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TITLE:

Tethered bilayer lipid membranes to monitor heat transfer between gold nanoparticles and lipid membranes

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KEYWORDS:

Tethered bilayer lipid membranes (tBLMs), Biosensor, Gold nanoparticles, Laser, Heat transfer, Membrane dynamics

SUMMARY:

This work outlines a protocol to achieve dynamic, non-invasive monitoring of heat transfer from laser-irradiated gold nanoparticles to tBLMs. The system combines impedance spectroscopy for the real-time measurement of conductance changes across the tBLMs, with a horizontally focused laser beam that drives gold nanoparticle illumination, for heat production.

ABSTRACT:

Here we report a protocol to investigate the heat transfer between irradiated gold nanoparticles (GNPs) and bilayer lipid membranes by electrochemistry using tethered bilayer lipid membranes (tBLMs) assembled on gold electrodes. Irradiated modified GNPs, such as streptavidin-conjugated GNPs, are embedded in tBLMs containing target molecules, such as biotin. By using

this approach, the heat transfer processes between irradiated GNPs and model bilayer lipid membrane with entities of interest are mediated by a horizontally focused laser beam. The thermal predictive computational model is used to confirm the electrochemically induced conductance changes in the tBLMs. Under the specific conditions used, detecting heat pulses required specific attachment of the gold nanoparticles to the membrane surface, while unbound gold nanoparticles failed to elicit a measurable response. This technique serves as a powerful detection biosensor which can be directly utilized for the design and development of strategies for thermal therapies that permits optimization of the laser parameters, particle size, particle coatings and composition.

INTRODUCTION:

The hyperthermic performance of irradiated gold nanomaterials offers a new class of minimally invasive, selective, targeted treatment for infections and tumors[1]. The employment of nanoparticles that can be heated by a laser has been used to selectively destroy diseased cells as well as providing a means for selective drug delivery[2,3]. A consequence of the photothermolysis phenomena of heated plasmonic nanoparticles is damage to the cell membranes. The fluid lipid bilayer membrane is considered a particularly vulnerable site for cells undergoing such treatments because denaturation of intrinsic membrane proteins as well as membrane damage can also lead to cell death[4], as many proteins are there to maintain the ionic potential gradient across cell membranes. While the ability to determine and monitor heat transfer at the nanoscale is of key interest to the study and application of irradiated GNPs[1,5-7], assessment and understanding of the molecular interactions between GNPs and bio-membranes, as well as the direct consequences of the laser-induced heating phenomena of embedded GNPs in biological tissues, are yet to be fully elucidated[8]. Therefore, a thorough understanding of the hyperthermia process of irradiated GNPs remains a challenge. As such, the development of a nanomaterial-electrode interface that mimics the natural surroundings of cells could provide a means by which to undertake an in-depth investigation of the heat transfer characteristics of irradiated gold nanoparticles within biological systems.

The complexity of native cell membranes is one of the significant challenges in understanding the irradiated GNPs interactions in cells. There have been various artificial membrane platforms developed to provide close simple bio-mimetic versions of natural lipid membrane architecture and functionality, including, but not limited to, black lipid membranes[9], supported planar bilayer membranes[10], hybrid bilayer membranes[11], polymer-cushioned lipid bilayer membranes[12] and tethered bilayer lipid membranes[13]. Each artificial lipid membrane model has distinct advantages and limitations with respect to mimicking the natural lipid membranes[14].

This study describes the employment of lipid membrane-coated electrodes as a sensor for assessing gold nanoparticle and lipid membrane interactions, using the tBLM model. The tBLM based biosensor detection scheme provides inherent stability and sensitivity[13] as tethered membranes can self-repair, unlike other systems (such as membranes formed by patch-clamp or liposomes) in which only a small amount of membrane damage results in their collapse[15-18]. Further, because tBLMs are of mm^2 dimensions, the background impedance is orders of magnitude lower than patch-clamp recording techniques, which enables a recording of changes

in basal membrane ionic flux due to nanoparticle interactions. As a result of this, the present protocol can contrast changes in membrane conductance by bound GNPs that are excited by lasers whose powers are as low as 135 nW/μm^2.

The system presented here provides a sensitive and reproducible method for determining precise laser parameters, particle size, particle coatings and composition needed to design and develop thermal therapies. This is critical for the refinement of emerging photothermal therapies, as well as offering valuable information for detailed mechanisms of heat transfer within biological systems. The presented protocol is based on previously published work[19]. An outline of the protocol is as follows: the first section defines the tBLM formation; the second section outlines how to construct the setup and align the excitation laser source; the final section illustrates how to extract information from the electrical impedance spectroscopy data.

PROTOCOL:

1. tBLMs electrodes preparation

1.1. Preparation of first monolayer coating

1.1.1. Immerse a freshly sputtered gold patterned electrode microscope slide in an ethanolic solution comprised of a 3 mM 1:9 ratio of benzyl-disulfide-tetra-ethyleneglycol-OH "spacer" molecules (benzyl disulfide comprised a four oxygen-ethylene glycol spacer, terminated with an OH group) and benzyl-disulfide (tetra-ethyleneglycol) n=2 C20-phytanyl "tethered" molecules. This creates the first layer coating to which a bilayer can be anchored.

NOTE: The gold electrode is made by evaporating 100 nm, 99.9995% gold (5n5 gold) film onto custom 25 mm x 75 mm polycarbonate slides[20].

1.1.2. Incubate electrodes with the first layer at room temperature for at least 1 h.

1.1.3. Rinse the gold electrodes by immersing in copious amounts of pure ethanol over 30 s.

1.1.4. Use the gold electrode slide with the first monolayer directly for the next step or store in a jar full of pure ethanol.

NOTE: To ensure the integrity of the first layer, minimize any direct contact to the gold portions of the slide

1.2. Assembling the first monolayer coated slide

1.2.1. Carefully take off one coplanar gold electrode slide from its container using tweezers, being sure not to make contact with the patterned areas where the tBLMs will form.

NOTE: Be mindful to identify the side of the slide onto which the gold is deposited.

1.2.2. Air dry slide for 1 – 2 min in to remove any residual ethanol.

1.2.3. Place gold electrode over a dry surface, ensure the gold electrode is correctly oriented with patterned gold surface facing up.

1.2.4. Peel the transparent adhesive layer cover from a thin laminate and place over the 6 channels to define each well.

1.2.5. Use a pressure roller to release any air between the slide and transparent adhesive layer, as shown in **Figure 1A**.

NOTE: The time required for this step will need to be optimized by the researcher. In this protocol, times are ranging from 2-3 min.

1.2.6. Introduce as soon as practicable (within 1-2 minutes) the second lipid bilayer to the assembled first monolayer coated electrode for self-assembly to avoid damaging the first layer.

1.3. Preparation of second lipid bilayer

1.3.1. Add 6 µL of 3 mM lipids of interest to the first well of the six wells slide. Do not let the edge of the micropipette tip touch the gold surface, which can damage the tethered chemistries on the electrode.

NOTE: The lipid mixture used in this work consisted of 3 mM 70% zwitterionic C20 diphytanyl-ether-glycero-phosphatidylcholine (DPEPC) and 30% C20 diphytanyldiglyceride ether lipids (GDPE) mixed with 3 mM cholesterol-PEG-Biotin in 50:1 molar ratio.

1.3.2. Introduce 6 µL of the lipid mixture to the other wells with a 10 s gap between each addition.

1.3.3. Incubate each well for exactly 2 min at room temperature before exchanging the lipid mixture over the electrodes with a buffer such as PBS. Space the times for the addition and buffer exchange 10 s apart so each well is incubated with the lipid for exactly 2 min each.

1.3.4. Wash 3 more times with 50 µL of PBS buffer (pH 7.0). Be sure to leave 50 µL of buffer over the electrodes at all times. Do not allow the electrodes to dry.

NOTE: Displacing the ethanol solvent with the aqueous solution in this way (the *solvent exchange method*) enables the rapid formation of a single lipid bilayer anchored to the gold electrode via the tethered chemistries.

1.4. Testing tBLM formation using electrical impedance spectroscopy (EIS) measurements

1.4.1. Insert prepared electrode slide into an AC impedance spectrometer (e.g., Tethapod). Ensure that the spectrometer is connected via a USB port to a computer running the software.

1.4.2. Open the software, click **Setup** and open **Hardware**.

1.4.3. Set the hardware settings to use 25 mV peak-to-peak AC excitation.

1.4.4. Set frequencies between 0.1 and 10,000 Hz with two steps per decade for rapid impedance measures press ok.

1.4.5. Click the **Setup** menu and open **Model**.

1.4.6. Use an equivalent circuit model that describes the tethering gold electrode as a constant phase element in series with a resistor describing the electrolyte buffer and a parallel resistor-capacitor network to describe the lipid bilayer, and press **OK**.

1.4.7. Press the **Start** button in order to start a real-time measurement of membrane capacitance (C_m) and membrane conduction (G_m). C_m values of typical tBLMs should be in the range of 12.5 nF to 15.5 nF for 10% tethered chemistries[21,22].

1.4.8. After running the protocol and finishing the experiment, save the data.

1.4.9. Repeat the measurement with the next well.

2. Laser irradiation

2.1. Experimental setup

NOTE: The custom-made system is set up for each tBLM well individually.

2.1.1. Perform experiments in a light-proof box to minimize laser hazardously.

2.1.2. Use an optics table to set up the experiment to reduce unwanted vibrations.

2.1.3. Place the impedance reader, where is the gold slide is connected, on an XYZ stage and elevate such that it sits in the path of the laser source.

2.1.4. Use coarse-fine focusing microscopic gearing to control the height of the laser source to achieve the appropriate precision.

2.1.5. Target the laser path along the longitudinal axis of the electrode slide.

CAUTION: Always wear suitable laser safety glasses and maintain good laser safety protocols.

2.1.6. Allow the selected tuned laser to stabilize before starting the experiment.

NOTE: A schematic of the experimental setup is illustrated in **Figure 2A**.

2.2. Alignment of laser and gold electrodes

NOTE: Before beginning, always assess the laser power output using a power-meter to ensure only very low wattages are delivered to the tBLMs.

2.2.1. Adjust either the laser path or the angle of the electrode such that the laser passes through the liquid covering the electrode and is just visible, evenly, at the gold surface.

2.2.2. Adjust the laser beam light position for each experiment by raising or lowering the laser beam source using the fine adjustment while observing changes in membrane conductance.

2.2.3. Lock the knob to secure the position of the laser path when there are no conductance changes are observed.

NOTE: Increased membrane conductance values will be generated when the laser interacts with the underlying gold electrode. It is, therefore, important to adjust the laser path such that no such interactions are possible.

2.3. Sample preparation

2.3.1. Prepare the laser beam light alignment (where there is no change in membrane conductance), as shown in **Figure 2**, position 3.

2.3.2. Add GNPs of interest (functionalized or bare) to the PBS buffer in which the tBLMs are immersed while the laser is switched OFF.

2.3.3. Mix the PBS buffer surrounding the tBLMs gently three times, being careful not to touch the electrode.

2.3.4. Incubate for 5-10 min at room temperature.

2.3.5. Turn the laser ON to irradiate sample, using the correct aligned laser beam light position as seen in **Figure 2**, position 3.

2.3.6. Use the appropriate combination of GNPs size, shape and concentration with laser light wavelength.

NOTE: The laser beam of set wavelength should couple to the corresponding GNP plasmon resonance frequency.

2.3.7. Record measured current continuously (real-time measurements).

2.3.8. Perform steps 2.2.1 – 2.3.7, omitting GNP addition for the control experiments.

3. Statistical data analysis and presentation

3.1. Export the data into a spreadsheet.

3.2. Extract the membrane conductance parameter versus time.

3.3. Use the recorded data after setting a laser beam light with the right position and prior to GNPs introduction.

3.4. Normalize data by dividing the measured membrane conductance over the baseline membrane conductance.

NOTE: This confirms that relative changes in membrane conduction values elicited by introduced irradiated GNPs.

3.5. Present data as plots of time (x-axis) versus normalized membrane conduction (y-axis).

4. Predict the amount of localized heat generated in the tBLMs from irradiated nanoparticles (thermal predictive model)

4.1. Solve the radiation transfer problem according to Dombrovsky[23], in order to calculate absorbed radiation power in irradiated nanoparticle solutions.

4.2. Calculate the heat generation by incorporating the heat source due to absorbed radiation into the energy equation.

NOTE: For a detailed explanation of the numerical analysis of heat generation in the tBLMs from irradiated nanoparticles and the nanomaterial-electrode interface, refer to [19].

REPRESENTATIVE RESULTS:

The gold substrate upon which tBLMs can be created is shown in **Figure 1**. A schematic of the experimental setup is presented in **Figure 2**.

Coplanar gold electrodes, as shown in **Figure 1A**, are made from 25 mm x 75 mm x 1 mm polycarbonate base substrate with patterned gold arrays. A transparent adhesive layer defines the six individual measuring chambers. The coplanar gold electrode allows the direct exposure of the laser light to tBLMs membrane. Each well of the electrode array contains a circle-shaped working electrode (area: 0.707 cm^2) and half-circle shaped counter electrode or coplanar electrode (area: ~ 0.725 cm^2), which are separated by a gap of ~2 mm. The transparent adhesive layer insulates the rest of the deposited gold from the bulk electrolyte. In contrast, the underlying

gold layout connects the working electrodes to contact areas outside the measuring chambers to provide the electrical connection to the EIS reader without the need for a reference electrode.

The laser path is aligned in a manner where it is interacting with the tBLMs and is scattered through the liquid buffer surrounding it, but not such that it can interact with the underlying gold substrate. This is easily determined via horizontal raising and lowering of the laser until the correct position is established. This position is just at the point where no changes in membrane conductance can be observed. Given that tBLMs are formed by attachment to a substrate layer of bulk gold, it seems likely that the changes in membrane conductance at position 1 and 2 in **Figure 2** are as a result of heat from interactions of the laser with nanostructures within the sputtered bulk gold layer. Thus, using the accurate position of horizontal light beam alignment focusing on eliminating interaction between the laser light and the bulk gold substrate found below the tBLMs.

Focusing the horizontal laser light directly towards the gold electrode causes an increase in membrane conductance, as presented in **Figure 2**, position 1 and 2. The precise laser position revealed negligible variation to the membrane conductance recordings during both periods of laser ON and laser OFF (**Figure 2B**, position 3). The GNP sample was added after establishing baseline recordings, as shown in **Figure 2**, position 3. The addition of streptavidin-conjugated 30 nm gold nanoparticles to tBLMs that contained biotinylated cholesterol showed a clear difference between the laser ON and OFF periods, as well as in comparison to position 3, with distinct increases in conductance amplitude during the laser ON phase (**Figure 2B**, position 4).

Figure 1: Schematic representation of the tethered bilayer lipid membrane (tBLM) model on a gold substrate. (**A**) Coplanar gold electrode slide with six wells, ultimately defined by the addition of a thin transparent adhesive layer. (**B**) The tBLM model comprises spacer (ethylene glycol chains ended with a hydroxyl group) and tethered molecules (ethylene glycol groups ended with hydrophobic phytanyl chain) tethers to the gold substrate surface to form the first layer. The second layer includes the non-tethered lipids. The modified figure was based on Cornell et al.[24].

Figure 2: Illustration of the assay set-up for alignment and corresponding measuring membrane conductance changes across tBLMs arising from laser illumination (λ = 530 nm). (**A**) Schematic representative of the different positions of horizontal laser alignment; where Position 1: laser light beam aligned with the gold substrate (when the laser was turned ON is indicated in red); position 2 the horizontal laser light mixed with membrane and gold substrate; position 3 laser light focused into the bulk fluid surrounding tBLMs; Position 4 laser beam light focused into the fluid surrounding the tBLMs in the presence of streptavidin-conjugated 30 nm spherical GNPs. (**B**) Normalized conductance recordings over time correspond to the different alignment positions. Positions 1, 2 and 3 measurements of tBLMs conductance in the absence of GNPs, whereas position 4 is a measurement of tBLMs conductance in the presence of streptavidin-conjugated 30 nm spherical GNPs. The membrane conduction values were normalized to the initial value of membrane conduction upon tBLMs formation. Results are representative of at least three independent experiments.

DISCUSSION:

This protocol describes the use of tBLM model with a coplanar electrode substrate in conjunction with a horizontal laser alignment set up that enables the real-time electrical impedance recording in response to laser irradiation of gold nanoparticles. The method of EIS recording presented here constructs a minimal list of experiments necessary to provide recording of ion current changes across the membrane, which corresponds to the heat generated by the coupled laser and gold nanoparticle interaction. There is a critical step in this protocol, which is the careful and precise alignment of the laser path towards the buffer surrounding bilayer lipid membrane.

The use of the tBLM model offers distinct electrical sealing properties that mimic natural lipid membranes characteristics[24]. tBLMs also provide an aqueous ionic reservoir region between the gold substrate and the subsequently formed membrane, where the tethered molecules and the spacer molecule had a thickness of 11 Å[25], and the bilayer lipid membrane thickness was around 6.5 nm[19]. This can offer space to incorporate membrane proteins, ion channels or other specific functionalized molecules[13,22]. The selection of 70% DPEPC and 30% GDPE lipids provides optimal sealing of bilayer lipid membrane to examine the electrical characteristics of tBLMs using EIS system[24]. Likewise, the introduction of cholesterol within the bilayer lipid membranes closely mimics native biomimetic model membranes. Cholesterol moieties improve the bilayer lipid membrane stability, as well as minimizing the membrane permeability to ions by providing high packing of the phospholipid bilayer[26,27]. Combining tBLMs with the EIS system provides indirect measurement of heat transfer between irradiated GNPs and bilayer lipid membranes. Further, the use of coplanar gold electrodes in this protocol enables the real-time EIS measurements without any interference from reference or counter electrodes.

Gold in the nanoparticle scale has different physical and optical characteristics to larger gold aggregates. The size and shape of nanoparticle access their bio-distribution, circulation lifetime and cell uptake, where nanoparticles of intermediate sizes (20-60 nm) exhibit maximum cell uptake as well as offer a high surface area to volume ratio, allowing for subsequent functionalization[28,29]. The implemented 30 nm GNP size in this study represented intermediate GNPs sizes, while the laser wavelength selection was according to the absorption peak of GNPs to yield the most efficient excitation, which consequently leads to heating. The laser illumination of tBLMs gold surfaces elevates membrane conduction peaks at the laser ON phase. This is proposed to be as a result of bulk gold surface nanostructures that interact with the laser, which would mask heat production phenomena following the addition of the GNPs[30]. To overcome this, the developed approach here GNPs are illuminated by using horizontal laser alignment across the lipid-buffer interface, as illustrated in **Figure 2**, position 3 and 4.

The protocols described here can be modified readily by altering the lipid composition of the membrane to mimic various natural cell types, or by altering the introduced GNPs size and shape such as 100 nm gold nanourchins with the corresponding laser beam light[19]. This can then be used to determine the impact of localized GNPs induced radiation on specific cell types.

In summary, this protocol serves as a robust detection biosensor to study interactions of in situ irradiated GNPs with model bilayer lipid membrane entities of interest to answer questions on

heat transfer phenomena. This will assist in developing more efficient photothermal therapies, as well as providing valuable information for detailed mechanisms of heat transfer within biological systems. This approach can be used as a tool for the prediction of the level of cell membrane destruction that can be experienced by these heated nanoparticles.

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DISCLOSURES:

The authors declare the following financial interests/personal relationships, which may be considered as potential competing interests: Prof Bruce Cornell is Director – Science and Technology at Surgical Diagnostics SDx tethered membranes Pty. Ltd.

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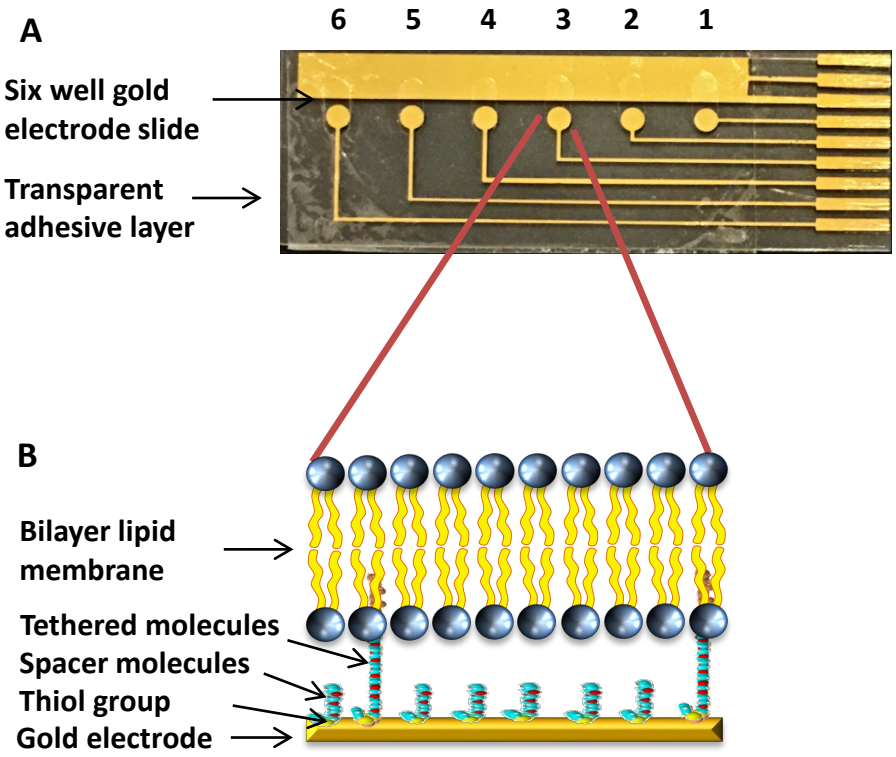
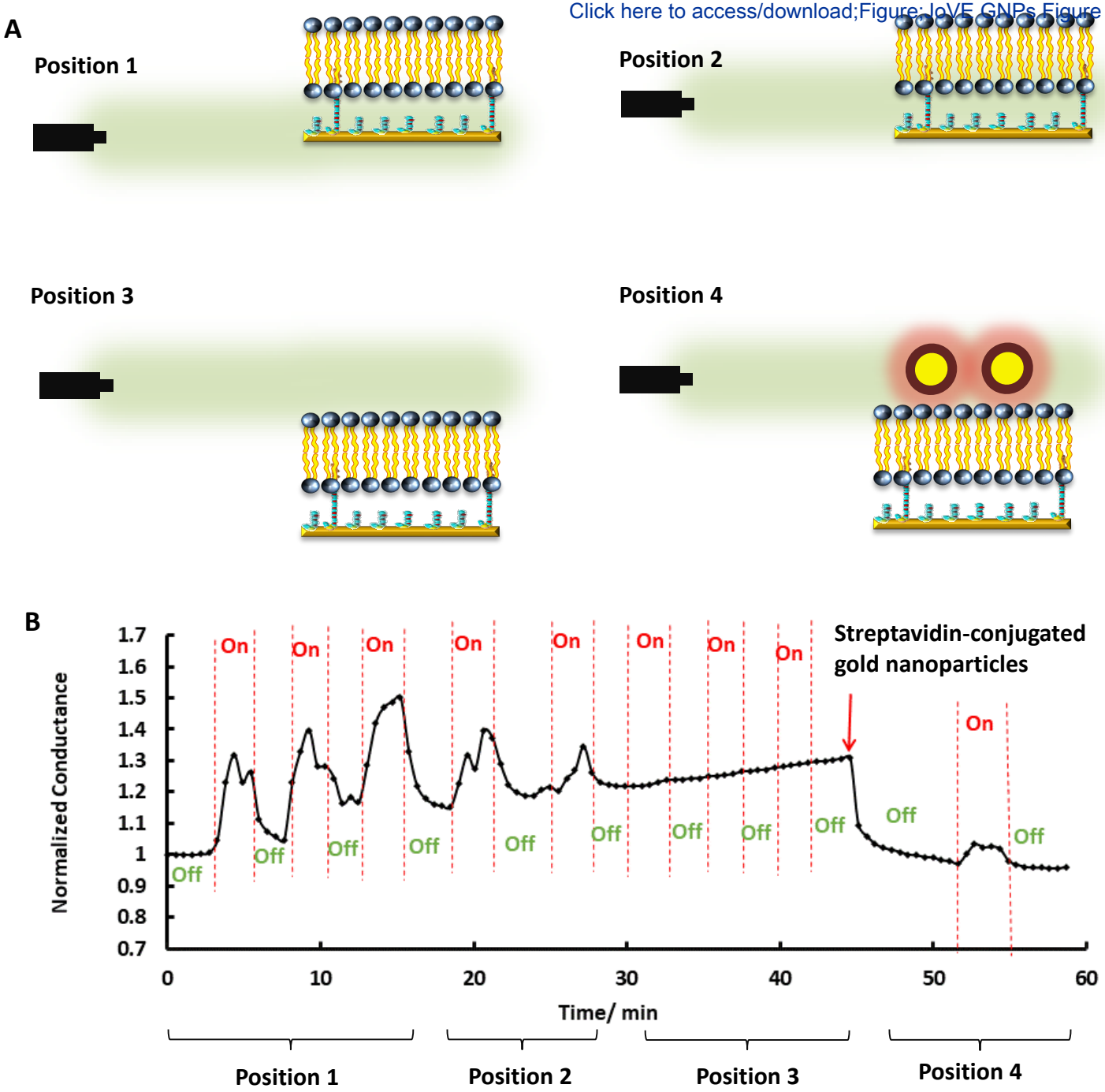


Figure 2 revised



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
30 nm diameter streptavidin-conjugated gold nanoparticles	Cytodiagnostics	AC-30-04-05	This is a streptavidin-conjugated GNPs product ready for use
30 nm diameter bare gold nanoparticles	Sigma-Aldrich	753629	This is a bare GNPs product ready for use
Cholesterol-PEG-Biotin (MW1000)	NANOCS	PG2-BNCS-10k	Dissolved in highly pure ethanol
C20 Diphytanyl-Glycero-Phosphatidylcholine lipids	SDx Tethered Membranes Pty. Ltd.	SDx-S1	1 ml glass vial containing 70% C16 diphytanyl phosphatidylcholine (DPEPC) and 30% C16 diphytanyl glycerol (GDPE) in 99.9% ethanol
Benzyl-disulfide-tetra-ethyleneglyc	SDx Tethered Membranes Pty. Ltd.	SDx-S2	Spacer molecules
Benzyl-disulfide (tetra-ethyleneglycol) n=2 C20-phytanyl	SDx Tethered Membranes Pty. Ltd.	SDx-S2	Tethered molecules
532 nm green laser continuous light	OBIS LS/OBIS CORE LS, China	ND-1000	The power of this laser was ~135 mW
tethaPod EIS reader	SDx Tethered Membranes Pty. Ltd.	SDx-R1	A reader of conductance and capacitance on six channels simultaneously

tethaPlate cartridge assembly	SDx Tethered Membranes Pty. Ltd.	SDx-BG	Materials to attach the slide with electrodes to the flow cell cartridge
Clamp and slide assembly jig	SDx Tethered Membranes Pty. Ltd.	SDx-A1	Materials to attach the slide with electrodes to the flow cell cartridge
Lipid coated coplanar gold electrodes	SDx Tethered Membranes Pty. Ltd.	SDx-T10	Coplanar gold electrodes are made from 25 mm x 75 mm x 1 mm polycarbonate base substrate with patterned gold arrays layout, then coated with benzyldisulphide, bis-tetraethylene glycol C16 phytanyl half membrane spanning tethers in a tether ratio of 10%
tethaQuick software	SDx Tethered Membranes Pty. Ltd.	SDx-B1	Software for use with tethaPod to process data and display conductance, impedance and capacitance measurements from the tethaPlate electrodes
99.9% Pure ethanol	Sigma-Aldrich	34963	Absolute, 99.9%
Phosphate buffered saline (PBS)	Sigma-Aldrich	P4417	pH 7

2nd Nov 2020
Editorial Office, JoVE

Responses to Editorial and Reviewer Comments:

We thank the editorials and reviewers for their constructive critique of our manuscript and detail the changes made regarding editorials' and reviewers' comments below.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
   The manuscript has been revised and corrections have been made.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and Note in the protocol section. Please use Calibri 12 points
   Formating changes have been made as requested.

3. Please ensure summary is within 10-50 word limit.
   The summary now is 48 words.

4. Please ensure the Introduction include all of the following with citation:
   a) A clear statement of the overall goal of this method
   b) The rationale behind the development and/or use of this technique
   c) The advantages over alternative techniques with applicable references to previous studies
   d) A description of the context of the technique in the wider body of literature
   e) Information to help readers to determine whether the method is appropriate for their application

   The Introduction has been revised following the instructions provided.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
   For example: tethaQuick software version; v2.0.56 from SDx Tethered 198 Membranes Pty Ltd, Roseville, Australia, tethaPod AC impedance spectrometer, Tethering solution from SDx Tethered Membranes Pty Ltd, Roseville, Australia, etc.
   All commercial language has been removed from the manuscript.

6. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. Please remain neutral in tone (e.g., limit the use of words like" innovative, novel, simple, fast, easy") when describing the procedure.
   The language has now been changed throughout the manuscript.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
   The manuscript has been revised and corrections have been made.

8. The Protocol should contain only action items that direct the reader to do something.
   The manuscript has been revised and corrections have been made.

9. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
   The manuscript has been revised and corrections have been made.

10. In JoVE Protocol format, only one NOTE can follow one step. "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Other details can be moved to the Discussion.
    The manuscript has been revised and corrections have been made.

11. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
    The manuscript has been revised and corrections have been made.

12. 1.1. Please include details about electrode microscope slide. Please include volume of ethanol used and time for one wash
    The 1.1 has been modified to reflect these comments.

13. 1.2.5: Rationale for using the laminate? How do you ensure this?
    The 1.2.5 has been modified to reflect these comments.

14. Will 1.1 come after 1.2? Please check.
    The steps checked 1.1 first, then 1.2, more illustration added.

15. 1..3.1 To the first well of gold electrode slide? Please use complete sentences and describe complete action throughout. How many wells are present per slide?
    The 1.3.1 section has been modified to reflect these comments.

16. 1.4, 3: Please include how each step is performed. Please include button clicks in the software, knob turns, command lines if any, etc. If using long scripts, please include as supplementary files.
    The 1.4.3 has been modified to reflect these comments.

17. 2.1: How do you set up the system?
    Further elaboration has been included at 2.1.

18. 2.2: At which step do you use the gold electrode slide. Please bring out clarity.
    Further elaboration has been included at 2.2.

19. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
    Pages have been highlighted.

20. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
    The Figures that were used in the manuscript have not been published previously.

21. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
    a) Critical steps within the protocol
    b) Any modifications and troubleshooting of the technique
    c) Any limitations of the technique
    d) The significance with respect to existing methods
    e) Any future applications of the technique
    The Discussion has been revised and corrections have been made.

Reviewers' comments:

Reviewer #1:

1. The abstract should be a clear window over what have been done, general descriptive statements seems not sufficient. It requires to include some details regarding methods and results;
   We agree with the suggestion of the reviewer. More details have now been added to the abstract.

2. Some details regarding buffers constituents and control/calibration procedures are suggested to be added;
   We have now included more details in sections 2.3.2; 2.3.8.

Reviewer #2:

1. Could you please explain the fabrication of gold patterned electrode microscope slide? And how it was proved in the manuscript?
   We thank the reviewer for alerting us to this omission. We have now included details of the gold fabrication in section 1.1.

2. Is there a significant difference in normalized conductance correspond to the different alignment positions (Figure 2B)?
   There are nanostructures and some bulk gold absorption within the sputtered gold that could significantly absorb and contribute to the heating of the membrane, which cause markable variation in the membrane conductance in positions 1 and 2. Achieving the correct laser alignment showed a significant difference between positions 1 and 2 compared to position 3 where there was no variation in membrane conductance. Irradiating GNPs position 4 showed a significant difference in comparison to position 3. Further elaboration has been included in the representative results section.

3. Please make more Discussion about normalized conductance recordings over time correspond to the different alignment positions (Figure 2B).
   More details are added to the Discussion.

Reviewer #3:

1. The work and Introduction will benefit from previous serious simulation of lipid membrane like : Tremi I, Anagnostopoulos D, Spyratou E, et al. Effect of 5-trans Isomer of Arachidonic Acid on Model Liposomal Membranes Studied by a Combined Simulation and Experimental Approach. J Membr Biol. 2018;251(3):475-489. doi:10.1007/s00232-018-0029-8 and review on Gomd nanoparticles and effects on lipids, proteins etc... And also Pharmacol Ther. 2017;178:1-17.

We agree with the assertion of the examiner that the addition of the work of Dimitriou, N. M. et al. would provide more GNPs properties. We have now added this reference accompanied with a further discussion.

2. Both in the title and the abstract of the manuscript authors refer to "heat transfer monitoring", "hyperthermia" and "real time recording of heat pulses", while in the end the only graph shown is that of the conductivity. Perhaps authors should be clearer on the properties that the reader is going to be presented with, because this writing might be misleading, allowing the reader to expect more results on these matters.
   The language has now been changed throughout the manuscript.

3. In the abstract authors claim that: "The technique was validated by swept-frequency electrical impedance spectroscopy and thermal predictive mathematical models". No mathematical models or any short detail on this matter is presented throughout the manuscript. In P. 2, Line 90 it is said that the protocol is based on previous published work in which the authors might have presented more details. However, authors should consider writing a few things about that as it looks like some analysis details are missing.
   We thank the reviewer for alerting us to this omission. We have now added a thermal predictive mathematical model to the manuscript in section 3.

4. P.1, Line 79-81 "The system presented here… and develop thermal therapies.". In this manuscript only one laser source is used and one type of particle. If previous work is done with different parameters it should be also added here.
   We appreciate the reviewer's concerns. Further Discussion has been included to reflect these comments.

5. P.3, Line 127-129: Authors should also add the abbreviation of lipids. Ex. zwitterionic C20 diphytanyl ether-glycero-phosphatidylcholine (DphPC) and C20 diphytanyldiglyceride ether lipids (GDPE). Also, since it is a protocol it could be also written here that lipids are soluble in ethanol and also that ethanol is preferred in polymer substrates (such as polycarbonate base substrate).
   We thank the reviewer for alerting us to this omission. We have now included the abbreviation of lipids in section 1.3.1. Further elaboration has been included in NOTE after section 1.3.1.

6. Does this lipid bilayer composition mimics natural lipid membranes? Cholesterol/lipid ratio is usually 40:60 in biomimetic model membranes. Authors could explain shortly why they used this lipid composition as a lipid bilayer. And also, why they used these nanoparticles. These could also be added in the end of the Introduction. For example 70% DphPC/30% GDPE usually provide optimal impedance seal.
   The tethered bilayer lipid membrane provides close mimic to the natural lipid membrane. In this work, the percentage of cholesterol was %2. Further elaboration has been included in the Discussion.

7. Do authors know the lipid bilayer thickness? Also, do they know the distance between the spacer molecules and the tether molecules?
   The tBLMs model used here contains tethered molecules and the spacer molecule thickness of 11 Å, where the bilayer lipid membrane thickness was around 6.5 nm. We have now added this information to the Discussion to reflect these comments.

8. Fig. 2B: SA-GNPs are probably referred to the streptavidin-conjugated gold nanoparticles. However, the abbreviation should be added in the text.
   We appreciate the reviewer's concerns. Figure 2B has been modified to reflect these comments.

9. Lase source/ wavelength should be written in the section 2 (Laser Irradiation) Writing should be consistent throughout the text. Ex. Tethering molecules are written also tether molecules or spacer molecules are written also spacers. Authors should either write Tether molecules, spacer molecules or tethers spacers (both in the text and figures).
   Section 2 has been modified to reflect these comments. The manuscript has been revised and corrections have been made for writing consistency.

10. Fig. 1B: Authors could also show with arrow the disulfide anchor that ties the membrane to the gold surface.
    We have revised and updated Fig. 1B.

11. P.6, Line 234-237 "The addition of … during the laser ON phase (Figure 2 B, position 4).": Authors here refer to on and off after the addition of streptavidin-conjugated 30 nm gold nanoparticles, but the Bracket in Figure 2B- position 4 only shows the laser on. Bracket should show both on and off in the case of nanoparticles.
    We have revised and corrected Fig. 2B.

12. The protocol reveals changes in membrane conductance and does not offer real time monitoring of heat transfer as its title and abstract state. The authors should reconsider their phrasing as it could be misleading.
    Wording has now been changed to clarify this point raised by the reviewer.

13. The abstract states that the technique was validated by thermal predictive mathematical models. These should be mentioned also in the main text.
    We have now added a thermal predictive mathematical model to the manuscript in Section 3.

14. Why the laser irradiance used is in the nW/µm2 range and not in the mW/µm^2 range, as discussed in p. 1 line 84? How this value was selected and how it was calculated?
    We are thankful for the reviewer for the constructive critique.
    The laser power is given as 135 mW, which would generate nW/µm^2. The laser intensity is calculated by Power/ Area.

15. Working in the micro-scale dimensions requires high precision and therefore an optics table should not be optional, as stated in p. 4 line 59.
    We thank the reviewer for alerting us to this omission. The 2.1.2 section has been modified to reflect these comments.

16. In all the pages, the revision date displayed at the bottom right seems rather old, is this accurate?
    We appreciate the reviewer's concerns; the date has now been updated.

17. What do the authors mean in section 2.3.8.? How many lasers were used and why recording current values before stabilization?
    The 2.3.8 section has been moved to (section 2.1.6) and modified to reflect these comments.

18. The legend of Figure 2 mentions wavelength to be 530 nm and not 532 nm, as presented in the materials table.
    Figure 2 legend has been modified to reflect these comments.

19. Figure 2 mentions SA-GNPs, but the "SA" is not defined.
    Figure 2 has been modified to reflect these comments.

20. The laser description in the materials table should not include irradiance, as this value is calculated in a specific area, probably not relevant to the set-up used.
    The irradiance has been removed from the manuscript.

21. A minor grammar - phrasing check is needed.
    The manuscript has been revised and corrections to grammar - phrasing have been made.