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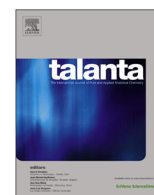

Ultra sensitive label free surface enhanced Raman spectroscopy method for the detection of biomolecules

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ABSTRACT

We present a proof of concept for a novel nanosensor for the detection of ultra-trace amounts of bio-active molecules in complex matrices. The nanosensor is comprised of gold nanoparticles with an ultra-thin silica shell and antibody surface attachment, which allows for the immobilization and direct detection of bio-active molecules by surface enhanced Raman spectroscopy (SERS) without requiring a Raman label. The ultra-thin passive layer (∼1.3 nm thickness) prevents competing molecules from binding non-selectively to the gold surface without compromising the signal enhancement. The antibodies attached on the surface of the nanoparticles selectively bind to the target molecule with high affinity. The interaction between the nanosensor and the target analyte result in conformational rearrangements of the antibody binding sites, leading to significant changes in the surface enhanced Raman spectra of the nanoparticles when compared to the spectra of the un-reacted nanoparticles. Nanosensors of this design targeting the bio-active compounds erythropoietin and caffeine were able to detect ultra-trace amounts the analyte to the lower quantification limits of 3.5×10^{-13} M and 1×10^{-9} M, respectively.

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1. Introduction

Raman spectroscopy is a rapid, non-destructive vibrational spectroscopy that relies on inelastic light scattering by analyte molecules and, therefore, provides a unique spectroscopic signature that potentially identifies the species. Sharp bands of good resolution, which make the technique ideal for distinguishing between closely related molecules, are also characteristic of Raman spectroscopy [1–6].

Surface enhanced Raman spectroscopy (SERS) is a Raman spectroscopic technique that significantly amplifies the inherently weak Raman signal. The phenomenon responsible for SERS occurs at the surface of coinage metals (Au, Ag or Cu), and the principal electromagnetic enhancement mechanism depends upon the nano-scale roughness features of the metal surface. Thus, SERS can potentially be observed either on roughened bulk metal surfaces or on the surfaces of metal nanoparticles (NPs). When incident light interact with the nanoparticles, it may induces collective oscillations of the electrons on the surface of the nanoparticles. These oscillations result in a substantial electromagnetic field known as the Localised Surface Plasmon Resonance (LSPR) [3]. The LSPR decays exponentially with the distance from the NP surface, and is reduced to virtually zero at 10 nm [6]. When the analyte molecule is within the effective range (<5 nm) [6] of the LSPR, a dipole moment is induced in the molecule. This leads to as large as a million fold Raman signal enhancement with a single isolated NP [5]. “Hotspots” are created when multiple NPs reside within each other's effective LSPR range (<1 nm is optimal) [3] and exhibit an extraordinary Raman signal enhancement as large as 1×10^{14}, allowing sensitivity of up to single-molecule detection [6].

Due to its high sensitivity, SERS has the potential to detect many bio-active molecules that normally exist in ultra-trace amounts. For example, SERS has been reported to detect proteins in biological samples [7]. SERS-based methods for protein detection can be divided into two types: (1) label-free and (2) Raman dye-labelled. The label-free strategy traditionally relies on directly adsorbing proteins onto bare metallic substrates and acquiring the vibrational information about the proteins themselves [8–10,11,12]

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by SERS. Raman dye-labelled methods detect proteins indirectly by monitoring the SERS signal of a Raman label (reporter) that is attached to the metallic SERS substrate. Metal NPs that are functionalised with various Raman dyes (Cy3, Cy5, MBA, DSNB, RhB) have been reported to indirectly detect target proteins [8,13,14].

The SERS detection of molecular species that do not have strong affinity for the SERS substrate is problematic. This is particularly true in complex fluids containing multiple species, in which competing moieties with higher affinities than the desired analyte could potentially bind to the substrate to the exclusion of other species. In addition, the competitive binding of multiple species to the metallic surface of the SERS substrate usually leads to a complex SERS spectrum in which the extraneous signals obscure the fingerprint of the target analyte [15]. To prevent non-specific binding to the metallic surface and protect the Raman label from cross-reacting with interfering molecules, SERS substrates used in bioanalytics are frequently coated with a passive layer. This passive layer can be made of polymer or silica, and is usually >20 nm thick [16–19]. The thickness of this layer, however, compromises the Raman fingerprint of the target analyte, and only indirect measurement is available through the detection of the Raman fingerprint of the reporter.

In this study, we developed a novel label-free SERS nanosensor for ultra-trace detection and analysis of both large and small bioactive molecules. Incorporation of an antibody-functionalized thin silica coating eliminates the need to incorporate a Raman reporter, avoids the interfering spectrum from a Raman label, and allows the observation of molecular vibrational information directly from the target analyte.

2. Experimental

2.1. Chemicals and antibodies

Trisodium Citrate, Silver Nitrate, (3-aminopropyl)-trimethoxysilane (APTMS), Sodium Silicate, 3-(trihydroxysilyl)-propylmethylphosphonate (THPMP), Glutaraldehyde and Ethylamine were purchased from Sigma Aldrich. Gold (III) chloride and Caffeine standard were purchased from ProSciTech, Queensland, Australia.

Human Urinary Erythropoietin International Standard (HuEPO IS, 2nd International Reference Preparation) and recombinant Human Erythropoietin International Standard (rHuEPO IS, 3rd International Reference Preparation) were sourced from the National Institute for Biological Standards and Control, UK.

Mouse monoclonal anti-Caffeine antibody (ab15221) was purchased from Sapphire BioScience Pty. Ltd., New South Wales, Australia. Mouse monoclonal anti-Erythropoietin (EPO) antibody (3F6) was purchased from MAIIA Diagnostics, Uppsala, Sweden.

Caffeine-free diet Coca-Cola (Coca-Cola, Australia) and Devondale skim milk (Devondale—MG, Victoria, Australia) were obtained from a supermarket and used to assess the blocking efficiency of the ethylamine blocking agent and the nonspecific binding of the respective antibody-functionalised nanoparticles.

2.2. Synthesis and characterization of antibody-functionalized nanoparticles

The bare gold NPs were synthesised by a modified literature method [20]. Briefly, 88.8 μL $AuHCl_4$ (aq, 0.5 M), 29 μL distilled water, 17 μL $AgNO_3$ (aq, 0.1 wt%) and 2 mL trisodium citrate (aq, 1 wt%) were stirred at room temperature for 3 min, then added to 20 mL of distilled water (95 °C). The mixture was then stirred and refluxed for 5 min to develop the NP colloid. To coat the gold NPs with silica, 131.25 μL of 1 mM aqueous APTMS were added to the previous colloid and stirred at room temperature for 15 min. Eighty-four μL of aqueous sodium silicate (0.54% v/v, pH10) were then added, and the solution was stirred for a further 5 min at room temperature, before raising the temperature to 90 °C and stirring for 20 min [21]. The silica-coated NPs were isolated by centrifugation and washed with ethanol, air dried then resuspended in distilled water.

To introduce terminal amino groups to the silica surface, 1.68 mL of aqueous THPMP (5% v/v) were mixed with the NP solution and stirred at room temperature for 1 h. The THPMP-primed NP solution was then mixed with 8 mL of 1 mM aqueous APTMS (adjusted to pH 5 with acetic acid) and stirred for 30 min at 35 °C. Glutaraldehyde terminals were then attached to the modified silica surface by amide linkage where the NPs were resuspended in glutaraldehyde (10% v/v in PBS (pH 7.4)) and sonicated for 30 min. Following the glutaraldehyde attachment, the NPs were isolated by centrifugation and washed with PBS (pH 7.4), air dried then resuspended in distilled water. The washing procedure was repeated after each of the subsequent manufacturing steps.

To functionalise the NPs with the antibody, the NPs were resuspended in the appropriate antibody solution in PBS (pH 7.4) and incubated at 4 °C overnight to develop amide linkages between the glutaraldehyde terminals and the amine groups of the antibody. The glutaraldehyde terminals serve as linkers between the antibody and the silica shell. To block any remaining unreacted glutaraldehyde terminals, the antibody-functionalised NPs were resuspended in 1.5 mL of 0.7% (v/v in PBS) ethylamine and incubated at 4 °C for 30 min.

2.3. Instrumentation and spectroscopic measurements

The NPs were characterised by UV–visible spectroscopy [Cary 100 spectrophotometer (Agilent Technologies, USA)] and high resolution transmission electron microscopy (HrTEM) [JEOL JEM-2100F TEM (JEOL, USA)].

For the Raman spectroscopy measurements, 10 μL of the analyte (caffeine or EPO) solution were mixed with an equal volume of the relevant antibody-functionalised NP solution. The mixture was then allowed to stand for 10 min for caffeine and 30 min for EPO, respectively. The standing times had been optimised for each analyte to ensure complete binding between the analyte and the antibody (data not shown). The NP solutions were then deposited on microscope slides and allowed to air dry. Raman spectra were collected on the Renishaw in Via Raman Microscope using an excitation wavelength of 785 nm and 5% of maximum (450 mW) laser power. Spectra were collected using a 50× objective lens over a wavelength range from 350 cm^{-1} to 1700 cm^{-1} using 10 accumulations of 10 s exposure times each for a minimum of six independent measurements. To confirm the efficient blocking of the unreacted glutaraldehyde linker and the selectivity of the antibody-functionalised NPs towards the relevant target analyte, 30 μL of either caffeine-free Diet Coke™ or skim milk were added to equivalent volumes of antibody-functionalised NP colloids (anti-caffeine antibody and anti-EPO antibody, respectively). The mixtures were allowed to stand for the optimised binding time, and then the NPs were isolated from solution by centrifugation and washed with PBS buffer until no sign of discolouration and/or cloudiness was observed. The washed NPs were re-suspended in 20 μL of PBS buffer, deposited on a microscope slides, and screened by Raman spectroscopy. These data were compared to the spectra of the relevant un-reacted antibody-functionalized NPs.

3. Results and discussion

The plasmonic properties of the bare and silica-coated NPs were characterised by UV–visible spectroscopy. Both bare and

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