Highly sensitive detection of dye-labelled DNA using nanostructured gold surfaces†

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Careful control of surface chemistry results in strong surface enhanced resonance Raman scattering from dye-labelled oligonucleotides assembled on nanostructured gold surfaces, releasing their potential as reliable enhancing surfaces.

Surface enhanced resonance Raman scattering (SERRS) has been demonstrated to be a highly sensitive technique for the detection of dye-labelled oligonucleotides.[1–3] Very high sensitivity has been obtained using metal nanoparticles. This has ultimately resulted in reports of the identification of single dye molecules in "hot spots" of high electric field gradients between nanoparticles.[4] However, in a typical colloidal analysis the process of aggregation is dynamic and it is necessary to control this in order to achieve sensitive, reproducible and linear concentration dependent results.[5] Although nanoparticles of a number of metals have been used for SERRS, in practice, silver and gold are often the most suitable materials.[6] Successful exploitation of the resonance element of the technique is important in obtaining the most sensitive measurements. This relies on good spectral overlap between the resonance excitation profile of the chromophore and the local surface plasmon properties of dynamic aggregates in solution at the excitation wavelength (λ_{ex}).[7]

The use of nanostructured surfaces as SERRS substrates is of great interest as they allow simple direct analysis and could allow SERRS to be applied where careful control of aggregation is not possible. For example, Vo-Dinh and co-workers have utilised a 9 nm thick silver island film to detect the breast cancer gene BRC*A1* by labelling with the dye Rhodamine B using a λ_{ex} of 632.8 nm.[8] This strategy involved the immobilisation of the complementary probe *via* mixed self-assembled monolayers (SAMs) of 1-mercaptoundecanol and 1-mercaptodecanoic acid. Surface attachment was achieved by the formation on the surface of a succinimidyl ester and subsequent reaction with amino modified DNA. The alkyl alcohols were needed in this SAM to act as spacers for the oligonucleotides allowing hybridisation to occur. In this case, silver was used rather than gold due to the higher levels of SERRS enhancement expected using 632.8 nm excitation. However, surface modification chemistry is more developed for gold and the possibility of surface degradation is reduced.

A potential drawback of randomly dispersed nanoparticle-based arrays of this type is the inconsistency of the enhancement factor across the film, resulting in variability in signal intensity. Recently, nanostructured gold surfaces (Klarite^{TM}) have been developed that utilise a standing wave generated in small surface wells ($\sim$1 μm in diameter) to enhance the effective magnitude of the incident and scattered photons leading to a 10^6-fold increase in signal over the bulk Raman intensity.[9] Studies using SAMs of benzenethiol have shown that the enhancement is very reproducible across the whole area of the substrate.[10] Typically the lowest observable concentrations from non-chromophoric species are around 10^{-6} to 10^{-7} M. To investigate the importance of the resonance aspect of SERRS and the attractiveness of these surfaces for bioanalysis, a series of experiments using dye-labelled oligonucleotides were conducted. These were directly analogous to nanoparticle based approaches to allow comparisons to be made.

Examining oligonucleotides labelled with dyes chosen for their spectral overlap with both the properties of the surface and the λ_{ex} resulted in a dramatic, resonance enhanced, increase in signal sensitivity of at least two orders of magnitude. When the dye-labelled oligonucleotide sequences were deposited directly onto the gold surface, clear unambiguous SERRS spectra from TAMRA, ROX, and BODIPY 650 could be recorded over and above any signal from the bases themselves. Matching the dye label ROX (λ_{max} 585 nm) with 632.8 nm λ_{ex} (Renishaw system 1000) gave the most sensitive results for this sequence. Examples of the spectra

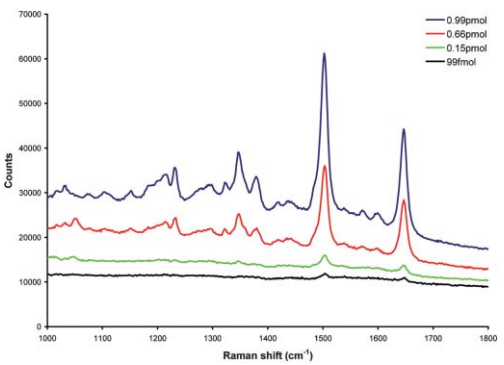

Fig. 1 Spectra of 500 nL oligonucleotide samples labelled with the dye ROX deposited onto Klarite^{TM} substrate. (Renishaw 1000, 632.8 nm λ_{ex}, 2.5 mW, 76% defocus, 1 × 10 s accumulation). No background correction has been applied.

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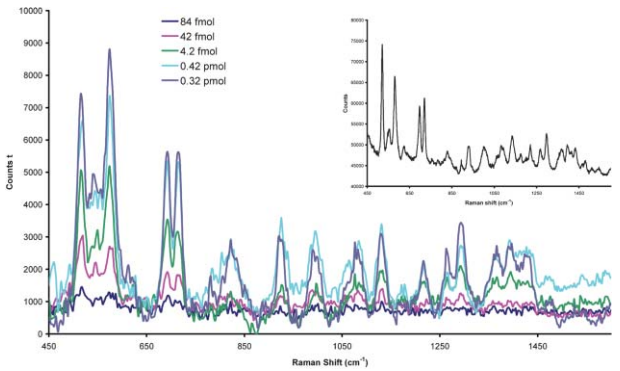

Fig. 2 Spectra of 500 nL oligonucleotide samples labelled with the dye Cy7 deposited onto Klarite™ surface modified with drying wells (Mesophotonics SE1000, 785 nm λ_{ex}, 1 × 10 s accumulation). Background correction has been applied. *Inset*: High resolution spectra of the most concentrated sample (Renishaw 1000, 785 nm λ_{ex}, 1 × 10 s accumulation). Background correction has been applied.

obtained are shown in Fig. 1. The most dilute solution that was detected and identified in this case was $\sim 1 \times 10^{-9}$ M.

The observed signal was spectrally identical to that observed when the same dye-labelled oligonucleotide was analysed using gold nanoparticles. When designing a SERRS analysis careful selection of the chromophore is necessary to achieve the most sensitive results. A large study recently carried out, using citrate reduced gold nanoparticles and a full range of dyes, has indicated that the best resonance enhancement is obtained when the λ_{max} of the dye is within 50 nm of the laser λ_{ex}.[6] Despite this, some benefit would be expected from using a chromophore with an absorbance maximum even further from the laser wavelength as the resonance excitation profile would be expected to decay in a Lorentzian profile. Theoretical and experimental studies of gold nanoparticle-arrays for SERRS have shown that the plasmon resonant frequency should have a maximum in the region between the excitation source and the absolute position of the enhanced Raman line.[11] Spectra could not be obtained when samples of a concentration lower than 10^{-7} M were tested using 514.5 and 406 nm λ_{ex}. Contributions from interband transitions of gold result in higher background and a much lower signal/noise ratio when excitation wavelengths in the green and blue are used (532, 514.5 and 406 nm). Gold surfaces, such as the ones used in this study, have broad plasmon resonances tuned in the red and near infrared wavelength regions (632.8 and 785 nm). The gold substrate plasmon resonance is dependent on the geometry and form factor of the surface texture and can be easily tuned by variation in the shape and depth of the wells.

Matching Cy7 (λ_{max} 748 nm) labelled oligonucleotides to a 785 nm laser (Mesophotonics SE1000 Raman spectrometer) yielded a further increase in sensitivity, when microwell structures were applied to the surface (Fig. 2). This modification may have the effect of concentrating the samples as it dries, allowing solutions as dilute as 1×10^{-11} M to be identified. At the concentrations tested, signals attributed to the DNA bases were not observed.[12]

The sample to sample variation is composed of the variation across the active area of the Klarite (*i.e.* the substrate) and the variability of the sample itself. With a SAM system (*e.g.* benzenethiol), the variability expressed as relative standard deviation (RSD) across the active area is typically 2–3%. Experimentally, the sample to sample RSD could be lowered at a cost of sensitivity by creating a broader laser spot using a beam expander. A trade-off between sensitivity (small spot) and signal consistency (larger spot) has to be established for each sample concentration and in this study it was found that exciting surface plasmons across a number of surface features (1 μm inverted pyramids) achieved the most consistent results.

At higher concentrations, in a drop coat test, the sample appears to dry in a visible 'coffee ring' pattern (when examined under a microscope). This implies that the dye containing component dries unevenly across the surface. This effect explains the higher RSD values shown for drop coat tests shown in Table 1. The majority of the signal probably arises from the first layer of molecules closest to the surface and any subsequent layers would contribute weakly to the SERRS signals and increase background fluorescence. Therefore, the deposition technique is critical in achieving reproducible results when using surfaces of this kind. As the active area has a gold coated surface, thiol based linker chemistries, similar to those used in surface plasmon resonance (SPR) technologies,[13] could be potentially combined with SERRS to improve reproducibility and to add functionality. Successful modification of the surface opens up the possibility of utilising the impressive sensitivity and multiplexing ability previously demonstrated using SERRS.[14]

Modification of the surface using a thioctic acid linker molecule enabled facile immobilisation of DNA capture strands, resulting in dramatically improved signal reproducibility. When dye-labelled oligonucleotides (BODIPY 650) were immobilised by hybridisation onto the surface using a SAM technique, no coloration was observed, yet the SERRS signal was strong and easily identifiable.

Capture experiments were performed by preparing a SAM based on a thioctic acid derived *N*-hydroxysuccinimide-ester linker according to the scheme shown in Fig. 3.

Hybridisation (at room temperature) of a dye-labelled target resulted in a strong SERRS response that allowed identification of the target dye in all tests. Density functional theory (DFT) studies of this linker using the DMol^3 module of Materials Studio (Accelrys, UK) indicate the linker occupies an area on the surface of around 50 nm^2 molecule^{-1}, due to a significant tilt angle. This is

Table 1 Overall sample to sample relative standard deviations observed during this study from samples of low concentration ($>1 \times 10^{-8}$ M)[a,b]

Sample and instrument	Deposition technique	RSD (%)
BODIPY 650 *Renishaw 1000*(50 μm spot size)	Drop coat	28.2
ROX *Renishaw 1000*(50 μm spot size)	Drop coat	34.9
Cy7 *Mesophotonics SE1000*(140 μm spot size)	Drop coat into microwell	26.7
BODIPY 650 *Renishaw 1000*(50 μm spot size)	Hybridisation onto prepared SAM	9.3

[a] SAMs of benzenethiol report RSDs of 2–3%. [b] $n > 10$ in all cases.

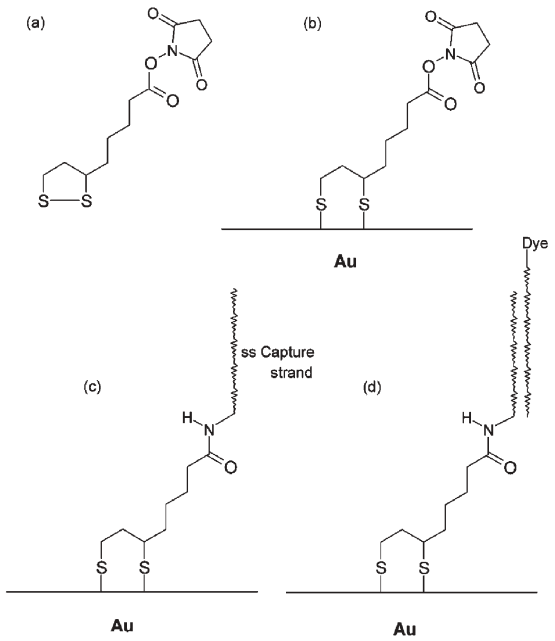

Fig. 3 Modification of Klarite surface with a oligonucleotide capture strand *via* a thioctic acid derived *N*-hydroxysuccinimide–ester linker; (a) 1-{[5-(1,2-dithiolan-3-yl)pentanoyl]oxy}-2,5-pyrrolidinedione, (b) linker bonded to the surface, (c) attachment of capture single-stranded DNA (ssDNA) strand, (d) hybridisation of dye-labelled complement.

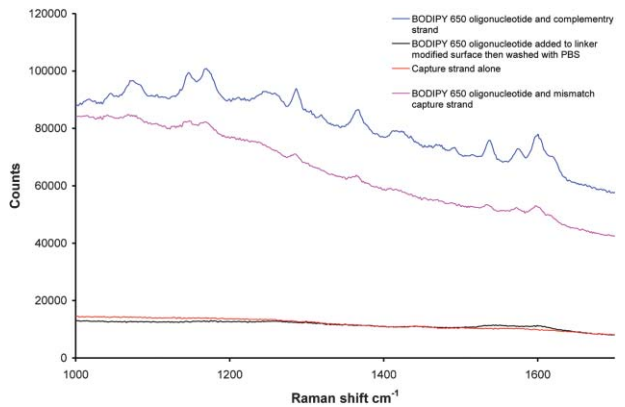

Fig. 4 Spectra obtained from dye-labelled DNA capture experiments (Renishaw 1000, 632.8 nm, 2.5 mW, 76% defocus, 1 × 1 s). No baseline correction applied.

greater than would be expected from a typically ordered alkyl thiol SAM and would explain why the oligonucleotides are not packed closely together to the extent where hybridisation is hindered significantly and a mixed SAM is necessary.[15]

Examples of the spectra obtained from capture experiments are show in Fig. 4. Target signal was not observed when the dye-labelled strand was added directly to the surface and then washed with phosphate buffered saline. As anticipated, a weaker signal can be observed from a SAM prepared with a mismatch strand as some hybridisation will still occur. This could be minimised by the careful selection of experimental temperature to be above the melt temperature (T_m) of the mismatch but below the T_m of the complement. The increased thermal stability of the thioctic acid based linker (with respect to equivalent monothiols) would facilitate this approach.

This series of experiments demonstrate that careful selection and use of a chromophore in experimental design can result in a significant increase in Raman signal from gold nanostructured surfaces in accordance with the resonance selection rules. When analysing samples of a low concentration the sample to sample reproducibility can be increased to an acceptable level by modification of the surface with a prepared SAM comprising oligonucleotide strands. Subsequent hybridisation of a dye-labelled complementary strand results in easily identifiable signals from the dye labels. The linker reported here potentially allows the easy attachment of other amine containing biomolecules to gold surfaces. Combining the effects described above will allow the design of surface based arrays that incorporate the versatility of gold surface chemistry with the sensitivity and multiplexing potential of SERRS.

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