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ARTICLE

Influence of Surface Coating on the Intracellular Behaviour of Gold Nanoparticles: A Fluorescence Correlation Spectroscopy Study

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In biomedical applications of nanoparticles (NP), the proper choice of surface chemistry is a crucial aspect in their design. The nature of the coating can heavily impact on the interaction of NPs with biomolecules, affect the state of aggregation, and ultimately determine their biological fate. As such, protein corona formation, and the aggregation behaviour of gold NPs (Au NPs) is studied here. Au NPs are prepared with four distinct surface functionalisations, namely: mercaptosuccinic acid (MSA), N-4-thiobutyroil glucosamine, HS-PEG_{5000} and HS-Alkyl-PEG_{600}. Corona fomation, aggregation, and the intracellular behaviour of the Au NPs are then investigated by means of Fluorescence Correlation Spectroscopy (FCS) in cell culture media and in live cells. To evaluate the state of aggregation, and the formation of a protein corona, the Au NPs are incubated in cell media and the diffusion coefficient determined via FCS. The *in vitro* behaviour is compared with the level of aggregation of the NPs in cells. Diffusion times of the NPs are estimated at different positions in the cell after a one hour incubation period. It is found that the majority of MSA and Glucose- Au NPs are present inside the cell as slowly diffusing species with diffusion times (τ_D) greater than 6000 µs (hydrodynamic diameter >250 nm). PEGylated Au NPs adsorb a small amount of protein and manifest low agglomeration both in media and in living cells. In particular, the HS-Alkyl-PEG_{600} coating shows an excellent correlation between lower protein adsorption, 4-fold lower compared to the MSA coated NPs, and limited intracellular aggregation. In the case of single HS-Alkyl-PEG_{600} coated NPs it is found that typical intracellular τ_D range from 500-1500 µs,indicating that these particles display reduced aggregation in the intracellular environment.

Introduction

In biomedical applications the surface coating of nanoparticles (NPs) is usually designed to minimise unspecific interactions, increase circulation, or to promote targeted delivery of the NPs to specific organs and cells, where they fulfill their therapeutic function. The biological fate, therapeutic efficacy, and toxicity of NPs are determined largely by their surface chemistry.[1-3]

In the biological environment NPs interact with surrounding biomolecules, resulting in the formation of a corona around the NPs, which can confer a new "biological identity" to the NPs[4-6] This new interface should then determine the interaction of the NPs with other biomolecules or cells. Often, the formation of a corona causes NP aggregation, but it can also reduce aggregation by stabilizing single NPs; thereby either enhancing or reducing cell uptake,[7] and effectively influencing their biodistribution and intracellular trafficking.[8-10] Moreover, the aggregation of NPs *in vivo* is often not desired and may lead to an accumulation in the lungs[11] or to their clearance by the mononuclear phagocyte system (MPS) thus, preventing the NPs from reaching a specific organ. In biomedical applications, it is crucial to avoid the unspecific NP accumulation in undesired organs, maintaining a prolonged circulation time and efficient renal clearance.[12]

Surface functionalisation is a common strategy to increase the circulation time and minimise unspecific cellular uptake of, NPs.

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The surface coating may consist of molecules characterised by low protein affinity thereby minimising protein corona formation and aggregation. There are several coatings currently available: glycans, polyethylene glycol (PEG), zwitterionic surfactants, *etc*. of which PEG is most commonlyfunctionalise used. PEG is associated with a higher affinity to water but low or no net charge that inhibits protein binding [13-16]. For PEG antifouling properties largely depend on PEG molecular weight andorientation on the surface. The orientation can be modified by placing a spacer between the NP surface and the PEG moiety. The proper choice of PEG ligand is therefore fundamental for achieving an effective antifoulingcoating. Several studies have been performed on PEG including; the influence of its molecular characteristics, the orientation/arrangement on NP, and its interaction with proteins.[7, 17, 18] However, little is known about its impact on the state of aggregation of NPs *in vitro* or in the intracellular environment.

An important issue in the design of a proper antifouling coating is to understand to what extent the antifouling character provided by the coating, is preserved upon NP uptake. Following uptake, NPs are often confined to acidic vacuoles with their trafficking in cells being affected by the interaction with the various biomolecules and cell components. All these interactions can affect the final NP surface composition, as well as the state of aggregation.[19, 20]

Fluorescence Correlation Spectroscopy (FCS) is a technique based on recording fluorescence fluctuations in a confocal volume, related to the diffusion of the fluorescent molecules. Diffusion times can be corelated to the size of the diffusing species, and also to the local micro-environmental conditions,i.e. local viscosity and temperature.[21-23] In recent years, FCS has frequently beenused to study the hydrodynamic size of NPs in different biological media[24-26] and to characterise protein corona structure and composition.[27, 28] In particular, by means of FCS it is possible to characterise the NP-protein affinity constants,[29, 30] the effect of different ligands on the protein coronaformation[31, 32] and the arrangement of some proteins around the NP surface.[33] Murray *et al.*[34] studied the intracellular dynamics of glucose functionalised Au NPs by means of FCS In this study it was shown that glucose functionalised NPs were not only present in intracellular vesicles forming large aggregates, but were also found to be located in the cytosol as a small homogeneousNPs with protein corona population or as small NPs aggregates.

In the work presented here, we aim to study the influence of the molecular characteristics of different ligands on gold (Au) NPs *in vitro*, evaluating the state of aggregation *in vitro* and during intracellular trafficking. Our aim is to understand whether or not the properties, conferred by the ligands, are retained by the NPs following uptake. We hope that these findings will aid in the future design of more efficient, target specific intracellular probes. Here we present a study of NPs with four distinct surface coatings, namely: 1) a long chain PEG (MW 5 KDa), PEG_{5000}; 2) an amphiphilic polymer composed of a short PEG (MW 600 Da) and an alkyl chain, Alkyl-PEG_{600}; 3) mercaptosuccinic acid; 4)N-4-thiobutyroil glucosamine. The two low molecular weight ligands are widely used as stabilising agents in aqueous solutions. *In vivo,* they confer a certain ability to reduce NP clearance by macrophages.[35-37] PEG_{5000} is widely used as an antifouling agent for NPs and surfaces,[38] while the Alkyl-PEG_{600} is interesting due to the simultaneous presence of an hydrophilic and an aliphatic chain which yields tighter ligand packing.[39] NP protein corona formation and aggregation in several media and stability as a function of time were studied by FCS, using properly labelled NPs. *In vitro,* FCS results were validated by transmission electron microscopy (TEM), dynamic light scattering (DLS) and UV-Vis spectroscopy. Finally, NP internalisation was tracked by FCS and confocal laser scanning microscopy (CLSM). Finally, four regions of interest were identified in the cells, within which FCS was employed to estimate the state of aggregation and the effect of the choice of ligand.

Results and discussion

in vitro Characterisation of NPs

Au NPs are widely employed in biomedical applications due to their high degree of biocompatibility. Au can form stable bonds with sulfur thereby offering multiple possibilities for functionalisation which, open various avenues for imaging, targeting, and to modify NPs via the use of thiols. In this work, Au NPs were produced according to a reported “one-pot” synthesis which, allows good control ove size and shape.[40] Four different molecules were selected to functionalise the Au NP surface (see Figure. 1): mercaptosuccinic acid, N-4-thiobutyroil glucosamine, HS-PEG_{5000}, and an amphiphilic HS-alkyl-PEG_{600} chain. Additionally, a fluorescent dye was attached to the NP surface to facilitate fluorescence studies (see Figure. 2a). A detailed description of all ligands and Au NPs synthetic procedures is included in the supporting information (sections S1†-S3†). NPs were characterised by means of Transmission Electron Microscopy (TEM), UV-vis spectra, and Dynamic Light Scattering (DLS). TEM showed that Au NPs range in diameter from approximately 14-22 nm (Fig. 2b). ATTO550 was chosen as fluorescent molecule, being characterised by a strong absorption, high fluorescence quantum yield and high thermal and photo-stability. HS-PEG_{5000}-NH_2 was introduced on the NP coating to covalently link ATTO550 and, as the dye is moderately hydrophilic and adds a positive charge to the coupled molecule, only 10% of the total ligand amount was substituted by the amino-PEG (Fig. S9-12). In the following discussion NPs will be distinguished in not fluorescent NPs: Au-MSA NPs, Au-Glucosamine NPs, Au-Alkyl-PEG_{600} NPs, and Au-PEG_{5000} NPs; and fluorescent labelled NPs: Au-MSA* NPs, Au-Glucosamine* NPs, Au-Alkyl-PEG_{600}* NPs, and Au-PEG_{5000}* NPs.

Table S5-7 show the hydrodynamic diameters of the AU NPs, for each of the four surface modifications used, as determined by three separate methods;: FCS, DLS and UV-vis (This reveals that the largest diameter particle is seen to be Au PEG_{5000} while the gluosamine Au NPs are seen to have the smallest diameter. This stands to reasonas the long hydrophilic PEG chains become

extended in aqueous solution. In the case of glucosamine it is likely that the ligand density is sufficiently low that it remains relatively unextended. The two other surface coatings showed relatively similar hydrodynamic diameters of 8 and 9 nm.

RPMI 1640 and full RPMI 1640 enriched with 10% fetal bovine serum (FBS) are commonly used media for cell culture. When NPs are exposed to these media, NP aggregation and the formation of a protein corona usually occur.[42] These events affect cellular uptake of NPs and highlight the need to better understand and characterise NPs in such environments. FCS is particularly powerful in this regard because of the possibility to perform experiments *in situ*.

Figure 3 shows FCS data with different correlation functions for the free dye, proteins and NPs. From the correlation functions we are able to discriminate even the variation in Au NPs HD in presence of FBS proteins.

In Fig. 4a we report the estimated hydrodynamic diameters for NPs dispersed in water, RPMI, and full RPMI. Given the dynamic nature of theprotein corona formation, we followed its evolution in a time window of one hour.[3, 9, 35,43] We chose to operate in this time window as it has been described as the most dynamic, where the majority of the protein-NP interactions take place. Au-MSA* NPs and Au-Glucosamine* NPs showed a significant increase in hydrodynamic diameter (>20 nm) in RPMI compared to water (Fig. 4a). This effect can be ascribed to a charge shield due to the high ionic strength of the media and to the presence of small molecules (i.e. lipids, sucrose), which can induce NP aggregation. Moreover, the high standard deviation suggests the formation of aggregates with heterogeneous dimensions. The effect of the medium observed on Au-Alkyl-PEG_{600}* NPs and Au-PEG_{5000}* NPs was almost negligible, excluding NP aggregation and suggesting a modification of the hydrodynamic shells caused by salts and small molecules present in the medium.

When the FBS solution is added to the NPs in RPMI (full RPMI), a reduction in hydrodynamic diameter is observed.. The addition of proteins by simple pipette mixing seems to promote disassembling of NP aggregates in favour of smaller, more stable NP-protein complexes. The effect is appreciable after just 5 minutes of incubation, in accordance with previously reported observations in literature.[9] This results primarily as a consequence of the electrostatic and steric stabilization of the NPs being enhanced by protein adsorption.[42] Once formed, the protein corona evolution was followed over time. However, in terms of diffusion times and derived hydrodynamic diameters, only

After this initial screening process, the Au NPs were then incubated with FBS, and the hydrodynamic diameters remeasured, in order to observe possible protein corona formation. Au-MSA* NPs were observed to increase in diameter by approximately 7 nm following a 1 hour incubation period (p-value <0.05). This may be attributable to protein corona formation however, it cannot be confirmed. The increase in diameter observed for Au-Glucosamine* NPs is not significant and can be explained by the formation of either an incomplete protein corona or, more probably, by a corona with a different composition and protein arrangement. The same could be applied to Au-PEG_{5000}* NPs whose data are in good agreement with the studies recently performed by Pelaz *et al*.[32] which reported a very limited final increase in hydrodynamic diameter for FePt NPs coated with a shell of poly (maleic anhydride-*alt*-dodecene) and PEG_{5000}. The authors reasoned that this small increment can be explained by the presence of a few protein molecules bound to PEGylated NPs or by the fact that proteins can partially penetrate into the PEG layers coating the NPs. Au-Alkyl-PEG_{600}* NPs did not show significant changes in their hydrodynamic ratio by the presence of proteins over the observation time. This finding suggests that the chosen ligands, and the amphiphilic PEG in particular, are suitable for limiting the interaction between proteins and the Au NPs while, also minimising protein corona formation. In order to quantify the total protein concentration in the protein corona a Pierce™660 Protein Assay was used. The results obtained are in close agreement with the latter observation and highlights the difference in the abundance of protein associated with the two PEGylated NPs (Fig. 4b). The amount of protein associated with the PEGylated NPs was calculated to be 4-fold lower when compared to MSA* NPs (1.5-2 g_{prot}/ g_{NP} versus 8 g_{prot}/ g_{NP}). DLS (Fig. S13†) and UV-vis measurements (Fig. S14-15†) also indicates that Alkyl-PEG_{600} inhibits protein complexation with the NP surface. Therefore, as extensively reported in the literature, it is reasonable to assume that a protein corona covering Au-MSA and Glucosamine NPs is formed with this corona consisting of a layer of proteins absorbed on the NP surface with different affinities. On the contrary, Au-PEG_{5000} NPs might present an incomplete corona or a partial penetration of proteins into the PEG coating, while the alkyl portion introduced in the shell of the Au-Alkyl-PEG_{600} NPs would hinder such kind of interaction, resulting in the formation of an incomplete or less defined corona (Fig. 4c).

Intracellular Dynamics of Au NPs

A549 cells were incubated with labelled Au NPs at a concentration of 15 µg/ml. FCS experiments were conducted after 30 min and one hour of incubation (Fig. 5a). The amount of NPs was chosen so as to allow fluorescence imaging, by avoiding the saturation of FCS signal. Measurements were performed in living cells with the laser power being kept at a level such as to minimise photo damage to cells.. Confocal fluorescence microscopy images allow for the comparison of NP uptake at different time points. Cells treated with Au-MSA* and Au-Glucosamine* NPs display increased uptake following a one hour incubation period.. This is more evident in the case of Au-MSA* NPs. On the contrary, both PEGylated NPs display lower uptake with respect to the non PEGylated NPs. Following this observation, four regions were identified for further investigation of intracellular NP diffusion, namely: the cytosol (CYT), the endoplasmatic reticulum (ER), bright spots located close to the cell membrane (BS1),and bright spots surrounding the nucleus (BS2)(Fig. 5b). FCS experiments performed on the cellular membrane and nucleus, show

poor, or no auto-correlation signals, with diffusion times associated with proteins (τ_D <500 µs) rather than NPs which, do not accumulate in these compartments (Fig. S16†). The first two tracks that usually showed bleaching (due to the initial excitation) and the tracks showing low signal and visible aggregates were excluded from the analysis (Fig. S17†). Moreover, a negative control was performed incubating free ATTO550 with the A549 cells. In this case the same data analysis procedure used with Au NPs has been applied demonstrating a different distribution of intracellular diffusing species (Fig S18† and Table S8-S9†).

Figure 6, 4 shows representative auto-correlation curves for Au-MSA* NPs (one for each region of interest) fitted with a 3D normal diffusion fitting model with two difusing components. This model, which provides good fits, allowed describing the species in the confocal volume grouping them in two main populations that, despite being a simplification, helps to appreciate the system complexity.

The different positions inside the cells where the auto-correlation curves were recorded are shown in the Figure 6. The tracks were fit by a two components equation that allows an estimation of the fractional contribution of each component to the total collected signal. We report the component that gave the largest contribution (fraction percentage ρ>0.5). For a detailed description of the statistical analysis of the tracks recorded, see ESI (Fig. S18-19†).

The fluorescent species in the confocal volume were characterised according to their diffusion time. They were grouped into four classes that were associated to different diffusing species: i) **500 < τ_D (µs) < 1500**: diffusion time of isolated free diffusing NPs; ii) **1500 < τ_D (µs) < 3000**: small aggregates of few NPs and/or NPs interacting with bio-macromolecules; iii) **3000< τ_D (µs) < 6000**: aggregates of NPs; iv) **τ_D (µs) > 6000**: large aggregates of NPs and/or NPs associated to cellular compartments. In Figure 7 we can see the FCS autocorrelation curves that correspond to an incubation period of 30 minutes for NPs in the distinct cellular regions/compartments.. As can be seen in the histograms, NPs coated with glucosamine were found in all compartments of the cell mostly as slowly diffusing species (red bars), with faster diffusing species found in the bright spots closer to the membrane (BS1) (green, yellow and orange bars), that reasonably might be associated to recently uptaken material. The other NPs are distributed inside the cells forming structures that are more heterogeneous. It is worth noting that in the case of Au-Alkyl-PEG_{600} NPs, species with diffusion times below 1500 µs (comparable to single diffusing NPs) have been detected in three compartments out of four (BS1, BS2, ER). We ascribe this result to the higher stabilising effect of the ligand, which is capable of partially inhibiting or slowing down NPs aggregation and/or association to cell compartments.

The difference between PEGylated and non-PEGylated NPs becomes more evident after one hour of incubation. We observe a significant shift towards a slowly diffusing species in all compartments of the cells treated with Au-MSA* and Au-Glucosamine* NPs (Fig. S19†). This behaviour is remarkable in the bright spots where either single NPs are confined in a small space enriched with biomolecules (cellular vesicles) or are present as aggregates (Fig. 8a-b).

The data referring to bright spots close to the membrane (BS1) suggest possible changes in the uptake pathways over time. We can speculate that after one hour of incubation, the protein corona formed around NPs increases in size, as discussed previously, and the accumulation of NPs on the membrane would bring to NPs uptake in the shape of visible aggregates >250 nm,[44] resolution limit of the CLSM, with diffusion times >6000µs. Our assumptions are in agreement with that extensively reported in literature regarding similar NPs following uptake through different pathways,[45-47] as a result of the size of the species that is one of its main determinants (Fig. S21-22†). In the case of Au-MSA* NPs, at first, they show a wide distribution of diffusing species in all compartments, comparable to the PEGylated NPs: 45% of the analysed tracks expressed diffusion times below 6000 µs in the BS1, 51% in the BS2, 50% in cytoplasm and 48% in endoplasmatic reticulum. As shown in Fig. 5a, we observed similarities in the aggregation behaviour after 30 min of incubation among MSA*, PEG_{5000}* and Alkyl-PEG_{600}* NPs, as they showed a lower amount of agglomeration compared to Au-Glucosamine* NPs. The intracellular behaviour of Au-MSA* NPs, following one hour of incubation, was observed to be more similar to that of Au-Glucosamine* NPs. This is typified by high uptake and the presence large bright spots, identified in confocal images as aggregates, indicative of limited intracellular colloidal stability. They were mainly detected as species with diffusion times greater than 6000 µs in BS1 (90% of the tracks), in BS2 (100%) and cytoplasm (79%). On the contrary, the distribution of diffusion times of Au-PEG_{5000} and Au-Alkyl-PEG_{600}* NPs was unchanged, instead expressing an overall higher colloidal stability both in media and in cell compartments. We assume that PEGylated NPs are uptaken partially through the formation of vesicles, seen as bright spots in the confocal images.. Looking at BS2, it is also possible to distinguish different behaviour between Au-PEG_{5000}* and Au-Alkyl-PEG_{600}* NPs. In fact, for Au-PEG_{5000}* NPs, after 1 hour of incubation, all the fluorescent species were found to display diffusion times greater than 3000 µs. On the contrary for Au-Alkyl-PEG_{600}* NPs, there were still populations with diffusion times in the range 500 < τ_D(µs) < 3000, 26% of the tracks below 3000µs and 51% < 6000µs, indicating the presence of single free diffusing nanoparticles or small aggregates. Moreover, the changes in diffusion time values and percentage distributions are minimal (Fig. S20†).

Conclusions

In summary, the intracellular dynamics of the NPs seems to reflect the properties of the coatings observed *in vitro*. In full RPMI glucosamine and MPS functionalized Au NPs presented a strong affinity toward proteins however, once internalised in cells, they show reduced stability and more prominent formation of aggregates and complexes with cellular macromolecules. On the contrary, PEGylated NPs, and in particular Alkyl-PEG_{600} Au NPs, which display lower affinity toward proteins shows a reduced propensity to form aggregates intracellular and greater stability.

Experimental

Materials and methods

$HAuCl_4 \cdot 3H_2O$, $AgNO_3$, hydroquinone, 4,4'-dithiodibutyric acid, glucosamine hydrochloride, butylamine, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N,N'-Dicyclohexylcarbodiimide (DCC), N-Hydroxysuccinimide (NHS), 1,4-Dithiothreitol (DTT) and NaOH were purchased from Sigma-Aldrich, and used without further purification. $HAuCl_4 \cdot 3H_2O$ was stored at 4° C, shielded from light, as 10 mM solution and NaOH was stored as 1 M water solution. $AgNO_3$ 10 mM and hydroquinone solutions were freshly prepared before each synthesis (avoiding exposure to light). HS-PEG_{5000}-OCH_3 and HS-PEG_{5000}-NH_2, purchased from Rapp Polymer GmbH, were used as received and stored under dry argon atmosphere at -20° C. HS-Alkyl-PEG_{600}-COOH (([1-mercaptoundec-11-yl]PEG_{600})-acetic acid) was synthesised by Chorisis srl. (Varese, Italy) following a literature reported procedure[39]. A549 (human lung cancer) were purchased from ATTC. Cells were cultured in RPMI 1640 medium from Lonza, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin from Sigma Aldrich.

The Au NPs were synthesised employing a 'one-pot' strategy described in Silvestri *et al.*[40]. The selected ligands were added directly to the reaction mixture, to stop the growing and cap the NPs. In this way a panel of four Au NPs characterised by different coatings were made. For all NPs, 10% of the surface was coated introducing a α-amino-ω-thiol PEG_{5000} to allow the subsequent labelling of the system by ATTO550 fluorescent dye (ATTO-TECH GmbH). We selected the long amino PEG chain in order to have a large distance between the metallic core and the fluorophore molecule, reducing the quenching effect due to the presence of the noble metal. We choose to cover 10% of the surface with α-amino-ω-thiol PEG_{5000} to have a good fluorescence signal with a slight modification of the NPs surface.

NPs were characterised extensively by TEM, UV-vis, DLS, fluorescence spectroscopy, FCS in media and in live cells. Proteins present in the corona were quantified by Pierce™660 Protein Assay. Cells were imaged by CLSM. For detailed procedures, see ESI.

FCS experiments in water. Stock solutions of ATTO 550 labelled Au NPs with a concentration of 1 mg/ml were prepared. Prior to each FCS measurement the solutions were sonicated for 5 min and double filtered on 0.22 µm regenerate cellulose syringe filters. This operation helps to remove the excess of free fluorophore that can remain electrostatically attached to the surface of the Au NPs. To perform the FCS measurements 25 µl of Au NPs were dissolved in 225 µl of MilliQ water. Each sample of NPs was measured in 3 independent experiments, comprised by 10 runs of 10 seconds each one. For each measurement session 50 nM water solutions of Rhodamine B and of ATTO 550 were measured to determine the structural parameter and the diffusion coefficient of the free dye.

FCS experiments in full RPMI cellular medium. Stock solutions of ATTO 550 labelled Au NPs with a concentration of 1 mg/ml were prepared. Prior to each FCS measurement the solutions were sonicated for 5 min and double filtered on 0.22 um regenerate cellulose syringe filters. This operation helps to remove the excess of free fluorophore that can remain electrostatically attached to the surface of the Au NPs. To perform the FCS measurements 25 µl of Au NPs were dissolved in 225 µl of a phenol red free RPMI solution, enriched with a 10% in volume of FBS. The FCS measurements were registered 10 min, 20 min, and 1h after the mixing of the solutions. Each NP sample was measured in 3 independent experiments, comprised by 10 runs of 10 seconds each one. As comparison, a FCS measurement was performed on bare NPs in RPMI. For each measurement session 50 nM water solutions of Rhodamine B and of ATTO 550 were measured to determine the structural parameter and the diffusion coefficient of the free dye. The diffusion coefficient of proteins was determined mixing 25 µg of FBS with a 50 nM solution ATTO 550 in RPMI.

FCS experiments in living cell. A549 cells were grown in RPMI media enriched with 10% of fetal bovine serum (FBS) and 1% of penicillin-streptomycin (PS) in a humidified atmosphere at 37 °C with 5% CO2. To perform FCS on live cells, 10.000 A549 cells were seeded on NuncTM Lab-Tek Chambered Coverglass (purchased from Thermo Fisher Scientific) and grown in a humidified atmosphere at 37 °C with 5% CO2 for 24 hours. The cells were incubated with 500 µl of 15 µg/ml solution of fluorescently labelled Au NPs for 30 and 60 minutes. The cells were washed twice with warm PBS. To perform the experiments in living cells, these were kept in Hepes 10 mM. The microscope objective used for FCS on live cells was a 40X water immersion objective. The excitation wavelength was 561 nm and cells were imaged in transmission mode. Before performing the measurements the ConfoCor and FCS x-y alignment was checked following the procedure of Altan- Bonnet[48] (Fig. S23). FCS measurements were done on at least 3 cells recording 20 runs of 10 second each in 8 distinct area inside the cell: 2 in the cytoplasm (CYT); 2 in the endoplasmatic reticulum (ER) and, whenever visible, 2 in brighter spots near to the membrane (bright spots 1, BS1) and 2 in

brighter spots near to the nucleus (bright spots 2, BS2). Moreover one experiment was performed on/close to the membrane and one on/close the nucleus.

CLSM imaging of cells. For imaging, cells were washed with PBS and the endoplasmatic reticulum stained by ER-Tracker Green (BODIPY FL glibenclamide) (Invitrogen, E34251) for 10 min at 37 °C.Two more washes were performed and cells were fixed by 3.7% paraformaldehyde (2 min at 37°C). After washing again, nuclei were stained by Hoechst 33342 Solution (20 mM) (Thermo Fisher Scientific) for 5 min at r. t. Cell were finally washed and kept in PBS. Stained cells were examined under the laser scanning confocal microscope (LSM 510 Meta confocal microscope Zeiss, Germany) equipped with a 63× oil objective lens (1.4 NA). Images were acquired sequentially to avoid cross-talk using excitation wavelengths 405, 488 and 561 for Hoechst, ER staining and ATTO550 respectively. Processing and overlaid with transmission images were performed with Image J free software.

Protein quantification assay. Proteins composing the corona were quantified by the Pierce™ 660 Protein Assay (Thermo Scientific). The protein's calibration curve was obtained by measuring BSA standard solutions ranging from 25 g/ml to 1000 g/ml. 100 l of protein solution was incubated with 1.5 ml of Pierce TM 660 Protein Assay Reagent for 1 h. The absorption spectra of the samples were registered employing a mixture of 100 l of PBS and 1.5 ml of Pierce™ 660 Protein Assay Reagent as baseline. The absorbance values at 660 nm were taken into account to build the calibration curve.

For the quantification of the PC proteins, 915 µg of NPs were incubated overnight in 1ml of pure FBS at 37 °C. The PC NPs were separated from the excess of proteins employing centrifugation (15000 RPM for 1h) over a layer of sucrose 0.7 M (700 l). The supernatant was removed and a pellet of NPs isolated. Each pellet was suspended in 100 µl of PBS and 1.5 ml of Pierce™ 660 Protein Assay Reagent was added and incubated for 1h. Absorbance at 660 nm was recorded on diluted samples in triplicates. Spectra of bare Au NPs were recorded to subtract the absorbance contribution at 660 nm.

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