tr>
<td>10</td>
<td>22.2 ± 5.1</td>
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<td>523.0</td>
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<td>-20.8 ± 5.2</td>
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</tr>
<tr>
<td>15</td>
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<td>524.5</td>
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<tr>
<td>150</td>
<td>159.1 ± 53.2</td>
<td>-24.6 ± 7.6</td>
<td>594.0</td>
</tr>
</tbody>
</table>
Conclusions

The optimization of exposure conditions (stability and purification) allowed us the study of the protein corona in citrate-stabilized AuNPs of different sizes. The proper combination of UV-vis spectroscopy, DLS, and zeta potential measurements has been proven as a potential tool to describe qualitatively the protein corona formation on the surface of AuNPs. These techniques need to be discussed together in order to avoid misleading interpretations. For example, the largest red-shift in the LSPR, measured by UV-vis is observed for the smallest particles, while DLS measurements show that these small particles form the thinnest corona. On the other hand, UV-vis spectroscopy and zeta potential do not predict the formation of a multilayer protein coating while DLS allows characterization of this process. Finally, all three techniques are effective in monitoring kinetic protein adsorption profiles. As a result we observed how both the kinetics of protein corona hardening and the characteristics of the protein corona are particle size-dependent, despite the question of debate remaining whether particle size determines the PC not only quantitatively but also in a qualitatively way.

The obtained results present relevant biological consequences as different NP–biological interactions take place at different time scales. Thus, tightly associated proteins may stay adherent to the particle when the particle is endocytosed from the extracellular fluid to an intracellular location, whereas proteins with a fast exchange rate are replaced by intracellular proteins during or after such a transfer. As a result, the same NP can give different biological responses depending on the portal of entry, history and preincubation in serum among others, thereby illustrating the importance of the study of size-dependences of the protein corona in a biological environment.

Experimental

Chemicals. Sodium citrate tribasic dihydrate (≥99%) and gold (III) chloride trihydrate HAuCl₄·3H₂O (99.9% purity) were purchased from Sigma-Aldrich. Fetal Bovine Serum, FBS (research grade, sterile filtered) and Dulbecco’s Modified Eagle Medium, DMEM (with 1000 mg/mL glucose and sodium bicarbonate, whiteout L-glutamine, sodium pyruvate, and phenol red, liquid, sterile-filtered), were purchased from Sigma. All reagents were used as received without further purification and all glass material was sterilized and dehydrogenated in an oven prior to use. Milli-Q water was used in the preparation of all solutions.

Nanoparticle Synthesis. Aqueous solutions of citrate-stabilized AuNPs with different sizes (3.5-150 nm) were synthesized according to the previously developed seeded-growth method in our group. Detailed synthetic procedure and full characterization of the resultant solutions can be found in the respective articles. Briefly, AuNPs with increasing sizes were obtained from an initial AuNP solution after different sequential steps of growth; consisting of sample dilution plus further addition of gold precursor. This means that the number of particles in the solution decreases as the size
increases. Consequently, the concentration of smallest particles was ~10^{14}, which decreased down to ~10^{10} for the largest 150 nm particles. All the particles were used within 20 days after synthesis. The use of aged particles may result in increased experimental variability, since after a large period of time the stability of the particles may be compromised by the degradation of the surfactant.

**Exposure to Cell Culture Media.** Complete cell culture medium (cCCM) consisted of DMEM supplemented with 10% of FBS. AuNPs and cCMM were mixed (1:10 by volume) and placed in an incubator at 37°C for different incubation times. As a result, the final concentration of AuNPs was that of the synthesis divided by the dilution factor: ~10^{13} NP/mL for the smallest particles and ~10^{9} for the largest particles (approximately 0.05 mM of Au). At these working concentrations, AuNPs were stable throughout the experiments and the number of proteins in larger excess with respect to the available particle surface area. After incubation, the samples were centrifuged between 2000 and 35000 g for 10-20 min. (the larger the size, the lower the speed) to remove the excess of unbound or loosely bound proteins on the NP surfaces followed by resuspension in the particle original medium (sodium citrate 2.2 mM). Any possible aggregated fraction resulting from the centrifugation process was removed by filtering the samples with a 200 nm-pore-size cellulose acetate membrane. In the case in which the AuNPs solution needed to be concentrated with respect to the synthesis concentration, such as for stability tests, a regenerated cellulose centrifugal filter (Millipore 10KDa) was used.

**Physicochemical Characterization of the NPs and NP-Protein Corona.** AuNPs and the time evolution of their coating by proteins were characterized before and after exposure to cCCM using different techniques. The proper combination of these techniques has been used in other similar studies by our group and proven to be reliable when performed with adequate controls. **TEM:** Diameter of the synthetized particles were obtained from analysis of TEM images acquired with a JEOL 10101 electron microscope operating at an accelerating voltage of 80 kV. Samples were prepared by drop-casting 10 microliter of the sample on a carbon-coated copper TEM grid and left to dry at room temperature. At least 500 particles from different regions of the grid were counted. In order to avoid aggregation of the particles during TEM grid preparation they were previously conjugated with 11-mercaptopoundecanoic acid. **UV-Visible Spectroscopy:** UV-Visible spectra were acquired with a Shimadzu UV-2400 spectrophotometer. 1 mL of sample was placed in a plastic cuvette, and spectral analysis was performed in the 300 to 800 nm range at room temperature. AuNPs exhibit a characteristic absorbance peak in this region, the so-called localized surface plasmon resonance (LSPR) band. The LSPR position is sensitive to the surrounding of the NPs at the molecular level, and therefore the changes in the close environment of the NPs (such as the protein adsorption) can be investigated using this technique. Water was taken as the reference for all samples. **DLS and zeta potentia measurements:** The hydrodynamic size and surface charge of the AuNPs before and after incubation in cCCM were determined by Dynamic Light Scattering (number mean)
and Laser Doppler Anemometry respectively, using a Zetasizer Nano ZS instrument equipped with a light source wavelength of 532 nm and a fixed scattering angle of 173°. Aliquots (0.8 mL) of the colloidal NP solutions were placed into specific plastic cuvette and the software was arranged with the parameters of refractive index and adsorption coefficient of gold, and solvent viscosity of water at 25 °C. Each value was the average of at least 3 independent measurements.

Acknowledgments

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Supporting Information

Full characterization of the cell culture media by UV-vis spectroscopy, dynamic light scattering and zeta potential, verification of the excess of proteins compared to the surface area of the nanoparticles and detailed purification steps of the particles.

References


(24) Casals, E., Pfaffer, T., Duschl, A., Oostingh, G. J., and Puntes, V. F. (2011) Hardening of the Nanoparticle-Protein Corona in Metal (Au, Ag) and Oxide (Fe3O4, CoO, and CeO2) Nanoparticles. Small 7, 3479-3486.


Supporting information for:

Size-dependent Protein-Nanoparticle Interactions in Citrate-Stabilized Gold Nanoparticles: The Emergence of the Protein Corona.

Jordi Piella, Neus G. Bastús and Víctor Puntes

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1. Characterization of complete cell culture media (cCCM)

Figure S1. Characterization of complete cell culture media (cCCM): DMEM supplemented with 10% of FBS. UV-vis spectrum (left) shows a strong absorbance in the region below 500 nm, while almost no absorbance is observed above this value, where the Localized Surface Plasmon Resonance peak characteristic of the AuNPs is usually located. Dynamic light scattering plot (DLS) (right) of the cCCM displaying an intense peak in the region between 5 and 15 nm attributed to the proteins. This peak may overlap with the AuNPs peak if they have not been previously purified. Finally, the zeta potential of the cCCM results in -11.2 ± 0.9 mV which is characteristic of the slightly negative charge of most of the proteins.

2. Excess of proteins in cCCM compared to the total surface area of AuNPs

For a specific colloidal solution of AuNPs, the average NP diameter ($\bar{D}$), average NP surface area ($\bar{S}$) and average NP mass ($\bar{M}$) can be calculated from the size distribution obtained by TEM analysis:

\[
\bar{D} \left( \frac{cm^2}{NP} \right) = \frac{\sum D_n}{n} \tag{1}
\]

\[
\bar{S} \left( \frac{cm^2}{NP} \right) = \frac{\pi \sum D_n^2}{n} \tag{2}
\]

\[
\bar{M} \left( \frac{mol s}{NP} \right) = \bar{V}_{NP}\delta M_W = \frac{4\sum (\frac{D_n}{2})^3}{3n} \delta M_W \tag{3}
\]

where $n$ and $D_n$ are the number and diameter of the particles that has been counted by TEM (at least 500 NPs are needed for a representative population), $\delta$ is the density of gold, 19.3 g/mL,
and $M_w$ the molecular weight, MW 197. Notice that a common error is to calculate $\bar{S}$ and $\bar{M}$ from $D$ (the polydispersity degree of the particles is erroneously omitted in this case). Finally, the concentration of gold ($C_{Au}$), obtained either from the concentration of precursor used in the synthesis of the particles and the yield of the reaction or by ICP-MS, can be translated to the number of NPs and the surface area in solution as follow:

$$\text{number of NPs} \left( \frac{NP}{L} \right) = \text{concentration of Au (M)} \cdot \frac{1}{M} \left( \frac{\text{mol}}{NP} \right)^{-1} \quad (4)$$

$$\text{surface area} \left( \frac{cm^2}{L} \right) = \text{number of NP} \left( \frac{NP}{L} \right) \cdot \bar{S} \left( \frac{cm^2}{NP} \right) \quad (5)$$

The total surface area in a standard synthesis procedure range from 2 to 30 cm$^2$/mL, which corresponds to $\sim 10^9$ NPs/mL for the larger particles (150 nm) and to $\sim 10^{13}$ for the smallest ones (3.5 nm).

On the other hand, the total number of proteins in cCCM is at least three orders of magnitude larger than that of the particles in solution. The total surface area they can cover upon adsorption on a surface is the result of multiplying their cross section (CS) for their concentration (C):

$$\text{area}_{\text{proteins}} \left( \frac{cm^2}{L} \right) = \sum (C_{\text{protein}} \cdot CS_{\text{protein}}) \quad (6)$$

For example, the triangular CS of the BSA in the folded conformation has been previously estimated to be 32 nm$^2 = 32 \cdot 10^{-14}$ cm$^2$ while their concentration in the cCMM is around $6 \cdot 10^{16}$ (0.1 mM). This represents enough BSA in the cCMM to cover a total area of 19300 (cm$^2$/mL); at least more than 5 $10^2$ times higher than that needed to fully surround the AuNPs surface in solution. Thus, it is reasonable to consider the number of proteins in the cCCM largely in excess with respect to the AuNPs, even if more than one layer of proteins is adsorbed on the surface of the particles, and the fraction depleted from the medium upon adsorption insignificant.
3. Number of washing steps for a proper DLS characterization

cCCM free of NPs gives a background peak in the range of 5-15 nm by DLS (see section 1), which is comparable to the hydrodynamic size of the small AuNPs used in the experiments here presented. Consequently, interferences from the unbounded proteins in solution when measuring the hydrodynamic size of the AuNPs by DLS is something that should not be ruled out if the particles has not been previously washed and dispersed in a solution free of proteins.

The interferences that free proteins in solution may induce to the DLS measurements\(^1\) can be estimated from their respective abilities to scatter light assuming Rayleigh scattering properties, in which scattering intensity of a specific molecule is proportional to its number in solution \((N)\) and its size raised to the six power \((D^6)\):

\[
I \propto \varepsilon \, N \, D^6
\]  

(7)

where \(\varepsilon\) is the refractive index for each molecule. The relative contributions of the unbounded proteins with respect to the AuNPs can be then quantified by defining a dimensionless parameter \((\gamma)\) as follow:

\[
\gamma = \frac{I_{NP}}{I_p} = \frac{\varepsilon_{NP} N_{NP} D_{NP}^6}{\sum \varepsilon_p N_p D_p^6} = \frac{0.47 N_{NP} D_{NP}^6}{\sum \varepsilon_p N_p D_p^6}
\]

(8)

where the refractive index for AuNP is \(\varepsilon_{NP} = 0.47\), while the \(\varepsilon_p\) is \(\sim 1.5\) for proteins. Previous studies with BSA have demonstrated that \(\gamma\) should be at least \(> 0.1\) to avoid the contributions of free proteins in the DLS measurements.\(^1\)

In this regard, rough calculations based on the approximate BSA concentration in cCCM (6·10\(^{16}\) protein/mL, HD 7 nm and \(\varepsilon_p = 1.447\)), demonstrated that \(\gamma \ll 0.1\) for AuNPs of 3.6 nm in the range of concentration here working (10\(^{13}\) NP/mL) and at least 2 cycles of washing were needed to avoid the contribution of unbound proteins (note that each washing step consist on replacing 980 uL of supernatant from a 1000 uL of solution decreasing the concentration of proteins in
solution by a factor of 50x). Indeed, based on the previous approximation the concentration of these particles should be in the range of $1 \cdot 10^{18}$ to be properly measured in cCCM without requiring any washing step. On the other hand, $\gamma$ already approach to that of 0.1 for AuNP of 60 nm at any concentration larger than $4 \cdot 10^{10}$, which is in the range of working concentration in our experiments. In agreement with that, DLS plot showed monomodal curves even in the absence of any washing steps for large AuNPs (Figure S2-S3).

![Figure S2. DLS plot of AuNPs measured in cCCM without purification of the particles.](image)

Particles of 60 nm and 150 nm show a monomodal curve, while particle of 30 nm, and below, present a bimodal curve due to interferences from unbound proteins in the medium.

![Figure S3. Hydrodynamic diameter measured by DLS.](image)

For 15 nm particles the measured diameter decreases after incubation in cCCM as a result of contributions from unbound proteins. For 60 nm particles unbound proteins in cCCM does not significantly contribute to the measured values.
4. Maximum number of proteins per NP considering a monolayer coating

The theoretical available number of binding sites (NBS) for a monolayer coverage of proteins (considering BSA as a model protein) on a NP with a diameter ($D$) is estimated by dividing the surface area of the NP ($\pi D^2$) by the triangular cross section of the folded BSA (32 nm$^2$):\(^2\)

\[
\frac{\frac{\mu_{\text{BSA}}}{N_\nu} - \frac{c_{S\text{BSA}}}{\text{deg}^2}}{\frac{22}{\text{mol}^2}} NBS = \frac{\pi D^2}{c_{S\text{BSA}}} = \frac{\pi D^2}{32} \tag{9}
\]

References
