JoVE52712

**Surface Enhanced Raman Spectroscopy Detection of Biomolecules Using EBL Fabricated Nanostructured Substrates**

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Dear Editors,

Thank you for peer reviewing of our manuscript. Please find below our list of changes and responses to the editorial and referee comments. The corresponding changes are introduced in the latest updated version of our manuscript, and highlighted in the reviewing mode.

Sincerely,

The Authors

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**► Response to editorial comments:**

1) All of your previous revisions have been incorporated into the most recent version of the manuscript. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. Please use this updated version for any future revisions. This version has been updated to reflect the recent edits you made to facilitate filming.

Unfortunately, the version contained in the JoVE submission site does not reflect the changes we did in response to the latest editorial request of revision of November, 17th. For this reason we took the liberty of using, instead, our more recent version (the one that we e-mailed to Sephorah Zaman, upon her request, on November, 19th).

2) Prior to peer review, the length of the Short Abstract is exactly at our 50 word limit. If, in response to peer review comments, changes are made to the Short Abstract, please ensure that the final length does not exceed 50 words.

The abstract has not been changed against the latest version of Nov. 19.

3) Prior to peer review, the highlighted portion of your protocol is close to or slightly over our 2.75 page highlighting limit. If, in response to peer review, additional details are added to the protocol, please adjust the highlighting to identify a total of 2.75 pages of protocol text (which includes sub-headings and spaces) that should be visualized to tell the most cohesive story of your protocol steps. The highlighting should include complete statements and not portions of sentences. See JoVE's instructions for authors for more clarification.

The length of the highlighted part has not been changed against the earlier version.

4) The words "Using manufacturer's instructions" were added to step 2.2.2 to indicate that this steps is performed using the instructions that come with the EBL instrument. If applicable, please add similar wording to step 2.2.1.

This change was already requested by editors, and addressed by us, at the previous round of revision (requested on Nov. 17, and addressed on Nov. 19).

5) Please print and sign the attached Author License Agreement, then scan and upload it with your manuscript files.

The electronic system does not allow us uploading the licence agreement. Instead, we e-mailed the signed and scanned agreement to Sephorah Zaman, upon her request, on Nov. 19. We would be happy to e-mail the licence agreement again if so required, or provide it by any different way available.

6) JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

As we indicated at earlier rounds of revision, we did include the DOIs wherever they exist.

**► Response to Reviewer #1 comments:**

The authors report the fabrication of Ag and Au using electron-beam lithography, and the characterization of the material after the previous reviewing process is actually very well presented; the description of the preparation and characterization procedures are satisfactory.

We would like to thank the reviewer for this positive feedback.

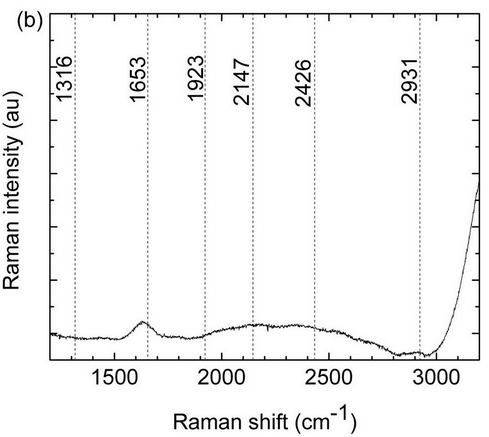
The concern of this referee is related to the SERS spectrum, mostly with the spectrum reported as D-glucose SERS spectrum. It is well known in the literature from the works by Petinger et alii that glucose loses structural integrity when adsorbing to Ag, resulting in hydrogenated carbon on the surface, which presents Raman/SERS spectra very similar to that reported by the authors. Important evidence is the presence of very broad bands covering the entire 1300-1600 cm-1 range, which is characteristic of amorphous carbon forming on the nanoparticle surface; this can be observed in Figures 5 and 10 of the manuscript Review 2.

We appreciate the importance of accurate interpretation of the SERS spectra. However, we strongly feel that confusion occurred in this case. The D-glucose spectra that appear in figure 5 are not SERS spectra. These are control Raman spectra of D-glucose solutions without any metal-containing substrates involved. Since there were no Ag surfaces, the spectra cannot be interpreted as the adsorption of glucose to Ag. We amended the first paragraph in our Results section (lines 339-348) and figure 5 caption to clarify that nanostructured substrates were not used to obtain the control Raman spectra in figure 5.

We would respectfully disagree with the statement by the referee that “very broad bands covering the entire 1300-1600 cm-1 range, which is characteristic of amorphous carbon forming on the nanoparticle surface … can be observed in Figures 5 and 10” based on the set of data that we have presented, and also the literature. The data available to us, as well as extensive literature (e.g. [34,35] and references therein), indicate that these bands (known as amide II bands) represent a superposition of peptide bond vibration modes from the protein immobilized on Ni surface between Ag features on nanostructured substrates, and are indicative of SERS enhancement of these modes in the vicinity of the noble metal nanostructures. Such bands are very weak for the protein in solution in the absence of SERS enhancement; they are absent on Ag pads without Ni surface available for the protein binding, but well pronounced on both nanostructured substrates with Ni surface exposed. In the revised version, we have detailed the lines 428-439 in the Results section to clarify this.

The results in Figure 9 are also striking; there are bands in 2280 and 2577 cm-1 that are characteristic of very specific groups (CN and SH, more specifically), or may be assigned as combination or harmonic bands. The presence of CN is very unlikely in proteins and SH moiety usually when present tend to bind directly to Ag or Au surfaces, with a consequent loss of the H, and no band from the SH is expected on SERS spectra.

We equally appreciate the concern regarding adequate interpretation of the SERS data for protein A in Design 1. We also fully agree that in large molecules such as proteins, assignment of particular bands to specific vibrations is often quite hypothetical, whereas some of the bands may correspond to a superposition of several vibrations. However considering that our Design 1 protein does not bind to any metal directly, but is immobilized on the MUA SAM, we really do not see why some SH bonds should not contribute to the SERS spectra. Nevertheless, we did not make any strong claims regarding these particular vibrations. Instead, we have included a control Raman spectrum of protein A in solution without substrate (figure 5a). This spectrum clearly shows several well-pronounced bands that are confidently attributable to the protein by the nature of the control experiment. Further to this, in Ref. [18] we also reported a SERS spectrum of MUA SAM-functionalized nanostructured substrate without protein A. We are happy to reproduce this SERS spectrum here:



*SERS spectrum of 50 nm pitch Au dots array on FS support, biofunctionalized by MUA SAM without protein A [18]. Vertical lines indicate the position of Raman bands for protein A in solution.*

When in figure 9c we compare the bands of our SERS spectrum from substrate-immobilized protein A with the control Raman spectrum of a solution, positions of the bands exhibit a strong similarity, although with slightly shifted wavelengths. In contrast, the substrate without protein shows an entirely different SERS pattern. This makes us confident that in figure 9, we observe a SERS spectrum of immobilized protein A.

With this in mind and paying attention to the spectral profile, this referee would have to argue that the presented spectra could be related to ripple from the spectrometer monochromator, unless additional experimental evidence is presented. If no experimental evidence clarifying the point raised above are presented, this referee could not recommend this manuscript for publication in the Journal of Visualized Experiments.

We hope that the comparison of our SERS spectrum of substrate-immobilized protein A from figure 9 with the control Raman spectrum of the protein in solution from figure 5a, as well as with the SERS spectrum of the substrate without the protein from Ref. [18], provide a sufficient support of the interpretations that we offer. We added additional clarification to lines 406-408 of the Results in the revised manuscript.

**► Response to Reviewer #2 comments:**

Summary: The manuscript demonstrates successfully the ability to use electron beam lithography to fabricate nanostructured arrays to produce high quality SERS.

We thank the reviewer for this comment.

Minor comments: 1. It seems based on illustration in figure 3 (design 2) that all immobilized biopolymers have ligands, but in text the ligands could be present or not at all. So it would be helpful in understanding the tests to include some [immobilized] biopolymers in figure 3 (design 2) that do not have a ligand bonded to it.

We appreciate the comment, and modified the caption to Figure 3 making it clearer that a system without ligand was also prepared for comparison.

2. Line 58: The author should reference the particular dicing saw that they used.

In Table of Equipment, we included an additional line specifying the dicing saw.

3. Line 61: Cleaning 1x1 cm dies in piranha etch would require special mounts/holders or mounting the dies onto to larger substrates so they can be safely handled while undergoing piranha etch. Nothing of that sort is mentioned. Since this is a methods article the authors should include minor steps that are important for the safety of somebody who would like to replicate their work.

We thank the referee for this suggestion, and included in step 1.2 new reference [29] of the pertinent safe operation protocol.

4. Line 78: Please also mention an approximate size of the droplet of PMMA. Since without that information the size of the drop depends the bore size of the dropper (which

can easily vary from one lab to another).

We agree that the size of PMMA droplet may somewhat vary across different labs. However in this case, critical for the outcome are the conditions of spinning and not the drop size. We feel that specifying size of the drop might restrict the users without necessity and drive the attention away from more crucial conditions, such as the speed and duration of spinning. As to the size of the pipette, the accompanying video would give an idea of what we have used to those who are interested.

5. Line 80, 86 and 87: What is the value of acceleration that they used for spinning? In other words the 'ramp value'.

In all spinning conditions, we used a 2 sec ramp time. To avoid confusion, we matched the wording in steps 2.1.1 and 2.1.3 to “2 sec ramp time”.

Major comments: 1. Electromagnetic enhancement of Raman scattering depends on size, shaping and spacing of metal nanostructures. Based on this fact, the authors chose EBL to make these nanostructures. However, based on figures 6 and figures 8 there is still some level non-uniformity (in nanometers) between the nanostructures. But perhaps only a certain level of uniformity needs to be achieved in this application? If this is so, then the authors should mention the resolution (± nm) they need from a particular nanofabrication process.

Every fabrication process involves some limitations of the resolution and level of uniformity, and so does EBL, as our figures 6 and 8 illustrate. How much uniformity is required for SERS bio-detection in general is a different and significantly more complex question. Determining these requirements for specific cases is still in the pipeline through the community. In this work, we describe how to use EBL (that provides an ultimate position control) to fabricate SERS substrates, and demonstrate that the control of inter-feature separations at the level of 10-20 nm or less might be required (see lines 604-618), although these requirements may depend on the application (lines 620-650). In the revised version, we amended our discussion (lines 667-669) to clarify that this technique allows the community to better control the size and position of nanostructures, thereby facilitating the identification of optimal substrate designs that may depend on the application.

2. Furthermore, the authors should provide more rationale for their array design. One thing that is not clear is that why do they start off with 25nm spacing between nanostructures in all three designs. Is that value based on some previous works?

Based on our previous works [16-20] periodic arrays of metal dots with a pitch of approximately 50 nm and inter-dot distance of roughly half the pitch or less, provide a reasonable initial trade-off between the ease of EBL fabrication and the functionality of the substrates for bio-detection. For patterns other than dots, the same inter-feature distance was chosen for consistency.

3. There is a lack of discussion of published literature that can be used instead of EBL to produce nanostructures. For example there are a variety of other methods to produce nanostructures for quality SERS (see Cao et al. 2013 Engineering Metal Nanostructure for SERS Application). It is important that the authors highlight/elaborate why they used EBL for their application in the light of these other methods of producing nanostructures for SERS.

We appreciate the request of clarifying the motivation of using EBL, and extended our discussion of the fabrication methods in lines 108-120. We also added the review by Cao et al. to our literature list.

**► Response to Reviewer #3 comments:**

This valuable article presents the fabrication and the characterization of biofunctionalized nanostructured surfaces involving aptamers or proteins. The figures are appropriate displayed the work and well referenced.

We appreciate this positive evaluation of our work by the referee.

Major concern:Row 201 "keV electron beam voltage" should change to "keV electron beam energy"

We changed the line in step 2.2.3 as requested.

2)Row161 please specify [the] concentrations of the H2SO4 and H2O2

The 1:3 proportion of H2S04 to H2O2 is indicated in the brackets right after the formula.

3)Row 182 "Bake the substrates ...for 3-5 min to" should change to"Bake the substrates ...for 3-5 min."

The typo in this line (step 2.1.2) was fixed over the previous round of revision.

4)For a better understanding of the figure 9, the Raman intensity scale should be placed vertical at the right side of the panel.

We entirely agree that the figures must be as self-explanatory as possible, and very much appreciate all advice toward this. However, the vertical axis in figure 9(b) has the meaning of physical coordinate (distance across the substrate), and the addition of similarly oriented Raman intensity legend bar leads to a confusion with more conventional Raman spectra. To avoid this confusion, we would prefer leaving the legend bar oriented horizontally. However in response to the comment, we amended the figure caption for more clarity.

**► In addition to the above, the following amendments have been also done:**

1. In step 4.1.5, “two-compartment Petri dish” was replaced with “multi-compartment Petri dish” since more common 4-compartment Petri dish was used for filming.
2. Typo was corrected in step 4.3.6 (“DBA” replaced with “PBS”).
3. In captions to figures 5a, 8, and 9 which were published earlier in Ref. [18], the copyright and permission notice is added. The permission for reproduction has been obtained from the publisher and can be provided to JoVE if so required.
4. In Acknowledgements, the names of team members who helped to prepare samples for filming were added.