Understanding the Kinetics of Protein–Nanoparticle Corona Formation

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ABSTRACT: When a pristine nanoparticle (NP) encounters a biological fluid, biomolecules spontaneously form adsorption layers around the NP, called “protein corona”. The corona composition depends on the time-dependent environmental conditions and determines the NP’s fate within living organisms. Understanding how the corona evolves is fundamental in nanotoxicology as well as medical applications. However, the process of corona formation is challenging due to the large number of molecules involved and to the large span of relevant time scales ranging from 100 μs, hard to probe in experiments, to hours, out of reach of all-atoms simulations. Here we combine experiments, simulations, and theory to study (i) the corona kinetics (over $10^{-3}$–$10^3$ s) and (ii) its final composition for silica NPs in a model plasma made of three blood proteins (human serum albumin, transferrin, and fibrinogen). When computer simulations are calibrated by experimental protein–NP binding affinities measured in single-protein solutions, the theoretical model correctly reproduces competitive protein replacement as proven by independent experiments. When we change the order of administration of the three proteins, we observe a memory effect in the final corona composition that we can explain within our model. Our combined experimental and computational approach is a step toward the development of systematic prediction and control of protein–NP corona composition based on a hierarchy of equilibrium protein binding constants.

KEYWORDS: protein–nanoparticle interactions, competitive protein adsorption, protein corona, FCS, DCS, SDS-PAGE, microscale thermophoresis, molecular simulation

The interaction of NPs with biological media is key in the transport of NPs across the cell membrane. When NPs are exposed to fluids that contain proteins and other biomolecules, part of those biomolecules are immediately adsorbed forming the so-called “protein corona”. This corona is believed to depend on the different biological environments crossed by the NP as well as on the current surroundings. Therefore, layers of adsorbed proteins that are formed while the NPs move from a biological milieu to another evolve as the concentration of protein and the media composition change.

Nowadays it is generally accepted that part of the proteins in the corona can remain for a relevant time on the NP surface (hard corona), possibly preventing the adsorption of other molecules. Other proteins, instead, dynamically exchange with those in solution (soft corona). Nevertheless, our knowledge about the dynamic exchange of the corona in response to changes in the composition of the milieu is still very limited. Due to the relevant role that the evolution of the corona plays in the way that NPs interact with biological systems, e.g., in their targeting of specific cellular receptors, it is crucial for any possible biological application to understand how the processes of protein adsorption and exchange occur in the corona. Hence, there is a need for an accurate modeling of the kinetics of the protein corona in order to decipher the biological identity of the NPs.
Coarse-grained (CG) molecular simulations have proven to be a powerful tool for the study of NPs interacting with biological systems at the macromolecular scale.\textsuperscript{10,11} Recently Vilaseca et al. developed a computational approach to simulate the adsorption of proteins on flat surfaces.\textsuperscript{12} These theoretical results suggest that the corona composition, after exposure to a multicomponent system, undergoes a relaxation scenario. In particular, fast-diffusing but weakly adsorbing proteins reach the surface first, but are replaced by strong-binding proteins next.

The mechanism of formation of the corona of the NP can be separated into two main stages: (i) the bare NP enters the biological environment and comes in contact with biomolecules that first adsorb forming the initial corona; and (ii) the corona composition starts to evolve due to competition between proteins. An atomistic description of such a complex mechanism of formation and evolution of the protein corona is at the moment computationally unfeasible and, more importantly, avoidable. Here we show that by combining experiments, computer simulations, and theory, we are able to develop an approach that allows us to predict the corona composition in a variety of cases. We present results for a three-component model plasma, and we describe how the approach can be extended in a systematic way to more complex protein solutions.

We consider a solution composed of different combinations of the following representative blood plasma proteins: human serum albumin (HSA), transferrin (Tf), and human fibrinogen (Fib). These proteins are very numerous in plasma and are present in the corona of silica (SiO$_2$) NPs.\textsuperscript{13} HSA is the most abundant protein in plasma, representing almost 55\% of its composition.\textsuperscript{14} It is a globular protein, with a small mass of 67 kDa, that regulates the osmotic pressure of plasma. Tf has a concentration in plasma of 3 mg/mL, with a mass of about 80 kDa. This protein transports iron through the body and maintains the iron homeostasis. Fib is a rod-like protein with a large mass of 340 kDa and has a key role in coagulation. Its concentration in plasma varies from 1.7 to 4.5 mg/mL.

We measure the affinity of each protein for the silica NPs using two independent approaches, namely differential centrifugal sedimentation (DCS) and microscale thermophoresis (MST). While the DCS characterization\textsuperscript{15} is performed by extracting the NPs from the solution, with MST we probe the interactions of NPs with proteins directly in solution. Using a combination of these two experimental techniques, we obtain reliable binding constants for each of the individual proteins interacting with silica NPs. Next, we use the measured NP affinities—and other known parameters—of the proteins to define a CG model that includes protein–NP and protein–protein interactions, up to three-body contributions. Yet the model is simple enough to allow us to perform molecular dynamics (MD) simulations for NPs in binary and ternary protein solutions up to 10 s. This time scale is out of reach of atomistic simulations and approaches the lower limit of the experimental time resolution. However, the relevant time scale for the experiments and the biological applications is as long as hours. Therefore, we develop an original theoretical approach, based on the non-Langmuir differential rate equation (NLDRE), to extrapolate the adsorption kinetics over hours. We test our theoretical predictions about the adsorption kinetics by using fluorescence correlation spectroscopy (FCS). With this technique, we label one protein species at a time and follow its binding on NPs precoated with other proteins. This allows us to monitor the adsorption kinetics and the displacement from a precoated corona by competitive binding. Finally, we verify our theoretical predictions about the relative composition of the hard corona using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

**RESULTS AND DISCUSSION**

**Binding Affinities Measurements in Single-Protein Solutions.** We prepare solutions of HSA, Tf, and Fib with silica NPs and measure by DCS the change in the sedimentation time with respect to conditions with no proteins (eq 3 and Figure 1a). By MST, we measure the normalized relative fluorescence $F_{norm}$ of fluorescently labeled NPs in a monocomponent protein solution under a thermal gradient (eq 4 and Figure 1b). We fit both quantities (Figure 1) with the law of mass action, eq S1 in Supporting Information (SI), in the limit of low NP concentration, whose solution $\Gamma_{eq}$ is the normalized surface coverage, as a function of the protein concentration $C_p$:

$$\Gamma_{eq}(C_p) = \frac{C_p}{K_D + C_p}$$

where $K_D$ for each of the three proteins separately (Table 1) is the only fitting parameter of the experimental data and marks the concentration at which $\Gamma_{eq} = 0.5$. The estimates from the two techniques agree in order of magnitude, with $K_{D_{HSA}} \ll K_{D_{Tf}} < K_{D_{Tf}}$. Hence we establish a hierarchy of the tendency to absorb on the NP surface, that for Fib is much larger than for Tf and for Tf larger than for HSA.
Table 1. Binding Affinity $K_D \equiv K^{off}/K^{on}$ Determined With DCS (center column) and MST (right column) for the Three Proteins Used in This Work$^{19}$

| protein | $K_D$ [μM] (DCS) | $K_D$ [μM] (MST) |
|---------|-----------------|-----------------|
| HSA     | 2.8 ± 0.2       | 2.4 ± 0.6       |
| Tf      | 0.65 ± 0.08     | 1.8 ± 0.4       |
| Fib     | $(11 \pm 2) \times 10^{-5}$ | $(2.2 \pm 0.9) \times 10^{-5}$ |

“The values for HSA coincide within the error bars and are consistent with previous literature.”$^{18,19}$ Those for Tf and Fib agree only in order of magnitude, however the MST measurements are possibly biased by agglomerates of NPs$^{27}$ and are based on less data and with larger noise (Figure 1) than the DCS measures. $K_D$ is expressed in μM = 10$^{-6}$ M.

The CG Model. Our goal is to unveil the molecular mechanisms that regulate the corona formation, and simulations are potentially helpful to this aim. However, a direct comparison with experiments is unfeasible with all-atoms simulations. Hence, we resort to a model where we coarse-grain mechanisms that regulate the corona formation, and in this way, we step up the size and duration of our MD simulations, approaching the experimental scale.

In our CG approach, we introduce effective potentials for protein-NP and protein-protein (two-body and three-body interactions) with implicit solvent. For the protein-NP effective interaction, we adopt a description within the framework of the well-established DLVO theory for colloidal dispersions (Figure 2a and eq S8 in SI).$^{18,19}$

For the two-body protein-protein effective interaction, we follow Vilaseca et al.$^{12}$ and consider an interaction potential that encodes two different conformations for each protein (eq S9 in SI and Figure 2b). This model, adopted to describe the competition among proteins near a surface, compares well with the experimental data without preassumptions about the adsorption mechanisms or the adsorption rates.

At high concentrations of protein, we introduce a three-body correction to the protein-protein interactions. We find that this term is essential to get simulation results consistent with the experimental data. This effective three-body interaction (eq S10 in SI) is due to correlations between pairs of proteins near the surface of the NP and could arise from conformational changes of surface-adsorbed proteins.

Table 2. Parameters for the CG Model of Proteins$^{4}$

|       | HSA | Tf  | Fib |
|-------|-----|-----|-----|
| $R_A$ [nm] | 2.7$^{38}$ | 3.72$^{16}$ | 8.5$^{19}$ |
| $R_B$ [nm] | 3.6$^{30}$ | 3.75$^{2}$ | 11.0$^{50,11}$ |
| $\epsilon$ [k_BT] | 2$^{12}$ | 2$^{12}$ | 2$^{12}$ |
| $M$ [kDa] | 67 | 80 | 340 |
| $\phi$ [mV] | $-15^{20}$ | $-10^{53,74}$ | $-20^{31}$ |
| $A_H$ [k_BT] | 9.75 | 8.4 | 7 |
| $N_{max}$ | 550 | 450 | 120 |

“$R_A$ and $R_B$ are the two characteristic length-scales of a protein in each conformation: $R_A$ is obtained from the maximum surface concentration of each protein, and $R_B$ is the hydrodynamic radius. $\epsilon$ is the repulsion energy between two adsorbed proteins at the shorter diameter distance in eq S9 in SI. $M$ is the mass of the protein (as specified by Sigma-Aldrich). $\phi$ is the zeta-potential in PBS. $A_H$ is the Hamaker constant, calibrated as explained in the text, for the DLVO interaction potential with silica NP in eq S8 in SI. $N_{max}$ is the maximum number of adsorbed molecules forming a full monolayer on the NP, as computed by simulations. We indicate the adopted units near the parameters and the used references, if applicable, near their values.

The model’s parameters (Tables 2 and S1 in SI) are all known but the DLVO’s Hamaker constant $A_H$ in eq S8 in SI. We estimate $A_H$ (Figure S3 in SI) based on our knowledge of the binding affinities $K^{Fib}_D$, $K^{Tf}_D$, and $K^{HSA}_D$ given by the eq 1 derived from the fit of the experimental data.

Competitive Protein Adsorption in Two-Component Protein Solutions. In order to test the competitive adsorption between different kinds of proteins, we consider solutions containing two among the three proteins, HSA, Tf, and Fib. To allow a better comparison between simulations and experiments, we follow a sequential protocol in which we introduce one kind of protein at a time into the initial NP suspension.

First, we perform simulations of silica NPs suspensions, at a concentration of 100 μg/mL, with HSA at different concentrations, chosen within the range of accessible experimental values. After equilibrating the precoating, we add to the solution Fib at 5 μg/mL concentration and study the adsorption kinetics of Fib (Figure 3).

Because $K^{Fib}_D \ll K^{HSA}_D$, we expect that Fib would displace the adsorbed HSA proteins. However, we find a strong dependence of the Fib adsorption kinetics on the concentration of HSA.

Figure 2. Schematic representation of the CG model. (a) The protein-NP interaction potential (continuous line, eq S8 in SI) as a function of the distance between the surface of the silica NP (sketched as a portion of a large sphere on the left) and the center of the protein (red line for HSA, blue for Tf, and green for Fib). For sake of clarity, in the panel we sketch only the HSA as an ellipsoid (on the right). (b) The protein-protein interaction potential (continuous line, eq S9 in SI) as a function of the distance between the centers of two HSA proteins. The dashed horizontal line marks the characteristic interaction energy $\epsilon$. Inset: sketch of two possible HSA conformations defining the characteristic distances $R_A$ and $R_B$ (both marked by dashed vertical lines in the main panel). (c) Snapshot of the simulation box showing the NP (golden sphere in the center) suspended in the protein solution (small spheres). The highlighted red zone corresponds to the buffer region, which we use to maintain the protein concentration constant as in the experimental setup.
The simulations clearly show that the rate of adsorption of Fib decreases for increasing concentration of HSA in solution. In particular, after 10 s of simulated time, we find that the Fib adsorbed on the NP decreases from \( \simeq 90\% \) to \( \simeq 35\% \) when the HSA concentration changes from 0 to 10 mg/mL, respectively.

To better understand this effect with the numerical approach, simulations much longer than those achievable within a reasonable time would be necessary. However, we develop an analytic theory that allows us to extrapolate our numerical results to physiologically relevant time scales (\( \sim 1 \) h). Our NLDRE theory is based on the law of mass action and differs from the standard Langmuir theory of adsorption because we do not assume constant adsorption rates. The essential parameters of our theory are the binding affinities and the adsorption/desorption rates of the molecules, that we assume to be dependent on each protein surface coverage and the concentration of NPs and proteins. As explained in SI, these parameters can be fitted from adsorption data at an early stage.

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We use the NLDRE theory to extrapolate the long-time behavior of the system (Figure 3) and predict that at the highest HSA concentration (10 mg/mL), it would take more than 5 min for Fib to displace HSA and to have more than 50%
of Fib adsorbed, despite the much higher tendency of Fib to adsorb on the NP surface. Our theory predicts that after 30 min, the Fib adsorption kinetics on NPs precoated with HSA at 10 mg/mL concentration is still relatively slower compared to pristine NPs (Figure 3) and that the saturation level is reached within the time frame of 100 min (Figure 3b).

In order to test our theoretical predictions, we perform experiments following the same protocol as in the simulations, i.e., adding Fib at 5 μg/mL concentration to silica NPs (at 100 μg/mL) precoated with HSA at different concentrations. We study the adsorption kinetics by estimating the Fib fraction bound with FCS. We verify that the Fib adsorption kinetics change due to the presence of competing proteins on the corona with an overall excellent agreement with our theoretical predictions (Figure 3a).

We repeat the experiment with different HSA incubation times, remove the adsorbed proteins from the NP surface, separate them using SDS-PAGE technique (Figure S4 in SI), and finally estimate the relative mass of each protein on the NP surface by densitometry (Figure 3b). We find that the experimental data follow our theory with very good agreement, confirming the predictive capability of the theory for binary solutions.

Furthermore, we test that the theory can be applied to other binary solutions. In particular, we repeat simulations, theoretical calculations, and experiments using Tf instead of HSA during the precoating step and then adding Fib in solution. Again, we find that our theory for binary solutions, based on short-time simulations, allows us to make predictions about the adsorption kinetics in excellent agreement with the experiments (Figure S5 in SI).

Competitive Protein Adsorption in Three-Component Protein Solutions. To verify if our approach can be extended in a systematic way to more complex protein solutions, we apply the same procedure to a ternary suspension with HSA, Tf, and Fib. In this case, we follow a three steps exposure protocol: (i) we first incubate NPs in HSA, (ii) we add Tf in solution expecting competition with HSA for the NP surface, and (iii) we finally add Fib that will compete with both Tf and HSA for the corona (Figure 4a).

We repeat the experiment with different HSA incubation times, remove the adsorbed proteins from the NP surface, separate them using SDS-PAGE technique (Figure S4 in SI), and finally estimate the relative mass of each protein on the NP surface by densitometry (Figure 3b). We find that the experimental data follow our theory with very good agreement, confirming the predictive capability of the theory for binary solutions.

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As in the case of the two-component solution, we test the theoretical prediction by FCS experiments. In particular, we measure the Fib fraction bound after incubating the NPs for 1 h with HSA and, later, for another hour with Tf, both at 0.07 mg/mL concentration (Figure 4a). The comparison of the experimental data with the theoretical prediction is, also for this ternary solution, very good (Figure 4b).

We find a similar good agreement between the experiments (Figure 4c) and the theoretical predictions when we consider the case of NPs sequentially precoated with HSA and Tf at higher concentrations (3.5 mg/mL each). In this case the theory predicts a large slowing down of the protein corona kinetics with respect to the case at lower HSA and Tf concentration, with a saturation of Fib only after ≈50 min (Figure 4c). Experiments confirm this prediction.

We observe that the competition with HSA and Tf makes the Fib adsorption slightly slower than the competition with solely HSA at a comparable total mass concentration. For example, for HSA and Tf at 0.07 mg/mL, the time needed for reaching 50% of Fib fraction bound is t ≈ 0.2 min (Figure 4b), while for HSA at 0.18 mg/mL, it is t ≈ 0.1 min (Figure 3). However, the time difference between the two cases is negligible when we compare the Fib adsorption kinetics for HSA and Tf at 3.5 mg/mL (Figure 4c) and for HSA at 7.0 mg/mL (Figure 3). This could be interpreted as a consequence of the fact that the binding affinities of HSA and Tf are comparable and both much higher than that of Fib, hence the Fib kinetics is regulated only by the total mass concentration of the competing proteins.

Furthermore, we find, both in simulations and experiments, that within the error bar, there is no difference in Fib kinetics if we incubate first with HSA and then add Tf or vice versa (Figure S7 in SI). However, our experiments show that the kinetics before the addition of Fib displays a memory effect if we change the incubation order, as we discuss in the next section.

**Memory Effects in Competitive Protein Adsorption of HSA and Tf.** After a preliminary screening showing differences in the kinetics when we invert the incubation sequence of HSA and Tf, we investigate experimentally the memory effect considering two different incubation protocols. In protocol A, we first incubate the silica NP for 1 h with HSA at 3.5 mg/mL, then we add Tf at the same concentration (3.5 mg/mL) for another hour. In protocol B, we invert the order of incubation, first Tf and next HSA, with the same concentrations and times. After each protocol, we wash the NP to remove unbound proteins, and next we remove the attached proteins and electrophoretically separate them inside a gel matrix (SDS-PAGE gel analysis, Figure 5a). Finally, after visualization, we quantify the relative abundance of each protein.

We find that the final amount of each protein depends on the protocol. Specifically, the first incubated protein is always more abundant in the corona at the end of the process (Figure 5b). We repeat the experiments for HSA and Tf at smaller concentration, 0.07 mg/mL (Figure S8 in SI), and find the same qualitative result, suggesting that the memory effect does not depend strongly on the initial protein concentrations. On the other hand, by adding Fib after each incubation protocol, we do not find any strong effect on the Fib adsorption (Figure S7 in SI), i.e., the memory effect occurs in our samples before the addition of Fib.

Based on these experimental evidence, we focus on investigating the possible mechanism causing the memory effect for the competitive adsorption between HSA and Tf. In particular, we compare the experiments with the results from our computational model. We observe that for the model defined by eqs S8 and S9 in SI, the two different incubation protocols lead to the same corona after a transient time (Figure S9 in SI). Therefore, the memory effect implies the existence of other interactions among proteins and NP beside those described by eqs S8 and S9 in SI. Hence we hypothesize that the protein adsorption on the NP induces a change in the protein–protein interaction. This change can be interpreted as a consequence of a protein conformational variation upon adsorption. Specifically, we assume that the change can be modeled by a three-body interaction between the proteins and the NP (eq S10 in SI).

We find that our hypothesis is sufficient to simulate the memory effect (Figure 5c,d). Hence the memory effect can be interpreted as a consequence of how the adsorption on the NP changes the interaction of the first-incubated protein with those adsorbed at a later time, e.g., due to conformational variations, hampering the replacement of the first by the latter proteins.

**CONCLUSIONS**

We study by experiments, simulations and theory the kinetics of the protein corona forming on 100 nm silica NPs suspended in a ternary solution made of HSA, Tf, and Fib. With the goal of developing a predictive computational model based on a limited knowledge of the protein–NP interactions, we first evaluate the NP binding affinities of each of these three proteins in monocomponent solutions by DCS and MST. We use the estimates of $K_D^HSA$, $K_D^Tf$, $K_D^HSA$, as parameters in a CG model for the protein–NP interactions and perform $\approx10$ s numerical simulations of the model for the competitive protein adsorption on silica NP in binary solutions. To extrapolate the Fib kinetics at physiologically relevant time scales (~1 h) and compare with FCS and SDS-PAGE techniques, we develop a NLDRE theory predicting a strong slowing down of Fib adsorption on HSA-precoated NPs compared to pristine NPs. While pristine NPs are covered with more than 50% of the Fib in solution within seconds, it takes more than 5 min when the NPs has been incubated with HSA at 10 mg/mL concentration. All our results show that the Fib kinetics slows down when the NPs are incubated with a higher HSA concentration. Therefore, the kinetic slowdown would become even more relevant for HSA concentrations as high as in human plasma (from 35 to 50 mg/mL). The analysis of the Fib adsorption kinetics on NPs after Tf incubation shows a similar slowdown and a similar good agreement between our theoretical predictions and the experiments. We expect a stronger kinetic effect when the competing proteins have similar affinities. For example, our preliminary results for Fibrinogen competing with Fibronectin show a kinetic effect lasting for tens of hours, a time-scale relevant for the evolution of the protein corona in NP uptake scenarios.

To test further the predictive power of our computational model, we perform $\approx10$ s numerical simulations of a three step exposure protocol, first incubating the NPs with HSA, then with Tf, and finally adding Fib. Next we extrapolate the results up to hours with the NLDRE theory. For this ternary solution, we predict a Fib adsorption kinetics that slows down with the total mass concentration of the two competing proteins in a fashion comparable to the case of the binary solutions. We understand this similarity as a consequence of the fact that both HSA and Tf have a binding affinity orders of magnitude higher than $K_D^HSA$. In this sense the relevant parameter determining the Fib adsorption slowdown is the total mass concentration of the protein.
competing proteins and not their relative amount. Also in this case, we test the theoretical predictions by comparing with FCS and SDS-PAGE techniques, and we find a very good agreement for the Fib kinetics, independent of the incubation order.

Nevertheless, a detailed experimental analysis of the kinetics before the addition of Fib shows a memory effect when we invert the order of precoating between HSA and Tf. The protein incubated first is always more abundant in the final corona. We realize that for reproducing this experimental feature, it is sufficient to add to our computational model a three-body interaction among the proteins and the NP. This additional term mimics the effect that the protein adsorption on the NP has on the protein–protein interaction. We interpret this mechanism as a consequence of possible irreversible degeneration of proteins at the NP surface.

In conclusion, by combining simulations and theory with limited experimental information on single-protein solutions, we are able to predict the protein corona composition in a ternary solution. We find evidence of memory in the corona formation when the environment changes, and we propose a mechanism that can account for this effect. Our results show that it is possible to develop an approach toward the prediction of the protein corona kinetics and composition in complex milieus that are changing with time. This is particularly relevant in those cases, e.g., in which a NP is traveling through the body. This knowledge is key for understanding how to modulate the protein corona. As a matter of fact, tuning the protein corona could be exploited to design specific NP properties. It can help to better engineer drug delivery carriers or a generation of biocomplexes for nanotheranostics. It may allow the development of patient-optimized NPs, making use of the fact that the protein corona will change when the NP is incubated in blood plasma extracted from patients with different diseases.

MATERIALS AND METHODS

Experimental Approach. Silica NPs (nominal diameter 100 nm) were purchased from Polysciences Inc. (cat no. 24041). FITC-labeled silica NPs were purchased from Kisker Biotech (cat no. PSi-G0.1). NPs were characterized by DLS to determine their size (Figure S10 in SI) and z-potential before use. Proteins (Fib, Tf, BSA) labeled with Alexa488 were purchased from Invitrogen (Life Technology) and treated as recommended from the supplier. Unlabeled Fib, holo-Tf, and HSA were purchased from Sigma-Aldrich.

Differential Centrifugation Sedimentation. DCS measures the sedimentation time of objects. It is then possible to calculate their diameter by assuming a value of the density of these objects (eq 2).15 When proteins or other molecules bind to the surface of NPs, they not only change their overall size but also the net density of the object. This causes a change in the sedimentation properties of the NP. It is convenient, from an experimental perspective, to assume a density of the core material and observe the change in apparent diameter as a function of protein concentration. The term apparent diameter is used as the size reported does not reflect the true size of the NP–protein complex, but it actually reflects the combination of changes in both the size and density which occur after the formation of the protein corona.

The diameter is computed as

\[ D = \left[ \frac{18\pi \ln(r_t/r_0)}{(\rho_{NP} - \rho_f) \eta \omega t_s} \right]^{1/2} \]  

where \( D \) is the particle diameter (cm), \( \eta \) is the fluid viscosity (poise), \( r_0 \) and \( r_t \) are the initial and the final radius of rotation (cm), \( \rho_{NP} \) is the particle density (g/mL), \( \rho_f \) is the fluid density (g/mL), \( \omega \) is the rotational velocity (rad/s), and \( t_s \) is the time required to sediment from \( r_0 \) to \( r_t \) (s).

Prior to DCS analysis, silica NPs were incubated in different concentrations of single protein solutions for 1 h at room temperature. After this, the solution was injected neat into the spinning DCS disk. The particles then sediment through a sucrose gradient at 30 °C ± 4 °C. Due to the different sedimentation rates of the free protein and the NPs, it is assumed that, upon injection, the NPs would be immediately separated from the surrounding protein as they pass through the disk. This gives the opportunity for proteins to desorb as the particles are no longer in equilibrium with their surroundings. The typical measurement time for these particles was on the order of 1–2 min.

After data acquisition, the changes in sedimentation can be rationalized by considering the object as more complex and modeling the sedimentation using a core–shell model (eq 3). This assumes that the object formed of a core of known size and density and a shell of protein of known density and variable thickness, with apparent diameter:

\[ D_f^2 = \frac{D^2 \rho_f + (D_f - D) \rho_c - D \rho_f}{D_f (\rho_c - \rho_f)} \]  

where \( D_s \) is the apparent diameter of the NPs, \( D_s \) and \( \rho_c \) are the NP core diameter and density, respectively, \( D_f \) is the total diameter of the core and the shell, and \( \rho_c \) and \( \rho_f \) are the shell and fluid densities, respectively. This equation describes a NP with a shell composed of species with a single density.15

In all cases the particles are assumed to be spherical and the layers homogeneous and discrete. For most cases, a progressive increase in shell thickness causes a nonlinear change in the sedimentation properties. However, we observe (Figure S1d in SI) that the reported shifts can be rationalized using eq 3: For this system and suitably small shifts (<12 nm), a linear approximation between the surface coverage of the NP and the protein concentration is acceptable. Hence, the apparent diameter, in this case, can be used to directly reflect the shell thickness of proteins. Assuming that the maximum saturation point of the curves is indicative of full surface coverage, the shell thickness can be normalized to represent a surface coverage percentage.

DCS is not biased by agglomerates, as it measures the time each particle takes to sediment. The time separation between the large agglomerates, that sediment first, and the individual particles, that sediment later, guarantees that even in the case of agglomeration, the single particle population can be monitored. In particular, by monitoring the main population peak in DCS, it is possible to follow the adsorption of protein (Figure S1a–c in SI), even with colloidal instability present, most obvious around the 50% coverage for Fib (Figure S1c in SI).

Microscale Thermophoresis. MST is a technique for binding studies and allows us to determine the binding affinities of the proteins to the NPs. In a standard assay, the binder (NP) is fluorescently labeled and kept at constant concentration, while the ligand (protein) is not. These conditions are very useful for proteins with low binding affinities or for measurements in complex fluids like plasma. As described in refs 20 and 21, we measure how the binding induces changes of the fluorescent signal in a thermal gradient by determining the relative fluorescence:

\[ F_{\text{norm}} = \frac{F_{\text{hot}}}{F_{\text{cold}}} \]  

where \( F_{\text{hot}} \) is the fluorescence after thermodiffusion and \( F_{\text{cold}} \) is the initial fluorescence. By fitting \( F_{\text{norm}} \) as a function of the protein concentration using a Hill equation (eq 1), we estimate the binding affinity. However, when there is NP aggregation, the technique averages agglomerates and single particles data, introducing noise in the affinity constants estimate.

MST measurements were performed on a Monolith NT 0.15 (NanoTemper, Germany) using 40% of blue LED (488 nm) and 1 V IR-laser power. Laser on and off times were set at 35 s and 5 s, respectively. Standard treated capillaries from NanoTemper were used. FITC-labeled silica NPs were used at a constant concentration of...
0.313 mg/mL, while for the protein of interest, a 1:1 dilution series was prepared.

**Fluorescence Correlation Spectroscopy.** FCS is a highly sensitive fluorescence technique that allows for the determination of the number and the size of particles simultaneously in solution. By diffusing into and out of the confocal volume, labeled particles, i.e., proteins, create fluctuations in the fluorescence intensity. The fluorescence signal is temporally correlated for the analysis. Two component fit to the time-correlation function \( G(\tau) \) is used to determine quantitatively the amplitude and the diffusion time of the fast freely diffusing proteins \( r_{\text{prot}} \) and the slow bound proteins \( r_{\text{prot-NP}} \). We follow the procedure described by Rusu et al.\(^\text{22} \) and Milani et al.\(^\text{2} \) using a two component fitting formula:

\[
G(\tau) = \frac{1}{N} \left[ (1 - y) \left( \frac{1}{1 + \frac{\tau}{r_{\text{prot}}}} \right) \left( \frac{1}{1 + \frac{\tau}{r_{\text{prot-NP}}}} \right) \right]^{1/2} + y \left( \frac{1}{1 + \frac{\tau}{r_{\text{prot}}}} \right)^{1/2}
\]

(5)

From these results, we can determine the fraction bound of proteins on the NP surface\(^7 \) that is

\[
f_b \equiv 1 - \frac{N_{\text{free}}}{N_0}
\]

(6)

where \( N_0 \) is the initial protein number from a fit in a monocomponent solution and \( N_{\text{free}} \leq N_0 \) is the amount of unbound protein after incubation with NPs.

FCS measurements were performed on a LSM10 microscope equipped with a Confocor2 unit (Carl Zeiss Jena, Germany), an argon laser (488 nm), and an acrylchromic 40X water-immersion objective with a NA of 1.2 (Carl Zeiss Jena, Germany). Fluorescence emission was separated from excitation light by using the corresponding bandpass filter 525/25 nm. All measurements were performed at room temperature (22° C) using NUNC eight-well slides (Thermo Scientific) and a sample volume of at least 200 μL. To avoid unwanted adsorption of proteins to the walls, the chambers were precoated with 5 mg/mL BSA for 1 h. Afterward the chambers were rinsed with Milli-Q to remove unbound BSA.

**SDS-PAGE.** SDS-PAGE is a method by which proteins in a complex mixture can be separated based on their molecular weight. This technique has been applied previously to study the proteins which make up the biomolecular corona.\(^5,2,2\) Briefly the proteins adsorbed on the NPs are removed from their surface, denatured, and loaded on a gel, whereby they are separated by applying an electric field. The protein bands can subsequently be visualized by staining the proteins with Coomassie Brilliant Blue dye.

Silica NPs (100 μg/mL, 0.5 mL) were incubated in different HSA concentrations (0.35–7 mg/mL) for 1 h at room temperature. After that, Fib was added to a final concentration of 5 μg/mL. The NPs were incubated with Fib for varying lengths of time (0–120 min), and the times reported correspond to the incubation time with Fib before hard corona preparation. The hard corona samples were prepared by removing the excess proteins from the sample, achieved through 4 successive cycles of centrifugation (20000 × g, 10 min) and resuspension in PBS. The final NP pellet was suspended in 10 μL PBS with an additional 5 μL loading buffer. The samples were subsequently boiled for 5 min before loading on a 6–4% discontinuous Tris-glycine gel. The samples were run for 1 h at 130 V. The gels were then extracted and fixed in 40% EtOH, 10% acetic acid for 1h. Following this, the gel was placed in 0.025% w/w Coomassie Brilliant Blue dispersed in 10% EtOH and 10% acetic acid. The gel was then left overnight to stain before imaging.

**Computational and Theoretical Approach.** For our computational and theoretical calculations we used a computer cluster with dedicated Graphical Processing Units (GPUs):

- CPU processors: 4× PCs with an INTEL i7-870 and 6 GB RAM, 1× PC with an INTEL i7-3770 and 8 GB RAM.
- GPU processors: 4× NVIDIA GTX 460, 2× NVIDIA GTX 660, 1× NVIDIA GTX 760, 1× NVIDIA Tesla C2075.

All machines were running under GNU/Linux Ubuntu 12.04. The programming codes were compiled using CUDA-C version 5.0 and GCC 4.6.

**MD Simulations.** We performed MD simulations of the CG model at constant volume and constant temperature, using a Langevin thermostat. We fixed the simulation box size based on the NP concentration, having one single NP in our volume (Figure 2c).

We kept a constant concentration of proteins in solution, regardless of the number of proteins adsorbed on the NP surface, adopting a method that mimics the experimental buffer. Specifically, we divided the system in two regions: the inner region, containing the NP with all the proteins concentrations fixed to the experimental values, and the outer region, which is used as a reservoir to control the concentration of proteins in the inner region. The outer region is not considered for the calculation of the observable quantities.

We compute the adsorption kinetics by counting the number of adsorbed proteins at every time-step. A protein is considered to be adsorbed when the minimum surface-to-surface distance between the protein and the NP is <0.5 times the specific protein’s radius.

**Rescaling of Numerical Time Scale to Real Time Scale.** Our CG calculations give us qualitative information about the kinetics, and only after the comparison with the experiments, we can extract the correct time scales and make our predictions quantitative. To match the time scales of simulations, we use numerical NLDRE solutions and experiments in, e.g., Figure 3, we fit the experimental results with the NLDRE solution of eq S4 in SI, assuming \( k_{\text{eff}}^{\text{exp}} \approx 0 \) and adjusting the value of \( k_{\text{eff}}^{\text{exp}} \). This assumption is justified by the large affinity of Fib to the NP. Furthermore, in eq S4 in SI also \( k_{\text{eff}}^{HSA} \) and \( k_{\text{eff}}^{HSA} \) are free parameters. Because we verify that the specific values of these two parameters do not affect the behavior of the Fib fraction bound, we assume \( k_{\text{eff}}^{HSA} = k_{\text{eff}}^{HSA} \). This procedure allows us to calculate the value of the Fib fraction bound at early stages, not available from experiments, within the NLDRE formalism with eq S3 in SI and match it with our computational results. Therefore, on the one hand, we match the NLDRE curve with the experimental fraction bound and, on the other hand, the NLDRE curve with the computational fraction bound. In this way we are able to calculate the scaling factor \( C_t \) (Table S1) in \( t_{\text{sim}} = C_t \times t_{\text{exp}} \), where \( t_{\text{sim}} \) is the simulation time and \( t_{\text{exp}} \) is the time in real units. We observe that this procedure leads to the same scaling factor \( C_t \) for data in Figures 3 and 4.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.6b04858.

Additional experimental data, theoretical results and methodological developments (PDF)

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Notes

The authors declare no competing financial interest.
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REFERENCES

(1) Monopoli, M. P.; Åberg, C.; Salvari, A.; Dawson, K. A. Biomolecular Coronas Provide the Biological Identity of Nanosized Materials. Nat. Nanotechnol. 2012, 7, 779–786.

(2) Lundqvist, M.; Stigler, J.; Cedervall, T.; Berggård, T.; Flanagan, M. B.; Lynch, I.; Elia, G.; Dawson, K. A. The Evolution of the Protein Corona Around Nanoparticles: A Test Study. ACS Nano 2011, 5, 7503–7509.

(3) Corbo, C.; Molinaro, R.; Parodi, A.; Toledano Furman, N. E.; Salvatore, F.; Tasciotti, E. The Impact of Nanoparticle Protein Corona on Cytotoxicity, Immunotoxicity and Target Drug Delivery. Nanomedicine 2016, 11, 81–100.

(4) Casals, E.; Pfäßer, T.; Duschl, A.; Oostingh, G. J.; Puntes, V. Time Evolution of the Nanoparticle-Protein Corona. ACS Nano 2010, 4, 3623–3632.

(5) Milani, S.; Bombelli, F. B.; Pitek, A. S.; Dawson, K. A.; Rädler, J.; Baldelli Bombelli, F. Reversible Versus Irreversible Binding of Transferrin to Polystyrene Nanoparticles: Soft and Hard Corona. ACS Nano 2012, 6, 2532–2541.

(6) Dell’Orco, D.; Lundqvist, M.; Oslakovic, C.; Cedervall, T.; Linse, S. Modeling the Time Evolution of the Nanoparticle-Protein Corona in a Body Fluid. PLoS One 2010, 5, e10949.

(7) De Simone, A.; Spadaccini, R.; Temussi, P. A.; Fraternali, F. Toward the Understanding of MNEI Sweetness from Hydration Map Surfaces. Biophys. J. 2006, 90, 3032–3061.

(8) Habash, M.; Reid, G. Microbial Biofilms: Their Development and Biological Impacts. Nat. Nanotechnol. 2011, 6, 223–228.

(9) salvati, A.; Pitek, A. S.; Monopoli, M. P.; Prapanon, K.; Bombelli, F. B.; Hristov, D. R.; Kelly, P. M.; Åberg, C.; Mahon, E.; Dawson, K. A. Transferrin-Functionalized Nanoparticles Lose Their Targeting Capabilities When a Biomolecule Corona Adsorbs on the Surface. Nat. Nanotechnol. 2013, 8, 137–143.

(10) Tavanti, F.; Pedone, A.; Macedoni, M. C. Competitive Binding of Proteins to Gold Nanoparticles Disclosed by Molecular Dynamics Simulations. J. Phys. Chem. C 2015, 119, 22172–22180.

(11) Ding, H.; Ma, Y. Design Strategy of Surface Decoration for Efficient Delivery of Nanoparticles by Computer Simulation. Sci. Rep. 2016, 6, 26783.

(12) Vilaseca, P.; Dawson, K. A. Frangese, G. Understanding and Modulating the Competitive Surface-Adsorption of Proteins Through Coarse-Grained Molecular Dynamics Simulations. Soft Matter 2013, 9, 6978–6985.

(13) Monopoli, M. P.; Walczyk, D.; Campbell, A.; Elia, G.; Lynch, I.; Baldelli Bombelli, F.; Dawson, K. A. Physical-Chemical Aspects of Protein Corona: Relevance to in Vivo and in Vitro Biological Impacts of Nanoparticles. J. Am. Chem. Soc. 2011, 133, 2525–2534.

(14) Anderson, N. L. the Human Plasma Proteome: History, Character, and Diagnostic Prospects. Mol. Cell. Proteomics 2002, 1, 845–867.

(15) Walczyk, D.; Bombelli, F. B.; Monopoli, M. P.; Lynch, I.; Dawson, K. A. What the Cell “sees” in Biomassience. J. Am. Chem. Soc. 2010, 132, 5761–5768.

(17) Kršetíč, Z.; Davidson, A. M.; Volk, M.; Lévy, R.; Brust, M.; Cooper, D. L. High-Resolution Screening of Monolayer-Protected Gold Clusters by Differential Centrifugal Sedimentation. ACS Nano 2013, 7, 8881–8890.

(18) Derjaguin, B.; Landau, L. Theory of the Stability of Strongly Charged Lyophobic Solids and of the Adhesion of Strongly Charged Particles in Solutions of Electrolytes. Prog. Surf. Sci. 1993, 43, 30–59.

(19) Verwey, E.; Overbeek, J.; van Nes, K. Theory of the Stability of Lyophobic Colloids: The Interaction of Sol Particles Having an Electric Double Layer; Elsevier Publishing Company: Amsterdam, The Netherlands, 1948.

(20) Seidel, S. A. I.; Dijkman, P. M.; Lea, W. A.; van den Bogaart, G.; Jerabek-Willemsen, M.; Lazic, A.; Joseph, J. S.; Srinivasan, P.; Baaske, P.; Simeonov, A.; Katritch, I.; Melo, F. A.; Ladbury, J. E.; Schreiber, G.; Watts, A.; Braun, D.; Duh, S. Microscale Thermophoresis Quantifies Biomolecular Interactions Under Previously Challenging Conditions. Methods 2013, 59, 301–315.

(21) Pippich, S.; Seidel, S. A. I.; Duh, S.; Uhlund, K.; Holthoff, H.-P.; Jenne, D.; Braun, D. Direct Detection of Antibody Concentration and Affinity in Human Serum Using Microscale Thermophoresis. Anal. Chem. 2012, 84, 3523–3529.

(22) Rusu, L.; Gambhir, A.; McLaughlin, S.; Rädler, J. Fluorescence Correlation Spectroscopy Studies of Peptide and Protein Binding to Phospholipid Vesicles. Biophys. J. 2004, 87, 1044–1053.

(23) Cedervall, T.; Lynch, I.; Lindman, S.; Berggård, T.; Thulin, E.; Nilsson, H.; Dawson, K. A.; Linse, S. Understanding the Nanoparticle-Protein Corona Using Methods to Quantify Exchange Rates and Affinities of Proteins for Nanoparticles. Proc. Natl. Acad. Sci. U. S. A. 2007, 104, 2050–2055.

(24) Linse, S. Systematic Investigation of the Thermodynamics of HSA Adsorption to N-Isopropylacrylamide/N-Tet-Butylacrylamide Copolymer Nanoparticles. Effects of Particle Size and Hydrophobicity. Nano Lett. 2007, 7, 914–920.

(25) Baier, G.; Costa, C.; Zeller, A.; Baumann, D.; Sayer, C.; Araujo, P. H. H.; Mailänder, V.; Musyanovych, A.; Landfester, K. BSA Adsorption on Differently Charged Polystyrene Nanoparticles Using Isothermal Titration Calorimetry and the Influence on Cellular Uptake. Macromol. Biosci. 2011, 11, 628–638.

(26) Brewer, S. H.; Glomm, W. R.; Johnson, M. C.; Knag, M. K.; Franzen, S. Probing BSA Binding to Citrate-Coated Gold Nanoparticles and Surfaces. Langmuir 2005, 21, 9303–9307.

(27) Becklin, M. C.; Schafer, S.; Dumelin, C. E.; Zenobi, R. Label-Free Determination of Protein-Ligand Binding Constants Using Mass Spectrometry and Validation Using Surface Plasmon Resonance and Isothermal Titration Calorimetry. J. Mol. Recognit. 2009, 22, 319–329.

(28) Mura-Galelli, M. J.; Voegel, J. C.; Behr, S.; Bres, E. F.; Schaaf, P. Adsorption/desorption of Human Serum Albumin on Hydroxyapatite: A Critical Analysis of the Langmuir Model. Proc. Natl. Acad. Sci. U. S. A. 1991, 88, 5557–5561.

(29) Adamczyn, Z.; Barbas, J.; Cieśla, M. Mechanisms of Fibrinogen Adsorption at Solid Substrates. Langmuir 2011, 27, 6688–6678.

(30) Armstrong, J. K.; Wenby, R. B.; Meiselman, H. J.; Fisher, T. C. the Hydrodynamic Radii of Macromolecules and Their Effect on Red Blood Cell Aggregation. Biophys. J. 2004, 87, 4249–4270.

(31) Adamczyn, Z.; Nattich, M.; Wasilewska, M.; Sadowska, M. Deposition of Colloid Particles on Protein Layers: Fibrinogen on Mica. J. Colloid Interface Sci. 2011, 356, 454–464.

(32) Macrì, E.; Sparta, A.; La Placa, M.; Ferretti, M. Adsorption of Lysozyme on Gold Surfaces: Influence of the Solution pH. J. Colloid Interface Sci. 2011, 356, 454–464.