Size matters: Size Dependency of Gold Nanoparticles Interacting with Model Membranes

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The rapid development of nanomaterials has led to an increase in the number and variety of engineered nanomaterials (ENMs) in the environment. Gold nanoparticles (AuNPs) are an example of a commonly studied ENM whose highly tailorable properties have generated significant interest through a wide range of research fields. In the present work, we report the first qualitative as well as quantitative experimental characterisation of the AuNP-membrane interaction. We investigate the interactions between citrate-stabilised AuNPs (diameters 5, 10, 25, 35, 50, 60 nm) and large unilamellar vesicles (LUVs) acting as a model membrane system. LUVs were prepared in two different formulations using 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). Our results show that the interaction between AuNPs and LUVs is size dependent; in particular, we reveal the existence of two AuNP's critical diameters which determine the fate of AuNPs in contact with a lipid membrane. The results provide a new understanding of the size dependent

interaction between AuNPs and lipid bilayers of direct relevance to nanotoxicology and to the design of NP vectors.

Engineered nanomaterials (ENMs) are used in a wide range of sectors including medicine, technology, cosmetics and food. The growing use of ENMs is raising serious concerns regarding their impact on human health ^{1,2}. In order to be able to distinguish between harmless and harmful ENMs, significant progress must be made in understanding the key initiating events at bio-nano interfaces and determining the NP properties relevant to these events. One of the key events contributing to nanotoxicity is the interaction of ENMs with phospholipid membranes. For this reason, lipid membranes such as phospholipid vesicles are commonly exploited as a minimal model system for investigating the biological impact of ENMs ³⁻⁵.

Among the many possible ENMs, gold nanoparticles (AuNPs) have emerged as promising candidates for biomedical diagnostic, therapeutic and photoelectrochemical applications ⁶⁻⁸ due to their facile surface chemistry, ease of synthesis and tuneable size. In the present work, we used citrate-stabilised AuNPs to study the physical chemical interactions between inorganic NPs and lipid model membranes. Citrate is a non-covalently bound capping agent and it can be readily displaced from the AuNP surface ^{9,10}. In this study, we investigated the size-dependence of the interaction between a range of citrate-stabilised AuNPs with model lipid membranes. We employed citrate-stabilised AuNPs with a diameter ranging from 5 nm to 60 nm (i.e. 5, 10, 25, 35, 50 and 60 nm) and two formulations of unilamellar liposome with a hydrodynamic diameter of 190 nm (characterization data of liposomes and AuNPs can be found in Supplementary Fig. 1 and 2). The liposomes were formed from POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) and DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine). Both POPC and DOPC are phosphocholine lipids with a zwitterionic head group and consequently a neutral net charge. However, they differ in the structure of their lipid tails. (Fig. 1a and 1b). Fluid-phase zwitterionic liposomes are expected to attractively interact with spherical AuNPs ^{5,11-13}. Lipid membranes are highly flexible, and the bilayer can be deformed due to NP adhesion on its surface. When a NP contacts a lipid bilayer, the NP-membrane

interaction can lead to different scenarios: NP internalisation within the membrane bilayer ¹¹, full NP engulfment (similar to the cellular non-specific endocytosis) ³ or NP adsorption on the external membrane surface ¹⁴. In the case of a tensionless membrane, this interaction process is ruled by the balance between bending and adhesion free energy ^{15,16}. The bending energy is a property of the membrane; however, the adsorption free energy depends on both the membrane and the nanoparticle. If the free energy per unit area could be measured, then it would be possible to predict if the NPs become fully wrapped by membrane lipids and hence enter the cell with potentially toxic consequences.

Although there is an extensive literature on biological and model membrane interactions with inorganic NPs, very little attention is given to quantitative studies ¹⁷. In the present work, we report the first qualitative as well as quantitative experimental characterisation of the AuNP-membrane interaction. Our aim is to better understand the role of size in determining the behaviour of NPs in contact with a lipid membrane (Fig. 1c). We show that citrate-stabilised AuNPs in the range from 5 nm to 60 nm interact with large unilamellar vesicles (LUVs) very differently. Smaller AuNPs (5 to 10 nm) tend to form aggregates on the bilayer surface or to intercalate between the outer and inner leaflets of the lipid bilayer, whereas larger AuNPs (25 to 35 nm) adsorb on the outer surface. For diameters between 50 and 60 nm the AuNP-membrane interaction is weak, characterised by few absorption events.

Fig. 1 | Schematic representation of the lipid chemical structure and AuNP-lipid interaction. Chemical structure of (a) DOPC and (b) POPC phospholipids. (c) Schematic of the AuNP-PC lipid interaction due to a combination of electrostatic and van der Waals interactions.

Main text

The fate of a nanoparticle in contact with a tensionless membrane is determined by the competing free energy cost of bending (k) the membrane to wrap the nanoparticle and the free energy gain (W) due to adsorption of the lipids on the NP surface. Theoretical and experimental studies identified the existence of a critical NP diameter whose value is defined by the ratio between these two energetic contributions: $dc \equiv 2\sqrt{2k/|W|}$ ^{15,18}. This value defines the minimum NP diameter needed to observe the NP's wrapping within the lipid bilayer. For example, the critical diameter for silica NPs has been determined to be around 20-25 nm, corresponding to a few mJ m^{-2} of adhesive strength^{19,20}. Note that the critical diameter changes depending on the nature of the nanoparticle surface. Molecular simulations^{21,22} have shown that for neutral crystalline silica surfaces the critical diameter, obtained from measurements of W , is ~ 16 nm while for amorphous silica is ~ 10 nm. For gold nanoparticles the critical diameter is much smaller (~ 4 nm) based on the simulation prediction of $W = 42 \text{ mN m}^{-1}$ ¹³ for a bare gold surface.

In this study, we investigate the effect of gold nanoparticle (AuNP) size on the interaction with lipid vesicles. We employed a broad size range of AuNPs: 5, 10, 25, 35, 50 and 60 nm, with two formulations of LUVs: POPC and DOPC. The chosen phospholipids form fluid membranes at room temperature with phase transition temperature at -18°C (DOPC) and -2°C (POPC). We first investigate the interaction between AuNPs and liposomes by dynamic light scattering (DLS). The DLS size distribution profiles and autocorrelation functions for all the lipid formulations investigated are illustrated in Supplementary Figure 3 and 4 respectively, while here we highlight the 10, 25 and 60 nm profiles of the POPC formulation. The DLS

investigations clearly show that the AuNP-membrane interaction is strongly size dependent. For the experiment, up to 5 subsequent injections of LUVs into the AuNP dispersion have been made, each increasing the surface area of LUV in solution (Fig. 2). Considering the higher light scattering properties of “hard” AuNP compared with “soft” lipid vesicles, we expect that the DLS signal will be dominated by the strongly scattering AuNPs. Interestingly, this was not the case for smaller AuNP sizes (5 and 10 nm). As shown in Figure 2a, the peak of the AuNP-LUVs mixture is shifted from the peak at the gold diameter to that of the LUVs after the first injection. We ascribed this effect to the strong interaction occurring between the smaller AuNP and the LUVs. As soon as the AuNPs interact with the LUVs, the AuNP signal shifts to that corresponding to the LUV-peak area, indicating a full AuNP-lipid bilayer interaction.

Fig. 2 | Size distribution and autocorrelation profiles of the interaction of AuNPs with LUVs. Size distribution and autocorrelation profiles of (a) 10 nm, (b) 25 nm and (c) 60 nm for the POPC formulation. All the profiles were obtained by subsequent injections of LUVs within an AuNPs dispersion, up to 5 injections (1st injection = dotted red line, 2nd injection = dashed blue line, 3rd injection = dashed green line, 4th injection = dashed violet line and 5th injection = dashed black line). Control samples were obtained by measuring the AuNP (orange) and LUVs (magenta) dispersions.

This effect can also be seen in the corresponding autocorrelation function profiles (Fig. 2, right hand side). After the LUVs were injected into the solution of small AuNPs (10 nm), we observed a drastic shift in the autocorrelation function to the longer decay times of LUV (Fig. 2a, right hand side plot). The size distribution and autocorrelation function profiles of medium-sized AuNPs (25 nm) interacting with LUVs show a progressive shift to larger sizes and longer decay times at every LUVs' injection. We identify this effect with the presence of two interacting NP populations in solution: free AuNPs and AuNPs absorbed by the LUVs. With every LUV injection, the population of free AuNPs progressively shrinks while the interacting population grows. Therefore, the DLS measurement profiles are the sum of these two differently sized populations and the size distribution profile and decay times fall in an intermediate region between the only AuNP and only LUV signals. Finally, the DLS profiles of larger AuNPs (60 nm) show a decrease in the AuNP-LUV interaction. Following the sequential injections of LUVs within the AuNP dispersion, the signal stays in the area of the strongly scattering AuNPs, indicating that the AuNP-LUV interactions are negligible at this AuNP size range.

Fig. 3 | Size distribution profiles obtained by injecting AuNP into LUV's dispersion, inverse injection. Size distribution profiles of AuNPs (orange), lipid vesicles (magenta) and mixture of AuNPs-lipid vesicles by injecting (a) smaller (10 nm), (b) medium (25 nm) and (c) larger (60 nm) size AuNPs into the LUVs dispersion.

Inverse experiments have also been performed by injecting AuNPs within the LUV dispersion (the DLS profiles for all the lipid formulations AuNPs' sizes are illustrated in Supplementary Fig. 5). The resulting size distribution peak of LUV-AuNP mixture stays unchanged in the LUV size-peak area (Fig. 3b, a and c). This indicates that the injected AuNPs (the minority population in solution) are all interacting with the LUVs due to the absence of the AuNP size peak even for larger AuNPs.

The AuNP-LUV mixtures characterised *via* DLS (Fig. 2 and Supplementary Fig. 3 and 4), following the first LUV injection, has been further analysed by transmission electron microscopy (TEM). The micrographs relative to these investigations are shown in Figure 4.

Fig. 4 | TEM micrographs of differently sized AuNPs interacting with POPC lipid model membrane. TEM micrographs of (a) small (5-10 nm), (b) medium (25-35 nm) and (c) large AuNP (50-60 nm) interactions with membrane. Scale bar 50 nm.

For the 5 and 10 nm AuNPs interaction with the liposomes' membrane, both POPC (Fig. 4) and DOPC (Supplementary Fig. 6), we observed two correlated outcomes: AuNPs aggregation on the liposome surface, particularly evident for 5 nm AuNP, and the formation of an inhomogeneous decoration of the lipid vesicles (Fig. 4a). In some cases, AuNP aggregation

occurred outside the lipid bilayer. For the 10 nm AuNP, an inhomogeneous and asymmetric 'pearl necklace' repartition of AuNPs around the lipid vesicle (Fig. 4b and Supplementary Fig. 6b) is observed. We understand this asymmetrical internalisation of the smaller AuNPs within the membrane as an 'unzipping' of the bilayer which has been already reported in experimental^{11,23} and computational studies²⁴ for small AuNPs. As soon as the AuNP enters in contact with the outer layer of the membrane, the AuNP is surrounded by a lipid corona with the hydrophobic tails facing the polar environment. This affinity mismatch drives the insertion of the first AuNP within the bilayer causing its acyl-chain dissociation and creating a void space in the proximity of the AuNP. This deformation is energetically unfavourable, and the energy cost is minimised by the inclusion of more NPs forming clusters. This effect explains the asymmetric distribution of AuNP around the liposomes and their adjacent inclusion as 'pearl necklace'.

For 25 and 35 nm AuNPs, the TEM micrographs in Fig. 4c-d clearly show a different outcome. The medium sized AuNPs stays absorbed on the surface of membranes, decorating the lipid vesicle. Since the radius of curvature is now larger than before, we might expect these NPs to be more easily wrapped by the membrane, the fact that they are not implies that the bending rigidity or the surface tension of the membrane increases at the point of contact and inhibits full wrapping. This is also the case for the larger AuNP (50 and 60 nm). However, the presence of absorbed AuNP was barely detectable in both TEM and DLS investigations.

These finding were confirmed for the AuNP-LUV mixture under cryo-EM. For this experiment up to 5 subsequent injections of LUVs into 5, 25 and 60 nm diameter AuNP dispersions were made as described for the DLS experiment. In order to reach a sample concentration suitable for the cryo-EM characterisation and deposition of LUVs, the mixtures were concentrated under gentle centrifugation at 6 rcf. to obtain LUVs' deposition. The greatest sedimentation was observed in the smaller AuNPs' mixture (i.e. 5 and 25 nm), which exhibited a visible red pellet indicating the presence of AuNP at the bottom of the Eppendorf where the LUVs are concentrated (Supplementary Fig. 7), while little sedimentation was observed in the larger AuNP-LUV mixture.

Fig. 5 | Cryo-EM micrographs showing the AuNPs-LUVs interaction. The AuNPs-LUVs mixture has been concentrated by gentle centrifugation and imaged by Cryo-EM. Micrographs of (a) 5 nm, (b) 25 nm and (c) 60 nm AuNP interaction with membrane. Scale bar 50 nm.

Cryo-EM micrographs of the resuspended pellets are shown in Figure 5. Smaller NPs (Fig. 5a) were distributed around the liposomes as a ‘pearl necklace’, confirming their internalisation within the bilayer as observed in the TEM analysis. Medium size AuNP (Fig. 5b) are instead

absorbed on the LUV's surface. Free AuNPs and AuNPs interacting with LUV are observed in the sample made with larger sizes (Fig. 5c).

Membrane disruption is one of the potential effects associated with nanotoxicity. For this reason, a fluorescence leakage assay was carried out to assess the degree of membrane disruption. Figure 6a shows the percentage of fluorescent dye leakage as a function of the AuNP diameter. For all the formulations investigated, the membrane leakage is negligible, and thus the assay was negative. To further investigate the possibility of a disruptive effect of the AuNP-LUV interaction on the membrane structure, the leakage assay has been repeated using AuNPs with a diameter size from 25 nm to 60 nm, at an increased concentration of AuNPs (Supplementary Fig. 8). Our results demonstrated that only 25 nm and 35 nm AuNPs at high concentrations can induce LUV leakage. However, it remains unclear if, at these experimental conditions, the leakage is caused by the AuNPs engulfment or the formation of transient pores in the membrane due to the AuNPs extracting lipid molecules.

Fig. 6 | AuNP-LUV quantification of interaction and membrane leakage assay results.

(a) The membrane leakage assay has been performed at the same conditions as all the data in order to detect any membrane disruption consequent to the interaction with AuNPs. (b) Enthalpy changes for the POPC (red) and DOPC (grey) formulations versus the AuNPs sizes.

The interaction between AuNPs and liposomes has been quantified using isothermal titration calorimetry (ITC) at the same concentration employed in the previous analyses. ITC is generally used to detect the temperature change during an interaction between reactive biomolecules (e.g. enzyme-substrate, protein-ligand, protein-ion) and it has been recently exploited for the detection of interactions between NPs and biomolecules such as proteins and amino acids ^{25,26}. Here, we exploit ITC as a powerful and highly sensitive technique to estimate the strength of the AuNP-LUVs interaction. In the experiment, the LUVs were titrated against the AuNPs dispersion. To quantify the heat response due to the AuNP-LUV interaction and compare it in the size range 5-60 nm, the ΔQ of interaction has been normalized to the AuNPs surface area to obtain ΔH (mJ m⁻²). The ITC results illustrated in Fig. 6b show that the interaction of AuNPs with LUVs is exothermic for all AuNP sizes. The interaction with the 5 and 10 nm AuNPs results in a smaller enthalpy change than the larger NPs which confirms that size determines how NPs interact with the liposome membranes. Note that the data reported here are enthalpies not free energies. To obtain the adhesion free energy of adsorption (W), we require the entropy change which could be obtained, for example, from simulation ¹³. While the smaller AuNPs are small enough to interact with single molecules in the bilayer and be located within the lipid bilayer of the membrane, larger sized AuNPs instead interact with the outer surface of the membrane only. This small difference in interaction between smaller and larger size is therefore detectable using ITC. For larger size AuNPs, the ITC detected a pronounced (~3 fold in enthalpy) difference between POPC and DOPC LUVs. POPC creates more ordered membrane-structures ^{27,28} compared with DOPC which results in an increase in the membrane bending rigidity for POPC membranes due to the difference in the degree of saturation of the lipid tail and chain length. These data demonstrate the sensitivity of the ITC technique to detect the influence of membrane composition on the interaction with inorganic nanoparticles. Membrane composition may indeed have serious implications to the susceptibility of different tissues/cells types to nanotoxicity.

The different mode-of-action of smaller sizes AuNPs (i.e. 5 and 10 nm) may be explained by the strong interaction between PC headgroups and AuNPs. As soon as the AuNP adsorbs on the bilayer, the AuNPs can extract lipid molecules from the outer layer of the membrane which behaves as a sticky layer and enhances the formation of AuNP aggregates. The effect of AuNPs aggregation in presence of lipids, has been further investigated by mixing DHPC micelles with 5 and 10 nm AuNPs (Fig. 7a). As a result, we were able to observe that in the presence of dispersed micelles in solution (Fig. 7b), lipids and AuNPs cluster together forming 'AuNP-chip-cookies' aggregates for both 5 and 10 nm AuNPs (Fig. 7c,d). The morphologies of the two AuNP-lipid formed aggregates are different for the two sizes. We suggest this effect is related to the difference in surface curvature of the two AuNP populations.

Fig. 7 | Smaller AuNPs interacting with micelles. (a) Schematic of the 5 and 10 nm AuNP interacting with lipid micelles and leading the formation of disordered aggregates AuNP/lipids (blue region in the cookie representation. TEM micrographs of (b) micelles forming 'AuNP chip cookies' aggregates with (c) 5 nm and (d) 10 nm AuNP.

Further investigation on the AuNP positioning in respect to the membrane has been carried out through spectroscopic measurements of the environment-sensitive probe Nile Red (NR). NR is embedded in the headgroup-tail interface of the lipid membrane. NR emission maxima changes as a function of solution polarity in a way that a blue shift in emission is attributed to a decrease in environment polarity. Whilst, a red shift in emission is attributed to an increase in environment polarity²⁹. Literature reports that 40 mol% addition of cholesterol to lipid

vesicles resulted in an 11 nm blue shift in NR emission maxima³⁰. This is attributed to NR situating deeper in the membrane (as confirmed via the parallax method where the change in NR fluorescence upon interaction with fluorescence quenchers located at different membrane depths) when cholesterol is present in the membrane. So, an alteration of the solvent environment of NR is an indication of the AuNP interaction at the headgroup/tail interface or further within the membrane. The AuNP interaction with the bilayer interface as a function of their size has been carried out employing 10, 25 and 60 nm diameter nanoparticles.

As can be seen from Figure 8a, only the addition of 10 nm AuNP resulted in a blue shift indicating a decrease in membrane polarity – an indication that nanoparticles are interacting within the membrane. Larger AuNPs (25 and 60 nm), however, gave a negligible shift in fluorescence emission maxima which is within experimental noise as indication of their peripheral interaction with the outer surface of membrane.

Fig. 8 | Change in Nile Red emission maxima as a function of AuNPs interaction with membrane. (a) A change in the emission maxima of the solvatochromic dye Nile Red shows a size-dependent interaction of gold nanoparticles with lipid membranes. (b) A reduction in Nile Red fluorescence intensity is only observed upon addition of 10 nm AuNP.

A 4-fold reduction in fluorescence intensity is also observed upon the addition of 10 nm NP (Fig. 8b). The literature reports a similar intensity drop upon the addition of cholesterol to

DOPC vesicles ³⁰, attributed to increased water penetration in the interfacial region of the membrane (where Nile Red is located) upon cholesterol addition. As it is expected that membrane remodelling upon introduction of small nanoparticles would result in increased water penetration, and Nile Red fluorescence reduces in more polar solvents, this intensity change further indicates a significant interaction between lipid membranes and 10 nm gold nanoparticles.

In summary, we show that citrate-stabilised AuNPs in the range from 5 nm to 60 nm interact with large unilamellar vesicles (LUVs) very differently (Fig. 9).

Figure 9. Schematic representation of the different outcomes arising from AuNPs-lipid membrane interactions. Changing the AuNP size from 5 nm to 60 nm, we observed four different types of outcomes: **(A)** AuNP (i) aggregation on the lipid bilayer surface and (ii)

internalisation within the lipid bilayer; **(B)** AuNP absorption on the membrane's outer surface; **(D)** AuNP-LUV interaction characterised by a few absorption events or absence of interaction.

Smaller AuNPs (5 to 10 nm) tend to form aggregates on the bilayer surface or to intercalate between the outer and inner leaflets of the lipid bilayer, whereas larger AuNPs (25 to 35 nm) undergo absorption events, while for diameters between 50 and 60 nm the AuNP-membrane interaction is minor, characterised by a few absorption events.

Conclusions

Investigating the physicochemical mechanisms of interaction between NPs and liposomes as a model membrane system is important for the understanding of key initiating events of nanotoxicity at the membrane interface. AuNPs are a promising engineered nanomaterial exploited for different applications due to their tuneable physical and optical properties. In this study, we employed a minimal model system to quantitatively and qualitatively investigate the strength of the AuNP-membrane interaction as a function of the AuNP size. We investigated the interaction of a wide range of AuNP size from 5 nm up to 60 nm in diameter, with artificial lipid vesicles as a model membrane. This model system has been chosen to mimic the interaction between nanoscale AuNPs and the surface of biological membranes. In all the experiments, we find that AuNPs interact differently with LUVs accordingly to their diameter. The experiments revealed the presence of two AuNP critical diameters at around 10 nm and 50 nm. Below 10 nm, the AuNP are internalised within the hydrophobic part of the bilayer while increasing the diameter size up to 50 nm, the AuNPs are absorbed on the LUV outer surface. Above 50 nm, the AuNP-LUV interaction is weak. While the smaller NPs interact strongly with the membrane and are, in some cases, completely wrapped as we expect from the gold critical diameter discussed in the introduction, increasing the NP diameter we only observed partial wrapping. The increase in membrane tension due to contact with the NP is the cause of the consequent inhibition of the wrapping as the AuNP diameter increases.

Our findings offer a deeper understanding of the AuNP-membrane interaction through a systematic study of NP size effect controlling NP-membrane interaction. Findings that can be useful to understand the key initiating events in the development of nanotoxicity and to design nanoparticle vectors for drug delivery.

Materials and methods

Chemicals were used as received unless otherwise indicated.

The spherical citrate-stabilised AuNPs were purchased from Nanopartz (Loveland, CO, USA) at a gold concentration of 0.05 mg mL^{-1} and citrate concentration of 3 mM.

Production of large unilamellar vesicles (LUVs). The employed lipids were DOPC and POPC from Avanti Polar Lipids (Alabaster, AL, USA). Lipid films were prepared by dissolving the lipid (13.6 μmol) in chloroform, before gently vortexing the solution for 1 minute. The solution was then evaporated under a stream of nitrogen to give a thin-lipid film, which was dried under vacuum overnight at room temperature. The dried lipid films were hydrated in a 3 mM citrate solution. To obtain unilamellar vesicles, the lipid suspension was freeze-thawed 6 times. Each freeze-thaw cycle consisted of freezing the sample in liquid nitrogen before thawing with a heat gun and vortexing for 60 seconds at room temperature. Samples were then extruded 21 times through a $0.2 \mu\text{m}$ polycarbonate filter (mini extruder from Avanti Polar Lipids, Alabaster, AL, USA) to yield unilamellar vesicles $\sim 200 \text{ nm}$ in diameter.

Transmission electron microscopy. A 2% uranyl acetate (UA) solution was used as a negative staining agent. 5 μL of AuNP/LUVs dispersion was deposited onto glow-discharged 200 square mesh copper grids (Agar scientific). After 1 minute, the grids were blotted with filter paper and then immersed into the UA staining solution for 20 s. Then, the grids were blotted again. Grids were imaged using a JEOL JEM-2100F fitted with a Gatan Orius SC 1000 camera (2x4k).

Cryogenic transmission electron microscopy. The AuNP-LUV mixtures were concentrated by centrifugation at 6000x g for 10 min, followed by removal of the supernatant and re-suspended in 10 µl 3 mM citrate solution. Samples were vitrified using a Vitrobot Mark IV (FEI) system under controlled temperature (21 °C) and humidity (100%). 4 µl of sample was deposited on Quantifoil copper grids with 2 µm holey-carbon on 200 square mesh (Agar scientific) and vitrified by plunging the grid into liquid ethane and transferred to liquid nitrogen. The grid is then quickly placed in a cryogenic stage and kept at -180 °C. Micrographs were collected using a Gatan 626 cryogenic holder on a FEI Tecnai 12 twin TEM operating at 120kV with a TVIPS F216 CCD camera.

Liposome-AuNP size distribution. The samples were analysed using a Malvern Panalytical dynamic light scattering instrument. The sample was crossed by a 120-mW He-Ne laser at 630 nm at a controlled temperature of 25 °C. The scattered light was measured at an angle of 173°. For the analysis, the samples were prepared at the same concentration used in the ITC analysis and then diluted with a 3 mM citrate solution at a final concentration of AuNPs of 0.1 mg mL⁻¹ into a final volume of 500 µl. The samples were analysed into a polystyrene cuvette (Malvern, DTS0012). All DLS data were processed using a Dispersion Technology Software (Malvern Panalytical).

Quantification of AuNP-membrane interaction via Isothermal Titration Calorimetry.

The liposome concentration was estimated as follows.

Firstly, the total number of lipid molecules per vesicle were calculated using the equation:

$$N_{tot} = 4\pi \frac{\left(\frac{d}{2}\right)^2 + \left(\frac{d}{2} - h\right)^2}{a_{lipid}} \quad (1)$$

Where d is the average diameter of vesicles obtained from light scattering measurements (**SI Fig.1**), h is the thickness of the lipid bilayer measured from the TEM micrographs (5 nm, **SI Fig.1**), a_{lipid} is the surface area per lipid molecule, which for DOPC/POPC is 71 Å². The number of liposomes per mL (N_{lip}) was finally calculated by using the equation:

$$N_{lip} = \frac{M_{lipid} \times NA}{N_{tot} \times 1000} \quad (2)$$

Where M_{lipid} is the molar concentration of lipid and NA is the Avogadro number. For a 2.5 mg mL⁻¹ lipid concentration, used in this study, gives a liposome concentration of liposomes per mL of 6.4 x10⁹.

Fluorescence vesicle leakage assay. Lipid films were hydrated in calcein buffer (50 mM calcein, 3 mM citrate pH 6.5) to a concentration of 10 mg mL⁻¹ and LUVs prepared as indicated above. The unencapsulated calcein was removed through size exclusion chromatography using a Sephadex G-50 column eluting in sucrose buffer (500 mM sucrose, 3 mM citrate). Calcein leakage was assessed by fluorescence spectroscopy, with the calcein fluorescence emission recorded in 96 well plates at $\lambda_{ex/em} = 494/514$ nm on a Cary Eclipse Fluorometer (Agilent Technologies, USA) on triplicate wells to obtain average fluorescent emission measurements for each vesicle composition. For all measurements, the fluorescence of a nanoparticle-free sample was used as a control to compare with AuNP-vesicle samples. The assay was firstly carried out at the employed concentration of the ITC measurements. Then, the calcein leakage was assayed to measure the effect of AuNP and vesicle concentrations change. For this purpose, a lipid concentration of 250 μ M was investigated over an increased nanoparticle amounts varying from 15 μ g to 5 μ g and a constant amount of 5 μ g AuNP was investigated over a lipid concentration change from 150 μ M to 75 μ M. After measurements, 10 (v/v)% Triton X-100 was added to each solution to lyse vesicles and determine the maximum fluorescence intensity for each well. Vesicles were left for 30 minutes to ensure complete lysis had occurred before recording final fluorescence readings. The measurement data were analysed by subtracting the control sample (vesicle solution without AuNPs) from the fluorescence intensities and normalising the result to the maximum fluorescence intensity.

Spectroscopic measurements of the environment-sensitive probe Nile Red. DOPC films (5 mg) containing 6 mol% Nile Red were prepared via mixing of components in chloroform

before solvent evaporation and drying of film overnight at RT in desiccator overnight. The lipid films were hydrated with 3 mM citrate buffer to a working concentration of 10 mg mL⁻¹, freeze-thawed 6 times to ensure membrane homogeneity and extruded 21 times through a 200 nm filter. Any unencapsulated Nile Red was removed through passage of extruded vesicles through a Sephadex G-50 column hydrated with 3 mM citrate, giving purified Nile Red vesicles ~5 mg mL⁻¹ concentration. Spectroscopy conditions: λ_{ex} = 476 nm, λ_{em} = 490-700 nm. PMT voltage = 800 V, scan rate = 120 nm min⁻¹, data interval = 1 nm, average time = 0.5 s and total volume 200 μ l. Data were plotted in OriginPro9. Applied standard Gaussian fit to each well to generate fit. All fits converged. Centre of peak taken as the emission maxima.

Acknowledgements

This work was supported by the European Union's Horizon 2020 research and innovation programme under Grant Agreement No. 686098 "SmartNanotox" (to C.C. and N.Q.) and Centre for an EPSRC Doctoral Prize Fellowship (to J.W.H.). NQ acknowledges useful conversations with M Schneemilch.

Author contributions

N.Q. and C.C. designed the experiments and performed the data analysis. C.C. designed and performed the DLS, TEM experiments. C.C. and J.W.H. designed and performed the leakage assay experiment. J.W.H. designed and performed the Nile red experiments. T.J.M. supplied preliminary AuNP samples and performed the spectroscopy analysis. C.C. and J.D.B. designed and performed the cryo-EM experiment. O.C. contributed to designing the experiments and discussion on the manuscript. C.C. and N.Q. wrote the manuscript with contribution from all authors.

Competing interests statement

The authors declare no competing financial interests.

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Size matters: Size Dependency of Gold Nanoparticles Interacting with Model Membranes

Supplementary Information

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Supplementary Figures and Legends

Supplementary Fig. 1 | Lipid vesicle characterisation. TEM characterisation of (a) DOPC and (b) POPC vesicles imaged in negative staining. (c) Size distribution profile of lipid vesicles with an average diameter of 190 nm. (d) The vesicle micrograph was analysed using imageJ software to measure the membrane thickness (~5 nm). Scale bars are 50 nm.

Supplementary Fig. 2 | (a) UV-vis absorption spectra of 5, 10, 15, 25, 35, 50 and 60 nm AuNPs solution and (b) TEM micrograph of 25 nm AuNPs. Scale bar is 50 nm.

Supplementary Fig. 3 | Size distribution profiles of the interaction of AuNPs with LUVs.

Size distribution profiles changing from (a, b) small sizes: 5-10 nm to (c, d) medium sizes: 25-35 nm and (e, f) large sizes: 50-60 nm for the POPC and DOPC formulations. All the profiles were obtained by subsequently injecting LUVs within an AuNPs dispersion up to 5 injections (1st injection = dotted red line, 2nd injection = dashed blue line, 3rd injection = dashed green line, 4th injection = dashed violet line and 5th injection = dashed black line). Control samples were obtained by singularly measuring the AuNP (orange) and LUVs (magenta) dispersions.

Supplementary Fig. 4 | Autocorrelation function profile of the interaction of AuNPs with LUVs. Autocorrelation function profiles changing from (a) small sizes: 5-10 nm to (b) medium sizes: 25-35 nm and (c) large sizes: 50-60 nm for the POPC and DOPC formulations. All the profiles were obtained by subsequently injecting LUVs within an AuNPs dispersion up to 5 injections (1st injection = dotted red line, 2nd injection = dashed blue line, 3rd injection = dashed green line, 4th injection = dashed violet line and 5th injection = dashed black line). Control samples were obtained by singularly measuring the AuNP (orange) and LUVs (magenta) dispersions.

Supplementary Fig. 5 | Size distribution profiles obtained by injecting AuNP into LUV's dispersion, inverse injection. Size distribution profiles of AuNPs (orange), lipid vesicles (magenta) and mixture of AuNPs-lipid vesicles by injecting (a) small sizes: 5-10 nm, b) medium sizes: 25-35 nm and (c) large sizes: 50-60 nm. Different formulations of liposomes are represented: POPC and DOPC.

Supplementary Fig. 6 | TEM micrographs of differently sized AuNPs interacting with DOPC lipid model membrane. TEM micrographs of (a) small (5-15 nm), (b) medium (25-35 nm) and (c) large AuNP (50-60 nm) interactions with membrane. Scale bar 50 nm.

Supplementary Fig. 7 | AuNPs-LUVs mixture after gentle centrifugation. The AuNPs-LUVs mixture has been concentrated by gentle centrifugation. Photo of the AuNPs-LUVs sedimentation after centrifugation of the mixture LUV and (a) 5 nm, (b) 25 nm and (c) 60 nm.

Supplementary Fig. 8 | Dye leakage assay at increased concentration of AuNPs. Study of calcein dye leakage from POPC (red), and DOPC (grey) lipid vesicles after interacting with increasing concentration of (a) 25, (b) 35, (c) 50 and (d) 60 nm AuNPs.