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Intracellular optical probing with gold nanostars†

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Gold nanostars are important nanoscopic tools in biophotonics and theranostics. To understand the fate of such nanostructures in the endolysosomal system of living cells as an important processing route in biotechnological approaches, un-labelled, non-targeted gold nanostars synthesized using HEPES buffer were studied in two cell lines. The uptake of the gold nanostructures leads to cell line-dependent intra-endolysosomal agglomeration, which results in a greater enhancement of the local optical fields than those around individual nanostars and near aggregates of spherical gold nanoparticles of the same size. As demonstrated by non-resonant surface-enhanced Raman scattering (SERS) spectra in the presence and absence of aggregation, the spectroscopic signals of molecules are of very similar strength over a wide range of concentrations, which is ideal for label-free vibrational characterization of cells and other complex environments. In 3T3 and HCT-116 cells, SERS data were analyzed together with the properties of the intracellular nanostar agglomerates. Vibrational spectra indicate that the processing of nanostars by cells and their interaction with the surrounding endolysosomal compartment is connected to their morphological properties through differences in the structure and interactions in their intracellular protein corona. Specifically, different intracellular processing was found to result from a different extent of hydrophobic interactions at the pristine gold surface, which varies for nanostars of different spike lengths. The sensitive optical monitoring of surroundings of nanostars and their intracellular processing makes them a very useful tool for optical bionanosensing and therapy.

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Introduction

Gold nanostars, which are anisotropic nanostructures with sharp tips, are promising candidates for a wide range of biotechnological and theranostic applications. Important examples of their utilization are photothermal applications,^{1–4} sensing,^{5–7} and imaging.^{8,9} Their localized surface plasmon resonances can be tuned in the visible and near-infrared wavelength range by modifying their size and spike morphology.^{10,11} The dependence of optical properties of gold nanostars on their geometry is getting better understood.^{12–15} The high enhancement in optical signatures, not only particularly visible by the strong enhancement of Raman scattering and surface-enhanced Raman scattering (SERS)^{16,17} but also

from the metal-enhanced fluorescence,¹⁸ as well as their two-photon luminescence^{3,19} makes them the basis of efficient optical labels for imaging and multifunctional theranostic tools.^{20–22} The high SERS enhancement factors of gold nanostars allow their use in SERS tags with different label molecules in animal cells, *e.g.*, for the tracing of cells in co-cultures²³ and targeting of antigens on cancer cells,^{24–26} or of nucleic acid sequences.²⁷ Conjugated with molecules that serve as reporter species, SERS from immobilized gold nanostars was used to retrieve information on the pH²⁸ or the oxygen level²⁹ from the region of the cell membrane and inside the cells. Signals of molecules in the cell membrane were reported from nanostars that are brought into the proximity of the membrane.^{30,31} Recently, SERS imaging data also indicated the internalization of receptor-bound gold nanostars by the cells.⁹ As with other nanoparticles, their surface composition and size change the uptake, processing, and toxicity of nanostars as well.^{3,32,33} Detailed knowledge on their surface properties when inside different types of cells, *e.g.*, on processing in a harsh endolysosomal environment or regarding integration into membranous structures, is needed to develop and optimize theranostic and biotech applications.^{34,35} Since the processing of nanomaterials in cells is determined by the biomolecular interactions at their surface, the molecular infor-

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As will be shown, direct interaction of the biomolecules with the gold surface occurs when the nanostars reside in endolysosomal structures in cells and their interactions are driven by hydrophobic interactions of proteins and lipids. Based on nanotomography imaging together with simulations of the electromagnetic enhancement of SERS and its comparison with the spectral data, the characterization of intra-endolysosomal aggregates was undertaken. Data obtained for the two different cell lines will be discussed in order to delineate the extent up to which the details of the surface interactions of the gold nanostars are determined by the cell culture, the intracellular environment, and by the nanostar morphology. The results underpin the potential role of label-free gold nanostars as probes of cellular biochemistry for a wide range of theranostic applications.

Gold(III) chloride trihydrate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), adenine, and 4-mercaptobenzoic acid (pMBA) were purchased from Sigma-Aldrich (Steinheim, Germany). Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum (FCS), and phosphate buffered saline (PBS) were purchased from Biochrom (Berlin, Germany).

Swiss albino mouse fibroblast cell line 3T3 (DSMZ, Braunschweig, Germany) and human colorectal carcinoma cell line HCT-116 (LGC Standards, Wesel, Germany) were grown in DMEM supplemented with 10% FCS and maintained in a humidified environment at 37 °C with 5% CO₂. Cells were incubated for 24 hours with the dilution of gold nanostars in DMEM-FCS of 1:10 (estimated nanostar concentration 5×10^{-12} M). For SERS, cells were grown on glass cover slips, washed two times with phosphate buffer before measurements to eliminate the remaining culture medium and nanostars, and kept in PBS. For 3T3 cells incubated with 25 mM HEPES nanostars, 602 spectra displaying SERS signal from 8 cells were obtained; for 50 mM HEPES nanostars, 120 spectra were obtained from 4 cells; for 75 mM HEPES nanostars, 68 spectra were obtained from 3 cells. For HCT-116 cells incubated with

25 mM HEPES gold nanostars, 245 spectra from 17 cells were collected. The SERS spectra were also measured from the cell culture medium using the nanostars at a ratio of 1:10 in DMEM-FCS and incubated for 24 h at 4 °C. For cryo soft X-ray nanotomography, cells grown on Formvar-coated tomography gold grids and incubated with the nanostars for 24 h were washed three times with PBS, excess of the buffer was removed with a filter paper, and the grid with the cells was plunge-frozen using liquid ethane.

SERS experiments

The SERS spectra were obtained using a single-stage spectrograph equipped with a CCD detector (Horiba, Munich, Germany) and a diode laser operated at 785 nm (Toptica, Graefelfing, Germany). To focus the excitation light and collect the Raman scattered light in 180 degree backscattering geometry, a 10× objective was used for the nanostar suspension samples and a 60× water immersion objective (N.A. 1.2) was used for the experiments with the culture media and the cells. The excitation intensity was $3 \times 10^5 \text{ W cm}^{-2}$ and $8 \times 10^5 \text{ W cm}^{-2}$ in these two experiments, respectively. All the spectra were obtained with an acquisition time of 1 s. Considering the whole recorded spectral range from 400 to 1800 cm^{-1} , the spectral resolution was 4–7 cm^{-1} . All spectrum pre-processing and band occurrence calculations were done using Matlab R2018a (The MathWorks, Inc.) and Wolfram Mathematica 12 software, respectively.

Cryo soft X-ray nanotomography

Vitrified cell monolayers with a thickness of approximately 10 μm were examined using a transmission X-ray microscope equipped with a cryostage.⁴⁹ X-ray microscopy was performed at beamline U41-PGM1-XM⁵⁰ at the electron storage ring BESSY II (Helmholtz-Zentrum Berlin für Materialien und Energie, Berlin, Germany). The tilt series of the individual cells were acquired at different angle ranges in increments of 1° at a pixel size of 9.8 nm (25 nm zone plate objective) and a photon energy of 510 eV. Depending on the sample thickness, the exposure time was adjusted for each tilt angle series between 1 and 15 s. The tilt series consists of up to 130 images. Tomograms were obtained by alignment of the corrected tilt series and tomographic reconstruction using the Etomo software (IMOD© 4.9.0, Colorado, USA). The reconstruction of the sample volume was carried out by back-projection or simultaneous iterative reconstruction technique (SIRT). The images of the tilt series were aligned using the intracellular gold nanostars as fiducial markers.

Finite difference time domain (FDTD) simulations

Simulations were carried out with Lumerical FDTD Solutions 8.6.3 software using a mesh of 0.2 nm for all nanostructures. For excitation, a plane wave was used with a wavelength of 785 nm, propagating along the negative z-axis direction. The frequency-dependent dielectric function of gold was taken from Johnson and Christy⁵¹ and the refractive index of the surrounding medium was set to 1.33. All the output images are normalized to the intensity of the excitation source.

Results and discussion

Plasmonic properties and SERS enhancement

The gold nanostars for intracellular application were synthesized *via* a two-step process using the HEPES buffer, which is commonly used in biochemistry. The synthesis consisted of a first step where mainly the seeds are formed, followed by the addition of gold ions in the second step, thus allowing further growth of the anisotropic structures.⁴⁷ In the reaction, the piperazine ring generates nitrogen-centered free radicals that reduce the gold ions⁵² and has a weaker adsorption on the (111) planes of the gold nanocrystals, thereby allowing preferential growth in this direction.^{11,45} The nanostar suspensions presented blue or green color depending on the HEPES concentration used for their synthesis. Fig. 1A shows the absorbance spectra of the gold nanostars synthesized with HEPES at

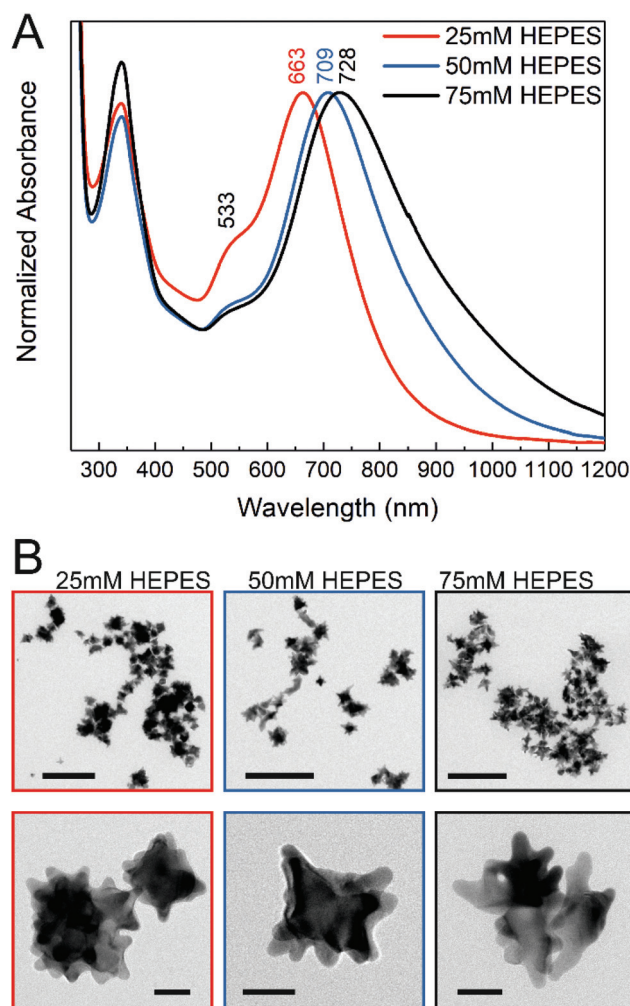


Fig. 1 (A) UV-vis-NIR spectra of gold nanostars synthesized with different HEPES concentrations. Maxima assigned to plasmon resonances are labeled for each nanostar condition. The maximum at 533 nm is common to all the spectra. (B) TEM micrographs of the nanostars. Color code is maintained. Scale bars: upper row 200 nm, lower row 20 nm.



The SERS enhancement of the gold nanostars was assessed using 4-mercaptobenzoic acid (pMBA) and adenine as the probe molecules under non-resonant excitation using a 785 nm laser. The spectra of pMBA (Fig. S3A†) are in good agreement with the spectra of this molecule obtained with other nanostar structures.^{17,53,56} The spectra obtained at different concentrations (Fig. S3A†) indicate that the orientation of pMBA on the metal surface does not change with altered concentration.⁵⁷ The intensity of the pMBA ring breathing SERS signal at 1078 cm⁻¹

Figure 1 is a scatter plot with error bars showing the SERS signal (cps) at 1078 cm⁻¹ as a function of pMBA concentration (M) for three different HEPES buffer concentrations: 25mM (red), 50mM (blue), and 75mM (black). The x-axis is logarithmic, ranging from 1E-8 to 1E-6 M. The y-axis ranges from 0 to 250 cps. The signal increases with concentration and plateaus at approximately 10⁻⁶ M. The 50mM HEPES buffer consistently yields the highest SERS signal across the measured concentration range.

pMBA Concentration (M)	25mM HEPES (cps)	50mM HEPES (cps)	75mM HEPES (cps)
1E-8	~20	-	-
~3E-8	~25	~35	~30
~5E-8	~40	~50	~45
~7E-8	~60	~75	~65
~1E-7	~100	~125	~110
~2E-7	~170	~210	~190
~5E-7	~220	~230	~225
~1E-6	~220	~215	~220
~2E-6	~225	~210	~210

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induces the formation of nanostar aggregates very efficiently and the major role of the electromagnetic enhancement as the contributor to SERS in these samples. The different signals must be a consequence of different properties of the aggregates, *e.g.*, due to a different spatial arrangement of the nanostars in the aggregates. They could be related to the slightly different morphologies of the individual nanostars in the different preparations as well as the HEPES concentration in the solution.

The formation of aggregates of the nanostars in cells is a very important aspect in their application, since processing and endolysosomal fusion and maturation take place.³⁷ To obtain an idea on the impact of aggregate formation of the nanostars on the distribution of the local optical fields that are responsible for the electromagnetic enhancement in SERS, we performed 3D-FDTD calculations on simplified geometrical models of the nanostars. In Fig. 4, the distribution of the intensity enhancement of the excitation field with respect to that of the incoming field at a wavelength of 785 nm, as used in the experiments here, is shown for nanostars with slightly different morphology. Similar to the samples in the experiment (*cf.* Fig. 1B and Fig. S1†), the model considers structures of a similar outer diameter but the spikes and the core have different sizes. Comparing the two geometries (Fig. 4A and B), the longer spikes give rise to high field intensity at their tips that will lead to a higher SERS intensity. With shorter spikes, the field intensity at the tips of the spikes is smaller in comparison, which is in full agreement with previous reports.^{10,12,13} There is also higher enhancement at the base of the longer spikes, close to the core (Fig. 4B). This supports the experimental data shown in Fig. 3, where a higher signal is observed for nanostars with longer tips. When considering two nanostars separated by a small gap (Fig. 4C and D) as a simple model for agglomeration, *e.g.*, in the cellular context, the localization of a high field intensity in the gap between the nanostars with shorter spikes is found (Fig. 4C) for the polarization of incident light along the long axis of the nanostar dimer, exceeding the intensity around the individual nanostar by ~ 3 orders of magnitude (compare Fig. 4A with Fig. 4C). This is similar to the distribution of the field around the dimers of spherical particles of the same size, showing the formation of a hot spot between the particles,^{63,64} as also confirmed in Fig. S6† for spherical nanoparticles of the same outer diameter. The field enhancement has a lower value and remains evenly distributed around the tips of the nanostars when the spikes are longer, with only a slightly higher field in the gap between those spikes that are aligned in the propagation direction of the excitation wave (Fig. 4D). The intensity values for the electric field in the gap between the particles with the longer spikes (Fig. 4D) show similar values to the values in the gap between the smooth gold spheres (Fig. S6†). The different possibilities of the tip-to-tip and tip-to-core plasmonic coupling of the nanostars in close proximity and the extent to which modes other than localized surface plasmon modes influence the spectrum when small changes occur in nanostar geometry¹³ can lead to a strong variation in the field

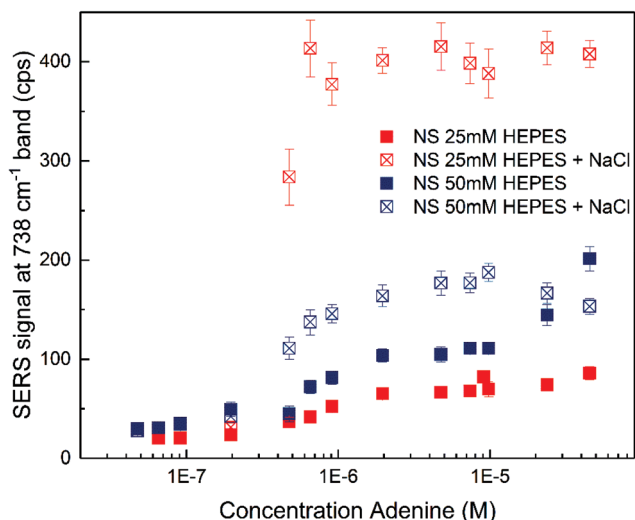


Fig. 3 SERS signal of the 738 cm^{-1} band in the spectrum of adenine as a function of adenine concentration for nanostars synthesized with different HEPES concentration in the presence and absence of NaCl. Each signal value was obtained by averaging 30 spectra; the error bars represent the standard deviation.

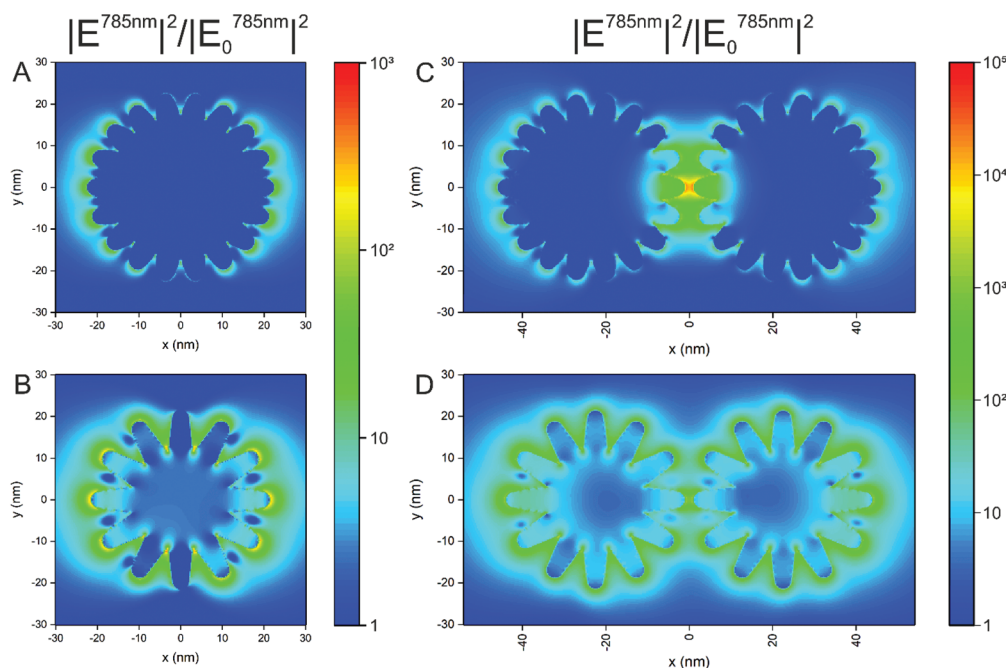


Fig. 4 Electric field intensity distribution (normalized to the intensity of the incident field) at 785 nm in the *xy* plane as obtained by FDTD simulation for (A) a single nanostar with 15 nm radius of the core and 7.5 nm long spikes, (B) a single nanostar with 10 nm radius of the core and 11.5 nm long spikes, (C) two nanostars with 15 nm radius of the core and 7.5 nm long spikes separated by a 2 nm gap, and (D) two nanostars with 10 nm radius of the core and 11.5 nm long spikes separated by a 2 nm gap. The excitation wavelength was set to 785 nm. The polarization of the incident plane wave was in the *x* direction. The monitor was placed in the equatorial plane. Common scale bar for (A) and (B) (middle), and common scale bar for (C) and (D) (rightmost).

distribution and the performance of the nanostars in an actual SERS experiment that uses more heterogeneous structures. Here, the small differences in the signal that occur when particles with longer spikes are aggregated by an external agent (Fig. 3, filled and open blue markings) are in good agreement with the smaller increase in the field intensity found for dimers of such structures compared to single particles (compare Fig. 4B and Fig. D). In contrast, the signal observed for the short-spike nanostars increases considerably not only compared to single particles (Fig. 3, open and filled red markings) but also to aggregated stars with longer spikes (Fig. 3, open red and blue markings), which is explained by the hot spots that are obtained in the gaps between the nanostars that have shorter spikes (Fig. 4C).

Biomolecular interactions at the nanostar surface in living cells

Three different nanostar samples were incubated with cells of the fibroblast cell line 3T3 by adding them to the cell cultures. When immersing them in the typical cell culture medium, here DMEM-FCS, the intensity of the signals ascribed to the HEPES at the surface diminishes, band ratios observed in the pristine nanostars spectra change, and bands that can be assigned to protein components in the culture medium become visible (compare Fig. S2A and S2B†). This indicates that the capping groups of the nanostars are easily interchangeable with the biomolecular content of the cell culture medium or other environments, such as their immediate cellu-

lar surroundings. Rather than being determined by the original capping agent, the uptake of the nanostars by the cells is therefore dictated by the acquired biomolecular corona, whose structure and modifications inside the cells can be characterized by SERS.^{38,41}

To study the nanostar–biomolecule interactions in the living cells, the SERS spectra were obtained after incubation of the cell cultures with gold nanostars for 24 h. After this incubation time, endolysosomes of many different maturation stages are known to form, which are distributed across the cytoplasm.³⁷ The average spectra with the nanostars synthesized at different HEPES concentration differ (Fig. 5A–C). To account for the known variability between the individual spectra from the same cell culture batch that is expected due to (i) the biological variation, (ii) the particular local composition in the environment of many different nanostars within each probed cell, and (iii) the variation that is inherent to each SERS experiment,^{42,65} the occurrence of bands in each individual spectrum was analyzed regardless of their absolute or relative intensities (Fig. 5D–F). The simultaneous analysis of the average spectra and the band occurrences is helpful to draw conclusions on the interactions between the biomolecules and particular types of nanoparticles, here, the nanostars of different preparations and their effect on cellular processing.³⁸ Many similarities are found that are indicative of a direct interaction of the proteins at the hard corona–gold interface of the nanostars with the occurrence of bands in the SERS spectra



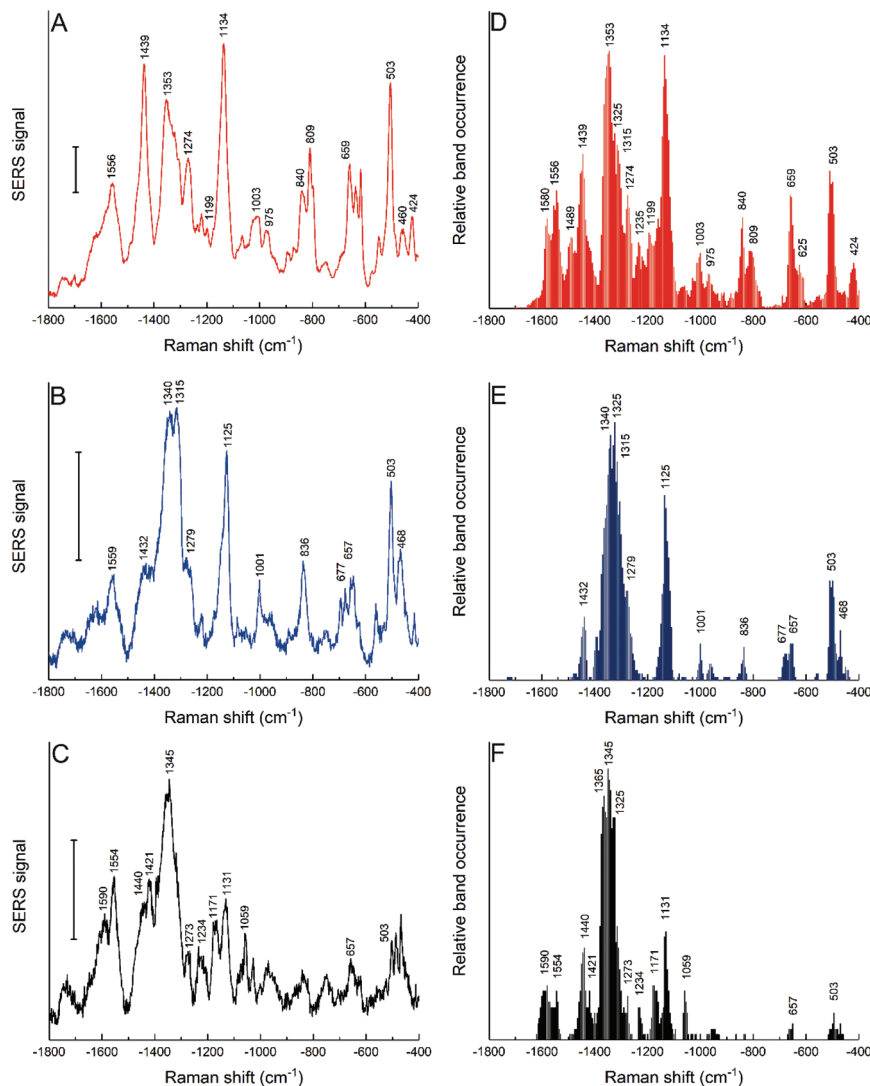


Fig. 5 SERS average spectra (left column) and relative band occurrence (right column) for 3T3 cells incubated for 24 h with gold nanostars synthesized with 25 mM HEPES (A and D), 50 mM HEPES (B and E) and 75 mM HEPES (C and F). Tentative band assignments are provided in Table S2.† Scale bars (A–C): 10 cps.

measured in cells incubated with spherical, citrate-stabilized gold nanoparticles.³⁸ The signals mainly attributed to proteins observed in all the cases are expected because of the known formation of a protein corona in the culture medium and its processing in the cells.⁴¹ The tentative assignments of the bands are given in Table S2.†

The intensity ratios for some bands change in the average spectra (Fig. 5A–C), which is in agreement with the frequency of finding the bands in the individual spectra (Fig. 5D–F). The results suggest that the biomolecules or functional groups in the surroundings of the nanostars inside the cells are similar but interact differently with the nanostars of each of the samples. As a first very obvious difference, the interaction of proteins with gold nanostars by their aromatic side chains must be named. Typical bands assigned to tryptophan at 1353 cm^{-1} and 424 cm^{-1} occur only in the spectra of the nanostars made at the lowest HEPES concentration of 25 mM

(Fig. 5D). Those of tyrosine, *e.g.*, represented by a characteristic signal at about 840 cm^{-1} in the data sets as well as the pronounced ring breathing vibration of phenylalanine at 1003 cm^{-1} are also obtained with the nanostars prepared at 50 mM HEPES concentration (Fig. 5D and E). However, no signals assigned to these amino acids appear in the spectra of the cells incubated with nanostars synthesized at 75 mM HEPES concentration (compare Fig. 5F with Fig. 5D and E), indicating that they are not located in the proximity of the gold surface due to different protein structure or interaction. Secondly, bands that are assigned to S–S stretching ($\sim 503 \text{ cm}^{-1}$) and C–S stretching modes ($\sim 650 \text{ cm}^{-1}$) that could be associated both with an intact secondary structure of the adsorbed proteins⁶⁶ as well as to the fragmentation of proteins in the corona of gold nanoparticles,⁴¹ depending on particular features, are found much more rarely in these samples as well (compare Fig. 5F with Fig. 5D and E). As a third element,



Such interfacial interactions can be related to the processing of gold nanoparticles by the cells³⁸ since the protein corona plays an important role as mediator between the pristine nanostructure and the cellular environment. The SERS data obtained with the nanostars here also support this. Since the nanostars carry the same molecular species at their surface when they are immersed in the cell culture medium regardless

The cells of the HCT-116 cell line in the same culture medium were also incubated with the nanostars obtained with the reduction of 25 mM HEPES, and both the average spectra (Fig. 6A) and the band occurrences were analyzed in all the individual spectra (Fig. 6B). In comparison to the spectra obtained with 3T3 cells with these nanostars (Fig. 5A and D), the bands assigned to aromatic rings from hydrophobic amino acid side chains at 1357 cm^{-1} , 424 cm^{-1} , 1003 cm^{-1} , and 840 cm^{-1} are much less abundant or are not shown at all (Fig. 6). A smaller frequency of occurrence is also observed for bands associated with C-S and S-S stretching modes of $\sim 650\text{ cm}^{-1}$ and $\sim 503\text{ cm}^{-1}$, respectively, which are found less often (compare Fig. 5D and 6B). This indicates that these functional groups interact less frequently with the nanostar surface in the HCT-116 cells than in the 3T3 fibroblasts, probably as a



To obtain an insight into the processing of the nanostars by the cells of both cell lines and to observe their interactions at the ultrastructural level, 3T3 fibroblast and HCT-116 cells were

incubated with the nanostars and plunge-frozen for analysis by soft X-ray nanotomography. In the samples of the 3T3 cells (Fig. 7A and S7A†), the nanostars are contained inside well-defined vesicular structures that are both single and multilamellar, indicating typical endolysosomal processing, and in many cases, are in close contact with the vesicles' membranes. The localization within and close to the membranous structures supports the observation of hydrophobic interactions of the proteins at the nanostar surface and of SERS signals that point directly towards the interaction of lipids with the gold in the 3T3 cells (Fig. 5). Since the uptake of nanostars is continuous throughout the 24 h incubation period, both individual nanostars and agglomerates are expected to be found inside the cells. Even though some single particles can be observed (Fig. 7A), a majority of the nanostars are a part of agglomerates of sizes between 100 and 450 nm (corresponding to ~10–1000 individual nanostars) without a particular spatial arrangement, which is in agreement with the strong SERS signals that were obtained for all the cells. These arrangements are similar to those found for spherical gold nanoparticles^{37,38} that, unlike the linear arrangements of silver nanostructures,⁷⁰ form agglomerates with their biomolecular environment. Interparticle gaps are visible, which indicate a loose configuration of the individual stars in the agglomerates in both the cell lines (Fig. 7A and B). No nanostars were found inside the nucleus or free in the cytosol, which indicates that during the incubation period, the nuclear membrane is not crossed by the particles and that the particles have not escaped the endolysosomal vesicles. In HCT-116 cells (Fig. 7B and S7B†), the distribution of particle accumulations is clearly different, with agglomerates larger in size (between 200 nm and 800 nm, corresponding to ~90–5700 individual nanostars) than those observed for the 3T3 cells (Fig. 7A and S7A†). In the HCT-116 cells, there are also single particles distributed in the cytoplasm, indicating that the nanostructures are endo-

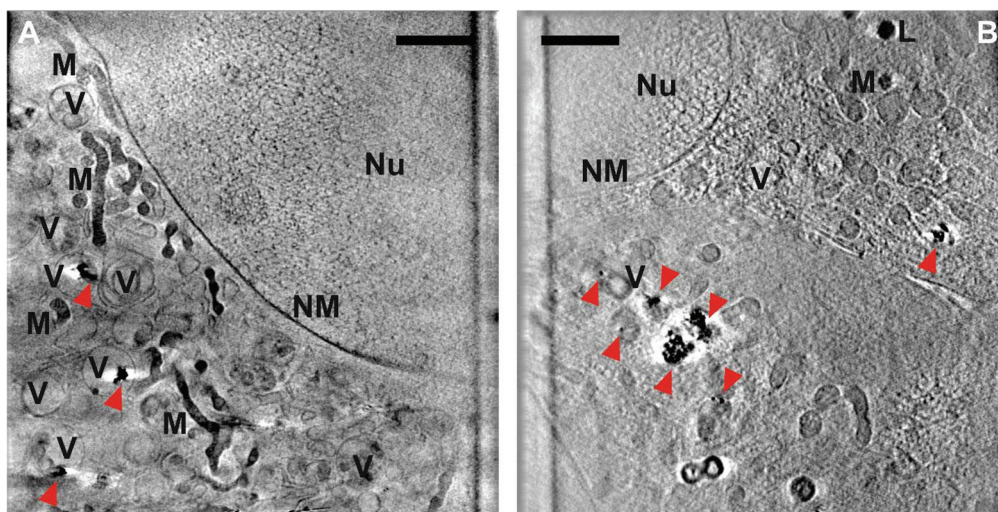


Fig. 7 Slices of X-ray tomographic reconstruction of (A) 3T3 and (B) HCT-116 cells incubated for 24 h with gold nanostars synthesized with 25 mM HEPES (cf. Fig. 1B). The red arrows indicate single particles or aggregates of nanostars. More examples are provided in Fig. S7.† Abbreviations: Nu, nucleus; NM, nuclear membrane; V, vesicles; M, mitochondria; L, lipid droplets. Scale bars: 2 μ m.

cytosed as individual nanoparticles, similar to other gold nanostructures.^{37,71,72}

The cellular compartments can be clearly visualized in the tomographic reconstructions, such as vesicular bodies, mitochondria, and lipid droplets. For both cell lines used, neither of the sampled cells show an obvious indication of cell death, such as dilated perinuclear cisternae,⁷³ suggesting that the uptake of nanostars does not induce adverse cellular responses. This is interesting, as the sensitivity of the HCT-116 cells towards incubation with citrate-stabilized gold nanoparticles in the same culture medium was recently demonstrated.³⁸ The absence of indicators of cell death here may be due to the different shape and specific structure and interactions of the corona of the nanostars, as revealed by the SERS spectra.

Conclusions

The cellular processing of un-labelled, non-functionalized gold nanostars synthesized by a mild, biocompatible method was studied, and their optical properties were assessed *via* experiments and by theory under conditions that resemble the situation in living cells. As evidenced by X-ray nanotomography, the uptake of single nanostars results in their processing through the endolysosomal pathway, where they are prone to aggregation, without the evidence of endosomal escape. The number and length of spikes were found to vary slightly for nanostars prepared at different concentrations of HEPES, at a very similar outer diameter of the nanostructures. While the enhancement of Raman scattering in non-resonant SERS experiments was very similar in the absence of aggregation of the nanostars, the optical properties of aggregates formed by the nanostars yielded very different effects, with higher enhancements found for nanostars of shorter spike length. The experimental data obtained with probe molecules outside the cells are in good agreement with the results of FDTD simulations under excitation conditions that are similar to those in the SERS experiments conducted in live cells. Non-resonant SERS spectra were obtained both in the presence and absence of aggregation, and under both conditions, the spectroscopic signals of the molecules are of similar magnitude over a wide range of concentrations, which is ideal for label-free SERS in cells and other complex environments. As indicated by the formation of nanostar aggregates in the endolysosomal system of 3T3 and HCT-116 cells, the high local fields in the aggregates of nanostars with shorter spikes found in the experimental and theoretical model must contribute significantly to the high SERS signals that were obtained from the endolysosomal system in the live cells. Understanding the endolysosomal processing and intra-endolysosomal aggregate formation has implications for the application of the nanostars in photothermal therapy and drug delivery.

The processing of nanoparticles and their surface species was studied by the qualitative analysis of SERS spectra incubated with the cells, independent of the (quantitative) enhancement of the Raman signals. A comparison of the bio-

molecular environment of the nanostars of slightly different geometry in the same cell line (3T3) indicated differences in the structure and interaction of the proteins in the hard corona of the nanoparticles, and also a different interaction with the membranous structures in the endolysosomal compartment. The very similar SERS data obtained in the cell culture medium with three different nanostar preparations, yet the very different spectral patterns in the endolysosomal system leads us to conclude that the specific molecular environment observed inside the cells is a consequence of post-endocytotic processing. In agreement with the important function of the protein corona to mediate between the physical properties of the pristine gold surface and the processing in the cellular ultrastructure,³⁸ the characteristic structure and interaction at the nanoparticle surface, which is evident from the spectra, are likely the result of small differences in the nanostar shape and geometry.

A comparison of the surface molecular structure and nanoparticle processing in the 3T3 and HCT-116 cell lines indicated differences in the size and density of the intra-endolysosomal nanostar agglomerates, connected to a different extent of hydrophobic interactions at the nanostar surface, which must be further elucidated with suitable lipid model systems as they will be important for the development of theranostic applications using gold nanostars. The similarities observed in the interacting functional groups and the structure of the protein corona of gold nanostars with that of the spherical gold nanoparticles in a previous study in the HCT-116 cell line³⁸ indicates the importance of similar, cell line-specific processing pathways that could also be related to the initial structure and processing of their protein corona.

Conflicts of interest

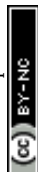
The authors declare no conflicts of interest.

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References

- 1 I. Baginskiy, T.-C. Lai, L.-C. Cheng, Y.-C. Chan, K.-Y. Yang, R.-S. Liu, M. Hsiao, C.-H. Chen, S.-F. Hu, L.-J. Her and D. P. Tsai, *J. Phys. Chem. C*, 2013, **117**, 2396–2410.



- 47 S. Saverot, X. Geng, W. Leng, P. J. Vikesland, T. Z. Grove and L. R. Bickford, *RSC Adv.*, 2016, **6**, 29669–29673.
- 48 C. A. Schneider, W. S. Rasband and K. W. Eliceiri, *Nat. Methods*, 2012, **9**, 671–675.
- 49 G. Schneider, P. Guttman, S. Heim, S. Rehbein, F. Mueller, K. Nagashima, J. B. Heymann, W. G. Muller and J. G. McNally, *Nat. Methods*, 2010, **7**, 985–987.
- 50 P. Guttman, S. Werner, F. Siewert, A. Sokolov, J.-S. Schmidt, M. Mast, M. Brzhezinskaya, C. Jung, R. Follath and G. Schneider, *Microsc. Microanal.*, 2018, **24**, 206–207.
- 51 P. B. Johnson and R. W. Christy, *Phys. Rev. B: Solid State*, 1972, **6**, 4370–4379.
- 52 A. Habib, M. Tabata and Y. G. Wu, *Bull. Chem. Soc. Jpn.*, 2005, **78**, 262–269.
- 53 C. G. Khoury and T. Vo-Dinh, *J. Phys. Chem. C*, 2008, **112**, 18849–18859.
- 54 H. de Puig, J. O. Tam, C. W. Yen, L. Gehrke and K. Hamad-Schifferli, *J. Phys. Chem. C*, 2015, **119**, 17408–17415.
- 55 W. Xi and A. J. Haes, *J. Am. Chem. Soc.*, 2019, **141**, 4034–4042.
- 56 Y. Liu, H. Yuan, A. M. Fales and T. Vo-Dinh, *J. Raman Spectrosc.*, 2013, **44**, 980–986.
- 57 A. Michota and J. Bukowska, *J. Raman Spectrosc.*, 2003, **34**, 21–25.
- 58 J. Kneipp, H. Kneipp, B. Wittig and K. Kneipp, *Nano Lett.*, 2007, **7**, 2819–2823.
- 59 B. Giese and D. McNaughton, *J. Phys. Chem. B*, 2002, **106**, 101–112.
- 60 E. Papadopoulos and S. E. J. Bell, *J. Phys. Chem. C*, 2010, **114**, 22644–22651.
- 61 F. Madzharova, Z. Heiner, M. Gühlke and J. Kneipp, *J. Phys. Chem. C*, 2016, **120**, 15415–15423.
- 62 V. Joseph, A. Matschulat, J. Polte, S. Rolf, F. Emmerling and J. Kneipp, *J. Raman Spectrosc.*, 2011, **42**, 1736–1742.
- 63 F. Madzharova, Z. Heiner, J. Simke, S. Selve and J. Kneipp, *J. Phys. Chem. C*, 2018, **122**, 2931–2940.
- 64 P. Nordlander, C. Oubre, E. Prodan, K. Li and M. I. Stockman, *Nano Lett.*, 2004, **4**, 899–903.
- 65 H. Xu, J. Aizpurua, M. Käll and P. Apell, *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top.*, 2000, **62**, 4318–4324.
- 66 G. P. Szekeres and J. Kneipp, *Analyst*, 2018, **143**, 6061–6068.
- 67 V. Živanović, Z. Kochovski, C. Arenz, Y. Lu and J. Kneipp, *J. Phys. Chem. Lett.*, 2018, **9**, 6767–6772.
- 68 J. Broda, J. Setzler, A. Leifert, J. Steitz, R. Benz, U. Simon and W. Wenzel, *Nanomedicine*, 2016, **12**, 1409–1419.
- 69 S. Salassi, F. Simonelli, D. Bochicchio, R. Ferrando and G. Rossi, *J. Phys. Chem. C*, 2017, **121**, 10927–10935.
- 70 D. Drescher, P. Guttman, T. Buchner, S. Werner, G. Laube, A. Hornemann, B. Tarek, G. Schneider and J. Kneipp, *Nanoscale*, 2013, **5**, 9193–9198.
- 71 M. Liu, Q. Li, L. Liang, J. Li, K. Wang, J. Li, M. Lv, N. Chen, H. Song, J. Lee, J. Shi, L. Wang, R. Lal and C. Fan, *Nat. Commun.*, 2017, **8**, 15646.
- 72 X. Xie, J. Liao, X. Shao, Q. Li and Y. Lin, *Sci. Rep.*, 2017, **7**, 3827.
- 73 M. K. Sakamoto, S. Mima, T. Kihara and T. Tanimura, *Anat. Rec., Part A*, 2004, **279**, 652–663.