Ultrafast Structural Dynamics of the Photocleavage of Protein Hybrid Nanoparticles

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ABSTRACT Protein-coated gold nanoparticles in suspension are excited by intense laser pulses to mimic the light-induced effect on biomolecules that occur in photothermal laser therapy with nanoparticles as photosensitizer. Ultrafast X-ray scattering employed to access the nanoscale structural modifications of the protein—nanoparticle hybrid reveals that the protein shell is expelled as a whole without denaturation at a laser fluence that coincides with the bubble formation threshold. In this ultrafast heating mediated by the nanoparticles, time-resolved scattering data show that proteins are not denatured in terms of secondary structure even at much higher temperatures than the static thermal denaturation temperature, probably because time is too short for the proteins to unfold and the temperature stimulus has vanished before this motion sets in. Consequently the laser pulse length has a strong influence on whether the end result is the ligand detachment (for example drug delivery) or biomaterial degradation.

KEYWORDS: protein-nanoparticle hybrids · photo-cleavage · thermal denaturation · pulsed X-ray scattering · nanobubbles

Biological application of nanoparticles include the hybridization with proteins, or specifically with antibodies for in-vitro and in-vivo labeling of the corresponding tissue containing the antigens. While nanoparticles may enhance the detection threshold for targeted tissues, they can also be used in a second step as local tools for tissue modification. It has been shown that laser-excited gold particles can enhance transfection through cell walls by transiently enhanced permeation of the cell walls.1,2 A large number of studies have focused on the effect of laser-heated particles, in particular of gold for cell destruction.3–5

The thermal kinetics of laser-excited metal nanoparticles is already well understood, which includes a plasmonic excitation of the conduction electron gas, a subsequent thermalization and coupling to the lattice, and the generation of heat. The heat dissipates with time constants in the nanosecond range into the local surrounding and thus into the adjacent biological tissue. Optical methods such as fluorescence lifetime methods or UV circular dichroism that probe the change in the plasmon resonance of the particles, in particular in the case of gold or silver particles, usually give only indirect access to the thermal state on the nanoscale without directly probing the primary structural modifications. On the other hand structural methods, such as ultrafast electron scattering6,7 and X-ray scattering have been successfully applied to study the photokinetics of small molecules,8–11 tertiary and quaternary protein movement,12 or the rich structural response in pure nanoparticle systems13–15

Here we show that in the model hybrid system of gold nanoparticle—bovine serum albumin (BSA) it is possible to follow the photoinduced structural response of the protein layer at a well-defined temperature and time scale both on a mesoscopic scale and for structural motifs of the proteins. Figure 1 visualizes two candidate reactions from particle heating: protein denaturation and protein cleavage from the surface. These two pathways will differ critically in the final impact on the protein in a practical application.

The study is conducted as a two-color pump–probe experiment with a laser pulse as ultrafast heat source and a temporally synchronized X-ray pulse as structural probe. Because of the low scattering cross section and the dilution of the samples, multiple shots have to be accumulated on the X-ray detector for obtaining meaningful data. At the same time the experiment has to be designed as single-shot excitation due to the irreversible nature of the protein excitation. The synchrotron-based scattering allows for a 100 ps time resolution.

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RESULTS AND DISCUSSION

Thermokinetics of the Excited Nanoparticles. The thermal kinetics of suspended nanoparticles following femtosecond laser excitation is quite well understood, both by optical spectroscopy and pulsed X-ray scattering. Heat dissipation can be understood down to the 10 nm scale by conventional macroscopic heat transfer, with one exception being that the interface resistance (Kapitza resistance) cannot be neglected. The interface resistance accounts for a finite probability of phonons crossing the particle—water interface due to the acoustic mismatch. The numerical approaches have shown to reproduce the heat dissipation fairly well, thereby allowing the quantification of the Kapitza resistance.

One practical aspect of this kinetics is that the heat transfer from the particle to the surrounding matrix (water or protein in the present case) is delayed. There will always be a step in temperature across the interface, so that the temperature reached in the surrounding layer will be considerably lower than the nanoparticle lattice. Figure 2 shows an example of such a calculation for bare gold particles, where an initial temperature rise of 500 K is assumed. The inset shows the temperature rise in the adjacent layer outside of the particle as a function of time delay after the laser excitation.

Implicitly it was assumed that the heat transfer equations are not directly temperature dependent, such as through temperature dependent heat conductivity. This has shown to be true in a temperature interval of some hundred K. However, one important nonlinearity is the explosive boiling of the water phase once the temperature surpassed the spinodal temperature for water. As consequence nanometric bubbles form symmetrically around the heated nanoparticles, which grow and collapse within a 1–2 ns time scale.

In SAXS one will therefore observe large scattering changes once the bubble formation threshold is surpassed, which can be seen in Figure 3. For the bare particles below (92 J/m²) the threshold fluence the difference invariant (excited minus non-excited) is almost zero except for a tiny negative signal within the first 100 ps after excitation, which reflects the transient heating and therefore slight density change of the surrounding water layer. At a fluence value far above (425 J/m²) the threshold a large transient is observable. It reflects the appearance and collapse of vapor bubbles around the particles. After the collapse the invariant almost (except from a tiny offset) returns to the nonexcited value, indicating the reversibility of the process.

A completely different picture is revealed for BSA-coated nanoparticles. At the same low fluence there is
already a strong transient change of the invariant, followed by a persistent difference. Here the absorption cross section increases slightly due to the stabilization of the plasmonic resonance of the particles in a medium with higher dielectric constant. Therefore a fraction of the particles already surpass the bubble formation threshold and some bubble nuclei are formed. The persistent change already indicates that the particle–shell structure has been perturbed permanently. At the higher laser fluence fully developed bubbles occur, followed by a larger persistent change.

**Protein Shell Expulsion.** While the bubble formation is an effect of the uncoated nanoparticles as well as the protein-coated nanoparticles, the persistent change is solely seen for the hybridized system. The bubble signature can be seen in Figure 4A for a fluence of 133 J/m². It can be modeled by only a vapor bubble around the particle of 1.45 times the particle radius. The main difference to the protein removal (and thus density reduction) is the amplitude of the difference signal. At higher bubble sizes the signal will shift in position as well. At the lower fluence (40 J/m²) a small modulation of the difference scattering is visible, which stems from a small density change due to heating. An analysis of the SAXS pattern at longer times (Figure 4B) after the transient evaporation process has decayed, shows an oscillatory structure, which is best explained by a complete expulsion of the BSA layer from the particle surface. A comparison is made for two different models of the modification of the BSA layer against the scattering difference. The first model assumes that BSA remains attached to the particles, but only its density is reduced while the thickness increases to mimic a transition from a folded protein to a random coil with extending strands of protein. In the second model the complete layer of BSA, having a density of 1.35 g/cm³, is removed from the particle surface. Although the first model can reproduce in general the sign of the oscillations with an increase of the protein layer from 9 nm thickness to 17 nm and accordingly reducing the density, a much better fit is obtained by model 2. Values for the (hydrated) density of BSA in the range of 1.35 g/cm³ have been reported, which agree well with our density fit. This implies that during the photoexcitation the protein shell is completely removed from the particle surface and does not recombine during the observation interval (1 μs). The static SAXS measurements have revealed a density change of the adsorbed shell of only 19% when adding BSA to the MCSA-coated particles. This slight discrepancy may, however, be due to a smaller density change with only BSA addition onto the MCSA terminated particles, while during the photodesorption the entire coating of BSA plus MCSA is expelled. The underlying breakage of the thiol bond during photoexcitation has been reported earlier. By a comparison of the long time signal to the signal at 250 ps and 40 J/m² it seems that the protein layer was not totally removed as neither model 1 nor model 2 fully explain the modulation in Figure 4A.

In consequence all structure changes are finished within the bubble dynamics interval, whereafter no further changes occur. Indeed the shape of the difference scattering in Figure 4 is independent of the applied laser fluence, and only the amplitude of its signal changes for low fluence values and for long delays. Using this fact the signal amplitude can serve as a quantitative measure of efficiency of protein expulsion. We can obtain the transient temperature rise that
Figure 5. Reaction probability derived from the signal amplitude in Figure 4B of the excited BSA-coated particles as a function of maximum temperature rise in the liquid shell around the nanoparticles (open circles) as well as a control experiment without BSA (crosses). A fit with a rate process function is shown as a red line. The bold blue line displays an analogue probability from the SAXS signal change during thermal denaturation.

the protein must have suffered within the first few hundreds of picoseconds, by using the calibration of temperature versus fluence.14

In Figure 5 the reaction probability (normalized signal amplitude) from Figure 4 has been plotted against the maximum temperature rise adjacent to the nanoparticles. The high temperature limit is used as a scaling factor to define a reaction (=desorption) probability from 0 to 1. A rate equation with a sigmoidal distribution describes the behavior well (see Supporting Information). The solid line shows a fit to the experimental data assuming this sigmoidal reaction probability. Following this result the protein layer is only expelled at a temperature rise of around 100–160 K in the water phase (50% value at 129 K), which agrees well with the bubble formation threshold, which would be located at 120 K for bare particles. Secondly and more interestingly the ΔS curves fall on top of each other when they are scaled with the inverse of the reaction probability. This means that the protein shell density is not affected at all in its structure below a temperature rise of some 70 K or even up to the threshold. Also shown in Figure 5 is the SAXS signal change for static heating of BSA-coated particles as a function of temperature, where the SAXS signal contains only information on the radial density distribution around the nanoparticles, and therefore the protein layer can only be modeled by a homogeneous layer. This certainly restricts the amount of information that one can obtain on the protein internal structure such as secondary or further folding motifs. However, at least the secondary motif can directly be addressed by X-ray scattering, keeping in mind that the helix structure is a well-defined longer range order in the protein. In some cases also tertiary or quaternary structure can be resolved for free proteins in solution23,24 even with high time resolution,12,25–27 The static wide angle scattering shows indeed a scattering maximum at around 1.35 Å⁻¹, which reflects the pitch of the α-helix structure in BSA. Additionally UV circular dichroism data prove that the helical content is unchanged upon the adsorption on gold particles, but reacts on thermal denaturation in a similar way as BSA in solution (static data shown in Figure S3A for the SAXS and Figure S3B for the WAXS part in the Supporting Information).

We have also performed pump–probe scattering with a larger scattering angle, which basically only differs from the SAXS experiment by the distance of the detector from the sample.14 The main features in these measurements are the change of the water structure factor due to heating and, in particular in the present case, due to expansion of the vapor bubbles and the powder scattering from the crystalline gold particles at Q positions of the (111), (200) and further peaks. Additionally the secondary protein structure gives rise to a scattering distribution at intermediate Q (between the SAXS region, >0.3 Å⁻¹, and the liquid scattering peaks, <1.5 Å⁻¹), which is subject to changes, if the protein structure is altered. Examples are given in Figure 6, which displays the scattering difference due to laser excitation of different laser fluences at 40, 63, and 140 J/m², which relate to temperature rises according to Figure 5 of 80, 120, and 180 K, respectively, in the range where the bubble formation is important for the protein expulsion. A theoretical calculation is performed, using a BSA analogue human serum albumin, entry 1BJ5 in the Protein Data Bank.28 The atomic coordinates are obtained from this data set and computed into a scattering curve using the program package CRYSOL,24 which is basically a computation of pairwise atomic scattering contributions by the Debye formula.29 From this data a structureless SAXS curve has been subtracted, that
possesses the same radius of gyration and density. By this procedure a transition from a folded protein to a random coil of the same extension is mimicked. The theoretical protein scattering curve can be scaled to absolute units with a standard scaling relation at the high Q limit of the overall water scattering and with the use of the amount of adsorbed protein in the suspension. While this model would not properly describe the mesoscale structure in the SAXS region, it would still allow us to monitor the secondary structural motif. This difference structure is overlayed on the time-resolved WAXS curves in Figure 6. One directly can see that in particular of the long time delays of 50 ns and 1 μs the region between 0.9 and 1.5 Å⁻¹ does not show any difference signal. This region is governed by the helix peak and does not change with laser fluence (that is whether or not the protein shell is intact or expelled) as would be predicted for the protein denaturation.

For the short time delay at 350 ps and 2 ns only the transient features from the water compression and the Bragg peak changes of the gold particles can be seen. The water structure change would have a minimum around 1.7 and a maximum around 2.3 Å⁻¹ while the first gold reflections are seen at 2.67 and 3 Å⁻¹ as peak shift (positive and negative feature around the nominal powder rings) and as somewhat reduced crystallinity at the high laser fluence for longer delays. In conclusion the wide angle data do not show any changes in the structural motif during transient heating, or during this process of protein expulsion around the bubble formation threshold, which suggests that the expelled proteins are not denaturated in terms of secondary structure. At the highest fluence and 350 ps delay the compressed water signal is, however, higher than the expected protein helix peak, so that one cannot exclude here a short time denaturation during expulsion. The later data from 2 ns is again flat, which do not support this possibility.

CONCLUSION

Gold nanoparticle hybridized with proteins have been photoexcited and analyzed by time-resolved structural techniques, yielding the fastest events in structure formation. Such hybrids are fascinating and yet challenging systems owing to their different levels of complexity both in static structure and photoinduced dynamics. By using pump–probe X-ray scattering, a detailed structural insight in the suspension dynamics and the reaction of the protein shell is achieved. The protein shell can be expelled through the bubble formation process, when surpassing the water spinodal temperature around the heated gold particles. Except for the shell removal no further modifications on the proteins could be observed. It indicates that the protein is relatively unsusceptible to very high, but brief (sub-nanosecond) temperature rise. Longer laser pulses and repetitive excitation on a similar system have been shown to modify the protein structural integrity. The interaction of proteins with inorganic particles acting as local antennae for selective targeting of tissue in biophysical applications is gaining a large amount of interest. Complemented by optical techniques, such as time resolved spectroscopies, the presented structural information by ultrafast time-resolved X-ray scattering in the SAXS and WAXS region can provide a valuable contribution to the understanding of dynamics on multiple length scales.

METHODS AND MATERIALS

Pump–Probe Setup. Laser-pump X-ray-probe was performed at the dedicated beamline ID09B at the European Synchrotron Radiation Facility in Grenoble, France. The setup has been described in detail elsewhere. In brief, an amplified femtosecond laser system (KMLabs) delivers 100 fs pulses of 780 nm
light at a 1 kHz repetition rate. The light is frequency doubled and focused on the liquid jet to about 0.15 mm diameter (full width at half maximum, fwhm). Near-field enhanced ablation from the particles was avoided by stretching the laser pulse temporally to about 3 ps by detuning the compressor of the laser amplifier stage. An X-ray mechanical chopper wheel selects X-ray flashes from the storage ring in synchrotron to the laser pulses. The 100-ps long X-ray pulses are quasi-monochromatic with a 2.6% bandwidth and a peak wavelength of 0.849 Å and focused onto the liquid jet by a toroidal mirror to about 80 μm fwhm. The scattering is recorded on a CCD (charge coupled device) camera化进程的表示 (MareCo CD 133) which itself is not time resolving, but records the stroboscopic probe of the structural relaxation as a function of the well-defined time delays between the laser and X-ray pulses. Mutual time delay of the pulses is done with minimal step size of 5 ps by electronic delay units.

The reaction is considered as irreversible, and therefore any portion of the liquid has only been cycled once through the laser-X-ray interaction point and then disposed. The liquid was pumped through an open X-ray capillary at sufficient speed to form a free-flowing liquid jet at the orifice so that interference of the nonflowing particles at the wall with the SAXS signal is avoided. In particular it has been observed that protein-covered particles adsorb on the glass wall (in contrast to bare particles) and are hit multiply by laser pulses, if the capillary is placed in the beam. The scattering from the accumulated debris compromises the scattering distribution considerably. This problem is avoided by using the free jet.

X-ray Small Angle and Wide Angle Scattering. Small angle X-ray scattering probes density inhomogeneities in the sample, in the range of nanometers to hundreds of nanometers, depending on the instrumental resolution.43,44 (see the Supporting Information (SI)). The situation is strongly simplified for scattering objects with spherical symmetry, where for compact gold particles of spherical shape and radius \( R \) an analytical function can be given for the form factor:

\[
F(Q) = 3 \sin(QR) / (QR) \cos(QR) / (QR)^3
\]

which is a function that shows oscillations at distinct values of the scattering vector \( Q \) which scale with \( R \). The inverse of the particle size—core—shell systems can be modeled with a function based on this sphere form factor. A model-independent and useful quantity for the (difference) scattering yield \( S(Q) \) is the Porod invariant:

\[
P = \int_0^{\infty} S(Q)Q^2 dQ = 2\pi^2 \Delta \rho R^2 \Phi(1 - \Phi)
\]

as it allows the derivation of the global scattering length density contrast \( \Delta \rho \) change after laser excitation without the need for assumptions on particle shape and size. \( \Phi \) denotes the filling fraction of the particles total particle volume within the suspension. \( \Phi \) serves only as a scaling factor for the absolute scattering cross section and is not important here.

The wide angle scattering probes the positional correlation between all atoms in the suspension. This encompasses the structure of the protein molecules, where the most prominent feature to be observed is the helix peak from the helical domains within the protein. Meanwhile the liquid scattering of water dominates the scattering and therefore limits the achievable signal-to-noise ratio in the difference scattering \( S(Q) \) between the excited system at delay \( r \) and the non-excitised system. The bulk water structure responds to changes of temperature and pressure caused by the energy deposition in the absorbing nanoparticles and heat transfer to water. In particular pressure changes caused by expanding vapor bubbles are seen in A.S.

Importantly all the contributions to the scattering can be modeled quantitatively by using the protein atomic coordinates from the Protein Data Bank and scaling to the water scattering, thus allowing a quantitative prediction of the expected changes due to bubble formation, water pressure change, or protein denaturation. Details of the analysis procedure is provided in the Supporting Information.

Particle Conjugation. Gold nanoparticles were prepared by the aurophilic chemical synthesis method described by Turkevich and optimized for variable size distribution and higher particle density.45,46 Here we produced particles from a 1.6 mM gold hydrochlorate solution with an average diameter of 15 nm. The size dispersion was around 14%. The batches of gold particle suspension were shipped in polyethylene bottles to the beamline at ESRF and conjugated on site with mercaptoacetic acid (MCSA, Acros Chemicals, final concentration 0.08 mM) and bovine serum albumin (BSA, Roth, final concentration 3 μM). The MCSA is supposed to act as electrostatic linker between BSA and the gold surface and as spacer. In the Supporting Information more detail on the protein adsorption is given.

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Supporting Information Available: Additional information is available on the details of the models used for the description of the SAXS and WAXS signals; the procedure of BSA adsorption on gold nanoparticles; static scattering data from the BSA—nanoparticle hybrids; the circular dichroism measurements on BSA-coated gold particles and the definition of the reaction probability. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES AND NOTES


