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Nanoparticle-Templated Self-Assembly Dynamics of Viral Capsids Probed by UV-Vis Absorbance Spectroscopy and X-Ray Scattering

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(Dated: October 4, 2018)

Viral capsid proteins have the remarkable ability to self-assemble around a cargo core, whether their genome, a polyelectrolyte or an inorganic nanoparticle. Although the equilibrium properties of such composite objects have been broadly investigated, little is known about the kinetic pathways leading to their assembly. By exploiting the plasmon resonance effect of gold nanoparticles, we show that the adsorption of viral proteins can be monitored through the redshift of the resonance wavelength. Kinetic measurements reveal that the process occurs in less than a second. The spatial arrangement of nanoparticles is probed by time-resolved small-angle X-ray scattering, and we observe the formation of transient aggregates with a lifetime in the hour range. The biocompatibility of gold nanoparticles induced by adsorbed proteins is of great importance for their use in biomedicine and the reported results should help better understand the processes of formation.

I. INTRODUCTION

The emerging study of biomolecular assemblies is an area of great fascination due to the astounding precision with which nanometer-sized objects are able to spontaneously assemble without any external intervention [1]. The assembly of viral proteins to form a capsid – i.e., the protein shell of a virus – is one such process [2–8]. Understanding the underlying mechanisms is fundamental in transferring the principles onto synthetic and macroscopic materials. A recent point of intrigue concerns how the self-assembly of viral proteins occurs around another material, e.g., the viral genome [9–18], a synthetic polymer [19–24] or a nanoparticle [25–33].

Gold nanoparticles (AuNPs) are of particular interest because they hold a lot of potential applications in photonics, electronics and biomedicine [34]. Once materials are reduced to nanometer sizes, a variety of interesting properties emerge, which are not seen in bulk materials. These distinctive optical and electromagnetic properties can be modified by changing parameters such as the size, shape and surface charge density of the nanoparticle in order to tune them for a particular function [35]. The existence of the surface plasmon resonance at the interface between a noble metal nanoparticle and the surrounding medium, serves to enhance the electromagnetic properties, notably absorption and scattering, in a way which is characteristic to each type of metal nanoparticle [36]. For biomedical applications in particular, it is crucial that nanoparticles are well stabilized because aggregation prevents them from diffusing through the tis-

sues and shortens their circulatory lifetime. Within this framework, the capsid formed by viral proteins around a synthetic core constitutes an efficient and biocompatible stabilizing agent as well as a protective barrier for the body.

The simplest viruses are made up of a capsid protecting the genome in the form of nucleic acids. Half of the viruses on Earth display an icosahedral symmetry and the capsid proteins are therefore arrayed onto an icosahedral lattice forming a two-dimensional crystal structure [37–39]. For example, brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV) are both single-stranded RNA viruses infecting plants, and their icosahedral capsid comprises 90 dimeric subunits that can self-assemble *in vitro* depending upon ionic conditions. The proteins interact with each other through a short-range hydrophobic and/or van der Waals attraction and a long-range electrostatic repulsion [40–42], while the subunit-genome, or subunit-cargo, interaction is realized by an electrostatic attraction. Daniel and coworkers [43] showed that the higher the surface charge density of ligand-stabilized AuNPs, the better their encapsulation into capsids derived from BMV. Theoretical investigations demonstrated that a threshold surface charge density must be exceeded and that the encapsulation efficiency is gradual due to charge regulation and size polydispersity of the nanoparticles [31]. He and coworkers [29] observed that the capsid of hepatitis B virus can withstand an increase of the nanoparticle diameter or adapt to it by generating packing defects.

While the equilibrium properties of empty or filled viral capsids have been thoroughly studied, the associated kinetic pathways, in contrast, are still poorly understood. Time-course static light scattering [44, 45], resistive-pulse sensing through nanofluidic channel [46], atomic force microscopy [47, 48], nuclear magnetic reso-

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nance [49], charge detection mass spectrometry [50] and time-resolved small-angle X-ray scattering (TR-SAXS) [51–55] have been used to shed some light on the self-assembly dynamics of empty or nucleic acids-filled capsids. The dynamics of AuNPs encapsulation is challenging to probe, because the adsorption of capsid proteins occurs very rapidly and, consequently, techniques with long acquisition times are ineffective. Malyutin and Dragnea [30] carried out a kinetic study by time-course light scattering and cryo-electron tomography on the encapsulation of AuNPs into BMV capsids. They reported a two-step mechanism where aggregates of nanoparticles are transiently formed upon the presence of proteins, then stable capsids enclosing nanoparticles bud off the aggregates.

In this work, we exploit the plasmon resonance effect of AuNPs to probe the adsorption of CCMV capsid proteins on ligand-stabilized AuNPs. We also study the aggregation mechanism proposed by Malyutin and Dragnea with TR-SAXS and we clearly detect the formation of aggregates, which are subsequently dissociated over a long timescale. We expect this study to promote further investigations on the self-assembly dynamics of nanoparticle-templated viral capsids and to help design systems useful to biomedical and imaging applications.

II. EXPERIMENTAL METHODS

A. CCMV virion purification

Cowpea chlorotic mottle virus (CCMV) is purified from infected blackeye cowpea leaves (*Vigna unguiculata*) following a protocol developed by Ali and Roossinck [56]. 140 g of infected leaves are homogenized with 300 mL of 0.15 M sodium acetate pH 4.8 in a blender, then 300 mL of ice-cold chloroform are added and the solution is stirred for 10 min. After 10 min of centrifugation at 10 000 $\times g$, the virions are precipitated by stirring the aqueous upper phase for 30 min in 20 mM NaCl and 8% of poly(ethylene glycol) (MW 8000). After another centrifugation at 10 000 $\times g$ for 10 min, the pellet is dissolved in 21 mL of 50 mM sodium acetate pH 4.8 and stirred for 1 h. The solution is centrifuged at 8000 $\times g$ for 10 min and the supernatant is centrifuged through a 20% (w/v) sucrose cushion at 150 000 $\times g$ for 2 h. The virions are collected in the pellet and stored in 50 mM sodium acetate pH 4.8 at -80 °C until use. Samples are deemed pure when the extinction ratio verifies $A_{260}/A_{280} > 1.60$.

B. Capsid protein purification

10 mg of purified virions are dissociated by a 24-h dialysis against 0.5 M CaCl_2 , 1 mM ethylenediamine tetra-acetic acid (EDTA) pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 50 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl pH 7.5.

After 18 h of centrifugation at 150 000 $\times g$, 1-mL aliquots of the supernatant are extracted and conserved if $A_{280}/A_{260} > 1.65$. The capsid proteins are kept in their assembled form in 0.5 M NaCl, 1 mM EDTA pH 8.0, 50 mM sodium acetate pH 4.8 at 4 °C until use within two weeks after purification. The protein concentration is estimated with an extinction coefficient $\epsilon_{\text{percent}} = 8.34$ at 280 nm.

C. Gold nanoparticle synthesis

We use three batches of gold nanoparticles (AuNPs), synthesized by different methods: Most UV-Vis absorbance experiments are done with AuNPs synthesized in toluene using oleylamine as a capping agent [57]. We study the effect of ligand charge (see Figure 3) on particles obtained by a seed-growth method in aqueous solution [58]. Particles used in TR-SAXS experiments (see Figure 5) are synthesized via an improved citrate method [59]. Particle sizes are similar in all batches.

Two types of ligands, similar to those used by Daniel *et al.* [43], are used to functionalize the AuNPs: a nonionic thiolated surfactant, (1-mercaptoundec-11-yl)hexa(ethylene glycol), HS- C_{11} -EG₆-OH, denoted as MUDOL and its acidic (carboxylated) counterpart, HS- C_{11} -EG₆-CH₂COOH, denoted as EG₆-COOH. The latter is purchased from ProChimia Surfaces (Poland). The AuNPs synthesized in water, initially stabilized by 0.5 mM CTAB, are incubated overnight with an excess of either ligand. Those synthesized in toluene are resuspended in THF, functionalized and then transferred to water. They are afterwards washed in pure water and stored at room temperature.

D. Encapsulation of AuNPs into viral capsids

CCMV capsid proteins in the form of dimeric subunits are initially dispersed in 0.5 M NaCl, 1 mM EDTA pH 8.0 and 50 mM Tris-HCl pH 7.5, while AuNPs are in pure water. For transmission electron microscopy and static extinction spectroscopy, subunits and AuNPs are dialyzed together against 50 mM NaCl, 1 mM EDTA pH 8.0 and 50 mM sodium acetate pH 4.8. Aniagyei *et al.* [27] found that acidic pH renders the adsorbed CCMV protein layer more compact at the surface of AuNPs than at neutral pH. However, EG₆-COOH-stabilized AuNPs are not fully stable at low pH. As a consequence, our attempts to carry out kinetics by mixing rapidly subunits and AuNPs in acidic buffer failed because the AuNPs precipitated before being stabilized further by the subunits. Therefore, adsorption kinetics and TR-SAXS experiments are performed by mixing subunits, AuNPs and pure water in such a way that the final ionic strength is about 0.1 M and the pH remains at 7.5. In all experiments, the final protein concentration is within 0.1-0.3

g.L⁻¹ and the subunit-to-AuNP molar ratio ranges from 180:1 to 3600:1 so that proteins are in large excess.

E. Transmission electron microscopy

5 μ L of sample solution is placed during 10 min on a carbon grid treated by glow discharge, and blotted with the edge of a filter paper. A drop of 1% (v/v) ammonium molybdate is deposited on the grid for 30 s, then removed with filter paper, and the grid is allowed to air dry for at least 10 min. Transmission electron microscopy (TEM) is performed with a JEOL JEM-2010 microscope equipped with a 200-kV field emission gun. The samples are imaged with magnifications comprised between $\times 20\,000$ and $\times 50\,000$ and the images are collected with a Gatan Ultrascan 4K CCD camera at a defocus comprised between 1.5 and 3 μ m.

F. UV-Vis absorbance spectroscopy

For static experiments, we measure the absorbance spectrum of dilute particles for wavelengths comprised between 400 and 1400 nm with an Agilent Cary 5000 spectrometer. Samples are placed in polystyrene cuvettes with a 10-mm optical path.

For adsorption kinetics, we use a Biologic SFM-3000 stopped-flow apparatus (France) equipped with a manual monochromator and a high sensitivity photomultiplier. The wavelength is set to 530 nm and the extinction is collected with a fixed time interval of 10 ms.

G. Time-resolved small-angle X-ray scattering

X-ray scattering measurements are performed with the SWING beamline at the SOLEIL synchrotron facility (Gif-sur-Yvette, France). The wavelength is set to $\lambda = 1.242$ Å and the sample-to-detector distance is 4 m, which provides scattering wavenumbers q ($q = 4\pi/\lambda \sin(\theta/2)$ where θ is the scattering angle) ranging from 2.3×10^{-3} to 0.28 Å⁻¹. Two-dimensional scattering images are recorded on an AVIEX large area CCD detector with an exposure time of 250 ms. The rapid mixing of AuNPs with subunits is carried out with a Biologic SFM-300 stopped-flow apparatus (France) equipped with three syringes and the flow is stabilized by a hard stop valve. The scattering intensities are converted into absolute units after subtracting the contribution of the buffer, and the uncertainties are calculated after circular averaging using the FOXROT software package.

III. RESULTS

A. Morphology of protein-coated AuNPs observed by electron microscopy

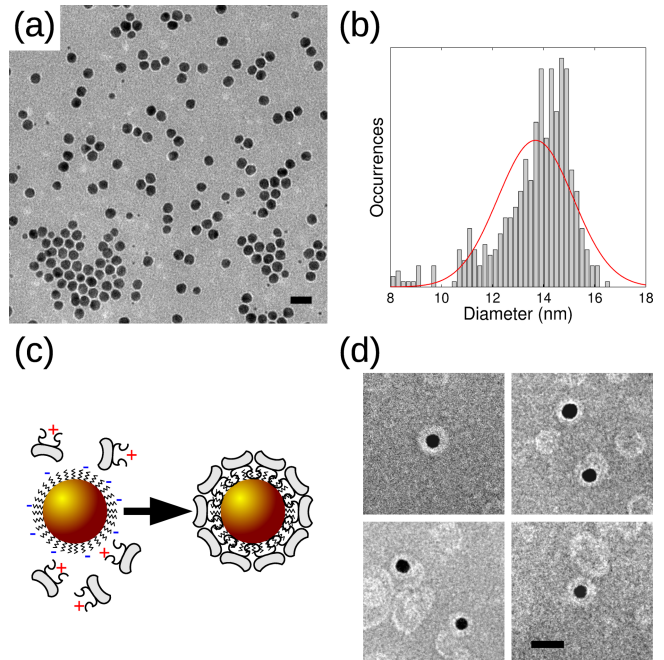


FIG. 1. Transmission electron microscopy of naked and protein-coated EG₆-COOH-stabilized AuNPs. (a) Naked AuNPs and (b) their size distribution inferred from TEM images. (c) Schematics depicting the coating of ligand-stabilized AuNPs by subunits. (d) Protein-coated AuNPs. Scale bars are 30 nm.

Figure 1(a) shows a TEM image of naked EG₆-COOH-stabilized AuNPs in pure water after synthesis. Note that all AuNPs used in this study are ligand-stabilized and naked AuNPs refers hereafter to as ligand-stabilized AuNPs without adsorbed proteins. Image analysis gives a diameter distribution of 13.7 ± 1.5 (mean \pm s.d.) nm [Fig. 1(b)], that is, the AuNPs are fairly monodisperse. At neutral pH and high ionic strength (0.5 M), CCMV capsid proteins are in the form of dimeric subunits. Each subunit carries two flexible, cationic arms rich in arginine. When EG₆-COOH-stabilized AuNPs and subunits are mixed at neutral pH and low ionic strength (~ 0.1 M), the cationic arms are electrostatically attracted by the anionic moiety of the ligands. As a result, the subunits are adsorbed at the surface of the nanoparticles and form a capsid with some defects [Fig. 1(c)]; when the pH becomes acidic, the capsids are consolidated. Figure 1(d) depicts such AuNPs encapsulated into viral capsids. The size of the capsids is close to that of the native virions, i.e., 28 nm, and since the subunits are in excess, several empty capsids or pieces of capsids are coexisting with the protein-coated AuNPs.

B. Redshift of plasmon resonance induced by capsid proteins

A homogeneous nano-sized gold sphere embedded in a uniform electric field has a polarizability α (defined as the ratio between the induced dipole moment of the sphere and the applied electric field) given by [60]:

$$\alpha = 3V \frac{(\epsilon_S - \epsilon_W)(\epsilon_{Au} + 2\epsilon_S) + f(2\epsilon_S + \epsilon_W)(\epsilon_{Au} - \epsilon_S)}{(\epsilon_{Au} + 2\epsilon_S)(\epsilon_S + 2\epsilon_W) + 2f(\epsilon_S - \epsilon_W)(\epsilon_{Au} - \epsilon_S)} \quad (1)$$

where $f = [R/(R + d)]^3$ with R the radius of the AuNP and d the shell thickness. ϵ_S denotes the relative permittivity of the shell layer.

Changes in α can be probed using UV-Vis absorbance spectroscopy, since the beam extinction E is proportional to the extinction cross-section: $E = N\sigma_{\text{ext}}l/\ln(10)$, where N is the number of nanoparticles per unit volume and l is the length of optical path. The extinction cross-section σ_{ext} is the sum of the contributions arising from absorption and scattering. The scattering contribution is here negligible due to the small size of the nanoparticles. Therefore, σ_{ext} is dominated by the absorption cross section σ_{abs} :

$$\sigma_{\text{ext}} \approx \sigma_{\text{abs}} = \frac{2\pi}{\lambda_W} \Im(\alpha) \quad (2)$$

with λ_W the wavelength of the incident light in water and $\Im(\alpha)$ the imaginary part of α . Despite its simplicity, the model is quite accurate and allows extracting reliable parameters via full-curve fitting of the extinction curves [61].

Figure 2(a) gives the calculated absorption cross sections [Eq. (2)] of AuNPs coated with a dielectric shell. Note that the refractive index n is related to the relative permittivity ϵ by $\epsilon = n^2$. ϵ_{Au} is a complex function of the wavelength and is derived from an analytical model (see Appendix). In the absence of a shell, i.e., when $n_S = n_W$, the absorption cross-section exhibits a plasmon resonance peak at a wavelength around 520 nm. As the refractive index of the shell increases, the peak shifts to higher values and the maximal amplitude increases. The shift of wavelength between the spectrum with no shell and that with $n_S = 1.4$ is 3 nm, and amounts to 8 nm when $n_S = 1.5$. The ratio of the maximal amplitudes is found to be 1.10 and 1.23, respectively. The refractive index of proteins is known to lie in the range of 1.45 and 1.65 [62], and the thickness of a CCMV capsid is 5 nm [63]. We expect thus to have similar effects on experimental measurements.

Figure 2(b) shows the experimental extinction spec-

tra measured on naked and protein-coated AuNPs. The spectrum of naked AuNPs is fitted with a model of gold spheres having a radius of 7 nm. The spectrum of

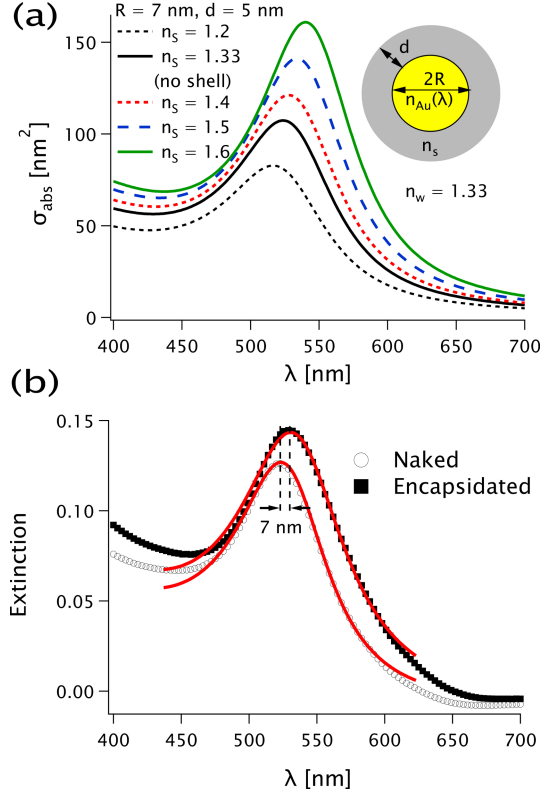


FIG. 2. UV-Vis absorbance spectroscopy of protein-coated AuNPs. (a) Calculated absorption cross-section of spherical AuNPs with a radius $R = 7$ nm and coated with a dielectric shell of thickness $d = 5$ nm with varying refractive indices n_S . (b) Experimental extinction spectra of naked and protein-coated AuNPs stabilized with EG₆-COOH ligands. The red solid lines are theoretical models fitting the measurements by using $R = 7$ nm, and either no shell (for the naked AuNPs, \circ) or a shell with $d = 5$ nm and $n_S = 1.44$ (for the encapsulated spheres, \blacksquare).

tra measured on naked and protein-coated AuNPs. The spectrum of naked AuNPs is fitted with a model of gold spheres having a radius of 7 nm. The spectrum of

protein-coated AuNPs is fitted with the same model of gold spheres, but coated with a 5-nm-thick protein layer, yielding a refractive index $n_S = \sqrt{\epsilon_S} = 1.44$. The shift of the resonance wavelength is found to be 7 nm. The fitting values are in agreement with the expected thickness and relative permittivity of a protein capsid.

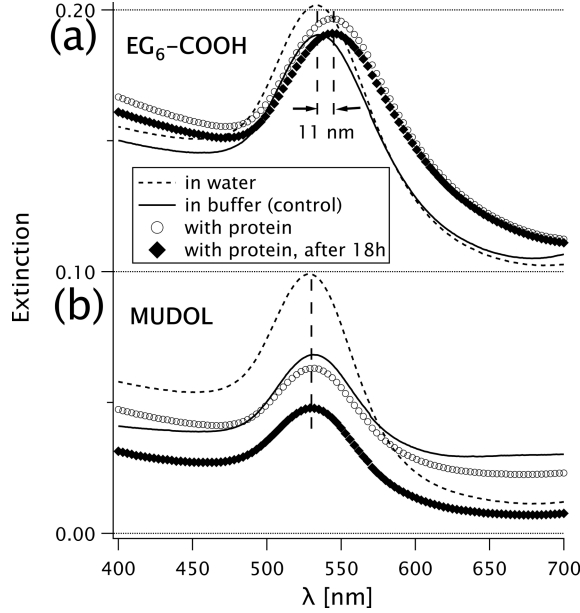


FIG. 3. Effect of ligands on the coating of AuNPs by proteins. Extinction spectra of AuNPs stabilized by (a) EG₆-COOH and (b) MUDOL ligands in pure water (dashed line), in buffer solution (solid line), in buffer solution just after mixing with capsid proteins (open discs), and in buffer solution 18 h after mixing with capsid proteins (full diamonds).

Figures 3(a) and (b) show the effect of ligands on the coating of AuNPs by viral proteins. The extinction spectra of AuNPs in pure water and in buffer solution are similar for both ligands, there is therefore no immediate effect of the buffer solution on AuNPs. More importantly, the extinction of EG₆-COOH-stabilized AuNPs in the presence of proteins exhibits a redshift of the resonance wavelength of 9 nm, whereas the resonance remains at the same wavelength for MUDOL-stabilized AuNPs with proteins. This difference demonstrates that proteins are adsorbed on EG₆-COOH-stabilized AuNPs but not on MUDOL-stabilized AuNPs: The surface charge density of AuNPs has to be negative and sufficiently high in absolute value to allow the adsorption of proteins, in good agreement with the findings reported by Daniel *et al.* [43]. The fact that the shift is 9 nm while it is 7 nm in the previous measurements [Fig. 2(b)] may arise from a slightly larger average thickness of the protein shell due to experimental variations in the subunit-to-AuNP molar ratios. The extinction of MUDOL-stabilized AuNPs in the presence of proteins after 18 h is significantly lower than immediately after mixing [Fig. 3(b)], unlike the case of EG₆-COOH-stabilized AuNPs in the presence of proteins [Fig. 3(a)]. Because MUDOL-stabilized AuNPs

have a neutral surface charge density and are not further stabilized by a layer of proteins, they massively aggregate due to strong attractive van der Waals forces and precipitate at the bottom of the tube. As a consequence, the concentration of AuNPs in the supernatant drops and so does the extinction.

C. Adsorption dynamics of proteins on nanoparticles

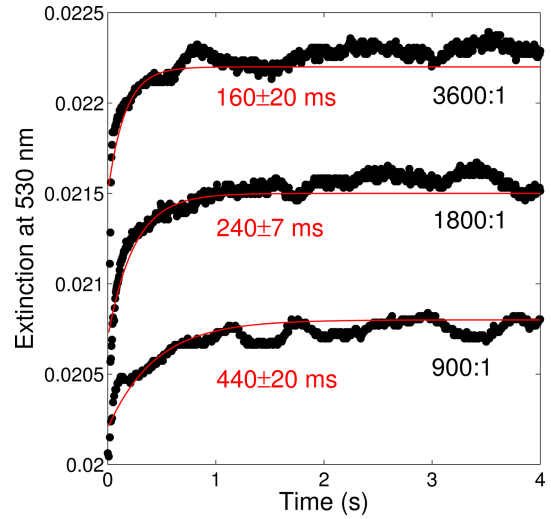


FIG. 4. Time-resolved extinction of protein-coated AuNPs, at various subunit-to-AuNP molar ratios. The red lines are exponential decay fits and the numbers in red are the corresponding decay times. The curves are shifted for clarity.

We investigate now the adsorption dynamics of proteins on EG₆-COOH-stabilized AuNPs in neutral buffer. Proteins and AuNPs are rapidly mixed and the extinction at 530 nm is monitored during the course of protein adsorption. As proteins are adsorbed on AuNPs, the wavelength of the plasmon resonance is gradually shifted from 520 nm to 530 nm in such a way that the extinction measured at 530 nm increases. Figure 4 gives the corresponding traces for three different molar ratios, with proteins in large excess. The adsorption occurs very rapidly, i.e., within a few hundreds of milliseconds. The adsorption timescale τ_{ads} decreases when the protein concentration c_S increases in such a way that $\tau_{\text{ads}} \propto c_S^{-0.8 \pm 0.2}$. A purely diffusion-limited process would yield $\tau_{\text{ads}} \propto c_S^{-1}$. The slight difference in the exponent may be due to a free energy barrier, most likely arising from the adsorbed subunits hindering the insertion of incoming free subunits through steric and electrostatic repulsions.

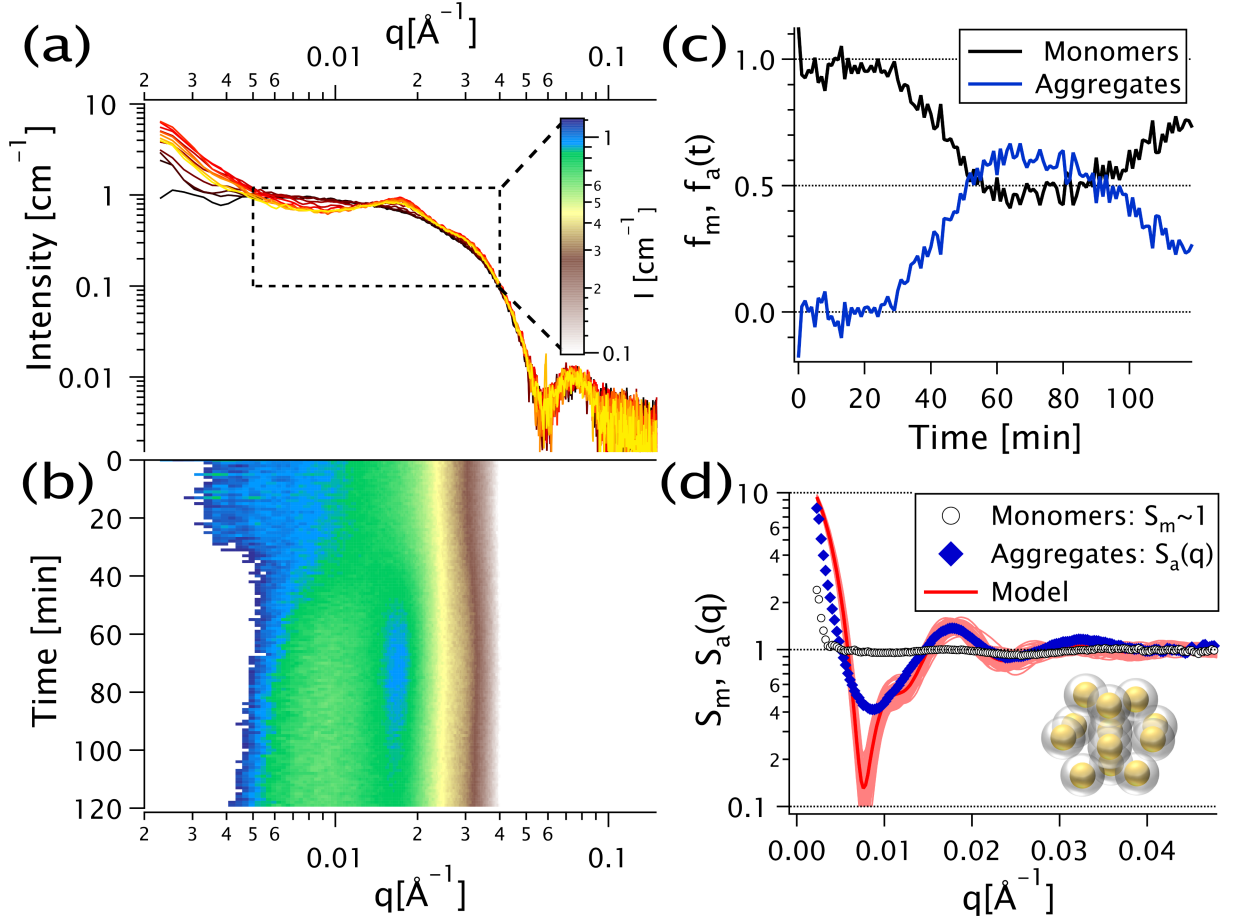


FIG. 5. Time-resolved small-angle X-ray scattering of EG₆-COOH-stabilized AuNPs coated by capsid proteins. (a) Scattering patterns collected at different time steps. (b) Two-dimensional representation of the scattering intensities as a function of time and wavenumber for the area delimited by the dashed box in (a). (c) Molar fractions of monomers (free protein-coated AuNPs) f_m and protein-coated AuNPs within aggregates f_a as a function of time inferred from singular value decomposition. (d) Corresponding structure factors of monomers $S_m(q)$ and aggregates $S_a(q)$. The light red lines are one hundred calculated structure factors of aggregates made of 13 particles separated by a distance fluctuating around 43 nm (3D model in inset) and the dark red line is their average. The protein concentration is 0.13 g.L⁻¹ and the subunit-to-AuNP molar ratio is 2640:1.

D. Assembly and dissociation dynamics of aggregates made of protein-coated AuNPs

Next, we probe the large-scale structures formed by protein-coated AuNPs by using TR-SAXS with a high brilliance synchrotron source. Because the electron density of gold is considerably higher than that of proteins, the scattering intensity arising from capsid proteins is negligible with respect to that due to AuNPs. However, if the protein-coated AuNPs transiently form aggregates as reported by Malyutin and Dragnea [30], the presence of the latter can be readily detected via the modulation they induce in the structure factor.

Figure 5(a) shows the apparition of a peak at $q \simeq 0.018$ Å⁻¹, due to loosely-packed AuNPs aggregates. The two-dimensional representation of Fig. 5(b) reveals that the peak (visible as a blue streak) appears about 50 min after mixing subunits and AuNPs, and disappears almost completely after 110 min. The isosbestic points on the

scattering intensities of Fig. 5(a) at $q \simeq 5.5 \times 10^{-3}$ and 0.013 Å⁻¹ suggest the presence of only two species: free (monomers) and aggregated protein-coated AuNPs, interconverting over the course of the process, in which case the scattering intensity $I(q, t)$ reduces to [64]:

$$I(q, t) \propto [f_m(t) + f_a(t)S_a(q)]P(q) \quad (3)$$

where f_m and f_a stand for the molar fractions of free protein-coated AuNPs and protein-coated AuNPs within aggregates, respectively. $S_a(q)$ is the structure factor of the aggregates and $P(q)$ is the form factor of AuNPs. $S_a(q)$ must be seen as a structure factor averaged over all aggregates, during the whole process. $P(q)$ is obtained from the first scattering pattern, when protein-coated AuNPs are still all free in solution. The molar fractions and $S_a(q)$ are estimated by retaining the two first components of a singular value decomposition [65]

performed on $I(q)/P(q)$. By rotating and rescaling the basis vectors in order to reproduce the flat structure factor of monomers, i.e., $S_m(q) \sim 1$, $f_m(t)$ can be estimated. $f_a(t)$ and $S_a(q)$ are then deduced by mass conservation. $f_m(t)$ and $f_a(t)$ are plotted in Fig. 5(c). The first aggregates appear about 30 min after mixing and their fraction continues to grow until $t \simeq 60$ min. At this moment, around 60% of AuNPs are within aggregates. Then, AuNPs are slowly released but 2 h after mixing there are still $f_a = 25\%$ in aggregated form.

E. Modeling the structure factor of the aggregates

The structure factor of the aggregates $S_a(q)$ is shown in Fig. 5(d). Since all interactions are presumably isotropic, we describe it based on a compact reference assembly comprising 13 particles, one in the center and the remaining twelve forming a shell around it. The distance between nearest neighbors is initially 43 nm, but the particles are then allowed to fluctuate at random around their equilibrium positions according to Gaussian distributions with standard deviation 6.45 nm along each space dimension (x, y, z). The structure factors corresponding to one hundred noise realizations are shown in light red and their average in dark red.

Despite its simplicity, the model is in qualitative agreement with the experimental data: the soft peak at 0.018 \AA^{-1} , indicative of a limited degree of order (as for an amorphous packing) is well captured. The discrepancies: a sharp minimum at 0.0076 \AA^{-1} and a rounded increase at lower q values, can be explained by the size polydispersity of the aggregates in solution. Note that the scattering signal is largely dominated by the gold nanoparticles and provides no direct information on the position and conformation of the proteins.

Given that the diameter of AuNPs is 15 nm as inferred from $P(q)$, EG₆-COOH ligand is 2 nm long at most, and the thickness of a CCMV capsid is 5 nm, the full diameter of protein-coated AuNPs is estimated to be 29 nm. In other words, the surfaces of protein-coated AuNPs within an aggregate are separated by a gap of 15 nm, most likely filled with capsid proteins. In another experiment where the subunit-to-AuNP molar ratio is reduced by a factor of two while keeping the subunit concentration identical, the structure peak is barely visible and there is almost no evolution of the scattering patterns. It suggests that the subunit-to-AuNP molar ratio as well as the subunit concentration have to be sufficiently high to induce the formation of transient aggregates.

IV. CONCLUSION

By combining UV-Vis absorbance spectroscopy and TR-SAXS, we probe the self-assembly of CCMV capsids templated by ligand-stabilized AuNPs with different temporal and spatial scales. The measurements allow

us to propose a three-step pathway: Right after mixing proteins and ligand-stabilized AuNPs, the proteins, driven by electrostatic interactions, adsorb on the AuNPs provided that the surface charge density is negative and high enough in absolute value. The adsorption occurs on the subsecond timescale and is accelerated when the subunit-to-AuNP molar ratio increases. In a large excess of subunits, a part of the protein-coated AuNPs form aggregates in coexistence with the remaining free protein-coated AuNPs within the first hour. The surface-to-surface distance between protein-coated AuNPs within an aggregate is sufficiently large to accommodate two or three layers of capsid proteins, and each aggregate comprises a dozen or so nanoparticles. Notice that the aggregates are soluble and do not precipitate at the bottom of the tube. Then, the aggregates slowly dissociate over a couple of hours by releasing individual protein-coated AuNPs. The latter are stable over long durations and electron microscopy images reveal a closed shell wrapping up each AuNP.

There is a need to better understand and subsequently, control the nonequilibrium self-assembly and self-organization dynamics of complex molecular systems [66]. Biological systems, spurred by the pressure of their own survival, have evolved to spontaneously build up large-scale, perfectly ordered structures in an error-free manner, despite an often adverse environment. The present work highlights the robustness and adaptability of viral proteins during the packaging of a cargo, as it efficiently occurs in host cell. It should promote further fundamental studies on bio-inspired systems, notably related to the physics of dynamical phenomena.

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Appendix: Analytical model of the dielectric function of gold

The relative permittivity of gold is approximated by the analytical model used by Etchegoin *et al.* [67]:

$$\epsilon_{\text{Au}} = \epsilon_{\infty} - \frac{1}{\lambda_p^2 \left(\frac{1}{\lambda^2} + \frac{i}{\gamma_p \lambda} \right)} + \sum_{i=1,2} \frac{A_i}{\lambda_i} \left[\frac{e^{i\phi_i}}{\frac{1}{\lambda_i} - \frac{1}{\lambda} - \frac{i}{\gamma_i}} + \frac{e^{-i\phi_i}}{\frac{1}{\lambda_i} + \frac{1}{\lambda} + \frac{i}{\gamma_i}} \right].$$

The first two terms come from the Drude model while the summation is over two terms which come from interband transitions. λ_p is the plasma wavelength, γ_p is the

damping expressed as a wavelength, A_i is the amplitude of each term, λ_i and γ_i are the wavelength and damping of the respective interband transitions. ϕ_i is the phase of each transition. The parameter values correspond to the Johnson and Christy data [68], with the exception of λ_1 , which we modify slightly to obtain better agreement with the spectrum of the naked particles: $\epsilon_{\infty} = 1.54$, $\lambda_p = 143$ nm, $\gamma_p = 14500$ nm, $A_1 = 1.27$, $\phi_1 = -\pi/4$, $\lambda_1 = 445$ nm (470 nm for Johnson and Christy), $\gamma_1 = 1900$ nm, $A_2 = 1.1$, $\phi_2 = -\pi/4$, $\lambda_2 = 325$ nm and $\gamma_2 = 1060$ nm.

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