Chemically Bound Gold Nanoparticle Arrays on Silicon: Assembly, Properties and SERS study of Protein Interactions

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ABSTRACT

A highly reproducible and facile method for formation of ordered 2 dimensional arrays of CTAB protected 50 nm gold nanoparticles bonded to silicon wafers is described. The silicon wafers have been chemically modified with long-chain silanes terminated with thiol that penetrate the CTAB bilayer and chemically bind to the underlying gold nanoparticle. The silicon wafer provides a reproducibly smooth, chemically functionalizable and non-fluorescent substrate with a silicon phonon mode which may provide a convenient internal frequency and intensity calibration for vibrational spectroscopy. The CTAB bilayer provides a potentially biomimetic environment for analyte, yet allows a sufficiently small nanoparticle separation to achieve a significant electric field enhancement. The arrays have been characterized using SEM and Raman spectroscopy. These studies reveal that the reproducibility of the arrays is excellent both between batches (< 10% RSD) and across a single batch (< 5% RSD). The arrays also exhibit good stability, and the effect of temperature on the arrays was also investigated. The interaction of protein and amino acid with the nanoparticle arrays was investigated using Raman microscopy to investigate their potential in bio-SERS spectroscopy. Raman of phenylalanine and the protein bovine pancreatic trypsin inhibitor, BPTI were studied using 785 nm excitation, coincident with the surface plasmon absorbance of the array. The arrays exhibit SERS enhancements of the order of 2.6 x 10^4 for phenylalanine, the standard deviation on the relative intensity of the 1555 cm^{-1} mode of phenylalanine is less than 10% for 100 randomly distributed locations across a single substrate and less than 20% between different substrates. Significantly, comparisons of the Raman spectra of the protein and phenlyalanine in solution and immobilized on the nanoparticle arrays indicates that the protein is non-randomly orientated on the arrays. Selective SERS enhancements suggest that aromatic residues penetrate through the bilayer inducing conformational changes in the protein.

INTRODUCTION

There is currently significant interest in methods for reproducibly fabricating well-defined metal nanoparticle arrays for applications ranging from sensing to photonics to surface enhanced spectroscopy.[1,2,3,4] A number of approaches have been explored which lead to stable and highly ordered nanostructured arrays including electron beam lithographic, nanosphere lithographic and chemical deposition methods.[5,6,7] Electron beam lithographic methods are probably the most effective means of achieving a high level of batch to batch reproducibility. However, these "top-down" fabrication approaches require significant infrastructure and do not lend themselves easily to mass production. Direct deposition of nanoparticles from solution onto solid substrates such as glass or metal is an attractive approach but highly ordered arrays can be difficult to create and reproducibility can be a problem.[8] However, Halas and co-workers elegantly demonstrated that 50 nm gold particles protected with cetyltrimethylammonium bromide (CTAB) can be drop-cast onto indium doped tin oxide (ITO) glass slides to produce close-packed, hexagonal monolayers of CTAB-coated gold nanoparticles with sub-10 nm inter-particle spacing.[9] Such gaps between adjacent metallic nanostructures have been found to result in a strongly enhanced interparticle fields upon optical excitation.[10] Reproducible SERS enhancements of up to 8 orders of magnitude for non-resonant chemically adsorbed analytes have been achieved using this approach. However, key issues remain with nanoparticle deposition methods. First, long-term stability may be poor due to self-aggregation and adventitious adsorption of impurities. Second, ordered assemblies over a microscopic length scale may be problematic and can for example be limited by the grain size of the substrate which for example in the case of glass or ITO rarely exceeds a micron or so. Here, we describe an approach in which close to atomically flat silicon is functionalized with a silane containing an available thiol group capable of binding to gold nanoparticles. The objective is to achieve relatively long-range order and stability in nanoparticle arrays for applications such as SERS.

Surface-enhanced Raman spectroscopy (SERS) has been the subject of extensive investigation because of its ability to combine ultra-sensitive detection with structural insights.[11,12] Typically SERS can enhance Raman signals of adsorbed non-resonant molecules by 5 to 6 orders of magnitude[1314]. However, significantly greater enhancements have been reported. The largest SERS signals are believed to come from " hot –spots", spatially localized surface plasmons resonances where the electric field of the exciting laser may experience huge local enhancements.[15,16,17,18,19,20] The main contribution to SERS is thought to be derived from the strong augmentation of the incident electromagnetic field due to resonance of the collective electron excitation localized on roughness features (surface plasmon)[21], while an additional weaker process may arise from resonant charge transfer to surface states from molecules chemically adsorbed onto the substrate. [22,23,24,25].

Whereas Raman spectroscopy can provide powerful insights into protein structure and conformation, proteins generally exhibit poor Raman cross sections necessitating prohibitively high concentrations of frequently very expensive protein for a given experiment. Therefore, surface enhanced Raman spectroscopy holds significant potential for biochemical analysis.[26,27,28] However, in the case of protein analysis, contact of a protein with a metal surface typically induces significant conformational change and even denaturation. One of our current interests is in creating substrates in which the metal surface modified in order to protect the protein structure from direct metal-protein interaction while keeping the protein sufficiently close to the surface to exploit the EM SERS effect.[29]

In the present work, we exploit CTAB protected gold nanoparticle arrays, a potential advantage of this approach is that the bilayer may offer some protection to native protein conformation while producing useful SERS enhancements. The silicon wafer substrate exhibits a strong and easily recognized phonon band at ~ 520 cm^{-1} offering a useful internal frequency, and under certain conditions, intensity, standard. Because in this present case, the gold nanoparticles are chemically bonded to the silicon surface, the arrays have very good long-term stability. In addition, because the silicon wafer is smooth relative to the dimensions of the nanoparticles, the batch to batch variability is very low. Finally, unlike for example glass or FTO, there is no interfering fluorescence background from the silicon substrate and the arrays as synthesized are consequently also free from fluorescence. Although we focus on the use of such substrates for SERS in this contribution, the materials and methods reported may find wider application across plasmonic enhanced sensing from other surface enhanced spectroscopies to fluorescence enhancement.

EXPERIMENTAL SECTION

Chemicals and Materials. Silicon wafers (Sigma), cetyltrimethylammonium bromide- CTAB, L-Phenylalanine, BPTI , 11-mercaptoundecanoic acid, (MUA), methanol, acetone, anal grade, isopropanol anal grade, 3-mercaptopropyl-trimethoxysilane were all obtained from Sigma-Aldrich, N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride (EDC), N,N"-dicyclohexylcarbodiimide and phosphorus buffer were obtained from Fluka. Citrate stabilized 50 or 100nm sized colloidal gold was purchased form British Biocell international (www.bbigold.com). Water (resistivity over 18 MΩ cm) purified using a Milli-Qplus 185 system was used throughout.

Surface preparation and modification. The solid substrate (silicon wafer of dimensions 1cm x 1cm) was cleaned by sequential ultrasonication in methanol, acetone and isopropanol for 15 min each. The wafer was then placed in an oven at 100º C for 15 minutes to ensure it was completely dry. The wafer was then silanized according to the protocol described by Majda et al.[30] Briefly, the cleaned, dried pieces of silicon were immersed in a mixture of water, isopropanol and either (3-mercaptopropyl) trimethoxysilane or (3-aminopropyl) trimethoxysilane and brought to reflux for 10 minutes. The wafer was then extracted with a tweezers, rinsed with isopropanol, blown dry with Ar and then cured in an oven at 107ºC for 8 min. This process was repeated twice.

The (3-aminopropyl) trimethoxysilane modified silicon wafers were then immersed into the 10^{-3} M solution of 11-mercaptoundecanoic acid (MUA) (pH =3.5) in the presence of a mixture of 0.002M EDC and 0.01m NHS (N-hydroxyl succinimide) for 3 hours to peptide couple the amine and MUA (Scheme 1).

The as-received 50 nm diameter colloidal gold solutions contained 4.5 x 10^{10} particles per mL. These particles were cetyltrimethylammoniurn bromide (CTAB) coated and concentrated according to the protocol reported by Wang et al.[31.] Briefly, the colloidal gold was incubated for an hour in a 1:1 ratio with 40 mM CTAB. Following which the mixture was centrifuged down and the solution decanted off, the nanoparticles were then resuspended in milli-Q water. This procedure was repeated twice to eliminate excess CTAB. The resulting CTAB coated gold nanoparticles were centrifuged a final time and resuspended in a volume of water that brought the final concentration to approximately 1×10^{12} particles mL^{-1}. The volume of the colloidal solution required to form a monolayer of nanoparticles on the surface was calculated. Typically, 24 1.5 ml eppendorf tubes were filled with 750µl each of 40 mM CTAB and gold colloid. After spinning and rinsing, the final volume from all tubes was condensed into a single eppendorf tube and brought to a final volume of about 50µl.

3 x 3μL droplets of this mixture were deposited on the silicon pieces and allowed to dry. This was repeated a second time. The substrates were then left for 12 hours under a nitrogen atmosphere at ambient temperature. The wafers were then extensively washed under sonication with propanol and then water. Prior to use the wafers were stored under nitrogen at room temperature, except in studies of temporal stability where they were stored in air at ambient temperature.

For phenylalanine a 0.1 M stock solution was prepared in 0.1 mM phosphate buffer and was subsequently serially diluted down to 60 μM. BPTI from bovine lung was purchased from Sigma and used without further purification. Solutions of BPTI were prepared at 45 mg mL^{-1} in phosphate buffer solution (pH 9.5).

The bare roughened gold substrate was prepared by redox cycling a gold electrode in KCl as reported previously.[32]

Physical Methods. Raman spectra were recorded on an HORIBA Jobin-Yvon Labram HR 2000 confocal Raman microscope using 785 nm excitation from a solid-state laser producing 4 mW of power at the sample. A 10x objective lens was used for SER spectra, and the spectra were acquired from accumulation of two spectra with 10 s integration time each.
Scanning electron microscopy (SEM) was performed with Hitachi S300N scanning electron microscope. An acceleration voltage of 20kV was employed.

Data Analysis. Baseline correction and band component analysis was undertaken using Labspec software (Horiba Jobin Yvon).
Curve Fitting of the Amide I spectral region (1590-1720 cm^{-1}) for BPTI was conducted using Labspec or Peakfit applying a Gaussian Lorentzian function. The band widths at half maxima were set at 25 cm^{-1} corresponding to bandwidth for highly regular α-helix or β-sheet standards reported by Anderson et al.[33] Spectral features between 1590 and 1630 cm^{-1}, not associated with the amide I region were also included in the fit since they shared a baseline with the amide I feature. A three component fit of the amide I band provided the best fit on the basis of visual inspection of the band sum and the fitted spectrum and a squared regression coefficient of R^2 greater than 0.995.

RESULTS AND DISCUSSION

Scheme 1 Schematic representation of the synthetic steps toward assembly of the nanoparticle arrays, where MUA is 11-mercaptoundecanoic acid and CTAB is cetyltrimethylammonium bromide and NHS is N-hydroxyl succinimide.

Fabrication. Scheme 1 illustrates the facile 3-step protocol used to produce the nanoparticle arrays. First, clean silicon wafer was modified via silanization with 3-(aminopropyl) trimethoxysilane. Second, 11-mercaptoundecanoic acid was peptide coupled to the 3-(aminopropyl)trimethoxysilane using N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride (EDC) coupling to produce silicon wafer modified with a long- alkyl chain with a terminal thiol group available to bind to the gold nanoparticles. Finally, CTAB modified gold nanoparticles were drop-cast onto the substrate and allowed to assemble overnight. The substrate was then washed with sonication. A second substrate was prepared in which the silicon wafer was modified with 3-(mercaptopropyl) trimethoxysilane, in order to compare the stability of the films when bound with a short and long chain thiol.[34]

The microstructure of the samples was studied by AFM or scanning electron microscopy (SEM). Figure 1 shows the SEM image of a monolayer array of 50 nm particles immobilized on silicon across a ten micron length scale. This figure shows that there are some areas of the surface that are unmodified but these regions are generally smaller than the spot size used in the Raman microscopy. The extent of continuous defect free coverage depends strongly on the nanoparticle concentration in the deposition solution and a more complete coverage can be obtained by repeating the deposition procedure and by concentrating the nanoparticle solution. If the modified substrates were not sonicated, regions containing multi-layers were observed following nanoparticle deposition, even with extensive washing. Washing coupled with sonication is extremely effective at removing these physically adsorbed layers, leaving only a single monolayer of nanoparticles that is chemically bound to the thiol terminated silicon. The inset shows an image at higher magnification in which individual nanoparticles can be seen. While most regions exhibit hexagonal close packing, there are single point defects and some regions of imperfect packing. This image highlights a key feature of immobilization approaches of this kind, i.e., that the thiol bond formation yields a surface that is stable over long periods (vide infra) because surface diffusion of the nanoparticles is inhibited, but defects anneal slowly.

Figure 1. SEM image of 50 nm CTAB-coated Au nanoparticles array on modified silicon substrate, approx 70µm^2. Insert, expanded region showing hexagonally close-packed monolayer array.

Raman Microscopy. In contrast to isolated 50 nm gold nanoparticles in solution where λ_{max} is approximately 520 nm, UV-vis diffuse reflectance spectra reveals that the 50 nm particle arrays exhibit a broad plasmon resonance band centered at 730 nm. This shift in λ_{max} indicates significant electronic interaction between the nanoparticles due to their close proximity. Given λ_{max} of the array plasmon, 785 nm was chosen as the Raman excitation line in order to achieve surface resonance enhancement of the Raman spectrum.

Figure 2 illustrates Raman spectra were recorded from different, separately prepared batches of Au arrays in the absence of analyte applied to the surface. The spectrum shows modes associated with both 11-mercaptoundecanoic acid (MUA) and CTAB. Typically, spectra were acquired by accumulating two spectra at 10 s integration each from randomly selected regions of the array. Figure 2 shows that 10 modes are observed in the spectral region 450 to 1700, their position and intensity show remarkably little inter-sample variability. For example, the intensity of the N-CH_3 sym bending mode observed at 1475 cm^{-1} varies by less than 5% between the four samples.

The major bands in the SERS spectrum arising from CTAB are located at the 1000-1170 cm^{-1} and 2800-3000 cm^{-1} spectral regions and are dominated by skeletal vibrations of C-C stretching modes and C-H vibration respectively[35,36], which are both sensitive to the conformation of the hydrocarbon chain.[37] In particular, the strongly enhanced feature at 765 cm^{-1} corresponds to C-Br stretching mode, whilst bands at approximately 999, 1040, 1110, 1306, 1476 and 1600 cm^{-1} are attributed to C-C stretching vibrations.[38] The assignments of bands in the 2800-3000 cm-^{1} region are well-established and have been used as a probe for lateral chain-chain interactions and conformational disorder[39]. For alkanethiols, it is possible to distinguish in the spectral region below 700 cm^{-1}, two or more well-separated bands originating from different rotational isomers: gauche at lower wavenumbers and trans at higher wavenumbers.[40,41,42,43] A strong band at 670 cm^{-1} is characteristic of a C-S stretching vibration associated with the MUA trans conformer and a very weak feature also appears at 645 cm^{-1} attributed to the gauche conformation of the S-C-C chain.

Figure 2. Raman spectra acquired from independently prepared 50 nm Au particle arrays. Insert shows the average CTAB spectrum in the range 2300-3400 cm^{-1}. Spectra were normalized relative to the Si intensity and offset on the intensity scale to enhance the clarity of presentation.

The very low relative intensity of this feature suggests that on these substrates MUA molecules exist in highly-ordered trans orientation. The very significant intensity of the C-S band indicates strong interaction between Au and S consistent with intercalation of the long-chain alkanethiol into the array and direct binding to the gold. In addition, a feature at 460 cm^{-1} which is attributed to the Au-S stretch appears for the nanoparticle arrays. Significantly, films formed using CTAB protected gold nanoparticles on a silicon surface functionalized with SAMs of 3-(mercaptopropyl) trimethoxysilane show very little signal arising from the silyl alkane thiol.[34] This observation suggests that these chains are too short to penetrate the CTAB layer and covalently attach the gold nanoparticles' consequently, these films are less uniform after washing/sonication and less stable over time, vide infra.

Nanoparticle Array Stability. A central goal of this work was to produce arrays which are physically and compositionally stable for extended periods. Figure 3 illustrates Raman spectra of the arrays obtained over an 80-day period where they are stored under ambient conditions in air out of direct light between measurements. These spectra show that there is no observable activity change over 40 days but that the signal is reduced by approximately 60% after 80 days. Stability is greater if stored under anhydrous N_2 under refrigeration. This stability contrasts sharply with other common SERS substrates where the spectral intensity has been reported to decrease dramatically over periods as little as a few hours.[44] In the system reported here, the enhanced stability most likely arise because the CTAB bilayer inhibits adsorption of adventitious impurities onto the surface and the chemical bonding of the nanoparticle to the substrate effectively blocks surface reconstruction on the nanoscale. Analogous arrays in which nanoparticles were assembled on silicon modified with a short chain thiol, (3-mercaptopropyl) trimethoxysilane, were stable under ambient conditions for only approximately 10 days.

These arrays were less uniform after washing/sonication and less stable over time, vide infra.[37]

Figure 3. Temporal evolution of SERS spectra of CTAB-coated gold nanoparticles on silicon substrate. Spectra were recorded from fresh sample (a), after 40 days (b) and 80 days (c) and normalized to area under the silicon phonon mode.

Figure 4. Raman spectra of bilayer protected gold nanoparticle arrays on silicon at (a) 20^{o}C (room temperature) (b) 50° C, (c) 90°C.

The effect of temperature on the films was also investigated; Figure 4 illustrates spectra of bilayer protected gold nanoparticle arrays on silicon at different temperatures. The samples were heated at a rate of 5°C/min from 20°C to 100° C. Figures 4a and b show that between 20 and 50° C, some small changes in the SER spectra are observed. Specifically, the intensity of the CTAB bands is reduced relative to silicon and the features are broadened. In particular, the relative intensity of features sensitive to the conformation of the hydrocarbon chain, the C-C symmetric stretch and CH wag modes of the hydrocarbon chain at 999 and 1013 cm^{-1}, change significantly between 50 and 90^{o}C. Overall, these changes are attributed to increasing disorder within the CTAB layers with increasing temperature. These changes were irreversible. On returning to room temperature the spectra were not restored. Conversely, the alkane thiol features remain essentially unchanged up to 50^{o}C. However, at 90°C there is significant weakening of intensity of both the CTAB bands (765, 999, 1110, 1270, 1475, 1535, 1600 cm^{-1}) and the MUA features. For example, Figure 4c shows that the C-S (str) band, 670 cm^{-1}, is significantly reduced in intensity relative to the Si phonon mode. In addition, the feature at 650 cm^{-1}, previously extremely weak, grows in at 90°C, indicative of the gauche conformation of alkane thiol as disorder in the layer increases.

Figure 5. SEM images of CTAB- coated gold nanoparticles on silica substrates taken after heating to 50^{o}C (a) and at 90°C (b).

Alkanethiol SAMs on planar and cluster gold surfaces have been shown to undergo melting prior to desorption within the temperature ranges used here.[45,46] These temperature induced spectral changes are irreversible.

Figure 5 shows SEM images of the array after heating to 50^{o}C and 90^{o}C.[47] Clearly after 90^{o}C there is significant aggregation of nanoparticles accompanied by desorption and multilayer formation. In addition, although the order of the remaining monolayer structures seems relatively unchanged the inter-particle distance appears to be reduced. This is consistent with the Raman spectroscopy and attributed to melting of MUA at high temperature accompanied by increased disorder in the CTAB bilayers.[48]

Interaction of Amino Acids and Proteins with the nanoparticle array.

Phenylalanine was used as a model non-resonant analyte to measure the interaction of aromatic amino acid residues with the arrays, as there was evidence that such residues were strongly interacting in protein, vide infra. Typically, 6 μl of a 6 μM solution of phenylalanine in phosphate buffer solution (pH=7) was dropped onto SERS platform, dried and washed with buffer solution. The surface coverage of amino acid was approximately 1.3 x10^{-10} mol cm^{-2} corresponding approximately to a monolayer.

Solution phase Raman spectra of the amino acid could not be achieved from the 6 μM deposition solution under the spectral acquisition conditions employed for the immobilized material. In sharp contrast, Figure 6b shows a well-defined spectrum is obtained for phenylalanine on the nanoparticle arrays. The solid and SERS spectra show the same features with minor shifts to the frequencies of some bands but also significant enhancement is observed particularly for modes at 749, 805, 847, 982, 1004, 1447, 1555 cm^{-1} and several weaker features (537, 621, 1034, 1215, 1620 cm^{-1}) all corresponding to phenylalanine[49],[50]. Although the background spectrum from the substrate contains features both from CTAB and MUA, it is highly reproducible and these contributions can be subtracted directly from the composite spectrum, without significant distortion of the spectrum.

The observed Raman and SERS bands with their assignment, based on previously published data[37,50], are summarized in Table 1.

Table 1. Assignment of bands observed in Normal Raman and SERS spectra of phenylalanine.

Solid	**SERS**	**Assignment**[38,50]
529m	537w	τ (CO) +δ (C=O)
623s	621w	Phe
752m	758s	δ_b(COOH)
803m	805s	ν(C-C), τ(C-C) and / or r (NH$_3$)
853	847	
	982s	
1006vs	1004s	Phen
1036	1034w	Phen
1218m	1215w	Phen
1357w	1354 s	Phen
1374 w	1372 s	
1450s	1447s	Phen and / or $\delta(C_{\alpha 1}H_2)$
1588m	1555s	Phen and ν_{as} (COOH)
1612	1620w	Phen

Abbreviations: Phe., phenyl ring ; m, medium; s, strong; w, weak; v, very

Importantly, the SERS spectra of phenylalanine on the gold nanoparticles arrays taken at different sites across the substrate were very reproducible. The standard deviation on the 1555 cm^{-1} phenylalanine mode is less than 10% for 100 randomly distributed locations across a substrate of approximately 5 mm^3 area.

We attempted to estimate surface enhancement factors (EF) of these particle arrays using following the relationship:

$EF=(I_{SERS/} I_{Raman}) / (N_{SERS/} N_{Raman})$,

where I_{SERS} and I_{Raman} are the measured SERS intensity of the adsorbed species on the gold nanoparticles and the measured Raman scattering intensity of the adsorbing species in bulk, respectively, and N_{SERS} and N_{Raman} are the number of the adsorbate molecules illuminated by the laser light to obtain the corresponding SERS and Raman spectra, respectively. I_{SERS} and I_{Raman} were measured at 1004 cm^{-1} (ring breathing mode) and N_{SERS} and N_{Raman} were calculated on the basis of the estimate of the concentration of surface species (1.3 $x10^{-10}$ mol/cm^2)[51]**,** density of bulk phenylalanine (0.358 g/cm^3), and the sampling areas (~ 1μm in diameter). On this basis, the calculated EF value is 2.6 $x10^4$. When determining the number of adsorbed molecules, it was assumed that the surface is covered with a complete monolayer of amino acid. This is the theoretical maximum number of molecules, and is likely to be an overestimate, consequently the EF reported is likely an underestimate rather than an over estimate of the actual value. Nonetheless the enhancement is considerably less than that reported for p-mercaptoaniline on CTAB gold nanoparticles on ITO reported by Halas et al, and suggests that unlike p-mercaptoaniline, intimate contact between the phenylalanine and the gold substrate does not occur.[9] Moreover the phenylalanine array spectrum is quite different to previously reported SERS spectra of phenylalanine deposited directly on gold and silver surfaces, where it has been demonstrated that direct surface interactions occur through the amino acid carboxylate.[52,53] In this present instance very strong enhancement of features at 1555, 1447, 1372, 1354, 847, and 758 cm^{-1} are observed, which are attributed to CH def, and aromatic ring stretch and def modes, suggesting penetration of the aromatic moiety into the bilayer and its orientation toward the surface.

Figure 6. Raman spectra of (a) bilayer protected gold nanoparticle arrays on silicon (b) 6 μl of a 6 μM Phenylalanine deposited on the array (background subtracted). (c) Raman spectrum of solid L-phenylalanine

Biological applications of metal nanostructured arrays include sensing and surface enhanced spectroscopy, however, electrostatic interactions between proteins and metal surfaces result in the preferential adsorption of positively charged protein domains onto metal surfaces. This preferential interaction can disrupt the native conformation of the protein fold, with a concomitant loss of protein function. In addition, protein surface interactions can lead to non-random orientation of the protein and although useful in some instances will tend to give a SERS spectrum which is very unrepresentative of the protein in solution.[54] Approaches to preservation of protein structure on metal substrates particularly for SERS, have included the use of a protein compatible film on the SERS substrate and adaptive silver films.[29,55,56] In the latter case metal vapor deposited onto a dielectric substrate restructure on interaction with protein, which is thought to limit alteration to protein structure. Such substrates show excellent signal enhancement permitting Raman spectroscopy of proteins at low concentrations/surface coverages and significant promise for protein detection. However, in previously reported studies the amide I stretch regions has not been investigated making it difficult to determine if the secondary structure of the protein has changed as a result of being immobilized.

We were interested to understand the nature of interaction of protein with the CTAB bilayer protected arrays. In particular, to assess if using a biomimetic bilayer would allow the protein to make close approach without intimate contact with the metal nanoparticles and how this interaction affected protein conformation. We studied the Raman spectrum of bovine pancreatic trypsin inhibitor, BPTI administered to the nanoparticle array to address these issues and, as illustrated in Figure 7, compared the Raman spectrum of BPTI in solution and on the substrate.

BPTI is a small protein consisting of 58 amino acids Six cysteine residues forming three disulfide bridges making it an attractive model system for probing undesirable substrate induced disulfide cleavage to form gold-sulfur bonds.

Key markers of secondary structure of in protein are amide stretching modes; both the amide I (1620-1700 cm $^{-1}$) and amide III (1220-1310 cm $^{-1}$) regions of the Raman spectrum [57].

Figure 7. Raman spectra of (a) bilayer protected gold nanoparticle arrays on silicon in presence of 10 μmol BPTI (background subtracted), (b) Raman spectrum of 45 μmol BPTI solution, (c) SERS spectrum of BPTI on an electrochemically roughened gold electrode.

Figure 7b shows the Raman spectrum of 45 μmol BPTI in phosphate buffer, pH 9.5 administered to the array. Spectral fitting of the amide I component of the spectrum, between 1600 and 1700 cm^{-1}, indicates approximately 28 % α-helix, 26% random coil and 46% β-sheet and turns, this is reasonably consistent with previous values reported from Raman spectroscopy and the small differences can be attributed to the high pH employed in this study.[58]. The Fermi doublet of tyrosine appear in ratio of 1.5 and a medium intensity band for the disulfide bridges appears at 510 cm^{-1} which is indicative of a g-g-g conformation of the CCSSCC dihedral angle as expected for BPTI .

The application of 6μL of 10μmol BPTI to the gold nanoparticle array results in a significant SERS enhancement of protein signal with significant changes to relative spectral intensities compared with solution. In particular modes in the spectral region 1550 to 1670 and in the region 1200 to 1370 cm^{-1} become very intense. These are attributed to aromatic amino acid residues indeed some similarly enhanced features were observed for the phenylalanine spectrum Figure 7, confirming this assignment. The relative intensity of the amide I band is small by comparison with the enhanced features, though it remains reasonably well resolved, curve fitting of the spectral envelope yields 8 % α-helix, 39% random coil and 53% β-sheet and showing α-helix conversion into random coil and β-sheet.[37]

However, Figure 7b shows the 510 cm^{-1} S-S mode is maintained indicating the disulfides do not undergo direct surface binding to the gold nanoparticles. For comparison, Figure 7c shows the Raman spectrum of BPTI directly adsorbed onto, electrochemically roughened Au electrode denatured the protein. This spectrum is dominated by enhancement of directly bound protein domains and little information on protein secondary structure is available. In particular the relative intensity of disulfide bond at 510 cm^{-1} is very significantly reduced and shifted to approximately 521 cm^{-1} indicating cleavage of some disulfides and distortion of the remainder to ggt confomers.[59]

We conclude overall, that the nanoarray substrate modifies the protein secondary structure without causing significant damage to the protein. There are two possible hypotheses for the interaction between BPTI and gold nanoparticles, strong electrostatic binding and CTAB displacement. In electrostatic binding the attraction between charged surface residues, (which is anticipated to be pH dependent, BPTI's isoelectric point is 9.4) of the BPTI and the (oppositely) charged passivating CTAB bilayer on the gold nanoparticle causes strong binding between protein and nanoparticle, wherein, the protein attaches itself to the layer on the gold nanoparticle, with little or no direct interaction between protein and gold surface. Conversely, if displacement is occurring the passivating layer is displaced by the protein allowing direct contact between nanoparticle and the substrate, functional groups derived from lysine (amine), cysteine (thiol), and others can bind directly to the gold surface. Given the partial loss of disulfide on bare gold and the gross structural changes about the remaining disulfides, it is unlikely, that direct surface contact is occurring. Electrostatic binding of the protein to the CTAB is the most likely scenario with, strong enhancement in aromatic amino acid residues due to intercalation of these residues through the layer as observed for phenylalanine. This non-random orientation of the protein is the likely origin of the protein secondary structural modification reflected in the amide I band.

CONCLUSIONS

A facile and highly reproducible synthetic method for formation of ordered 2 dimensional arrays of CTAB bilayer protected 50 nm gold nanoparticles bonded to silicon wafers was described. The silicon wafers were chemically modified with long-chain alkane thiol to which CTAB protected gold nanoparticles were chemically bonded. SEM showed long range order of the arrays and Raman spectroscopy reflected the excellent reproducibly of the substrates both between batches and across a single batch. The arrays also exhibit very good stability, the Raman spectrum of a single given substrate changed by less than 5% over a 5 week period when the substrates were stored under ambient, light free conditions. Under application of temperatures in excess of 50^{o}C the CTAB bilayers were irreversibly disrupted with some nanoparticle aggregation and above 90^{o}C melting of the silicon binding alkane thiol occurred resulting in extensive nanoparticle aggregation. The behavior of amino acid phenylalanine and protein BPTI in contact with the arrays was studied using Raman spectroscopy. The SERS spectrum of phenylalanine was quite different to those reported previously for this amino acid on bare metal and was attributed to intercalation of the aromatic head group into the CTAB bilayer. An enhancement of 2.6 $x10^{4}$ was calculated for the phenyl mode at 1003 cm^{-1}. Similarly, BPTI did not appear to make intimate contact with the gold nanoparticles, and the cysteine disulfide bonds were preserved, unlike BPTI in contact with bare gold, where gross structural changes were evident. However significant enhancement of aromatic amino acid residues indicate strong intercalation of these residues through the CTAB bilayer. Conformational changes to the protein lead to an increase in disordered random coil conformation which is attributed to strong electrostatic interactions between the protein and CTAB bilayer. Investigations are currently underway to investigate the effect of protein charge on conformational change induced by the CTAB bilayer.

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