Journal of Visualized Experiments

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

--Manuscript Draft--

Manuscript Number:	JoVE52712R3
Full Title:	Surface Enhanced Raman Spectroscopy Detection of Biomolecules Using EBL Fabricated Nanostructured Substrates
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Bio-functionalized surfaces; Proteins; aptamers; molecular recognition; nanostructures; electron beam lithography; surface-enhanced Raman spectroscopy.
Manuscript Classifications:	10.1.637.512: Nanostructures; 4.12: Amino Acids, Peptides, and Proteins; 4.13.695.578.424.224: Aptamers, Nucleotide; 5.5.196.822.860: Spectrum Analysis, Raman; 5.5.472: Immobilization; 5.5.601.123: Biosensing Techniques; 7.2.842: Physicochemical Phenomena; 93.33.44: lithography (circuit fabrication)
Corresponding Author:	Maria Stepanova University of Alberta Edmonton, Alberta CANADA
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	ms1@ualberta.ca
Corresponding Author's Institution:	University of Alberta
Corresponding Author's Secondary Institution:	
First Author:	Robert F. Peters
First Author Secondary Information:	
Other Authors:	Robert F. Peters
	Luis Gutierrez-Rivera
	Steven K. Dew
Order of Authors Secondary Information:	
Abstract:	Fabrication and characterization of conjugate nano-biological systems interfacing metallic nanostructures on solid supports with immobilized biomolecules is reported. The entire sequence of relevant experimental steps is described, involving the fabrication of nanostructured substrates using electron beam lithography, immobilization of biomolecules on the substrates, and their characterization utilising surface-enhanced Raman spectroscopy (SERS). Three different designs of nano-biological systems are employed, including protein A, glucose binding protein, and a dopamine binding DNA aptamer. In the latter two cases, the binding of respective ligands, D-glucose and dopamine, is also included. The three kinds of biomolecules are immobilized on nanostructured substrates by different methods, and the results of SERS imaging are reported. The capabilities of SERS to detect vibrational modes from surface-immobilized proteins, as well as to capture the protein-ligand and aptamer-ligand binding are demonstrated. The results also illustrate the influence of the surface nanostructure geometry, biomolecules immobilization strategy, Raman activity of the molecules and presence or absence of the ligand binding on the SERS spectra acquired
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a	

certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	
If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter.	Dec 24, 2014

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

AUTHORS:
Robert F. Peters
Department of Electrical and Computer Engineering
University of Alberta
National Institute for Nanotechnology
National Research Council of Canada
Edmonton, AB
rfpeters@ualberta.ca

Luis Gutierrez-Rivera
Department of Electrical and Computer Engineering
University of Alberta
National Institute for Nanotechnology
National Research Council of Canada
Edmonton, AB
legutier@ualberta.ca

Steven K. Dew
Department of Electrical and Computer Engineering
University of Alberta
National Institute for Nanotechnology
National Research Council of Canada
Edmonton, AB
steven.dew@ualberta.ca

Maria Stepanova
Department of Electrical and Computer Engineering
University of Alberta
National Institute for Nanotechnology
National Research Council of Canada
Edmonton, AB
ms1@ualberta.ca

CORRESPONDING AUTHOR: Maria Stepanova

KEYWORDS:
Bio-functionalized surfaces, proteins, aptamers, molecular recognition, nanostructures, electron beam lithography, surface-enhanced Raman spectroscopy.

SHORT ABSTRACT:
We describe the fabrication and characterization of nano-biological systems interfacing nanostructured substrates with immobilized proteins and aptamers. The relevant experimental steps involving lithographic fabrication of nanostructured substrates, bio-functionalization, and surface-enhanced Raman spectroscopy (SERS) characterization, are reported. SERS detection of surface-immobilized proteins, and probing of protein-ligand and aptamer-ligand binding is demonstrated.

LONG ABSTRACT:

Fabrication and characterization of conjugate nano-biological systems interfacing metallic nanostructures on solid supports with immobilized biomolecules is reported. The entire sequence of relevant experimental steps is described, involving the fabrication of nanostructured substrates using electron beam lithography, immobilization of biomolecules on the substrates, and their characterization utilising surface-enhanced Raman spectroscopy (SERS). Three different designs of nano-biological systems are employed, including protein A, glucose binding protein, and a dopamine binding DNA aptamer. In the latter two cases, the binding of respective ligands, D-glucose and dopamine, is also included. The three kinds of biomolecules are immobilized on nanostructured substrates by different methods, and the results of SERS imaging are reported. The capabilities of SERS to detect vibrational modes from surface-immobilized proteins, as well as to capture the protein-ligand and aptamer-ligand binding are demonstrated. The results also illustrate the influence of the surface nanostructure geometry, biomolecules immobilization strategy, Raman activity of the molecules and presence or absence of the ligand binding on the SERS spectra acquired.

INTRODUCTION:

Capabilities to develop and characterize conjugate nano-biological systems interfacing solid nanostructures and biological polymers are becoming increasingly important to further advances in next-generation bio-sensing and bio-actuation technologies[1,2]. This involves multi-disciplinary studies across a number of research fields, such as the fabrication of pertinent solid-state components (micro-or nano-electrodes, nano-engineered coatings, nanowires, or nanoparticles)[2,3,4]; immobilization of biomolecules on the surfaces to create desired bioconjugates[5,6,7]; and monitoring nano-biological interfaces[1]. In most cases, the selection of optimal fabrication, bio-functionalization, and characterization methods is strongly inter-related. Clearly, the choice of nanofabrication techniques would be driven by the requirements of the solid state components of the system, being largely dependent on the detection method, which in turn is determined by the nature of the biopolymers involved and the purpose of monitoring the interface.

Out of a broad variety of techniques applied to characterize bioconjugate systems[1,3], surface enhanced Raman spectroscopy (SERS) has emerged as a highly promising method for the detection of chemical and biological species on surfaces[8,9,10,11]. SERS employs inelastic scattering of monochromatic light by surface-immobilized biomolecules (Figure 1) allowing the capture of unique signatures corresponding to molecular vibrations. This capability to distinguish among different molecules without involving labels, complex chemistry, or time-consuming steps, makes SERS a potentially very efficient method of bio-detection. Another important advantage of SERS is its high sensitivity. The excitation of localised surface plasmons by light interacting with noble metal nanostructures (SERS substrates) increases dramatically the intensity of Raman scattering by the analyte, allowing the detection of very small amounts of molecules, from monolayers down to the single-molecule limit[8,9,10,11]. Finally, most biomolecules require aqueous solutions to be stable. Because water often has limited Raman activity, background signal from aqueous samples is minimized[9]. Applications of SERS have exhibited an exponential increase over the last decade[10]. However, a much discussed challenge of SERS is that the

electromagnetic enhancement of Raman scattering depends critically on the size, shape, and spacing of metal nanostructures where plasmonic waves are induced[11,12,13]. In order to achieve efficient and reproducible SERS measurements, control over the substrate geometry is required at the nanoscale dimensions.

[Place Figure 1 here]

Numerous methods employed to fabricate SERS substrates[11,12,13] can be roughly classified into bottom-up and top-down methods. Methods of the first type employ various processes of self-assembly or directed chemical synthesis to produce nanostructures. Often addressed examples include immobilization of monodisperse nanoparticles on solid supports[11,12,13], thermal, sputter, or electrochemical deposition of roughened metal films[11,12], and various chemical synthesis methods[13]. Although such techniques tend to be relatively simple and inexpensive, most of them are challenged by a lack of control over the location of the structures, and limited sample-to-sample reproducibility.

In contrast, top-down lithography techniques employ manipulable instruments such as particle beams to create desired patterns on surfaces. One of the most often used nanolithography methods, electron beam lithography (EBL), offers superb control over features down to below 10 nm and also a flexibility to allow for different substrate designs on solid supports[11,12]. In EBL, a beam of electrons focused down to a spot of a few nanometers in diameter scans across a surface of an electron sensitive material (resist) causing a chemical change in exposed regions. For positive tone resists such as polymethylmethacrylate (PMMA), electron beam exposure results in scission of the polymer chains composing the resist, leading to an increased solubility in an appropriate solvent (developer). The process of electron-beam lithography includes spin-coating of a uniform layer of resist on a substrate; exposure of the targeted resist material in a vacuum chamber with an electron beam; and development of the sample to remove the soluble regions.

Dielectric supports underneath metallic nanostructures, such as fused silica, have been shown to significantly increase the intensities in SERS due to localization of plasmonic waves compared to other materials such as silicon[14,15]. However EBL patterning on dielectric substrates, especially at the nanoscale, involves significant challenges due to charge build-up during exposure. Previously, we have shown[16,17] that these difficulties can be overcome by placing conductive polymer layers above the resist. Figure 2 shows a schematic of the overall fabrication process using EBL exposure and development followed by metal deposition and liftoff to produce metallic nanostructures on fused silica supports.

[Place Figure 2 here]

In this paper, we present the entire sequence of process steps involving SERS substrates fabrication by EBL, bio-functionalization of the substrates, and collection of the Raman spectra. Three designs explored in our recent works[18,19,20] are addressed (see Figures 3 and 4, and Table 1). In Design 1, recombinant protein A is immobilized on bio-functionalized Au nanostructures on a fused silica (FS) support[18], and SERS detection of the protein is demonstrated. In Design 2, recombinant glucose-binding protein[22,27,28] with and without the ligand (D-glucose) is immobilized

by means of histidine tags in spaces between Ag nanostructures on Ni-coated FS[19], and the binding of glucose to the protein is detected. In Design 3, thiolated dopamine-binding DNA aptamer[20,24] is immobilized on Au nanostructures on FS, and the binding of dopamine by immobilized aptamer is demonstrated. Inclusive of all relevant experimental steps from substrate preparation to Raman spectra acquisition, and representative of different biomolecules and strategies of immobilization, these examples are useful for a broad variety of applications, from exploration research interrogating nano-biological interfaces by SERS to the development of SERS biosensors of small molecules employing protein- or aptamer-ligand binding as a recognition method.

[Place Figure 3 here]
[Place Figure 4 here]
[Place Table 1 here]

PROTOCOL:

1. Substrate Preparation

1.1) Use a semiconductor dicing saw to cut a fused silica (FS) wafer into 1 cm × 1 cm or smaller dice.

1.2) Clean samples in a piranha solution (H_2SO_4:H_2O_2, 3:1, 'CAUTION strong oxidizer') bath[29] for 15 min, then rinse the dice in deionized water and dry in nitrogen.

1.3) Place the samples on a hotplate face up at 180 °C for 15 min. Cool the samples after removing from the hotplate to room temperature.

1.4) For Designs 1 and 3, proceed to step 2.

1.5) For Design 2, place the sample in an electron beam evaporation chamber and coat with a 10 nm layer of Ni.

2. Fabrication of Nano-Patterned PMMA Masks Using Electron Beam Lithography (EBL)

2.1) Spin-coat the PMMA resist and conductive layers on the substrates.

2.1.1) Use a wafer spinner with a vacuum chuck and place samples individually in the centre on the chuck. Place 1 drop of polymethylmethacrylate (PMMA) resist on the centre of the samples using a glass pipette and spin at 3500 RPM for 60 sec with a 2 sec ramp time.

2.1.2) Bake the substrates at 180 °C for 3-5 min. After baking the substrates, cool the samples to room temperature.

2.1.3) With the substrates cooled and returned to the spinner chuck, spread a drop of conductive polymer on the substrate. Spin the substrate for 40 sec at 3000 RPM with a 2 sec ramp time. Bake samples at 80°C for 1 min.

2.2) Perform EBL exposure according to the standard procedure[16,17,18,19,30].

2.2.1) Using manufacturer's instructions, prepare an exposure design employing feature doses from Table 1 with the smallest possible beam step size.

2.2.2) Load the sample into the electron beam lithography chamber. If the EBL system does not have autofocusing, use a small scratch away from where the pattern is to be exposed and away from the bead edge for focusing.

2.2.3) Using manufacturer's instructions, perform the required focusing and astigmatism correction as well as write field alignment as appropriate, and expose the sample. To allow for proper exposure profile and best pattern quality, use a 30 keV electron beam energy and 7.5 µm aperture for the exposures.

2.3) Remove the conductive polymer and develop exposed samples.

2.3.1) Prepare a beaker with deionised (DI) water for removing the conductive polymer and a second beaker with a developer mixture (IPA:H_2O, 7:3), and stir for 5 min at room temperature. Prepare isopropanol (high purity) in a third beaker as a rinse agent.

2.3.2) Using tweezers, place the samples into the water for 3 sec to remove the conductive polymer film, then place the sample into the developer and move the tweezers slowly up and down for 20 sec. Immediately transfer the substrates to the isopropanol and rinse for 10 more seconds, then dry the sample with nitrogen.

3. Noble Metal Nanostructures Fabrication

3.1) Load the samples upside down into the electron beam evaporator system to allow for the evaporated metal to be deposited on the front face of the samples. Deposit a 10 nm thick Au layer onto the samples for Designs 1 and 3, and a 10 nm thick Ag layer for Design 2 at a rate of approximately 0.1 nm/sec.

3.2) Fill a sonication system to the recommended height with water and fill a separate beaker with acetone. Place a sample face up in the bottom of the beaker and allow the sample to soak for 10 min. Holding the beaker, place it into the water bath and allow the height of the acetone to match the height of the water and turn on the sonication system. Allow sonication to occur for up to 60 sec.

3.3) Using the same procedure as detailed in step 3.1, prepare uniform Au and Ag pad substrates by deposition of 10 nm thick metal films on FS (Designs 1 and 3) and Ni-coated FS (Design 2) substrates skipping step 2.

4. Bio-Functionalization of Substrates

4.1) Prepare Design 1 samples:

4.1.1) Prepare a 1 mM solution of 11-mercaptodecanoic acid (MUA) in ethanol at room temperature. Sonicate for 10 min.

4.1.2) Immerse proper nanostructured substrate in the solution of MUA for 48 h. Rinse the sample with ethanol three times and dry for 5 min at room temperature.

4.1.3) Prepare a 75 mM solution of N-ethyl-N'-(3-(dimethylamino) propyl) carbodiimide (EDC) in DI water. Prepare a 15 mM solution of N-hydroxysuccinimide (NHS) in DI water.

4.1.4) Using a micropipette deposit 100 µl of NHS on the Au on the substrate, and immediately add 100 µl of EDC on the same area. Incubate for 1 min to activate the self-assembled monolayer (SAM) of MUA.

4.1.5) Place a 100 µl drop of protein A solution (47 µM) on the same area of the substrate and store the sample for 24 h at 5°C in a multi-compartment Petri dish with 1 ml of DI water in another compartment, and with a sealed cover.

4.1.6) Rinse the sample in DI water 3 times by continuously stirring the samples in separate beakers for 20 sec in each beaker. Do not let the samples dry after rinsing or during the rinse.

4.1.7) Proceed to step 5.

4.2) Prepare Design 2 samples:

4.2.1) Prepare a 0.9 mM solution of glucose binding protein (GBP) in potassium phosphate buffer (K_2HPO_4, 25 mM, pH 7.5). Prepare a 100 mM solution of D-glucose in the buffer.

4.2.2) Mix 30 µl of 100 mM D-glucose solution and 30 µl of 0.9 mM GBP solution using a 1 ml plastic microtube container and a micropipette. Incubate for 30 min.

4.2.3) Deposit 20 µl of the ligand-free GBP solution and of the ligand-bound GBP solution each on the prepared substrates using a micropipette. Store the samples at 5°C for 24 h in a Petri dish with a sealed cover.

4.2.4) Rinse the samples 3 times in the potassium phosphate buffer solution at room temperature.

4.2.5) Proceed to step 5.

4.3) Prepare Design 3 samples:

4.3.1) Dilute the dopamine binding aptamer (DBA) solution to a concentration of 1 µM using the TRIS EDTA buffer with a final pH of 7.4.

4.3.2) Prepare a dopamine solution to a 5 µM concentration by measuring the dopamine powder on an analytical balance and mixing it in the phosphate buffered saline (PBS) with a stir bead for 5 min.

4.3.3) Deposit a 20 µl drop of the DBA solution on the surface of each substrate and let the samples sit for 1 h at room temperature with a cover over the Petri dish.

4.3.4) Rinse the samples 3 times in a potassium phosphate buffer (K_2HPO_4, 25 mM, pH 7.5).

4.3.5) Place the samples upright on top of a cleanroom grade wipe to dry the backside while maintaining a film on the front side of the substrate. Set a sample aside as a control.

4.3.6) Place a 5 µl drop of the dopamine solution on the surface of the existing drop of the PBS buffer solution on the remaining samples with the desired dopamine concentrations. Incubate the sample for 10 min.

4.3.7) Place a drop of the dopamine solution on the Au pad surface without aptamer. Incubate for 10 min.

4.3.8) Rinse the samples in the potassium phosphate buffer solution 3 times.

5. Raman Spectroscopy

5.1) Place each sample in a water-proof chamber to avoid evaporation by laser exposure.

5.1.1) Fill a plastic syringe with chemically inert high vacuum grease, place samples on glass slides and dispense a few millimeters of grease surrounding the samples without touching the samples.

5.1.2) Place a microscope coverslip on top of the substrates and gently press down to form a seal, creating a thin liquid interface between the substrates and the coverslips without allowing the buffer to come in contact with the vacuum grease.

5.2) Using an optical Raman microscope system, obtain a focus on the surface of the metal nano-patterned region to be sampled without turning on the laser.

5.3) Perform Raman sampling[18,20] with a laser intensity between 2.5 – 2.7 mW with a total duration of less than 20 seconds to prevent damage to the sample with an objective of 10X magnification. Acquire Raman spectra for samples from Designs 1, 2, and 3 using the excitation wavelengths as indicated in Table 1. Also acquire control Raman spectra for solutions of protein A, GBP, and D-glucose, and for dopamine powder employing glass slides without metal nanostructures as supports for comparison.

REPRESENTATIVE RESULTS:

Collecting control Raman spectra for the main components, including free proteins in solution and free ligands in solution or in powder form without using metal-containing substrates, is important to enable a proper comparison as well as for interpretation purposes. Figure 5a presents a typical Raman spectrum for free protein A in DI water on a glass slide without nanostructured substrates. Two bands with the highest Raman intensity, the band at 2931 cm^{-1} and at 1091 cm^{-1}, correspond to vibrations involving C-H and C-S bonds, respectively. Other bands with a lower Raman

intensity such as 563 cm^{-1}, 1450 cm^{-1}, 1653 cm^{-1} and 2426 cm^{-1}, can be attributed to a superposition of vibration modes[18,33,34,35]. Control Raman spectra for the ligand free GPB in buffer solution with three different concentrations, 0.3, 0.9 and 1.3 mM, are shown in Figure 5b. In the figure, the broad band around 3400 cm^{-1} corresponds to the solvent[36], whereas the band at 2935 cm^{-1} represents vibrations involving C-H bonds of the protein[33,34]. Figure 5c shows the high wavelength Raman spectrum for D-glucose in buffer solution for different concentrations: 1, 6, 100, 200, and 400 mM. When the concentration of glucose is increased, C-H bonds vibration bands arise at 2890 cm^{-1} and 2960 cm^{-1} [19]. Control Raman spectra of dopamine in crystal form obtained with both 532 nm and 780 nm excitation wavelengths are shown in Figure 5d. Much of the Raman spectrum comes from the benzene ring bending and C-H bond stretches of the molecule[20,37]. Some of the bands at around 3000 cm^{-1} are only observed at the 532 nm but not 780 nm excitation wavelength.

[Place Figure 5 here]

In order to obtain SERS spectra for surface-immobilized biomolecules, substrates comprising metallic nanostructures on fused silica supports are fabricated as described in steps 1-3. The quality of fabricated substrates is monitored using scanning electron microscopy (SEM). The standard SEM procedures are described elsewhere[16,17,18,19,20] and not included in the present protocol. Figure 6 shows representative SEM images of Au and Ag nano-dots and nano-hexagon like structures (panels a-d), as well as non-structured Au and Ag pads (panels e and f, respectively). The next steps involve immobilization of biological material on the nano-structured substrates employing three designs listed in Table 1, and acquisition of their SERS spectra. In order to maintain an aqueous environment during Raman imaging, each sample is placed in its corresponding solution (see Table 1), capped with a thin glass cover, and sealed as illustrated in Figure 7.

[Place Figure 6 here]
[Place Figure 7 here]

In Design 1, recombinant protein A is immobilized on the substrates functionalized by a self-assembled monolayer of 11-mercaptodecanoic acid (MUA) in DI water[18]. The substrates in this design comprise three arrays of Au dots with a 50 nm pitch and varying inter-dot distances on fused silica (see Figures 6a and 8). The process of protein immobilization starts with the formation of a SAM on the substrates. To obtain covalent binding between the SAM and the protein, the carboxylic acid groups of SAMs are transformed into amine reactive NHS-ester by treatment with a mixture of N-ethyl-N'-(3-(dimethylamino) propyl) carbodiimide (EDC) solution and N-hydroxysuccinimide (NHS) solution in DI water. Immobilization of protein A occurs by displacement of the NHS group by lysine residues of the protein[38]. An example of imaging samples for Design 1 with protein A immobilized on Au nanostructures is shown in Figure 9. Figure 9a presents an optical microscope image of the samples, which comprise three arrays of bio-functionalized Au nanodots with different inter-dot gaps (see also Figures 3a and 8), and Figure 9b shows the Raman spectral mapping over these arrays. It can be seen that the highest Raman intensities are found for Array I where the inter-dot gaps are the narrowest, whereas lower intensities are obtained for Array III with the widest inter-dot gaps. This can be explained by a stronger plasmon coupling effect produced by higher electric fields in the narrow spaces between the dots[18]. Figure 9c shows the strongest SERS spectra obtained

for Arrays I and II. The spectra exhibit several bands (1630 cm^{-1}, 1964 cm^{-1}, 2280 cm^{-1}, 2577 cm^{-1}, and 2916 cm^{-1}) in proximity to the Raman modes of free protein A in solution seen in Figure 5a. Attributable to vibrations of various bonds found in proteins, these bands either appear at similar locations in both immobilized protein and in solution, or are slightly shifted to somewhat higher wavenumbers when immobilized. In contrast, SERS spectra of similar nanostructured substrates functionalized by MUA SAM without the protein show an entirely different pattern,[18] confirming that figure 9 represents SERS mapping of surface-immobilized protein A.

[Place Figure 8 here]
[Place Figure 9 here]

In Design 2, recombinant glucose binding protein (GBP)[22] complexed with D-glucose (ligand) is immobilized on the appropriate substrates in potassium phosphate buffer solution. Samples with immobilized ligand-free GBP are also prepared for comparison[19]. In this design, glucose-binding protein is attached to surface by means of a histidine tag, which binds well to Ni but not to noble metals[6]. Since the substrates comprise arrays of Ag nano-dots, nano-hexagons, and unstructured Ag pads on Ni-coated FS (Figures 6b, 6d, and 6f, respectively), one can expect most of immobilized protein molecules to be located in gaps between Ag nanostructures where Ni coating is available. The Raman spectra obtained for immobilized glucose-free and glucose-bound GBP are shown in Figures 10a and 10b, respectively. All these spectra exhibit a broad band at approximately 3300 cm^{-1}, which corresponds to the buffer solution[36]. The spectra obtained with an unstructured Ag pad contain only this single band and do not show any protein vibration modes, confirming that immobilized protein is not found on the Ag surface as expected. In contrast, the spectra obtained with arrays of Ag nano-dots and nano-hexagons exhibit bands around 1550 cm^{-1} and 2900 cm^{-1}, which represent the analyte[33,34]. In particular, the broad band around 1550 cm^{-1}, known as the amide II band, is attributable to peptide bonds vibrations in proteins[34,35]. In the case considered, this band represents a superposition of the vibration modes from GBP immobilized on Ni surface between Ag features, and is indicative of SERS enhancement of these modes in the vicinity of noble metal nanostructures when the substrates containing nano-dots or nano-hexagons are used. This band is very weak for the protein in solution in the absence of SERS enhancement (Figure 5b) and absent on Ag pads without Ni surface available for the protein binding, but it is well pronounced for nanostructured substrates with some Ni surface accessible for the protein to bind. However, even more important for the present study are the other, narrower bands around approximately 2900 cm^{-1} that can be attributed to C-H bond wibrations[33,34]. The spectrum of glucose-free GBP shows a pronounced band at 2933 cm^{-1} with the nano-dots substrate, and a weak but discernible band at a similar wavelength with the nano-hexagons substrate (Figure 10a). Distinct from the case of glucose free protein, the SERS spectra of glucose-bound GBP shown in Figure 10b exhibit two bands corresponding to C-H bonds vibrations regimes, at 2850 cm^{-1} and 2910 cm^{-1}. The bands are well pronounced in the spectrum of glucose-bound GBP on nano-hexagons substrate, and they also can be seen in the spectrum of GBP on nano-dots substrate. The band at 2850 cm^{-1} is reasonably close to the 2890 cm^{-1} one in the control Raman spectrum from D-glucose in solution, and therefore it can be attributed to glucose bound to the protein, whereas the other band (at 2910 cm^{-1}) is attributable to C-H bond vibrations of both the protein and glucose. One can

conclude that difference of SERS signatures from glucose-free and glucose-bound substrate-immobilized GBP is observable in this region, and C-H bond vibrations of protein-bound glucose are detectable employing the design described.

[Place Figure 10 here]

In Design 3, the customized dopamine binding aptamer (DBA) with thiol termination[24] is immobilized on the substrate in tris(hydroxymethyl)aminomethane (TRIS) ethylenediaminetetraacetic acid (EDTA) buffer solution, and the dopamine is then bound to the immobilised aptamer[20]. The substrates for this design contain arrays of Au nano-hexagons on FS (Figure 6c). Unstructured Au pads (Figure 6e) are also used for control purposes. Since DNA is intrinsically fluorescent[39], 780 nm excitation wavelength is used in Design 3 to reduce this factor. In this design, the recognition element (aptamer) is not Raman active in the region of Raman shifts considered in Figure 11, whereas dopamine shows a significant Raman activity in this region. Since signal from samples exposed to only dopamine without immobilized aptamer shows no resultant dopamine bands[20], the observed SERS bands are expected to originate from aptamer-bound dopamine. Figure 11 compares the SERS spectra of immobilized aptamer on gold nanostructures before and after the addition of dopamine. As expected, immobilized dopamine-free aptamer does not exhibit Raman bands. In contrast, a number of pronounced Raman bands are observed for dopamine-bound immobilized aptamer. The positions of most bands in Figure 11 are close to those in crystalline dopamine, albeit with differences in amplitudes.

[Place Figure 11 here]

Figure Legends:

Figure 1:
Scheme of surface-enhanced Raman spectroscopy.

Figure 2:
Scheme of electron beam lithography, metal deposition, and liftoff process steps employed to fabricate metallic nanostructures on dielectric substrates[16-20].

Figure 3:
Schemes of three representative designs using different biomolecules, methods of immobilization, and substrate materials: (a) - protein A immobilized on the noble metal nano-dots functionalized by a self-assembled monolayer (SAM) of 11-mercaptodecanoic acid (MUA) in DI water; (b) – histidine-tagged glucose binding protein (GBP) complexed with D-glucose immobilized on the substrate surface between noble metal nano-dots; (c) – thiol-terminated dopamine binding aptamer completed with dopamine (DBA) immobilized on noble metal nano-dots. See further details in Table 1. In Design 2 illustrated by panel (b), a sample without the corresponding ligand was also prepared for comparison.

Figure 4:
Biomolecules employed in three designs: (a) – protein A; (b) – glucose binding protein and D-glucose; (c) – dopamine binding DNA aptamer and dopamine. The protein tertiary structures in (a) and (b) are taken from Protein Data Bank, PDB ID

1BDD[21] and 2HPH[22], respectively, and drawn with VMD for LINUXAMD64, version 1.9.1[23]. The aptamer secondary structure in (c) is predicted from the sequence[24] using ValFold[25] software and drawn with PseudoViewer 3.0[26]. The letters G, A, T, and C correspond to guanine, adenine, thymine, and cytosine nucleotides, respectively.

Figure 5:
Control Raman spectrum of protein A in DI water obtained at 532 nm excitation wavelength[18](a); Raman spectra of ligand-free glucose binding protein in buffer solution obtained at 532 nm excitation wavelength[19] (b); Raman spectra of D-glucose in buffer solution obtained at 532 nm excitation wavelength[19] (c); and spectra of dopamine powder obtained at 532 nm and 780 nm excitation wavelengths[20] (d). All the spectra are regular Raman spectra of solutions (a,b,c) and powder (d) on glass slides without nanostructured substrates. In (d), the assignment of Raman shift regimes to various molecular vibrations was done using General Atomic and Molecular Electronic Structure System (GAMESS)[31] and MacMolPlt[32] software as detailed elsewhere[20]. Reprinted panel (a) with permission from[18] American Vacuum Society.

Figure 6:
Scanning electron microscope (SEM) images of 10 nm thick Au and Ag surface nanostructures employed as SERS substrates: (a,b) – arrays of nanodots; (c,d) – arrays of nano-hexagons; (e,f) – unstructured pads. Substrates with Au structures (left) employ FS supports, whereas substrates with Ag structures (right) use a 10 nm thick Ni coating on the FS. The images were obtained as described elsewhere[16,17,18,19].

Figure 7:
Scheme of water-proof chamber for Raman imaging of bio-functionalized samples in solution.

Figure 8:
SEM images of three arrays of Au nanodots on FS substrate used in Design 1[18] The arrays have the same 50 nm pitch and slightly different dot radii resulting in different widths of inter-dot gaps. This is achieved by applying different EBL exposure doses to produce PMMA masks for the three arrays (see Table 1). Higher exposure doses result in wider holes in PMMA masks, allowing for larger Au dot sizes after metallization and liftoff. Reprinted with permission from[18] American Vacuum Society.

Figure 9:
SERS imaging of substrate-immobilized protein A in Design 1[18]. (a) – Optical microscope image of the sample, comprising three arrays of Au nanodots with 50 nm pitch and different inter-dot gaps on a fused silica substrate (see also Figure 6), bio-functionalized as shown in Figure 3a. (b) – Raman mapping of the sample. (c) – SERS spectra from dot Array I and II. In panel (b), the vertical axis represents the distance across the substrate, the horizontal axis represents the Raman shift, and the legend bar indicates the Raman intensities. Vertical dashed lines in panels (b) and (c) represent benchmark Raman bands of free protein A in solution and (*) in panel (c) indicates SERS bands from Array I. The Raman spectra were obtained 532

nm excitation wavelength. Reprinted with permission from[18] American Vacuum Society.

Figure 10:
SERS spectra of ligand-free (a) and ligand-bound (b) glucose-binding protein immobilized on three different substrates in Design 2[19]. The spectra were obtained with 780 nm excitation wavelength.

Figure 11:
SERS spectra of ligand-free (purple line) and ligand-bound (blue line) dopamine-binding aptamer immobilized on Au nano-hexagon substrates in Design 3[20]. The red line shows a control SERS spectrum of dopamine powder.

Table Legends:

Table 1:
Three designs of nano-biological systems.

DISCUSSION:

SERS is gaining a recognition as an extremely powerful technique of bio-detection offering many unique advantages. The relation with molecular vibrations allows selectively identifying "fingerprints" of specific analytes from SERS spectra, whereas the extremely high sensitivity makes it possible detecting very small amounts of the analyte[9,10,11,36]. Furthermore, SERS is a nondestructive technique that is also relatively insensitive to water, and thereby it is very well suited for probing biological materials in their natural aqueous environment[9]. The results presented emphasize these advantages as well as further demonstrate strong potential of SERS as a very flexible label-free technique of bio-detection. In three designs employing monolayers of different substrate-immobilized biomolecules, Raman modes have been detected that could be confidently attributed to the particular analytes. That the detection of these biomolecules, or their respective ligands, have been demonstrated employing planar surfaces of fused silica as the support for SERS substrates, makes the designs compatible with current electronics and microfluidics settings, promising numerous applications in relation with emerging bio-electronic architectures interfacing biological materials with surfaces of electronic and electrochemical devices[2,3]. Importantly, in two of three designs SERS detection has been demonstrated for specific binding of small molecules, such as glucose and dopamine, employing monolayers of the surface-immobilized protein and aptamer, respectively, as the recognition elements.

However, several aspects should be taken care of in order to achieve an efficient SERS bio-detection in the "on-chip" setting. First of all, a well-known challenge that is common for most biomolecules is their propensity to degrade, particularly when exposed to non-natural conditions such as dry environment or intense laser light. Throughout the protocol, we have emphasized the importance of always keeping the bio-functionalized samples immersed in appropriate solutions during the entire experiment, from preparation of the samples to the acquisition of Raman spectra. For the latter, a custom water-proof chamber has been designed (Figure 7) to avoid evaporation of the liquid during laser exposures. The duration of exposure and laser

intensity should also be limited as described in step 5.3 of the protocol to avoid damage of the samples.

The outcomes of the SERS detection are found sensitive to the geometry of the substrate employed, and particularly the inter-feature separation of the metallic nanostructures. As it follows from Figures 8 and 9, the SERS intensity of Design 1 samples depends strongly on the width of the gaps between Au nano-dots on fused silica. Out of three arrays of Au nanodots tested in this design (Figure 8), the highest Raman intensity is achieved with Array I, which has the narrowest gaps between the Au features and therefore it provides more efficient electromagnetic field enhancement. As Figure 9 illustrates, control of inter-feature separations at the level of 10-20 nm or less is required. Employing EBL for fabricating SERS substrates, as demonstrated here, provides an efficient resolution specifically for controlling the widths of inter-feature gaps. With a positive-tone EBL resist such as PMMA, the size of holes in PMMA masks can be varied by simply changing the exposure doses. After lift-off this results in different sizes of fabricated metal dots, and the width of gaps between the dots may be tuned as desired by selecting proper EBL exposure doses[18].

The other challenge is optimization of SERS substrate geometry for specific bio-detection application. Although the enhancement effect increases with a decrease of the inter-feature gaps, the relatively large size of biological molecules imposes limitations on how narrow the gaps may be. This is evident from the results for Design 2, where the immobilization method is such that the protein efficiently binds only to the surface between noble metal dots, but not to the dots themselves (see Figure 3b). As it follows from Figure 10, the SERS spectra for unstructured Ag pads do not show any bands from the analyte. Although the pads exhibit a nano-crystalline structure with very thin inter-island gaps (see Figure 6f) these gaps are too narrow to accommodate a protein molecule. Yet another dimension of complexity is added when protein-ligand binding has to be detected. In Figure 10, the SERS C-H bands are more pronounced in the spectra from ligand-bound GBP than in the ligand-free one, which may be hypothetically explained by a change in the GBP conformation upon binding of D-glucose[27,28], resulting in a more rigid structure with increased Raman activity. If one compares the two nanostructured substrates, the C-H band from ligand-free protein is stronger in SERS spectra obtained with the nano-dots substrate, whereas both the protein and glucose C-H bands from ligand-bound protein are more pronounced with the nano-hexagons substrate. Two factors are expected to result in these differences[19], the availability of space between Ag features where the GBP could bind to Ni, and the susceptibility of the ligand-bound and ligand free protein to the electromagnetic enhancement of the Raman scattering in “hot spots” between these features. On one hand, the nano-dots pattern offers a relatively larger inter-feature area where Ni coating is available for the protein to bind, which may explain a more pronounced C-H band observed for glucose-free GBP on Ag nano-dots substrate. On the other hand, due their non-uniform structure (see Figure 6d), Ag nano-hexagons might be prone to show a stronger electromagnetic enhancement in narrow gaps between Ag islands within nano-hexagons[19] resulting in stronger C-H vibration bands from glucose-bound GBP on the nano-hexagons substrate. Some details of this interplay require further verification, and optimization of SERS substrates for complex analytes involving large proteins such as the GBP is still in the pipeline.

Clearly, SERS detection of ligand binding employing immobilized biomolecules as a recognition element is facilitated when only the ligand is Raman active in a selected region, whereas the other components are not. This is the case of Design 3, where pronounced SERS bands of aptamer-bound dopamine are obtained (Figure 11). The aptamer-dopamine pair exhibits excellent specificity and the SERS spectrum comprises pronounced bands without any significant background signal.

Future advance of the label-fee SERS technology would involve extensive tests of biomolecules' SERS signal enhancement with a broad range of different surface nanostructure designs. The usage of direct-write electron beam lithography to fabricate various nanostructures with a superb level of control over size, shape, and inter-feature separation, combined with the sample preparation protocols presented here, would facilitate comparison and cross-validation of the results obtained by different research groups. This would address the major challenge of reproducibility when SERS substrates are fabricated employing alternative "bottom up" methods[11,12,13], allowing for a better control of metal nanostructure's size and position toward a reliable identification of optimal substrate design for a broad variety of applications. Scalability of these techniques may subsequently be improved by combining EBL with complementary nanolithography methods such as nanoimprint lithography[20] toward future mass-production of nanoscale designs optimized employing the tunable EBL techniques.

ACKNOWLEDGMENTS:

The authors would like to thank: David Wishart, Valentyna Semenchenko, Mark McDermott, Michael Woodside, and Albert Cao for their help in developing and preparing the protein conjugates as well as the DNA aptamer; T. M. Fahim Amin, Mosa Sharmin Aktar, and Trevor Olsen for their assistance in the sample preparation, Jonathan Mane for his assistance in generating images of the molecular structures; and the funding sources including the National Research Council of Canada – National Institute for Nanotechnology (NRC-NINT), Natural Sciences and Engineering Research Council of Canada (NSERC), and the University of Alberta for supporting the work.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES

1. Sapsford, K.E., Tyner, K.M., Dair, B.J., Deschamps, J.R., Medlintz, I.L. Analyzing Nanomaterial Bioconjugates: A Review of Current and Emerging Purification and Characterization Techniques. *Anal. Chem*. **83,** 4453-4488, doi: 10.1021/ac200853a (2011).
2. Walcarius, A., Minteer, Sh.D., Wang, J., Lin, Yu, Merkoçi, A. Nanomaterials for Bio-Functionalized Electrodes: Recent Trends. *J. Mater. Chem. B.* **83**, 4453-4488, doi: 10.1039/c3tb20881h (2011).

3. Kim, J., *et al.* Applications, Techniques, and Microfluidic Interfacing for Nanoscale Biosensing. *Microfluid. Nanofluid*. **7**, 149-167, doi:10.1007/s10404-009-0431-8 (2009).
4. Rassaei, L., Singh, P.S., Lemay, S.G. Lithography-Based Nanoelectrochemistry. *Anal. Chem*. **83**, 3974-3980, doi: 10.1021/ac200307n, (2011).
5. Wong, L.S., Khan, F., Micklefield, J. Selective Covalent Protein Immobilization: Strategies and Applications. *Chem. Rev.* **109**, 4025-4053, doi: 10.1021/cr8004668 (2009).
6. Ley, C., Holtmann, D., Mangold, K.-M., Schrader, J. Immobilization of Histidine-Tagged Proteins on Electrodes. *Colloids and Surfaces B: Biointerfaces.* **88**, 539-551, doi:10.1016/j.colsurfb.2011.07.044 (2011).
7. Kim, D., Herr, A.E. Protein Immobilization Techniques for Microfluidic Assays. *Biomicrofluidics* **7**, 041501:1-47, doi:10.1063/1.4816934 (2013).
8. Anker, J.N., Hall, W.P., Lyandres, O., Shah, N.C., Xhao, J., Van Duyne, R.P. Biosensing with Plasmonic Nanosensors. *Nature Materials.* **7**, 442-453, doi:10.1142/9789814287005_0032 (2008).
9. Bantz, K.C., et al. Recent Progress in SERS Biosensing. *Phys.Chem.* **13**, 11551-11567, doi:10.1039/c0cp01841d (2011).
10. Sharma B., Frontiera R.R., Henry A.-I., Ringe E., Van Duyne, R.P. SERS: Materials, Applications, and the Future. *Mater. Today.* **15**, 16-25, doi: 10.1016/S1369-7021(12)70017-2 (2012).
11. Kleinman S.L., Frontiera, R.R., Henry, A.-I., Dieringer, J.A., Van Duyne, R.P. Creating, Characterizing, and Controlling Chemistry with SERS Hot Spots. *Phys.Chem.Chem.Phys*. **15**, 21-36, doi:10.1039/c2cp42598j (2013).
12. Fan, M., Andrade, F.S., Brolo, A.G. A Review on the Fabrication of Substrates for Surface Enhanced Raman Spectroscopy and their Applications in Analytical Chemistry. *Anal. Chim*. *Acta* **693**, 7-25, doi:10.1016/j.aca.2011.03.002 (2011).
13. Cao, Y., Li, D., Jiang, F., Yang, Y., Huang, Zh. Engineering Metal Nanostructure for SERS Application. *J. Nanomater*. **123812**, 1-12 doi: 10.1155/2013/123812 (2013)
14. Glembocki O., Rendell, R., Alexson, D., Prokes, S., Fu, A., Mastro, M., Dielectric-Substrate-Induced Surface-Enhanced Raman Scattering. *Phys. Rev. B*. **80**, 085416:1-6, doi:10.1103/physrevb.80.085416 (2009).
15. Merlen, A., et al. Surface Enhanced Spectroscopy with Gold Nanostructures on Silicon and Glass Substrates. *Surf. Sci*. **605**, 1214-1218, doi:10.1016/j.susc.2011.04.004 (2011).
16. Muhammad, M., Buswell, S.C., Dew, S.K.; Stepanova, M. Nanopatterning of PMMA on Insulating Surfaces with Various Anticharging Schemes Using 30 keV Electron Beam Lithography. *J. Vac. Sci. Technol. B.* **29**, 06F304, doi:10.1116/1.3636367 (2011).
17. Peters, R., Fito, T., Gutierrez-Rivera, L., Dew, S.K., Stepanova, M. Study of Multilayer Systems in Electron Beam Lithography. *J. Vac. Sci. Technol. B* **31,** 06F407, doi:10.1116/1.4827813 (2013).
18. Gutierrez-Rivera, L., Peters, R., Dew, S., Stepanova, M. Application of EBL Fabricated Nanostrucutred Substrates for SERS Detection of Protein A in Aqueous Solution. *J. Vac. Sci. Technol. B* **31,** 06F901, doi:10.1116/1.4821800 (2013).
19. Gutierrez-Rivera, L., Peters, R., Dew, S., Stepanova, M. Surface-Enhanced Raman Spectroscopy Detection of Protein-Ligand Binding Using D-glucose and

Glucose Binding Protein on Nanostructured Plasmonic Substrates. *To be published* (2014).
20. Peters, R. Fabrication and Testing of Surface-Enhanced Raman Spectroscopy Substrates for the Detection of Biomolecules. MSc Thesis, University of Alberta (2014).
21. Gouda, H.,Torigoe, H.,Saito, A.,Sato, M.,Arata, Y.,Shimada, I. Three-Dmensional Solution Structure of the B Domain of Staphylococcal Protein A: Comparisons of the Solution and Crystal Structures. *Biochemistry.* **31,** 9665-9672, doi:10.1021/bi00155a020 (1992).
22. Cuneo, M.J.,Johnson, S.J.,Beese, L.S.,Hellinga, H.W. High Resolution Structure of E. Coli Glucose/Galactose Binding Protein Bound with Glucose. *Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank,* doi**:**10.2210/pdb2hph/pdb (2009).
23. Humphrey, W., Dalke, A. and Schulten, K., VMD - Visual Molecular Dynamics, *J. Molec. Graphics.* **14**, 33-38, doi:10.1016/0263-7855(96)00018-5 (1996).
24. Walsh, R., DeRosa, M.C. Retention of Function in the DNA Homolog of the RNA Dopamine Aptamer. *Biochem. Biophys. Res. Comm.* **388**, 732-735, doi:10.1016/j.bbrc.2009.08.084 (2009).
25. Akitomi, J., Kato, S., Yoshida, Y., Horii, K., Furuich, M., Waga, I. ValFold: Program for the Aptamer Truncation Process. *Biomed. Inf.* **7**, 38-40, doi:10.6026/97320630007038 (2011).
26. Han, K., Lee, Y., Kim, W. PseudoViewer: Automatic Visualization of RNA Pseudoknots. *Bioinformatics.* **18**, S321-S327, doi:10.1093/bioinformatics/18.suppl_1.s321 (2002).
27. Dwyer, M.A., Hellinga, H.W. Periplasmic Binding Proteins: a Versatile Superfamily for Protein Engineering. *Curr. Opin. Struct. Biol*. **14**, 495-504, doi:10.1016/j.sbi.2004.07.004 (2004).
28. Benson D.E., Conrad D.W. Design of Bioelectronic Interfaces by Exploiting Hinge-Bending Motions in Proteins. *Science.* **293***,* 1641-1644, doi:10.1126/science.1062461 (2001).
29. Bozic, S., Chorzempa, J. Pirahna Cleaning (University of Alberta, 2011) http://www.nanofab.ualberta.ca/wp-content/uploads/downloads/2011/04/ Pirahna_SOP.pdf
30. Mohammad, M. A. *Raith 150^{TWO} SOP* (University of Alberta, 2011) www.nanofab.ualberta.ca/wp-content/uploads/downloads/2011/09/ Raith_150TWO_SOP.pdf
31. Schmidt, M.W., *et al.* General Atomic and Molecular Electronic Structure System. *J. Comput. Chem.* **14**, 1347-1363, doi:10.1002/jcc.540141112 (1993).
32. Bode, B.M., Gordon, M. S., MacMolPlt: a Graphical User Interface for GAMESS, *J. of Mol. Graph. Mod*. **16**, 133-138, doi:10.1016/s1093-3263(99)00002-9 (1998).
33. Bright A., Devi, T.S.R., Gunasekaran, S, Spectroscopical Vibrational Band Assignment and Qualitative Analysis of Biomedical Compounds with Cardiovascular Activity*. Int. J. Chem. Tech. Res.* **2**, 379-388 ISSN : 0974-4290 (2010).
34. Bandekar, J. Amide Modes and Protein Conformation. *Biochim. Biophys. Acta* 1120, 123-243, doi:10.1016/0167-4838(92)90261-b (1992).
35. Barth, A., Zscherp, C. What Vibrations Tell About Proteins. *Quarterly Reviews of Biophysics.* **35**, 369–430 doi: 10.1017/S0033583502003815 (2002).

36. Chrimes A.F., Khoshmanesh Kh., Stoddart, P.R. Mitchell A., Kalantar-Zadeh K. Microfluidics and Raman Microscopy: Current Applications and Future Challenges. *Chem. Soc. Rev.* **42**, 5880-5906 doi: 10.1039/C3CS35515B (2013).
37. Park, S.-K., Lee, N.-S, Lee, S.-H. Vibrational Analysis of Dopamine Neutral Base based on Density Functional Force Field, *Bull.-Korean Chem. Soc*. **21**, 959-968 (2000).
38. Briand, E., Salmain, M, Compère, C., Pradier, C.M. Immobilization of Protein A on SAMs for the elaboration of immunosensors. *Coll. Surf. B: Biointerfaces* **53**, 215-224, doi:10.1016/j.colsurfb.2006.09.010 (2006).
39. Lakowicz L.R., *et al.* Radiative decay engineering: 2. Effects of Silver Island Films on Fluorescence Intensity, Lifetimes, and Resonance Energy Transfer. *Analytical biochemistry* **301**, 261-277 doi: 10.1006/abio.2001.5503 (2002).

Figure 1
Click here to download Figure: Fig1.pdf

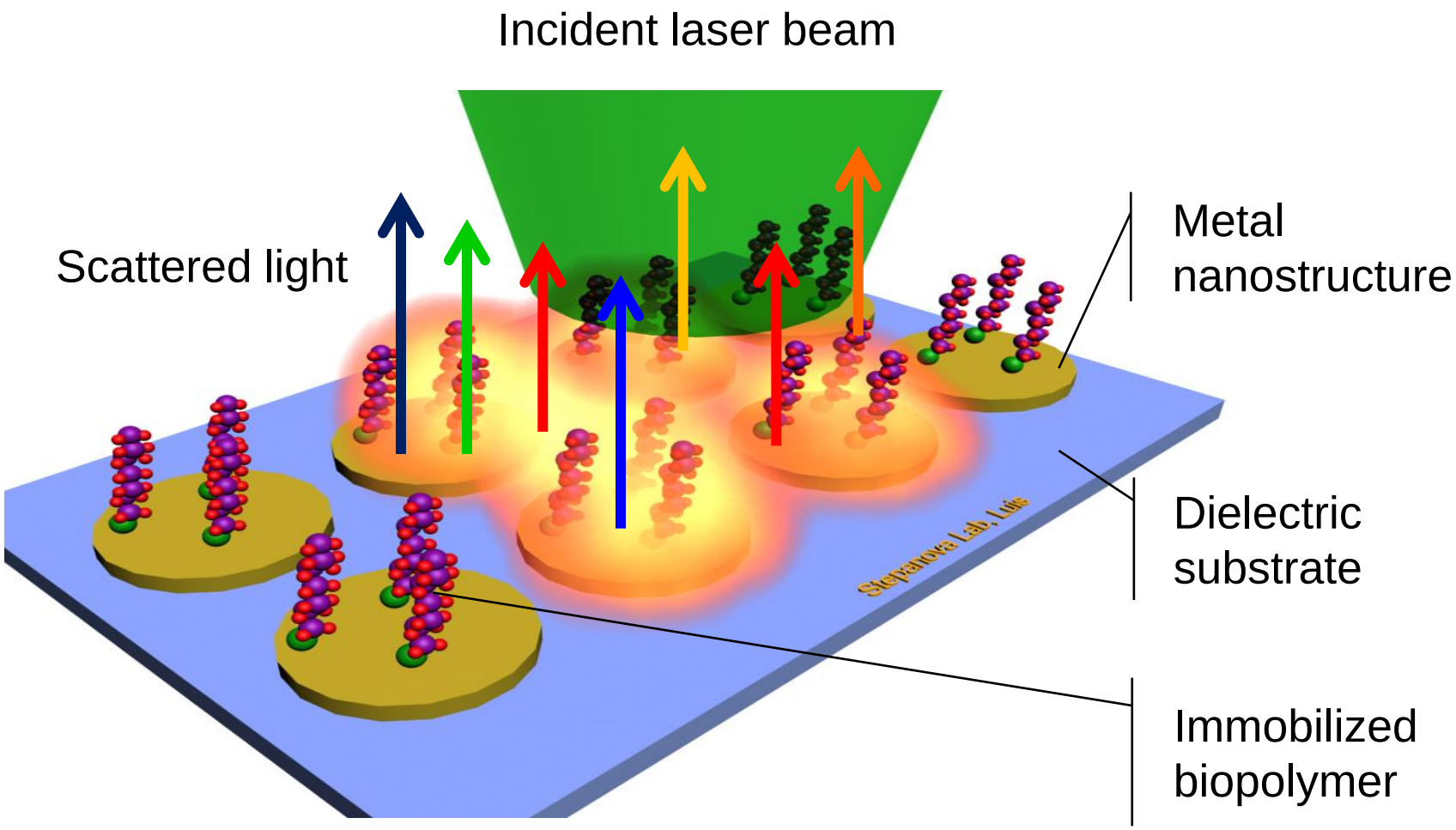


Figure 2
Click here to download Figure: Fig2.pdf

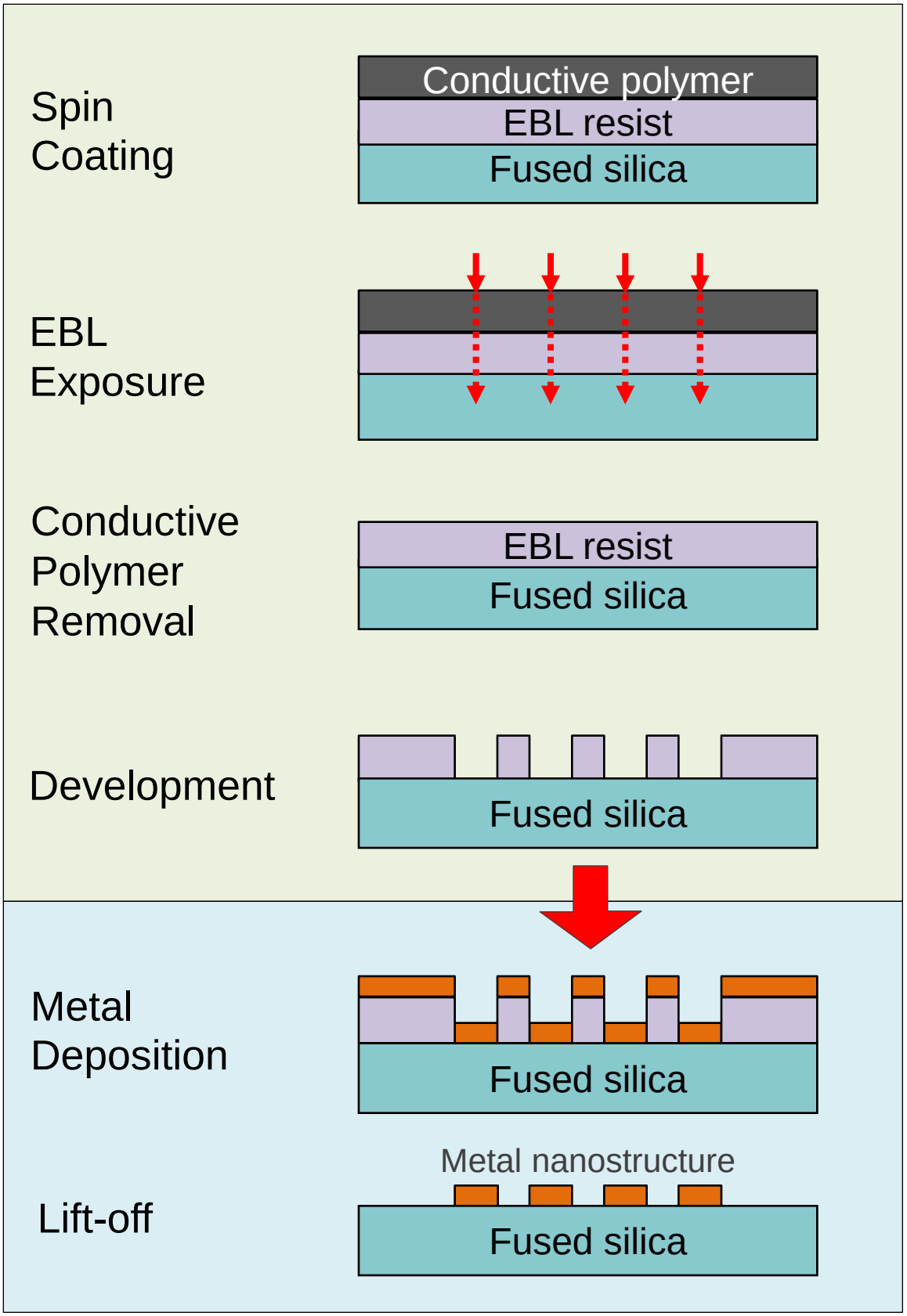


Figure 3
Click here to download Figure: Fig3.pdf

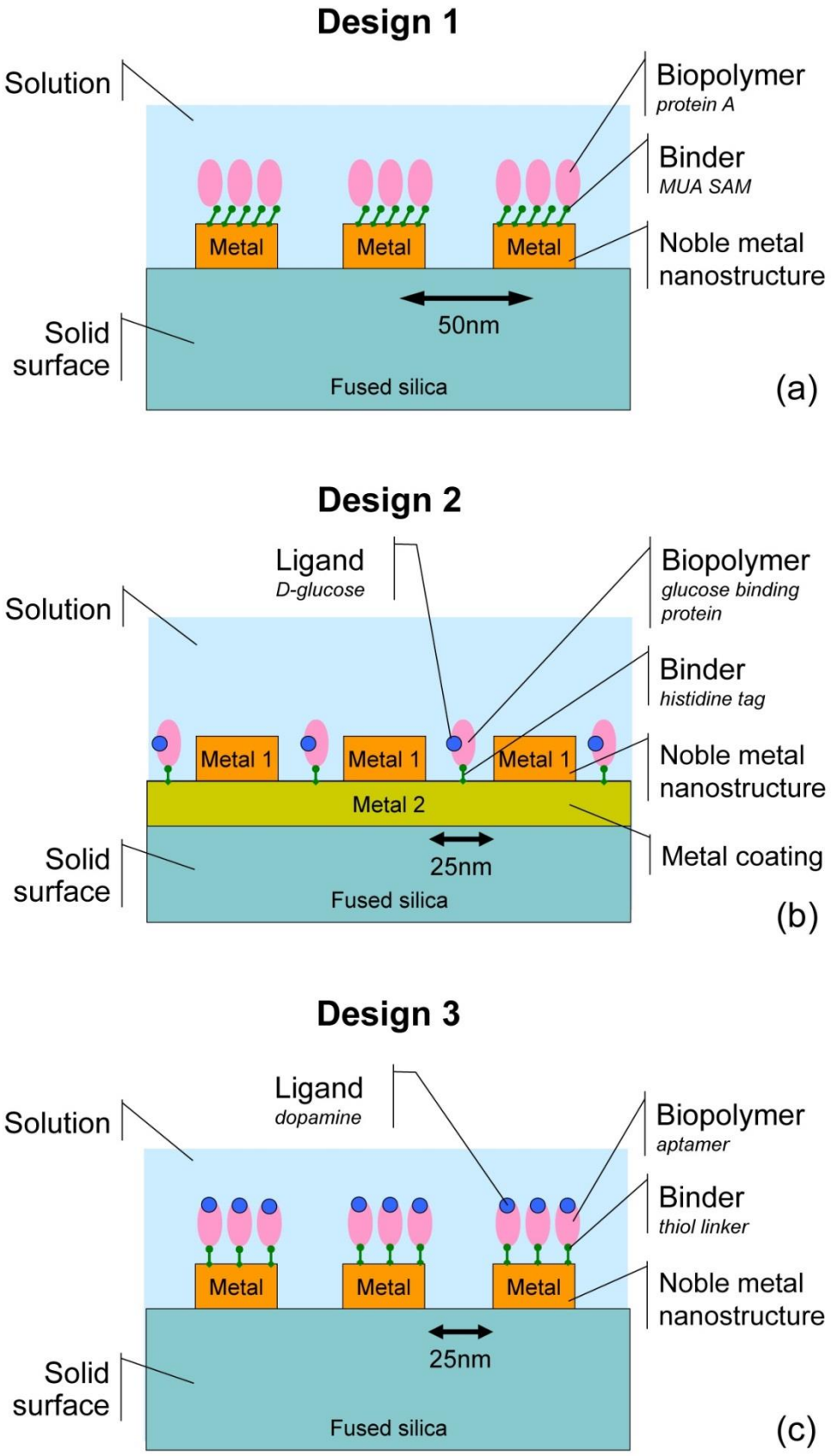
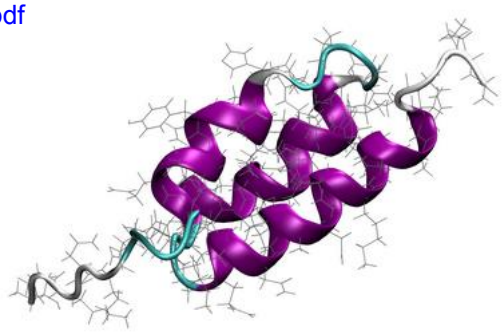
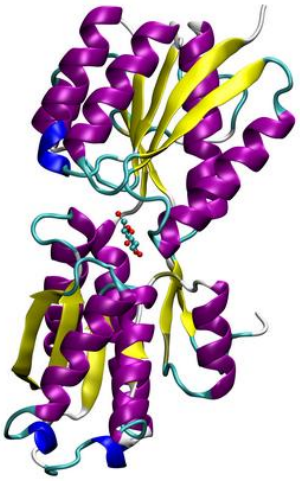


Figure 4
Click here to download Figure: Fig4.pdf

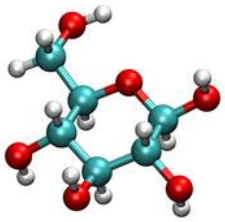


(a)

Protein A

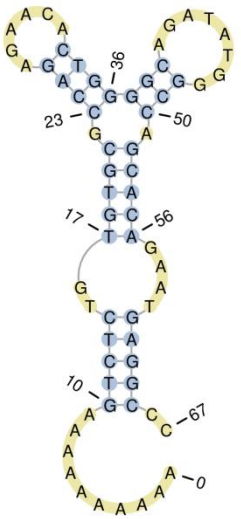


Glucose
binding protein

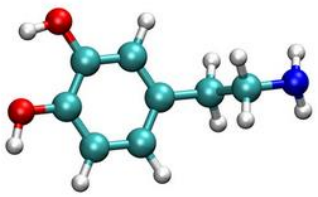


(b)

D-glucose



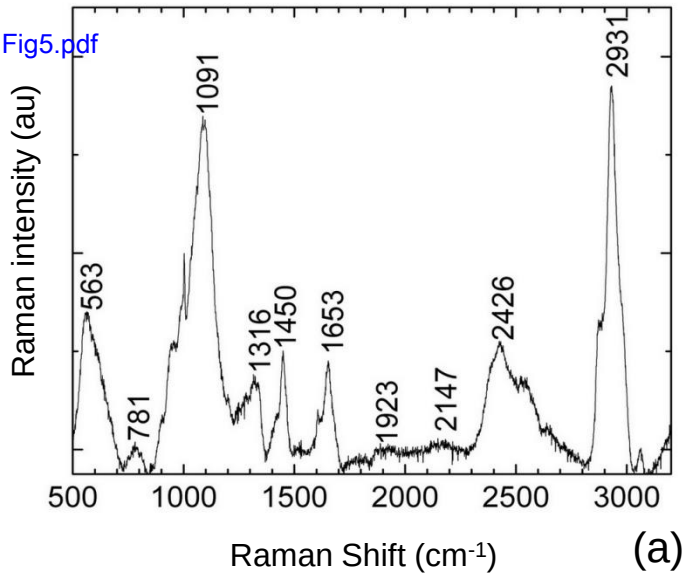
Dopamine
binding aptamer

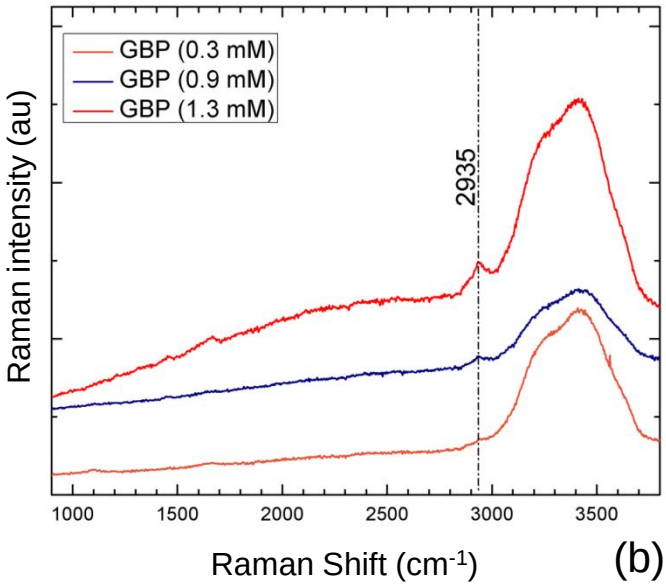


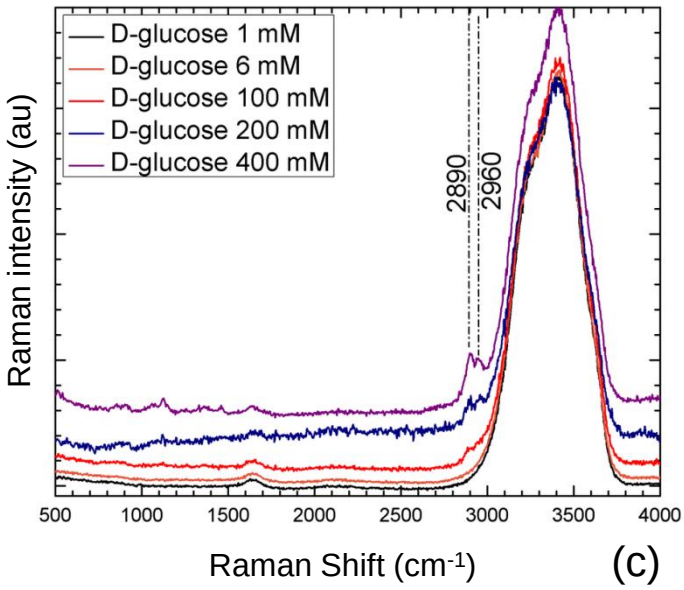
(c)

Dopamine

Figure 5
Click here to download Figure: Fig5.pdf







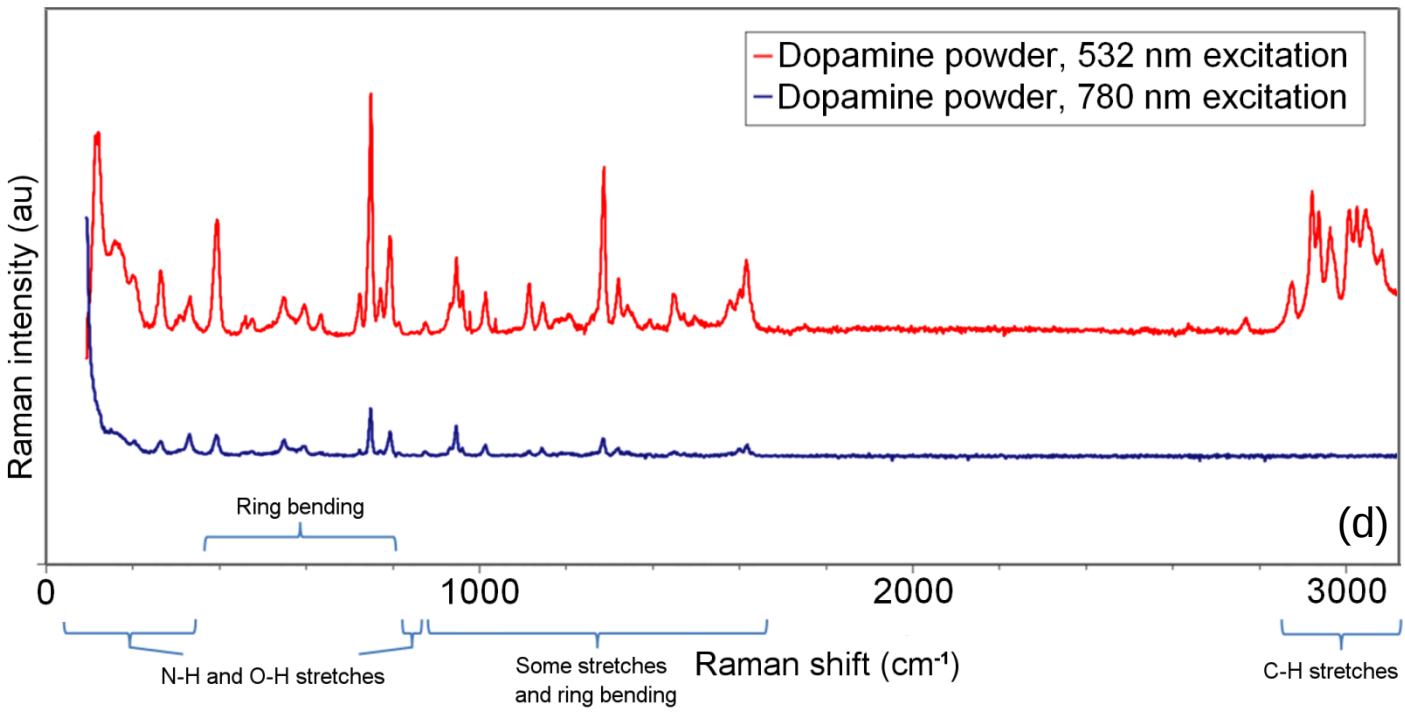


Figure 6

Click here to download Figure: Fig6.pdf

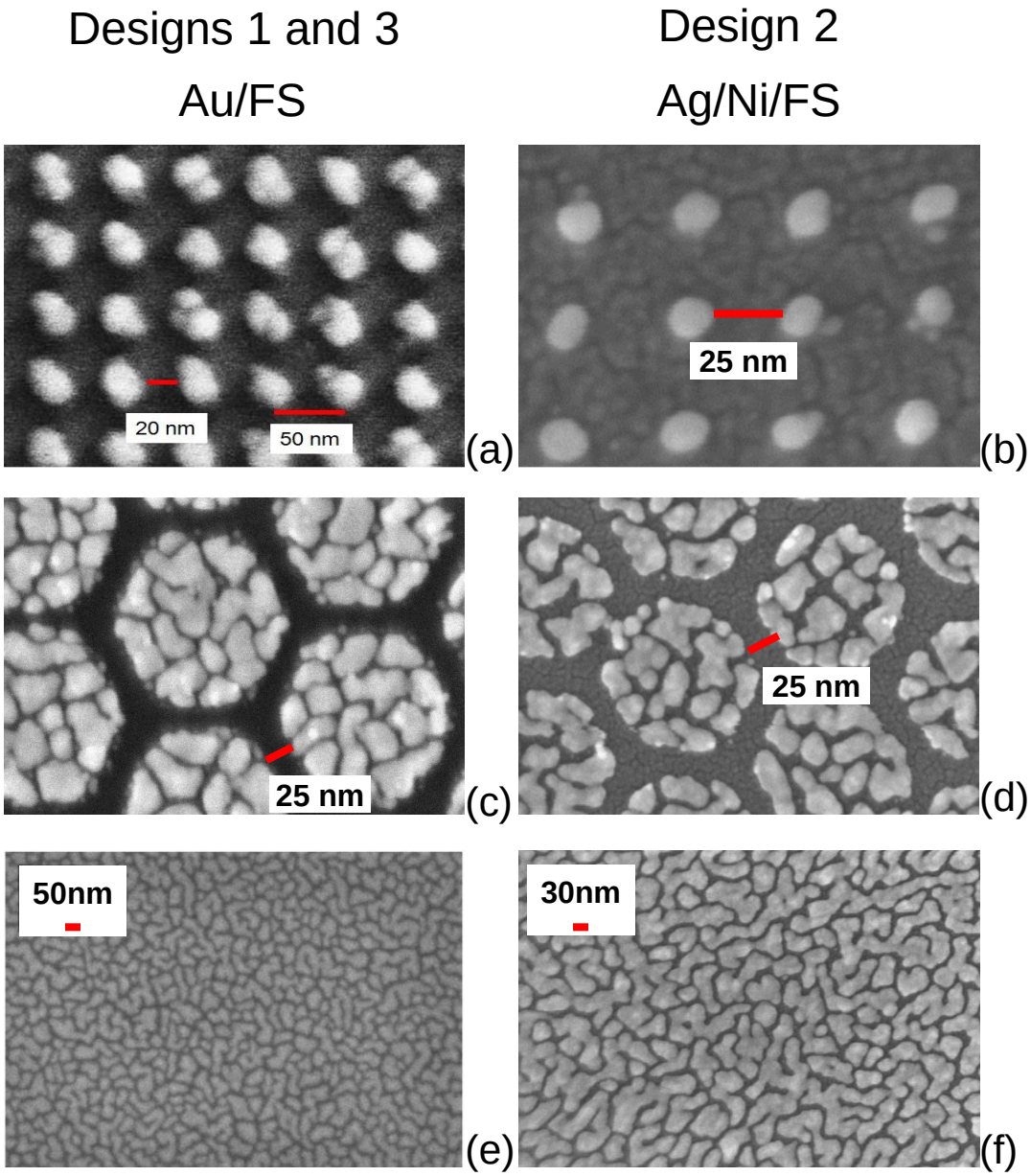


Figure 7
Click here to download Figure: Fig7.pdf

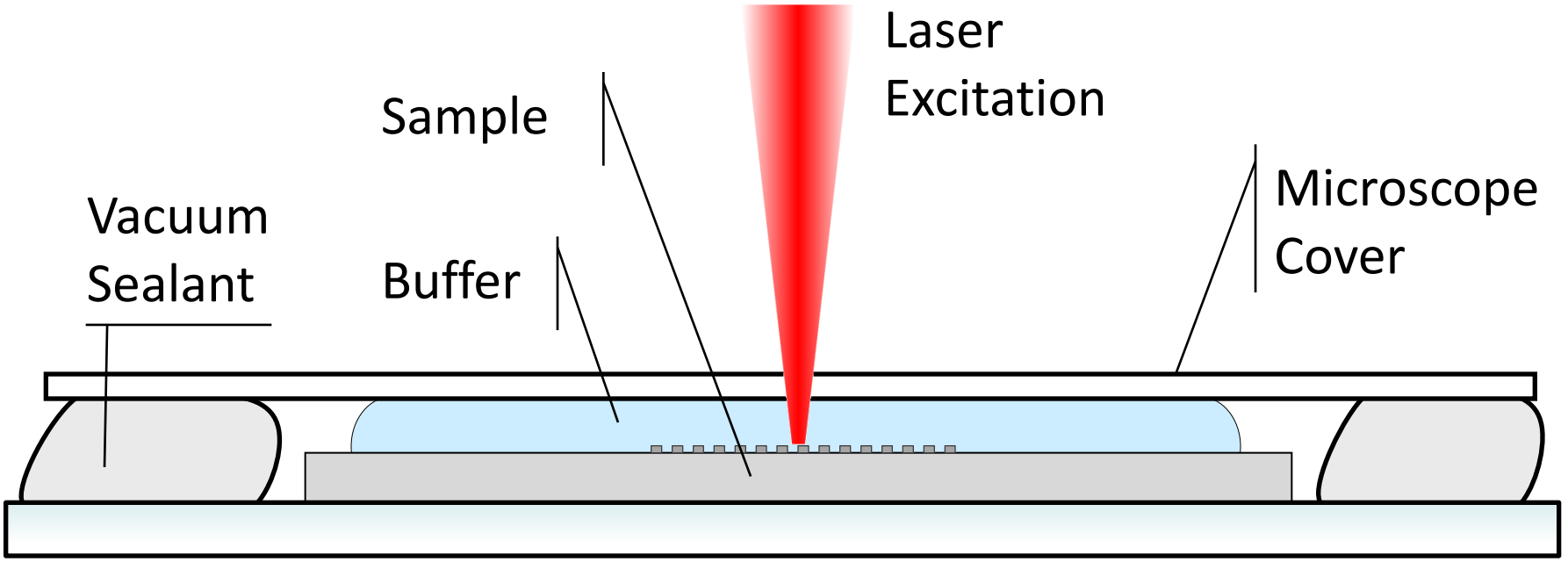


Figure 8

Click here to download Figure: Fig8.pdf

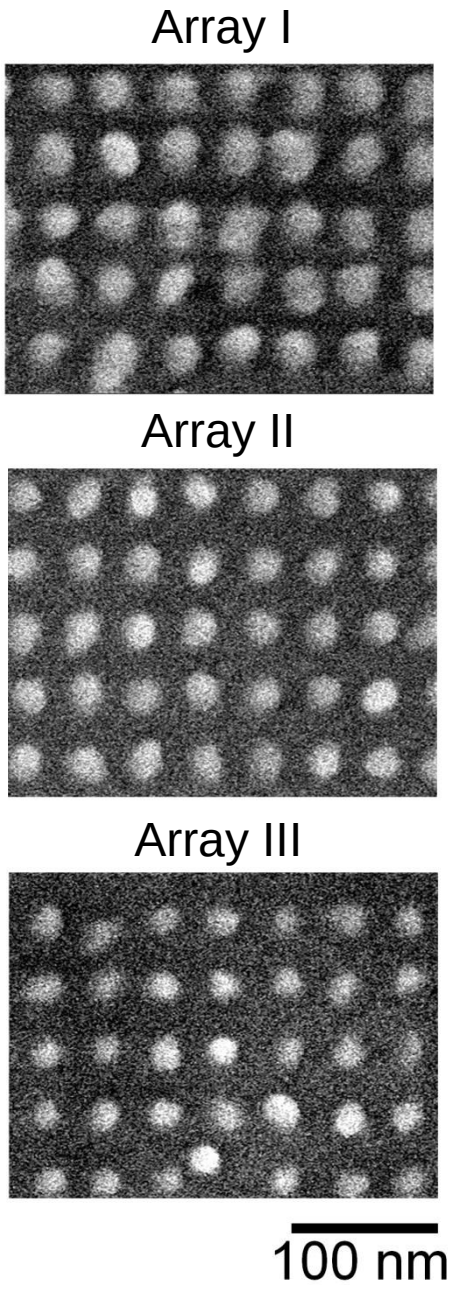


Figure 9

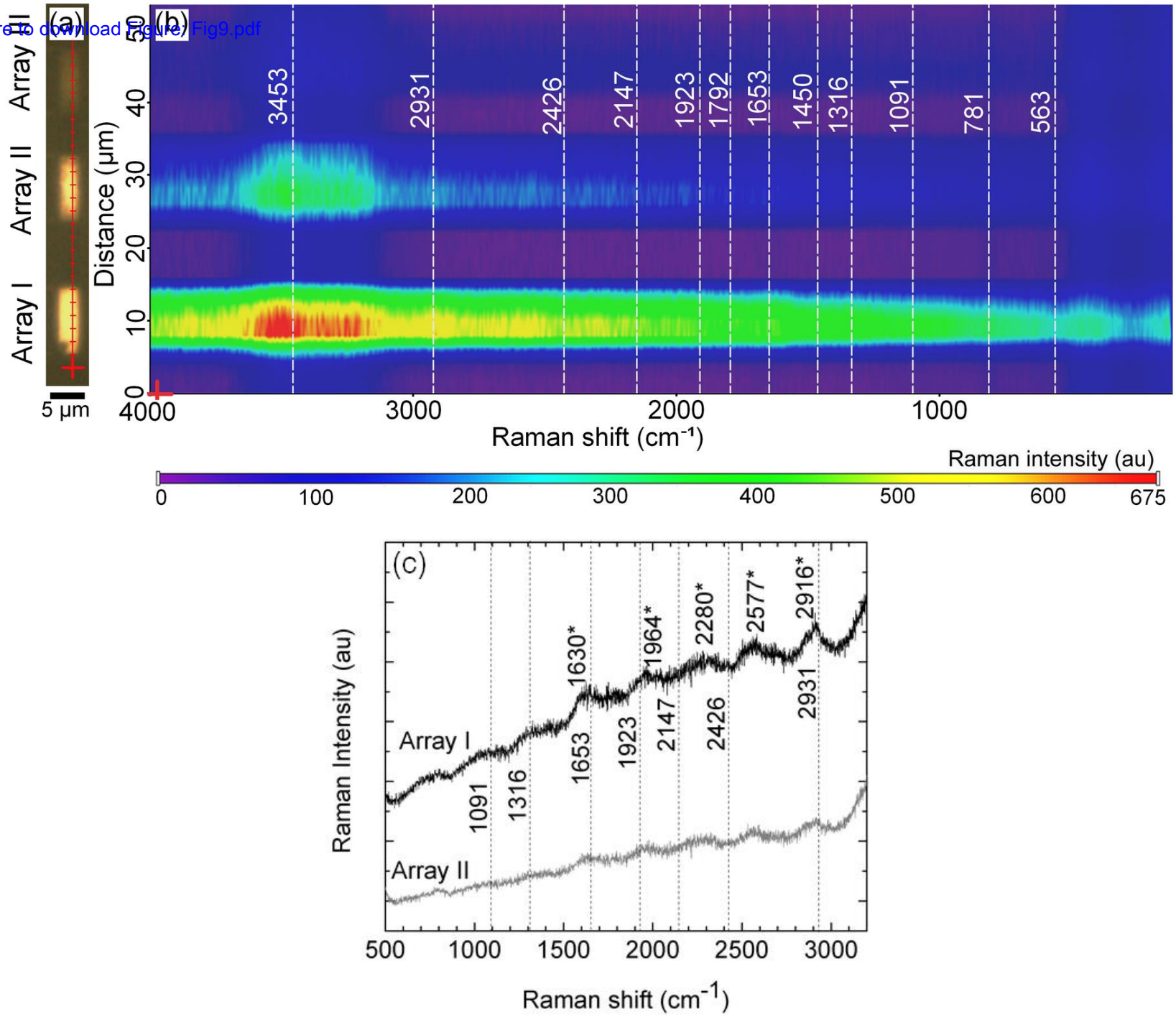
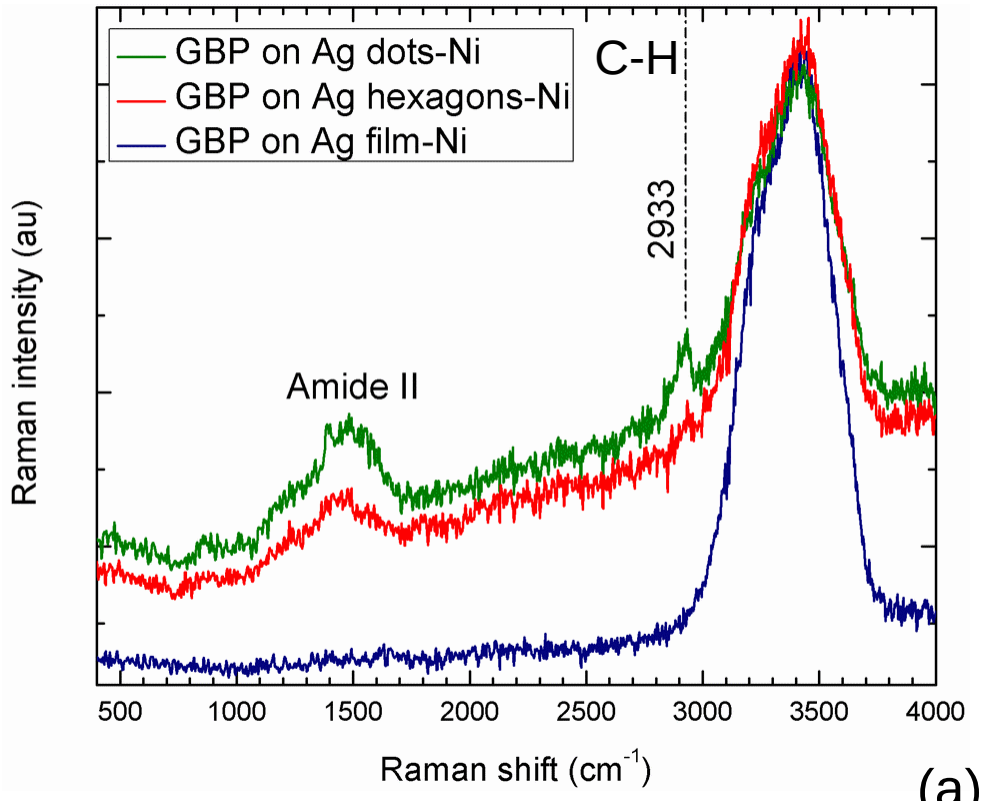
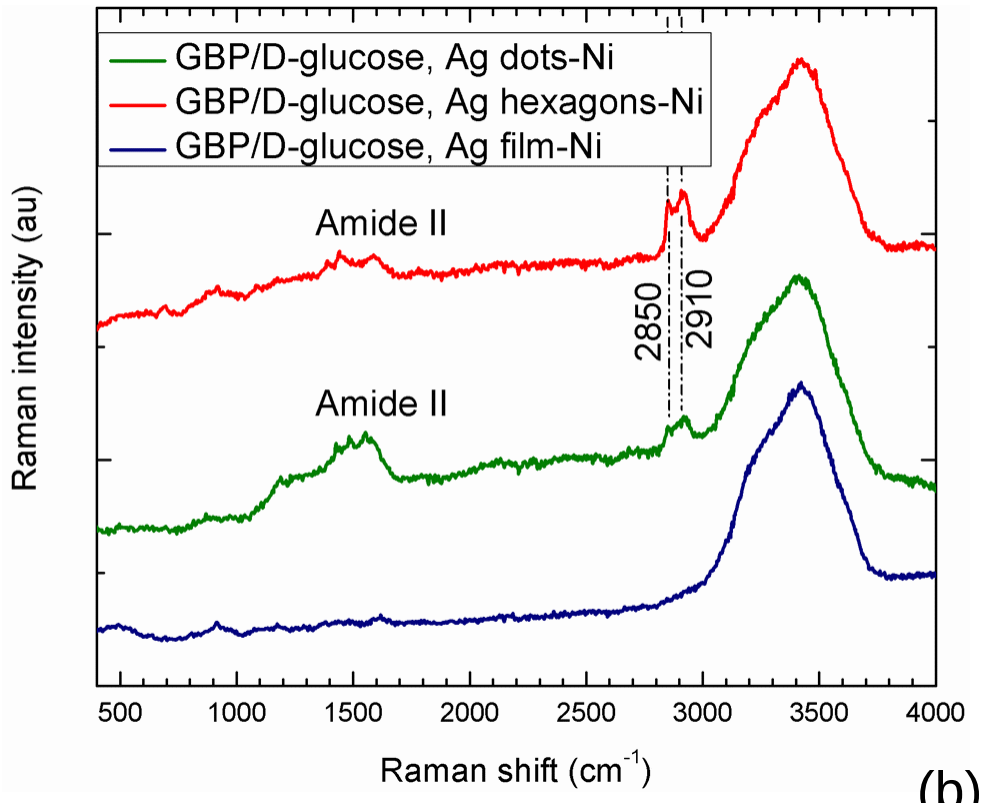


Figure 10
Click here to download Figure: Fig10.pdf



(a)



(b)

Figure 11
Click here to download Figure: Fig11.pdf

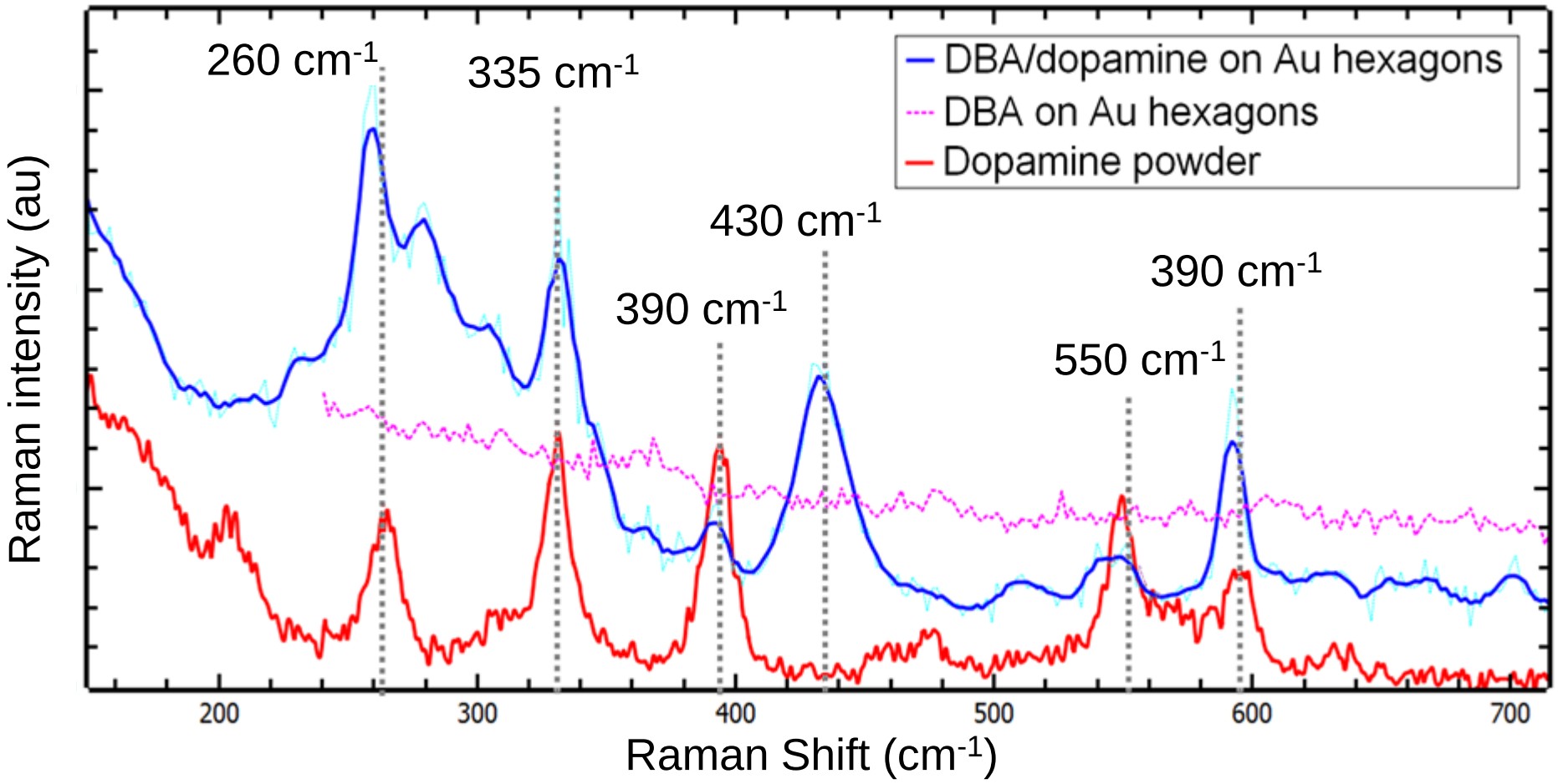


Table 1
Click here to download Table: Table 1.xlsx

	Design 1	**Design 2**	**Design 3**
Biopolymer	Protein A	Glucose binding protein (GBP)	Dopamine binding aptamer (DBA)
Binder	11-Mercaptoundecanoic acid (MUA) self-assembled monolayer (SAM)	Histidine tags	Thiol linkers
Ligand	None	D-glucose	Dopamine
Solution	Deionised (DI) water	Potassium phosphate buffer	Tris(hydroxymethyl)aminomethane (TRIS) and ethylenediaminetetraacetic acid (EDTA) buffer; Phosphate buffered saline (PBS)
Substrate	Au structures on FS	Ag structures on Ni-coated FS	Au structures on FS
Patterned area	4 μm x 10 μm	4 μm x 8 μm	4 μm x 10 μm
Pattern	Au dots, 50 nm pitch	Ag dots, 40 nm pitch Ag hexagons, 200 nm pitch Ag unstructured pads	Au hexagons, 200 nm pitch Au unstructured pads
EBL exposure doses	Dots: Array I 120 μC/cm^2 Array II 96 μC/cm^2 Array III 72 μC/cm^2	Dots: 105 μC/cm^2 Hexagons: 170 μC/cm^2	Hexagons: 180 μC/cm^2
Laser excitation wavelength	532 nm	532 nm	780 nm

Table of Materials

Click here to download Excel Spreadsheet- Table of Materials/Equipment: Table of Materials.xlsx

Name of Material	Company
11-Mercaptoundecanoic acid (MUA)	Sigma Aldrich (www.sigmaalrich.com)
Conductive polymer	Mitsubishi Rayon (www.mrc.co.jp)
D-glucose	Collaborator Lab.
Dopamine	Collaborator Lab.
Dopamine binding aptamer (DBA)	Integrated DNA Technologies Inc. (www.idtdna.com)
Fused silica wafers	Mark Optics www.markoptics.com
Glucose binding protein (GBP)	Collaborator Lab.
High vacuum grease	Dow Corning (www.dowcorning.com)
Hydrogen Peroxide 30%, H_2O_2	J.T. Baker
N-ethyl-N'-(3-(dimethylamino) propyl) carbodiimide (EDC)	Sigma Aldrich www.sigmaaldrich.com
N-Hydroxysuccinimide (NHS)	Sigma Aldrich (www.sigmaaldrich.com)
Potassium phosphate buffer	Collaborator Lab.
Phosphate buffered saline (PBS)	Collaborator Lab.
Polymethylmethacrylate (PMMA) 950 A2	MicroChem (www.microchem.com)
Recombinant protein A	Protein Mods Inc. (www.proteinmods.com)
Sulfuric acid 96%, H_2SO_4	J.T. Baker
Tris(hydroxymethyl)aminomethane (TRIS) and	Sigma Aldrich (www.sigmaaldrich.com)

Catalogue number	Comments
450561 ALDRICH	Used for surface functionalization in Design 1
aquaSAVE-57xs	A 70 nm thick layer is used as anti-charging coating for EBL exposures
	Ligand in Design 2
	Ligand in Design 3
5'- /Thiol Modifier C6 S-S/ AAAAAAAAAA GTCTCTGTGT GCGCCAGAGA ACACTGGGGC AGATATGGGC CAGCACAGAA TGAGGCCC-3'	Biopolymer in Design 3
PDB ID 2HPH (www.ncbi.nlm.nih.gov/protein/gi|145579532)	Biopolymer in Design 2
	Used to seal water-proof chamber, step 5.1
	Used for pirahna solution, step 1.2
03450 FLUKA	Used for immobilization of biopolymer in Design 1
130672 ALDRICH	Used for immobilization of biopolymer in Design 1
	Buffer used in Raman sampling
	Solvent in Design 3
	A 90 nm thick layer is used as EBL positive tone resist
PDB ID 1BDD (www.rcsb.org/pdb/explore/explore.do?structureId=1bdd)	Biopolymer in Design 1
	Used for pirahna solution, step 1.2
T9285 SIGMA	Buffer in Design 3

Table of Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: Table of Equipment _PR.xlsx

Equipment	Company
Dicing saw	Diamond Touch Technology Inc. (17301 W Colfax Ave # 152, Golden, CO)
Electron beam evaporator	Kurt J. Lesker (www.lesker.com)
Electron beam evaporator	Johnsen Ultravac (JUV) (www.ultrahivac.com)
Microscope cover slips (25 mm)	Fisher Scientific (www.fishersci.ca)
Microscope slides (3×1 in.)	Fisher Scientific (www.fishersci.ca)
Raith 150^{TWO} EBL exposure system	Raith Inc. (www.raith.com)
Raman microscope	Thermo Scientific (www.thermoscientific.com)
Sonicator system	Branson (www.bransonic.com)
Spinner	Brewer Spinner and Hotplate (www.brewerscience.com)

Catalogue number	Comments
	Used to cut FS wafer, step 1.1
	Used for Au and Ag evaporation
JuV E-gun	Used for Ni evaporation
12-545-102	Used in water-proof chamber, step 5.1
	Used in water-proof chamber, step 5.1
Raith 150^{TWO} system	Used for EBL exposures, step 2.2
Nicolet Almega XR	Used for Raman spectroscopy, step 5.3
	Used for liftoff and solutions mixing
Cee 200X and Cee 1300X	Used to spin-coat PMMA and conductive polymer, step 2.1

This piece of the submission is being sent via mail.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Surface Enhanced Raman Spectroscopy Detection of Biomolecules

Author(s): R.F. Peters, L. Gutierrez-Rivera, S.K. Dew, M. Stepanova

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☐ Standard Access ☒ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Maria Stepanova

Department: Electrical and Computer Engineering

Institution: University of Alberta

Article Title: Surface Enhanced Raman Spectroscopy Detection of Biomolecule

Signature: M. Cal Date: Oct. 13, 2014

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Rebuttal Comments
Click here to download Rebuttal Comments: List_of_Changes_PR.docx

JoVE52712

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

Robert F. Peters
Luis Gutierrez-Rivera
Steven K. Dew
Maria Stepanova

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

==========

► 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.

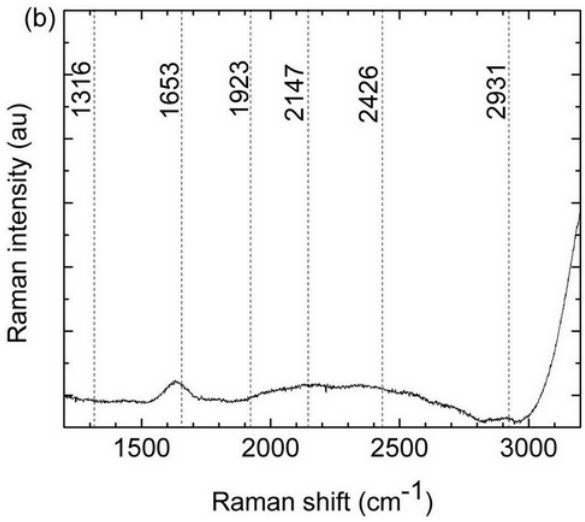
We appreciate the importance of accurate interpretation of the SERS spectra. However, we strongly feel that confusion occurred in this case. The D-glucose spectra that appear in figure 5 are not SERS spectra. These are control Raman spectra of D-glucose solutions without any metal-containing substrates 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 H_2S0_4 to H_2O_2 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.