Supporting Information for:

Interaction of Nanoparticles with Lipid Membranes:

a Multiscale Perspective

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<table>
<thead>
<tr>
<th>Supplementary Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characterization of Giant Unilamellar Vesicles (GUVs) and Large Unilamellar Vesicles (LUVs) from POPC</td>
<td>S2</td>
</tr>
<tr>
<td>Protein Corona isolation</td>
<td>S4</td>
</tr>
<tr>
<td>Protein corona characterization</td>
<td>S5</td>
</tr>
<tr>
<td>NP@Ct and NP@PC physicochemical characterization</td>
<td>S6</td>
</tr>
<tr>
<td>Selection of NP model concentration (determination and cytotoxicity)</td>
<td>S8</td>
</tr>
<tr>
<td>GUVs’ membrane permeabilization upon interaction with NPs: FCS experiments with Alexa 568</td>
<td>S10</td>
</tr>
<tr>
<td>GUVs’ membrane permeabilization upon interaction with NPs: FCS experiments with Tamra</td>
<td>S12</td>
</tr>
<tr>
<td>FCS characterization of the mobility of the fluorescent lipid Liss Rhod PE inside the GUVs’ bilayer</td>
<td>S14</td>
</tr>
<tr>
<td>Supplementary Materials and Methods</td>
<td>S15</td>
</tr>
</tbody>
</table>
Characterization of Giant Unilamellar Vesicles (GUVs) and Large Unilamellar Vesicles (LUVs) from POPC

Supplementary Figure S1: GUVs and LUVs were prepared right before each experiment, as described in the Supplementary Materials and Methods; the GUVs were resuspended in PBS 10 mM prior to use and allowed to settle for two hours on the well bottom before exposure to NPs. Panel (a) reports a representative Fluorescence Correlation Spectroscopy (FCS) profile for Liss Rhod PE diffusing inside a POPC GUVs’ bilayer. As reported in the confocal scanning laser microscopy (CLSM) image in panel (b), FCS measurements were performed focusing the laser beam on the GUVs pole, indicated by the yellow viewfinder (⊕). The experimental curve (black dots) and the curve fit, according to a 2D normal diffusion model (red continuous line), were obtained after a calibration procedure (c) employing a 50 nM solution of Alexa 568 (D = 332μm²s⁻¹ in water at 25°C). This procedure allows to determine the parameters of the detection volume, according to equation (2) and to accurately estimate the 2D diffusion coefficient of Liss Rhod PE inside the bilayer, as D = 8.0 ± 0.9 μm²s⁻¹. Panel (d) (e) (f) report CSLM images of POPC GUVs fluorescently labeled with Liss Rhod PE ((d), λ excitation 561 nm λ emission 571 nm – 650 nm), β-Bodipy
((f), $\lambda_{\text{excitation}}$ 488 nm $\lambda_{\text{emission}}$ 498 nm – 530 nm) and unlabeled POPC ((e), transmission). Panel (g) reports the physiochemical characterization of POPC LUVs dispersed in PBS through $\zeta$-potential and dynamic light scattering (DLS) measurements, which yield a $\zeta$-potential of -4.9 mV and a mean hydrodynamic diameter ($D_h$) of 140 nm, respectively.
**Protein Corona isolation**

**Supplementary Figure S2:** Experimental protocol adopted for the isolation of NP@PC (gold circles surrounded by a red halo), obtained through incubation of NP@Ct (gold circles) in 10% FBS (red ellipses) or 10% TAMRA labeled FBS (red ellipses with stars). Further details on FBS labeling and Protein corona separation are provided in the Supplementary Materials and Methods. Briefly, NP@Ct were mixed and incubated with the different protein sources for one hour in order to let the proteins adsorb and exchange citrate molecules on the NP surface (left panel). After obtaining stable NP@PC complexes (middle), centrifugation and two rinsing steps of the resulting pellet were performed to eliminate the protein excess, giving stable NP@PC or NP@LabPC complexes (right panel).
**Protein corona characterization**

![Image of SDS-PAGE and Dot Blot](image)

**Supplementary Figure S3**: Biochemical characterization of NP@PC and NP@LabPC complexes. Panel (a) shows on the left a representative fluorescence image of the SDS-PAGE separation of the proteins eluted from NP@PC and NP@LabPC complexes, obtained as described in the Supplementary Figure S2, and their respective supernatants. The gel image was acquired by using an epi-UV short pass illumination and a UV short pass filter, as described in Supplementary Materials and Methods. In particular, the first and the third lanes contain the proteins eluted from NP@LabPC and NP@PC while the second and the fourth lanes contain the proteins present in the supernatant of the last wash of the NP@LabPC and NP@PC, respectively. The red box indicates the residual fluorescence dye left after the chromatographic cleaning procedure. (Wang et al. 2013)

The right part of the panel presents a Silver Staining of the same samples loaded and processed by SDS PAGE. Panel (b) reports fluorescence Dot Blot of the AuNP protein corona complexes obtained with labeled (NP@LabPC) or unlabeled FBS (NP@PC) spotting drops of the two solutions on a parafilm, illuminating the solution on a transilluminator and acquiring images using a long pass filter.
Supplementary Figure S4: (a) Left panel: AFM image of as synthesized NP@Ct adsorbed on mica. Isolated NP@Ct as well as NP@Ct clustered in multimers can be observed. Size analysis performed on the NP and NP-cluster height gives the distribution displayed in the right panel.
which results centered at 15 nm. (b) UV-Vis spectra of NP@Ct redispersed in water (black line) or PBS (dotted line) and of NP@PC after purification from a PBS solution of labeled FBS (dashed line) or unlabeled FBS (dashed dotted line) and resuspension in PBS. The red arrow points out the red shift of the LSPR peak due to protein adsorption on NP surface, highlighted in the figure by the formation around the NP (yellow circle) of a protein corona (red halo), while the NP dispersion in PBS is characterized by a red-shifted broader SPR peak due to the formation of some NP clusters. Panel (c) reports a reflection confocal image of NPs in the GUV medium obtained as reported in Supplementary Materials and Methods. Panels (d-e) report the $\zeta$-potential ($\zeta$ Pot), the hydrodynamic diameter ($D_h$) and the polydispersity of NP@Ct obtained through a cumulant analysis stopped at the second order for NP@Ct dispersed in water and through Laplace inversion with the CONTIN algorithm for NP@Ct dispersed in PBS. In this latter case, the presence of some salt-induced NP@Ct aggregation (as emerged from UV-Vis spectra, panel (b)) was taken into account by analyzing the ACFs through the CONTIN algorithm, as further reported in the Supplementary Materials and Methods. The aggregation extent appeared quite low just after NPs dispersion preparation, as inferred from the distribution of hydrodynamic diameters of NPs, and increases with time (data not shown), both in terms of aggregate sizes and number of aggregates, with respect to primary NPs. Panel (f) reports the $\zeta$-potential, the hydrodynamic diameter, and the PDI of NP@PC dispersed in PBS. As suggested by the UV-Vis results, the formation of the protein corona leads to an increase of the hydrodynamic diameter and decrease of $\zeta$-potential.
Selection of NP model concentration

**AuNPs-GUVs relative concentration evaluation.**

The amount of AuNPs’ was chosen in order to reach saturation of the of the GUVs’ surface. Therefore, a controlled excess, roughly 2:1, of NPs’ surface with respect to the external surface area of GUVs was employed.

The final concentration in the measurement chamber is \([NP] = 3.5 \text{ nM}\) (determined through the UV-Vis absorption, see materials and methods paragraph) \([lipids] \approx 20 \text{ µM}\) (from the initial lipid amount employed for the electroformation, assuming the completeness of the electroformation process and the absence of air bubbles in the electroformation chamber. Approximately one half of the lipids (10 µM) will be localized in the external leaflet, since the bilayer thickness, about 4-5 nm, is negligible with respect to the average diameter of 25 µ of the GUVs. The surface area of a NP with hydrodynamic radius \(r \approx 15 \text{ nm}\) (considering only the metallic core of NPs, without taking into accounts neither ligands nor the protein corona shell) is \(NP_{cs} 4 \pi r^2 \approx 2826 \text{ nm}^2\).

Considering a lipid cross section \(lipid_{cs} \approx 0.5 \text{ nm}^2\), the NP surface is approximately doubled with respect to the full coverage of the external bilayer leaflet.

**In-Vitro NP Toxicity evaluation**

Viability of HeLa cells upon exposure to NP@Ct and NP@PC at 3.5 nM was evaluated via a metabolic assay (MTT assay). Results, reported below in Supplementary Figure S5, exclude any toxicity.

**Experimental details**

HeLa epithelial cells were seeded with a density of 1x10⁴ cells/well in 96-well-plates with a final volume of 100 µL growth medium (DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin) and incubated for 24 hours in an incubator (37°C, 5% CO₂). Subsequently, cells were washed with PBS twice, and incubated in DMEM. After that, cells were incubated with a 3.5 nM solution containing respectively NP@Ct or NP@PC. As control experiments, cells were incubate respectively in NP free hydrogen peroxide supplemented DMEM and 10% FBS supplemented DMEM.

After incubation of two hours with NPs, the cells were washed with PBS and a DMEM solution out of 10% MTT was added to the each well (total 100 µl/well). After incubation time of one hour with MTT, the solution was replaced with DMSO and absorbance spectra of the solutions were
measured, using a 96-well plate reader following the method described in the guidelines provided with the kit.

**Supplementary Figure S5:** Viability of HeLa cells after exposure to NP@Ct and NP@PC in a serum depleted medium (DMEM). Cells were incubated with the different NPs for two hours and their viability was then evaluated *via* a metabolic assay (MTT assay). The values reported are an average over 4 x 2 independent tests and were normalized to the values recorded for the control experiments without NPs. Control experiments obtained after exposure of cells to hydrogen peroxide (H\(_2\)O\(_2\)) and serum supplemented medium (complete medium) are also reported.
GUVs’ membrane permeabilization upon interaction with NPs: experiments with Alexa 568

Supplementary Figure S6: (a) Alexa diffusion was monitored through FCS and the experimental curves were fitted according to a one-component 3D normal diffusion model (equation 2). Alexa diffusion coefficient (D, μm²·s⁻¹) and concentration (C, nM) in different conditions are reported: first, Alexa was incubated for two hours with GUVs (in sucrose/PBS solution, as described in the Supplementary Materials and Methods section) and its 3D diffusion was monitored outside (Alexa/GUV outside) and inside (Alexa/GUV inside) GUVs’ lumen. Alexa penetration inside GUVs’ lumen is around 10% (1.9 nM) with respect to the external concentration (19 nM). As expected, the diffusion coefficient value of Alexa monomers, which in H2O at 25°C amounts to 332 μm²·s⁻¹, is decreases in sucrose/PBS buffer, due to the higher viscosity (around 210 μm²·s⁻¹, both outside and inside GUVs’ lumen). In the presence of AuNPs in the incubation medium, Alexa penetration inside GUVs’ lumen (NP@Ct Alexa/GUVs inside and NP@PC Alexa/GUVs inside) is

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<th>C (nM)</th>
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</thead>
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<td>Alexa / GUV inside</td>
<td>1.9±0.4</td>
<td>208±9</td>
</tr>
<tr>
<td>Alexa / GUV outside</td>
<td>19±1</td>
<td>217±10</td>
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<tr>
<td>NP@Ct Alexa/ GUV inside</td>
<td>2.0-38.5</td>
<td>5.0-220</td>
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<tr>
<td>NP@Ct Alexa / GUV outside</td>
<td>21±3</td>
<td>190±20</td>
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<tr>
<td>NP@PC Alexa / GUV inside</td>
<td>2.4-20.2</td>
<td>190±20</td>
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<tr>
<td>NP@PC Alexa / GUV outside</td>
<td>21±2</td>
<td>202±10</td>
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increased with respect to the external concentration (NP@Ct Alexa/GUVs outside and NP@PC Alexa/GUVs outside) both for citrated and corona-covered NPs. Moreover, a high variability in Alexa concentration inside GUVs’ lumen can be detected (from 2.0 to 38.5 nM for NP@Ct Alexa/GUVs inside, from 2.4 to 20.2 nM for NP@PC Alexa/GUVs inside). This variability is represented in the histogram (b), which reports the percentage ratio values of fluorophore concentration inside/outside GUVs’ lumen for different GUVs in the absence (black bar) and in the presence of NP@Ct (orange bars) and NP@PC (purple bars). The height of the histogram bars represent the frequency of each measured value. While internal/external concentration values found for NP@PC / GUVs samples never exceed 100% and Alexa is clearly found in its monomeric form inside GUVs’ lumen (D 190 µm² s⁻¹), internal/external concentration values exceeding 100% are found for NP@Ct / GUVs, and can be related to the adsorption of several Alexa molecules on NP aggregates which are found inside GUVs’ lumen (D 5.0 µm² s⁻¹). Each experiment was carried out on three different batches measuring Alexa diffusion at least inside ten different GUVs for each batch.
GUVs’ membrane permeabilization upon interaction with NPs:

experiments with Tamra

Supplementary Figure S7: (a) Tamra-labeled serum (LabFBS) and Tamra-labeled NP@PC (NP@LabPC) were prepared as described in the Supplementary Materials and Methods. Here the FCS characterization of NP@LabPC is reported: (a) representative ACFs acquired for Tamra-labeled serum (LabFBS, black markers) and curve fit (red line). The best fit was obtained with a two components 3D normal diffusion model (equation 2), with a fast component corresponding to free Tamra monomers \( D_1 = 280 \, \mu m^2 s^{-1} \), see table panel d) and a slow one corresponding to Tamra bound to serum proteins \( D_2 = 31 \, \mu m^2 s^{-1} \), see table panel d). The diffusion coefficients of each species were kept as fixed parameters to improve the robustness of the fitting. (b) Free Tamra
experimental ACF (black markers) compared to NP@LabPC washing solution (green markers) and curve fit (red line) according to a one component 3D normal diffusion model (free Tamra, $D_1 = 280 \mu m^2 s^{-1}$, see table panel d, equation 2) and to a two components ($D_1 = 280 \mu m^2 s^{-1}, D_2 = 31 \mu m^2 s^{-1}$ see table panel d) 3D normal diffusion model (equation 3). The fitting results on NP@LabPC washing solution, which requires a two component diffusion model to describe the experimental data (being the diffusion coefficient of each component, free Tamra and Tamra bound to serum proteins, kept as fixed parameters to improve the robustness of the fitting) highlight the presence of unbound Tamra molecules as well as free labeled serum proteins in NP@LabPC dispersion, suggesting to employ a three component model (corresponding to NP@LabPC, LabFBS and free Tamra coexisting species) for the analysis of FCS curves of NP@LabPC. (c) Representative NP@LabPC experimental ACFs (black markers) and curve fit (red line) according to a three components 3D normal diffusion model with the diffusion coefficient of each component (free Tamra $D_1 = 280 \mu m^2 s^{-1}$, Tamra bound to serum proteins $D_2 = 31 \mu m^2 s^{-1}$, Tamra bound to NPs corona $D_3 = 5.6 \mu m^2 s^{-1}$) kept as fixed parameters to improve the robustness of the fitting. $D_3$ diffusion coefficient of NP@LabPC was estimated from the hydrodynamic radius ($R_h$) of NP@LabPC obtained through DLS, by employing the Stokes-Einstein equation: $D = (kT)/(6\pi \eta R)$. (e) Histogram reporting the percentage ratio values of fluorophore concentration inside/outside GUVs’ lumen for different GUVs in the absence (black bar) and in the presence (purple bars) of NP@LabPC. The height of the histogram bars represent the frequency of each measured value.
FCS characterization of the mobility of the Liss Rhod PE inside the GUVs’ bilayer

Supplementary Figure S8: (a, b) Representative experimental ACFs of Liss Rhod PE diffusing in the GUVs’ bilayer on GUVs’ poles measured after two hours incubation at room temperature. for bare GUVs (red markers), and for GUVs incubated with NP@Ct (yellow markers) and NP@PC (purple markers). (a) Curve fitting (continuous lines) and related fit residuals (inset) of GUVs (red lines), GUVs/NP@Ct (yellow lines) and GUVs/NP@PC (purple lines) according to a 2D normal diffusion model with one diffusing component (equation 4). Clearly, this model is not able to describe the experimental data when GUVs are incubated with NPs, both citrated and corona-coated. (b) Comparison between curve fitting (continuous lines) and related fit residuals (inset) of GUVs (red lines) according to a 2D normal diffusion model with one diffusing component (equation 4) and of GUVs/NP@Ct (yellow lines) and GUVs/NP@PC (purple lines) according to a 2D normal diffusion model with two diffusing components (equation 5), with the diffusion coefficient of the fast component kept fixed $D_1 = 8 \, \mu\text{m}^2\text{s}^{-1}$, i.e. the value found for bare unperturbed GUVs. (c) fitting results, in terms of diffusion coefficient ($D_{1,2} , \mu\text{m}^2\text{s}^{-1}$) and weight factor ($f_{1,2}$) of each diffusion component. While a one component 2D diffusion model perfectly describes the motion of Liss Rhod PE within bare POPC GUVs, in the presence of NPs, both citrated and corona-covered, a slower component ($D_2 = 1.5-1.9 \, \mu\text{m}^2\text{s}^{-1}$) coexists with the faster, unperturbed component, highlighting the structuring of the bilayer into regions characterized by different fluidity.
Supplementary Materials and Methods

Chemicals: H\text{AuCl}_4\cdot3\text{H}_2\text{O} (\geq 99.9\%), sodium citrate dihydrate (> 99%), Sucrose (99.5%), Phosphate Buffer Saline (PBS) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO). Fetal Bovine Serum (FBS) is from Lonza (Basel Switzerland). Low Bind Protein tubes were from Eppendorf (Hamburg, DE). AnaTag 5-Tamra protein labeling kit is from Anaspec (Fremont, CA). 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl (Liss Rhod PE) is from Avanti Lipids (Alabaster, AL). 2-(4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (β-Bodipy) and Alexa 568 are from Invitrogen (Carlsbad, CA). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) is from Avanti Lipids (Alabaster, AL). Silver staining kit is from Cosmobio Co (Tokyo, JP). All chemicals were used as received. Milli-Q grade water was used in all preparations.

Synthesis of gold nanoparticle (NP@Ct): Gold nanoparticles (NP@Ct) were synthesized following the classical Turkevich method. Briefly, 2 mL of 1 wt% trisodium citrate aqueous solution were rapidly injected into 20 mL of H\text{AuCl}_4 1 mM boiling solution under vigorous stirring. The formation of NPs is indicated by the color of the solution, which turns from the original pale yellow to burgundy. After 15 minutes the solution was cooled down by means of a water-ice bath. NP@Ct were stored at 4 °C. NP@Ct dispersion were centrifuged (5 min @ 500 rcf) at 15°C in order to remove aggregates prior to use. Nanoparticle size distribution was evaluated, as shown in Supplementary Figure S4, by AFM measurements following the methods described in the NIST guidelines (the guide can be found at http://ncl.cancer.gov/NCL_Method_PCC-6.pdf).

Determination of AuNPs Concentrations: The concentration of AuNPs was determined via UV-Vis spectrometry, using the Lambert-Beer law \( E(\lambda) = \varepsilon(\lambda)lc \) taking the extinction values \( E(\lambda) = 1.4 \) at \( \lambda = 519 \) nm i.e. at the LSPR maximum and the extinction coefficient \( \varepsilon (\lambda) = 4 \cdot 10^8 \text{M}^{-1}\text{cm}^{-1} \).
for gold NPs according to methods described in the literature. (Chanana et al. 2013; Haiss et al. 2007)

**FBS labeling:** FBS labeling was carried out using the AnaTag 5-Tamra protein labeling kit. Briefly, a solution containing 15 mg of protein was prepared from the FBS stock solution by diluting with PBS. This solution was mixed with a conjugation buffer provided in the commercial kit, and then incubated with the fluorescent dye solution (10 μl of the dye solution) for one hour at r.t.. The protein-Tamra conjugates were then purified through a size exclusion column provided with the kit, which guaranteed the elimination of most unbound probe molecules, with a residual percentage of free Tamra. This small amount of free Tamra (revealed through FCS measurements, Supplementary Figure S2, Table T1) was retained for the experiments on GUVs’ membrane permeabilization (manuscript, Figure 2) in order to contemporary investigate GUVs’ membrane penetration capability of NP@PC, LabFBS and free Tamra. Tamra labeled FBS was stored at 4°C.

**Preparation of Gold Nanoparticle-corona complexes (NP@PC and NP@LabPC):** NP@Ct were coated with FBS proteins (Tamra Labeled or unlabeled) by a simple ligand exchange process that leads to the formation of the protein corona (See Supp Fig 2). (Monopoli et al. 2011) Briefly, 100 μl of a 3 nM solution of NP@Ct were resuspended in 1 ml PBS containing 10% FBS (Tamra labeled or not). The solution was then incubated for one hour at room temperature. After the incubation NP@PC and NP@LabPC were purified via centrifugation (10000 rcf, 30 min). (Monopoli et al. 2013) After two washing steps with PBS, NP@PC or NP@LabPC were resuspended in 100 μl of PBS and employed for the experiments.

**SDS-PAGE and Dot-Blot:** Immediately after the last centrifugation step, the NP@PC or NP@LabPC pellet was resuspended in protein loading buffer [62.5 mM Tris-HCL pH 6.8, 2% (w/v) SDS, 10% glycerol, 0.04 M DTT and 0.01% (w/v) bromophenol blue], it was then boiled for
five minutes at 100 °C and an equal sample volume was loaded in 10% gel polyacrylamide gel. Gel electrophoresis was performed at 120 V, 400 mA for about one hour each, until the proteins neared the end of the gel. The gels were using the COSMOBIO silver staining kit. For Dot blot, NP@PC and NP@labPC were spotted on parafilm and imaged. Gels and dot blots were scanned using a G:Box Chemi XR5 gel doc system (Syngene, Frederick, MD).

**GUVs and LUVs preparation:** Giant Unilamellar Vesicles were prepared through electroformation.(Angelova et al. 1992; McIntosh 2007; Nappini et al. 2011) Briefly, a POPC (-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) 2.5 mg/ml stock solution in CHCl$_3$ was prepared and 0.1% (for confocal microscopy imaging) or 0.01% (for Fluorescence Correlation Spectroscopy experiments) with respect to the total lipid amount of the fluorescent dyes β-Bodipy and Liss Rhod PE was added. 10 μl of the stock solution were deposited on each of two ITO-coated glass slides, on the conductive side. Chloroform was dried under vacuum for two hours and a dry lipid film on each sheet was obtained. The electroformation chamber was prepared sandwiching the sheets with an O-ring separating the lipid films. The chamber was filled with an aqueous solution of Sucrose 300 mM, and the electrical contact between the sheets was provided by putting on each sheet a copper tape connected to a pulse generator, set at a sinusoidal alternating voltage of 10 Hz frequency and 2 Vpp amplitude for two hours. GUVs were employed within 24 hours after preparation for CLSM and FCS experiments.

For Small Unilamellar Vesicles preparation the proper amount of POPC was dissolved in chloroform/methanol 6:1 (v/v). 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) PBS 10 mM by vigorous vortex mixing. To prepare Unilamellar vesicles (ULV) with narrow distribution, the dispersion was then tip-sonicated for 30 minutes. Dynamic Light Scattering and Zeta Potential were then employed to estimate vesicles’ size and surface charge.
**Dynamic Light Scattering:** DLS measurements at $\theta = 90^\circ$ and Zeta Potential determination were performed using a Brookhaven Instrument 90 Plus (Brookhaven, Holtsville, NY). Each measurement was an average of ten repetitions of one minute each and repeated ten times. The ACFs were analyzed through the cumulant fitting stopped to the second order for samples characterized by a single, monodisperse population, allowing an estimate of the hydrodynamic diameter of particles and of the polydispersity index. For polydisperse samples (NP@Ct in PBS buffer), which show the formation of some aggregates just after samples preparation, the experimental ACFs were analyzed through the Laplace inversion according to CONTIN algorithm. The relative weight of each population (nanoparticles or aggregates) is reported by number of objects forming each population.

**Zeta Potential:** Zeta potential measurements were performed using a Zeta Potential Analyzer (Zeta Plus, Brookhaven Instruments Corporation, Holtsville, NY). Zeta potentials were obtained from the electrophoretic mobility $u$, according to Helmholtz-Smoluchowski equation: $\zeta = (\eta/\varepsilon) \times u$ with $\eta$ being the viscosity of the medium, $\varepsilon$ the dielectric permittivity of the dispersing medium. The Zeta Potential values are reported as averages from ten measurements.

**UV-VIS:** UV-Vis spectra were measured with a JASCO UV-Vis spectrophotometer. Briefly, NP@Ct or NP@PC/ NP@LabPC were resuspended in the appropriated solvent (water or PBS) and the absorbance of the solution was then measured.

**Confocal Laser Scanning Microscopy (CLSM):** CLSM experiments were carried out with a laser scanning confocal microscope Leica TCS SP2 (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a 63x water immersion objective. The 488 nm laser line was employed to detect β-Bodipy fluorescence ($\lambda$ excitation 488 nm, $\lambda$ emission 498 nm - 530 nm); the 561 nm laser line was employed to detect Liss Rhod PE and Tamra fluorescence ($\lambda$excitation 561 nm, $\lambda$ emission 571nm).
the 633 nm laser line was employed to detect AuNP scattering (λexcitation 633 nm, λ emission 620 nm -640 nm). For confocal microscopy and FCS experiments 30 µl of GUVs’ dispersion in Sucrose 300 mM prepared as previously described were put in a measurement chamber (Lab-Tek® Chambered # 1.0 Borosilicate Coverglass System, Nalge Nunc International, Rochester, NY USA ) and diluted with 170 µl of PBS. Finally, 100 µl of NPs dispersion were added. GUVs and NPs were then incubated at r.t. for two hours, before the acquisition of the images.

**Fluorescence Correlation Spectroscopy (FCS):** FCS measurements were carried out with a ISS module (ISS, Inc.1602 Newton Drive Champaign, IL, USA) equipped with two APD with 500-530 nm and 607-683 nm BP. FCS measurements were carried out by exciting the fluorescent probe (Alexa 568 or Tamra or Liss Rhod PE) at 561 nm and acquiring the fluorescence emission between 607 and 683 nm. FCS is based on the analysis of the time fluctuations of the fluorescence intensity of a selected fluorescent probe, due to concentration fluctuations inside a small determined detection volume.(Ries & Schwille 2012; Koynov & Butt 2012). The autocorrelation function of the fluorescence intensity (G(τ)) is thus calculated as a function of the fluctuations of the signal from the averaged value, δI(t) = I(t)−<I(t)>, as:

\[
G(\tau) = \frac{<\delta I(t)\delta I(t+\tau)>}{<I(t)>^2}
\]  

(1)

The models employed for the analysis of the autocorrelation functions (ACFs) take into account the shape and the exact size of the detection volume. Generally the volume is approximated as 3D-ellipsoidal Gaussian shape with axial (z₀) and lateral (w₀) defining parameters. These parameters can be determined through calibration, by employing a reference fluorescent dye with well-known diffusion coefficient. For the calibration, we employed a 50 nM standard solution of Alexa 568 (D = 332 µm²s⁻¹ in water at 25°C, see a representative FCS curve reported in the Figure S1). For a
three-dimensional Brownian diffusion mode in a 3D Gaussian volume shape, the ACFs profiles can be analyzed according to equation (2):

$$G(\tau) = \frac{1}{<c> \pi^{\frac{3}{2}} w_0^2 z_0} \left(1 + \frac{4D\tau}{w_0^2}\right)^{-1} \left(1 + \frac{4D\tau}{z_0^2}\right)^{-\frac{1}{2}}$$

(2)

With $<c>$ the averaged fluorophore concentration (nM), $D$ the diffusion coefficient of the probe ($\mu$m$^2$s$^{-1}$). The same equation was employed to monitor Alexa 568 diffusion in the experiment of membrane permeabilization upon interaction with NPs (Figure 2, manuscript). In the case of a multicomponent system the measured autocorrelation function is a weighted sum of the autocorrelation functions of each component.(Milani et al. 2012) In our study, for the analysis of NP@LabPC we take into account a fast component, corresponding to free Tamra monomers, an intermediate component, corresponding to Tamra bound to serum proteins (LabFBS) and a slow component, corresponding to Tamra bound to NPs’ protein corona shell. The general expression for the autocorrelation function of the fluorescence intensity is in this case described by equation (3)

$$G(\tau) = \frac{1}{<c> \pi^{\frac{3}{2}} w_0^2 z_0} \sum f_i \left(1 + \frac{4D_i\tau}{w_0^2}\right)^{-1} \left(1 + \frac{4D_i\tau}{z_0^2}\right)^{-\frac{1}{2}}$$

(3)

being $f_i$ the weight factors of each $i$ diffusing component with diffusion coefficient $D_i$. The diffusion coefficient of the three components were kept fixed in the fitting procedures, to improve curve fit robustness. Fit results are reported in the Figure S6.

The general scaling law which expresses the mean square displacement of a molecule ($<r^2>$) as a function of time is $<r^2> = Dz2t^\alpha$, being $z$ the dimensionality of the diffusing medium and $\alpha$ the anomalous ($\alpha \neq 1$) or normal ($\alpha = 1$) diffusion parameter, which takes into account for variation from the pure Brownian linear dependence of the mean square displacement of a given species from time. An important research field where FCS technique is powerful, thanks to the combined single-molecule sensitivity and the possibility to carry out spatially resolved investigations on complex systems is the study on confined diffusion (being $z < 3$, thus 1D or 2D diffusion) and on locally
structured environments, where a fluorescent probe undergoes hindered anomalous subdiffusion (being \(\alpha < 1\)). Many efforts have been accordingly devoted to the development of FCS studies on the 2D diffusion of fluorescently labeled lipids along planar lipid membranes. (Chiantia et al. 2009; Macháň & Hof 2010) Giant Unilamellar Vesicles have a diameter (from few to few tens microns) considerably larger than the detection volume \((w_0 \approx 0.2 \, \mu m \text{ and } z_0 \approx 1 \, \mu m\) are the exact parameters determined after each experimental session through the described calibration procedure), thus the motion of the fluorescent probe (Liss Rhod PE) within the membrane is observed through FCS as a 2D-confined diffusion. In this case, the exact alignment of the lipid bilayer with the detection volume is critical to obtain a reliable absolute value of the diffusion coefficient of the fluorescent probe within the bilayer. We performed FCS measurements on GUVs’ top and determined the alignment of the bilayer and the center of the detection volume with the maximum steady-state fluorescence intensity. GUVs’ were prepared in a sucrose aqueous solution and diluted with PBS. The higher density of sucrose determines the spontaneous sinking of the GUVs on the bottom of measurements’ chamber. FCS measurements were then performed after two hours incubation at r.t., in order to avoid unwanted fluctuations of the free-standing bilayer. Moreover, we verified that our FCS data on Liss Rhod PE diffusion along bare POPC GUVs’ bilayer is consistent with the literature data on a similar system. (Guo et al. 2008) The FCS curves acquired for bare POPC GUVs were in fact successfully analyzed through a pure Brownian one-component 2D confined diffusion (equation (4)).

\[
G(\tau) = \frac{1}{N} \left(1 + \frac{4D\tau}{w_0^2}\right)^{-1}
\]

(4)

After incubation with NPs, the ACFs acquired for POPC GUVs in the same experimental conditions are characterized by higher time decays. Moreover, a change in the ACFs’ shape can be described by a 2D diffusion model, with two diffusion components (equation (5)).

\[
G(\tau) = \sum_i \frac{1}{N} f_i \left(1 + \frac{4D_i\tau}{w_0^2}\right)^{-1}
\]

(5)
Being the fast component fixed as the diffusion coefficient value found for Liss Rhod PE inside bare POPC GUVs in the absence of NPs, to improve the robustness of the fitting, a slower 2D diffusion component comes out, attributable to the coexistence of microdomains characterized by higher rigidity within POPC membrane, upon interaction with NPs.

References


S22


Wang, F. et al., 2013. The biomolecular corona is retained during nanoparticle uptake and protects the cells from the damage induced by cationic nanoparticles until degraded in the lysosomes. Nanomedicine : nanotechnology, biology, and medicine. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23660460.