Rational design of plasmonic nanostructures for surface enhanced spectroscopy

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NANYANG
TECHNOLOGICAL
UNIVERSITY

**RATIONAL DESIGN OF PLASMONIC
NANOSTRUCTURES FOR SURFACE
ENHANCED SPECTROSCOPY**

ZOU CHANGJI

**SCHOOL OF MATERIALS SCIENCE AND
ENGINEERING**

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Doctor of Philosophy

2014

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ABSTRACT

Plasmonics has emerged as a promising and extensively researched field due to its advantages of confining electromagnetic radiation by metal nanostructures and has found wide applications including sensors, wave guides, solar cells, lithographical methods, etc. However, the current applications related to the biological system are still limited, and possess tremendous possibilities and great potential to improve the performance. This dissertation focuses on the rational design and development of plasmonic nanostructures that can push the limit of its plasmonic application, especially related to surface enhanced spectroscopies, as they are widely adopted for useful analysis of different biological components. Through such approach, improvements can be achieved for the enhanced signal to noise ratio, additional information for spectroscopic interpretation, or potentiometric range for analysis, which builds up the platform to extend to other related systems as well.

Firstly, a simple strategy based on the synergistic modulation of inter-particle and substrate-particle interaction has been applied to the large-scale fabrication of two-dimensional (2D) Au and Ag nanoparticle arrays. By controlling the electrical double layer thickness of the colloidal particles and lowering the basicity of the aminosilane modified substrate, the nanoparticle arrays could be formed with a wide range of inter-particle distances, and tunable plasmonic properties. Methylene blue was selected to demonstrate that these arrays can be employed as wavelength-selective substrates for multiplexed acquisition of surface-enhanced Raman scattering (SERS) spectra and metal enhanced fluorescence (MEF) spectra.

Cytochrome c is a redox protein that plays a crucial role in the electron transport within the respiratory chain. SERS spectra and surface enhanced infrared absorption spectra (SEIRAS) were both collected for cytochrome c adsorbed on two layers of gold nanoparticle modified surface. External potential was applied to cytochrome c electrochemically and changed its redox state. During this process, the conformational change was monitored and analyzed by two-dimensional correlation spectroscopy which combines both the IR and Raman spectra. The method of 2D correlation analysis is very powerful to convolute spectra with overlapping bands. There are many approaches especially in IR for deconvoluting bands such as phase sensitive detection (PSD), etc., but there is no method yet available to deconvolute both Raman and IR and simultaneously plot them together in one spectrum. This is important since IR and Raman are methods which are complementary to each other. Through 2D correlation analysis, the components that undergo conformational change can be highlighted, and their sequential relationship was also identified.

Roughened silver surface prepared by electrochemical oxidation and reduction cycles was also used to study cytochrome c with its B band resonance before. But one limitation for the silver substrate is that it can be easily oxidized in the electrochemical environment and hinders its application. To address this challenge, a thin gold layer was sputtered onto the colloidal assembly of silver nanoparticles on the silver substrate to increase its potentiometric range. Good enhancement factor and resonance condition from the silver structure were also maintained as revealed from the SERS spectra of its potential titration.

In conclusion, rational design of plasmonic substrates has been tailor to achieve the targeted application with different biological molecules. Tunable

plasmonic resonance, two dimensional correlation analysis, and extended potentiometric range have been realized to extend the existing platforms for better performance, more information that can be obtained, and wide application.

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Chapter 1 INTRODUCTION

1.1 Introduction to Plasmonics

1.1.1 What is Plasmonics

Plasmon is the collective oscillation of free electrons between the metal and dielectric interface, excited by the electromagnetic waves. It gives the noble metals such as gold and silver their unique properties of confining or amplifying light at the scale that breaks the refraction limit of conventional optics. There have been tremendous applications that can be developed from this simple light-metal interaction phenomenon, and contribute to the field now known as plasmonics.

As shown in Figure 1-1, plasmonics can be basically classified into two types: localized surface plasmon resonance (LSPR) and propagating surface plasmon (PSP). For a localized SPR (Figure 1-1A), the dimension of the nanoparticles or nanostructures is much smaller than excitation wavelength. The electrical component of the electromagnetic radiation will exert a force on the negatively charged conduction electrons, and make them oscillate back and forth in the alternating manner. When the excitation frequency matches the resonance condition of the metal electrons, the light will be localized and great amplification of the signal can also be achieved to serve as the mechanism of certain surface enhanced spectroscopy effect.

For the propagating surface plasmon, it is generated by coupling the incident electromagnetic wave to the metallic surface (usually Au, Ag, or Cu) or nanowires through prism, waveguide or grating coupler.¹ The propagation of the plasmon is in the x and y directions, and the intensity will decay exponentially in

the z direction to the dielectric for about 200 nm.²⁻⁴ The resonance condition is very sensitive to the dielectric environment and surface topography, hence it can be utilized for SPR sensing to achieve very good sensitivity.

Figure 1-1 Schematic illustration of (A) localized surface plasmon resonance and (B) a surface plasmon polariton.⁵ Reproduced with permission from Reference 5.

1.1.2 Synthesis And Assembly Of Plasmonic Structures

In order to utilize the useful properties of plasmonic interactions, different sizes, shapes, and pattern of the nanoparticles or assembly have to be achieved and controlled in the precise manner. And there has been tremendous work that has been dedicated to the development of this field. Basically it can be categorized into two approaches: bottom up and top down, each with their own advantages and limitations. For the bottom up approach, it means that plasmonic nanoparticles of different size, shape, and composition can be achieved by chemical synthesis. The advantage of this approach is that large scale of nanoparticles can be synthesized in solution with good control of uniformity and reproducibility. Basically, it is not time-consuming and doesn't require sophisticated equipment.

As shown in Figure 1-2, several common nanoparticle shapes are listed according to their evolution pathways.⁶ For gold and silver, their plasmonic

behavior is relatively strong, hence is mostly explored. As a general means, the precursor of the metal atoms are introduced into the solution in their ionic form. Certain reducing agents, such as sodium citrate are then introduced to reduce the metal precursor to their elementary form. Due to the excess of the metal atoms in the solution, they have to nucleate to reduce the energy level they possess, and to form a cluster of atoms called seeds. During the nucleation process, if no imperfection is introduced, it will form single crystal seed. Then capping agents were introduced to selectively adsorb on the metal cluster facets. Due to the hindrance of incoming metal atom during the seed growth, different facets of the nanoparticles will have different growing speed. As a result, different shapes of the nanoparticles can be achieved. On the other hand, if lattice imperfection was introduced at the seeding stage, twinned plan can be formed and to develop more sophisticated structures like beams, pentagonal rods, plates, etc.

Figure 1-2. Evolution pathways of different nanoparticle shape synthesis.⁶

Reproduced with permission from Reference 6.

The significance of forming different shape of the nanoparticles is that the resonance condition can be tuned with different shapes, hence modes of the plasmon.^{7, 8} This is beneficial when the resonance of the nano structures can be in the same range of the excitation wavelength, hence the maximum of the plasmon signal can be achieved. Moreover, by controlling the growth time of the nanoparticles, the size of them can also be varied, which is in relation with the fact that different modes of plasmon oscillation can be supported. Generally, when a particle is small, it can only support single mode of plasmon, and the extinction behavior is dominated by absorption. But when the particles grow bigger, multiple modes can be supported as characterized by multiple peaks or broaden peaks in the extinction spectra, and the scattering component becomes dominant other than the absorption part. The chemical synthesis of nanoparticles not only enables us to

gain more understanding of the fundamental physics and chemical in the process, but also play an important role in the plasmonic property achievement and in the possible assembly process.

Besides the bottom up approach, the top down approach is also required in certain scenarios of applications. It basically means the lithographic method, such as photolithography, e-beam lithography, nanosphere lithography etc. (Figure 1-3) As compared to chemical synthesis, the lithographic method is better utilized to develop patterning on the surface, and to achieve repeating nanostructure assembly with excellent uniformity. It also, on the other hand, is more time-consuming and more expensive since special instruments have to be involved. In order to combine the benefits of both two approaches, hybrid method that integrates the bottom up and top down approaches were also used to generate desired nanostructures for specific applications.^{9, 10}

Figure 1-3 Lithographic nanofabrication for large-scale arrays of nanostructures.(A) Nanosphere lithography.¹¹ Reproduced with permission from Reference 11. (B) E-beam lithography.¹² Reproduced with permission from Reference 12.

1.2 Application of Plasmonic Platform to Biological Related Field

In this thesis, the focus will be in the field of how to utilize the plasmonic platform to meet the challenges in the biological field. Extensive research effort has been carried out mainly in three application fields: sensing, imaging and therapy. Plasmonics has also contributed to other fields such as wave-guiding¹³, super lenses¹⁴, active plasmonics¹⁵, etc. which are beyond the scope of this thesis. Several excellent review articles have been published to discuss the achievements and progress of the study, and the future directions and challenges.¹⁶⁻¹⁸

1.2.1 Biosensing

Biosensing is the important and fundamental step for the diagnostic of a disease, and provides valuable information for the treatment of the patient. There are usually a group of requirements for a good biosensor and diagnostic technique including sensitivity, selectivity, cost, time of testing, ease of storage and accessibility, etc. Some of the traditional sensors' performance still needs improvement. For example, ELISA has been established a gold standard for immunoassay testing, but it requires relatively long time since multiple incubation and washing steps are involved. Some lateral flow assays have been designed for rapid test, but the sensitivity was not as low as ELISA. For nuclear acid testing, Polymerase chain reaction (PCR) was very effective and has good sensitivity, but it requires sophisticated instruments which are not always available in some developing countries and point-of-care settings. Hence, novel materials and sensor design have been continuously explored to push the limit for the current sensor performance.

Molecules and peptides are relatively small, and may not have direct biological functions. But they are extensively studied for plasmonic materials

mainly due to their simplicity as a model molecule, ease to be fabricated by design. Surface-enhanced Raman spectroscopy has been extensively studied as a direct application for plasmonic materials. Several Raman and fluorescence dyes such as methylene blue¹⁹, para-mercaptoaniline (pMA)²⁰, Rhodamine 6G²¹, perylene tetracarboxylic diimide (PTCDI)²² were used due to their conjugation capability with gold or silver surface. As shown in Figure 1-4, it shows methylene blue to be used as involving in antimalarial drugs.²³

Figure 1-4 Methylene blue (MB) as a redox-cycling phenothiazine drug in vivo.²³
Reproduced with permission from Reference 23.

For the other biologically relevant molecules, such as dithiol molecules including glutathione and cysteine, LSPR refractive sensing was used to detect them based on gold nanorod.²⁴ However, one drawback for such small molecules is lack of selectivity, since there is no specific receptor for them, unlike antibody/antigen and biotin/streptavidin. For the other special group of biomolecules, peptides (simply a chain of amino acids), they do not necessarily possess biological function, but due to their capability to be custom engineered, they are also used extensively for plasmonic study. Oligopeptides were combined with gold nanoparticles to achieve the colorimetric detection of Mercury ions.²⁵ In

the other report, peptide engineered gold nanoparticles were designed to induce reversible aggregation upon the presence of Zn ions.²⁶

Antibody and antigen are important proteins that can function as receptors in the cell membrane, trigger for immune response or signal regulator for biological functions. Some antibody/antigens are identified to be biomarkers for pathogenic study, diagnostics or target for target therapy, such as HER2 for breast cancer²⁷, PSA for prostate cancer²⁸, ADDL for Alzheimer's disease²⁹, etc. For the DNA, it is closely related with gene transcription, translation, etc., which gives it biological importance. Also due to technological maturity for DNA design and synthesis, they are used extensively for complementary DNA detection or structural design. As shown in Figure 1-5, cytochrome c can adopt different reorientations on different SAM layer on the gold electrode.³⁰

Figure 1-5 Different orientations of cc absorbed to differently modified gold surfaces. A: cc absorbed on a SAM of MUA (11-mercaptoundecanoic acid). B: cc absorbed on a SAM of 2-mercaptoethanol.³⁰ Reproduced with permission from

Reference 30.

Numerous analytes have been detected using plasmonic structures and substrates. They are mostly based on three basic sensing principles: refractive

index sensing³¹, plasmonic NP coupling³², surface enhanced spectroscopy^{33, 34}. Different sensors can be developed based on the same principle. The challenge may be how to smartly design your system for real applications, such as clinical diagnostic and device making. For the ultimate single molecule detection, the low signal to noise ratio is a challenge, but this may not be necessary for biomedical applications. In order to perform sensing in the physiological condition, the visible light scattering from the biological tissues should be avoided, which makes the nanorods or nanoshells attractive candidates since they have the resonance condition in the near-infrared range. One other critical issue is to prevent unspecific binding other than the desired target in the complex physiological fluids. One recent example has been attempted to use gold nanorods for hepatitis B detection.³⁵ The HBs antigen was detected by conjugating the antibody to the gold nanoparticle, and then the surface was blocked by BSA to prevent unspecific binding. As shown in Figure 1-6, with the presence of the analyte, the resonance of the gold nanorods exhibited a red shift ranging from 4.3 to 30 nm, to distinguish from the negative controls.

Figure 1-6. (A) TEM of gold nanorods for hepatitis B detection. (B) UV-Vis spectra showing a red shift with the addition of the analyte.³⁵ Reproduced with permission from Reference 35.

1.2.2 Imaging

The ability of the plasmonic materials to scatter light in the visible and near-infrared region has also been utilized for imaging diagnostics. One of the most commonly utilized techniques is the dark field imaging which can be used to track and locate a series of biological process including cancer cell imaging^{36, 37}, cell mitosis and cell division³⁸, etc. However, most of the imaging techniques have been performed in *in vitro*, since a good contrast can only be achieved when the particle size is larger than 100 nm. On the other hand, it has been tested that the particle size 20 – 70 nm would give the optimum blood circulation time, and higher uptake and localization in tumors. To address this challenge, large density of small particles can be introduced which can be aggregated to increase the scattering efficiency, but higher density of nanoparticles would also increase the toxicity and cell damage, which prevent this technique to be applied *in vivo*. As shown in Figure 1-7, the uptake of the plasmonic nanoparticles could enhance the

contrast of the imaging, but too high concentrations (0.4 nM in this case) of the nanoparticles would decrease the clustering and increase the rate of cell death.³⁹

Figure 1-7. Still pictures taken at 0 and 22 hours of HSC-2 cells incubated with 0, 0.05, 0.1, 0.4 nM nuclear-targeting AgNPs.³⁹ Reproduced with permission from Reference 39.

Besides the direct observation of the plasmonic nanoparticles for imaging, the coupling of the plasmonic particles could also be utilized and observe some unconventional phenomenon that cannot be studied by traditional approaches. For example, Ag dimer was used to probe the compartmentalization dynamics of cells as plasmonic ruler.⁴⁰ pH sensitive molecules were modified on nanoparticles and introduced into the cells to measure the localized pH change as shown in Figure 1-8.⁴¹ Cells are also grown on Ag island film for surface enhanced fluorescence study.⁴² So far, plasmonic materials are mainly used for imaging or concept demonstration. The challenge may be how to correlate the data with the real biological function mechanism.

Figure 1-8 Probing and imaging pH in single live cells after 60 min of incubation time with SERS nanosensors. ⁴¹ Reproduced with permission from Reference 41.

Other imaging techniques have also been made possible by utilizing the plasmonic nanoparticles. For example, the tumor cell can be imaged by Raman spectroscopy with the potential of multiplexed labeling and detection by conjugating different Raman dyes.⁴³ Sometimes even passive accumulation (meaning no antibody conjugation, hence no bio-specificity) was used to increase the contrast. For example, the nanoshells can scatter more in the near-infrared region than the normal tissues, hence can be used for Photoacoustic Tomography in the Rat Brain⁴⁴, or optical coherence tomography (OCT) for photothermal cancer therapy⁴⁵. To further enhance the capability of nanoparticles for biomedical use, the nanoshells were combined with iron oxide nanoparticles and clinically proven fluorescence dye ICG to achieve specific targeting (through antibody conjugation), imaging with increase contrast (NIR scattering of nanoshells), photothermal therapy (NIR absorption), as well as increase contrast for MRI imaging (through iron oxide NPS) and fluorescence imaging (through fluorescent dye). The current challenge may be the clinical trial for such products such as immuno-response, dose effect, toxicity test, etc. The technology for the materials

platform has already been developed, unless the absorption or scattering cross section can be greatly improved to increase the efficiency.

1.2.3 Therapy

Biosensing is an important step for the diagnostics of the disease, but to cure the illness, therapy is also required where plasmonic materials have made several contributions to this field. The most widely used method is the photothermal therapy, which utilizes the non-radiative property of the plasmonic nanoparticles. Besides absorption and scattering of light, the plasmonic materials could also induce heating around them. Once the temperature increase is highly localized around the nanoparticles, tissue (usually tumor) ablation could be achieved. For example, the photothermal response based on gold nanoshells has been extensively studied and was also under FDA trial in the past decade.¹⁸ Specific antibodies have been conjugate to the nanoshells against the human brain tumor cell lines. Such test has been done in vitro with good sensitivity and selectivity for complete removal of cancer cells at pM level of nanoshells.

One other therapeutic application of plasmonic materials is drug delivery both in vitro and in vivo. Recognition ligands and cytotoxic drugs can be conjugated to the surface of the plasmonic nanoparticles. Certainly polymer layer could also be introduced as protection or for controlled release of the loaded drugs. Depending on the size of the nanomaterials and drug molecules, the circulation half-life could be shortened to enable rapid clearance by the renal systems. Porous structures are usually desired for the drug loading and some of the carrier design involves magnetic materials to control the localized delivery.¹⁸ Xia and coworkers made use of nanocages coated with poly(N-isopropylacrylamide) (pNIPAAm) to modulate the drug release upon the stimuli of light or temperature.⁴⁶ Nanorods

coated with mesoporous silica shell and poly(NIPAAm-co-N-hydroxymethyl acrylamide) were also loaded with doxorubicin (DOX) for the use in chemophotothermal therapy in vitro with glioma cells.⁴⁷ The other drug release stimuli also include pH, ion level, mechanical strain, etc.¹⁸

Figure 1-9. Schematic illustration (A) and TEM characterization (B) of the controlled-release system.⁴⁶ Reproduced with permission from Reference 46.

Gene therapy has also been successfully demonstrated to improve the performance with the help of plasmonic nanomaterials. This is promising to be an alternative to the current chemotherapy and opens the possibility for personalized medicine. The conjugation on the plasmonic nanoparticles surface plays an important role in the transfection efficacy in the cellular environment. To achieve this, poly(ethyleneimine) (PEI) was assembled layer-by-layer on AuNP surface to enhance the cell uptake and prevent aggregation.^{48, 49} The size and shape of the nanoparticles also affect the delivery efficiency across mammalian cells. For the commercial aspect, Mirkin's group has developed several products based on their core competency. Polyvalent oligonucleotide-gold nanoparticles were used for

intracellular gene regulation.⁵⁰ High density lipoproteins (HDL) were synthesized with cholesterol binding ability to control the coronary heart disease (CHD).⁵¹ Since their products are based on spherical gold nanoparticles, which are only utilized for the size control capability, surface chemical binding capability, and fluorescence quenching capability (whose mechanism is often not explained in the plasmonic way). They are not actually used for their plasmonic properties, since their resonance peak is around 520 nm, which will be mainly scattered by biological tissues. Some of them still need to be improved for real clinical applications.

1.3 Surface Enhanced Spectroscopy

1.3.1 Surface-Enhanced Raman Spectroscopy

Spectroscopic methods have useful to characterize chemical or biological molecules based on their energy level and corresponding molecular characteristics. The Raman process is complementary to the infrared process but with different selection rules. However, the scattering cross section of the Raman process is much smaller as compared to infrared spectroscopy, which hinders the development and application of this technique.³¹

In 1977, Richard Van Duyne discovered that the molecules adsorbed on the roughened silver surface gave tremendous Raman signal, much larger than their surface area proportions.⁵² They proposed that the enhancement came from the amplification of the electromagnetic radiation due to the roughened silver surface. It was later found that the shape tip of the nanoparticles can also behave as an antenna to concentrate the light through

the “lightening” effect. More often, the coupling between two or more nanoparticles were identified to give tremendous enhancement of the incident laser intensity. By some means, even single molecule detection was possible through surface enhancement Raman spectroscopy. In vivo SERS measurement was also developed for non-invasive diagnostics as shown in Figure 1-10.⁵³

Figure 1-10 Experimental setup used for in vivo SERS measurements in rats. CCD, charge-coupled device; BP, band-pass.⁵³ Reproduced with permission from Reference 53.

1.3.2 Surface Enhanced Infrared Absorption Spectroscopy

As one other important branch of the surface enhanced spectroscopies, surface enhanced infrared absorption spectroscopy also utilizes the confinement effect of the plasmons and amplifies the infrared signal as compared to the conventional setup.^{54, 55} For example, attenuated total reflectance (ATR) has been used in conjunction with the infrared spectroscopy to increase optical path length through which the IR beam can interact with the analyte at the interface, and proved to be beneficial for investigating biological samples.³⁰ A prism is usually coated with a layer of gold which serves the layer to support the plasmon. Then the laser excitation

is coupled to the prism and to induce the plasmon generate through the effect of total internal reflection. This experimental setup is very useful for the flow system analysis of biological analyte adsorption and desorption process to characterize the binding constant and kinetics, etc.^{30, 56} The side of gold layer is easily accessible for various chemistries to anchor the acceptor molecule, which the analyte molecule can be introduced through the flow channels, which can induce a change in the resonance angle and to achieve very low detection sensitivity. The gold layer can also be used as an electrode for spectro-electrochemical applications.⁵⁷ Artificial membrane system could be built through his tag chemistry and the quality can be checked by impedance spectroscopy.⁵⁸

1.3.3 Metal Enhanced Fluorescence

Metal enhanced fluorescence is also explored as the plasmonic platform is combined with the fluorophore for enhancement of signal generation.^{59, 60} Lakowicz et al proposed that for metal enhanced fluorescence, there would be an additional pathway for the excited electron to return to the ground state.⁶¹ As a result, the lifetime of the fluorescence process will be shorted, usually as a complementary proof of the enhancement effect of plasmonic structures.

Figure 1-11. Modified Jablonski diagram in free space and in the presence of a metal.⁶² Reproduced with permission from Reference 62.

As shown in Figure 1-11, E and E_m represent the excitation rate without metal and the metal-enhanced excitation rate; Γ is the radiative rate of the fluorophore; Γ_m represents the radiative rate due to the metal proximity; k_{nr} is the non-radiative rate. In the absence of metal, the quantum yield Q_0 and lifetime τ_0 are represented by:

$$Q_0 = \Gamma / (\Gamma + k_{nr}) \quad \tau_0 = (\Gamma + k_{nr})^{-1}$$

When a fluorophore is near a metal surface, the quantum yield Q_m and lifetime τ_m are represented by:

$$Q_m = \frac{\Gamma + \Gamma_m}{\Gamma + \Gamma_m + k_{nr}} \quad \tau_m = (\Gamma + \Gamma_m + k_{nr})^{-1}$$

Hence as the additional radiative rate Γ_m increase, the quantum yield increases while the lifetime decreases. There is also a distance regime that distinguishes the enhancement and quenching phenomenon on the scale of tens of nanometers. If the molecules are closely attached to the surface, it would fall into the fluorescence quenching regime, while beyond that, the fluorescence enhancement will take into effect.⁶³

1.4 Two Dimensional Correlation Spectroscopy

1.4.1 Definition

Two dimensional NMR spectroscopy has been established in the 1970s to provide more information than one dimensional NMR spectroscopy. In analogue to that, Noda proposed a generalized method to correlation spectroscopic data in a two dimensional way as an extended tool for more complicated analysis. As a general scheme, the study system can be subject to a series of external perturbation including mechanical force, temperature change, potentiometric titration, etc. Then the change of the system is probed by spectroscopic way such as infrared, Raman or UV-Vis spectroscopy, etc. Consequently, the generated series of spectra is called dynamic spectra $\hat{y}$, and defined as:

$$\tilde{y}(\nu, t) = \begin{cases} y(\nu, t) - \bar{y}(\nu) & \text{for } -T/2 \leq t \leq T/2 \\ 0 & \text{otherwise} \end{cases}$$

where y is the raw spectra and $\bar{y}$ is the reference spectra. The change of the sample typically occurs in the cycle such as during a redox change, a sinusoidal mechanical variation, etc. The reference spectra can be taken as the time averaged spectra or the a representative static spectra. Then, the dynamic spectra is Fourier transformed to generate the spectra $\hat{Y}$.

$$\begin{aligned} \tilde{Y}_1(\omega) &= \int_{-\infty}^{\infty} \tilde{y}(\nu_1, t) e^{-i\omega t} dt \\ &= \tilde{Y}_1^{\text{Re}}(\omega) + i\tilde{Y}_1^{\text{Im}}(\omega) \end{aligned}$$

The conjugate of the Fourier transform $\hat{Y}^*$ at the other wave number is generated by:

$$\begin{aligned}\tilde{Y}_2^*(\omega) &= \int_{-\infty}^{\infty} \tilde{y}(\nu_2, t) e^{+i\omega t} dt \\ &= \tilde{Y}_2^{\text{Re}}(\omega) - i\tilde{Y}_2^{\text{Im}}(\omega).\end{aligned}$$

Then, we come to the definition of the two dimensional correlation intensity as:

$$\Phi(\nu_1, \nu_2) + i\Psi(\nu_1, \nu_2) = \frac{1}{\pi T} \int_0^\infty \tilde{Y}_1(\omega) \cdot \tilde{Y}_2^*(\omega) d\omega.$$

Where the real part Φ is called the synchronous correlation spectrum and the imaginary part Ψ is called the asynchronous correlation spectrum. The resultant typical spectra is shown in Figure 1-12.⁶⁴

Figure 1-12. Synchronous (left) and asynchronous (right) spectra generated from dynamic spectra subject to sinusoidal mechanical perturbation.⁶⁴ Reproduced with permission from Reference 64.

1.4.2 Synchronous Spectrum

In the synchronous Peak spectrum, autopeaks were formed along the diagonal line to show the major changing peaks in their intensity. Cross peaks are also formed off the diagonal line between the peaks that have simultaneous intensity

change with each other. The correlation peak would be positive if the corresponding two peaks are changing their intensities in the same directions. The correlation peak would be negative if the corresponding peaks are changing their intensities in the opposition directions. The intensity is proportional to the extent of intensity change of corresponding peaks.

1.4.3 Asynchronous Spectrum

In the asynchronous spectrum, there will be no auto peaks formed. Since Hilbert transformation is used to shift the phase of dynamic spectra, the sequential relationship is introduced between the correlating peaks. According to Noda's rule, if the asynchronous peak is positive, it means that ν_1 re-orientates before ν_2 . If the asynchronous peak is negative, it means that ν_1 re-orientates after ν_2 . However, this relationship is reversed if the synchronous peak at the same position is negative.

1.5 Research Scope, Motivation And Objective

It has been illustrated in the previous sections that plasmonics is a very promising field with its unique properties and capabilities that have been utilized the many fields of applications. The motivation of this thesis is how to develop useful plasmonic structures that enhance the performance, and take advantage of the capacities of plasmonic materials to address the challenges in the selected biological field. To do so, we have elaborated the current status and remaining questions at each level of the biological system. And our focus is biological molecules and proteins based on our expertise and desired problem to solve. The objectives of this thesis include:

- To develop plasmonic structures that provide good signal to noise ratio with respect to different laser excitation wavelengths for surface enhanced spectroscopy.
- To utilize the enhanced signal to noise ratio from plasmonic structures to analyze redox proteins that play an important role in the biological process
- To apply two dimensional correlation analysis for sights of information that can be generated from this approach
- To develop the plasmonic substrate that can achieve the desired resonance condition while maintaining reasonable potentiometric range for redox studies

1.6 Organization of The Thesis

This thesis consists of five chapters. Chapter 1 provides the literature review that gives the overview of the field with the emphasis on the remaining challenges and necessity of the current work. The basic theory of plamonics is introduced with the different synthesis and assembly techniques. Then the current state of plasmonic applications to the biological field was reviewed to identify the remaining questions. Surface enhanced spectroscopy is introduced as an useful tool to characterized many biological small and macro molecules. And the foundation of the two dimensional correlation is elaborated to establish the applicability to the study system. Chapter 2 introduces the work of two-dimensional gold and silver nanoparticle assembly to achieve the tunable behavior of the substrate with the laser excitation. Chapter 3 presents the two dimensional correlation study of

cytochrome c by combining the surface-enhanced Raman spectroscopy and surface enhanced infrared absorption spectroscopy on a two layer gold surface to reveal the conformational change of the protein during the redox transitions. Chapter 4 demonstrates the sputtering of a thin gold layer on the silver plasmonic substrate would increase the potentiometric range of the structure for electrochemical redox studies while maintaining good resonance condition to achieve the signal to noise ratio. Last , but not lease, final conclusions and future recommendations will be introduced in Chapter 5.

Chapter 2 SYNERGISTIC MODULATION OF SURFACE INTERACTION TO ASSEMBLE METAL NANOPARTICLES INTO TWO-DIMENSIONAL ARRAYS WITH TUNABLE PLASMONIC PROPERTIES

2.1 Introduction

The assembly of ordered arrays of plasmonic nanoparticles to form active plasmonics is an emerging field that creates, studies, and utilizes localized surface plasmon resonance (LSPR) at the small length scales. It has attracted a lot of attention due to their promising applications for optoelectrics,⁶⁵ catalysis, sensors,⁶⁶ and therapeutics.^{67, 68} The unique optical, electrical, and magnetic properties of ordered arrays of plasmonic nanoparticles are due to coupling of LSPR and thus depend strongly on inter-particle distance,^{31, 69, 70} which promise desirable platforms to integrate ordered nanoparticle arrays into active plasmonic devices. To address the requisite issues, the top-down lithographic techniques have been developed, such as e-beam lithography has been used to fabricate metallic “bowtie” nanoantennas consisting of two opposing tip-to-tip Au triangles, which exhibit tunable plasmonic properties dependent with gap distance. However the limitations arise from low efficiency, cost, and surface roughness of resulting small features (sub-100 nm) over a large area, which is a prerequisite for commercial fabrication process.

Alternatively, various bottom-up methods, such as colloidal approaches based on template-directed and interfacial self-assembly concepts, providing intriguing alternatives to assembling plasmonic nanoparticle into ordered arrays with desirable properties. Especially, interfacial self-assembly without any

template assistance has received considerable attention to construct large area two-dimensional (2D) ordered arrays with controllable inter-particle distance. For example, well-ordered and close-packed 2D metal nanoparticle arrays with controllable inter-particle distance have been extensively studied and can be prepared by controlled evaporation of the solvent or in a Langmuir-Blodgett trough.⁷¹⁻⁸⁰ However, using such method, the nanoparticle surface is capped with organic molecules, or polymers, which requires the etching or replacement process for the practical applications. The crucial challenge therefore is how to cost-effectively fabricate large area 2D ordered arrays in a green and environmentally benign way.

Recently, the charge-stabilized colloidal nanoparticles assembly on the modified substrate has provided a facile control of ordered particle array across large-scale surface. Negatively charged colloidal nanoparticle, such as citrate-stabilized nanoparticle, is most used building block due to their homogenous size monodispersity, shape controllability, and capability of facile surface functionalization. While it was reported that chemical modification by using polymers or monolayers of molecules with amino or thiol end groups on the surface has been used to enhance the specific affinity between particle and substrate, which played a key role for the successful particle arrays, such as homogeneity, ordering and aggregates, etc.^{81, 82} Additional, the thickness of electrical double layer of colloidal nanoparticle in the solution can be adjusted by varying the ionic strength, which is an another key factor in tuning the inter-particle distance and influencing the system's stability.

However, there still is a lack of understanding of how synergistically impacts the particle assembly by particle interaction and surface confinement of

the substrate to nanoparticle. Here we demonstrate that the interplay of substrate surface confinement to nanoparticle and inter-particle interaction provides synergistic control of 2D uniform arrays of Au and Ag nanoparticles on aminosilane modified substrate. The surface confinement of the substrate to nanoparticle could redistribute the double electric layer charges on particle surface which results in the increase of the thickness of double electric layer of particle and increasing of inter-particle distance of 2D nanoparticle arrays on the substrate.

Therefore, a wide range of inter-particle distance of 2D nanoparticle arrays on the substrate can be tuned based on synergistic impact of surface confinement of the substrate to nanoparticle. The resultant 2D metal nanoparticle arrays with tunable inter-particle distance exhibit the tunable and explicit plasmonic properties which induce the multiplex surface enhanced spectroscopy (SERS and SEF) response that corresponds to the presence of molecular analyte. This is very desirable for matching the plasmon resonance conditions for both the laser excitation and probed molecule, which can be helpful for various applications in multiplexed spectroscopic sensors, integrated devices and antennas etc.

2.2 Experimental Section

2.2.1 Materials

HAuCl₄, sodium citrate, APTMS were purchased from Sigma-Aldrich and used as received. PTCDI-EA and Methylene blue was purchase from Merck. 50K PMMA was purchased from MicroChem.

Amino-functional silane (APTES) was fabricated on a SiO₂/Si, quartz or glass substrate by a vacuum vapor method. Cleaned substrates were placed in an

airtight vial, which contained one drop of APTES. The vial was heated at 70 °C for 3 hours in vacuum oven. As a consequence, the APTES molecules are organized in a layer with their free amino groups away from the substrate. The modified substrates were immersed into pure water 8-10 hours in order to lower the basicity of amino groups on the substrate. The Au nanoparticles (13 nm) were prepared by sodium citrate reduction of H_{Au}Cl₄ solution.⁸³ The Ag nanoparticle (30 nm) with citrate modification was purchased from Ted Pella Inc. Au and Ag nanoparticle were centrifuged and re-dispersed in DI water solution to get rid of the excess citrate before assembly. 1M NaCl was used to adjust the different ionic strength into centrifuged particle solution.

The treated substrate was then immersed into Au or Ag nanoparticle solutions for 8-10 hours, after which it was rinsed with DI water, N₂ blow dried. The substrate was incubated in 1 μM Methylene blue ethanol solution overnight, rinsed with ethanol, and then N₂ blow dried for SERS experiment. To prepare the sample for metal enhanced fluorescence experiment, 5% 50K PMMA diluted in anisole was spin coated at 3000Rpm for 60s. The thickness is about 30nm. Then 0.1 mM PTCDI-EA aqueous solution and 0.01 mM MB aqueous solution was prepared, and the immersion time is 5 s and 300 s respectively. Then the substrate was rinsed with water and air dried.

2.2.2 Characterization

SEM measurements were performed on a JEOL SEM 7600F electron microscope, with the accelerating voltage of 10kV. UV-Vis absorption spectroscopy was measured with Shimadzu UV2501. SERS measurements were carried out on the WITec Alpha 300 system, with 100× objective (NA = 0.9

Olympus Japan). Samples were excited using two different lasers sources of 633nm (HeNe laser, Research Electro-optics Inc, United States) and 532nm (Nd:YAG laser, WITec, Germany). The power density was 10^4 W/cm^{-2} , with the acquisition time 1s.

2.2.3 Calculation of SERS enhancement factor

We used the following formula to calculate the SERS enhancement factor:

$$\text{Enhancement Factor} = \frac{I_{\text{SERS}}}{I_{\text{pure}}} \cdot \frac{N_{\text{pure}}}{N_{\text{SERS}}}$$

In order to calculate the number of pure MB molecules without the SERS effect N_{pure} , we use pure MB powder as sample, and assume that the laser illumination volume is 1 uL^3 . Hence, N_{pure} is calculated as follows:

$$N_{\text{pure}} = \frac{\rho V}{M_w} \cdot N_A = \frac{\frac{500 \text{ kg}}{\text{m}^3} \cdot 1 \mu\text{L}^3}{\frac{319.86 \text{ g}}{\text{mol}}} \cdot 6.02 \times 10^{23} / \text{mol} = 9.41 \times 10^8$$

In order to calculate the number of MB molecules on our two dimensional metal particle array with the SERS effect N_{SERS} , we assume that the gold nanoparticle surface is covered with one layer of MB molecules, and the coverage is about 30%. The illuminated area on the surface is about $1 \mu\text{m}^2$, and the inter-particle distance is d. The area occupied by MB molecule is 0.8 nm^2 .⁸⁴

Hence, N_{SERS} can be calculated using the formula:

$$N_{\text{SERS}} = \frac{1 \mu\text{m}^2}{\pi \left(\frac{d}{2}\right)^2} \cdot \frac{4\pi(13 \text{ nm})^2}{0.8 \text{ nm}^2} \cdot 30\%$$

I_{SERS} is the integrated area under the peak around 1621 cm^{-1} . With the values of N_{pure} , I_{pure} , N_{SERS} , I_{SERS} known, the enhance factor can be easily calculated.

2.3 Results and Discussions

2.3.1 Assembly of Gold Nanoparticle Array

In a typical experiment, the substrate surface was first modified with a monolayer of aminopropyltriethoxysilane (APTES), which provided uniform positively charged amine groups away from the substrate. It is well known that the properties of substrate modification play a key role for the homogenous and order assembly of Au nanoparticle on the surface.^{81, 82} Before use to assemble Au nanoparticles, the modified substrates are always immersed into pure water 8-10 hours in order to lower the basicity of amino groups on the substrate. It is worth pointing out after pure water immersion, the Au nanoparticles can be assembled on the surface with high homogenous distribution without aggregation (Figure 2-1).

Figure 2-1. SEM images of 2D Au assembly on silicon substrate with (A) high magnification and (B) low magnification after pure water immersion.

Otherwise high basicity of amino group without immersion treatment will destroy the double electric layer of charged-stabilized nanoparticle, which induce the aggregation and less homogenous distribution of Au nanoparticles on the

surface (Figure 2-2). Therefore lower the basicity of aminosilane modified substrate is a key factor for the uniformly distributed assembly of nanoparticle.

Figure 2-2. SEM images of 2D Au assembly on silicon substrate with high magnification without pure water immersion.

Furthermore inter-particle distance of 2D Au particle (13 nm) arrays can be tune by adjusting the ionic strength of the solution of 0.5 mM, 3.3 mM, 6.6 mM, 10.0 mM, 13.3 mM and 16.6 mM, respectively (Figure 2-3). As the ionic strength increased, the distance between different nanoparticles decreased. The particles were well separated and uniformly distributed on the wafer surface with decreasing inter-particle distance. At the ionic strength of 16.6mM, small clusters began to form since the electrical double layer thickness at this condition was sufficiently thin, which decreased the stability of the system. Obvious color change can be observed by naked eyes for large-scale 2D Au particle arrays on the glass wafer as shown in the optical images (Figure 2-3 inlet) due to the inter-particle coupling effect.

Figure 2-3. SEM images of 2D Au assembly with tunable inter-particle distance by adjusting the ionic strength of the solution of 0.5 mM, 3.3 mM, 6.6 mM, 10.0 mM, 13.3 mM and 16.6 mM, respectively and their corresponding optical images (inlet).

The color intensity also correlated well with the particle densities. The distribution of the inter-particle distance (particle to particle surface) is shown in Figure 2-4A. All curves are fitted using Gaussian distribution for the inter-particle distance histograms, measured by the Matlab graphic analysis programs. It is found that the standard deviation is the same as shown as in Figure 2-4B for the different ion strength except 0.5 mM in this range of values (the distribution of distances is normalized by the mean distance), which indicated good control of uniform inter-particle distance with tunable trend.

Figure 2-4. (A) The normalized inter-particle distance distribution fitted with Gaussian distribution at different salt concentrations. (B) The distribution of distances is normalized by the mean distance.

It is well known that in the solution the effective diameter of the nanoparticles is determined by the diameter of the rigid core (r_{core}) and the thickness of the double charge layers ($1/\kappa$), which further determines the inter-particle distance w (Figure 2-5A). The thickness of electrical double layer around the colloidal nanoparticles is controlled by ionic strength of the system, which can be varied depending on the ionic strength of the solution. In a more detailed examination of the Helmholtz and Gouy-Chapman model, the thickness of the adouble layer shell ($1/\kappa$) in the aqueous system is given by the following equation:⁸⁵

$$\kappa^2 = \frac{4\pi e^2}{\epsilon kT} \sum_i n_{i0} Z_i^2 \quad (1)$$

$$w = 2 \frac{1}{\kappa} \quad (2)$$

where w is the inter-particle distance without surface confinement between two adjacent Au nanoparticles. In this experiment, the particles are

confined onto the surface by electrostatic attraction, so the charge distribution of the electric double layer of Au particle should be changed.

Figure 2-5. Schematic illustration of the inter-particle distance of negatively charged colloidal gold nanoparticles assembly on positively charged silicon surface (B), as compared to the solution phase (up) (A)

Predicting the exact distribution may be tedious, thus in order to simplified the redistribution, we make the following assumption. First the net charge of the whole Au nanoparticle should be keep similar before and after particle is confined onto the surface. Therefore the net charge of Au nanoparticle within the double layer on free solution should be the sum of upper and lower net charge of the particle when the particle is confined on the charged surface plus some excess charge that counterbalancing the effect on the interface between particle and surface as shown the following equation:

$$\int_{V_A} \rho_A = \int_{V_{B_upper}} \rho_{B_upper} + \int_{V_{B_lower}} \rho_{B_lower} + \int_{A_{interface}} \rho_{interface} \quad (3)$$

where A is the particle in the free solution, B is the particle is confined onto the surface, ρ is the volume charge density, and V is the volume of counterbalance ions. Due to the strong electrostatic attraction, the upper part of the double layer of the particle B supposes to be a hemisphere (Figure 2-5B). It is assumed that the charge counterbalance happens in the near bottom region and has negligible effect to the rest of the system. So that, the net counter ions charge of this upper part should be equal to the one of B through the following equation:

$$\int_{V_A} \rho_A = \int_{V_{B_upper}} \rho_{B_upper} \quad (4)$$

where $\rho_A \approx \rho_{B_upper}$. According to the above assumption and simplification, the inter-particle distance of the nanoparticles is determined from the following equation:

$$V_A = \frac{4}{3}\pi\left(\frac{1}{k}\right)^3 - \frac{4}{3}\pi r_{core}^3 \approx V_{B_upper} = \frac{1}{2}\left(\frac{4}{3}\pi\left(\frac{w}{2}\right)^3 - \frac{4}{3}r_{core}^3\right) \quad (5)$$

$$w' = 2\left(2\left(\frac{1}{K} + r_{core}\right)^3 - r_{core}^3\right)^{\frac{1}{3}} - 2r_{core} \quad (6)$$

where w' is the inter-particle distance between two adjacent Au nanoparticles when particle is confined on the substrate, r_{core} is the radius of Au nanoparticle. As a result, the thickness of double layer of Au particle on the surface is thicker than that of particle in the solution and the inter-particle distance will increase as compared to the case in pure colloidal solution.

The experimental measured inter-particle distances of Au nanoparticle assembly at the ionic strength (0.5 mM – 16.6 mM) are in good agreement with the fitted curve according equation 6, which is all larger than inter-particle distances of Au nanoparticle without surface confinement (Figure

2-6A). It results in a wide range of particle to particle surface distance from 53 nm to 13 nm than ever reported in the literature, which can exhibits continuous and tunable plasmonic properties as shown in Figure 2-6B.

Figure 2-6. The measured and fitted results of the inter-particle distance with the salt concentrations. (A) UV-Vis spectra of gold nanoparticle 2D assembly array.

(B)

The peak around the 520 nm corresponds to the single nanoparticle plasmonic mode; it kept increasing because the amount of the nanoparticles per unit area increased with the decreasing inter-particle distance, hence absorption intensity increased. At the ionic strength of 6.6 mM, the 2D assembly became purple in color and a new band appears at 605 nm, suggesting the coupling of Au nanoparticle due to the decreasing inter-particle distance. The 2D arrays became blue in color and the plasmon band at 605 nm experienced a further red-shift.

The intensity increased with the increasing the ionic strength with the decreasing inter-particle distance. At the high ionic strength of 16.6 mM, the

extinction intensity at 520 nm decreased and red shifted indicated the strengthened coupling effect between Au nanoparticles with the close inter-particle distance. Above results show the wide range of inter-particle distance plays an important role in the plasmonic properties of Au particle arrays, results in continuous and tunable plasmonic properties which is expected to induce the wavelength-selected multiplex surface enhanced spectroscopy (SERS and SEF).

2.3.2 Surface-Enhanced Raman Spectroscopy of Gold Nanoparticle Array

Two commercial lasers with visible (532 nm and 633 nm) excitation were selected to compare the SERS and SEF response. Vertical lines were drawn on the absorption image of 2D Au particle arrays as a guidance to show the laser excitation used to measure the SERS and MEF response that corresponds to the presence of molecular analyte (Figure 2-6B). It is expected the multiplex response in SERS and SEF due to the plasmon resonance conditions matching for both the laser excitation and detected molecule.

Figure 2-7. The SERS spectra of gold nanoparticle arrays (A) with 633 nm laser excitation, (B) with 532 nm laser excitation.

Methylene blue as the widely used Raman label was select to compare SERS response on 2D Au particle arrays with tunable inter-particle distance. Figure 2-7A and Figure 2-7B show the SERS spectra measured on 2D Au nanoparticle arrays with tunable inter-particle distance at 633 nm and 532 nm irradiation wavelength, respectively. Raman peak at 1621 cm^{-1} of methylene blue (MB) corresponds to the intense C-C ring stretch mode, and was used as the characteristic peak for our SERS study.⁸⁶ Multiplex SERS responses for 2D Au particle arrays with tunable inter-particle distance at 633 nm and 532 nm irradiation wavelengths are summarized in Figure 2-8A and Figure 2-8B. The Raman signal intensity at 1621 cm^{-1} at 633 nm laser irradiation gradually increased with the increasing ionic strength and reached maximum with the highest ionic strength (16.6 mM), which correlates well with plasmonic resonances (Figure 2-6B).

Figure 2-8 the enhancement factor calculated for (A) 633 nm and (B) 532 nm laser wavelengths for the gold nanoparticle arrays

It is direct evidence that Raman is closely related with the scattering intensity of the light, since Raman signal is proportional to incoming and outgoing electromagnetic radiation intensity to the power of 4, that is $I \propto |E|^4$. As the irradiation wavelength moves to 532 nm, 2D Au particle arrays at ionic strength of 13.3 mM produces SERS signal with highest intensity (Figure 2-8B), which is also matched to the plasmonic resonances of 2D Au particle arrays. The detail calculation about enhancement factor can be found in the supporting information. The enhancement factor with the 633 nm laser is on the order to 10^4 - 10^5 , but for the enhancement factor with the 532 nm laser, it became lower, on the order of 10^2 to 10^3 . The main reason is the label molecule (MB) can achieve more resonance with the 633 nm for the resonance Raman condition than the 532 nm laser. These results indicate 2D Au particle arrays with tunable plasmonic properties demonstrate both strong signal response and wavelength-selected multiplex SERS responses, which making them attractive for multi-wavelength imaging and tagging applications.

2.3.3 Metal Enhanced Fluorescence of Gold Nanoparticle Array

2D Au nanoparticle arrays with tunable inter-particle distance were expected to exhibit the multiplex enhanced fluorescence responses. The tunable plasmon resonances of Au 2D assembly in visible range also allow us to carry out SEF at 532 nm and 633 nm irradiation wavelength. In order to maximize the fluorescence enhancement, it is important to design nanostructures that have plasmonic resonances whose frequencies coincide with the absorption and emission spectral windows of the emitter. Therefore two fluorescence emitters were chosen in the experiment, namely *N,N*-Di(2-aminoethyl)-3,4,9,10-perylene tetracarboxylic diimide (PTCDI-EA) and methylene blue (MB). The 2D Au particle array at the 0.5 mM ionic strength was used for PTCDI-EA enhancement at 532 nm laser irradiation, since its plasmon resonance is matched to the electronic absorption spectra of the PTCDI-EA in solution and the resonance with the 532 nm laser line (Figure 2-9A).

Figure 2-9 (A) Normalized absorption for Au nanoparticle array and PTCDI-EA;
(B) Metal enhanced fluorescence for PTCDI-EA;

We spin coated a thin layer of PMMA (35 nm) on the glass substrate with or without 2D Au particle array, which can not only provide a porous surface for

PTCDI-EA molecules absorption; also it keeps the fluorescence dyes away from the gold nanoparticles with a distance to prevent the fluorescence quenching. Clear enhancement of the fluorescence from the PTCDI-EA on the 2D Au particle array at the 0.5 mM ionic strength was observed as shown in Figure 2-9B. The enhanced emission of PTCDI-EA on the 2D Au particle array shows a maximum at 548 nm and a shoulder at 590 nm, which are quite similar with the fluorescence of PTCDI-EA in solution (Figure 2-10) indicating the PTCDI-EA monomer emission enhancement.

Figure 2-10 Fluorescence spectrum of PTCDI-EA molecules in solution

The lifetime of the PTCDI-EA molecules on the 2D Au particle array decreased to 0.86 ns compare to pure PTCDI-EA molecules without the metal enhancement as the fluorescence intensity was increased, to further prove the metal enhanced fluorescence capability (Figure 2-11A). In order to keep coincidence with the absorption of MB molecules, the blue-color 2D Au particle array at 13.3 mM ionic strength was chosen to match its resonance condition with 633 nm laser excitation. The SEF phenomenon was also observed due to the Au particle array's enhancement effect as shown in Figure 2-11B. Hence, by tuning the inter-particle distance with a selection range, we are able to select the proper

substrate to match the resonance condition of the fluorescence dye to achieve the best signal magnification.

Figure 2-11 (A) Fluorescence lifetime for PTCDI-EA on different substrates. (B) Metal enhanced fluorescence for methylene blue.

2.3.4 Assembly of Silver Nanoparticle Array

In the same principle, the silver nanoparticles assembled onto the surface showed decreasing inter-particle distance as the ionic strength was increased from 0.05 mM to 1 mM (Figure 2-12A). Obvious color change can be observed from yellow to orange by the naked eyes, as seen in Figure 2-12A inset. The distribution and the standard deviation of the inter-particle distance are shown in Figure 2-12B and Figure 2-12C.

Figure 2-12 (A) SEM pictures of silver nanoparticle 2D assembly arrays and their corresponding photo images (inlet). (B) The normalized inter-particle distance distribution fitted with Gaussian distribution at different salt concentrations. (C) The distribution of distances is normalized by the mean distance.

Similar to the case of the gold nanoparticle confinement on the surface, the experimental measured inter-particle distances of Ag nanoparticle assembly at the ionic strength (0.05 mM – 1 mM) are also in good agreement with the fitted curve according equation 6, which is all larger than inter-particle distances of Ag nanoparticle without surface confinement (Figure 2-13A). The UV-Vis absorption spectra of 2D silver nanoparticle arrays are shown in Figure 2-13B. The peak around the 445 nm corresponds to the single silver nanoparticle plasmonic mode, it kept increasing and red-shift with the increasing of ionic strength due to the coupling between the silver nanoparticles with decreasing inter-particle distance.

Figure 2-13 (A) The measured and fitted results of the inter-particle distance with the salt concentrations. (B) UV-Vis spectra of the silver nanoparticle arrays.

2.3.5 Surface-Enhanced Raman Spectroscopy of Silver Nanoparticle Array

By addition of silver nanoparticle two dimensional arrays, the resonance condition that we can tune span most of the visible wavelength range. Multiplex SERS activity is also observed for 2D Ag particle arrays with tunable inter-particle distance. The SERS spectra of methylene blue on Ag nanoparticle array is shown in Figure 2-14.

Figure 2-14 The SERS spectra for the silver nanoparticle 2D arrays (A) at 633 nm laser excitation, (B) at 532 nm laser excitation

Raman intensity reached maximum at the highest ionic strength (1 mM), where strongest plasmonic resonance occurred, for both 633 nm and 532 nm laser irradiation (Figure 2-15). The 532 nm laser can achieve much higher enhancement

factor than 633 nm, which is due to the plasmonic resonance peak of 2D Ag particle assembly is closer to irradiation wavelength at 532 nm than 633 nm (Figure 2-13B).

Figure 2-15 the calculated enhancement factor for the silver nanoparticle 2D assembly using (A) 633 nm and (B) 532 nm laser exciations.

2.4 Conclusions

We have successfully demonstrated the synergistic control of 2D uniform arrays of Au and Ag nanoparticles on the substrate by the interplay of substrate surface confinement to nanoparticle and inter-particle interaction, which result in a wide range of inter-particle distance of 2D particle arrays on the substrate as confirmed by SEM and the obvious color change of the substrate. The resultant 2D Au and Ag nanoparticle arrays with tunable inter-particle distance exhibit the tunable and explicit plasmonic properties which induce the multiplex surface enhanced spectroscopy (SERS and SEF) response that corresponds to the presence of molecular analyte. This strategy provides a green and environmentally benign approach for using a colloidal nanoparticle to produce high quality large area 2D ordered arrays, which have potential applications in multiplexed spectroscopic sensors, integrated devices, cell culture and antennas etc.

Chapter 3 TWO-DIMENSIONAL HETEROSPECTRAL CORRELATION ANALYSIS OF THE REDOX-INDUCED CONFORMATIONAL TRANSITION IN CYTOCHROME C USING SURFACE-ENHANCED RAMAN AND INFRARED ABSORPTION SPECTROSCOPIES ON A TWO-LAYER GOLD SURFACE

3.1 Introduction

Cytochrome c (cyt c) is an important heme protein that functions as an electron carrier within the electron transport chain. As a model system, it has attracted intensive research attention using different characterization techniques.^{54, 87-93} Electrochemistry has been successfully used as an important tool to study the functionality of the protein by providing an external potential to mimic the electron transfer process.^{94, 95} Unfortunately, electrochemistry provides little structural information. X-ray crystallography and NMR spectroscopy, on the other hand, are well-known structural characterization techniques that have been applied to the cytochrome c system.^{88, 96} Although very useful, they are not applicable for dynamic studies such as the ones encountered here due to the lack of temporal resolution. Moreover, it is not possible for all the proteins to form a well-ordered crystal.

Spectroscopic methods also have been applied by several groups; UV-Vis reflectance spectroscopy was used to study the electron transfer rate⁹⁷⁻⁹⁹, and Edmiston et al. utilized fluorescence spectroscopy to determine the molecular orientation when the protein was bound to a surface.⁹¹ Second-order harmonic spectroscopy has been used to determine the adsorption kinetics.¹⁰⁰ Quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) have also been used in

combination of electrochemistry techniques to study the electron transfer process of cytochrome c on gold electrode modified with different self-assembled monolayers.¹⁰¹

Vibrational spectroscopy is very powerful for characterization of the hydrogen bonding pattern and secondary structure of proteins and polypeptides. Ataka et al. studied cytochrome c on different self-assembled monolayer (SAM) structures using SEIRAS difference spectroscopy.¹⁰² Spiro et al. attempted the complete assignment of cytochrome c through isotope labeling.¹⁰³ A complicating factor though is that cyt c possesses the unique characteristic that its backbone is strongly infrared active but hardly detected using Raman spectroscopy, whereas the heme center is Raman active but gives rise to a very weak infrared signal. Thus, the protein structure or conformational change cannot be entirely described by either technique alone, which sometimes has led to ambiguous conclusions that are still a topic of debate. Hence both techniques, Raman and infrared are complementary techniques as they reveal information of both the protein backbone and the redox center.

Figure 3-1. The protein structure of cytochrome c. The heme (in blue) is Raman active, while the protein backbone (in yellow and orange) is infrared active.

Two-dimensional (2D) correlation spectroscopy was first developed by Noda in 1989 and has triggered many research efforts thereafter.¹⁰⁴⁻¹⁰⁷ It utilizes a mathematical method to plot the series of dynamic spectra generated by external perturbation (temperature, time, potential, etc.) on a two-dimensional scale, hence generating two types of plots, namely, synchronous and asynchronous. This technique is advantageous because it can resolve overlapping peaks and provide information about the sequence of dipole reorientation/conformational change etc. For example, Wu et al. utilized 2D IR spectroscopy to study the thermal unfolding of ribonuclease A upon reduction,¹⁰⁸ and Ozaki et al. used 2D Raman spectroscopy to study the molecular interaction between chemical components in pharmaceutical tablets.¹⁰⁹ Heterospectral 2D IR-Raman analysis also has been investigated by Noda et al.¹¹⁰ for the study of N-methylacetamide.

However, most of the 2D correlation studies have been used to explore the same structural segment of the molecule. To the best of our knowledge, the present work is the first attempt to apply 2D heterospectral correlation analysis to address different parts of the target protein, which is possible because of the special characteristics of cytochrome c stated above. A two-layer gold surface prepared by thermal evaporation and electroless deposition was utilized for the enhancement of the spectroscopic signal. Hetero 2D would allow us to correlate the changes in the infrared and Raman spectra. Additionally, by combining IR and Raman spectroscopy, the sequential pattern of the conformational changes in cyt c can be probed by applying the sequential rules of 2D correlation spectra. Molecular dynamics calculations are also performed to examine the interaction between the functional units probed by the hetero 2D correlation, and to reveal details of intra-molecular interactions.

3.2 Experimental Section

3.2.1 Materials

Gold(III) chloride trihydrate ($\text{AuCl}_3 \cdot 3\text{H}_2\text{O}$, 99.999%), hydroxylamine hydrochloride ($\text{NH}_2\text{OH} \cdot \text{HCl}$, 99%), cytochrome *c* from bovine heart, sodium perchlorate, 3-aminopropyltrimethoxysilane (APTMS), and 2-mercaptoethanol were purchased from Sigma-Aldrich. Dipotassium phosphate was purchased from Merck, Darmstadt. All chemicals were used without further purification.

3.2.2 Preparation of the Two-Layer Gold Surface and Cytochrome *c* adsorption

A two-layer gold surface was prepared using the optimal conditions described in our previous work^{57, 111}. Briefly, a 30 nm gold film was deposited by electrothermal evaporation onto an ATR-crystal after a thorough rinse with ethanol and sonication. To grow the second gold layer, the substrate was immersed in 50 mL of an aqueous solution containing 0.4 mM hydroxylamine hydrochloride to which 500 μL of an aqueous solution of gold (III) chloride trihydrate (0.3 mM) was added. After 2 min, an equivalent aliquot of the AuCl_3 solution was added. This process was repeated 5 times such that the sample remained in the growth solution for a total of 10 min. The sample was removed from the growth solution, rinsed with water, and dried in a stream of nitrogen. Thereafter, the sample was immersed in an aqueous solution of 2-mercaptoethanol (ME) (1 mM) for 15 minutes and then rinsed with water. Subsequently, cyt *c* was adsorbed for 60 min using a solution of cc (0.35 mM) in PBS buffer (20 mM K_2HPO_4 and 10 mM NaClO_4 , pH 7).

3.2.3 Spectroelectrochemical Measurements

All electrochemical measurements were recorded in a homemade liquid cell using an Autolab instrument (PGSTAT302) equipped with an ECD module amplifier for low currents, an ADC750 module for rapid-scan measurements, and a SCANGEN module for analog potential scanning (Eco Chemie, B.V., Utrecht, Netherlands). All measurements were collected using a three-electrode configuration with the gold film as the working electrode, an Ag/AgCl, KCl_{sat} reference and a platinum wire as the counter electrode. All electrode potentials are reported relative to the standard hydrogen electrode (SHE).

The IR measurements were performed as described previously¹¹¹. The spectroelectrochemical cell was mounted on top of a trapezoidal silicon ATR-crystal required for a single reflection in the attenuated total reflection spectroscopy (ATR) mode. The IR beam of the FTIR spectrometer (VERTEX 70 FTIR spectrometer, Bruker, Karlsruhe, Germany) was coupled to a prism at an angle of incidence $\Theta = 60^\circ$. The total reflected beam IR intensity was measured using a photovoltaic MCT (mercury cadmium telluride) detector. For the static measurements, the mirror velocity was 120 kHz, the resolution was 4 cm⁻¹, and 1000 scans were collected for one spectrum during a measurement time of 10 min. All spectra were measured using parallel polarized light. The spectra were analyzed using the software package OPUS 6.5 (Bruker, Karlsruhe).

The Raman experiments were performed on the combined system of WITec confocal Raman microscope using the 532 nm line of a Nd:YAG laser with circular polarization. It corresponds to the Q electronic transition of the heme. The laser beam was coupled to a confocal Raman microscope (Alpha 300, WITec)

equipped with a water immersion objective (Nikon 60*, NA = 1.0, WD = 2.0). Thus, the laser beam was focused and the scattered light was filtered by an edge filter and guided to the spectrometer (UHTS 300, WITec) by a 600 grooves/mm grating to provide the spectral resolution. The recorded spectrum was imaged onto a thermoelectrically cooled EMCCD detector (WITec). The Raman spectra were acquired using a 15 s integration time with a laser power density of $2.6 \times 10^6 \text{ W/cm}^2$ with spot size of $\sim 1 \text{ }\mu\text{m}^2$. The SERS spectra were analyzed using the software packages WITec control 1.54 and WITec project 2.06.

3.2.4 2D Correlation Calculations

The synchronous and asynchronous 2D plots of the infrared, Raman, and heterospectral correlations were calculated using the software 2D Shige © Shigeaki Morita, Kwansei-Gakuin University, 2004-2005. The calculated data was re-plotted in Origin for better visualization. The color bar shows the different intensities of the 2D correlation peaks, which indicates the extent of correlation between x and y variables.

3.2.5 Molecular Dynamics

The simulations were based on the crystal structure of the oxidized form of bovine heart cytochrome *c* (PDB code: 2B4Z) using the GROMOS11¹¹² software and the GROMOS 54A7¹¹³ force field. The crystal structure was modified by removing the covalent bond between the heme and His18 and Met80, while adding the thioether bridges to the heme vinyl groups through Cys14 and Cys17. The simulations of the reduced form used the same initial structure but different charge parameters on the heme. The GROMOS++¹¹⁴ package was used for simulation analysis. The dihedral based secondary structure classification was based on the

work of Hollingsworth *et. al.*¹¹⁵ The precise simulation settings are described in the supporting information.

3.3 Results and discussions

3.3.1 General Considerations

Cytochrome c is a small size (12,000 Daltons) highly conserved protein that is found in viruses, plants humans, etc. Its primary structure is made up of 100 amino acids, though many higher order organisms consist of 104 amino acids. It is an important component in the electron transport chain, and that is loosely bound to the inner membrane of mitochondria. It consists of 5 α -helices, 2 beta strands, and some turn structures, which can be detected by infrared spectroscopy and assigned to specific portions of the protein.¹⁰²

In our previous work, a two-layer gold surface was used to enhance the SEIRAS signal of adsorbed cyt c, and we found that a growth period of 10 min for the second gold layer provided optimal conditions.¹¹¹ Identical surfaces were prepared for both the SEIRAS and SERS to ensure similar experimental conditions. As shown in Figure 3-2, the atomic force microscopy images clearly show that the initial surface is smooth, with an average roughness of 1.9 nm. After 10 minutes, the surface became rough due to the growth of a second layer of gold nanoparticles into islands and the average surface roughness increased to 6.9 nm. Such two layer gold surface has been utilized before by our group in a 2D correlation study of cytochrome c oxidase and cytochrome c.^{30, 93} The motivation of the present work is to advance the understanding of the interaction between the

heme group and the surrounding protein backbone upon changing the electrode potential.

Figure 3-2 AFM images of the two layer gold surfaces with different second gold layer growth time: (A) 0 min, meaning before the growth of the second gold layer (B) 10 mins.

The two-layer gold surface was modified with 2-Mercaptoethanol (ME) prior to adsorption of cyt c. Hydroxyl-terminated SAM has been suggested to weakly affect the conformational characteristics of cyt c upon adsorption.¹⁰² Moreover, cyclic voltammetry data shows that cyt c is successfully adsorbed onto the two-layer gold surface. The oxidation peak (cathodic peak) at 280 mV and the reduction peak (anodic peak) at 203 mV agree well with the previously reported values³⁰ (Figure 3-3).

Figure 3-3 Cyclic voltammetry of Cytochrome c adsorbed to a two-layer gold surface shows two characteristic peaks of Cytochrome c at 203 mV and 280 mV, proofing that the protein is adsorbed to the two layer gold surface.

The infrared difference spectra of cyt c have been studied before using this same methodology and surface architecture (Figure 3-4).³⁰

Figure 3-4 The infrared difference spectra from potentiostatic titration of cc by ATR-IR-spectroscopy. Potential differences applied to the immobilized protein are varied stepwise from -100 mV to 350 mV and are plotted with different baselines.

Detailed assignments can be found in Ref ¹⁰². The main infrared peaks and the corresponding assignments discussed in the text are summarized in Table 3-1.

Table 3-1 The infrared peaks and corresponding assignments as discussed in the 2D correlation spectra. ¹⁰²

Reduced state	Oxidized state	Assignment	Designation	Residues
	1552	Amide II	β -turn type III	
1624		Amide I	extended β -strand	37-40, 57-79
	1658	Amide I	β -turn type II and/or α -helix	32-35, 35-38
	1672	Amide I	β -turn type III	14-19, 67-70
1692		Amide I	β -turn type III	14-19, 67-70

As the potential increases, the peaks at 1552 cm^{-1} and 1658 cm^{-1} increase in intensity, and one additional peak appears at 1672 cm^{-1} . These peaks are characteristic for oxidized cyt c and correspond to the amide II band of a type III β -turn, the amide I band of a type II β turn and/or α -helix and type III β -turn, respectively. Simultaneously, the peak at 1692 cm^{-1} , corresponding to the amide I band of the type III β -turn (reduced state), decreases in intensity with increasing electrode potential (Figure 3-4).

It possesses a sigmoid appearance (Figure 3-5) Taken together, the sigmoid appearance of the 1692 cm^{-1} intensity clearly indicate that cyt c undergoes a change from the reduced state to the oxidized state.

Figure 3-5 Infrared peak intensity change at 1692 cm^{-1} (black) and 1552 cm^{-1} (red) with the increasing potential.

For the SERS measurement, a 532 nm laser is used as the excitation source to match the Q electronic transition of the heme instead of the B band. This is preferred since gold surface is used to achieve maximum signal enhancement. The SERS spectra show no obvious changes until +50 mV vs. SHE, as can be observed in Figure 3-6. As the potential increased further, all of the peaks decrease in intensity except for the ν_{10} peak at 1621 cm^{-1} , which increases. The ν_{10} peak corresponds to the C-C asymmetric stretching mode, $\nu(\text{C}_\alpha\text{--C}_m)_{\text{asym}}$. A sigmoid behavior is also observed for the 1364 cm^{-1} peak intensity, as shown in Figure 3-6 (inset).

Figure 3-6 Evolution of the SERS active peaks surface with potential (from -100 mV - 500 mV vs. SHE) for cytochrome c adsorbed to the 2-mercaptoethanol SAM-modified two-layer gold substrate. The black arrows are used to label the peaks that increase or decrease in intensities as the potential increases. (Inset: The sigmoid behavior of intensity change of Raman peak at 1364 cm^{-1}).

Again this sigmoid curve shows that the redox transition can be followed by the spectral intensity change, Note that unspecific re-orientation of the protein on the surface won't give such sigmoid behavior.³⁰ For detailed Raman peak assignment we refer to the work by Spiro et al ¹⁰³, which are in good agreement with the peaks found in our experiment. The selected peaks and their corresponding assignment are shown in Table 3-2 as will be discussed in relation to the 2D correlation plots.

Table 3-2 Raman peaks and corresponding assignments as discussed in the 2D correlation spectra.¹⁰³

Raman shift (cm ⁻¹)	Assignment	Stretching motion
1314	$\nu_{21} - \delta(\text{C}_m - \text{X})$
1364	$\nu_4 - \nu(\text{Pyr half-ring})_{\text{sym}}$
1397	$\nu_{20} - \nu(\text{Pyr quarter-ring})$
1587	$\nu_{19} - \nu(\text{C}_\alpha\text{C}_m)_{\text{asym}}$

3.3.2 2D IR

Overlapping peaks arising from different functional groups make it difficult to analyze the infrared peaks of cyt c, thus hindering accurate band assignments and reliable determination of the conformational changes of the protein. Second-derivative or Fourier deconvolution analyses have been applied to address this problem,^{89, 116} but some of the findings and conclusions do not agree with the results from X-ray crystallography¹¹⁷ and NMR studies⁸⁸. Reaction-induced infrared difference spectroscopy also has been developed both for the bulk and the SAM layer of cyt c, and the ability to enhance the spectral resolution was demonstrated using an enzymatic reaction.^{102, 118} The concept of the difference spectra suits the basis of 2D correlation spectroscopy, which also utilizes an external perturbation (light, voltage, etc.) to generate dynamic spectra for analysis.

The use of 2D correlation spectroscopy spreads the spectrum in the second dimension and facilitates the deconvolution of overlapping peaks. Thus it can help enhance the spectral resolution.¹¹⁹ Although vibrational features of different molecular origin may contribute in a similar manner to the spectra in the static case, they may behave differently (either delayed or accelerated) in the case of a dynamic change, which can be distinguished by 2D correlation. Thus, 2D correlation is an effective tool for deconvoluting overlapping peaks.⁶⁴

The synchronous and asynchronous 2D infrared correlation plots of cyt c were calculated using the 2D Shige[®] software package from the series of 1D IR spectra generated with stepwise increased potentials from -100 mV to +500 mV. The peaks that undergo changes in intensity result in correlation peaks in the 2D plots, whereas the peaks that remain constant result in no or very small correlation peaks. The autopeaks along the diagonal are all positive in a synchronous 2D IR plot (Figure 3-7) and reveal the major peaks of the 1D infrared spectra. However, these peaks exhibit different degrees of intensity change during the potential increase. In the synchronous 2D IR plot, the intensity of the autopeak at 1692 cm⁻¹ is more pronounced than that at 1658 cm⁻¹ (Figure 3-7), suggesting that the sequence of peptide groups responsible for the 1692 cm⁻¹ autopeak are influenced to a greater extent by the applied potential than those responsible for the 1658 cm⁻¹ autopeak. By examining the IR peak assignment¹⁰², it leads to the conclusion that the amide I band of the type II β -turn and/or α helix (1658 cm⁻¹) and the amide I band of the type III β -turn (1692 cm⁻¹) originate from different regions of the protein backbone. Thus, different regions of the protein backbone can be probed by changing the applied potential, as revealed by the synchronous 2D IR plot.

Figure 3-7 Synchronous 2D IR correlation plots from a SEIRAS analysis of cyt c adsorbed to a two-layer gold surface.

Cross peaks in the synchronous 2D IR plot are also indicative of modes with coupled or related origin. For example, the strong correlation peak at (1552 cm^{-1} , 1692 cm^{-1}) indicates that the spectral features at these two positions may be related, and most likely belong to the same segments of the protein backbone – the type III β -turn, (Table 3-1). The negative peak sign (dark blue), however, suggests that the peak intensities at the two positions change in different directions. The fact that one of them belongs to the reduced state and the other one the oxidized state clearly suggests that the redox transition was successfully recorded.

The asynchronous plot (Figure 3-8) provides additional useful information about the sequential relationship between the changes in intensity of peaks with different spectral origins, thus making it useful for peak deconvolution. The spectra do not include any autopeaks, which is a distinct characteristic feature of an asynchronous 2D spectrum.

Figure 3-8 asynchronous (B) 2D IR correlation plots from a SEIRAS analysis of cyt c adsorbed to a two-layer gold surface.

We observed a strong negative cross peak at (1658 cm^{-1} , 1692 cm^{-1}) and a strong positive cross peak at (1692 cm^{-1} , 1658 cm^{-1}). By applying Noda's rules for analyzing 2D correlation spectra¹⁰⁶, the peptide groups corresponding to the amino acids in the type II β -turn and/or α helix (represented by the peak at 1658 cm^{-1}) change their conformation before those in the type III β -turn (represented by the peak at 1692 cm^{-1}). Since the signal change of the amino residues represented by the IR peak 1692 cm^{-1} occurred after those represented by the IR peak 1658 cm^{-1} , the amino acids 14-19 and 67-70 may re-orient to the surface first followed by those at positions 32-38 (Table 3-1). We conclude by comparing existing literature¹⁰² observations with our findings that the changes seen in the 2D IR plots can be attributed to conformational changes within the protein backbone.

3.3.3 2D Raman

Prior to the heterospectral 2D correlation analysis, the 2D Raman correlation spectra were calculated from the series of 1D Raman spectra at increasing potentials to probe how the Raman active heme group responds. The synchronous 2D Raman correlation plot is shown in Figure 3-9. In addition to the positive autopeaks, most of the cross peaks were also positive, which indicates that all of the peak intensities decreases during the potential increase except for the peak at 1621 cm^{-1} , which resulted in a negative peak in the 2D spectrum and, therefore, increased in intensity. This synchronous 2D Raman correlation agrees well with the observation from the 1D Raman spectra shown in Figure 3-6.

Figure 3-9 Synchronous 2D Raman correlation plot from the SERS spectra of cytochrome c on gold.

According to the peak assignment by Spiro et al.,¹⁰³ the Raman peaks at 1397 cm^{-1} and 1587 cm^{-1} correspond to vibrational modes of the pyrrole quarter ring and the C–C bond, respectively (Table 3-2). The pyrrole quarter ring is located next to the iron center of the heme, whereas the C–C bond is located

further away from the heme center. As can be observed from the synchronous 2D Raman correlation spectrum, the peak intensity of the 1587 cm^{-1} autopeak is much more pronounced than that of the autopeak at 1397 cm^{-1} . This observation indicates that the $\nu(\text{C}_\alpha-\text{C}_m)_{\text{asym}}$ mode (corresponding to the peak at 1587 cm^{-1}) has a stronger correlation to the change in potential than the $\nu(\text{pyrrole quarter ring})$ has (corresponding to the peak at 1397 cm^{-1}). This is in agreement with literature¹²⁰ and our MD simulation suggesting that the heme unit is prone to re-orient (bend upward or downward) with the applied potential. Hence the $\nu(\text{C}_\alpha-\text{C}_m)_{\text{asym}}$ mode is, as expected, more sensitive to conformational changes than the $\nu(\text{pyrrole quarter ring})$ mode. The two peripheral propionic acid groups, that are a part of the heme structure, also can establish strong interactions with neighboring amino acid residues via hydrogen bond/electrostatic interaction. Thus different modes respond differently to applied potential because of changes in the local environment.

The asynchronous 2D Raman correlation plot, in contrast, does not exhibit significant peaks. Instead, the signals appear within the noise level (Figure 3-10). The lack of significant features is most likely due to the fact that the corresponding peaks may exhibit frequency shifts in addition to pure intensity changes. This induces noise in the 2D spectra. Thus the asynchronous correlation analysis is typically more sensitive to noise than the synchronous analysis was.⁶⁴

Figure 3-10 Asynchronous 2D Raman correlation spectrum showing only peaks at noise level.

According to Noda¹⁰⁶, the asynchronous correlation intensity vanishes when all reorientations of the two dipole-momentum transitions occur in phase with the external perturbation. Hence, all Raman-active regions of cyt c are expected to react at the same rate following a potential perturbation due to the rigid structure of the heme center. This is consistent with the conclusion that the bonding distance between the six ligands with the heme center iron ion doesn't change during the redox process, as revealed by recent X-ray investigation.^{117, 121}

3.3.4 Hetero 2D

Thus far, only autologous IR- or Raman-active modes have been correlated. We introduce also a heterospectral correlation analysis of the infrared and Raman spectra representing different parts of the protein. Such 2D spectra combining these two complementary techniques could serve as a visualization aid to describe more complete conformational change of cyt c during the redox process. The synchronous and asynchronous 2D heterospectral correlation plots are again calculated using the 2D Shige[©] software and the result is shown in Figure 3-11 and

Figure 3-12. The infrared frequency is chosen as the x-axis, and the Raman shift as the y-axis, which makes the coordinate system as (IR peak position, Raman peak position) in the hetero 2D plot. These 2D heterospectral correlation plots are no longer symmetrical about the diagonal line, which is in contrast to the autologous IR-IR or Raman-Raman 2D spectra. Hence, all peaks had to be interpreted as cross correlation peaks.¹²²

Figure 3-11 Synchronous 2D heterospectral correlation plots generated from the SEIRAS and SERS dynamic spectra of cyt c on gold.

The synchronous 2D heterospectral correlation plot, Figure 3-11, shows that the infrared peak at 1658 cm^{-1} forms strong positive peaks with the main Raman peaks, and that the strongest peaks are formed with the Raman peaks at 1314 cm^{-1} and 1587 cm^{-1} . There are also positive peaks formed under the IR peak at 1552 cm^{-1} with these Raman peaks. For the correlation under the peak at 1692 cm^{-1} , two negative peaks are observed, which are not on the same scale as the positive peaks.

As mentioned before, an examination of the band assignments suggests that the IR peaks at 1692 cm^{-1} and 1552 cm^{-1} both can be attributed to the type III beta-

turn, with the 1692 cm^{-1} peak corresponding to the reduced state and the 1552 cm^{-1} peak to the oxidized state. During the potential change from -100 mV to $+500\text{ mV}$ vs. SHE, cyt c undergoes a redox change from the fully reduced state to the fully oxidized state, which is reflected well in the synchronous 2D heterospectral correlation plot by the negative sign of the correlation peak. The IR peak at 1658 cm^{-1} corresponding to the type II beta-turn and/or α helix in the oxidized state involves the residues 32-38. These residues can interact directly with the propionate groups of cyt c and thereby exhibit a strong correlation with the heme during the redox process. The different intensities of the correlation peaks also indicated a large extent of reorientation.

Interestingly, the 1692 cm^{-1} peak, which corresponds to the amino acids near His 18 that is ligated to the heme iron, resulted in smaller correlation peaks than the peak at 1658 cm^{-1} did. A possible interpretation of these data could be that the strong coupling between His 18 and the heme prevents the two moieties from changing configuration and/or reorient. In contrast, the residues associated with the propionate groups have more freedom to move, thus inducing more reorientation during the redox process. The correlation peaks between the IR peak 1692 cm^{-1} (corresponds to the amino acids 14-19, including the His-18) with the Raman peaks are either absent or very weak. On the other hand, the correlation peaks between IR peak 1658 cm^{-1} are strong in intensity and show different peak sign as compared to the peaks under IR peak 1692 cm^{-1} . This indicates a different coupling mechanism between the contributing amino acid residues and the heme center.

The asynchronous 2D heterospectral correlation plot is shown in Figure 3-12. We assume that the general rules developed by Noda are valid for all kinds

of 2D correlation methods, and can be applied to our data as described in the following. The sign of the asynchronous 2D heterospectral correlation peak is positive at $(1692\text{ cm}^{-1}, 1587\text{ cm}^{-1})$, whereas the sign of the peaks at the same position in the synchronous hetero 2D plot indicate that the sequential relationship for this set of data has to be inverted¹⁰⁶. Thus, by applying the Noda's rules to the hetero 2D plots, the residues represented by the IR peak at 1692 cm^{-1} would reorient after the heme, which is represented by the Raman peak at 1587 cm^{-1} . This analysis allows us to visualize the dynamics of the redox process of cyt c as follows: the heme moiety first undergoes a conformational change, because it directly coordinates to the iron ion involved in the redox process. This is followed by a reorientation of the His 18 residue that is ligated to the heme iron.

Figure 3-12 Asynchronous 2D heterospectral correlation plots generated from the SEIRAS and SERS dynamic spectra of cyt c on gold.

We also observed two additional peaks in the asynchronous 2D heterospectral correlation plot at $(1692\text{ cm}^{-1}, 1364\text{ cm}^{-1})$ and $(1692\text{ cm}^{-1}, 1397\text{ cm}^{-1})$, which are absent in the synchronous 2D heterospectral correlation plot. Based

on the peak assignment, the Raman peaks at 1364 cm^{-1} and 1397 cm^{-1} correspond to the stretching modes of $\nu(\text{Pyr half-ring})_{\text{sym}}$ and $\nu(\text{Pyr quarter-ring})$, respectively. According to these findings we propose that, upon oxidation the heme undergoes a vibrational change that reflects a change in the distance between the pyrrole group and His 18. Additional to the described findings, we could show with the help of MD calculations, that there is stronger coupling between the heme structure and the protein backbone shown in Figure 3-13.

Figure 3-13 Normalized distribution of the heme – His18 (dashed) and heme – Met80 (solid) distances during MD simulations. Blue and black lines belong to oxidised state simulations, red and orange lines belong to reduced state simulations

The broad synchronous peak at $(1658\text{ cm}^{-1}, 1314\text{ cm}^{-1})$ is split into three separate peaks in the asynchronous plot at 1624 cm^{-1} , 1658 cm^{-1} , and 1672 cm^{-1} , respectively, because they are of different origins and display different sequential interactions with the applied external potential. Again, these IR peaks correspond to the amide I bands of the extended β -strand, the type II β -turn and/or α helix and the type III β -turn, respectively. A similar splitting behavior can be observed with the Raman peak 1587 cm^{-1} , but not with the Raman peaks 1364 cm^{-1} and 1397 cm^{-1} . A closer examination of the Raman vibrational mode assignments (shown in

Table 3-2) reveals that the peaks showing splitting (1314 cm^{-1} and 1587 cm^{-1}) correspond to the pyrrole side chain, while those showing no splitting (1364 cm^{-1} and 1397 cm^{-1}) correspond to either pyrrole half-ring or quarter-ring vibrations, respectively. This clearly shows that, asynchronous 2D heterospectral correlation analysis can be used to deconvolute overlapping peaks originating from segments of different rigidity, molecular environment, etc.

The potential dependent response for amino acid 14-19 and 67-70, as represented by the infrared peak 1692 cm^{-1} , is out of phase of the heme center, as shown from the strong hetero asynchronous correlation between IR peak 1692 cm^{-1} and all the Raman peaks. The same conclusions can be drawn from the strong correlation between the Raman peaks at 1314 and 1587 cm^{-1} with all the infrared peaks in the amide I region. This indicates that the conformation change of these two Raman vibrational modes are out of phase with those represented by the infrared amide I peaks. The amide II peak at 1552 cm^{-1} is assigned to the same amino acids as the amide I peak at 1692 cm^{-1} , but they represent different redox states. That's why they observed same degree of asynchronous change, but in different directions.

The correlation peaks between the IR peak 1692 cm^{-1} and the Raman peaks, which are weak or absent from the synchronous spectra, increase their intensity in the asynchronous spectra. On the other hand, the correlation peaks between the IR peak 1658 cm^{-1} and the Raman peaks, which are strong and positive in the synchronous spectra, disappear or become weak in the asynchronous spectra. This could indicate a change of the bond angle between the two axial ligands His-18 and Met-80, and also the position shift of the propionate group, since the different

correlation may indicate the change of relative positions. This is also consistent with the findings by XRD and NMR.^{117, 121, 123}

3.3.5 Molecular Dynamics

To confirm our observations and conclusions, molecular dynamics simulations were also performed in collaboration with Prof Oostenbrink (BOKU University). Because there are no prominent changes seen in the overall secondary structure associated with the oxidation, the secondary structure dynamics for each residue are examined. The amount of time each residue spent in a particular secondary structure class was determined for both the oxidized and reduced forms. Those residues for which this time varied by at least 5% (or less if they were close to a previously monitored residue) are collected in Table 3-3.

Table 3-3 Residues identified based on secondary structural differences in different oxidation states. Percentages are shown for each residue stand for the time spent in β -turn type III (turn III) , extended β -strand (EBS), β -turn type II (turnII) and α -helix structures respectively

Difference in Secondary Structure (DBSSP)												
Residue number	Oxidised turn III %	Reduced turn III %	Difference %	Oxidised EBS %	Reduced EBS %	Difference %	Oxidised turnII %	Reduced turnII %	Difference %	Oxidised helix %	Reduced helix %	Difference %
14	59.9	59.6	0.3	0.0	0.0	0.0	0.0	0.0	0.0	9.2	6.2	3.0
15	28.0	35.8	-7.8	0.0	0.0	0.0	0.0	0.0	0.0	61.6	55.0	6.6
16	48.5	53.9	-5.4	0.0	0.0	0.0	0.0	0.0	0.0	27.6	24.5	3.1
17	0.0	0.0	0.0	13.2	4.4	8.8	0.0	0.0	0.0	0.0	0.0	0.0
18	0.0	0.0	0.0	15.0	33.0	-18.0	0.0	0.0	0.0	0.0	0.0	0.0
19	0.0	0.0	0.0	2.2	2.4	-0.2	0.0	0.0	0.0	13.9	12.1	1.8
21	0.0	0.0	0.0	0.0	0.0	0.0	2.2	2.4	-0.2	21.6	0.4	21.2
22	0.5	0.0	0.5	0.9	0.0	0.9	14.0	72.5	-58.5	0.2	0.0	0.2
23	0.0	0.0	0.0	2.0	0.0	2.0	0.9	0.0	0.9	0.0	0.0	0.0
32	0.0	0.0	0.0	0.0	0.0	0.0	0.4	3.4	-3.0	0.0	0.0	0.0
33	0.0	0.0	0.0	0.0	0.0	0.0	75.1	69.2	5.9	0.2	0.0	0.2
34	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
43	0.0	0.0	0.0	0.0	0.0	0.0	14.0	10.9	3.1	0.0	0.0	0.0
44	0.0	0.0	0.0	0.0	0.0	0.0	32.8	39.8	-7.0	0.0	0.0	0.0
45	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
52	4.4	1.5	2.9	0.0	0.0	0.0	0.0	0.0	0.0	93.7	97.1	-3.4
53	20.9	10.5	10.4	0.0	0.0	0.0	0.0	0.0	0.0	73.7	86.7	-13.0
54	24.1	7.6	16.5	0.0	0.0	0.0	0.0	0.0	0.0	69.7	73.4	-3.7
55	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	-0.4
67	23.5	29.0	-5.5	0.0	0.0	0.0	0.0	0.0	0.0	72.7	67.4	5.3
68	24.2	31.9	-7.7	0.0	0.0	0.0	0.0	0.0	0.0	71.0	64.4	6.6
69	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
70	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	79.6	74.6	5.0
75	0.0	0.0	0.0	0.0	0.0	0.0	77.2	82.2	-5.0	0.0	0.5	-0.5
76	0.0	0.0	0.0	0.0	0.0	0.0	85.0	85.6	-0.6	0.0	0.0	0.0
77	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
78	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	19.2	16.8	2.4
79	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

The residue assignments from the 2D heterospectral correlation spectroscopic analysis are consistent with the residues that experienced a change in their secondary structure assignments or interaction dynamics with the heme propionates as shown in Figure 3-14.

Figure 3-14 (A) Amino acids with changed secondary structure dynamics (orange) and heme hydrogen bonding (purple) based on MD simulations. (B) Amino acids in the amide I region detected using 2D SEIRAS and associated with the IR peaks at 1658 cm^{-1} (blue) 1692 cm^{-1} (red) and 1624 cm^{-1} (yellow). Green parts are unmarked, and the heme is shown in grey, oxygen and nitrogen atoms are colored red and blue, respectively

The simulations show a decreased amount of time spent in the β -turn type III structure, including residues 14-17 (-7%) and 67-69 (-7%), during oxidation of the protein, which agrees well with the assignment peaks at 1692 and 1552 cm^{-1} (Table 3-2). An unassigned region of residues 52-55 took up the β -turn type III at an increased proportion (+ 10%) as shown in Table 3-3. This region was also identified to change upon oxidation in horse heart cyt c.⁸ In our simulations it is associated with propionate hydrogen bonding/electrostatic interaction (Table 3-4),

rather than with direct interaction through His18 or Met80, as the other two regions are. This is also reflected in the asynchronous hetero 2D plot shown in Figure 3-12, that the helix structure differ from the beta turn structure in molecular environment; hence they can be distinguished by combining the hetero 2D analysis and MD simulation.

Table 3-4 Residues identified based on different hydrogen-bond dynamics towards the heme between oxidised and reduced state MD simulations. Pr1 and Pr2 stand for propionate 1 and propionate 2 functional groups of the heme residue respectively.

H-bonding of Heme Propionate groups					
Donor	Acceptor		Ox. H-bond	Red. H-bond	Difference %
ARG 38	Heme	Pr1	0.05	0.24	-18.8
GLY 41	Heme	Pr1	0.61	0.53	8.1
ASN 52	Heme	Pr1	0.58	0.27	30.6
ASN 52	Heme	Pr2	0.37	0.75	-38.3
THR 78	Heme	Pr2	0.94	0.58	36.2
LYSH 79	Heme	Pr2	0.41	0.35	6.4

The IR peak at 1658 cm^{-1} is assigned to β -turn type II and/or α helix structure, and to the two consecutive turns at residues 32-35 and 35-38.⁸ During the MD simulations both turns are observed, but the change in secondary structure is very small (3 and 6 %) upon oxidation, which is in good agreement with NMR and X-ray observations. On the other hand, the turns changed H-bonding dynamics with the heme (Arg38), and established important hydrogen bond interactions with the regions 14-19, and 21-23 (Asn31).

Additionally the distance of the heme and His18 was also monitored in the different oxidation states (Figure 3-13). Although the average distance changes only slightly, the smaller charge of the reduced heme center allowed for more fluctuations, in good agreement with the stronger coupling observed in the hetero 2D correlation analysis.

3.4 Conclusions

We have successfully performed a 2D correlation analysis upon changing the redox state of cyt c adsorbed on a two-layer gold surface. 2D infrared correlation and 2D Raman correlation spectroscopies were performed to enhance spectral resolution and to provide a deeper understanding of the sequential changes occurring in the various portions of the protein.

Specifically, we have performed three types of correlation analysis, namely, autologous IR vs. IR, Raman vs. Raman and heterologous IR vs. Raman. Our data indicate that different segments of the protein respond differently to change in the electrode potential and reoriented sequentially. For example, it is found that the heme groups reoriented and changed conformation/interactions prior to the rest of the protein backbone. Some peaks that are not readily observed in the 1D spectra can be distinguished using a 2D correlation analysis. Peaks arising from different spectral origins or different molecular environments can be differentiated by their dynamic change under an external perturbation. Thus, the oxidation of cytochrome c can be readily monitored using 2D correlation analysis. There is also a good agreement between the results of the 2D analysis and the molecular dynamics

simulations, e.g. the distance study between the His18 and the heme is reflected in the 2D correlation spectra to reveal the conformation change of the protein.

These findings are remarkable because they show a clear fragmentation of sequential order, which indicates that the conformational changes induced by the oxidation occurred at different rates for different parts of the protein backbone. The example of a well-studied system, such as cytochrome *c*, has effectively demonstrated the potential of 2D heterospectral correlation spectroscopy for further applications on more complex membrane redox proteins.

Chapter 4 MODIFICATION OF A SILVER SUBSTRATE FOR ADVANCED SPECTRO-ELECTROCHEMICAL APPLICATIONS OF SERR SPECTROSCOPY

4.1 Introduction

Surface-enhanced Raman spectroscopy (SERS) is a powerful analytical tool in many fields of science, such as chemistry, medicine and the life sciences.¹²⁴⁻¹²⁷ Although the Raman scattering cross section is extremely small in the order of magnitude of 10^{-30}cm^2 , the sensitivity can be dramatically improved by introducing the surface enhancement effect of roughened surfaces first discovered by Fleischmann et al.^{128, 129}

It is well known that two different mechanisms are responsible for the enhancement effect, the chemical enhancement effect (CEE)⁵² and the electromagnetic enhancement effect (EMEE)¹³⁰. The former mechanism is based on charge transfer interactions between the substrate and the adsorbed analyte and therefore only takes place in the case of adsorbed molecules on the surface while EMEE occurs for all molecules close to the substrate and is based on the amplified electromagnetic fields generated upon excitation of the localized surface plasmons of nanostructured metals. Thus EMEE usually provides larger contribution to enhancement, observed experimentally. For this purpose many different fabrication methods such as nanoparticle adsorption on metals¹³¹, nanosphere lithography¹³², combining colloidal assembly with lithography¹³³ have been used successfully for the investigation of various molecules.

An additional enhancement of the signal is achieved when the frequency of the laser light is chosen to be in resonance with an electronic transition of the molecule.¹³⁴ This method called resonance Raman scattering is commonly used to investigate holo-proteins such as heme proteins for example, which have a strong absorption in the near UV region due to a π - π^* electronic transition with the resulting Soret-band.^{92, 135-138}

Electrochemically roughened silver surfaces are usually employed leading to particularly high sensitivities when Raman scattering is excited in the region of the Soret band at around 410 nm.^{92, 139} However, the electrochemical method comprising corrosive currents alternating between anodic and cathodic directions results in fractal surfaces of uncontrolled geometry. The highest control of geometry is possible using lithography methods for structure fabrication. Because of its intrinsic resolution, electron beam lithography can create various complex structures on very small scale, but is not yet appropriate for mass production, due to limitations of single structure generation.^{140, 141}

Having a simple production method a silver surface modified with Ag nanoparticles (NPs) of controlled size and dispersity allows us still a good control over the geometry and the enhancement effect. These modified surfaces were reproducibly obtained with an enhancement factor of 10^5 , four times higher than the best electrochemically roughened silver surface.¹³¹ Concerning electrochemically-controlled SERRS measurements however, a further general drawback of Ag surfaces became apparent. The potential window is limited in the positive direction to + 300 mV due to anodic dissolution of Ag. Au electrodes have a larger potential range than Ag thus making it more stable for electrochemical

purposes than silver, which unfortunately forms silver oxide in the positive potential range leading to degradation of biopolymers and corruption of the surface-enhanced Raman signal.¹⁴² Therefore, many efforts have been made to fabricate SER-active Au-Ag hybrid systems with surface plasmon resonances in the near UV region combining the specific advantages of Au and Ag. However, the fabrication of these structures is usually very complicated and only a few of them were able to achieve an enhancement effect below 550 nm.¹⁴²⁻¹⁴⁶

In this study we have shown that gold adlayers of 5 nm thickness obtained by a particular sputtering process, behave electrochemically almost as a uniform gold electrode. Ag NPs covered with an adlayer of gold, on the other hand, still give rise to the SERR effect with sufficient optical transparency of the gold adlayer and the resonance of the Ag NPs in the near UV region. This is investigated in the present study, using cytochrome *c* (cc) as the benchmark system for spectro-electrochemical measurements of heme proteins.

4.2 Experimental Section

4.2.1 Fabrication of Silver Nanoparticles

Silver nanoparticles (AgNPs) were synthesized as described previously¹³¹; briefly 8.25 ml 37% formaldehyde solution was added to 250 ml of an aqueous AgNO₃ solution (10 mM). The mixture was heated during vigorous stirring to 100°C for at least 15 minutes. Then 4 ml of an aqueous solution of citric acid (33 mM) was added. The temperature was kept constant at 100°C until the color changed in the first minutes to a bright yellow, eventually changing into a darker greenish yellow, indicating a suspension of AgNPs.

4.2.2 Preparation of Template Stripped Silver (TSS) Surfaces

TSS was prepared according to the method known for template stripped gold (TSG).¹⁴⁷ A silver layer of 45 nm was deposited by thermal evaporation on freshly cleaned silicon slides. The evaporation rate was kept constant below 0.1nm/s. Subsequently, cleaned glass slides (pure white) were glued to the silver layer using glue designed for optical purposes having a refractive index of 1.59 at 589 nm (EPO-TEK 353ND-4). The silicon slide was stripped shortly before functionalization of the smooth silver surface.

4.2.3 Surface Modification of TSS

Self-assembled monolayers (SAMs) were formed by immersion of the TSS slides into an aqueous solution of cysteamine (CA) (10 mM) for 1 hour. After thorough rinsing with water the modified TSS slides were immersed into the suspension of Ag NPs for 15 hrs. The long immersion time was required due to the varying concentration of formaldehyde in the solution. The silver slides were then rinsed with water again and dried in a stream of Ar before a gold film of 5 nm thickness was deposited by DC magnetron sputtering (Leybold Univex 450 C) in pure Argon atmosphere, from 3-in Au targets (William/CERAC) with a purity of 99.95%¹⁴⁸. Thereafter, the slides were immersed into a solution of mercaptoethanol (ME) (10 mM) for at least 1 hour to form a SAM of ME. Finally, after rinsing with water, cc was adsorbed on the functionalized Ag/AgNPs/Au electrode from a solution of cc (350 μ M) in PBS buffer (20mM K₂HPO₄ / 100 mM NaClO₄ / pH=7) for at least 30 min.

4.2.4 Measurement of Reflectance Spectra of the Structured Surfaces

For the measurements of the reflectance spectra a 75 W Xenon lamp from LOT-Oriel was used as a light source (see Figure 4-1). Two polarizers were used to adjust the intensity of the light and the light was focused on top of the surface with an objective (Olympus PL, 10 X, WD = 10.5, NA = 0.25) after passing through a beam splitter. The reflected light was collected by the same objective and was then directed to a spectrometer (Andor Shamrock 303i with a grating 150 l/mm and a spectral resolution of 1.034 nm) by the beamsplitter. The resulting reflection spectra have all been referred to TSS substrates.

Figure 4-1 Setup for reflectance spectra measurements

4.2.5 Simulation Method

In order to understand the reflection measurements, simulations employing the finite difference time domain (FDTD) method were performed. The software package Meep1.2, available under the general public license, solves the Maxwell

equations for a discrete geometry. To simulate the reflection spectra, the substrate was excited by a plane wave with normal incident. The light source had a Gaussian distribution of wavelengths, ranging from 200 to 5000 nm, mimicking a white light source, similar to the Xenon lamp. The resulting reflection from the substrate was normalized by the reflection of a flat TSS surface.

4.2.6 Raman Experiments

The setup used for Raman experiments has been described in detail previously.⁹² All Raman experiments were performed using the 406.7-415.4 nm multiline of a Kr⁺ laser (Innova 90C-K, Coherent). After passing an interference filter for 413 nm the laser beam was coupled into a confocal Raman microscope (LabRam, HR800, HORIBA Jobin Yvon) equipped with a water immersion objective (Olympus LUMPLFL, 100 X, WD = 1.5, NA = 1, BFOBJ). By this means the laser beam was focused on top of the modified TSS/AgNPs/Au surface immersed in PBS. The scattered light was filtered by a holographic notch filter and guided to a 1800 grooves/mm grating providing spectral resolution of 3 cm⁻¹. The spectrum was imaged onto a liquid nitrogen cooled back-illuminated CCD detector (Symphony, Jobin Yvon).

A custom made measuring cell (Figure 4-2) was used for the SERRS experiments. For the electrochemical measurements the cell was provided with a platinum wire used as a counter electrode (CE), and a reference electrode (RE) (Ag/AgCl, saturated KCl), while the TSS was acting as a working electrode (WE). Different from various experiments in literature¹³¹ the working electrode is not moved during the measurements. A software controlled (Gpes, Autolab)

potentiostat (Autolab, PGSTAT302, Eco Chemie, B.V., Utrecht, Netherlands) was used for modulation of the potential applied to the protein and all potentials are quoted versus SHE.

Figure 4-2. Raman measuring cell for spectro-electrochemical investigations.

4.2.7 Scanning Electron Microscopy (SEM)

SEM images of the modified TSS/AgNPs/Au slides were taken using a Zeiss SUPRA 40 microscope. The SEM images were then analyzed using the ImageJ for particle detection and counting and Gimp 2.8 for image processing.

4.3 Results and Discussions

4.3.1 Sputtering of the Gold Layer on the Silver Substrate

The adsorption of the silver nanoparticles on the silver surface was done as previously described to yield optimum results regarding the enhancement factor.¹³¹ Due to the adsorption of Ag NPs on the Ag surface, the resonance peak is better defined around the Soret band of cc absorption as compared to the resonance peak

on the electrochemically roughened surface. Moreover the reproducibility of the surface with Ag NPs is also improved. By controlling the adsorption time, we can not only control the surface coverage of the particles but we can also tune the ratio between well separated and aggregated NPs. SEM images of the TSS/AgNPs modified surfaces showed a distribution of NPs similar to the one found previously for the Ag surface optimized with respect to the enhancement factor (EF) (Figure 4-3A).

After sputtering the gold layer on top of the Ag/AgNPs surface, the particle distribution did not change, which means there was no loss of particles due to the sputtering process and the surface morphology was preserved. (see Figure 4-3B) For a better visualization of the ratio between separated particles and particle clusters, they are colored in green and red, respectively. The number of well separated particles and clusters was 68 and 119 NPs/ μm^2 , respectively, corresponding to 77 and 176 NPs/ μm^2 found in our previous work under then optimized conditions¹⁴⁹. This time, however, we had to use a prolonged assembly time, 15 hrs, due to varying concentrations of formaldehyde in the solution. The exact thickness of the sputtered Au adlayer was determined by sputtering a 5 nm thick continuous Au layer on the surface of a silicon wafer. The thickness was confirmed by ellipsometry measurements (data not shown) to be $5.5 \text{ nm} \pm 1.0 \text{ nm}$.

Figure 4-3 SEM pictures of (A) Ag/Ag NP surface and (B) Ag/Ag NP/Au surface.

The lower row shows the same SEM images with separated particles and particle clusters colored as green and red, respectively. Scale bar: 200 nm.

4.3.2 Resonance Condition of The Ag/Ag NP/Au Surface

The resonance condition of the Ag/Ag NP/Au surface has been tested both experimentally and by simulation. (Figure 4-4) The reflectance spectrum for the colloidal Ag NPs on TSS covered with a 5nm thick gold layer, shows a broad asymmetric absorption peak with a sharp falling edge at 400 nm, while the rising edge is rather smooth. The exciting laser wavelength of 410 nm lies within the plasmon resonance region. These findings are supported through FDTD simulations, using a discrete model of a regular grating of Au covered Ag nanoparticles. The model consists of Ag particles with a radius of 30 nm within the unit cell of the grating, which has a period of 200 nm. An ideal plane light source is placed 1000 nm above the surface and the spatial resolution of the model is 3.33 nm. The surroundings are modeled with a refractive index of $n = 1.45$, representing the dielectric environment of the adsorbed citric acid.¹⁵⁰

This simulation model results at least in principle in a similar reflection spectrum, even though the sharp edge at 400 nm is not reflected by the model. The regular grating excites a plasmonic resonance at 290 nm, which interferes with the peak from the localized surface plasmons (LSP) of the nanoparticles and causes the deviation between simulation and experiment in the region of the measured fast falling edge. The smooth raising edge is in good agreement with the measurement, even though the measured peak at 860 nm cannot be found in the simulations. Since the adsorption of the nanoparticles results partly in NP clusters which are not uniform in size and shape, the real topography and the simulated ones are not equivalent. Therefore the simulations are only reflecting the predominant effects and represent a crude explanation attempt for the measured reflectance spectra.

Figure 4-4 Reflectance spectrum of the substrate containing AgNP with a 5nm thick gold coating sputtered on top measured (red) and simulated (black), and normalized to reflection of a TSS substrate.

4.3.3 Extended Potentiometric Range

Cyclic voltammograms (CV) of *cc* are shown in Figure 4-5, on the modified Ag/AgNP/Au as compared to an Ag/AgNP surface. In the latter case it can clearly be seen that silver surface has been oxidized above 300 mV, most likely to form silver oxide, which is known to seriously decrease the enhancement factor.¹⁵¹ In this important potential range, the oxidization of Ag dominates the CV measurements and excludes the electrochemical investigation of *cc* on Ag/Ag NP surfaces. In the case of the Ag/AgNPs surface modified by the gold adlayer, on the other hand, could be extended to +400 mV (Figure 4-5). Hence a complete CV of *cc* could be measured with the reduction peak at 210 mV, and the oxidation peak at 300 mV, resulting in a peak separation of 90 mV and a redox potential $E^0 = 255$ mV, which is very well in the range reported in the literature.¹⁵²

Moreover, the gold adlayer reduces the well-known photodegradation of protein layers by the incident laser beam, which is a serious problem in Raman scattering. This results in a better handling and a simpler cell design for spectroelectro-chemistry. Due to the improved stability we were able to use TSS instead of massive silver electrodes which had to be rotated during the measurement to prevent the photodegradation of the protein.⁹² Since the Ag NPs are in direct contact with both the Ag surface and the gold layer, it maintains the good conductivity which is essential for electrochemistry. Hence, the Ag/Ag NP/Au electrode can be readily used as a study system for holo proteins.

Figure 4-5 Cyclic voltammogram of *cc* adsorbed on a SAM of Mercaptoethanol on the Ag NP functionalized surface with (black) and without (red) 5 nm thick Au film.

4.3.4 Surface-Enhanced Raman Spectroscopy of Cytochrome C

In order to perform a spectro-electrochemical titration of *cc*, *cc* was again adsorbed on a SAM of mercaptoethanol. Changing the applied potential stepwise between -50 to +400 mV, SERR-spectra were recorded on Ag/AgNPs/Au surface (Figure 4-6). Four Raman bands can be observed between the spectra range 1300 and 1650 cm^{-1} , which correspond to the ν_2 , ν_3 , ν_4 , ν_{10} vibrational mode of the porphyrin rings.¹⁵³ The most prominent band (ν_4) is observed around 1361 to 1374 cm^{-1} , which corresponds to the distortion mode of the pyrrole half-ring. Since this band is very sensitive to the redox state of the central metal ion,¹⁰³ the transition from the reduced to the oxidized state can be deduced from the ν_4 band shifting from 1361 to 1374 cm^{-1} . This corresponds to our previous studies on *cc* on AgNPs modified silver electrodes.¹³¹

Figure 4-6 Raman spectra showing potential titration of cc using Ag/Ag NP/Au electrode.

In order to compare the enhancement effect of the Ag/Ag NP developed before and the new Ag/Ag NP/Au surface quantitatively, the SERR spectra of cc on both surfaces with no external potential applied was shown in Figure 4-7. The intensity of the ν_4 band resulting from a distortion mode of the pyrrole half-ring is used to compare the enhancement effect on both surfaces. Even though the absolute intensity of the SERR spectra on the Ag/Ag NP surface is 2.5 times higher than that on the Ag/Ag NP/Au surface, the background noise level is also higher. Hence, taking into account the signal to noise ratio, the Ag/Ag NP/Au surface signal is even two times higher than then Ag/Ag NP surface. Even though the Au layer is absorbing intensity in the spectral region of the laser the Ag nanoparticles on TSS still contribute to 40% of the SERS signal.

Figure 4-7 Spectra comparison of cc on different surfaces, when no potential applied

In any case, the optical response of the modified surface is still dominated by the local plasmons of the AgNPs, although the intensity of the single particle peak is quenched to a certain extent by the thin Au layer. Hence we could expect from the reflectance spectra of the Ag/AgNPs/Au surface that the enhancement factor due to resonance with the exciting light is decreased, but still high enough for appropriate Raman measurements with a signal to noise ratio high enough for spectro-electrochemical applications. The geometrical properties of the NPs, on the other hand do not change much, thus we can conclude that the electromagnetic enhancement effect remains stable. The chemical enhancement effect, also known as charge transfer effect, however, could be different on the pure Ag/AgNPs and Ag/AgNPs/Au surface, due to different interactions between a pure silver and an Au coated silver surface, which could eventually influence the orientation of the assembled analytes for example cc on the two surfaces.

4.4 Conclusions

We have shown that the introduction of a 5 nm Au layer on an Ag surface with Ag nanoparticles significantly improves the stability of the surface and makes it more suitable for electrochemical measurements due to an extended potential window. The comparison of the SEM images of both surfaces showed that the nanoparticle distribution remained the same before and after sputtering the Au layer. FDTD simulation of the reflectance spectra indicated that the resonance position, for the surface with assembled particles, is predominantly influenced by the diameter of the single particles. Therefore the simulations were able to give a rough explanation for the measured reflectance spectra.

Cyclic voltammetry measurements revealed that applying higher positive voltages up to 400 mV was possible on the new surface, allowing to use this surface for spectro-electrochemical investigation of complex heme proteins. The comparison of the SERRS measurement on both Ag/AgNP/Au surface and Ag/Ag NP surface showed that the new surface maintains 40% of the enhancement due to the introduction of the Au adlayer, which is sufficient to obtain a very good signal to noise ratio in the cc titration measurement.

In summary, we have designed a modified surface for spectro-electrochemical applications, exhibiting the advantages of the Ag surface combined with the increased stability of the Au adlayer. Since Raman spectroscopy has recently drastically profited from technical development, we foresee that SERRS technique with our newly developed substrate may be widely used for the investigation of complex redox proteins such as horseradish

peroxidase, glucose oxidase, cytochrome P450, very important for potential biosensing- or drug screening-applications.

Chapter 5 CONCLUSIONS AND RECOMMENDATIONS

In this thesis, the rational design of plasmonic nanostructures for surface enhanced spectroscopy has been developed to address the challenges of the biological field. Spectroscopic techniques have been proved to be very useful in characterizing chemical and biological molecules such as methylene blue, cytochrome c, etc. Plasmonic substrate can greatly enhance the signal to noise ratio in the spectroscopic analysis through its unique properties of confining and amplifying electromagnetic radiation. By developing each configuration of the plasmonic structures, they can be used to achieve the tunable behavior with the laser excitation, better signal to noise ratio to correlate vibrational spectroscopic data for conformational change study, and extended potentiometric range for redox proteins using electrochemistry and surface-enhanced Raman spectroscopy. The main conclusions drawn from this project are shown as below.

Firstly, it has been have successfully demonstrated the synergistic modulation of the 2D uniform arrays of Au and Ag nanoparticles on a substrate through the interplay of inter-particle and particle-substrate interactions, which resulted in a wide range of inter-particle distances between the nanoparticles on the substrate, as confirmed by SEM and the obvious color change of the substrate. The resultant 2D Au and Ag nanoparticle arrays with tunable inter-particle distance exhibited tunable and explicit plasmonic properties and induced multiplex SERS response in the presence of molecular analytes. This strategy provides an approach towards producing high quality, large area 2D ordered arrays using

colloidal nanoparticles, which have potential applications as multiplexed spectroscopic sensors, integrated devices, cell culture and antennas

Two layer gold surface was also developed to utilize its roughened surface to generate plasmonic hot spots for surface-enhanced Raman spectroscopy, and surface enhanced infrared absorption spectroscopy. Cytochrome c, an important protein in the electron transport chain, was selected as the study system since its protein backbone is infrared active and its heme center is Raman active. Such selection rules have made two-dimensional correlation spectroscopy very useful to analysis this protein as it gives a more overall picture of the protein's conformational change during the redox change. It was shown that different components of the protein would have different experience from the potential by the electrode, and the conformational change between the functional groups can be distinguished to possess sequential relationship as the redox state of cytochrome c was changed. Because the heme is closest to the iron center, it can be demonstrated from the asynchronous hetero 2D spectra that it will re-orientate before the protein backbone, as the electrode potential was increased from negative to positive ones.

The previously developed silver nanoparticle adsorbed on silver surface was also improved by concerning that pure silver structure is not stable in the electrochemical environment and has limited range in the positive potential side for redox studies. A thin layer of 5 nm gold was sputtered onto the silver substrate and it was shown from the SEM characterization that the configuration of the silver nanoparticles didn't show obvious change before and after the sputtering process, which is desirable to achieve the B mode resonance with the laser excitation. The potentiometric range was increased from +300 mV to +400 mV vs.

SHE, and could finally complete the redox cycle when using cytochrome c as the analyte.

Hence to conclude, plasmonic nanostructures have been rationally designed and fabricated for the study of selected biological molecules. The performance of the surface enhanced spectroscopy has been improved by achieving tunable behavior with the laser excitation, increasing the signal to noise ratio, and widening the potentiometric range. Such platforms can be readily extended to develop other plasmonic substrate or other desired analyte.

Recommendation of future work

With the establishment of the plasmonic nanostructures stated in previous session, they can be tailored and modified to suit the purpose of other related application as well. For example, the two dimensional gold and silver substrate have shown their capability of tunable behavior with the excitation. Two-dimensional surface as also serve as a great support for tethered bilayer lipid membranes, which is a useful and convenient biomimetic way to resemble the real membrane system in cells or cell organelles. We can also construct the plasmonic nanoparticle array in three-dimensional form to achieve an even closer configuration for the cells and organelles.

As shown in Figure 5-1, the three-dimensional silica and gold core satellite structure can be developed by substituting the silicon wafer surface with the silica nanoparticles. The surface chemistry is similar. By tuning the ionic strength in the solution, the inter-gold-nanoparticle distance can also be tuned to achieve different degrees of coupling. The resonance condition can be seen by the optical images that they exhibit different colors which can be distinguished by naked eyes.

Different core sizes of the silica nanoparticles can also be synthesized and used to achieve single silica gold core satellite probe that is visible under the conventional microscope for surface-enhanced Raman spectroscopy.

Figure 5-1 SEM pictures of colloidal core satellite structures and their corresponding colloid solution colors

Two-dimensional correlation spectroscopy can also be applied to different analyte, or the same analyte cytochrome c but from different perspectives. For example, the work presented in this thesis focused on the conformation change of cytochrome c at different applied potentials. We also can study the system under the time resolved conditions, meaning at different time scales. By utilizing an acoustic optical modulator, the electrochemical and laser pulses can be synchronized and induce the population of cytochrome c to evolve between the oxidized species and reduced species. Useful information is possible to be obtained during this process.

Besides cytochrome c, cytochrome c oxidase is an important membrane protein that is involved in the electron transport chain. It has more complex

structures that consist of four redox centers and two sub units of protein backbones. It has been successfully studied in a protein tethered bilayer lipid membrane manner, and the correlation study by combining the vibrational spectroscopic data can also generate a number of correlation peaks that can be carefully analyzed for useful interpretations.

Chapter 6 LIST OF PUBLICATIONS

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[#] Equal contribution
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