Fabrication of biosensor arrays by DPN and multiple target detection by triple wavelength fast SERRS mapping

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ABSTRACT

We have used Dip-Pen Nanolithography (DPN) to create biosensor arrays on micro- and nano-structured surfaces. Bespoke linker and labelling materials enables detection of biological molecules by surface enhanced resonance Raman scattering (SERRS). Careful DPN-directed placement of the biological species or capture chemistry, within the array, facilitates rapid read out via ultra fast Raman line mapping. Writing DPN features that complement the spectroscopic collection geometry allows the lateral resolution and detection speed to be optimised. Applied in a DPN directed array format, we show that SERRS offers several advantages over conventional fluorescence detection. The information rich nature of the SERRS spectrum allows multiple levels of detection capability to be embedded into each pixel, further increasing the information depth of the array. Effective practical application of multiple target detection by SERRS in a plasmonic array format is demonstrated, using suitable dye labels and resonant wavelengths of excitation.

Keywords: DPN, SERS/SERRS, Plasmonics

1 INTRODUCTION

Surface enhanced [resonance] Raman scattering (SE[R]RS) is a technique whereby signals from suitable molecules are enhanced enormously by close proximity to high electric field gradients at metal surfaces, in some cases to the extent where scattering from single molecules can be observed.[1] The technique is flexible and has been demonstrated to be effective in number of biodiagnostic applications including gene probes,[2,] and DNA detection[3]. A significant part of the overall enhancement in SERRS derives from the additional 'resonance' with the molecular chromophore. The resonance-enhanced spectrum from a reporter dye is often less complex in appearance than would normally be expected from a larger molecule as only selected vibronic states are enhanced (to a lesser or greater extent) when probed with a single wavelength of excitation (λ_{ex}). Therefore, in many cases only narrow SERRS lines (~0.5 nm) that conform to Raman, resonance and surface selection rules are observed. This is a significant advantage of the technique when applied in real assay a number of characteristic bands within each dye class are enhanced to a greater extent than other materials in the matrix. Two notable examples of this are the distinctive N=N modes that can be observed in the family of SERRS azo dyes,[4] and the unique carbonyl modes of the squarylium type reporter.[5] A further advantage of SERRS is that the excitation wavelength (λ_{ex}) can be selected anywhere in the optical range and wavelength selectivity can be observed using some combinations of dye reporters.[6]

A number of effective SERS surfaces have been reported in recent years, including those made by nanosphere lithography,[7] silver metal island films [8] and nanostructured gold surfaces.[9, 10] Herin, we demonstrate the effective combination of DPN and fast line scanning spectroscopy to gold SERS surfaces to create effective and efficient biosensor arrays.

2 EXPERIMENTAL

DPN was performed using an NScriptor™ (Nanoink, Skokie, IL) instrument and an environmental chamber to control temperature and humidity. Klarite™ SERS substrates were obtained from D3 Technologies (Glasgow, UK) and modified with self assembled monolayers appropriate to each experiment before use. In a number of experiments solutions for lithography were prepared using "Just Add DNA" solutions (Nanoink), typically 1% B in A solution being used as a start point for optimization in each case. HPLC purified thioctic acid modified oligonucleotide sequences were purchased from ADT Bio (Southampton, UK) and used without further purification. The areas surrounding the DPN-written active features could be modified with materials of the generic form thiol-aklyl-polyethylene glycol, to prevent non specific binding of oligonucleotides. Self-assembly steps were typically

performed over 15 minutes in a sealed chamber. Effective hybridizations were achieved at room temperature by immersion slide into solutions containing the target oligonucleotides in PBS buffer within a sealed hybridization chamber.

SERRS spectroscopy was performed using a Renishaw *InVIA* Raman system (Renishaw, UK) coupled to an inverted microscope (Leica, Germany) equipped with a Streamline™ mapping stage. Three laser wavelengths of excitation were used; a 632.8 nm (HeNe, ~30 mW), 785 nm (diode, ~180 mW) and 830 nm (diode, ~170 mW). The laser at the surface was line focused and attenuated as appropriate to the experiment (typically >0.5 mW, using a delivery optic with NA > 0.5).

3 RESULTS AND DISCUSSION

3.1 Surface chemistry and DPN

It has been recently demonstrated that very sensitive SERRS measurements from dye labelled oligonucleotides could be recorded from a commercially available SERRS surface (Klarite™, D3 Technologies, UK).[11] Furthermore, careful control of the surface chemistry (as apposed to drop coating) lead to a dramatic reduction in the relative standard deviation (~40% to ~10%) of the signal obtained from the positive control region of the substrate area. Following a similar methodology, active areas of the SERS surface were coated with a thioctic acid derived linker. 3' Amino modified oligonucleotides with 5' dye modifications could then be immobilized *via* an inbuilt N-hydroxy succinimidyl (NHS) ester *via* facile chemistry. These idealised self-assembled films resulted in strong SERRS signals, typically up to 25,000 counts per second against a background of 200-300 in blank regions that had not been modified with the linker. Surface passivation of designated blank regions was necessary when using amino modified oligonucleotides, as the interaction between the primary amine and the gold surface is strong enough to result in SERRS signals even after 10 washes (10 ml 0.5 M PBS). 5' Dye labelled oligonucleotides could be deposited directly by this method in order to study the efficiency of the surface modification. Alternatively, a stand could be immobilized that could be used to capture by hybridized, a second dye labelled oligonucleotide or a target strand and a dye labelled complement in a three strand sandwich approach.

All three schemes described above were transferred to direct DPN writing of thioctic acid (and thiol) modified oligonucleotide sequences, resulting in similar strengths of signals from areas as small as 10 x 10 microwells. In an indirect approach the thioctic acid NHS linker could be written directly by DPN (with similar flexibility to mercaptohexadecanoic acid) and deposition of the primary capture strand could be achieved by NHS coupling to the 3' primary amine. However in this case it was necessary to process the written features rapidly as the exposure to water vapour in the DPN process could lead to hydrolysis of the material rendering it ineffective. A number of alternative bespoke surface chemistries, suitable for DPN, are currently under investigation and will be reported shortly.

DPN writing was performed in single surface microwells (~1.3 μm diameter) to establish the whether effective SERRS, suitable in magnitude and spectral resolution, could be obtained the smallest features on the surface (shown below in figure 1). DPN writing of DNA arrays prepared in this manner could also scaled up using multipen arrays to cover larger areas (up to 800 x 800 μm).

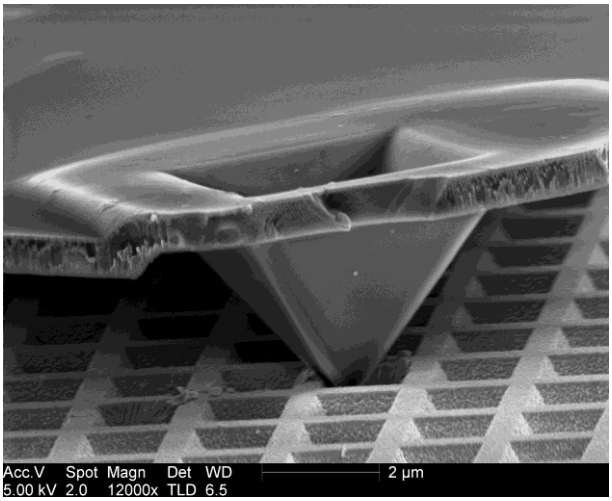

Figure 1 A-frame tip interaction with (Klarite™) SERS surface demonstrating how single plasmonic microwell can be selectively functionalized by DPN.

3.2 Spectroscopic detection by SERRS

The high sensitivity of the SERRS method allowed detection of dye-labeled oligonucleotide capture from single plasmonic array "pixels" ~1 μm^2 in area (examples of which are shown unprocessed in figure 2). Additionally, the information rich nature of the SERRS spectrum allows multiple levels of detection capability to be embedded into each pixel, further increasing the information depth of the array. Individual spectral components from dyes similar in structure (such as Cy3.5™ and Cy5™) could be easily identified.

In idealized conditions, a large number of dye reporters resonant at the red and near IR could be written by DPN and identified using SERRS. These included IR700, Bodipy

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650, Cy3.5™, Cy5™, Cy5.5™, ROX, TAMRA and a number of modified squarylium materials. Using suitable combinations of dye labels wavelength selectivity could be observed e.g. Cy3.5™ (λ_{ex} 632.8 nm) and Cy5.5 (λ_{ex} 785 nm). Therefore, effective practical application of multiple target detection by SERRS in a plasmonic array format could be demonstrated. Although standard Klarite surface were studied in this case the versatility of the surface plasmon derived SERRS response can be further optimized by tuning the aspect ratio of the surface features. Spectral differentiation could between features could be achieved either by curve fitting of multi-component analysis of each spectra.

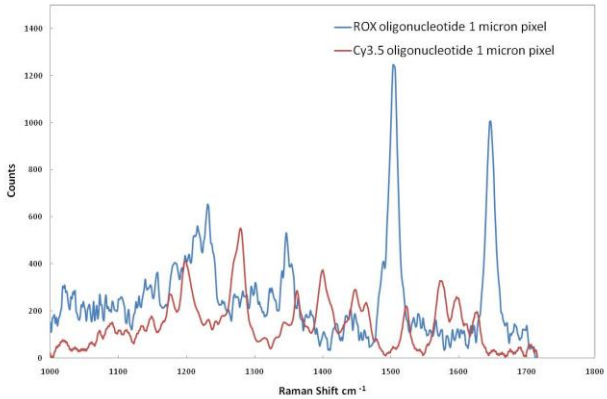

Figure 3 Unprocessed SERRS spectra obtained from ROX and Cy3.5™ dye-labelled oligonucleotide sequences written into single plasmonic microwell.

To demonstrate that the massively scalable potential of DPN written arrays[12] is not fundamentally incompatible with SERRS rapid multi wavelength SERRS readout of array features and arrays was performed. Fast SERRS mapping on the surfaces was achieved using a Streamline™ Raman system (Renishaw, UK). Optimization of the system and procedure allowed large areas of the slide to be analyzed quickly. The whole surface of a Klarite™ slide (4 mm x 4 mm) could be examined in under 10 minutes using 200 μm steps. This allowed the location of positive control areas to be quickly identified. Areas of interest could be examined at the maximum resolution for the surface (i.e. 1.3 μm steps) using higher power magnification (although the minimum step size of the could be as small as 100 nm). At higher magnifications a typical 25 x 25 μm array area could be scanned in high detail within a few minutes. The use of controlled surface chemistry results in highly efficient SERRS, which in turn, allows the spectrometer to operate at the shortest effective accumulation time per pixel. This is key to rapid scanning times and the use of low laser powers to avoid sample damage.

The Streamline™ mapping system works by line focusing the laser incident on the surface and rapidly scanning simultaneously collecting spectra from the whole area of the charge coupled device, collecting ~1000 spectra per minute. The method is naturally suited to the Klarite surface as plasmon derived enhancement is obtained from areas adjacent to each individual pixel. The strongest enhancement occurs where a standing wave are set up in the base of each microwell. This means that the use of DPN to write in the base of each well results in an efficient deposition technique in this case as little material is wasted coating surrounding areas.

The fact that the surface plasmons are able to travel some distance across the gold surface means that in order to achieve effective lateral resolution from a single dye reporter it is necessary to leave a spacing of one or two 'blank' microwells between active sites (~5 μm maximum). However, the fact that SERRS provides such distinct spectra from different dye reporters, that can be overlaid whilst retaining a quantitative response, means that array features can be placed by DPN in closer proximity than the optical method would normally allow. Writing DPN features that complement the spectroscopic collection geometry allows the lateral resolution and detection speed to be optimized. Massively parallel DPN has been recently demonstrated, capable of covering areas > 1 cm^2, and the geometry of these MEMS-generated DPN arrays can be tailored to the form factor of detecting substrates. This flexibility is key to enhancing the throughput of this combined technique by many orders of magnitude.

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