nature portfolio

Peer Review File

Hybrid Lipid-AuNP Clusters as Highly Efficient SERS substrates for Biomedical Applications

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The fabrication of “LipoGold Tags”, i.e. Lipids containing gold nanoparticles and raman active molecules that can be used as SERS tags, is presented in this manuscript. The idea and findings are interesting, however there are several aspects that are not clear and need to be considered. Moreover, the encapsulation of hydrophobic molecules in liposomes is a common approach for drug encapsulation applications, and the inclusion of NPs into them is also not a novel methodology, thus the novelty of system is doubtful.
In general, based on the cryoTEM images, during the synthesis of the LipoGold Tags, the attachment of the gold NPs seems to be not very homogeneous and controlled. The selected lipid is the one is the DOPC, however in the TEM images, this one present aggregated NPs…Even if authors show a small variation from Batch-to-batch when measuring the Raman intensity at 1069 cm−1, it seems that free AuNPs could be also be in the mixture, DLS measurements of the liposomes including the NPs should be added for all the 6 batches that were measured for the comparison.
Moreover, the information relation of the SERS measurements is quite scarce. I recommend including in the Figure captions the measurement conditions, such as laser, acquisition time, power density of the laser…etc. The SERS mapping of cells should be also included in Figure 7b.
In Figure S20 the cells measured seem to be death. If we compare the morphology of the cells taken with the fluorescent confocal microscope Figure 7 and these ones, it is obvious that they do not look the same and they look unhealthy.
It is obvious that the produced SERS tags are expected to be used to measure biological tissues, but the excitation wavelength used (633 nm) is not he best one, it would be better to used 785 nm one which has better penetration depth in biological samples.
Authors say in the introduction that the use of Raman active molecules to functionalize AUNPs and obtain SERS tags is a complex process (page 3 around line 77), which can lead to unstable NPs, due to in some cases the organic nature of the Raman active molecules. However, there are many examples of stable SERS tags functionalize with organic RRs which can be obtained using a phase transfer procedure from an aqueous solvent to an organic one, acting the RRs as coating agents as well. Thus, the affirmations related to this topic in the introduction, in my opinion are inappropriate.
References should be improved, some important references and authors from the SERS tags field are missing.
Overall, I think that the presented work is of interest, but further work is needed to merit being published in Nature Communications.

Reviewer #2:
Remarks to the Author:
The authors report a new method for fabrication of SERS tags by firstly loading Raman reporter molecules in liposomes, and then fabricating liposome-Au NPs assemblies to enhance the Raman signals. They compared the effect of different lipid compositions (DOPC, POPC, DPPC, and DSPC) on NPs aggregation and SERS intensity, and found AuNPs-DOPC show the highest SERS enhancement. Then they varied both RRs and lipid vesicles' concentrations to optimize the SERS enhancement, and eventually employed CT-B-modified LipoGold tags to specifically detect intracellular GM1 alterations, which can distinguish healthy donors and patients affected by infantile GM1 gangliosidosis. Overall, this new fabrication method is smart, and the experiments were carefully designed. However, this method did not show significant advance in comparison with the existed techniques of SERS tags fabrication from my point of view: 1) the enhancement factor is not superior to the other tags; 2) It seems that the liposome also generates some SERS signals (Figure 3b, Figure S11b, Figure S14b), which might compromise the multiplexing capability of the SERS tags; In a previous article (ACS Nano, 2022, 16, 10341-10353), 26-plexed SERS multiplexing had been achieved. Other comments are as follows.
1) How are the Raman reporter molecules distributed around the Au NPs? In a typical SERS tag, the RRs attach to the metal NPs through the –SH and form a monolayer, is it the same situation in this platform?
2) In Figure 7a, it seems that the cell nucleus was also stained by CT-B-LipoGold.

Reviewer #3:
Remarks to the Author:
In this study novel and extremely simple synthetic route to create SERS probes so-called LipoGold tags of remarkable efficiency, versatility, and facile functionalization was presented. The approach assumes the spontaneous association of citrate-capped AuNPs on lipid vesicles. The proposed method overcomes currently limiting SERS probe fabrication factors such as reproducibility, and optical and colloidal stability. The proposed liposomal surface provides a platform for simple bioconjugation with antibodies, enabling precise targeting specificity
It is worth mentioning that all sections of the manuscript describe all conducted experiments in detail, which will allow for the repetition of each procedure in any other laboratory. Additionally, each section contains a deep discussion of the obtained results.
The article is divided into a few logically organized chapters. The authors start with the preparation of so-called LipoGold Tags. The Au NPs are formed by the Turkevich

method, the NPs characterization is in SI, because such a procedure has been well known for many years. The preparation of LipoGold Tags is in my opinion most important and innovative part of the manuscript. The presented approach is based on great and novel ideas realized in a good manner. The authors tested four various liposome types. The authors introduce the Figure, with a schematic idea of the process and all necessary characterization results based on Uv-Vis and cryo-TEM measurements. The collected data are carefully examined and discussed in detail. I like the Figures showing results with interpretation. This type of Figure improves and clarifies the manuscript. Of course, the authors include studies about reproducibility and stability.

I have just a few small suggestions for the next section entitled Multiplexing. In this section, the authors used three different Raman reporter molecules. So far, all previous tests were done on p-MBA, here authors introduce new RR so-called RR2 and RR6. I Wonder about the numeration of RR molecules. In the whole text, there is no information about RR3, RR4, or RR5 molecules. Additionally, I did not find the full names of applied compounds. However, as in previous parts, the conducted research is well planned, the discussion is deep and Figure 5 summarizes obtained results.

In the next section authors studied the possibility of bioconjugation based on pegylated phospholipid with a reactive carboxylic acid moiety (here one can find the full compound name). Presented research confirmed SERS activity after bioconjugation. The authors also showed an in-vitro test of LipoGold Tags in SHSY-5Y cell culture. Application of RR6 allows distinguishing characteristic peaks because such peaks fall in the biologically silent region. Finally described nanostructures were successfully applied in the detection of GM1 alteration.

In my opinion, the key result of the presented paper is the fabrication method of LipoTags. The proposed approach is innovative. The presented results showed in-depth analysis followed by careful discussion and interpretation. I strongly believe that this manuscript has great significance in the field of novel types of SERS tags for various (also in-vitro) measurements). The paper cited all relevant work in the field.

I strongly agree that the presented results can be a milestone in the field of AuNP cluster-based SERS nanoprobes. In my opinion, the presented manuscript has to be accepted for publication in a prestigious scientific journal, I just wonder if so long and detailed manuscript will fit a communicating type of article.

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The fabrication of "LipoGold Tags", i.e. Lipids containing gold nanoparticles and Raman active molecules that can be used as SERS tags, is presented in this manuscript.

Comment 1:

The idea and findings are interesting, however there are several aspects that are not clear and need to be considered. Moreover, the encapsulation of hydrophobic molecules in liposomes is a common approach for drug encapsulation applications, and the inclusion of NPs into them is also not a novel methodology, thus the novelty of system is doubtful.

Response:

We thank the Reviewer for this comment, which prompted us to clarify some aspects.

We concur that the inclusion of hydrophobic molecules or of nanoparticles in liposomes for drug encapsulation is already a mature field of research that has led to the development of various applications and commercial products. The research here presented is not aimed at increasing the already abundant number of examples in this field, but rather to introduce an entirely different approach towards Surface-Enhanced Raman Spectroscopy (SERS) applications, combining liposomes and gold nanoparticles (AuNPs). We believe that one of the main qualities of our system lies in its simplicity and easy preparation through spontaneous assembly of well-known building blocks. Therefore, the novelty is in the nano architecture and not in the ingredients. Control over the interaction between gold and liposomes, i.e on the architecture of the adducts, is achieved by modulating the liposomes composition and concentration, resulting in colloidally stable hybrids with extremely reproducible SERS enhancement and well-defined physicochemical properties. We can obtain precise control on the plasmonic and SERS properties via compositional and stoichiometric adjustment. In addition, the use of liposomes facilitates post-functionalization which enables target specificity. In essence, we would like to stress the novelty of our approach, which leads to top-of-the-line SERS probes in a very easy and flexible way. We appreciate the opportunity to further clarify these points.

Action undertaken:

To stress the novelty of the work and underline the state-of-the-art on NPs-liposome interactions the following sentences have been modified:

From:

"The formation of such AuNPs-vesicle hybrids combines the biocompatibility and encapsulation capabilities of lipid scaffolds with the diverse properties of inorganic NPs, such as electric, optical, magnetic, and catalytic functionalities."

To:

“The formation of such AuNPs-vesicle hybrids combines the biocompatibility and encapsulation capabilities of lipid scaffolds, extensively employed for embedding hydrophobic and hydrophilic drugs, with the diverse properties of inorganic NPs, such as electric, optical, magnetic, and catalytic functionalities.”

From:

“Here, we leverage these previous findings building upon our fundamental knowledge on the spontaneous clustering of citrate-capped AuNPs on synthetic lipid vesicles, to develop a novel class of nanomaterials, LipoGold tags, that emerge as flexible, highly efficient, and colloidally stable SERS probes.”

To:

“Here, we leverage these previous findings on liposomes, NP-vesicle interaction, and spontaneous clustering of citrate-capped AuNPs on synthetic lipid vesicles, to develop a novel class of nanomaterials, LipoGold tags, that emerge as flexible, highly efficient, and colloidally stable SERS probes.”

From:

“Overall, this work presents a straightforward and powerful strategy, based on simple and rapid self-assembly for creating AuNPs-vesicle hybrid materials for various diagnostic and imaging purposes, addressing current challenges in the fabrication of metallic nanoarrays for SERS applications.”

To:

“Overall, this work presents an entirely novel and highly straightforward strategy, based on simple and rapid self-assembly for creating AuNPs-vesicle hybrid materials for SERS applications, addressing current challenges in the fabrication of metallic nanoarrays.”

Comment 2:

In general, based on the cryoTEM images, during the synthesis of the LipoGold Tags, the attachment of the gold NPs seems to be not very homogeneous and controlled. The selected lipid is the one is the DOPC, however in the TEM images, this one present aggregated NPs…Even if authors show a small variation from Batch-to-batch when measuring the Raman intensity at 1069 cm−1, it seems that free AuNPs could be also be in the mixture, DLS measurements of the liposomes including the NPs should be added for all the 6 batches that were measured for the comparison.

Response:

We agree that in the Cryo-EM image selected for the main text, some aggregated particles can be seen. Although such aggregates are only visible in that image (for instance, they are not visible in the other images included in the supplementary information), we are aware that individual vesicles can show inhomogeneities. However, it’s essential to consider that cryo-

EM does not provide ensemble-average information since 6-7 imaged vesicles cannot represent the full picture of the system. To address concerns regarding homogeneity and control, we have incorporated additional analyses utilizing complementary and ensemble-averaged techniques such as dynamic light scattering (DLS) and UV-vis spectroscopy. These techniques offer ensemble-average insights into the system, crucial for comprehensive understanding.

Action Undertaken:

We have supplemented the manuscript with new data showcasing the reproducibility of plasmonic properties and particle size across six distinct sample. As shown in figure S11 in SI (and figure 1 in the present response), the plasmonic and DLS variations are minimal and within experimental uncertainty. In addition, we Laplace-inverted the DLS autocorrelation profiles with Contin. The size distributions are represented as weighted on intensity and number of particles (the new data have been included in figure S11 of the revised version of the supplementary information). Remarkably, our analyses reveal no evidence of free nanoparticles in the system and all the different analyses employed display a single monodisperse particle distribution of about 150 nm (hydrodynamic size consistent with the formation of the AuNPs crust on the membrane of 100 nm liposome).

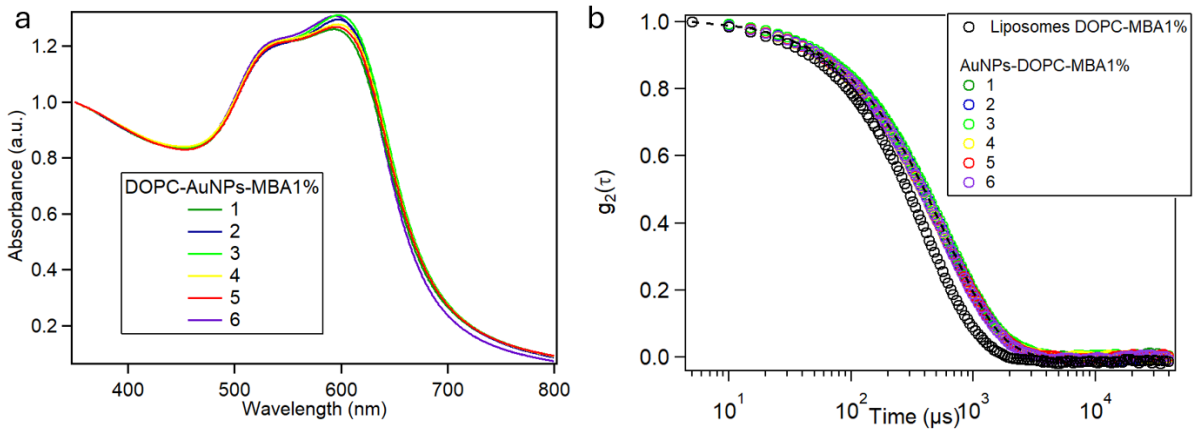


Figure 1: (a) UV-vis spectroscopy of 6 different of DOPC(MBA)-AuNPs hybrids; (b) DLS autocorrelation functions of 6 different of DOPC(MBA)-AuNPs hybrids and DOPC(MBA) liposomes with black dashed line representing the Contin fitting.

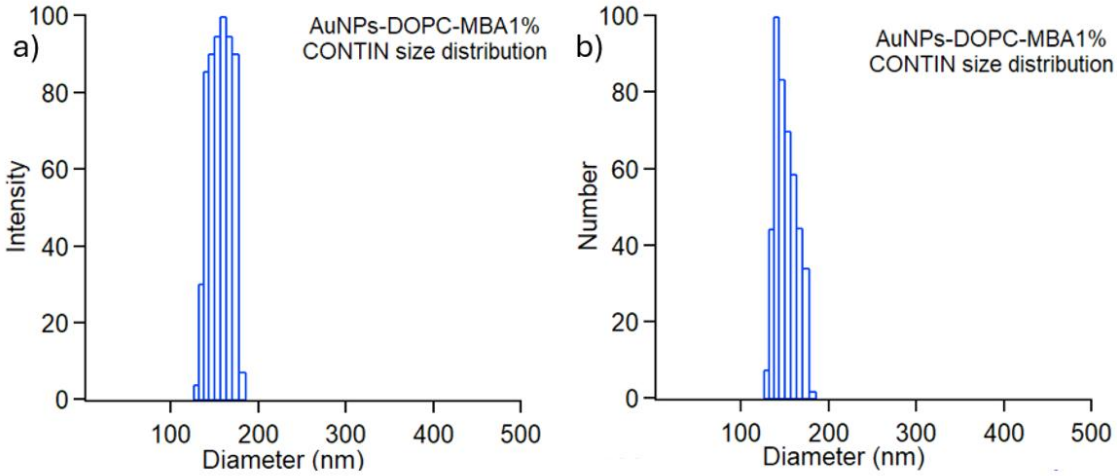


Figure 2: (a) Size distributions based on scattering intensity and particle number (b) of DOPC(MBA)-AuNPs hybrids from CONTIN analysis.

A fully new section (including the new figure S11) has been added in the Supporting information file:

"Plasmonic and size reproducibility of DOPC(MBA)-AuNPs

The reproducibility of DOPC(MBA)-AuNPs suprastructures was additionally assessed via UV-vis spectroscopy and DLS analysis comparing plasmonic variations and particle size of six different batches after 30 minutes of incubation of AuNPs with DOPC(MBA) liposomes. As shown in figure S11, plasmonic and DLS variations are minimal. The autocorrelation curves have been fitted through a Contin algorithm and the size distribution has been shown both weighted on scattering intensity and particle number (see figures S11 c) and d) respectively). The analysis shows the presence of a single size population peaked at about 150 nm (size consistent with the size of liposomes with a AuNPs crust)."

As a side comment, we would like to add that we share the Reviewer's concern about reproducibility in terms of size and size distribution of the adducts. We consider a precise (and thermodynamically driven) control over the nanostructure of the hybrid system, one of the key features and advantages of LipoGold Tags, and we devoted considerable experimental effort to unveil the mechanistic aspects. In an ongoing study, we combine experiments and simulation to explain the plasmonic response of a series of synthetic liposomes of different composition and different ratios. An interesting result (whose implications lie beyond the scopes of this manuscript, but overall strengthen the degree of our structural control) is the emergence of an isosbestic point, i.e. specific wavelength at which two or more absorbing species in a solution exhibit the same absorbance value, as a function of liposomes concentration. The presence of the isosbestic point indicates the coexistence of two distinct species: aggregated and freely dispersed AuNPs in a precise range of NP/liposome ratios (the ratio can be controlled varying the concentration of liposomes). The isosbestic wavelengths depend on the composition of liposomes and correspond to a precise average NP-NP distance, characteristic for a given lipid composition. In summary, this will be further evidence of our ability to control the structural features of the adduct choosing the appropriate

composition and concentration, as also confirmed by the reproducibility across different batches.

Comment 3:

Moreover, the information relation of the SERS measurements is quite scarce. I recommend including in the Figure captions the measurement conditions, such as laser, acquisition time, power density of the laser…etc.

Response:

We agree with the Reviewer's recommendation.

Action Undertaken:

Following the Reviewer's suggestion, information about the SERS measurements is added in the Figure captions in the Revised version of the Manuscript.

To make these points clearer the following sentences have been modified:

From:

"Figure 2. (a) Raman-SERS spectra acquired for different kinds of liposomes (DSPC, DPPC, POPC and DOPC) with 4-MBA molecules embedded in the bilayer. (b) Comparison between the Raman Intensity at 1060 cm-1 and UV-Vis signal at 600 nm. (c) Graphical representation of the different interactions between AuNPs and various types of liposomes (DOPC, POPC,DPPC, DSPC). (d) Average enhancement factors (EFs) for clusters of colloidal AuNPs conjugated with 4-MBA (red shaded area) from the literature (a 46 ; b 47; c 23); cluster of colloidal AuNPs conjugated with 4-MBA and immobilized onto different substrates (green shaded area) from the literature (d 48 ; e 49 ; f 50); and cluster of colloidal AuNPs interacting with liposome DOPC(MBA) (blue shaded area) presented in this work. "

To:

"Figure 2. (a) Raman-SERS spectra acquired for different kinds of liposomes (DSPC, DPPC, POPC and DOPC) with 4-MBA molecules embedded in the bilayer. Excitation wavelength: 638 nm; accumulation time: 60s; number of acquisitions: 2; objective magnification: 10x. (b) Comparison between the Raman Intensity at 1060 cm-1 and UV-Vis signal at 600 nm. (c) Graphical representation of the different interactions between AuNPs and various types of liposomes (DOPC, POPC,DPPC, DSPC). (d) Average enhancement factors (EFs) for clusters of colloidal AuNPs conjugated with 4-MBA (red shaded area) from the literature (a 46 ; b 47; c 23); cluster of colloidal AuNPs conjugated with 4-MBA and immobilized onto different substrates (green shaded area) from the literature (d 48 ; e 49 ; f 50); and cluster of colloidal AuNPs interacting with liposome DOPC(MBA) (blue shaded area) presented in this work. "

From:

"Figure 3. (a) Raman spectra of AuNPs interacting with liposome dispersion of DOPC(MBA) at different concentrations; (b) Raman spectra of AuNPs interacting with DOPC liposome dispersion with different MBA concentrations; (c) Raman-SERS spectra of six different

batches of LipoGold tags; (d) Batch-to-batch Raman intensity variation for the 1069 cm−1 and 1577 cm-1 peaks calculated from the Raman-SERS spectra reported in (c). “

To:

“Figure 3. (a) Raman spectra of AuNPs interacting with liposome dispersion of DOPC(MBA) at different concentrations; (b) Raman spectra of AuNPs interacting with DOPC liposome dispersion with different MBA concentrations; (c) Raman-SERS spectra of six different batches of LipoGold tags; (d) Batch-to-batch Raman intensity variation for the 1069 cm−1 and 1577 cm-1 peaks calculated from the Raman-SERS spectra reported in (c). Excitation wavelength: 638 nm; accumulation time: 60s; number of acquisitions: 2; objective magnification: 10x.”

From:

“Figure 4. (a) Time evolution of the Raman and (b) UV-visible spectra of DOPC(MBA)-AuNPs LipoGold tags aqueous dispersions; (c) Comparison between the time evolution of Raman Intensity at 1060 cm-1 and UV-vis signal at 600 nm; (d) Time evolution of the DLS autocorrelation functions of DOPC(MBA)-AuNPs LipoGold tags.”

To:

“Figure 4. (a) Time evolution of the Raman and (b) UV-visible spectra of DOPC(MBA)-AuNPs LipoGold tags aqueous dispersions; (c) Comparison between the time evolution of Raman Intensity at 1060 cm-1 and UV-vis signal at 600 nm; (d) Time evolution of the DLS autocorrelation functions of DOPC(MBA)-AuNPs LipoGold tags. Excitation wavelength: 638 nm; accumulation time: 60s; number of acquisitions: 2; objective magnification: 10x.”

From:

“Figure 5. (a) Graphical model representing the three SERS probes constituted by three different RRs; (b) Raman-SERS spectra of DOPC(RR)-AuNPs with (colored curves) and without (black curves) the presence of AuNPs. Spectra backgrounds are removed by subtracting the profile of water samples with a 638 nm excitation laser at the same power and same acquisition parameters (c) UV-Visible spectra of DOPC(RR)-AuNPs after mixing; (d) Dynamic light scattering (DLS) curves obtained by measuring DOPC(RR)-AuNPs after 30 minutes of incubation.”

To:

“Figure 5. (a) Graphical model representing the three SERS probes constituted by three different RRs; (b) Raman-SERS spectra of DOPC(RR)-AuNPs with (colored curves) and without (black curves) the presence of AuNPs. Spectra backgrounds are removed by subtracting the profile of water samples with a 638 nm excitation laser at the same power and same acquisition parameters. Excitation wavelength: 638 nm; accumulation time: 60s; number of acquisitions: 2; objective magnification: 10x. (c) UV-Visible spectra of DOPC(RR)-AuNPs after mixing; (d) Dynamic light scattering (DLS) curves obtained by measuring DOPC(RR)-AuNPs after 30 minutes of incubation.”

From:

“Figure 6. (a) Graphical scheme reporting the functionalization protocol of liposomes-AuNPs surface with antibodies, exploiting the EDC/NHS coupling mechanism; (b) Fluorescence correlation spectroscopy (FCS) measurements of antibodies (Ab(I)) and DOPC-AuNPs@antibody conjugates; (c) Graphical scheme reporting the principle of coupling between antibodies(II) attached to the glass slide and antibodies(I) conjugated to the AuNPs-liposome surface (Ab(I)-DOPC(MBA)-PEG-AuNPs); (d) Raman-SERS spectra of liposomes with 0% (red curve), 1% (blue curve) and 5% (magenta curve) PEG molecules with and without antibodies attached to the surface of the glass slide through a sandwich-like immunoassay. (e) Raman intensity at 1577 cm-1 for each sample at different quantities of PEG, with and without antibodies. Error bars are standard deviations. (n = 3; error bars represent s.d., NS>0.05; *p<0.05; **p<0.01; *p<0.001; an unpaired Student’s t-test was performed).”

To:

“Figure 6. (a) Graphical scheme reporting the functionalization protocol of liposomes-AuNPs surface with antibodies, exploiting the EDC/NHS coupling mechanism; (b) Fluorescence correlation spectroscopy (FCS) measurements of antibodies (Ab(I)) and DOPC-AuNPs@antibody conjugates; (c) Graphical scheme reporting the principle of coupling between antibodies(II) attached to the glass slide and antibodies(I) conjugated to the AuNPs-liposome surface (Ab(I)-DOPC(MBA)-PEG-AuNPs); (d) Raman-SERS spectra of liposomes with 0% (red curve), 1% (blue curve) and 5% (magenta curve) PEG molecules with and without antibodies attached to the surface of the glass slide through a sandwich-like immunoassay. Excitation wavelength: 532 nm; accumulation time: 60s; number of acquisitions: 2; objective magnification: 10x. (e) Raman intensity at 1577 cm-1 for each sample at different quantities of PEG, with and without antibodies. Error bars are standard deviations. (n = 3; error bars represent s.d., NS>0.05; *p<0.05; **p<0.01; *p<0.001; an unpaired Student’s t-test was performed).”

From:

“Figure 7. (a) Representative confocal microscope images of SHSY-5Y cells incubated with free CT-B, CT-B- LipoGold, and bare LipoGold. Scale bar is 50 um in all cases; (b) SERS spectra from random regions acquired for control cells (red line), in the presence of bare LipoGold (gray line) and CT-B- LipoGold (blue line) (c) Histogram showing the quantitative values of RR6 Raman intensity at 2210 cm-1. Error bars are standard deviations. (n = 3; error bars represent s.d., NS>0.05; *p<0.05; **p<0.01; *p<0.001; an unpaired Student’s t-test was performed).”

To:

“Figure 7. (a) Representative confocal microscope images of SHSY-5Y cells incubated with free CT-B, CT-B- LipoGold, and bare LipoGold. Scale bar is 50 um in all cases; (b) SERS spectra from random regions acquired for control cells (red line), in the presence of bare LipoGold (gray line) and CT-B- LipoGold (blue line). Excitation wavelength: 532 nm; accumulation time: 60s; number of acquisitions: 2; objective magnification: 60x. (c) Histogram showing the quantitative values of RR6 Raman intensity at 2210 cm-1. Error bars are standard deviations. (n = 3; error bars represent s.d., NS>0.05; *p<0.05; **p<0.01; *p<0.001; an unpaired Student’s t-test was performed).”

From:

“Figure 8. Fibroblasts from WT and from patients affected by infantile GM1 gangliosidosis incubated with CT-B-LipoGold and analysed with flow cytometry and Raman microscopy. Cells were fixed, permeabilized and incubated for 45 min with 5 µg/mL of CT-B or 5 µg/mL of CT-B-LipoGold, previously dissolved in distilled H2O. (a) The flow cytometry analysis shows a significant variation in the side scatter area (SSC-A) of WT cells labelled with CT-B-LipoGold compared with WT incubated with CT-B. (b) MFI/MFIWT mean values are obtained by dividing the MFI of a distribution by the mean MFI obtained from a control. MFI/MFIWT mean values increase significantly in patients’ cells compared to WT, and comparably when CT-B was uncoupled or coupled to LipoGold. n=3, >1000 cells were analysed for each n. Student’s t-test *** $p \leq 0.001$. (c) Averaged Raman-SERS spectra from 10 single cells acquired for WT in the presence of CT-B (green line) and CT-B-LipoGold (red line), and averaged Raman-SERS spectra from 10 single cells acquired for patient sample in the presence of CT-B (blue line) and CT-B-LipoGold (grey line). (d) Histogram showing the quantitative values of Raman intensity at 1060 cm-1. Error bars are standard deviations. (n = 10; error bars represent s.d., NS>0.05; *p<0.05; **p<0.01; *p<0.001; an unpaired Student’s t-test was performed).”

To:

“Figure 8. Fibroblasts from WT and from patients affected by infantile GM1 gangliosidosis incubated with CT-B-LipoGold and analysed with flow cytometry and Raman microscopy. Cells were fixed, permeabilized and incubated for 45 min with 5 µg/mL of CT-B or 5 µg/mL of CT-B-LipoGold, previously dissolved in distilled H2O. (a) The flow cytometry analysis shows a significant variation in the side scatter area (SSC-A) of WT cells labelled with CT-B-LipoGold compared with WT incubated with CT-B. (b) MFI/MFIWT mean values are obtained by dividing the MFI of a distribution by the mean MFI obtained from a control. MFI/MFIWT mean values increase significantly in patients’ cells compared to WT, and comparably when CT-B was uncoupled or coupled to LipoGold. n=3, >1000 cells were analysed for each n. Student’s t-test *** $p \leq 0.001$. (c) Averaged Raman-SERS spectra from 10 single cells acquired for WT in the presence of CT-B (green line) and CT-B-LipoGold (red line), and averaged Raman-SERS spectra from 10 single cells acquired for patient sample in the presence of CT-B (blue line) and CT-B-LipoGold (grey line). Excitation wavelength: 638 nm; accumulation time: 120s; number of acquisitions: 2; objective magnification: 10x. (d) Histogram showing the quantitative values of Raman intensity at 1060 cm-1. Error bars are standard deviations. (n = 10; error bars represent s.d., NS>0.05; *p<0.05; **p<0.01; *p<0.001; an unpaired Student’s t-test was performed).”

Comment 4:

The SERS mapping of cells should be also included in Figure 7b.

Response:

We appreciate the Reviewer’s suggestion on the SERS mapping of cells in Figure 7b. Additional experiments were performed to address this aspect of our study.

Action Undertaken:

To answer the Reviewer's concerns, SHSY-5Y fixed cell cultures were incubated with LipoGold for 90 minutes and then thoroughly washed with water to remove unbound SERS probes. Then, 272 SERS spectra were acquired on a single cell, as reported in the image acquired in bright field. The Raman intensity of the peak at 1060 cm^{-1} of MBA was used to construct the SERS map shown in the figure 3. Each SERS spectrum was collected with a 638 nm laser, acquiring for 30 seconds and an objective with 60x magnification capability. Our findings reveal MBA Raman peaks distribution following the distinctive shape of the cell. We acknowledge that these results are preliminary and represent an early stage of our investigation. We envision that further exploration and refinement of this methodology could unveil even more detailed and conclusive insights into the cellular structures under investigation.

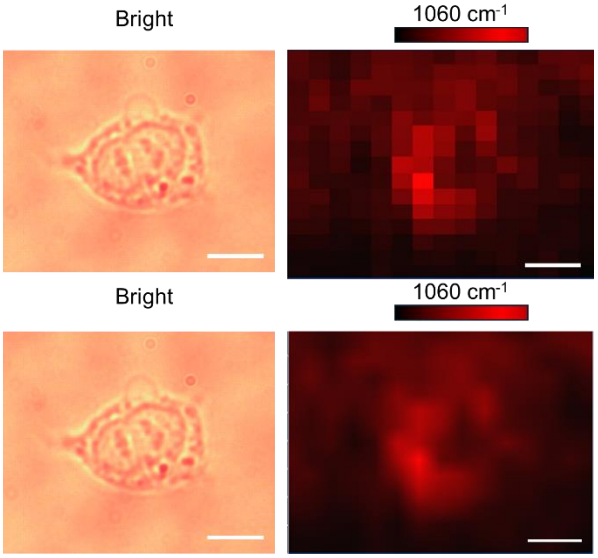


Figure 3: SERS mapping of SH-SY5Y cell treated with LipoGold for 90 min. Scalebar is 10 um in all cases. Measurements were performed with the following parameters: laser 638 nm, 20 mW laser power, 30 s acquisition time, 1 accumulation, 272 data points, Y step 2 um and X step 3 um, objective magnification: 60x. Top panel represents bright field image of the analysed cell; SERS map generated using the intensity at 1060 cm-1. Bottom panel represents bright field image of the analysed cell; SERS map generated using the intensity at 1060 cm-1 when a post-processing smoothing filter is applied.

Comment 5

In Figure S20 the cells measured seem to be death. If we compare the morphology of the cells taken with the fluorescent confocal microscope Figure 7 and these ones, it is obvious that they do not look the same and they look unhealthy.

Response:

We thank the Reviewer for the comment that stimulated us to clarify our experimental conditions.

The cells in Figure S20 have been detached with trypsin, fixed and permeabilized (and are thus dead, as correctly noted by the Reviewer), a standard procedure needed for carrying out flow cytometry analysis. It is normal that adherent cells assume a round-shaped morphology after detachment. On the other hand, cells analyzed in Figure 7 were grown on glass coverslips and fixed without previous detachment, as commonly performed in confocal microscopy imaging.

Action undertaken:

To make this point clearer we added a sentence in the legend of Figure S21, now renamed Figure S23 in the Revised version of Supporting Information.

The caption of figure S23 has been changed from:

“a) Wide-field image of deposited patient cells on CaF2 substrate; b) Raman-SERS spectra acquired from 10 individual cells of patient sample.”

To:

“a) Wide-field image of deposited patient cells on CaF2 substrate, after their detachment with trypsin, fixation and permeabilization; b) Raman-SERS spectra acquired from 10 individual cells of patient sample.”

Comment 6:

It is obvious that the produced SERS tags are expected to be used to measure biological tissues, but the excitation wavelength used (633 nm) is not he best one, it would be better to used 785 nm one which has better penetration depth in biological samples.

Response:

We thank the Reviewer for the insight, and we agree that one of the possible uses of these SERS tags is for measure biological tissues. In response, we would like to emphasize that our LipoGold system offer the flexibility to operate at 785 nm, as highlighted in the revised version of the supporting information (Figure S6). In this figure we compare Raman measurements of LipoGold(MBA), using both 638 nm and 785 nm excitation sources while maintaining constant the other working parameters (laser power, acquisition time, number of accumulations, objective magnification, grating). The Raman characteristic peaks of MBA are well visible for both wavelengths, even if the Raman intensity at 785 nm is comparatively lower. Our rationale for employing the 638 nm excitation wavelength is based on the resonance optimization of the plasmonic profile of the nanoparticles, particularly when aggregated on the liposomes, as illustrated in Figure 1b. This resonance optimization at 638 nm is crucial for enhancing the overall performance of our system, especially in applications involving 2D cell cultures or capturing liquid biomarkers. Furthermore, we are actively engaged in optimizing our system further. This includes exploring the implementation of different nanoparticles, such as nanostars, whose plasmon resonance is shifted towards higher wavelengths. By tuning the resonance to wavelengths offering better penetration depths, we aim to enhance the suitability

of our system for tissue measurements. We appreciate your input, which contributes to the continuous improvement of our research.

Action undertaken:

The figure referenced has been added in the supplementary information file (Figure S6)

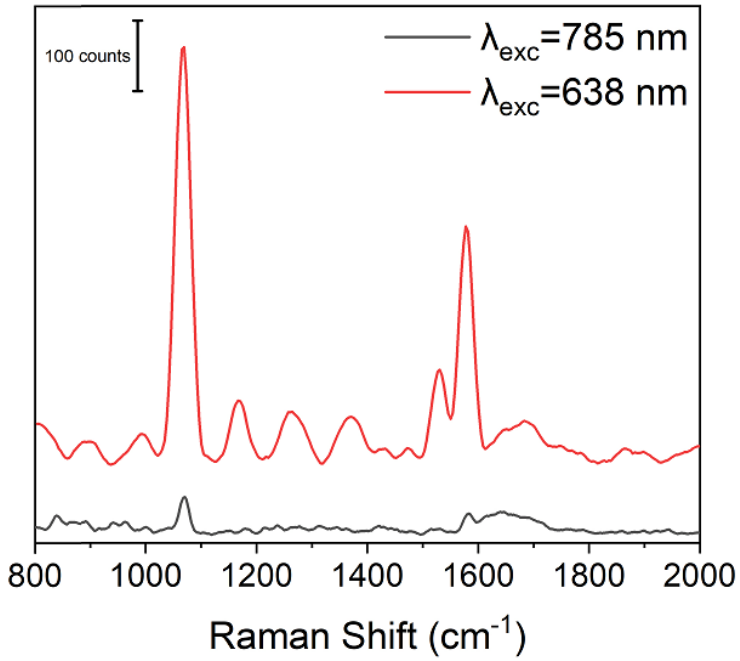


Figure 4: Raman spectra of DOPC liposomes containing MBA acquired with different laser wavelengths (638 and 785 nm), keeping constant all the working parameters.

Comment 7:

Authors say in the introduction that the use of Raman active molecules to functionalize AUNPs and obtain SERS tags is a complex process (page 3 around line 77), which can lead to unstable NPs, due to in some cases the organic nature of the Raman active molecules. However, there are many examples of stable SERS tags functionalize with organic RRs which can be obtained using a phase transfer procedure from an aqueous solvent to an organic one, acting the RRs as coating agents as well. Thus, the affirmations related to this topic in the introduction, in my opinion are inappropriate.

Response:

We agree with the Reviewer that our affirmation might be too harsh. It is true that there are examples of stable SERS tags functionalized with organic Raman reporter molecules (RRs), often achieved through phase transfer procedures from aqueous to organic solvents. However, it's important to note that the stability of such systems can vary depending on multiple factors including the specific nature of the RRs and the synthesis method and experimental protocols employed need to be optimized per each different organic molecule (, https://doi.org/10.1016/j.jcis.2022.12.099). Our intention in highlighting the complexity of the

process in the introduction was not to imply that stable SERS tags cannot be achieved with organic RRs, but rather to emphasize the challenges and considerations involved in ensuring the stability and functionality of AuNPs functionalized with such molecules. In our study, we presented an extremely simple and thermodynamically driven approach and we have demonstrated that the protocol's simplicity remains largely unaffected by changes in RRs, with minimal impact on the results obtained (minimal size and plasmonic variations as a function of the raman reporter). We believe that addressing these challenges remains a significant challenge in the field of SERS tag development. Our work contributes to this ongoing effort by providing insights into the robustness and versatility of our synthesis protocol.

Action undertaken:

For these reasons the sentence in the introduction have been modified from:

“Although numerous strategies have been proposed for fabricating efficient SERS tags with high targeting efficiency and SERS intensity, as well as long-term colloidal stability,[27–31] these methods often involve time-consuming and multiple-step syntheses, leading to NP aggregates lacking in reproducibility and structural stability, usually dependent on the RR chemical structure.[26] In summary, a simple, reproducible, and versatile strategy for developing RRs-AuNPs clusters as SERS probes is still lacking.”

To:

“Numerous smart and effective strategies have been proposed for fabricating efficient SERS tags with high targeting efficiency and SERS intensity, as well as long-term colloidal stability. However, these methods often involve time-consuming and multiple-step syntheses that may lead to NP aggregates lacking in reproducibility and structural stability, usually dependent on the RR chemical structure. In the present work, we propose a simple, reproducible, and versatile strategy for developing RRs-AuNPs clusters as SERS probes simultaneously ensuring high SERS performances and structural and colloidal stability.”

The sentence in the conclusions have been modified from:

“This strategy holds the promise to overcome the main challenges currently limiting SERS probe fabrication, specifically addressing synthetic simplicity, reproducibility, and optical and colloidal stability.”

To:

“This strategy holds the promise to overcome the common challenges in SERS probe fabrication, specifically addressing synthetic simplicity, reproducibility, and optical and colloidal stability.”

Comment 8:

References should be improved, some important references and authors from the SERS tags field are missing.

Response:

We agree with the Reviewer.

Action undertaken:

In response, we have updated the list of references to include additional sources, particularly focusing on key authors and references in the SERS tags field. We trust that these revisions enhance the comprehensiveness of our manuscript and provide a stronger foundation for our work.

[15] 10.1016/j.talanta.2018.04.009

[16] doi.org/10.1002/cnma.201500221

[17] doi: 10.7150/ntno.30924

[22] doi.org/10.1021/acssensors.9b00321

[23] doi.org/10.1021/acsnano.0c04368

[30] doi.org/10.1021/acs.bioconjchem.0c00022

[38] doi:10.1038/NNANO.2011.79

Overall, I think that the presented work is of interest, but further work is needed to merit being published in Nature Communications.

Reviewer #2 (Remarks to the Author):

The authors report a new method for fabrication of SERS tags by firstly loading Raman reporter molecules in liposomes, and then fabricating liposome-Au NPs assemblies to enhance the Raman signals. They compared the effect of different lipid compositions (DOPC, POPC, DPPC, and DSPC) on NPs aggregation and SERS intensity, and found AuNPs-DOPC show the highest SERS enhancement. Then they varied both RRs and lipid vesicles' concentrations to optimize the SERS enhancement, and eventually employed CT-B-modified LipoGold tags to specifically detect intracellular GM1 alterations, which can distinguish healthy donors and patients affected by infantile GM1 gangliosidosis. Overall, this new fabrication method is smart, and the experiments were carefully designed. However, this method did not show significant advance in comparison with the existed techniques of SERS tags fabrication from my point of view:

Comment 1:

T*he enhancement factor is not superior to the other tags;*

Response:

We appreciate the Reviewer's comments and acknowledge that the enhancement factor (EF) of our LipoGold nanoconstructs may not surpass all existing tags in the literature. However, we strongly believe our novel class of tags introduces distinctive advantages. It is important to note that our LipoGold offers a SERS enhancement that is, at the very least, comparable to the most effective tags reported in current literature. Compared to the current state of the art, embedding the hydrophobic Raman reporter molecules within the lipid bilayer is a unique strategy to protect the RRs from the external environment and prevent degradation as well as to overcome non-negligible issues of RRs-ligands competition that can often result in uncontrolled aggregation and irreproducibility. Here, the Lipo-Gold Tags preparation (thermodynamically driven) is fast and remarkably straightforward. Control over the interaction between gold and liposomes is achieved by modulating the liposome's composition and concentration, resulting in colloidal adducts with consistently reproducible SERS enhancement and physicochemical properties. In addition, as shown in the manuscript, the use of liposomes as scaffolds to template the gold clustering facilitate additional functionalization and enables the introduction of target specificity. This versatility opens up possibilities for parallel sensing (probes) as well as therapeutic applications (drugs). We believe that the combination of ease of synthesis, reproducibility, biofunctionalization versatility, and the broad spectrum of loaded molecules positions our LipoGold nanoconstructs as a promising and valuable addition to the existing repertoire of SERS tags.

Comment 2:

It seems that the liposome also generates some SERS signals (Figure 3b, Figure S11b, Figure S14b), which might compromise the multiplexing capability of the SERS tags; In a previous article (ACS Nano, 2022, 16, 10341-10353), 26-plexed SERS multiplexing had been achieved.

Response:

We agree with the Reviewer that the potential interference of liposome-generated SERS signals should be taken into account. To address this concern, we would like to emphasize that our LipoGold tags are designed with Raman reporter molecules (RRs) specifically incorporated to generate Raman characteristic peaks of significantly higher intensity compared to any background signals originating from the liposomes. To further enhance the multiplexing capabilities and mitigate potential interference, one effective strategy involves selecting RRs with at least one characteristic peak that does not overlap with those originating from the LipoGold nanoconstructs. This could be easily achieved by exploiting the emerging class of bioorthogonal RRs that, as reported in the original version of the Manuscript, exhibit Raman peaks in the biological-silent Raman region, where no interference is observed from the biomolecules or bio-inspired constructs. Notably, we have recently reported a straightforward method for the synthesis of novel bioorthogonal tags. This method allows for the generation of a palette of four molecules, but can ideally be expanded to create a versatile library of bioorthogonal tags by tuning the number and chemical nature of bioorthogonal emitting groups conjugated to the probe backbone [please refer to: Dallari et al. Int. J. Mol. Sci. 2022, 23(10), 5573 and Hu et al. Nature Methods volume 15, 194–200 (2018)]. In summary, leveraging bioorthogonal RRs represents a promising avenue to achieve robust multiplexing capabilities without compromising the accuracy and specificity of our SERS tags.

We thank the Reviewer for highlighting this important consideration, which aligns with our vision towards advancing the efficacy and versatility of our technology.

Action Undertaken

To better clarify the multiplexing capabilities of the LipoGold tags, the concept of bioorthogonality has been highlighted in the revised version of the Manuscript in the section "Functional properties of LipoGold tags: multiplexing"

"RR2 and RR6 were specifically designed to have bioorthogonal peaks, falling in the biological silent region (1800-2500 cm-1), that is free of background interference thus resulting in a higher signal-to-noise ratio. "

Comment 3:

How are the Raman reporter molecules distributed around the Au NPs? In a typical SERS tag, the RRs attach to the metal NPs through the –SH and form a monolayer, is it the same situation in this platform?

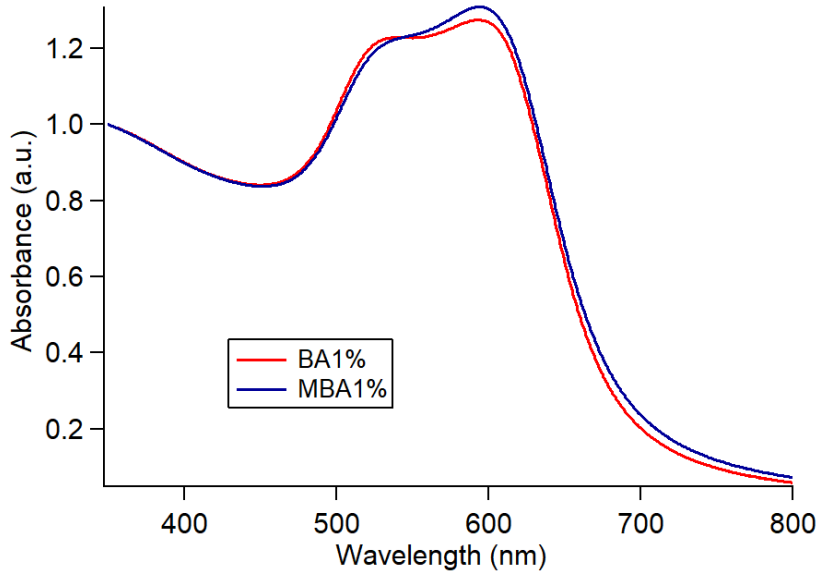
Response

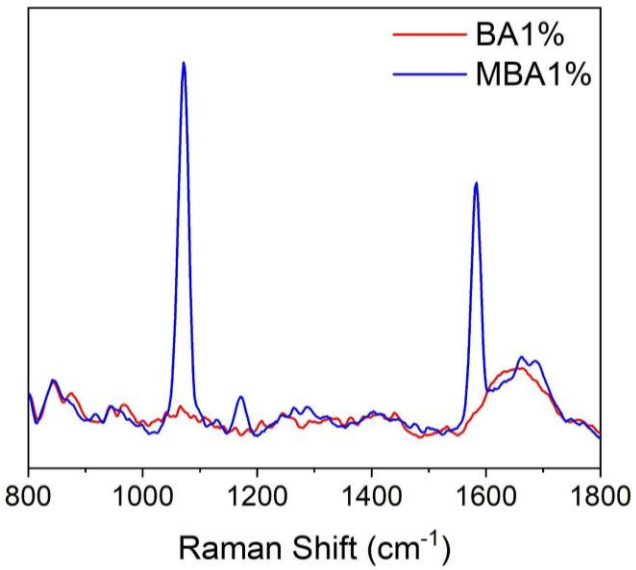
We truly thank the Reviewer for raising this point, which gave us the opportunity to extend the investigation on the interaction between the liposomes, the Raman reporter molecules and the gold nanoparticles, to gain deeper understanding of their interfacial behavior within the construct. In our system, hydrophobic molecules can diffuse within the lipid bilayer. In the initial version of the manuscript, we proposed a hypothesis suggesting that the thiolated moiety of RRs could potentially covalently bind to the gold surface after the spontaneous aggregation of AuNPs on the liposome surface. However, prompted by this remark, we conducted further experiments to validate this hypothesis.

Specifically, we prepared liposomes containing benzoic acid (BA) and monitored the plasmonic and Raman variations following AuNPs aggregation. Remarkably, while the plasmonic features of the samples remained relatively unaffected by the presence of thiolated groups, the Raman signal corresponding to BA-containing liposomes vanished. This outcome strongly suggests that the enhanced Raman signals indeed arise from the interaction between thiol moieties and AuNPs.

Action Undertaken

We have incorporated these novel findings into the revised supplementary information file (Figure S10) and have discussed them within the main and supplementary texts. This additional data further strengthens our understanding of the distribution and interaction of RRs around AuNPs within our platform.





According to these new relevant data, the main text has been modified from:

“This enhancement can be attributed to a close interaction between MBA and AuNPs, driven by the MBA thiol pendant group.”

To:

“This enhancement can be attributed to a close interaction between MBA and AuNPs, driven by the MBA thiol pendant group. To corroborate such hypothesis, we monitored plasmonic and Raman variations of AuNPs interacting with liposomes containing Benzoin Acid (BA). As shown in SI Figure S10, while the plasmonic features of the DOPC-AuNPs are not dramatically affected by the presence of the BA or MBA molecules, the Raman signal for BA containing liposomes disappears (see section 3.2 in SI for further information).”

A fully new section (including the new figure S10) has been added in the Supporting information file:

“Effect of thiolated moieties in the SERS enhancement of MBA-DOPC tags”

To prove that the SERS enhancement can be attributed to a close interaction between MBA and AuNPs, driven by the MBA thiol pendant group, we compared plasmonic and Raman signal arising from AuNPs clustered on DOPC liposome encapsulating Mercaptobenzoic acid (MBA) and benzoic acid (BA) that can be distinguished only for the thiol moiety.

Briefly, liposomes containing benzoic acid (BA) were prepared and plasmonic and Raman variation after the AuNPs aggregation were monitored. As shown in figures, since the plasmonic spectra are not dramatically affected by the presence of the thiolated molecules, while the Raman signal for BA containing liposomes disappears, we can hypothesize that the enhanced Raman signals arise from the interaction between thiol moieties and AuNPs.”

Comment 4:

In Figure 7a, it seems that the cell nucleus was also stained by CT-B-LipoGold.

Response:

We thank the Reviewer for spotting this issue. After the standard GM1 staining with CT-B the cell nucleus should not appear labeled. The nucleus of figure 7a was probably the result of a cell fixation artifact.

Action undertaken:

We repeated the experiment and replaced the panel (a) of figure 7, showing a larger field of view with several cells, where the nuclei are clearly not labeled by CT-B-LipoGold.

Reviewer #3 (Remarks to the Author):

In this study novel and extremely simple synthetic route to create SERS probes so-called LipoGold tags of remarkable efficiency, versatility, and facile functionalization was presented. The approach assumes the spontaneous association of citrate-capped AuNPs on lipid vesicles. The proposed method overcomes currently limiting SERS probe fabrication factors such as reproducibility, and optical and colloidal stability. The proposed liposomal surface provides a platform for simple bioconjugation with antibodies, enabling precise targeting specificity

It is worth mentioning that all sections of the manuscript describe all conducted experiments in detail, which will allow for the repetition of each procedure in any other laboratory. Additionally, each section contains a deep discussion of the obtained results.

The article is divided into a few logically organized chapters. The authors start with the preparation of so-called LipoGold Tags. The Au NPs are formed by the Turkevich method, the NPs characterization is in SI, because such a procedure has been well known for many years.

The preparation of LipoGold Tags is in my opinion most important and innovative part of the manuscript. The presented approach is based on great and novel ideas realized in a good manner. The authors tested four various liposome types. The authors introduce the Figure, with a schematic idea of the process and all necessary characterization results based on Uv-Vis and cryo-TEM measurements. The collected data are carefully examined and discussed in detail. I like the Figures showing results with interpretation. This type of Figure improves and clarifies the manuscript. Of course, the authors include studies about reproducibility and stability.

I have just a few small suggestions for the next section entitled Multiplexing.

Comment 1:

In this section, the authors used three different Raman reporter molecules. So far, all previous tests were done on p-MBA, here authors introduce new RR so-called RR2 and RR6. I Wonder about the numeration of RR molecules. In the whole text, there is no information about RR3, RR4, or RR5 molecules. Additionally, I did not find the full names of applied compounds. However, as in previous parts, the conducted research is well planned, the discussion is deep and Figure 5 summarizes obtained results.

In the next section authors studied the possibility of bioconjugation based on pegylated phospholipid with a reactive carboxylic acid moiety (here one can find the full compound name). Presented research confirmed SERS activity after bioconjugation.

The authors also showed an in-vitro test of LipoGold Tags in SHSY-5Y cell culture. Application of RR6 allows distinguishing characteristic peaks because such peaks fall in the biologically silent region. Finally described nanostructures were successfully applied in the detection of GM1 alteration.

In my opinion, the key result of the presented paper is the fabrication method of LipoTags. The proposed approach is innovative. The presented results showed in-depth analysis followed by careful discussion and interpretation. I strongly believe that this manuscript has great significance in the field of novel types of SERS tags for various (also in-vitro) measurements). The paper cited all relevant work in the field.

I strongly agree that the presented results can be a milestone in the field of AuNP cluster-based SERS nanoprobes. In my opinion, the presented manuscript has to be accepted for publication in a prestigious scientific journal, I just wonder if so long and detailed manuscript will fit a communicating type of article.

Response:

We thank the Reviewer for the suggestion. The RR-X bioorthogonal molecules, as mentioned in the comment 2 from Reviewer 2, were synthesized by implementing a straightforward method recently published by our group [I*nt*. J. Mol. Sci. 2022, *23*(10), 5573; https://doi.org/10.3390/ijms23105573] and the nomenclature was kept. For sake of clarity, the full chemical name of the RR2 and RR6 compounds have been reported in the revised version of Materials and methods section as following:

RR2: 4-nitro-N-(2-mercaptoethyl)benzamide

RR6: 4-(phenylethynyl)-N-(2-mercaptoethyl)benzamide

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The revised version of the manuscript still presents several points that are not clear enough in my opinion.
Related to novelty authors have explained that the system itself what is novel as SERS tag but not the components used i.e. liposomes and AuNPs...Still is not so clear for me that the approach is so novel. I recommend authors including in the introduction a better explanation highlighting the novelty of the system and the advantages over other SERS tags already available.
In response to comment 4: why SHSY-5Y cells were fixed. Why do authors not use alive ones if the goal is to label cells and image them? I recommend doing this experiment with alive cells and improve Raman mapping showing not only 1 cell but several ones to see the efficacy of the tags in a whole culture.
In response to comment 5: Again, why authors use always fixed cells? The advantage of doing SERS is that one can do live imaging over time...otherwise which are the advantages over techniques commonly used to image cells cultures? Moreover, in terms of detection another advantage is multiplexing but also authors did not show any Raman mapping in a co-culture. Ideally instead of using only SHSY-5Y cells the experiments should be done using a mixture of cells from healthy and diseased donor and the LipoGold tags should only target the specific one. Moreover. In Figure 7 Raman mappings showing the same area that colocalize the SERS signal and the fluorescent signal from the LipoGold should be included.
In response to comment 6: In answer to comment 6. A considerable decrease of signal can be observe using 785 nm laser. This should be done with the different Raman reporters as MBA is one that gives a high signal, but I think is interesting to see what happens with other ones.
Overall, I think that the presented work is of interest, further work is needed to merit being published in Nature Communications.

Reviewer #2:
Remarks to the Author:
I am satisfied with the response and the corrections. Thus, I recommend its acceptance at the current stage.

Reviewer #3:

Remarks to the Author:
The authors modified the manuscript according to all of my suggestions. Now I can recommend the manuscript for publication in Nature Communications.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

1) The revised version of the manuscript still presents several points that are not clear enough in my opinion.

Related to novelty authors have explained that the system itself what is novel as SERS tag but not the components used i.e. liposomes and AuNPs…Still is not so clear for me that the approach is so novel. I recommend authors including in the introduction a better explanation highlighting the novelty of the system and the advantages over other SERS tags already available.

We thank the reviewer for raising this point, which allows further sharpening of the introduction.
While liposomes and nanoparticles have indeed been studied and utilized in various applications, we emphasize that our manuscript reports on a novel application of these components. Specifically, we present the development of LipoGold tags as hybrid lipid-AuNP clusters for highly efficient surface-enhanced Raman scattering (SERS) substrates in biomedical applications. The novelty lies not in the individual components themselves, but rather in their synergistic combination for this purpose. This results in unique advantages for SERS-based imaging and detection. We believe that these findings fill a specific gap, as it is well-reported in literature that obtaining stable AuNPs as well as AuNPs-clusters for SERS is challenging. The SERS tags need to be stable, easy to functionalize with Raman reporters and targeting moieties, and reproducible. Typically, Raman reporter molecules have to compete with the other components of the surface. Often, they are exposed towards the environment and can undergo degradation or detachment from the metal surface. In addition, when exposed on the surface, RRs affect NPs stability, while their molecular composition modifies the Raman tag synthesis. The most common approaches that overcome these drawbacks are usually based on long and tedious synthesis and are dependent on the RR structure. Considerable efforts need to be devoted to synthesizing efficient Raman tags, limiting their development and spreading. Here, based on our knowledge on the spontaneous clustering of citrate-coated gold nanoparticles on lipid vesicles, we designed a system where the interplay between different interfacial forces enables building-up an efficient, versatile and multifunctional SERS probe via simple self-assembly steps, that overcome all the drawback mentioned. Specifically, three distinct interfacial phenomena lead to the spontaneous formation of the final construct:
(i) with a similar strategy as the common inclusion of hydrophobic drugs in liposomal vectors, the hydrophobic Raman reporter molecules are included in the lipid membrane, due to hydrophobic interactions. This allows avoiding exposure of RRs towards the water solution, improving solubility and avoiding significant sample-to-sample differences especially when changing RR structure.

(ii) liposomes loaded with the Raman reporter are exposed to citrate-coated gold nanoparticles, leading to the controlled clustering of AuNPs on the liposomal surface and the formation of SERS hotspots, according to a mechanism investigated in recent studies.
(iii) finally, the Raman reporter, which is freely diffusing inside the liposomal membrane, is able to encounter the AuNPs clusters and attach to them via its thiol moiety, ensuring the close proximity between the Raman reporter and the SERS hotspots, which is necessary for efficient SERS enhancement.
These three steps are spontaneous and obtained by mixing Turkevich AuNPs with liposomes with the correct composition. To the best of our knowledge, this is one of the easiest protocols to produce SERS tags with all the mentioned benefits. The simplicity of the protocol, together with all the resulting advantages, represent the strength and the novelty of the manuscript.

We revised the introduction to provide a clearer explanation of the novelty of our system and highlight its advantages over existing SERS tags.

Action undertaken:

The introduction has been modified from:

Here, we leverage these previous findings on liposomes, NP-vesicles interaction, and spontaneous clustering of citrate-capped AuNPs on synthetic lipid vesicles, to develop a novel class of nanomaterials, LipoGold tags, that emerge as flexible, highly efficient, and colloidally stable SERS probes.
to:
Here, based on the spontaneous clustering of citrate-coated gold nanoparticles on lipid vesicles, we designed a novel system (LipoGold tags) where the interplay between different interfacial forces enables building-up an efficient, versatile and multifunctional SERS probe via simple self-assembly steps, that overcome all the drawbacks mentioned. Specifically, three distinct interfacial phenomena lead to the spontaneous formation of the final construct:
(i) the hydrophobic Raman reporter molecules are included in the lipid membrane, due to hydrophobic interactions. This allows avoiding exposure of RRs towards the water solution, improving solubility and avoiding significant sample-to-sample differences especially when changing RR structure.
(ii) liposomes loaded with the Raman reporter are exposed to citrate-coated gold nanoparticles, leading to the controlled clustering of AuNPs on the liposomal surface and the formation of SERS hotspots, according to a mechanism investigated in recent studies.
(iii) finally, the Raman reporter, which is freely diffusing inside the liposomal membrane, is able to interact with the AuNPs clusters and attach to them via its thiol moiety, ensuring the close proximity

between the Raman reporter and the SERS hotspots, which is necessary for efficient SERS enhancement.
The novelty of this approach lies not in the individual components themselves, but rather in their synergistic combination and the unique advantages they offer for SERS-based imaging and detection.

2) *In response to comment 4: why SHSY-5Y cells were fixed. Why do authors not use alive ones if the goal is to label cells and image them?*

Concerning the use of fixed cells, we acknowledge the reviewer's point and the potential benefits of utilizing live cell imaging.
Living cells can be used for direct imaging without fixation and permeabilization when the species of interest are located on the surface of the cell. However, during the time of incubation with the specific fluorescent probe, the cell is active and dynamic processes such endocytosis and plasma membrane re-modelling continue. If the incubation with the probe is quick, in the order of a few minutes, imaging can be performed live, without much perturbation. In the experiment in figure 7 we used fixed cells because the incubation was relatively long, 90 min, during which cells can in principle even undergo mitosis in addition to endocytosis. Our aim was not to simply label the cells, but to demonstrate the specific binding and stability of the functionalized LipoGold on the surface of the cells, in the most direct and straightforward way, without additional caveats due to endocytosis of the LipoGold that could make the interpretation of the results less clear.
In the case of fibroblasts, cells must be fixed and permeabilized as the primary accumulation of GM1, which has to be detected either with fluorescence and flow cytometry, or with LipoGold and SERS, is intracellular in the pathological mutant.
Action undertaken:
In order to improve clarity, we added the following paragraph in the Revised version of the Manuscript:

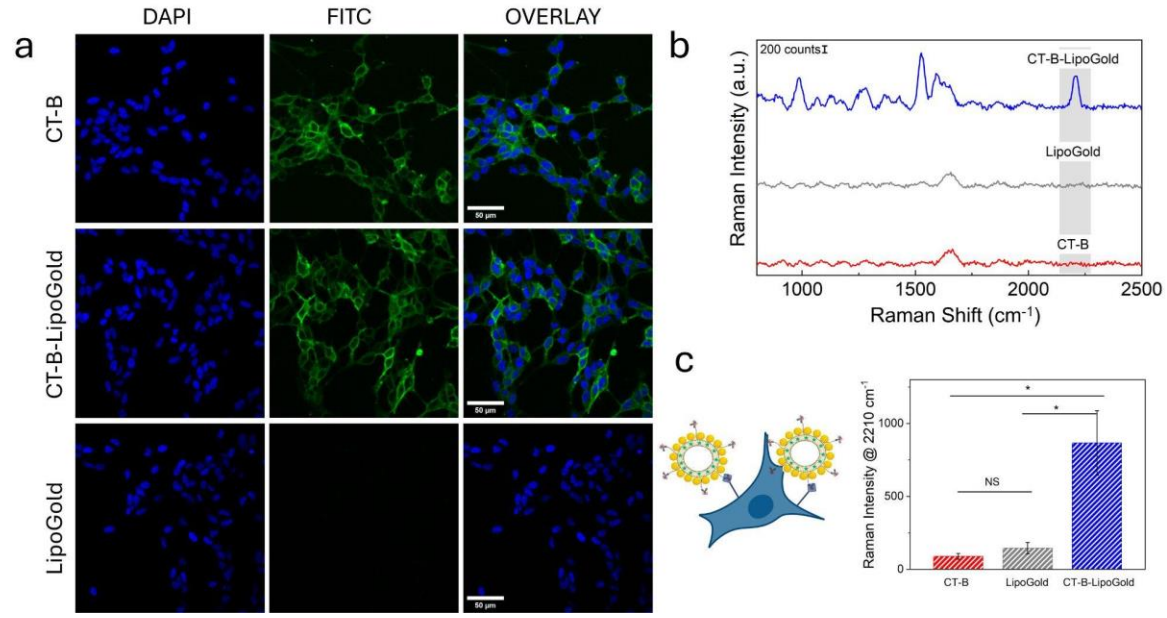
"For this experiment, cells were previously fixed to avoid non-specific endocytosis, mitosis and even plasma membrane remodeling during the relatively-long incubation (90 minutes)"

3) *I recommend doing this experiment with alive cells and improve Raman mapping showing not only 1 cell but several ones to see the efficacy of the tags in a whole culture.*

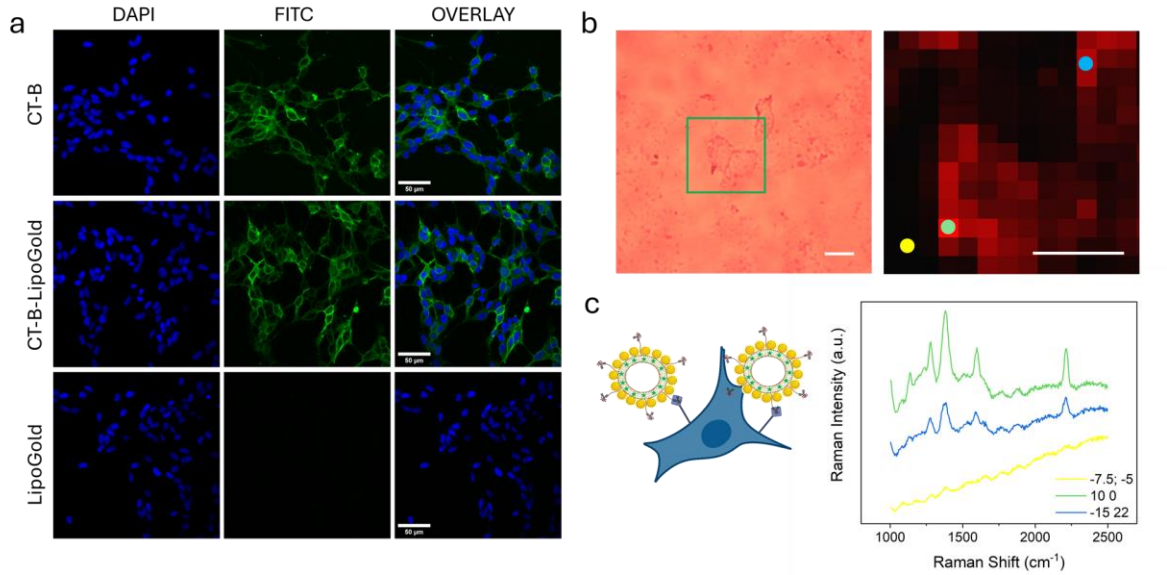
We agree with the reviewer that showing Raman maps from multiple cells would be beneficial for the manuscript. For these reasons, we repeated the experiment reported in figure 7 with the LipoGold tags and collected multiple Raman maps. Raman maps have been built using the peak located at 2100 cm^{-1} in the silent region of the cells, characteristic of RR6.

Action undertaken:

In this revised version, the Raman maps have been included in the manuscript, both in the main text and in the supplementary information file. Figure 7 has been modified from:



to:



The main text was accordingly modified from:

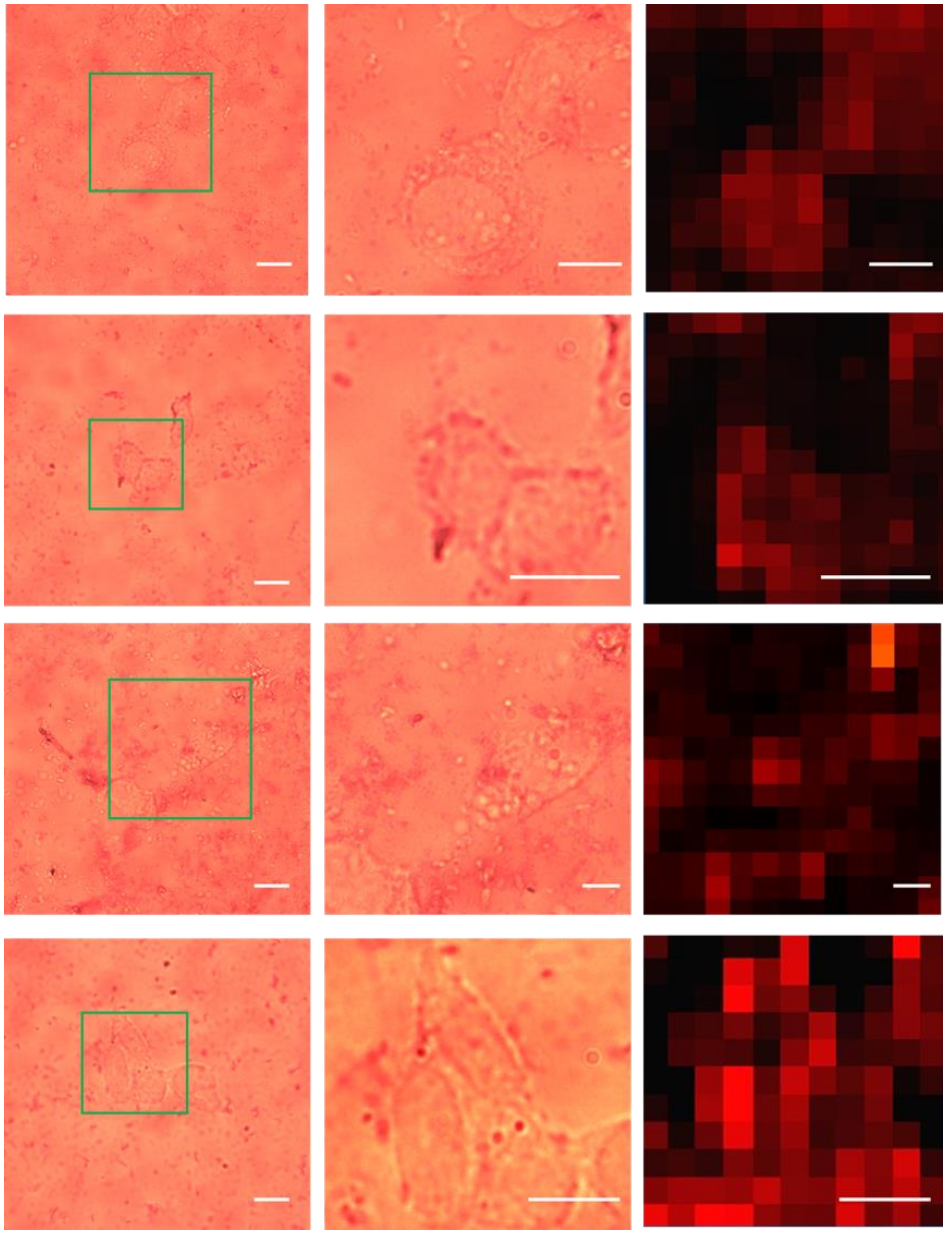
As shown in the confocal images in Figure 7a, the intense green fluorescent signal from FITC was observed on the surface of cells incubated either with the free tag or CT-B-DOPC(RR6)-PEG-AuNPs, while no signal was observed when incubated with bare SERS probes (DOPC(RR6)-PEG-AuNPs). This demonstrated the high selectivity and specificity of the liposome-based SERS nanoprobes even when

working with complex matrices. Subsequently, the FITC signal was exploited to localize the SERS constructs and Raman spectra from the cell were acquired. Figure 7b shows representative SERS spectra acquired from the control cells (red line), bare DOPC(RR6)-PEG-AuNPs adducts (gray), and CT-B-DOPC(RR6)-PEG-AuNPs (blue line). The SERS spectra revealed the presence of the strong narrow peak characteristic of the RR6 mainly coming from the cell membranes only when NPs-liposomes tags are bioconjugated with CT-B (Figure 7c). The successful colocalization of fluorescence and Raman signal paves the way for the exploitation of these DOPC(RRs)-AuNPs constructs for immuno-SERS microscopy.

To:

As shown in the confocal images in Figure 7a, the intense green fluorescent signal from FITC was observed on the surface of cells incubated with the free tag CT-B-LipoGold (RR6). In contrast, no signal was observed when incubated with bare SERS probes LipoGold (RR6). This result demonstrated the high selectivity and specificity of the liposome-based SERS nanoprobes even when working with complex matrices. The bright field image (figure 7b) displays the analyzed cells and their corresponding SERS map. The green rectangle represents the area scanned with Raman confocal microscopy used to build the map by exploiting the SERS intensity of the peak located at 2210 cm^{-1}, in the biological silent region. For example, figure 7c reports SERS spectra collected from three different points on the map. The color of each spectrum corresponds to the respective dot shown in figure 7b. Other SERS maps are reported in Figure S24, revealing the possibility to identify cell morphology by mapping the localization of the strong narrow peak characteristic of RR6 when NPs-liposomes tags are bioconjugated with CT-B.

Figure S24 was added in the supplementary file:



4)*In response to comment 5: Again, why authors use always fixed cells? The advantage of doing SERS is that one can do live imaging over time...otherwise which are the advantages over techniques commonly used to image cells cultures?*

As already stated in response 2, the use of fixed cells is instrumental in preventing non-specific endocytosis and other biological mechanisms that could lead to internalization, without necessarily ensuring the specific targeting of the synthesized probes. The primary aim of the experiment is to demonstrate the targeting abilities of the produced Raman tags. Moreover, live imaging is not the sole advantage of Raman maps. Fluorescence imaging often suffers from photobleaching, where the fluorescent signal diminishes over time due to prolonged exposure to light. In contrast, SERS signals do not bleach, allowing for longer observation times and repeated measurements without signal loss.

Additionally, SERS can detect single molecules and is more sensitive than fluorescence in many cases. SERS imaging typically has lower background interference compared to fluorescence imaging, where autofluorescence from the sample or surrounding medium can obscure the signal of interest. However, it's important to underline once again that our intent is not to prove that SERS imaging or sensing is superior to other approaches. Rather, we aim to introduce a novel and straightforward approach for producing and using Raman tags.

5)Moreover, in terms of detection another advantage is multiplexing but also authors did not show any Raman mapping in a co-culture. Ideally instead of using only SHSY-5Y cells the experiments should be done using a mixture of cells from healthy and diseased donor and the LipoGold tags should only target the specific one. Moreover. In Figure 7 Raman mappings showing the same area that colocalize the SERS signal and the fluorescent signal from the LipoGold should be included.

Multiplexing refers to the capability of quantifying different analytes during the same single measurement. It is not fully clear to us how this can be related with co-culturing cells. In our case, we used cells from healthy and affected donors to validate the potential of LipoGold tags to distinguish between the two populations, and their diagnostic use in general terms. The experiment proved with both fluorescence, scattering, and Raman analysis, that the lipoGold tags accumulation in affected donor cells is highly different. The present experiment is a proof of concept aimed at strengthening the usability of the LipoGold tags, as, despite these tags are extremely simple to prepare and functionalize, they still work in complex environment and detection experiments. As mentioned, the experiment is only a proof of concept to validate the LipoGold tags applicability and their use can be optimized for many other applications. Considering this, a co-culturing experiment as the one proposed by the referee could be certainly interesting, in particular for discriminating cancer from normal cells, but it is out of the scope of this manuscript.

Action undertaken:

As discussed for question 3, we included in the main text the Raman maps. However, the microscopes used for Raman and fluorescence measurements are two different instruments and colocalization between fluorescent and Raman signals of the same field-of-view cannot be performed. Besides this instrumental hurdle, we believe that such experiment would not be of high relevance for the scopes of the present manuscript, in terms of additional information on the LipoGold tags.
As already mentioned in the answer to question 2, the focus of the present work is to fill the gap between SERS tag production and biological tests. We believe that the presented tags can facilitate the production of SERS tags since it doesn't require any specific synthetic expertise.

In response to comment 6: In answer to comment 6. A considerable decrease of signal can be observe using 785 nm laser. This should be done with the different Raman reporters as MBA is one that gives a high signal, but I think is interesting to see what happens with other ones.

We thank the reviewer for the insightful comment. As mentioned, the considerable decrease in signal is expected, since the resonance frequency of the utilized AuNP clusters matches the excitation wavelength of the 638 nm laser. It is true that higher wavelengths are preferred for deeper penetration of the laser. To achieve a higher SERS effect for our LipoGold tags, the plasmonic resonance of the gold clusters needs to be shifted to higher wavelengths.

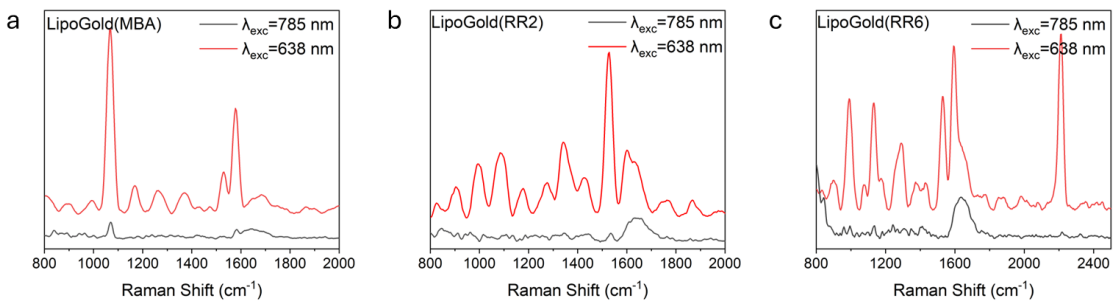
We propose two main strategies for a future work to achieve this shift:

1. Using Larger NPs: Employing larger nanoparticles (20-30 nm) could result in a more red-shifted plasmonic peak due to their clustering properties.
2. Using Anisotropic Metallic NPs: Utilizing anisotropic metallic nanoparticles, such as rods, cubes, or stars, could also achieve the desired red shift.

Our next experiments will focus on applying larger particles and exploring different shapes to obtain a plasmonic signal closer to 785 nm, which will allow better penetration in biological samples.

Action undertaken:

We agree with the reviewer that the decrease in signals observed using the 785 nm laser should be demonstrated for other Raman reporters (RRs) as well. Therefore, we have collected Raman spectra for LipoGold tags embedding RR2 and RR6. A similar elevated decrease of Raman signal has been found for RR2 and RR6 as well. The data have been included in the revised version of the supplementary information file in Figure S6.



Overall, I think that the presented work is of interest, further work is needed to merit being published in Nature Communications.

Reviewer #2 (Remarks to the Author):

I am satisfied with the response and the corrections. Thus, I recommend its acceptance at the current stage.

We are grateful for the positive feedback from Reviewer #2 and are pleased that the revisions were satisfactory.

Reviewer #3:

Reviewer #3 (Remarks to the Author):

The authors modified the manuscript according to all of my suggestions. Now I can recommend the manuscript for publication in Nature Communications.

We thank this Reviewer and are pleased that she/he finds it suitable for Nature Communications.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
I still think that the novelty is not well justified, there are already reports that present lipomes with AuNPs used as SERS tags, as can e seen in https://www.thno.org/v12p1870.pdf, SERS Tags for Biomedical Detection and Bioimaging., for example.
Moreover, the response related to fixing the cells is not convincing for me. There are many reports that label the membrane with SERS tags or fluorophores done in live, but of course is more complicated but more interesting than fixing cells...This way you can study the tag-cell interaction over time. Moreover, If authors say that fibroblast detection need to be done intracellularly, that I do not understand the problem of leaving the cells uptaking the tags, the cells normally dive 1 one day so one can more or less know when to do the incubation that most of the population is not dividing...
Overall, I think that the presented work is of interest, but is not novel enough to be published in Nature Communications.

Reviewer #1 (Remarks to the Author):

I still think that the novelty is not well justified, there are already reports that present lipomes with AuNPs used as SERS tags, as can e seen in https://www.thno.org/v12p1870.pdf, SERS Tags for Biomedical Detection and Bioimaging., for example.

Moreover, the response related to fixing the cells is not convincing for me. There are many reports that label the membrane with SERS tags or fluorophores done in live, but of course is more complicated but more interesting than fixing cells…This way you can study the tag-cell interaction over time. Moreover, If authors say that fibroblast detection need to be done intracellularly, that I do not understand the problem of leaving the cells uptaking the tags, the cells normally dive 1 one day so one can more or less know when to do the incubation that most of the population is not dividing…

Overall, I think that the presented work is of interest, but is not novel enough to be published in Nature Communications.

Response:

We respectfully disagree with the reviewer's comment regarding the novelty of the manuscript. As detailed in the previous revision, we have thoroughly justified the novelty of our approach in comparison to existing methods. In addition to the previous comments, we can say the innovation of the present work extends beyond the mere combination of AuNPs and liposomes. We have developed a new, highly reproducible protocol that is significantly simpler also compared to the few discussed in the suggested review. The simplicity of our method is founded on a deep and comprehensive understanding of how individual parameters influence the spontaneous assembly of AuNPs. This knowledge has been accumulated through years of dedicated research. Despite its simplicity, we have demonstrated the potential of our probes through proof-of-concept experiments in Raman imaging and detection. We firmly believe that our strategy is unique and offers significant advantages from multiple perspectives (please refer to the previous revision).

Regarding the need for cell fixation:

In the experiments that we carried out cells could not be synchronized (this would have needed starvation, a stressing condition for the cells, and we did not want to introduce any additional variable) and division could take place anytime along 24 hours. Moreover, with respect to the objectives of the manuscript, it is not clear how performing the live experiments as suggested by referee 1 could add more value to the results obtained with fixed cells. The standard experiments, which were used as reference, to evaluate changes in intracellular GM1 content with CTXB in fibroblasts from patients involves the fixation and permeabilization of the cells (Capitini et al Biomedicines. 2022 Aug 12;10(8):1962. doi: 10.3390/biomedicines10081962, Tonin et al. Sci Rep. 2019 Nov 27;9(1):17684. doi: 10.1038/s41598-019-53995-5), while the surface labelling was demonstrated to be not sufficient to detect differences (Tonin et al. Sci Rep. 2019 Nov 27;9(1):17684. doi: 10.1038/s41598-019-53995-5).

Action undertaken:

To make clearer the need of fixed and permeabilized cells, the following sentence has been changed from:

In contrast to the experiment reported in Figure 7, fixed cells were permeabilized to enable the detection of intracellular GM1, as already previously implemented.

To:

In contrast to the experiment reported in Figure 7, fixed cells were permeabilized to enable the detection of intracellular GM1, since surface labelling alone was previously demonstrated to be not sufficient to detect differences.