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## Gold nanoparticles interacting with synthetic lipid rafts: an AFM investigation

Microscopy

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#### Summary

Inorganic nanoparticles (NPs) represent promising examples of engineered nanomaterials, providing interesting biomedical solutions in several fields, like therapeutics and diagnostics. Despite the extensive number of investigations motivated by their remarkable potential for nanomedicinal applications, the interactions of NPs with biological interfaces are still poorly understood. The effect of NPs on living organisms is mediated by biological barriers, such as the cell plasma membrane, whose lateral heterogeneity is thought to play a prominent role in NPs adsorption and uptake pathways. In particular, biological membranes feature the presence of rafts, that is segregated lipid micro and/or nanodomains in the so-called liquid ordered phase  $(L_0)$ , immiscible with the surrounding liquid disordered phase  $(L_d)$ . Rafts are involved in various biological functions and act as sites for the selective adsorption of materials on the membrane. Indeed, the thickness mismatch present along their boundaries generates energetically favourable conditions for the adsorption of NPs. Despite its clear implications in NPs internalisation processes and cytotoxicity, a direct proof of the selective adsorption of NPs along the rafts' boundaries is still missing to date. Here we use multicomponent supported lipid bilayers (SLBs) as reliable synthetic models, reproducing the nanometric lateral heterogeneity of cell membranes. After being characterised by atomic force microscopy (AFM) and neutron reflectivity (NR), multidomain SLBs are challenged by prototypical inorganic nanoparticles, that is citrated gold nanoparticles (AuNPs), under simplified and highly controlled conditions. By exploiting AFM, we demonstrate that AuNPs preferentially target lipid phase

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boundaries as adsorption sites. The herein reported study consolidates and extends the fundamental knowledge on NPs-membrane interactions, which constitute a key aspect to consider when designing NPs-related biomedical applications.

#### Introduction

Despite the impressive technological advancement in the design of 'smart' inorganic nanoparticles (NPs), their impact on biological systems and related toxicity are still poorly understood (Nel *et al.*, 2009; Henriksen-Lacey *et al.*, 2017), limiting their effective clinical translation. The interaction of engineered nanomaterials, either intentionally or inadvertently released into the environment, with living organisms is mediated by biological barriers, such as cell plasma membranes, which primarily determine NPs biological fate and cytotoxicity (Beddoes *et al.*, 2015). Therefore, the interaction of NPs with biological interfaces is a key research topic, aiming at the safe use of nanotechnology and maximisation of its potential in therapeutics and diagnostics (Mendozza *et al.*, 2019; Zendrini *et al.*, 2020).

In this framework, lipid-based synthetic model membranes are useful platforms to mimic biological interfaces under simplified conditions, allowing for the identification of key determinants regulating nano-bio interactions (Gkeka *et al.*, 2013; Simonelli *et al.*, 2015; Su *et al.*, 2018). Supported lipid bilayers (SLBs) are often used as 2D biomembrane models (Richter *et al.*, 2006; Hardy *et al.*, 2013), enabling to precisely tune their physicochemical properties and avoiding the complications related to the 3D nature of biological membranes. They also represent versatile and promising platforms for the development of biosensors (Nikoleli *et al.*, 2018) and technological assays for biological applications (Worsfold *et al.*, 2006).

In addition, multicomponent SLBs models allow studying the lateral compositional heterogeneity that characterises

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most biological membranes. The existence of discrete lipid domains in natural membranes was questioned for a long time before its direct experimental assessment (Munro, 2003). Recently however, advanced experimental techniques have provided convincing evidence that the self-organisation of lipids and proteins can induce subcompartmentalisation in cell membranes (Lingwood & Simons, 2010), which is thought to have a profound impact on their biological function (Sezgin et al., 2017). A specific case of lateral organisation is represented by lipid rafts, defined as micro and/or nanodomains, enriched in lipids such as cholesterol, sphingomyelin, saturated glycerophospholipids and glycosphingolipids: these lipids segregate in the so-called liquid-ordered phase  $(L_{o})$ . which is immiscible with the surrounding liquid-crystalline (disordered,  $L_d$ ) phase (Kovnova & Tenchov, 2013). This phase heterogeneity induces a thickness mismatch between neighbouring domains and the consequent, ergonically unfavourable, exposure of hydrocarbon regions to water, which results in an energetic cost, due to interfacial energy (Heberle et al., 2013). Rafts are thought to participate in the formation and targeting of nano-sized biogenic lipid vesicles (e.g. extracellular vesicles, EVs) (Busatto et al., 2020). They are also actively involved in multiple membrane processes, for example, they act as structural platforms for organising protein machinery (Lingwood & Simons, 2010), they can preferentially associate with specific membrane proteins (Simons & Ikonen, 1997) and represent centres for the assembly of signalling molecules. From a mechanical point of view, the presence of phase boundaries and, hence, bilayers thickness mismatches, generates deformations and increases membrane permeability (Kuzmin et al., 2005; Rawicz et al., 2008; Sheikh & Jarvis, 2011). All these structural perturbations promote the selective adsorption of materials on the membrane; indeed, as pointed out by Hamada et al. (2012), lateral heterogeneity, promoted by the presence of micro-sized lipid rafts, regulates the adsorption of nano/microparticles, with the larger ones preferring the  $L_d$  phase-domains and the smaller ones being localised in the L<sub>o</sub> phase-domains of cellsized lipid vesicles. These selective NPs adsorption pathways are also present in the case of nano-sized lipid segregated domains and can be studied exploiting liposomes with tuneable rafts size (Heberle et al., 2013). However, investigating the interaction of NPs with nanometric lipid rafts remains a major challenge, mainly hindered by the small size of the segregated domains, which makes standard optical techniques not suitable for the task. Recent studies demonstrated that gold nanoparticles (AuNPs) adsorb more strongly to phase-separated multicomponent lipid bilayers; in particular, they are believed to preferentially target phase boundaries, due to the intrinsic negative curvature that characterises these regions (Melby et al., 2016; Sheavly et al., 2019).

To the best of our knowledge, this behaviour has only been investigated by computational studies (Sheavly *et al.*, 2019) and experiments involving quartz crystal microbalance (QCM)

(Melby *et al.*, 2016), which provide important but indirect evidences. In summary, the preferential adsorption of AuNPs along the boundaries of nano-sized lipid domains has never been directly observed.

To fill this gap, we exploit Atomic Force Microscopy (AFM), to directly visualise the preferential adsorption of AuNPs on the phase boundaries of multicomponent SLBs, presenting both an  $L_d$  and an  $L_o$  phase-like domains and previously characterised by neutron reflectivity (NR). The  $L_d$ domains are mainly composed of 1,2-dioleoyl-sn-glycero-3phosphocholine (DOPC) with two unsaturated hydrocarbon chains that hinder molecular packing, while the  $L_0$  domains are mainly composed of 1,2-dipalmitoyl-sn-glycero-3phosphocholine (DSPC) lipids; cholesterol molecules occupy the free volume between the lipid acyl chains (Toppozini et al., 2014; Sezgin et al., 2017). The quantitative localisation and morphometry of AuNPs adsorbed on the SLB reveal important information regarding their interaction with the lipid matrix. The study corroborates the already theorised differential NPslipid interaction taking place at the phase boundaries of lipid rafts. The presented results could help the development of future NPs-based applications that involve their adsorption on membranes characterised by nanoscale phase segregations.

#### Materials and methods

#### Materials

Tetrachloroauric (III) acid ( $\geq$ 99.9%), trisodium citrate dihydrate ( $\geq$ 99.9%), methanol (99.8%), CHCl<sub>3</sub> ( $\geq$ 99.9%), NaCl ( $\geq$ 99.5%) and CaCl<sub>2</sub> (99.999%) were provided by Sigma-Aldrich (St. Louis, MO, USA). The same for 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) ( $\geq$ 98.0%), cholesterol ( $\geq$ 99.5%) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) ( $\geq$ 98.0%). All chemicals were used as received. Milli-Q grade water was used in all preparations.

#### AuNP preparation

Anionic gold nanospheres of 16 nm in size were synthesised according to the Turkevich-Frens method (Turkevich *et al.*, 1951; Frens, 1973). Briefly, 20 mL of a 1 mM HAuCl<sub>4</sub> aqueous solution were brought to boiling temperature under constant and vigorous magnetic stirring. Two millilitres of 1% citric acid solution were then added and the solution was further boiled for 20 min, until it acquired a deep red colour. The nanoparticles solution dispersion was then slowly cooled down to room temperature.

### Vesicle preparation and SLB formation for neutron reflectivity measurements

*Vesicle preparation.* The proper amount of a DOPC/DSPC/ cholesterol mixture (39/39/22 mol%) was dissolved in chloroform and a lipid film was obtained by evaporating the solvent under a stream of nitrogen and overnight vacuum drying. The film was then swollen and suspended in warm (50°C) 100 mM NaCl water solution by vigorous vortex mixing, in order to obtain a final 0.5 mg mL<sup>-1</sup> lipid concentration. The resultant multilamellar vesicles (MLVs) were tip sonicated with a Digital Sonifier Model 450 (Branson, Hampton, NH, USA), provided with a Horn Tip (diameter 25.4 mm), in an intermittent-pulse mode (5 s), with a power of 400 W (amplitude 50%), for 15 min to obtain a homogeneous dispersion of unilamellar vesicles (ULVs).

Surface cleaning procedure. DOPC/DSPC/cholesterol single lipid bilayers were formed on  $50 \times 80 \times 15 \text{ mm}^3$  Silicon mirrors (Andrea Holm GmbH, Tann, Germany; roughness  $\leq 5$  Å). Substrates were preliminary rinsed in either ultrapure water and ethanol, in order to remove organic residues. After that, they were bath sonicated treated for 30 min in ethanol with a Bandelin DL 102 3L bath sonicator (Bandelin Ultraschall seit 1955, Berlin, Germany), followed by other 30 min in ultrapure water (Millipore Simplicity UV). The surfaces were then cleaned with a Novascan PSD-UV8 UV/ozone plasma (Boone, IA, USA) for 30 min and rinsed in ultrapure water. Finally, they were dried with nitrogen gas and stored in ultrapure water, ready for the deposition.

*Vesicle fusion and SLB formation.* CaCl<sub>2</sub> was added to the vesicle dispersion, reaching a final concentration of 10 mM, just before the injection in the NR measuring cell. This was performed in order to promote their adhesion to the support and their subsequent disruption. Vesicles were left incubating for 30 min; then, the saline buffer was switched to  $D_2O$  to promote the vesicle disruption and SLB formation. The use of  $D_2O$  instead of ultrapure water ensures a better resolution of the lipid structures for the NR measurements.

#### Vesicle preparation and SLB formation for AFM measurements

*Vesicle preparation.* The proper amount of a DOPC/DSPC/ cholesterol mixture (39/39/22 mol%) was dissolved in chloroform and a lipid film was obtained by evaporating the solvent under a stream of nitrogen and overnight vacuum drying. The film was then swollen and suspended in warm (50 °C) ultrapure water solution by vigorous vortex mixing, in order to obtain a final 0.5 mg mL<sup>-1</sup> lipid concentration. The resultant multilamellar vesicles in water were subjected to 10 freeze-thaw cycles and extruded 10 times through two stacked polycarbonate membranes with 100 nm pore size at room temperature, to obtain unilamellar vesicles with narrow and reproducible size distribution. The filtration was performed with the Extruder (Lipex Biomembranes, Vancouver, Canada) through Nuclepore membranes.

*Surface cleaning procedure.* All reagents were purchased from Sigma-Aldrich Inc (www.sigmaaldrich.com, St. Louis,

MO, USA). DOPC/DSPC/Chol supported lipid bilayers were formed on microscopy borosilicate glass coverslips (Menzel Gläser). Glass slides were first immersed in a 3:1 mixture of 96%  $H_2SO_4$  and 50% aqueous  $H_2O_2$  ('oxidising piranha') solution for 2 h in order to remove any organic residue present on their surface. Then, the slides were cleaned in a sonicator bath (Elmasonic Elma S30H, Distrelec, Lainate, MI, Italy) for 30 min in acetone, followed by 30 min in isopropanol and 30 min in ultrapure water (Millipore Simplicity UV). Glass slides were then cleaned with air plasma for 15 min (air plasma cleaner PELCO easiGlow, Ted Pella Inc., Redding, CA, USA) and incubated in ultrapure water for 10 min in order to maximise the number of reactive silanols present on the surface. Finally, they were dried with nitrogen gas and stick to a magnetic disk, ready for the lipid solution deposition.

Vesicle fusion and SLB formation. A 100  $\mu$ L droplet of buffer solution was first spotted on the SiO<sub>2</sub> slide. The buffer solution consisted of CaCl<sub>2</sub> 200 mM diluted 1:10 in KCl 100 mM. A 10  $\mu$ L droplet containing the lipid mixture was then added to the buffer droplet and left incubating at room temperature for 30 min in order to promote the vesicle adsorption on the surface. After that, the droplet was removed and replaced by a 100  $\mu$ L droplet of ultrapure water which was then left incubating for other 15 min. AuNPs deposition on the SLB was obtained by adding 5  $\mu$ L of a 7.8 nM AuNPs dispersion to the ultrapure water droplet and leaving it to incubate for 10 min. After the system equilibrated, the large droplet was gently removed and the slide was inserted in the AFM fluid cell for the measurements.

*Neutron reflectivity measurements.* NR measurements were conducted at the REFSANS Horizontal TOF reflectometer of the Helmholtz-Zentrum Geesthacht located at the Heinz Maier-Leibnitz Zentrum in Garching, Germany (Kampmann et al., 2006; Moulin & Haese, 2015). Neutrons in the wavelength range 3.0–21.0 Å were used to carry out the measurements. Two incident angles, namely 0.60° and 3.00°, allowed collecting data in the range  $0.007 < Q/Å^{-1} < 0.22$ . The arrival times and positions of scattered neutrons were detected on a Denex 2D 500  $\times$  700 mm<sup>2</sup> multiwire <sup>3</sup>He detector (pixel size  $2.1 \times 2.9 \text{ mm}^2$ , efficiency 80% at 7 Å, gamma sensitivity  $< 10^{-6}$ ) positioned at 4.5 m from the sample. The detector was installed in a liftable vacuum tube in order to reach exit angles up to 5.2° at the maximum elongation. In order to receive sufficient statistics, a counting time of about 4 h for the measurement was chosen. The software MOTOFIT (Nelson, 2006) was employed for the analysis of the NR curve. Details on data analysis are reported in the SI.

#### AFM measurements

*AFM setup.* All AFM experiments were performed at room temperature on a Bruker Multimode 8 (equipped with

Nanoscope V electronics, a sealed fluid cell and a type JV piezoelectric scanner) using Bruker SNL-A probes (triangular cantilever, nominal tip curvature radius 2–12 nm, nominal elastic constant 0.35 N m<sup>-1</sup>) calibrated with the thermal noise method (Hutter & Bechhoefer, 1993). The AFM fluid cell was filled with a saline buffer solution, consisting of KCl 100 mM, which has the main effect of reducing the Debye length that characterises the electrical double layer (EDL) interaction region between AFM tip and SLB (Müller *et al.*, 1999). In this way, better image resolution can be achieved.

AFM imaging. Imaging was performed in PeakForce mode. In order to minimise deformations or rupture events induced by the scanning probe, the applied force setpoint was kept under 200 pN range. Feedback gain was set on values high enough to obtain optimal image quality but low enough to prevent the introduction of noise signals that would otherwise interfere with the resolution of the different lipid domains, having a height difference of  $\sim 1$  nm. The average height value of all bare substrate zones was taken as the baseline zero height reference. Image background subtraction was performed using Gwyddion 2.53.16 (Nečas & Klapetek, 2012). In order to map the edges of lipid rafts and AuNPs, height ranges were manually optimised to define two image masks, the first only containing all Lo domains, the second singling out all NPs. Once both types of objects were correctly selected by appropriately chosen masks, a Gwyddion built-in function was used to automatically detect edges, and the resulting images were exported. Finally, the exported images containing the edges of either Lo domains or NPs, originally present in the same AFM image, were superimposed to reveal all NPs-lipid domains edge overlaps. To estimate the degree of preferential adsorption of NPs along the rafts' edges, we calculated the ratio between the number of NPs adsorbed along the boundaries and the total amount of NPs present in the images.

#### **Results and discussion**

#### Formation of supported lipid bilayers containing lipid rafts

The formation of a continuous planar bilayer [DOPC/DSPC/ cholesterol (39/39/22 mol%)], covering the vast majority of the supporting surface, was achieved through vesicle fusion and characterised by NR. Briefly, as described in the Materials and Methods section, liposomes in a saline buffer were mixed with a low amount of  $CaCl_2$ , injected within the measurement chamber and left adsorbing on the support (a clean Si crystal). The presence of  $Ca^{2+}$  ions in solution promotes the crowding of vesicles on the surface by reducing the repulsive interactions between liposomes with surface charge. As reported by Richter *et al.* (2006), when a critical vesicle coverage is reached, the stress on the vesicles becomes sufficient to induce their rupture; in our case the phenomenon was also favoured by the additional osmotic shock, coming from the replacement of the saline buffer with ultrapure water. The edges of the newly-formed SLB are energetically unfavourable and cause the rupture of other surface-bound vesicles. If the density of adsorbed vesicles is sufficiently high, these cascade phenomena can lead to the complete surface coverage.

Given its ability to probe large sample areas (tens of millimetres), neutron reflectivity (NR) was herein applied to probe the effective formation of a homogeneous SLB and its structure along the normal to the SLB plane. Figure 1(A) shows a representative NR profile measured for the SLB in D<sub>2</sub>O (green circles), together with the fitting curve (red continuous line). The curve was analysed with MOTOFIT and, consistently with the literature (Montis et al., 2016, 2020; Luchini et al., 2019), it was possible to model the profile of the SLB as a stack of five layers (see scheme in Fig. 1B): the silicon oxide layer, a layer of solvent  $(D_2O)$ , a layer for the polar headgroups in contact with the support (inner heads), a layer for the lipid chains (chains) and, finally, a layer for the polar headgroups in contact with the solvent (outer heads). Each layer is characterised by a defined contrast (the scattering length density, SLD), thickness (*d*), roughness ( $\rho$ ) and hydration (solvent %). The curve fitting results are reported in Table 1. The overall thickness of the bilayer is  $\sim 5 \text{ nm}$  (given by the sum of the thickness values related to the inner and outer heads, plus the lipid chains). The negligible hydration (0.1%) of the lipid chains layer indicates that the surface was almost completely covered by a homogeneous lipid bilayer. The analysis of the experimental data allowed reconstructing the entire profile of the SLB along the normal to the surface (see Fig. 1B).

While NR provides information on the average structure with respect to the bilayer normal, AFM can be used to resolve in detail the in-plane rafts morphology (Milhiet et al., 2001; Yuan et al., 2002; Mingeot-Leclercq et al., 2008; Cai et al., 2012). The SLB was formed on functionalised borosilicate glass coverslips, by injecting the liposomes (this time suspended in ultrapure water) in the buffer solution where they experienced an osmotic imbalance across the membrane, decreasing their pressurisation (please refer to 'Materials and methods' section for the details). As a result, following the adhesion to the substrate, liposomes will deform adopting more oblate shapes (Ridolfi et al., 2019), increasing the area occupied by each vesicle and favouring the previously described vesicle fusion mechanism. As shown in Figure 1(C), consistently with NR data the surface is almost completely covered by a lipid bilayer, which presents nanometric domains of different heights, with the brighter areas corresponding to thicker membrane regions and the darker ones to thinner SLB portions. Accordingly, the height distribution of Figure 1(D)confirms the presence of two distinct lipid phase-like domains, with height values of  $h_d = 3.7$  nm and  $h_o = 4.7$  nm, in good agreement with the results obtained by Heberle et al. (2013) on the same vesicle preparation. This thickness mismatch can be ascribed to the coexistence of two lipid phases of different composition, dictating variations in the membrane's height



Fig. 1. Characterisation of the multicomponent SLB formed from DOPC/DSPC/cholesterol (39/39/22 mol%) liposomes by vesicle fusion. (A) Neutron Reflectivity profile (green circles) and best fit (continuous red line) corresponding to the SLB in D<sub>2</sub>O; from the fitting analysis the average bilayer thickness is ~ 5 nm. (B) Scattering length density (SLD) profile, describing variations of the SLD along the direction perpendicular to the bilayer. (C) Representative AFM topography of the SLB. The bilayer uniformly covers the surface, displaying both the  $L_o$  (brighter thicker regions) and  $L_d$  phases (darker thinner regions) as segregated domains. The reported scalebar is 1 µm. The 500 × 500 nm micrograph (bottom inset) displays the small hole in the bilayer that allowed flattening the image with respect to the SiO<sub>2</sub> surface. Two perpendicular height profiles were traced, horizontally and vertically, across the whole image (top inset); the profiles confirm the presence of the two distinct lipid phases covering the surface. (D) Height distribution obtained from the AFM image; the two distinct peaks, centred at  $h_d = 3.7$  nm and  $h_o = 4.7$  nm, describe the different heights that characterise the  $L_d$  and  $L_o$  phase, respectively.

(Lewis & Engelman, 1983; Petrache *et al.*, 2000; Bleecker *et al.*, 2016): in particular, membrane thickness was found to increase with length or degree of saturation of the lipid tails (Lewis & Engelman, 1983; Petrache *et al.*, 2000). Here the thicker domains can be associated with the  $L_o$  phase, which is enriched with cholesterol and DSPC, that is a fully saturated long chain lipid. On the contrary, thinner regions correspond to the  $L_d$  lipid phase mainly composed of DOPC, which is characterised by a shorter tail length and two chain unsaturated bonds. After having properly flattened the image, by the application of a mask (see Fig. S4), it is possible to determine the area fractions occupied by each of the two phases. Heberle *et al.* (2013) reported the area fraction corresponding to the  $L_d$  phase-like domains for liposomes of the very

calculations on SLBs at 28°C are in line with those findings, giving a  $L_d$  area fraction of 0.50. Results also suggest that the SLB formation did not significantly modify the amount of  $L_d$  and  $L_o$  lipids, originally present in the unfused vesicles and that the lipid phase behaviour is not affected by the presence of the solid support. The presented results strengthen the essential role of AFM in providing comprehensive morphological details on structure of rafted membranes. In the following paragraph, we extend the existing literature on AFM-based rafts characterisations (Milhiet *et al.*, 2001; Yuan *et al.*, 2002; Mingeot-Leclercq *et al.*, 2008; Cai *et al.*, 2012), by studying the structure of lipid rafts following their interaction with AuNPs.

same composition to be 0.52 (at a temperature of  $20^{\circ}$ C); our

**Table 1.** Curve fitting results of NR data obtained with MOTOFIT. The reported fitting parameters are referred to the three layers composing the bilayer [inner heads referred to the layer of polar headgroups in contact with the support, lipid chains referred to the hydrophobic region of the SLB, outer heads referred to the layer of polar headgroups in contact with the solvent (i.e. D<sub>2</sub>O)]. For each layer four parameters are reported: *d* (Å), the thickness of the layer;  $\rho$  (Å), roughness of the layer; SLD (10<sup>-6</sup> Å<sup>-2</sup>), scattering length density of the layer (calculated from the layer composition); solvent % D<sub>2</sub>O penetration in each layer.

Layer name	$d(\text{\AA})$	$\rho ({\rm \AA})$	$\rm{SLD}(10^{-6}\rm{\AA}^{-2})$	Solvent %
Inner heads Lipid chains	$5\pm 2$ $38\pm 3$	$\begin{array}{c} 2\pm1\\ 1\pm1 \end{array}$	$1.87 \\ -0.18$	$5 \pm 1$ $0.1 \pm 0.1$
Outer heads	$6\pm 2$	$2\pm 1$	1.87	$16\pm4$

## Interaction of AuNPs with lipid rafts: localisation of AuNPs at the boundaries

In order to investigate the interaction of 16 nm citrated AuNPs (please refer to Materials and Methods for AuNP synthesis and to SI for AuNPs characterisation details) with the lipid rafts present in the SLB, 5  $\mu$ L of the NPs dispersion were injected in the ultrapure water buffer. Different literature reports connect the presence of phase segregation within the lipid bilayer to the selective adsorption of NPs along the domains boundaries (Melby *et al.*, 2016; Sheavly *et al.*, 2019); however, a direct proof of this interaction is still missing to date. AFM represents one of the few techniques that could provide the sufficient resolution to simultaneously resolve the height difference between the two lipid domains ( $\sim 1$  nm) and the morphology of AuNPs. Despite the high resolution

provided by AFM, the measurement remains challenging, as the spontaneous attachment of AuNPs to the probe (Fig. S5) can often lead to imaging artefacts. In order to overcome this problem, the AFM fluid cell was filled with the same saline buffer used for SLB formation and the force SetPoint was kept on very low values (lower than  $\sim 200$  pN). The use of the saline buffer as imaging solution should compensate the tip-sample electrical double-layer repulsion (Müller et al., 1999) and limit the attachment of the NPs to the probe. In order to identify the portions of lipid bilayer characterised by the presence of AuNPs, images of  $5 \times 5 \,\mu\text{m}$  regions were initially acquired. Figure 2(A) shows a representative AFM topography of the SLB following the NPs injection. The bigger spherical objects represent vesicles that still have to fuse within the bilayer, while the smaller ones are the AuNPs. which seem to be homogeneously distributed above the SLB.

From a simple AFM topography, small lipid vesicles can be confused with AuNPs or AuNPs clusters; this could introduce statistical noise to the localisation and morphometrical analysis. We recently developed an AFM-based nanomechanical characterisation able to discriminate lipid vesicles from objects with the same morphology but different mechanical behaviour (Ridolfi et al., 2019). This method evaluates the deformation that lipid vesicles undergo once adsorbed on a surface, by calculating their contact angle ( $\alpha$ ). Through the measurement of  $\alpha$  and by assuming that the surface area of the vesicles is preserved upon adsorption, it is also possible to evaluate the diameter that characterises the unperturbed vesicles in solution (called Diameter in solution). As described in Figure 3, lipid vesicles are characterised by a narrow distribution of contact angles over a wide range of sizes (Diameter in solution), while AuNPs present a narrow size distribution and



(B)



Fig. 2. (A) Representative AFM topography of the SLB following the interaction with AuNPs. Lipid rafts are still visible as differently shaded areas. The larger and heterogeneous spherical objects represent unfused vesicles while the smaller ones are the AuNPs that have been homogeneously adsorbed on the lipid bilayer. Scalebar is 1  $\mu$ m. (B) Transmission electron microscopy (TEM) image of the AuNPs that were used in the experiments, scalebar is 100 nm (please refer to the SI for details regarding TEM characterisation).



Fig. 3. Plot representing the distributions of contact angle vs solution diameter of either vesicles (blue circles) and AuNPs (yellow circles). Vesicles data have been obtained from the liposomes present in Figure 2(A) while the AuNPs data come from micrographs like the ones reported in Figure 4(A). Even though adsorbed on the SLB, liposomes show their nanomechanical fingerprint: a narrow contact angle distribution over a wide range of sizes. Their average contact angle is  $\sim 54^{\circ}$  hence describing highly deformed shapes, possibly due to the SLB formation procedure. AuNPs display narrow distributions for both their size and contact angle, with average values of 14 nm and 109°, respectively.

higher contact angle values. This enables the easy singling out of the AuNPs and their exclusive inclusion in the next analysis.

In order to precisely determine whether the NPs targeted specific locations on the lipid matrix, the size of the scanned region was further reduced. In Figure 4(A), representative images, with sizes of  $\sim 600 \times 600$  nm, illustrating the SLB decorated by AuNPs have been reported. The micrographs of Figure 4(A) constitute the direct proof of the AuNPs selective adsorption along the segregated phase boundaries.



**Fig. 4.** (A) Representative AFM micrographs that clearly display the selective adsorption of AuNPs along the boundaries of the lipid rafts (brighter regions of the SLB that correspond to the Lo lipid phase). From the images it is also possible to distinguish between isolated and clustered NPs. All scalebars are 100 nm. (B) Contour images obtained from the micrographs. Black lines represent the rafts edges while gold circles define the contours of the AuNPs. The gold NPs edges are always in contact with at least one of the lines describing the lipid segregated phase boundaries.



Fig. 5. Schematic representation of the configuration used to evaluate, from a conceptual point of view, the contact angle that would characterise an AuNP with a diameter of 14 nm, adsorbed on a rigid flat surface and surrounded by a  $\sim$  5 nm lipid bilayer.

In the free image processing software Gwyddion 2.53.16. the sequential application of different masks allowed mapping the edges of either the lipid rafts and NPs shown in Figure 4(A)and, hence, obtaining a clearer indication of their relative positions. In Figure 4(B) the contour images of NPs and rafts have been superimposed with different colours, to highlight that AuNPs preferentially targeted the boundaries of the two lipid phases; indeed, the lines describing their shapes are always in contact with the edges of the lipid rafts. To estimate the degree of preferential adsorption of NPs along the rafts' edges, we calculated the ratio between the number of NPs adsorbed along the boundaries and the total amount of NPs present on the SLB, finding that 91% of the NPs were located along the edges. These results prove the hypothesis that phase boundaries represent energetically favourable niches for lipid-NPs interactions. As previously discussed elsewhere (Sheavly et al., 2019), NPs adsorption induces bilayer bending, which entails an energy penalty that increases the free energy associated with the overall process. This energy penalty is almost completely reduced along the phase boundaries, where the local negative curvature of the membrane, caused by the thickness mismatch between the two lipid phase-like domains minimises the free energy associated with the NPs adsorption (Sheavly et al., 2019).

#### Inclusion of AuNPs within the lipid bilayer

AuNPs have a diameter of 16 nm (refer to SI for details), which is close to the average height measured with AFM imaging  $(14 \pm 2 \text{ nm})$ . This suggests that after adsorbing on the SLB, AuNPs probably penetrate the bilayer and reach the SiO<sub>2</sub> surface. This result further extends the characterisation of NPs–lipid interaction and corroborates our vision of rafts' boundaries as regions of increased permeability (Kuzmin *et al.*, 2005; Rawicz *et al.*, 2008; Sheikh & Jarvis, 2011), where the membrane can easily wrap around the adsorbed NPs. Recent findings (Montis *et al.*, 2020) confirm these results, suggesting that free-standing lipid bilayers can bend around the AuNPs surface, guided by citrate-lipid ligand exchange at the interface. All the above hypotheses are confirmed by the evaluation of the AuNPs contact angle with respect to

the SLB. As suggested by Vinelli *et al.* (2008), the contact angle of a perfectly spherical, non-deformable (under the considered forces) object should be  $180^\circ$ , while we measured a substantially lower value. These apparent discrepancies can be rationalised by a careful morphological analysis, as detailed below.

The size of AuNPs is comparable with the tip radius; hence, the effect of tip convolution should be taken into account. This was performed by assuming the NPs as perfectly spherical and non-deformable objects with heights that coincide with their actual diameters. This is a reasonable assumption given that, during an AFM measurement, the error along the vertical direction is negligible compared to the ones in the scanning plane. As a consequence, all the measured radii were then corrected by  $\sim 6$  nm (corresponding to half the difference between the average NPs height and diameter measured by AFM). The NPs average contact angle, calculated with respect to the SLB and by using the corrected radii, gave a value of 109°, which is in very good agreement with the result that can be obtained from a simple geometrical model (Fig. 5), featuring a 14 nm spherical and undeformable NP immersed in a  $\sim$ 5 nm lipid bilayer. For that case,  $\alpha$  would be equal to 107°; this last result confirms that AuNPs penetrated the lipid bilayer and reached the underlying substrate.

#### Conclusion

The presence of lipid rafts within the cell membrane has been linked with multiple important biological functions, like the formation and targeting of lipid nanovesicles. The thickness mismatch that originates between the different immiscible segregated domains is thought to generate mechanical stresses that enhance the membrane permeability along these regions. We herein exploited atomic force microscopy to investigate the preferential adsorption of AuNPs along the phase boundaries of SLBs, generated from DOPC/DSPC/cholesterol (39/39/22 mol%) liposomes. Different works in the literature suggested a selective adsorption of AuNPs along the boundaries of lipid segregated domains, but a direct observation of this phenomenon is still missing to date. AFM allowed us to probe the existence of nanometric lipid rafts on the newly

formed SLB and to spot the presence of NPs along their edges, hence providing a direct proof of this preferential adsorption pathway. In addition, we provided useful details about the experimental procedures that could significantly improve the reliability of AFM imaging; indeed, one of the major challenges hindering this type of measurements is the frequent tip contamination, caused by the attachment of the NPs to the AFM probe. We showed that the use of a saline buffer as imaging solution within the AFM fluid cell leads to optimal image quality and strongly reduces tip contamination events. Then, through the application of an AFM-based morphometric nanomechanical characterisation, it was also possible to further investigate the reorganisation of the lipid bilayer, as a consequence of the AuNPs adsorption. We found out that the lipid matrix wrapped around the NPs, allowing them to penetrate within the hydrophobic region until reaching the rigid SiO<sub>2</sub> surface of the slides. The theoretical calculation of the morphological parameters describing this phenomenon is in perfect agreement with the experimental results and further corroborates our interpretation. Further studies will focus on extending this characterisation to membranes with varying compositions and employing NPs of different core and/or size.

#### Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. The authors declare no competing financial interests.

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#### **Supporting Information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1**. Experimental SAXS profile (markers) obtained for citrated AuNPs and curve fit (solid black line) according to the Schulz spheres model from the NIST package SANS Utilities.

**Fig. S2.** UV-Vis absorption spectra of citrated AuNP dispersion (after 1:5 dilution in water).

**Fig. S3.** AFM topography representing AuNPs either isolated or clustered, adsorbed on a bare mica substrate.

Fig. S4. AFM topography of the SLB obtained from DOPC/DSPC/Chol (39/39/22 %w/w) liposomes.

Fig. S5. AFM topography showing the effects of tip contamination on the imaging of AuNPs adsorbed on the lipid bilayer. **Table. S1.** Chemical formula, molecular volumes and corresponding scattering length densities of species relevant to this study