

Design and development of multifunctional Raman active noble metals nanoprobe for the detection of malaria and tuberculosis biomarkers

BY

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Declaration

I declare that this thesis “Design and development of multifunctional Raman active noble metals nanoprobe for the detection of malaria and tuberculosis biomarkers” is my own work, aided by my supervisors submitted to the School of Chemistry, Faculty of Science, University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination in any other university, and all the sources used or quoted have been indicated and acknowledged with complete references.

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Abstract

Surface enhanced Raman spectroscopy (SERS) has emerged as a surface sensitive vibrational technique that leads to the enhancement of the Raman scattering molecules on or close to the surface of a plasmonic nanostructure. The enhancement is found to be in orders of 10^4 to 10^{15} , which allows the technique to be sensitive enough to detect a single molecule.

In this study, we report on the synthesis of different sizes of gold and silver nanoparticles (AuNPs and AgNPs) and gold nanorods (AuNRs). These are functionalized or co-stabilized with different stoichiometric ratios of HS-(CH₂)₁₁-PEG-COOH and alkanethiols (Raman reporters), i.e.; HS-(CH₂)₁₁-NHCO-coumarin(C), HS-(CH₂)₁₁-triphenylimidazole (TPI), HS-(CH₂)₁₁-indole (HSI), HS-(CH₂)₁₁-hydroquinone (HQ) to form mixed monolayer protected clusters (MMPCs). The alkanethiols were chosen as Raman reporters to facilitate the self-assembled formation of monolayers on the metal surface, thus resulting in stable MMPCs. The optical properties and stability of MMPCs were obtained using ultraviolet-visible (UV-vis) spectrophotometry and a zeta sizer. Size and shape of the as-synthesized nanoparticles were obtained using transmission electron microscopy (TEM). The tendency of thiol-capped nanoparticles to form self-assembled ordered superlattices was observed. Their Raman activities were evaluated using Raman spectroscopy, with the enhancement factor (EF) being calculated from the intensities of symmetric stretch vibrations of C-H observed in the region of about 2900 to 3000 cm⁻¹ in all SERS spectra. In all four different alkanethiols (Raman reporters), smaller size metal nanoparticles (14 nm for AuNPs and 16 nm AgNPs) showed higher EF compared to 30 and 40 nm metal nanoparticles. The EF was observed to increase proportionally with stoichiometric ratios of alkanethiols from 1%

to 50%. The prepared MMPCs with small sizes were used as a SERS probe for the detection of malaria and tuberculosis biomarkers.

The conjugates of monoclonal anti-Plasmodium Lactate Dehydrogenase (anti-pLDH) pan-malaria and Protein A with four different alkanethiols were used to capture 1 mg/mL of recombinant *Plasmodium Vivax Lactate Dehydrogenase* (Pv-LDH) and or *Mycobacterium Tuberculosis* 38 kDA (MTB), respectively. The capturing of the antigens with the conjugates was confirmed by an immunochromatographic test and Raman spectroscopy. Immunochromatographic tests confirmed the presence of the coloured test line which revealed the interaction of the immobilised antigens and antibodies on the conjugates, whereas Raman spectroscopy confirmed the presence of the alkanethiols' vibrational peaks on the conjugates which confirmed the capturing of the antigen. The intensity of vibrational peak by Raman spectroscopy at 1020 cm^{-1} was found to be dependent on the alkanethiols used, and on the captured concentration of an antigen.

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Additional publications

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Abbreviations

AgMMPCs	Silver mixed monolayer protected clusters
AgNPs	Silver nanoparticles
a.u	arbitrary units
AIDS	Acquired immunodeficiency syndrome
AuMMPCs	Gold mixed monolayer protected clusters
AuNPs	Gold nanoparticles
AuNRs	Gold nanorods
BSA	Bovine serum albumin
C	HS-(CH ₂) ₁₁ -NHCO-coumarin
CCD	Charged coupled device
CE	Chemical enhancement
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
EDC	N-ethyl-N-[dimethylaminopropyl] carbodiimide
EF	Enhancement factor
EM	Electromagnetic enhancement
FT-Raman	Fourier transform Raman
HIV	Human immunodeficiency virus
HOMO	Highest occupied molecular orbital
HQ	HS-(CH ₂) ₁₁ -hydroquinone
HSI	HS-(CH ₂) ₁₁ -indole
IgG	Immunoglobins
LOD	Limit of detection
LUMO	Lowest unoccupied molecular orbital
mAbs	Monoclonal antibodies
MGITC	Malachite green isothiocyanate

mg/ml	Milligram per millimetres
mL	Millimetres
MMPCs	Mixed monolayer protected clusters
MPCs	Monolayer protected clusters
mV	Millivolt
NHS	N-hydroxysuccinimide
NHSS	N-hydroxysulphosuccinimide
NIR	Near-infrared
nm	Nanometres
NPs	Nanoparticles
OD	Optical density
PABs	Polyclonal antibodies
PEGs	Poly ethylene glycol
PMT	Photomultiplier tubes
QDs	quantum dots
SAMs	Self-Assembled Monolayers
SERRS	Surface-enhanced resonant Raman scattering
SERS	Surface enhanced Raman scattering
SPR	Surface plasmon resonance
TB	Tuberculosis
TEM	Transmission electron microscope
TPI	HS-(CH ₂) ₁₁ -triphenylimidazole
UV-vis	Ultraviolet–visible spectroscopy
WHO	World Health Organization

Chapter One

1.0 Motivation of the study

Nanoscience and Nanotechnology offer the opportunity to design multifunctional nanostructures for various applications because of the possibility of combining more functionalities into a single entity on the nanoscale in an unprecedented way. These multifunctional nanostructures not only monitor and detect the cellular changes associated with disease pathogenesis, but also offer possible treatment of these diseases at cellular level [1-3]. Recently, the multidisciplinary developments in the fields of chemistry, physics and biology have led to the rational design and use of multifunctional nanostructures for biomedical applications, such as cell imaging, drug delivery, diagnosis and therapeutics [3-6]. In diagnostics, development of immunoassays has been an active field in the past years due to accurate methods imparted by antibody specificity [7-9]. In last two decades, significant efforts have been put on improving the analytical performance of immunoassays, with the majority effort focusing on lowering the detection limit using novel detection modalities, such as Surface enhanced Raman scattering (SERS) [8-12].

Surface enhanced Raman scattering is a surface sensitive spectroscopic technique that leads to the enhancement or detection of vibrational molecules near the plasmonic nanostructures, greatly extending the role of Raman spectroscopy. Generally, Raman scattering is a relatively weak optical process that provides information about the unique vibrational modes of molecules; this phenomenon was discovered by an Indian physicist in 1928[13]. The scattering that occurs in Raman spectroscopy can be categorized into either elastic (Rayleigh) or inelastic (Stokes or Anti-Stokes shift) as shown in Figure 1.1. After the discovery by Raman, the scattering was assumed to be of the same energy as the incident radiation, termed elastic scattering. Inelastic scattering refers to the Stokes and

Anti-Stokes scattering which result in a change in energy. Stokes scattering is observed when the energy of the emitted photon is lower than the energy of the incident radiation, and anti-stoke result from higher energy of the emitted photon. Stokes-shift Raman scattering is dominant by anti-stokes shift.

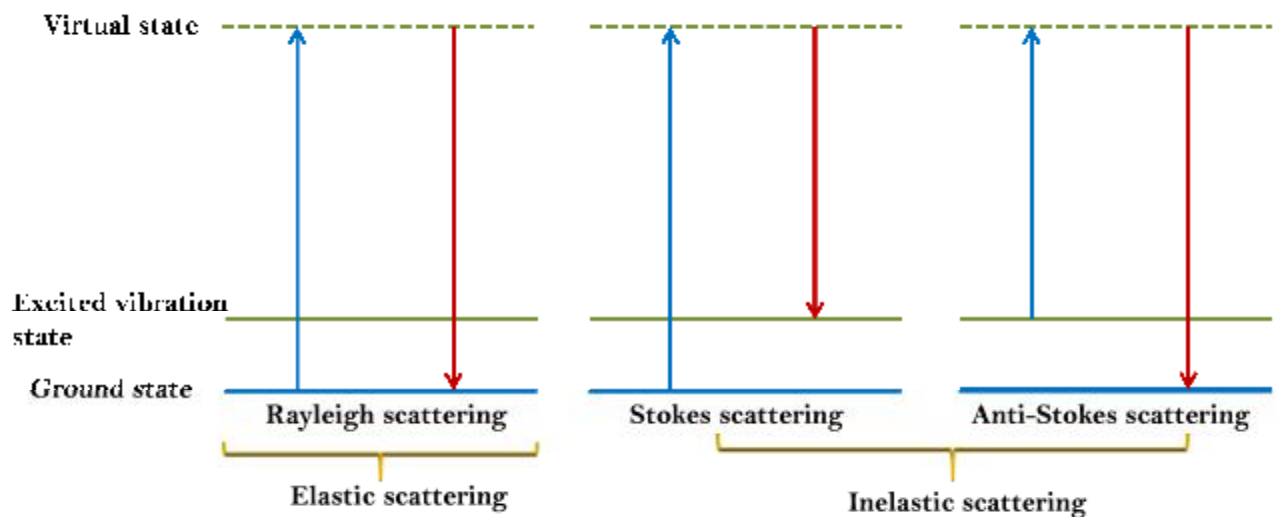


Figure 1.1: Energy diagram showing the difference between Rayleigh scattering and Raman scattering (Stokes and Anti-Stokes) [14]

A Raman spectrometer consists of a light source, monochromator, sample holder and the detector. Several factors affect the analysis on Raman spectroscopy including the signal to noise ratio, stability of the instrument, fluorescence impingement and resolution. The development of the effective fourier transform (FT)-Raman spectrometers with red or near-infrared laser sources solved the problem of fluorescence impingement that affects the signal. The improvement on the sensitiveness of the detectors in conjunction with coupling of optical filters and microscopes enhanced the capacity of analysis [15]. Raman instrumentation is based on two technologies; dispersive Raman and FT-Raman, shown in Figure 1.2. The two technologies differ in terms of the laser source and also by the way in which the scattering is detected [16-18].

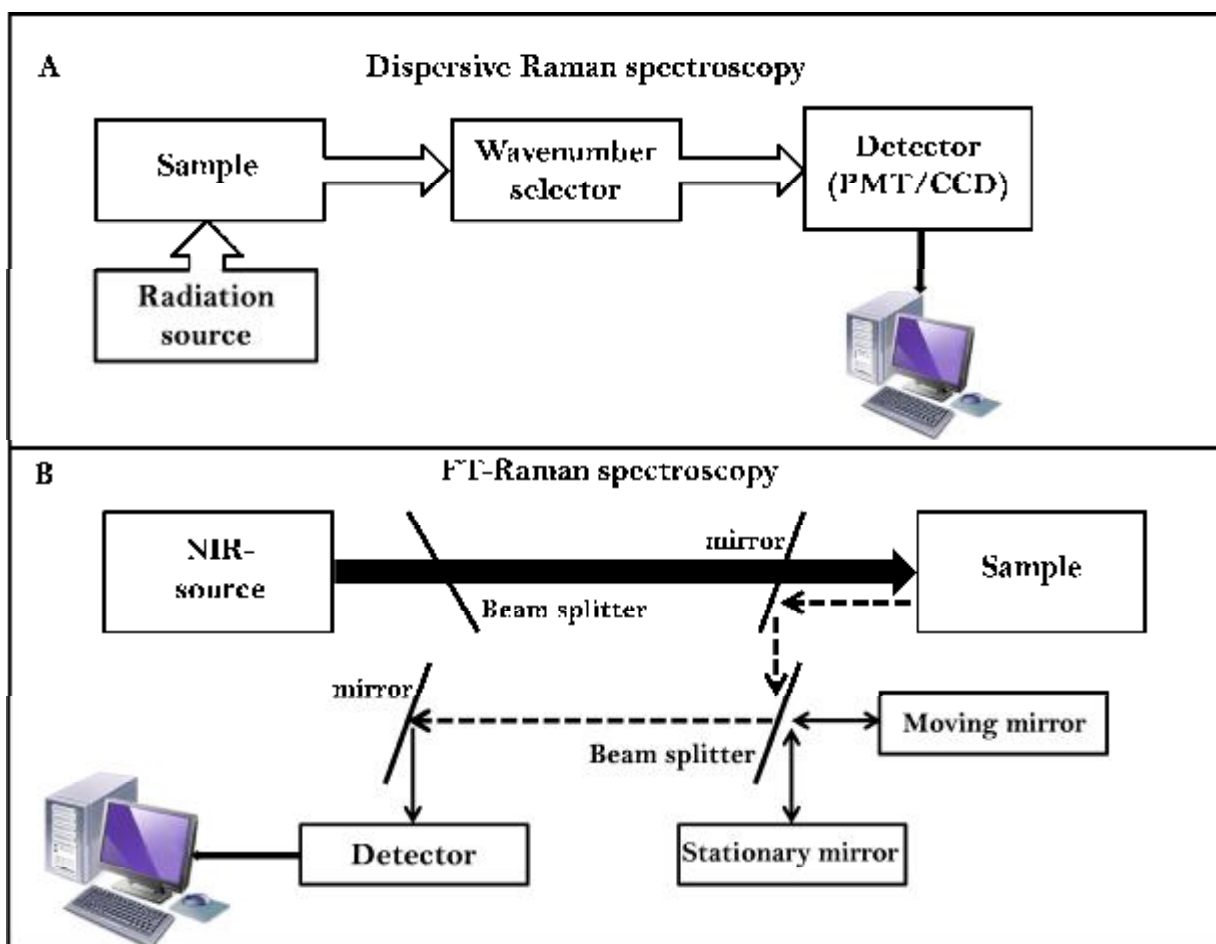


Figure 1.2: Raman instrumentation (a) dispersive Raman and (b) FT-Raman spectroscopy

In the early 1970s, Fleischman and co-workers discovered that the Raman signals from pyridine were significantly enhanced when adsorbed on a roughened silver electrode [19], and SERS was born. SERS has been applied in a number of analyses ranging from biochemistry to life sciences [20-26]. The classic application of SERS is the direct sensing of various analytes attached onto a metallic substrate, yielding both qualitative and quantitative information [27].

The nanotechnology evolution has enabled the design of novel SERS probes that combine metallic nanostructures and specific organic Raman reporter molecules. Such SERS-active probes produce strong, characteristics Raman signals and can be used to indirectly detect

the target molecules by using laser Raman spectroscopy demonstrating optical labelling functions similar to those of external chromophores, such as organic dyes and fluorescent quantum dots (QDs). However, this kind of probe has the ultra-sensitivity, multiplexing, and quantitative abilities of the SERS technique, and it shows extraordinary features for bio-analysis. The development of the multifunctional optical SERS-based detection technique in the life sciences bears some advantages over the other techniques, namely [28,29];

- ▼ Improved photo-bleaching resistance.
- ▼ Narrow vibrational band signatures.
- ▼ Excitation with a single laser source even in the red region which minimises fluorescence background.
- ▼ Rich spectral information with enhanced sensitivity.
- ▼ Facilitated multiplexed immunoassays.

Despite tremendous interest to exploit such properties, the setback to bring the SERS-based detection technique onto practical application has been due to the challenges such as;

- ▼ Robustness and reproducibility of metallic nanoparticles used as substrates.
- ▼ Chemical and biological compatibility of metallic nanoparticles which require controlled chemical functionalization.
- ▼ Activity loss of biomolecules conjugated to metal nanoparticles which mostly depend on the stability of metallic nanoparticles.

These challenges were avoided by using alkanethiols as Raman reporters which lead to the formation of highly stable mixed monolayer protected clusters (MMPCs). The incorporation of polyethylene glycol (PEG) layer influenced water solubility, biocompatibility, stability and also lowers the non-specific binding.

1.1 Aims and approaches to the study

The aim of the project was to design and develop the multifunctional Raman active probes of AuMMPCs and AgMMPCs using difference Raman reporters. The developed SERS probes were used to design an immunoassay for malaria and tuberculosis. Several objectives were set-out to achieve the aim of the study as follows;

1. To synthesize and characterize different sizes and shapes gold and silver nanoparticles.
2. To functionalize these nanoparticles with the monolayer bearing –COOH group and one of the four Raman reporters to form mixed monolayers protected clusters (MMPCs).
3. To conjugate the functionalized MMPCs with specific biomarkers for the detection of malaria and tuberculosis.
4. To test the prepared Raman probes as SERS probes for diagnosis of malaria and tuberculosis.

1.2 Thesis outline

The format of the thesis is as follows:

Chapter one-Motivation of the study

The chapter briefly gives the motivation of the study, history of the Raman technique which leads to the discovery of Surface enhanced Raman spectroscopy. The chapter also presents the outline of the thesis.

Chapter two-Literature review

This chapter presents the general literature associated with surface enhanced Raman scattering, in particular, the evolution, fundamental mechanisms and how the varied parameters used, i.e. affect the metal nanoparticle size, shape and the choice of the Raman reporter. The chapter also focuses on the literature of adsorption of alkanethiols on a metallic surface to form self-assembled monolayers, the conjugation strategies of the biomolecules and the applications of SERS probes.

Chapter three-Synthesis and functionalization of different gold nanoparticles sizes with different Raman reporters

This chapter focuses on the synthesis and characterization of different gold nanoparticle (AuNP) sizes and their functionalization with four Raman reporters to form gold mixed monolayer protected clusters. The synthesized AuMMPCs were evaluated for Raman activities.

Chapter four-Synthesis and functionalization of gold nanorods with different Raman reporters

The chapter presents the synthesis and functionalization of gold nanorods (AuNRs) with four different Raman reporters and their Raman activities. A part of this chapter was published as: *Mbuso Mlambo, Phumlani S. Mdluli, Poslet Shumbula, Siyasanga Mpelane, Nosipho Moloto, Amanda Skepu and Robert Tshikhudo. Synthesis and characterisation of mixed monolayer*

protected gold nanorods and their Raman activities. Materials Research Bulletin 48 (2013) 4181–4185.

Chapter five-Synthesis and functionalization of different silver nanoparticles sizes with different Raman reporters

This chapter focuses on the synthesis and characterization of different silver nanoparticle (AgNP) sizes and their functionalization with four Raman reporters to form silver mixed monolayer protected clusters. The synthesized AgMMPCs were evaluated for Raman activities.

Chapter six-Design and development of gold and silver nanoparticles SERS immunoassay for the detection of malaria and tuberculosis biomarkers

The chapter reports the design and development of SERS immunoassays using gold and silver nanoparticles functionalized with four different Raman active alkanethiols. The immunoassays were used to detect malaria and tuberculosis.

Chapter seven-Conclusions and future prospects

This chapter gives the general conclusions based on the objectives highlighted at the beginning of the thesis and the future prospects of the study.

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Chapter Two

General literature review on SERS

2.1 Introduction to Surface-Enhanced Raman scattering/spectroscopy

The SERS effect is about amplifying or enhancing the Raman signals (almost exclusively coming from molecules) by several orders of magnitude. The amplification of the signals in SERS comes through the electromagnetic interaction of light with metals; this produces large amplifications of the laser field through excitations known as plasmon resonances. The molecules must be adsorbed on a metal surface or be in a close proximity to benefit. The coinage surface enhanced Raman scattering or SERS, is summarized in the following three foundations of the effect:

- ▼ Surface (S): SERS is a surface sensitive technique; the molecules must be on or close to the metal surface. This is a major point for applications of SERS, the compound to be attached must bear the functional groups capable of binding the metal substrate.
- ▼ Enhanced (E): The signal enhancement is provided by the plasmon resonances in the metal substrate. The magnitude of enhancement is determined by the optical properties of the metal used, as the interaction with light will differ from metal to metal.
- ▼ Raman (R): The technique involves measuring the Raman signals of molecules (SERS probes or analytes). Raman spectroscopy is the study of inelastic light scattering as shown in Figure 1.1, and it provides information on the vibrational structure of molecules.

- V** The final S in SERS represents scattering or spectroscopy, depending on whether one prefers to emphasize the optical effect (scattering) or the technique (spectroscopy).

2.2 Fundamental theory of SERS

The fundamental theory of SERS has been well documented in the form of excellent reviews and books [1-11]. There are two well understood primary theoretical mechanistic models used to explain SERS, a long-range electromagnetic (EM) enhancement and the short-range chemical enhancement (CE) models.

2.2.1 Electromagnetic enhancement

The EM enhancement occurs when the incident light illuminates a metal surface, causing the collective oscillations of the electrons of a metal nanoparticle called surface plasmon resonance (SPR) [12]. When the wavelength of the incident light matches the SPR wavelength, the metal nanoparticle will radiate a dipolar field and be coherent with the exciting electric field. The process results in redistribution of the local field and a greater enhancement of the EM field at a specific position of the nanostructure called a ‘hot spot’. The molecules that are near or adsorbed on the hot spot will experience greater enhanced incident intensity that will excite the Raman modes. The Raman signal scattered will be enhanced in the same way, yielding greater total output. When both the incident light and the scattered photon of molecules are in resonance with the plasmon frequency, the SERS signal is enhanced by a 10^4 enhancement [7]. The selection rule for the EM enhancement is distance-dependant, it only applies to those molecules on or close to the metal surface and the EM enhancement is chemically non-selective [1]. The EM enhancement is also a main contributor to the SERS effect [5, 7] and dependent on the inherent properties of the metal nanostructure (material type, size and shape) [13].

2.2.2 Chemical enhancement

The second mode of enhancement is related to the short-range mechanism of the chemical nature, and is known to contribute an order or two of magnitude of enhancement to the Raman signal intensity [14-16]. CE involves the interaction between the chemisorbed molecules and the metal surface, and can further be explained in two ways. The first explanation is that the molecule (adsorbate) to the metal surface induces a novel charge transfer intermediates that have larger Raman cross sections than those of molecules unadsorbed [3]. The other explanation is that the lowest unoccupied molecular orbital (LUMO) and highest occupied molecular orbital (HOMO) of the chemisorbed molecules fall symmetrically about the Fermi level of the metal surface, and the excitation of half energy is enough to make a transition. Thus, charge transfer between a metal surface and an adsorbate can produce Raman excitation photons [2,17]. CE is achieved by altering the scattering cross section of an adsorbate on a metal surface, thus the enhancement is dependent on the chemical features of an adsorbate [7].

2.3 SERS probes and SERS substrates

There are many parameters that can be varied in SERS experiments. However, only two parameters will be addressed in this thesis, i.e.; (i) the molecular species to be detected, and (ii) the metallic structures onto which they adsorb. The above parameters are independent to a certain extent, although some degree of compatibility is crucial.

2.3.1 SERS substrates

A SERS substrate is defined as a metallic structure that supports or gives the strongest plasmon resonances leading to the largest enhancement or amplification. The SERS substrates can be distinguished in two different types of enhancement, (i) those that provide a relatively uniform enhancement on the surface and (ii) those with large variations. The

latter exhibits highly localized positions of very high enhancement, suitable for use in single-molecule detection. The former is vital for reproducibility in applications.

Since SERS enhancements arise from a resonant response of the substrate, the enhancement is strongly dependent on the excitation wavelength. This is an indication of SERS enhancement varying with excitation wavelength, and to a lesser extent with a Raman shift of the modes. To conclude; any given SERS substrate will exhibit good enhancements in a limited excitation wavelength range. Recently, researchers have opted to use metal nanoparticles rather than the roughened metal, with gold and silver nanoparticles being mostly studied. Metal nanoparticles act as a structural scaffold and as a Raman signal amplifier for the synthesis of SERS probes. In general, their size distribution, geometry, chemical composition, and surface chemistry influence the Raman enhancement ability. The effect of changing the optical properties of metal nanoparticles or nanocluster-based SERS probes have been recently summarized by Wang *et al.* [18] as shown in Figure 2.1. In this work, gold nanoparticles, silver nanoparticles and gold nanorods were studied. The unique properties of these metal nanoparticles (Au and Ag) and nanorods-based SERS substrates are explained herein.

2.3.1.1 Gold and Silver nanospheres.

Gold and silver nanoparticles are the most widely studied Raman enhancing substrates. AuNPs are synthesized by the reduction of HAuCl_4 with sodium citrate as described by Frens [19]. AuNPs prepared by the above method have offered many advantages, such as production of controlled size distribution, long-term stability, and biocompatibility, which makes them interesting for various applications.

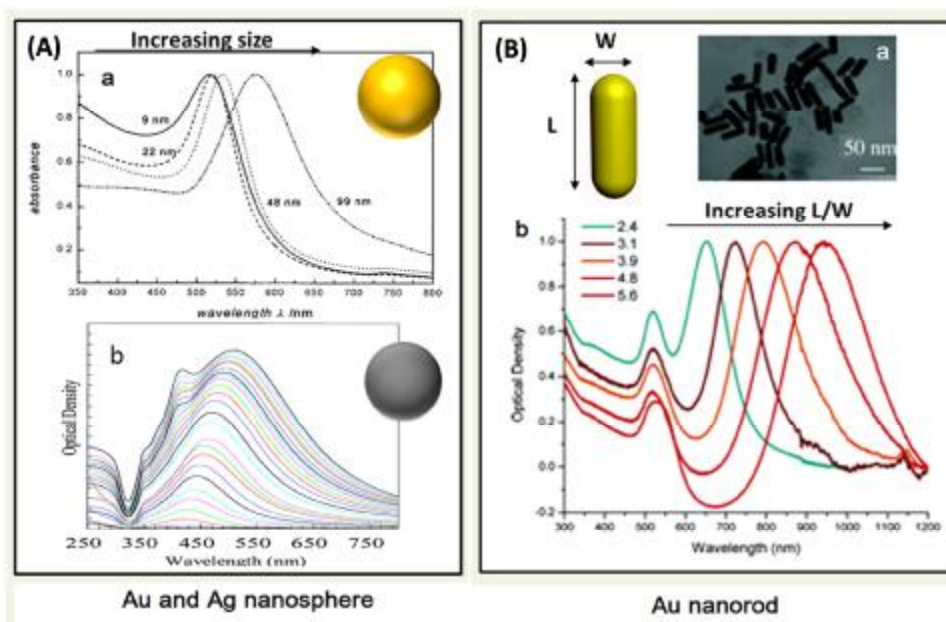


Figure 2.1: Representative substrates used for the synthesis of SERS probes. (A) (a) UV-vis absorption spectra of different sized AuNPs in water. The particle sizes are 9, 22, 48, and 99 nm, respectively [28]. (b) Extinction spectra of different sized AgNPs. The particle sizes are 29, 34, 37, 44, 48, 52, 58, 61, 75, 78, 92, 97, 105, 113, 120, and 136 nm, respectively [29]. (B) (a) TEM image of gold nanorods (AuNRs) of aspect ratio 3.9. Surface plasmon absorption spectra of AuNRs of different aspect ratios (b), showing the sensitivity of the strong longitudinal band to the aspect ratios of the nanorods [20].

Beside AuNPs which have been mostly studied for biological applications or drug delivery by SERS [21-23], AgNPs have also been employed for SERS studies. The most widely used AgNPs for SERS applications are usually prepared by the reduction of AgNO_3 either with sodium citrate at boiling condition as reported by Lee-Meisel [24] or with hydroxylamine hydrochloride at room temperature as reported by Leopold *et al.* [25]. As shown in Figure 2.1 A (b), the maximum SPR position of both AgNPs types is between 400-600 nm, and red-shifts with increasing particle size [26, 27]. AgNPs have been

reported to display a large plasmonic effect compared to gold nanoparticles rendering a more efficient Raman signal-enhancing material, with SERS signals higher than those of similar gold nanostructures by 10 to 100-fold [28]. The contributing factor to the high plasmonic nature of AgNPs is their possession of a d-s band gap in the UV region, which promotes less damping of the plasmon mode [29, 30]. The size of a nanomaterial plays a crucial role in determining SERS signal enhancement capacity [31-34]. Firstly, the intensity of the electromagnetic field is strongly dependent on the number of electrons excited and, the volume of the nanostructure [28]. Secondly, the use of bigger sized nanoparticles is inappropriate since they result in larger radiation damping effects, thereby decreasing the enhancement factor. The reported optimum size range for efficient Raman signal-enhancing is 30-100 nm [4]. Silver nanoparticles are also known to have poor biocompatibility, uncontrollable size distributions, and only short-term stability [35]; hence, AuNPs are normally used in designing SERS tags.

2.3.1.2. Gold Nanorods.

The absorption spectrum of AuNRs have two bands; a weak transverse band in the visible region which appears in the same region as that of AuNPs corresponding to electron oscillations along the short axis and a strong longitudinal band in the longer wavelength region, corresponding to electron oscillation along the long axis [36]. AuNRs have attracted much attention due to their tuneable, longitudinal plasmon resonance that can be achieved by changing the aspect ratio [37]. By simply varying the silver nitrate concentration during the growth process, the longitudinal plasmon resonance shifts from the visible to the NIR region as the rod's aspect ratio increases from 2.4 to 5.6, as shown in Figure 2.1C [20]. Besides, AuNRs have a high theoretical permicrometer absorption coefficient that is more than an order of magnitude higher than that of nanoparticles [38]. These advantages have enabled AuNRs to be used in many bio-applications, including

molecular and cell imaging, in vivo tumour detection, and photothermal therapy [38-41]. The aspect ratio also plays an important role in the Raman enhanced effect: The SERS signals of 4-mercaptopyridine attached on AuNRs with an aspect ratio of 1.6 are much stronger than those of AuNRs with an aspect ratio of 4.5 when using a 632.8 nm laser as the excitation source [42].

2.3.2 SERS probes

A good SERS probe is defined as the compound which is adsorbed (covalent) on the metallic surface, and its vibrating or scattering molecules are highly or largely amplified. SERS probes are classified by two characteristics;

Intrinsic Raman properties: The intensity of Raman scattering can vary by many orders of magnitude depending on the molecules being investigated and the incident laser wavelength. The Raman scattering is generally intense for molecules with electronic energies close to the exciting laser energy, resulting to resonant Raman scattering (RRS).

Probe/metal interaction: For any given compound to be classified as a good SERS probe, it must not only be a good Raman scatterer, but should have the ability to adsorb efficiently on the SERS substrate. Table 2.1 shows the summary of the reported different SERS probes or Raman reporters bearing functional groups with high and low affinities for metal substrates.

One of the most crucial steps in the synthesis of SERS probes is to select the best Raman reporter to conjugate or to be adsorbed on the SERS substrate. Several principles are considered in the selection of the Raman reporter(s) and the deposition method to be followed in preparing the SERS probes with strong and stable signals.

Since the two enhancement mechanisms (discussed in section 2.4) are mostly distance-dependant, the Raman reporter molecules need to be attached or in a close proximity to the surface of the SERS substrate for optimum enhancement to occur. The interaction between the Raman reporter molecules and the metal surface should be strong enough to prevent desorption during further modification or conjugation of biomolecules for application. In the case of Au and Ag, nitrogen- or sulphur-containing molecules are often used because of their strong affinity to these metal surfaces.

- ▼ The Raman reporter molecules must have a large Raman cross section to produce strong SERS signals.

In some instances, optical absorption of an ideal Raman reporter to be chosen plays a role in deciding the excitation wavelength, if the two matches would lead to surface-enhanced resonant Raman scattering (SERRS) which may cause the enhancement factor to be further enhanced 100 times [43].

- ▼ An ideal reporter(s) to be chosen must form uniform layers or monolayers or even mixed monolayers on the surface of a metal nanostructure since the SERS signal is also influenced by the manner and the number of the reporters encapsulated on the probe.

Table 2.1: Summary of the reported Raman reporters bearing functional groups [18]

Type	Probe/Raman reporter	Linking mode	Advantages	Disadvantages
Nitrogen-containing cationic compounds	Crystal violet, rhodamine B, rhodamine 6G, Nile blue	electrostatic force N-Au (Ag) interaction	cheap large Raman cross section	weak affinity to metal weak signal stability hard for further probe surface coating
Sulfur-containing compounds	3,3'-diethylthiadicarbocynine iodide malachite green isothiocyanate tetramethylrhodamine-5-isothiocyanate rhodamine-5-(and-6-) isothiocyanate	S-Au (Ag) interaction	large Raman cross section strong binding affinity to metal good surface chemistry for targets attachment	limited types hard to form SAMs
small thiol-containing compounds	4-aminothiophenol 4-methylbenzenethiol 2-naphthalenethiol benzenethiol	S-Au (Ag) interaction	cheap strong binding affinity to metal few Raman bands which is good for multiplexing	small Raman cross section

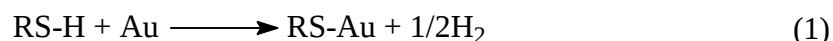
- ▼ The last important principle to consider on choosing the suitable Raman reporter is to select the one(s) with relatively few bands to avoid the overlapping of peaks especially when more than one reporter is used.

While the Raman reporter strongly influences the quality of the SERS probes, there are only a few studies that have investigated novel Raman reporters. The key problem is that researchers prefer to use the well-studied molecules and focus more on the metal substrates. Olivo and co-workers have reported a combinatorial synthesis and screening of a triphenylmethane compounds library for the improvement of a highly sensitive SERS probe [44-48]. The group found at least 13 compounds having a stronger SERS signal than crystal violet. The modified lipoic acid enabled a strong binding to gold surface, and the probes showed a better signal stability than those of malachite green isothiocyanate (MGITC) [45,46].

2.3.2.1 Self-Assembled Monolayer (SAMs) Protected clusters

It has been noted that the formation of uniform layers of Raman reporters on the surface of a metal substrate improves the SERS amplification. The difficulty of attaching uncharged high density reporters without inducing aggregating self-assembled monolayer structures of Raman reporters was developed [49-52]. These Raman reporters possess a thiol group to anchor to the metal surface and a carboxyl group which is used as a linkage to biomolecules, rendering them good for applications. The molecules acted as both signal generator and the stabilizer of nanoparticles. The thiol-anchored metallic clusters known as monolayer protected clusters (MPCs) have been known since the late 80s [53]. However, Brust *et al.* [54] have demonstrated the first practical formation of stable, isolable gold MPCs. They reported the classical two-phase method for the preparation of very small gold clusters coated with alkanethiolate ligands. The adsorption of alkanethiols on gold and silver [55,56] nanoparticles is the most studied and versatile approach towards the

formation of MPCs, because the thiol head group covalently binds to a metallic surface while the alkyl spacer stabilizes the MPCs through a Van der Waals interaction. The adsorption of thiols is believed to lead to a formation of a covalent Au-S bond. The mechanism of Au-S bond formation from alkanethiols has been generally assumed to involve hydrogen evolution [57,58] as shown in Equation 1.



The mechanism of Au-S bond formation may involve two steps, oxidative addition of an S-H bond to a gold surface and reductive elimination of hydrogen [57]. The alkanethiolate monolayer coverage provides two key functions: shielding the particles from agglomeration and providing a scaffold for the attachment of functional molecular entities. Capping agents on nanoparticle surfaces can be substituted by stronger ligands, similarly to the thiol ligand by replacing the citrate ions on a gold surface [59-61], as shown in equation 2,



where x and n are the numbers of entering (stronger) and original (weaker) ligands, respectively. This type of ligand-exchange reaction renders a significant means of chemical functionalization of nanoparticles with the potential exploitation of their electronic and optical properties. Further functionalization of MPCs through ligand exchange results into multifunctional mixed monolayer-protected clusters (MMPCs) [62]. Mixed monolayer systems enhance the versatility of the monolayer system, allowing multiple functionalities to be attached onto the outer layer of a metallic core. These systems provide control over a variety of properties, from solubility in organic or aqueous solvents to specific molecular recognition [63]. The synergy can be used to create complex functional devices, including redox active, electronic or magnetic storage devices, solution based sensors and highly efficient catalysts [63]. The advantage of MMPCs is that they have potential applications

in catalysis [64], microelectronics [65], optics [66], magnetic [67] and chemical recognition [68]. Biological applications of MPCs or MMPCs require specific surface functionalization and water solubility. SAMs application in SERS is diverse and includes surface chemistry and substrates modification. The use of SAMs in SERS has been investigated in various ways; SAMs have been used in SERS to verify the EM enhancement mechanism [69,70]. Several researchers have showed than using shorter SAMs' chains yielded stronger Raman signals than longer SAMs' chains [70-73]. This was because shorter chains were closer to the metal substrate hence they exhibited a stronger interaction with the EM field. SERS has been used to study the SAMs to verify the mechanism of the thiol-metal surface interaction [72,74-77]. Raman and SERS measurements of SAMs (alkylthiols), revealed that the S-H vibration at $\sim 2500\text{ cm}^{-1}$ disappeared when SAMs were adsorbed on the metal surface. However, the Raman peak observed at $\sim 200\text{ cm}^{-1}$ associated with metal to sulphur vibrations confirm the formation of SAM via the cleavage of the S-H bond. Another confirmation of SAM formation on SERS was the high intensity C-S vibration band observed at $\sim 650\text{ cm}^{-1}$ due to its closeness to the metal substrate [72,78].

2.3.2.2 Attachment of targeting molecules

Bioconjugation can be described as any procedure that links a nanoparticle to a biomolecule under mild conditions [79]. The synthetic procedures to produce these metal nanoparticles have been achieved in different ways but the coupling with biomolecules has been limited to few protocols based on the non-covalent and covalent attachment [80-82].

2.3.2.2.1 Non-covalent attachment

The most common non-covalent approach to immobilise enzymes onto the surface of SAMs has been via electrostatic binding which was first reported by McLaren *et al.* [83,84] in the late 1950s. They investigated the kinetics of an enzyme embedded on a charged membrane. This method provides the potential for control over the orientation of the immobilised

protein molecules depending on the charge distribution of the protein. The major drawback of electrostatic binding is that the strength of the bond is dependent on the solution conditions. Changes in ionic strength and pH can cause the protein to be lost from the surface of the SAMs [85].

Schlereth and Kooyman [86] have also immobilised enzymes non-covalently on the SAMs surfaces using a biospecific affinity ligand. In this approach the enzyme electrode interface is constructed via a stepwise fabrication process. One of the advantages of immobilising using specific ligands is that the orientation of the enzyme can be controlled with appropriate ligand design. The versatility of affinity ligands for immobilising enzymes depends on how easily ligands can be synthesised for other biomolecules and how strongly the biomolecule remains bound to the SAMs surface.

2.3.2.2.2 Covalent attachment

Covalent attachment uses cross-linkers to form a covalent bond between molecules and proteins. Cross-linkers are molecules that contain reactive terminals/ends (-OH, -COOH, -NH₂) that binds specific functional groups of proteins or other molecules. Cross-linkers can be either homobifunctional or heterobifunctional. Homobifunctional cross-linkers have one or two identical reactive groups and are often used in a one-step reaction to cross-link proteins to each other, to other molecules, and to stabilize quaternary structures. A popular reaction is carbodiimide coupling which couples amines to carboxylic acids. In the reaction, N-ethyl-N-[dimethylaminopropyl] carbodiimide (EDC) converts the carboxylic acid into a reactive intermediate which is susceptible to attack by amines. In some cases EDC with N-hydroxysuccinimide (NHS) or N-hydroxysulphosuccinimide (NHSS) is used as it produces a more stable reactive intermediate which has been shown to give a greater reaction yield [87]. Heterobifunctional cross-linkers have two different reactive groups, allowing sequential conjugation. They can be used to conjugate amines which sometimes are present

at the active protein site for modification. This sometimes may lead to a loss of activity. The most widely used heterobifunctional linkers are those having an amine-reactive succinimidyl ester (NHS-ester) at one end and sulfhydryl-reactive group on other end. One example is the surface modification of gold PEGylated particles with an amino-reactive end coupled with a heterobifunctional crosslinker 4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt or sulfo-SMCC bearing a terminal maleimide functionality [88].

2.4 SERS applications

Since the discovery of SERS in the last few decades, it has been a subject of intense research activity. This can be confirmed by an increasing number of publications published every year since 1975, as shown in Figure 2.2. The breakthrough of single-molecule detection using SERS reported by Kneipp *et al.* [89] in 1997 spiked a great interest in SERS as observed in Figure 2.2. The interest amongst the researchers was to try to develop SERS-based sensors [90,91] verifying the single-molecule detection claim [92,93]. The growth on SERS publications since its discovery until now has been drawn from different areas of research as shown in Figure 2.3.

SERS has emerged as a powerful tool in the detection of several analytes ranging from ions [94], proteins [95], nucleic acids [96], pathogens [97] and currently hot topics include live-cells [98], tissue imaging [91] and in vivo imaging [49] techniques. This thesis focuses on the bio-analytical applications of SERS, specifically on the detection of biomolecules. Taking an advantage of the richness of Raman signals and single-molecule detection sensitivity, researchers have successfully applied SERS probes in more complicated multiplex and ultrasensitive immunoassays of biomolecules.

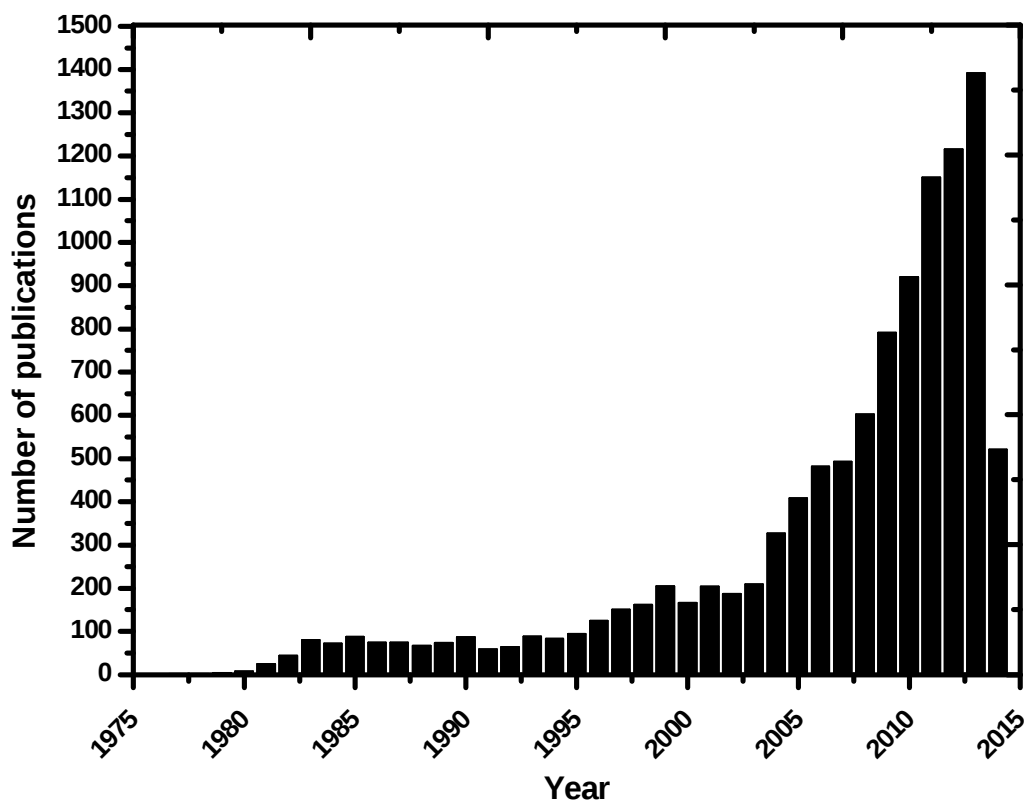


Figure 2.2: Graphical representation of the SERS publications since 1974, the data was extracted from Scopus database (www.scopus.com) on the 15/05/2014. The title search performed was Surface enhanced Raman and the acronym “SERS”.

The successful application follows a good design of SERS probes which is generally composed of four parts; a metal nanostructure to act as SERS substrate, an organic Raman reporter molecule, a protection layer and a spacer to attach targeting molecules as shown in Figure 2.4.

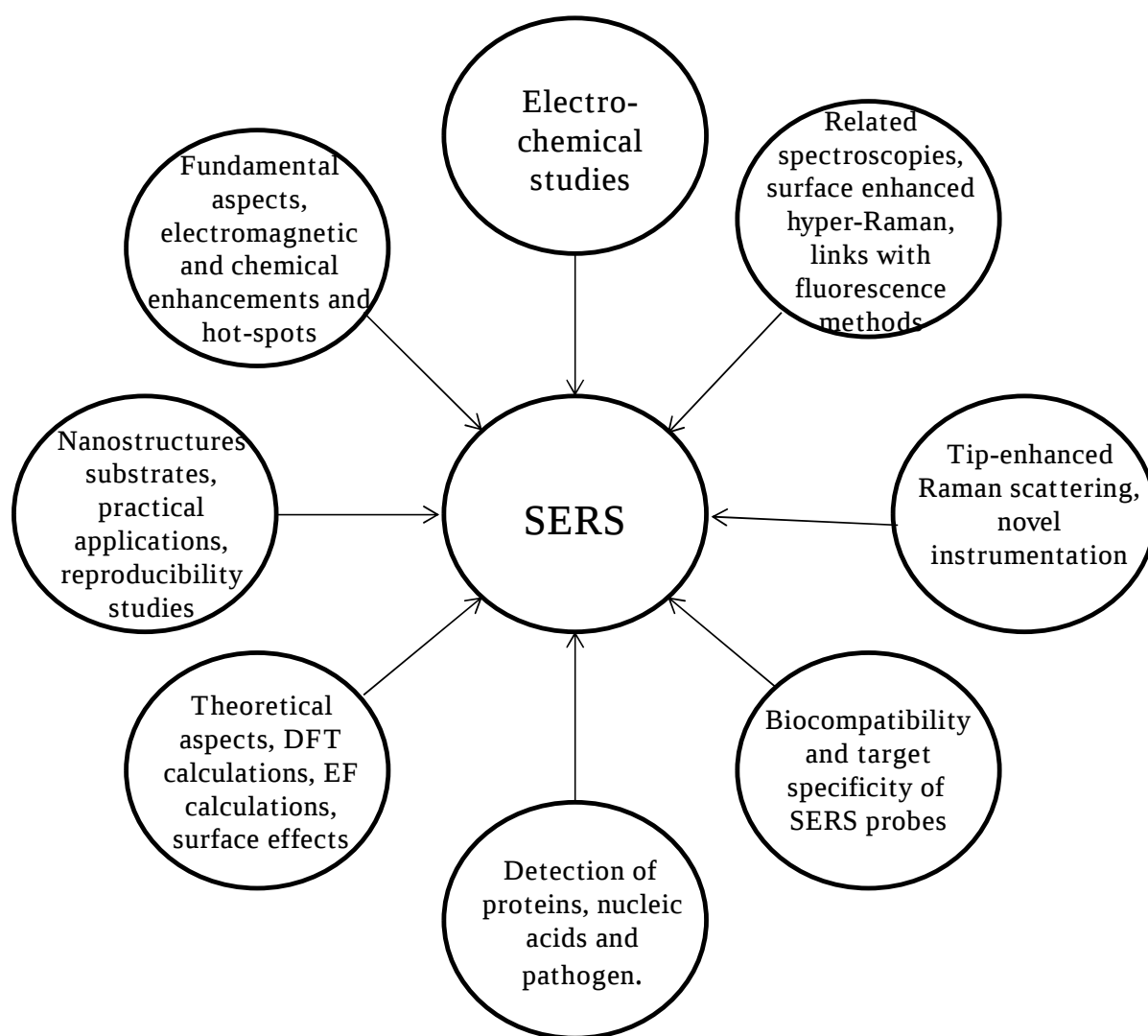


Figure 2.3: Schematic representation of some main areas contributing to SERS publications.

After designing the stable and biocompatibility SERS probe, an immunoassay is developed for the detection of biomolecules. The schematic design for SERS immunoassay followed in this work is shown in Chapter 6, Figure 6.2. Immunoassay simple refers to the biochemical test that measures the presence or concentration of a macromolecule in a solution through an antibody or an immunoglobulin. The first biomolecule to be detected using SERS immunoassay was an antigen. In a typical experiment, polyclonal antibodies (PABs) are immobilised on the solid substrate and then the antigen and monoclonal

antibodies (mAb)-conjugated SERS probe are added in sequence. After washing off the non-specific binding agents and free SERS probes, the antigen can be identified by measuring the SERS signal [99].

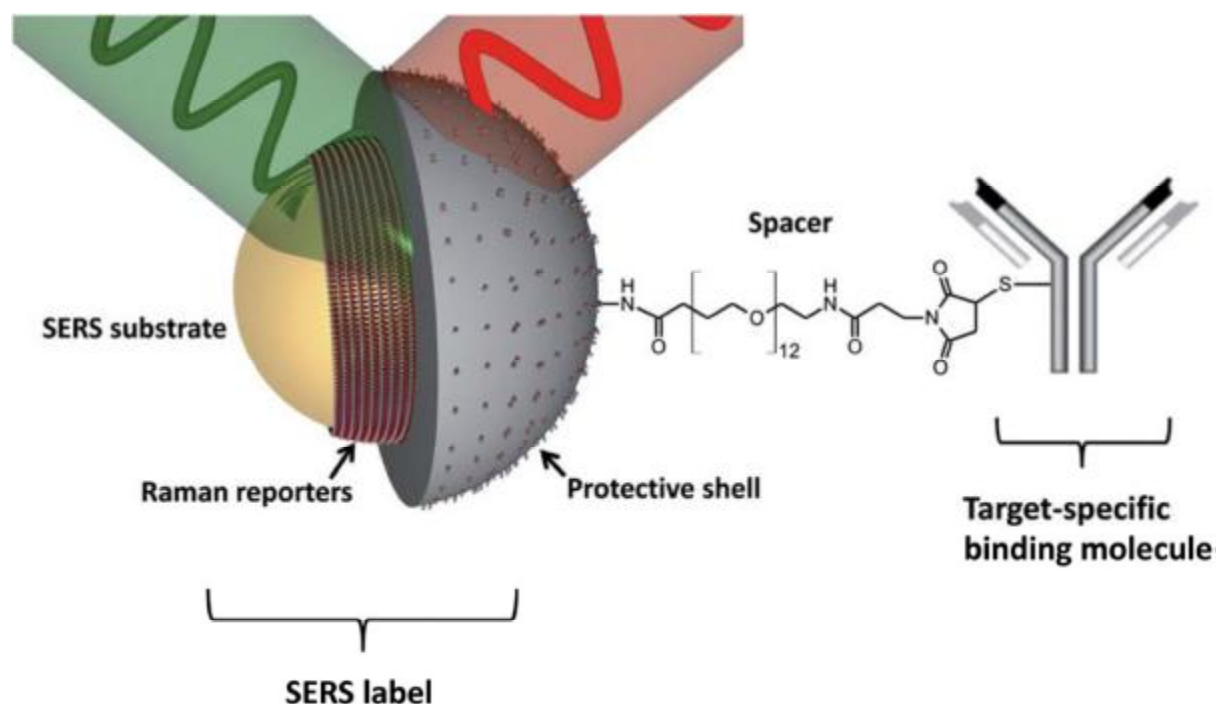


Figure 2.4: Components of SERS probes: SERS substrate (a metal nanostructure), Raman reporter (SAMs), Protective shell (biocompatibility and water solubility) and target-specific molecule (specific biomarkers for targeted detection) [100].

A multiplex detection of human interleukin (IL)-2 and IL-8 was achieved via this method [101]. The use of SAMs by this method have been reported by Ni *et al.*, using thiophenol (TP), 2-naphthalenethiol (NT) and 4-mercaptobenzoic acid (MBA) as SERS reporter molecules for a readout method in a dual-analyte sandwich immunoassay [102]. The antibodies were adsorbed on the gold surface while the reporters chemisorbed as thiolates. The assay was successful in detecting rat IgG and rabbit IgG. Data indicated that a small amount of weakly adsorbed antibody may desorb from one reporter and re-adsorb to another reporter in the two-component reporter suspension. This led to a spectral

signature similar to non-specific adsorption for a multiplexed assay. This co-immobilization of a reporter and antibodies was challenged by particle aggregation. The use of a bifunctional reporter with a large scattering cross-section and reactive terminal to covalently couple an antibody to prevent non-specific adsorption has been reported [69,103,104]. 5,50-dithiobis(succinimidyl-2-nitrobenzoate) (DSNB) is a bifunctional reporter with a large scattering cross-section due to the nitro stretch [$\nu(\text{NO}_2)$]. Its disulphide moiety is cleavable and can react with gold to form two thiolate layers. The use of DSNB places the nitro group in close proximity with the gold surface addressing the sharp decay in the enhanced electric field as the distance from the surface increases. The succinimidyl groups of DSNB are used to covalently tether antibodies to the particles [105-107]. The conjugate system showed the detection was at femtomolar level [95]. In 2005, Park reported a combination of the two designs, resulting in detection levels comparable to the DSNB-based reporter while offering a more facile route for modification [108]. The method involved the thiolate derived from the bifunctional compound dithiobis(succinimidyl undecanoate) (DSU), which has disulfide and succinimidyl groups like DSNB but is an inherently weak scatterer. Thus, the second thiolate is selected to have a large Raman cross-section (4-nitrobenzenethiol) forming two thiolated layers. The design was challenged by the low detection of *Escherichia coli* at low concentration although the antigen binding specific was improved.

The sandwich immunoassay has been further developed for quantitative analysis of SERS probes conjugated to target biomarkers [109,110]. Wang *et al.*, used the goat anti-hIgG and hIgG as a model system for protein detection [110]. A substrate coated with goat anti-hIgG was exposed to a solution containing different concentrations of hIgG. After incubation, the sample was immersed into a solution of goat anti-hIgG-conjugated to AuNRs-embedded silica SERS probes. The hIgG first bound to the corresponding goat

anti-hIgG that was immobilised on the substrate. Then, it could capture the same antibody that was labelled on the SERS probe. Thus, the amount of hIgG could be reflected by the SERS signal with a limit of detection (LOD) of 0.01 ng.ml⁻¹. A summary of other reports on the detection of biomolecules are shown in Table 2.2, adopted from Wang *et al.* [18].

Table 2.2: Summary of biomolecules detected by SERS probes [18]

Detection sample	substrate	Raman reporte(s)	dtetection limits	ref
glutathione	AgNPs	rhodamine 6G, crystal violet	1 μM	[111]
		5,5'dichloro-3,3'-disulfopropylthiacynine		
glucose, lactose and glucuronic acid	AgNPs	rhodamine B	1 nM	[112]
melamine	AuNPs	4-MPY	0.1 ppb	[113]
	AuNRs	DNBA	0.38 mg.L^{-1}	[114]
adenosine triphosphate	Au nanostar	MGITC	12.4 pM	[115]
rabbit IgG	AuNPs	4-MBA	1-0.1 fg.mL^{-1}	[116]
mouse IgG	Au-Ag-C core shell NPs	4-MBA	100- 10 ng.mL^{-1}	[108]
		p-aminothiophenol, thiophenol		
thrombin	AgNPs	DBDT	100 pM	[117]
	AuNPs	rhodamine 6G	0.5 nM	[118]
	AuNPs	4-MPA, 4-MPY and RBITC	220 pM	[119]
	Au-Ag-C core shell NPs	4-MPA	10 fM	[120]
	AgNPs	DBDT, BDT and 2,6-NDT	100 pM	[121]
prostate-specific antigen	AuNPs	DNBA, rhodamine G	1 pg.mL^{-1}	[122]
inteleukin-2 (IL-2) and IL-8	AgNPs	8-azaadenine, benzoyladenine		[123]
avidin	AgNPs	Coomassie brilliant blue dyes		[100]
human IgG	AuNRs	4-aminothiophenol	0.1 ng.mL^{-1}	[124]
	AuNP nanocluster	4-MBA	100 fg.mL^{-1}	[125]
mucin protein MUC4	AuNPs	4-nitrobenzethiol	1 ng.mL^{-1}	[126]
carcinoembryonic antigen	$\text{SiO}_2@(\text{AgNPs}/\text{PEI})_5$	4-aminobenzenethiol (4-ABT)	0.1 pg.mL^{-1}	[127]
DNA, oligonucleotides derived from West Nile virus genome	AuNPs	DSNB	10, 00 pM	[128]
multiple pathogen DNAs	AuNPs	Cyanine5 and TAMRA	10 pM to 10 nM	[129]
synthetic model ssDNA	AuNPs	Rox dye	10,00 pM	[130]

Abbreviations MGITC= malachite green isothiocyanate; 4-MPY= 4-mercaptopyridine; 4-MBA= 4-mercaptopbenzoic acid;

DBDT= biphenyl-4,4'-dithiol; RBITC= Rhodamine B isothiocyanate; BDT = 1,4-benzenedithiol; 2,6-NDT = 2,6-naphthalenedithiol;

DNBA= 5,5'-dithiobis(2-nitrobenzoic acid); DSNB, 5,5'-dithiobis(succinimidyl-2-nitrobenzoate); TAMRA= carboxytetramethylrhodamine.

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Chapter Three

Synthesis and functionalization of different gold nanoparticle sizes to mixed monolayer protected clusters with different Raman reporters

3.1 Introduction

Since the discovery of the SERS effect, research based on the impact of different parameters and properties of the metal nanostructured substrates used in SERS experiments have been conducted. Metal nanoparticles exhibit an SPR absorption that is dependent on various factors such as dielectric constants of metals and their surfaces, interparticle distances, size and shape of nanoparticles [1-3]. The excitation of the SPR absorption results in a strong local electromagnetic field enhancement in the vicinity of the nanoparticles. It is this local electromagnetic field enhancement that is responsible for the major enhancement in the Raman spectra of molecules near or attached to the surface of metal nanoparticles [3]. There are four major factors that contribute to the total SERS enhancements effect at varied experimental conditions. These factors include (i) electromagnetic enhancement resulting from the excitation of a metal, (ii) charge transfer from a metal to an adsorbate resulting from excitation of the metal, (iii) a resonance mechanism where the excitation corresponds to an electronic transition in the molecule, and, (iv) an off-resonance enhancement due to a metal-adsorbate interaction [4].

Kelly *et al* [3] and Pustovit *et al* [5] showed that the increase in particles size increases the local EM, but as the particle increases, the particles absorb less light and scatter more through inelastic scattering and this should decrease the overall SERS intensity. The dependence of EM contribution of SERS enhancement on nanoparticle size has been well investigated in theoretical studies [3,5-10]. Experiments on arrays made from metal nanoparticles of different sizes have been reported [1,11,12]. It was reported that the inter-

particle coupling played a significant role in the distribution of the local field in addition to the size effect [13-16]. Zheng *et al.* have shown, using nanoparticles in solutions, that SERS experiments can be probed without inter-particle coupling effects [17]. SERS experiments carried out using nanoparticles in solution have also revealed that addition of the analyte can cause some aggregation, which can lead to a significant increase in the SERS enhancement using both experimental [18-20] and theoretical [21,22] studies. Aggregated nanoparticles also show an extended plasmon band to longer wavelengths which provides optimum enhancement of excitation and scattering fields in the near-infrared region.

The design of a SERS substrate requires a Raman reporter that will be adsorbed on the surface and then show enhanced vibrations in the Raman spectroscopy experiment. The use of alkanethiols as Raman reporters has attracted much attention due to the strong affinity of a metal for the sulphur atom. Various thiol-functionalized metallic clusters (Au, Ag, Pt, Pd), have been synthesized [23,24], and thiolated Au clusters have received more attention. In this study, four alkanethiols with different number of phenyl rings bearing a number of functional groups were used as Raman reporters. The choice of these alkanethiols was informed by their thiol head group to facilitate the formation of the metal-sulphur bond, the functional groups on the phenyl rings to enhance their Raman spectra. These alkanethiols have not been used for the SERS effect. In this chapter, the effect of AuNP size with four Raman active alkanethiols was investigated.

3.2 Experimental

3.2.1 Materials

Hydrogen tetrachloroaurate trihydrate (Sigma- Aldrich 99.9%), tri-sodium citrate (ACE AR, 99%), HS-(CH₂)₁₁-PEG-COOH, HS-(CH₂)₁₁-PEG-OH, HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, HS-(CH₂)₁₁-hydroquinone were obtained from ProChimia Surfaces (Poland).

3.2.2 Instrumentation

High purity water with resistivity of 18.1 Ω M was obtained from a Milli-Q Advantage water system purchased from Millipore (USA) and was used in all the experiments. Samples were purified using a Hettich MIKRO 22R centrifuge. Absorption spectra of AuNPs were recorded on a Lambda 35 UV-vis spectrometer. Transmission electron microscopy (TEM) images were obtained using a JEM-2100F at 200 kV. The TEM grids were prepared by depositing approximately 10 μ l of the solution obtained after centrifugation and allowed to dry in air. Raman spectra were acquired using a PerkinElmer Raman Station 400 benchtop Raman spectrometer. The excitation source was a near-infrared 785 nm laser (100 mW at the sample), with a spot size of 100 μ m. A spectral range of 100-3200 cm^{-1} was employed. The detector was a temperature controlled Charged Coupled Device (CCD) detector (-50 $^{\circ}$ C) incorporating a 1024 x 256 pixel sensor. The spectra were processed using Spectrum software supplied by PerkinElmer (Bucks, UK). The zeta potential measurements were acquired using a Malvern ZetaSizer.

3.2.3 Synthesis, functionalization and conjugation of Au nanoparticles

3.2.3.1 Synthesis of citrate gold nanoparticles

In a typical experimental procedure, an aqueous solution of tri-sodium citrate (0.04 M) was added to a boiling aqueous solution of tetrachloroaurate (250 mL, 1 mM). The mixture was allowed to boil for five minutes with vigorous stirring, and then the mixture was removed

from heat and stirred continuously for a further three hours. To obtain different sizes of AuNPs, different volumes of tri-sodium citrate aqueous solution (25, 12 and 2.1 mL) were added.

3.2.3.2 Functionalization of gold nanoparticles

The resulting different sizes of AuNPs were filtered, and then 40 mL aliquots of each size (AuNPs) were treated with 200 μ L of HS-(CH₂)₁₁-PEG-COOH (8 mg/mL). Each 40 mL aliquot of the AuNPs coated with HS-(CH₂)₁₁-PEG-COOH, were further divided into two aliquots of 20 mL each, to be co-stabilized with different percentages of HS-(CH₂)₁₁-NHCO-coumarin (1% or 50%, respectively). For AuNPs to be stabilized with 1% HS-(CH₂)₁₁-NHCO-coumarin, 0.02 mg of HS-(CH₂)₁₁-NHCO-coumarin was dissolved in 1 mL of methanol and then thoroughly mixed with HS-(CH₂)₁₁-PEG-COOH (1.98 mg) in 1 mL of methanol. For stabilization with 50 % HS-(CH₂)₁₁-NHCO-coumarin, 1 mg of each alkanethiol was dissolved in methanol and swirled for a few minutes. The mixtures of different percentages (1% and 50% HS-(CH₂)₁₁-NHCO-coumarin) were each added to 20 mL of AuNPs, and stirred at 800 rpm for three hours, at room temperature. A similar procedure was followed for the preparation of AuMMPCs using other Raman active alkanethiols i.e.; HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone.

3.2.4. Density Functional Theory (DFT) calculations and models

All calculations were carried out with Gaussian 03 programs [25] within the DFT framework. In this study the ligand-metal system structures of gold were optimized using the Becke's three parameter hybrid functional [26] with the Lee *et al.* [27] (B3LYP) correlation functional employed with the electron core potential basis set LANL2DZ developed by Hay and Wadt [28-30]. For gold (Au), the LANL2DZ set electron core

potential simulates the 60 of the total 79 (Au) electrons. The remaining 19 electrons are described by an electron basis sets consisting of a (8s6p4d) set of primitive Gaussian type functions contracted to the [3s3p2d]. For C, H, N, S and O the LANL2DZ basis consists of a (10s5p) set contracted to the (3s2p) set, while for H a (4s) set contracted to the (2s) basis set was used.

3.3 Results and discussion

The general scheme for the synthesis of different size gold mixed monolayer protected clusters (AuMMPCs) from citrate stabilized gold nanoparticles is shown Figure 3.1. The synthesized AuNPs stabilized with citrate were firstly coated with HS-(CH₂)₁₁-PEG-OH to prevent aggregation as shown in equation 1 (Figure 3.1). The AuMMPCs were prepared by mixture of 1% and 50% ratios of each of the Raman active alkanethiols with an acid functionalized alkanethiol under a high stirring speed, displacing some of the HS-(CH₂)₁₁-PEG-OH moieties on the surface of AuNPs, as shown in equation 2 (Figure 3.1). The summary of SPR bands of all different AuNPs functionalized with different Raman active alkanethiols in different stoichiometric ratios are shown in Table 2.1. The AuNPs are classified as 14, 30 and 40 nm throughout this section. The measured average diameters are shown in the TEM discussion

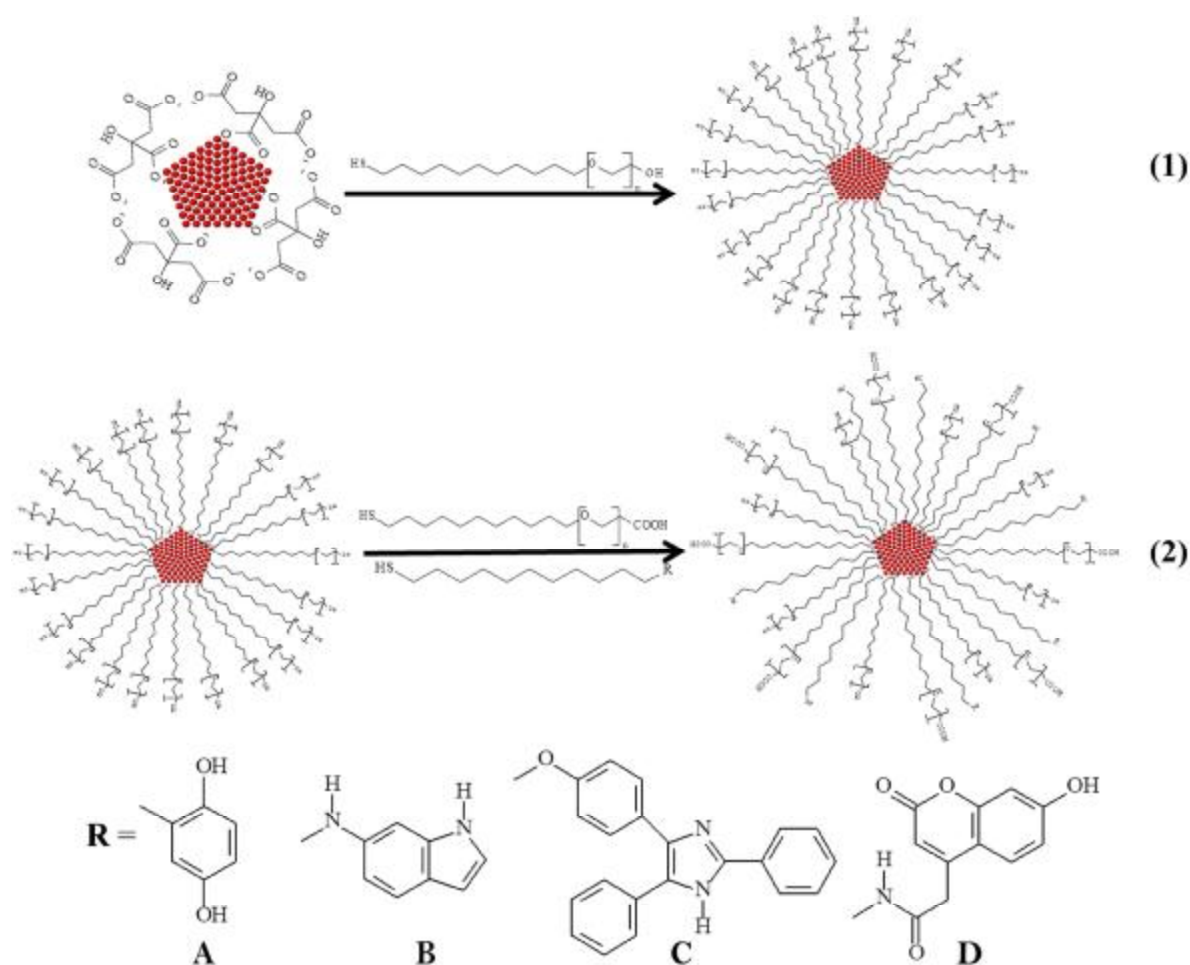


Figure 3.1: General scheme for the synthesis of citrate-AuNPs and their functionalization to from AuMMPCs using (a) $\text{HS}-(\text{CH}_2)_{11}$ -hydroquinone (b) $\text{HS}-(\text{CH}_2)_{11}$ -indole, (c) $\text{HS}-(\text{CH}_2)_{11}$ -triphenylimidazole and (d) $\text{HS}-(\text{CH}_2)_{11}$ -NHCO-coumarin.

The SPR bands shown in Table 2.1, display a shift to longer wavelength as the AuNP size increases; this is consistent with Mie's theory in which the absorption increases with an increase in the size of the AuNPs [31-33]. The absorption spectra of these bands are shown in the supplementary data (Figure S3.1 to S3.4), which reveal the redshift of an SPR bands.

Different AuNPs sizes functionalized with stoichiometric ratios of $\text{HS}-(\text{CH}_2)_{11}$ -NHCO-coumarin are presented in Figure S3.1. The SPR bands for the 1% and 50% ratios AuNPs

of 14 nm are shown in Figure S3.1 (a), and revealed a significant red-shift. The bands shifted from 519 nm to 523 (1%) and 525 nm (50%). The absorption spectra of AuNPs of 30 and 40 nm with different ratios of HS-(CH₂)₁₁-NHCO-coumarin are presented in Figure S3.1 (b) and (c). Similar results as described above were observed. A slight red-shift of the SPR bands was observed from 528 nm (30 nm) and 529 nm (40 nm) to 533 (1%) and 534 nm (50%), respectively as the stoichiometric ratios of HS-(CH₂)₁₁-NHCO-coumarin were added on the AuNPs surfaces. The shift to the longer wavelength of the SPR bands is attributed to the change in their dielectric environment.

Similar trend of SPR shifts to longer wavelength was also observed for the other three stoichiometric ratios of HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, HS-(CH₂)₁₁-hydroquinone as shown in their absorption spectra in the supplementary data (Figure S3.2, S3.3 and S3.4).

The other noticeable features in the absorption spectra of all four different Raman active alkanethiols was the broadening of the SPR peak of the sample made with 50% stoichiometric ratio and attributed to the presence of the carboxyl terminated group ligands which enhances the tendency of ligand intermolecular hydrogen bonding. This may cause aggregation and broadening of SPR absorption peak [34].

Figure 3.2 shows the particle distribution histograms of the citrate stabilized AuNPs with an average diameters of 14 nm, 30 nm, and 40 nm. These different AuNPs sizes were used to synthesize AuMMPCs with different Raman active alkanethiols. The TEM images which were used to measure the average diameters are shown in Figures 3.3. The TEM images of different sized AuMMPCs functionalized with 1% and 50% HS-(CH₂)₁₁-NHCO-coumarin are also shown in Figure 3.3, revealing well dispersed particles observed for all samples of gold nanoparticles.

Table 3.1: Summary of SPR bands of AuNPs and AuMMPCs

	Au14nm	519
	Au30nm	528
	Au40nm	529
Coumarin	Au14 1%	523
	Au14 50%	525
	Au30 1%	533
	Au30 50%	533
	Au40 1%	534
	Au40 50%	534
TPI	Au14 1%	525
	Au14 50%	527
	Au30 1%	533
	Au30 50%	533
	Au40 1%	533
	Au40 50%	536
HSI	Au14 1%	524
	Au14 50%	526
	Au30 1%	532
	Au30 50%	533
	Au40 1%	532
	Au40 50%	537
HQ	Au14 1%	524
	Au14 50%	524
	Au30 1%	532
	Au30 50%	532
	Au40 1%	532
	Au40 50%	532
SPR= surface plasmon resonance		

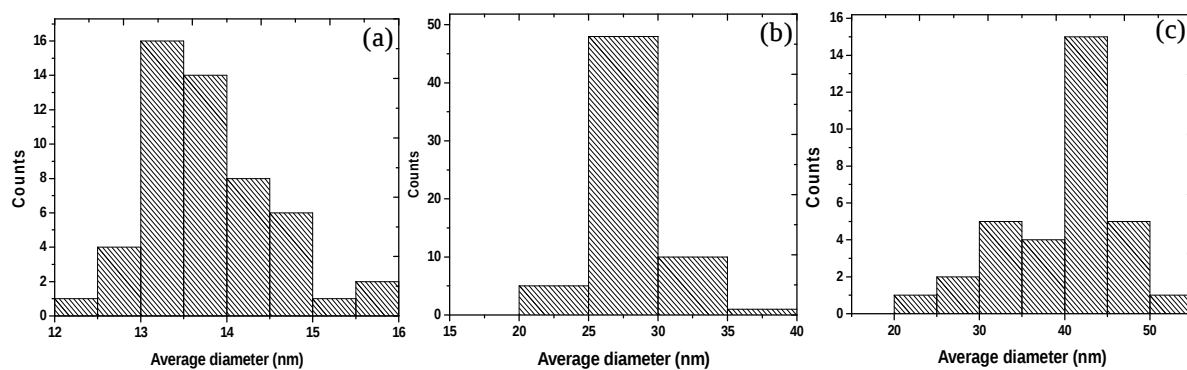


Figure 3.2: Particle distribution histograms of citrate stabilized AuNPs with average size of (a) 14 nm, (b) 30 nm, and (c) 40 nm.

There was no aggregation observed, however a noticeable feature observed was the clumping of AuNPs as higher stoichiometric ratio was added in larger AuNPs of 30 nm and 40 nm as shown in Figures 3.3. The TEM images of AuMMPCs functionalized with different stoichiometric ratios of HS-(CH₂)₁₁-triphenylimidazole are shown in Figure 3.4. TEM images of AuMMPCs functionalized with different stoichiometric ratios of HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone are shown in the supplementary data (Figure S3.5 and S3.6). The images revealed no signs of aggregation in AuMMPCs prepared with both alkanethiols. All the as-prepared AuMMPCs from the four different alkanethiols (HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone) revealed a well-known tendency of thiol-capped nanoparticles to form self-assembled ordered superlattices [35]. The predominant feature in all TEM images was the different interparticle distances which depended on the alkanethiol used compared to the citrate stabilized gold nanoparticles.

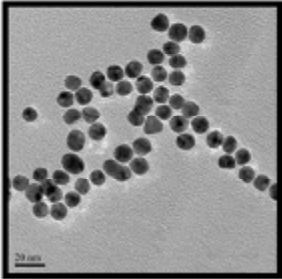
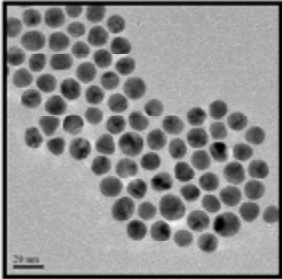
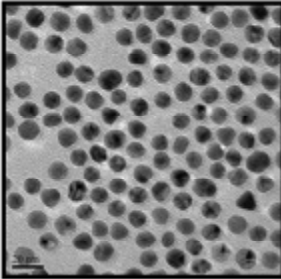
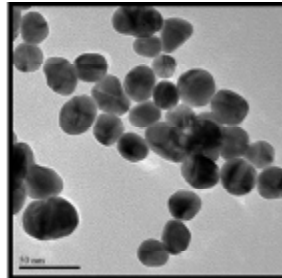
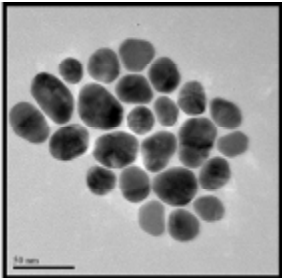
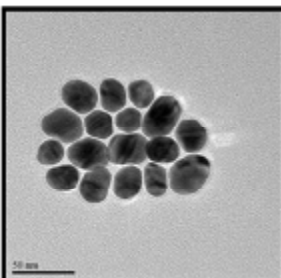
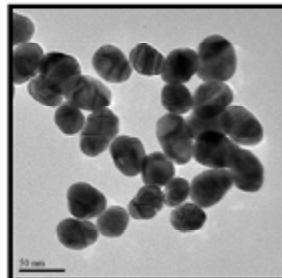
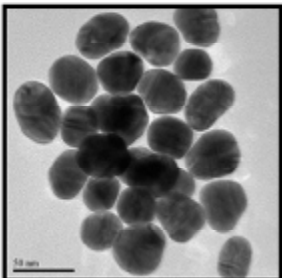
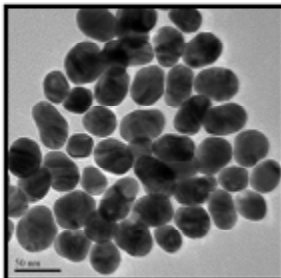
Ligands	Citrate	1% Raman reporter	50% Raman reporter
Sizes 14 nm			
30 nm			
40 nm			

Figure 3.3: TEM images of AuNPs and their corresponding AuMMPCs of 1% and 50% of HS-(CH₂)₁₁-NHCO-coumarin

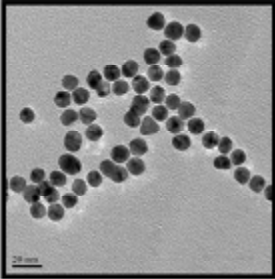
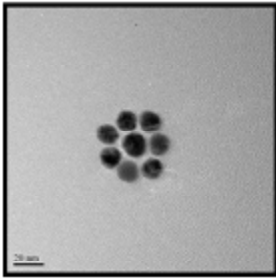
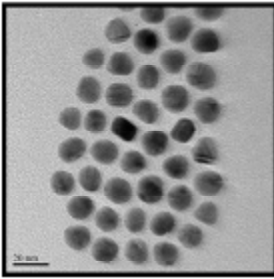
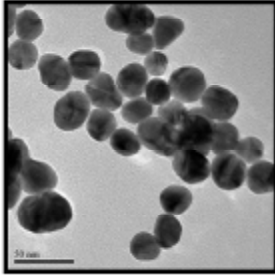
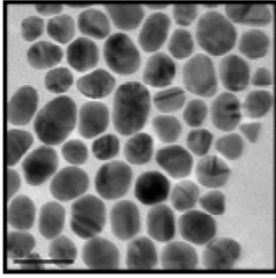
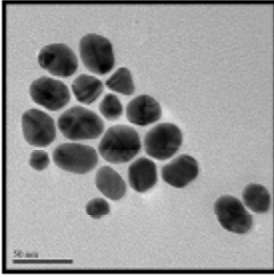
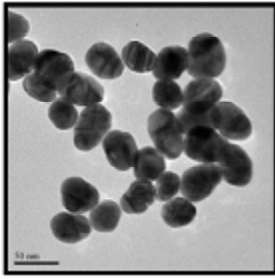
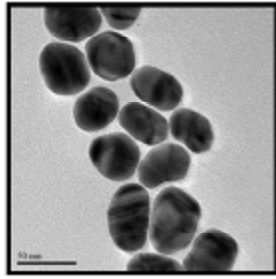
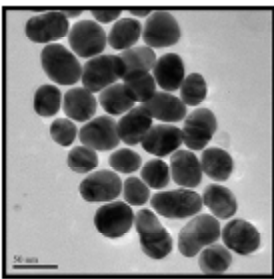
Ligands	Citrate	1% Raman reporter	50% Raman reporter
Sizes 14 nm			
30 nm			
40 nm			

Figure 3.4: TEM images of AuNPs and their corresponding AuMMPCs of 1% and 50% of HS-(CH₂)₁₁-triphenylimidazole.

The stability of all AuNPs of different sizes functionalized with alkanethiols was evaluated using the Zetasizer. Zetasizer is a good analytical tool to probe the dielectric charge as a result of surface changes due to ligand attachment on the surface of nanoparticles. The particle arrangement or surface charge is determined by the closeness of the Zeta potential to zero. It is important to note that nanoparticles can either be stabilized electrostatically (ionic) or sterically where a covalent bond is formed between the surfactant/ligand with metal. The values of Zeta potentials of all AuNPs and AuMMPCs are shown in Table 3.2. The Zeta potential graphs of all particles are shown in the supplementary data (Figure S3.7 to S3.16). The acidic citrate-stabilized AuNPs of different sizes revealed high stability potentials of -42.4 mV (14 nm), -28.7 mV (30 nm) and -40.9 mV (40 nm).

The functionalization of citrate-capped AuNPs with four different alkanethiols via the adsorption of thiol moiety led to formation of a sulphur to metal bond, displacing the citrate ions on the gold surface. The thiol head group is a weak acid, will therefore be expected to show a weak negative zeta potential, while the displaced citrate ions will attract the H^+ from the cleaving H-S bond. Weak negative zeta potentials were observed for all different alkanethiols, as shown Table 3.2.

Zeta potential has been known to be influenced by pH, ionic strength and pH-dependant ionizable functional groups (both acidic and basic) that can undergo dissociation and protonation [36-39]. The different functional groups present on the Raman active alkanethiols and HS-(CH₂)₁₁-PEG-COOH showed no significant effect on the Zeta potentials of the AuMMPCs. The -COOH end group have the pK_a value of 4.5, the introduction of the weak acidic thiol groups could have lowered the pH of the AuMMPCs. The influence of a weak acid -COOH end group on the zeta potential of AuMMPCs was investigated and the graphs are shown in Figure 3.5.

Table 3.2: Values of Zeta potentials of AuNPs and AuMMPCs

Sample		Zeta Potential (mV)
	Au14nm	-42.4
	Au30nm	-28.7
	Au40nm	-40.9
Coumarin	Au14 1%	-1.26
	Au14 50%	-5.21
	Au30 1%	-2.58
	Au30 50%	-2.58
	Au40 1%	-22.3
	Au40 50%	-18.5
TPI	Au14 1%	-39
	Au14 50%	-29.6
	Au30 1%	-12.5
	Au30 50%	-8.5
	Au40 1%	-16
	Au40 50%	-16.8
HSI	Au14 1%	-30.1
	Au14 50%	-30.1
	Au30 1%	-14.5
	Au30 50%	-4.05
	Au40 1%	-19.3
	Au40 50%	-33.3
HQ	Au14 1%	-27
	Au14 50%	-26.7
	Au30 1%	-10.6
	Au30 50%	-11
	Au40 1%	-16
	Au40 50%	-15.4

The strong negative zeta potential of AuMMPCs showed weak negative values for 50% carboxylate group in all Raman active alkanethiols. The decrease of zeta potential suggested that the surface charge of AuNP is become less negative. The further increase of a weak acidic –COOH percentage from 50% to 99% displayed no significant change on the potential. The noticeable change varied with size of AuNPs and the Raman active alkanethiol.

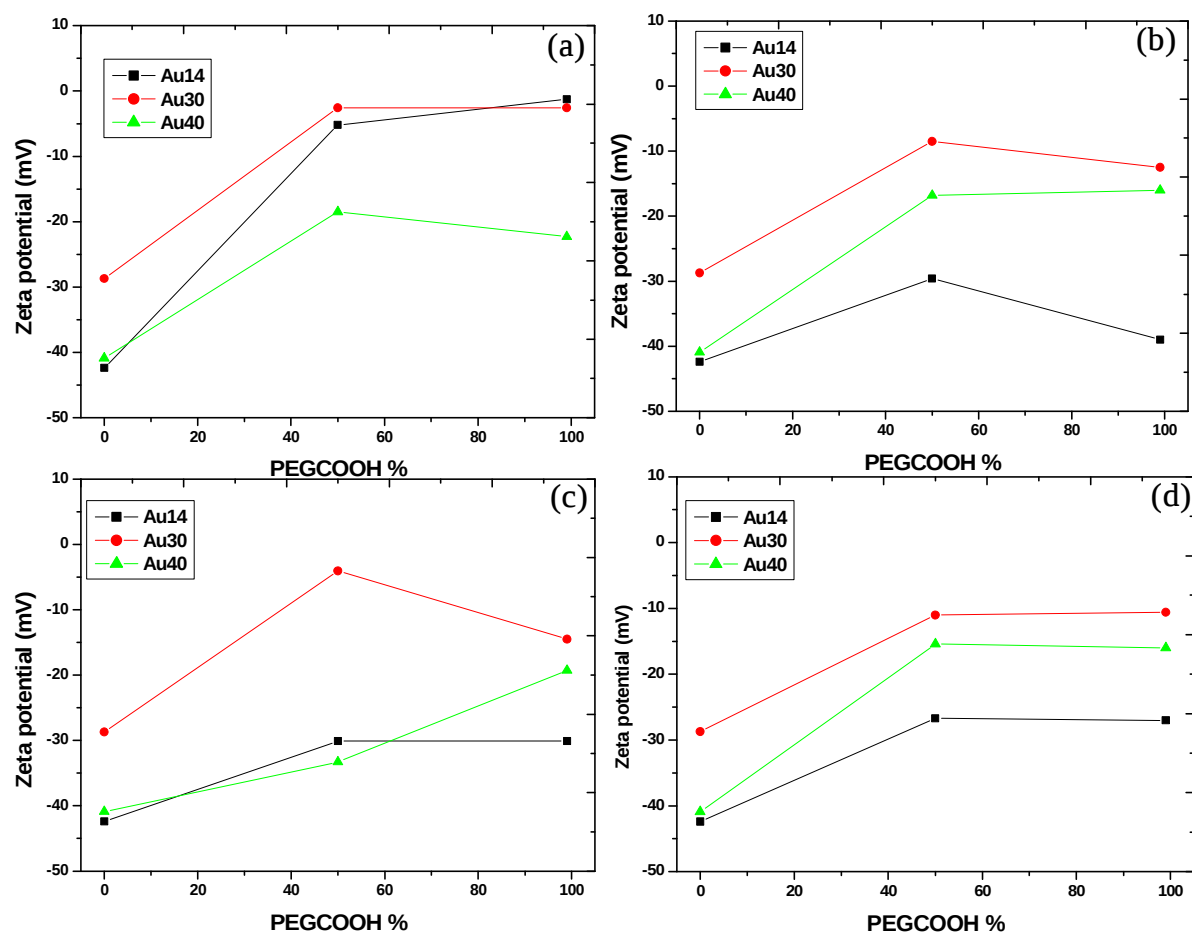


Figure 3.5: Zeta potential graphs of AuMMPCs of (a) HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole and (d) HS-(CH₂)₁₁-hydroquinone against the -COOH percentages.

The evaluation of Raman activities of all the prepared AuMMPCs with four different Raman alkanethiols in different stoichiometric ratios (1 and 50%) were carried out using a Raman spectrometer with a near-infrared 785 nm laser. The adsorption for the different alkanethiols on the gold nanoparticle's surface was theoretically calculated using Density Functional Theory (DFT) calculations. All the calculations were carried using the Gaussian 03 program, and theoretical Raman spectra obtained were correlated with experimental Raman spectra. The optimised geometries of four different alkanethiols adsorbed on gold nanoparticle used to obtain the theoretical Raman spectra are shown in

Figure 3.6, (a) Au-S-(CH₂)₁₁-NHCO-coumarin, (b) Au-S-(CH₂)₁₁-triphenylimidazole, (c) Au-S-(CH₂)₁₁-indole and (d) Au-S-(CH₂)₁₁-hydroquinone. These models were designed to indicate the adsorption of all these derivatives on gold atoms; this assumption was validated with theoretical Raman studies which were compared to the experimental Raman spectra for all these derivatives. The optimised geometries of free or unmodified alkanethiols are shown in the supplementary data as Figure S3.19.

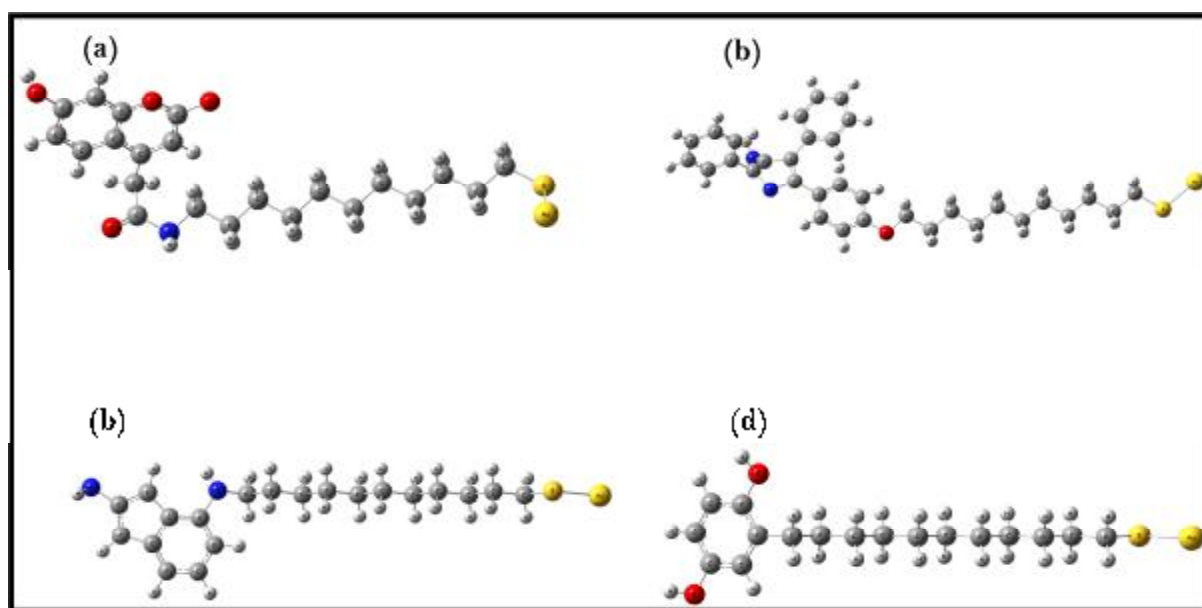


Figure 3.6: Optimized geometries of (a) Au-S-(CH₂)₁₁-NHCO-coumarin, (b) Au-S-(CH₂)₁₁-triphenylimidazole, (c) Au-S-(CH₂)₁₁-indole and (d) Au-S-(CH₂)₁₁-hydroquinone

The DFT calculated spectra of four different alkanethiols adsorbed on the gold nanoparticle's surface and their corresponding free alkanethiols theoretical spectra are shown in Figure 3.7. Figure 3.7, depicts (a) the calculated Raman spectra of HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole and (d) HS-(CH₂)₁₁-hydroquinone. The theoretical Raman spectra of free alkanethiols are represented by (i) and the alkanethiols adsorbed on gold surface by (ii). All the theoretical spectra depict the disappearance of ν (-S-H) vibrations observed in the region above $\sim 2600\text{ cm}^{-1}$ upon the

interaction with a gold surface. This results in a new vibrational peak in the region above 200 cm^{-1} associated with $\nu(\text{Au-S})$. This phenomenon was also noted in the experimental Raman spectra in Figures 3.8, 3.9 and Figures S3.20, S3.21. This indicated that as the alkanethiol adsorbs on the surface of gold nanoparticle, it is deprotonated.

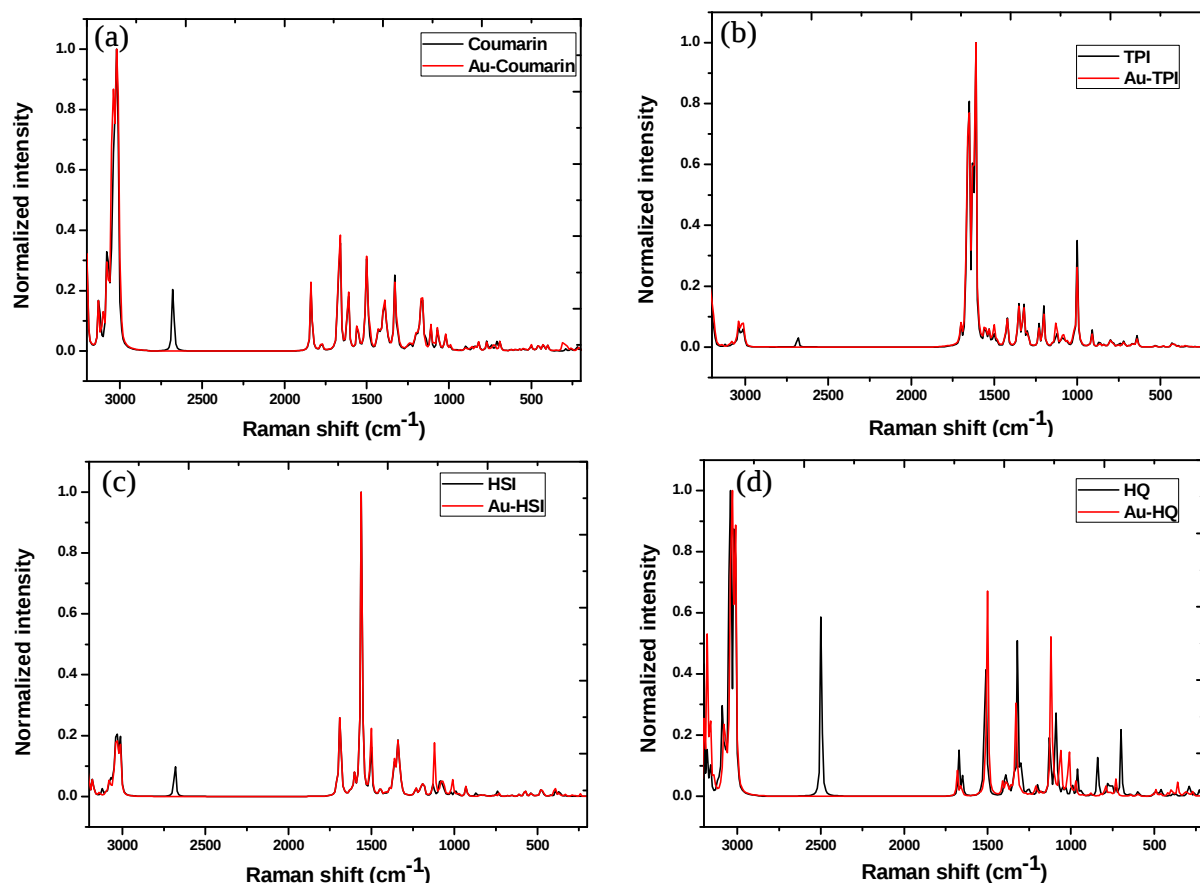


Figure 3.7: DFT calculated Raman spectra of AuMMPCs of (a) $\text{HS}-(\text{CH}_2)_{11}\text{-NHCO-coumarin}$, (b) $\text{HS}-(\text{CH}_2)_{11}\text{-triphenylimidazole}$, (c) $\text{HS}-(\text{CH}_2)_{11}\text{-indole}$ and (d) $\text{HS}-(\text{CH}_2)_{11}\text{-hydroquinone}$.

The summary of all the vibrational peaks obtained for experimental AuMMPCs observed from four Raman active compounds are shown in the supplementary data as Table S 3.1 to S3.4. The SERS spectra of AuMMPCs prepared from different stoichiometric ratios of $\text{HS}-(\text{CH}_2)_{11}\text{-NHCO-coumarin}$ with three different sizes of AuNPs are shown in Figure 3.8, (a)

14 nm, (b) 30 nm and (c) 40 nm. Figures 3.8 (a) to (c), (i) represent the free- HS-(CH₂)₁₁-NHCO-coumarin, (ii) and (iii) are different stoichiometric ratios of HS-(CH₂)₁₁-NHCO-coumarin 1% and 50% respectively. All the spectra revealed the disappearance of the S-H vibration at 2570 and 2590 cm⁻¹, attributed to the interaction of HS-(CH₂)₁₁-NHCO-coumarin via the thiol-end with the AuNPs' surface which was in agreement with the theoretical Raman spectra. The evolution of the new vibrational peak in the region 200 to 300 cm⁻¹ was attributed to an Au-S vibration. This is consistent with reported alkanethiols SERS spectra [40,41]. The SERS spectrum of HS-(CH₂)₁₁-NHCO-coumarin improved as the stoichiometric ratio was increased from 1% to 50%, attributed to an increase of the adsorbing molecules contributing to the enhancement or amplification of the signal regardless of the size of AuNPs. All SERS spectra also revealed a high enhancement of symmetric and asymmetric vibrational bands of C-H bonds in the region 2900 to 3000 cm⁻¹ (*) and 1400 to 1500 cm⁻¹ (#), respectively. A summary of other vibrational bands is presented in the supplementary data, Table S3.1. SERS spectra of AuMMPCs of different stoichiometric ratios of HS-(CH₂)₁₁-triphenylimidazole with different AuNPs sizes are shown in Figure 3.9 (a) 14 nm, (b) 30 nm and (c) 40 nm. Free- HS-(CH₂)₁₁-triphenylimidazole is represented by (i) in Figures 3.9 (a) to (c). The increase in stoichiometric ratios of HS-(CH₂)₁₁-triphenylimidazole revealed some improvements in the spectra. Similar trends to Au-S-(CH₂)₁₁-NHCO-coumarin were observed as S-H vibrations disappeared and a new band evolved in the 200 cm⁻¹ region attributed to a metal-sulphur bond. A notable feature observed was the broad peak in the region of 1500 cm⁻¹, showing the enhancement of the phenyl rings of HS-(CH₂)₁₁-triphenylimidazole. Table S3.2, in the supplementary data, summarizes the existence of other peaks which also confirm the interaction of gold with the thiol-end of HS-(CH₂)₁₁-triphenylimidazole. SERS spectra of the two alkanethiols i.e.; HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone are shown in the

supplementary data as Figure S3.20 and S3.21, respectively. The SERS spectra in Figures S3.20 and S3.21 revealed similar trends to SERS spectra of HS-(CH₂)₁₁-NHCO-coumarin and HS-(CH₂)₁₁-triphenylimidazole for HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone.

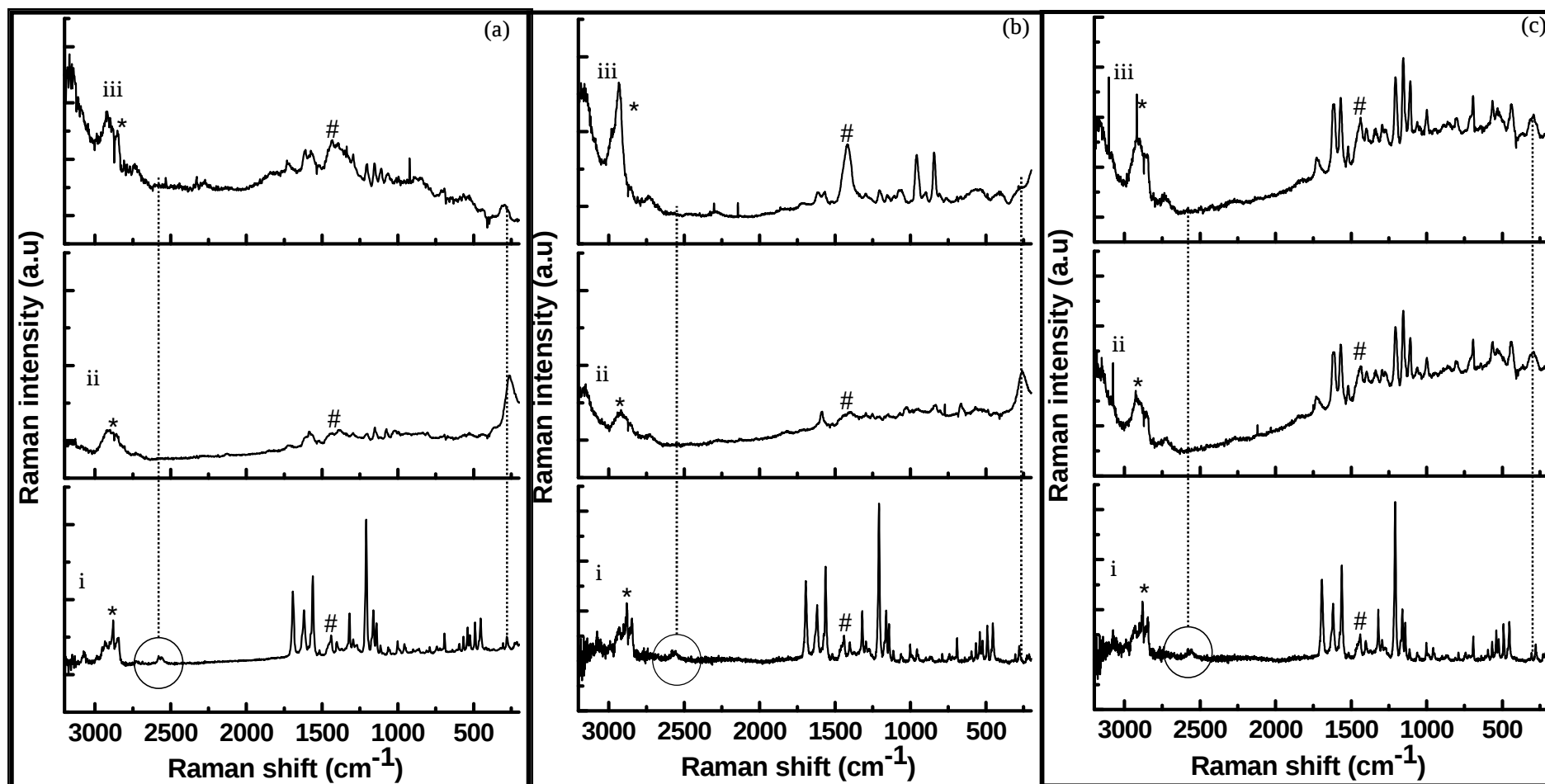


Figure 3.8: Raman spectra of AuMMPCs of different stoichiometric ratios of HS-(CH₂)₁₁-NHCO-coumarin prepared with different AuNPs sizes (a) 14 nm, (b) 30 nm, and (c) 40 nm. The circled peak is the vibrational band of S-H, (* and #) denotes the symmetric and asymmetric bands of C-H bonds

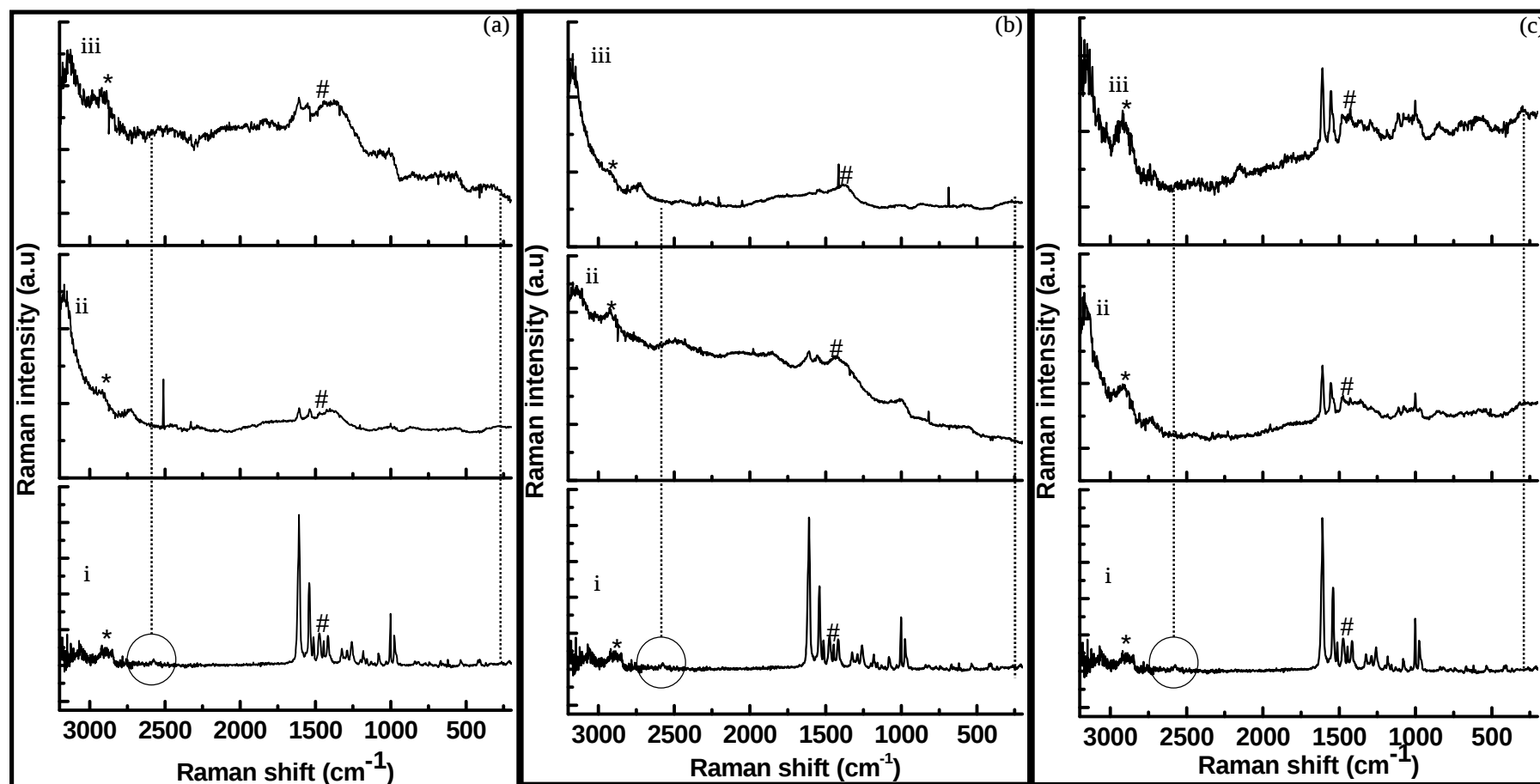


Figure 3.9: Raman spectra of AuMMPCs of different stoichiometric ratios of HS-(CH₂)₁₁-triphenylimidazole prepared with different AuNPs sizes (a) 14 nm, (b) 30 nm, and (c) 40 nm. The circled peak is the vibrational band of S-H, (* and #) denotes the symmetric and asymmetric bands of C-H bonds

In Figure S3.20, the AuMMPCs of HS-(CH₂)₁₁-indole showed broad peaks with little or no improvement as the 1% and 50 % stoichiometric ratios were added. In both Figures (S3.20 and S3.21), S-H vibrations disappeared, while an Au-S vibration appeared in the lower wavenumber region. The summary of the peaks for both alkanethiols are shown in the supplementary data as Table S3.3 and S3.4.

The most utilized method to evaluate the sensitivity of the any Raman reporter is to calculate the enhancement factor (EF) [42]. The calculated EF values of all four alkanethiols adsorbed on different AuNPs sizes are shown in Figure 3.10. Under the same collection conditions (including laser power, accumulation time, and exposure time) the EF can be calculated using equation (1)

$$EF = (I_{surf}/I_{bulk}) * (N_{bulk}/N_{surf}) \quad (1)$$

where I_{surf} and I_{bulk} are the intensities of the vibrational mode in the SERS and the vibrational mode in the Raman spectrum, respectively [43]. N_{bulk} represents the number of molecules probed on the Raman spectrum (free alkanethiol), while N_{surf} represents the number of molecules probed using SERS (alkanethiol with Au substrate). N_{bulk} can be expressed using equation (2):

$$N_{bulk} = Ah\rho/m \quad (2)$$

where A , h , ρ , and m are the laser spot area, the focal length, the density of solid (HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone. The molecular weights (405,55 g/mol; 498, 72 g/mol; 318,52 g/mol and 310.5 g/mol), respectively. N_{surf} can be expressed using equation (3):

$$N_{surf} = 4\pi r^2 CAN \quad (3)$$

where r , C , A and N are the average radius of the Au nanoparticles, the surface density of the alkanethiol, the area of the laser spot, and the surface coverage of the Au nanoparticles (particles μm^{-2}), respectively. Substituting equations (2) and (3) in (1), results in the following;

$$EF = (I_{\text{surf}}/I_{\text{bulk}}) * (Ah\rho/4\pi mr^2CAN) \quad (4)$$

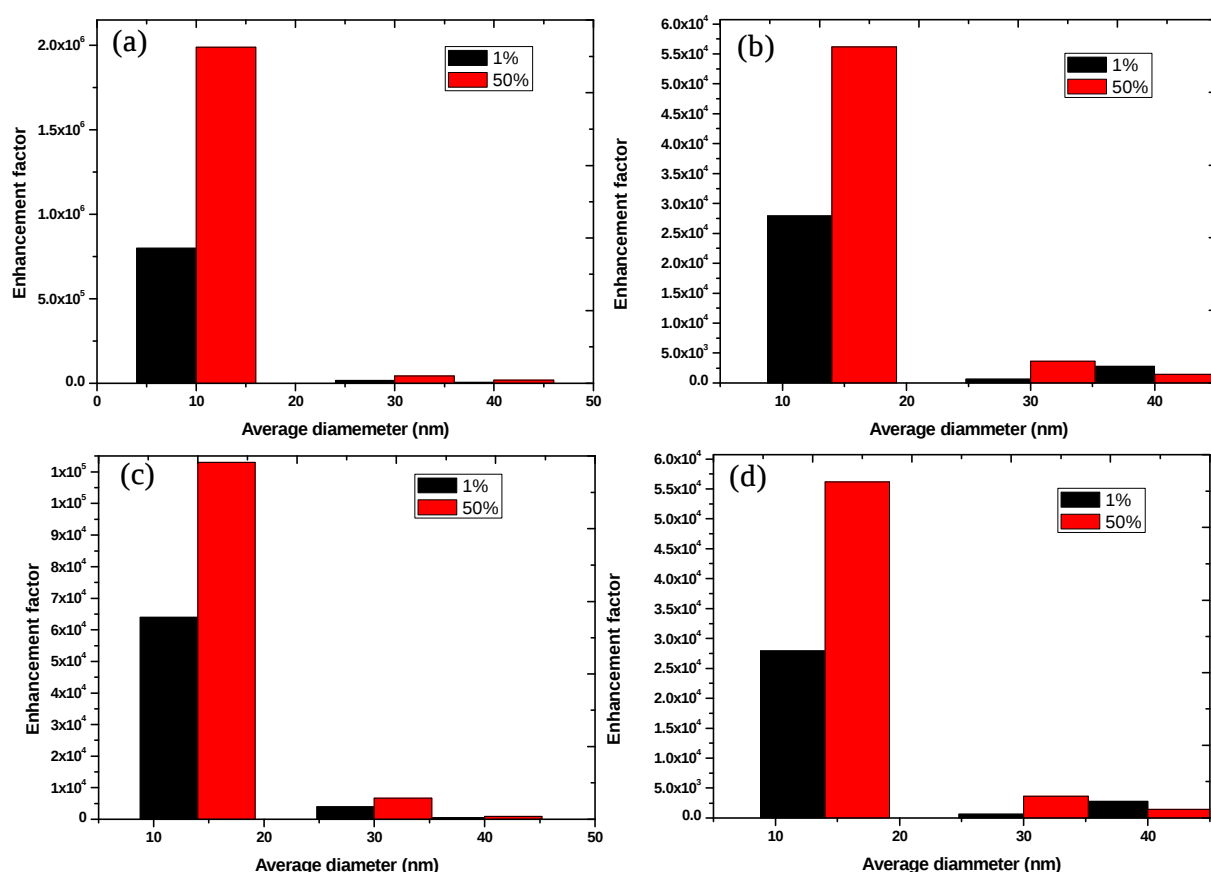


Figure 3.10: Bar graphs correlating the calculated EF's of all alkanethiols (a) HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole and (d) HS-(CH₂)₁₁-hydroquinone adsorbed on AuNPs with different sizes.

The EF values were calculated from the intensities of symmetric stretch vibrations of C-H observed in the region 2900 to 3000 cm^{-1} in all SERS spectra. The bar graphs shown represent the calculated EF's from different alkanethiols, with Figure 3.10 (a) showing the EF's of HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole and (d) HS-(CH₂)₁₁-hydroquinone. All the graphs show an increase in EF as the stoichiometric ratios were increased from 1% to 50%. A decrease in the calculated EF was observed as the AuNPs increases from 14 nm to 40 nm. The higher calculated EF's on smaller AuNPs (14 nm) suggest that there is a higher number of energetically favoured site on the preferred lattice plane compared to bigger sizes of AuNPs. A higher number of energetically favoured sites allow adsorption of more molecules, thus increasing the number of molecules contributing to the enhancement or amplification of the signal. This is contrary to what most researchers observed, however Pustovit *et al* also observed the same trend [5]. Amongst the four alkanethiols used, the highest calculated EF's were observed for AuNPs of 14 nm size with a 50% stoichiometric ratio of an alkanethiol and HS-(CH₂)₁₁-PEG-COOH. The obtained highest calculated EF's observed in descending order were HS-(CH₂)₁₁-NHCO-coumarin (2.0×10^6), HS-(CH₂)₁₁-indole (1×10^5), HS-(CH₂)₁₁-hydroquinone (8.0×10^4), and HS-(CH₂)₁₁-triphenylimidazole (6.0×10^4). Such an order suggests that the phenyl ring(s) and the bulkiness of an alkanethiol influence the enhancement or amplification of the Raman signal. The smallest calculated EF for HS-(CH₂)₁₁-triphenylimidazole is attributed to the bulky nature of the alkanethiol. The shielding effect results in molecules being adsorbed; hence fewer molecules contribute to the enhancement and thus a smaller EF is measured compared to other alkanethiols. The closeness of the phenyl rings in HS-(CH₂)₁₁-NHCO-coumarin to the alkane chain promotes electron delocalization to these rings resulting in a stronger enhancement or strong vibrational peaks as observed in the 1400 to 1500 cm^{-1} region as shown in Figure 3.9. The strong enhancement along the phenyl rings can also be recognised by the enhancement of the C=O

vibrational peak in the region 1700 cm^{-1} , as observed in Figure 3.9 (c). The HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone showed higher calculated EF's than HS-(CH₂)₁₁-triphenylimidazole simply because of their less bulky nature, promoting the adsorption of more molecules.

3.4 Conclusions

The stable AuMMPCs of different AuNP sizes functionalized with different stoichiometric ratios of alkanethiols (HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, HS-(CH₂)₁₁-hydroquinone).and HS-(CH₂)₁₁-PEG-COOH) were successfully prepared. The stability of AuMMPCs was confirmed by the observation of a sharp SPR bands in their UV-vis spectra. The TEM images and the Zeta potentials also revealed no signs of aggregation. The Raman activities showed an improvement in the intensity of the vibrational peaks as the stoichiometric ratios were increased from 1% to 50%. SERS spectra of all Raman reporters confirmed the interaction was via the thiol-end of the alkanethiol. The calculated EF's revealed HS-(CH₂)₁₁-NHCO-coumarin as the best Raman reporter, followed by HS-(CH₂)₁₁-indole, and HS-(CH₂)₁₁-hydroquinone. The HS-(CH₂)₁₁-triphenylimidazole showed the smallest calculated EF, due to its bulky nature. The calculated EF's were observed to decrease with an increase in AuNP size.

3.5 References

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Chapter Four

Synthesis and characterisation of mixed monolayer protected gold nanorods and their Raman activities

4.1. Introduction

Metal nanorods (NRs) have received widespread interest due to their unique one-dimensional structure; their unusual optical, electrical, and catalytic properties, and their use in chemical sensing, cellular imaging, and therapeutics [1-3]. Specifically, noble metal gold [4-8], silver [9], platinum [10, 11], and palladium [12-15] nanorods have garnered great interest, among which AuNRs have been most extensively studied, due to their plasmonic activity. Most conventional synthetic routes to produce gold nanorods rely on solution-phase reactions such as seed-mediated growth [7, 8]. This synthetic route proved to be successful in producing AuNRs with different aspect ratios thereby rendering them with the desired properties. In this route, gold seeds, with a size range of 3-4 nm, are first synthesized by chemical reduction of a gold salt with a strong reducing agent in the presence of a capping agent. These seeds are then added to a solution containing more metal salt, a weak reducing agent, and a surfactant-directing agent. These seeds serve as nucleation sites for the anisotropic growth of AuNRs. This protocol gives fairly monodispersed and stable gold nanorods [16-18]. The quality of the seed particles can affect the growth mechanism of the nanorod. In general, smaller seeds lead to more monodispersed nanorods [6, 19]. Various capping agents such as cetyltrimethylammonium bromide (CTAB) [18], benzyldimethylhexadecylammonium chloride [5], tetraoctylphosphineoxide [20, 21], oleic acid [22], etc, have been successfully employed for the creation of rod-shaped particles. However, CTAB has been extensively used. Gold nanorods stabilized with cationic quaternary ammonium surfactants are positively charged, because ammonium surfactants form a bilayer on the surfaces of AuNRs, with the

ammonium head-groups of one monolayer facing the nanorod surfaces [16]. The presence of the surfactant bilayer makes AuNRs very stable when dispersed in aqueous solutions.

Evidence for rod-shaped particles is usually obtained by TEM, but for gold, the optical spectra of the plasmons are very informative. Nanorods show double plasmon bands, commonly ascribed to light absorption (and scattering) along both the long axis, known as the longitudinal plasmon band, and the short axis known as the transverse plasmon band. Thus, the particle shape dictates on how the wavelengths of light can be absorbed, and elastically scattered. AuNRs of an aspect ratio ranging from 2 to 5, display plasmon bands with tuneable maxima from ~ 700 to 900 nm [23, 24], while high aspect ratio nanorods exhibit a longitudinal plasmon band past 1200 nm [5, 17, 25].

A much stronger and more sensitive surface plasmon band for AuNRs compared to spherical particles [26-29] and the ability to form two dimension assemblies [30] make gold nanorods suitable substrates in surface enhanced Raman spectroscopy (SERS) and sensor applications. Therefore, the knowledge on the surface structure of gold nanorods is essential for SERS system development. Gold nanorods have a larger surface area compared to spherical nanoparticles, thus allowing adsorption of a larger number of target molecules on the surface and also a high possibility of yielding more hot spots [31]. These enhanced fields of gold nanorods offer increased sensitivity for chemical sensing modalities such as SERS, in which the Raman intensity from molecules increases enormously, if the molecules are within ~ 10 nm of a metal nanoparticle surface [5, 32]. One of the significant advances in the development of SERS is the detection of Raman scattering from a single molecule, as first achieved by two independent groups [33, 34]. To date, a SERS enhancement factor of over 10 orders of magnitude may be realized, allowing single-molecule detection even under non-resonant Raman excitation [35, 36]. The SERS effect originates primarily from the giant electromagnetic field resonating with the plasmon at the

surface of a metal nanostructure (silver, gold, etc) [37, 38], as well as from the chemical enhancement owing to the charge transfer resonance between the analyte and the substrate [38]. SERS is of great potential in (bio)-chemical analyses because of its high sensitivity and fluorescence-quenching capability [39]. The presence of CTAB poses a huge threat in utilizing AuNRs for biological applications since CTAB, which is a cationic detergent, shows high cytotoxicity [40-42]. However, Connor *et. al.* reported that the cytotoxicity is not due to the nanorod-bound CTAB layer, but due to the excess CTAB left in the solution, which can be removed by centrifugation [43]. Thus, activation of AuNRs by replacing CTAB with a biocompatible and functionalization-friendly stabilizing agent is essential for the realization of functional nanorod probes that can be used in different application studies.

To further widen the applications of AuNRs in SERS technique, it is crucial to fabricate SERS-active metallic nanostructures with both high enhancement performance, and good reproducibility. This chapter reports the synthesis of Raman active AuNRs achieved by replacing CTAB molecules on the surface of a nanorods by mixture of four different Raman reporters (HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, HS-(CH₂)₁₁-hydroquinone) and HS-(CH₂)₁₁-PEG-COOH through ligand exchange. These SERS active AuNRs can potentially be used to develop a highly sensitive SERS diagnostic probe due to a gold nanorod Raman enhancement, and the terminal –COOH in HS-(CH₂)₁₁-PEG-COOH can be used to attach biomolecules for various applications.

4.2. Experimental section

4.2.1 Chemicals

Cetyltrimethylammonium bromide (CTAB, 99%), L-ascorbic acid (AA, >99%) and sodium borohydride (NaBH₄, 99%) were all used as purchased from Aldrich. Silver nitrate (AgNO₃, A.R.) was from Fluka and all other chemical used were obtained as stated in Chapter 3.

4.2.2 Preparation of Au Seeds

In a typical procedure, 250 μl of an aqueous 0.01 M solution of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was added to 9.75 ml of 0.1 M CTAB solution in a glass beaker. The solutions were gently mixed by swirling the beaker. The solution appeared bright brown-yellow in colour. Then, 600 μl of an aqueous 0.01 M ice-cold NaBH_4 solution was added all at once, followed by rapid stirring for 2 minutes. The solution developed a pale-brown-yellow colour. The solution was then transferred into a centrifuge tube and kept at room temperature for 2 hours.

4.2.3 Preparation of Au nanorods

In a typical experiment, 9.5 ml of 0.1 M CTAB was added to 75 μl of 0.01 M AgNO_3 , followed by the addition of 500 μl of 0.01 M $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ and then gently mixed by swirling. The solution appeared bright brown-yellow in colour. Then 110 μl of 0.1 M AA was added to it. The solution became colourless upon addition and mixing of the AA. Finally, 70 μl of seed solution was added, and the reaction was left undisturbed in an oven at 40 $^\circ\text{C}$.

4.2.4 Functionalization of gold nanorods

In a typical experiment, 2 ml of each centrifuged AuNRs were transferred to two 10 ml test tubes. Different stoichiometric ratios of $\text{HS}-(\text{CH}_2)_{11}\text{-NHCO-coumarin}$ and $\text{HS}-(\text{CH}_2)_{11}\text{-PEG-COOH}$ (1 and 50 %) were used to co-stabilize nanoparticles with CTAB. For 1% a mixture of $\text{HS}-(\text{CH}_2)_{11}\text{-NHCO-coumarin}$ (0.02 mg) and (2 mg) of $\text{HS}-(\text{CH}_2)_{11}\text{-PEG-COOH}$ in methanol, was added to 2 ml centrifuged CTAB gold nanorods and mixed using a shaker for 24 hours. The same procedure was used for a 50 % mixture of $\text{HS}-(\text{CH}_2)_{11}\text{-NHCO-coumarin}$ (1 mg) and (1 mg) $\text{HS}-(\text{CH}_2)_{11}\text{-PEG-COOH}$ in methanol. Similar procedure was applied for the functionalization of AuNRs using the other three Raman active alkanethiols, i.e. $\text{HS}-(\text{CH}_2)_{11}\text{-triphenylimidazole}$, $\text{HS}-(\text{CH}_2)_{11}\text{-indole}$ and $\text{HS}-(\text{CH}_2)_{11}\text{-hydroquinone}$.

4.2.5 Instrumentation

The characterization techniques were used as stated in chapter 3.

4.3 Results and discussions

A seed-mediated method to make AuNRs was adopted from an earlier publication [7]. Two steps were followed to fabricate monodispersed and stable nanorods as shown in Figure 4.1. In the first step, sodium borohydride, which is a strong reducing and nucleating agent was employed to reduce gold ions to form monodispersed spherical nanoparticles acting as seeds for nanorods. In the second step these nuclei were then added to a solution containing a CTAB surfactant stabilized gold complex in the presence of ascorbic acid and silver nitrate for efficient growth of one-dimensional AuNRs. .

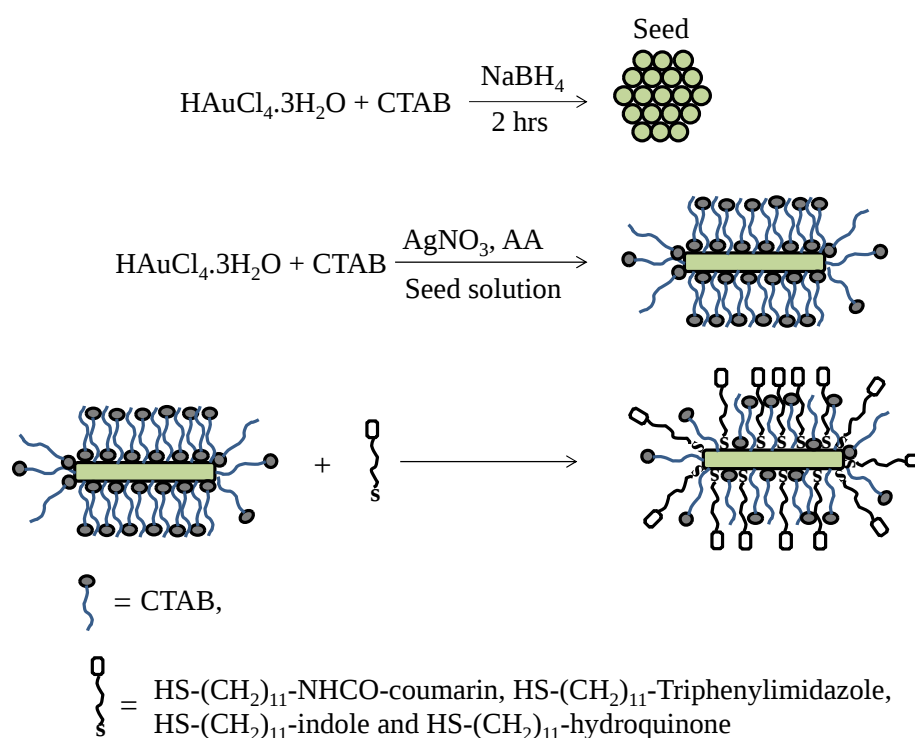


Figure 4.1: General synthetic scheme of gold nanorods MMPCs.

The last step represents the functionalization of CTAB-capped AuNRs with HS-(CH₂)₁₁-PEG-COOH and four different Raman active alkanethiols (HS-(CH₂)₁₁-NHCO-coumarin,

HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, HS-(CH₂)₁₁-hydroquinone). Figure 4.2 (a) shows the absorption spectra of AuNRs, together with (i) unmodified CTAB-gold nanorods, (ii) 1% HS-(CH₂)₁₁-NHCO-coumarin and (iii) 50% HS-(CH₂)₁₁-NHCO-coumarin. The peaks corresponding to the transverse surface plasmon due to electron oscillations perpendicular to the rod were observed in the region of 500 to 530 nm. The longitudinal surface plasmon peak corresponding to electron oscillations parallel to the rod was observed around 731 nm. The longitudinal surface plasmon peak for CTAB-capped nanorods was red-shifted to 743, whilst a red-shift to 747 nm for both 1% and 50% HS-(CH₂)₁₁-NHCO-capped nanorods was observed. This observation is associated with changing the dielectric environment of the surface of the gold nanorods as HS-(CH₂)₁₁-NHCO-coumarin is added. The thiol on the HS-(CH₂)₁₁-NHCO-coumarin molecule is very reactive and as a result, CTAB molecules can be easily displaced via the ligand exchange mechanism. Although HS-(CH₂)₁₁-NHCO-coumarin molecules possess high affinity for gold nanorods, it is known that the surface will not be completely covered by HS-(CH₂)₁₁-NHCO-coumarin as a certain percentage of CTAB traces are expected, as demonstrated in Figure 4.1. However, this displacement is mainly driven by the concentration of the incoming ligand-(HS-(CH₂)₁₁-NHCO-coumarin). This was observed by a direct red shift of the longitudinal surface plasmon to 747 nm as the stoichiometric ratio of HS-(CH₂)₁₁-NHCO-coumarin was increased.

Figure 4.2 (b), (c) and (d) show the absorption spectra of AuNRs capped with three Raman active alkanethiols, compounds namely; HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone, respectively. All spectra revealed no distinct features on adding different percentages onto the surface of the gold nanorods. The only noticeable feature was the blue shift of the longitudinal surface plasmon peak from 743 nm to approximately 700 nm. The shift to lower wavelength signifies a change in the aspect ratio

of gold nanorods which might be associated with a complete change of the morphology of a nanorod to a sphere like nanoparticle. There was no change in the transverse surface plasmon peak of AuNRs capped with these three Raman active compounds as observed in the region of 500 to 530 nm.

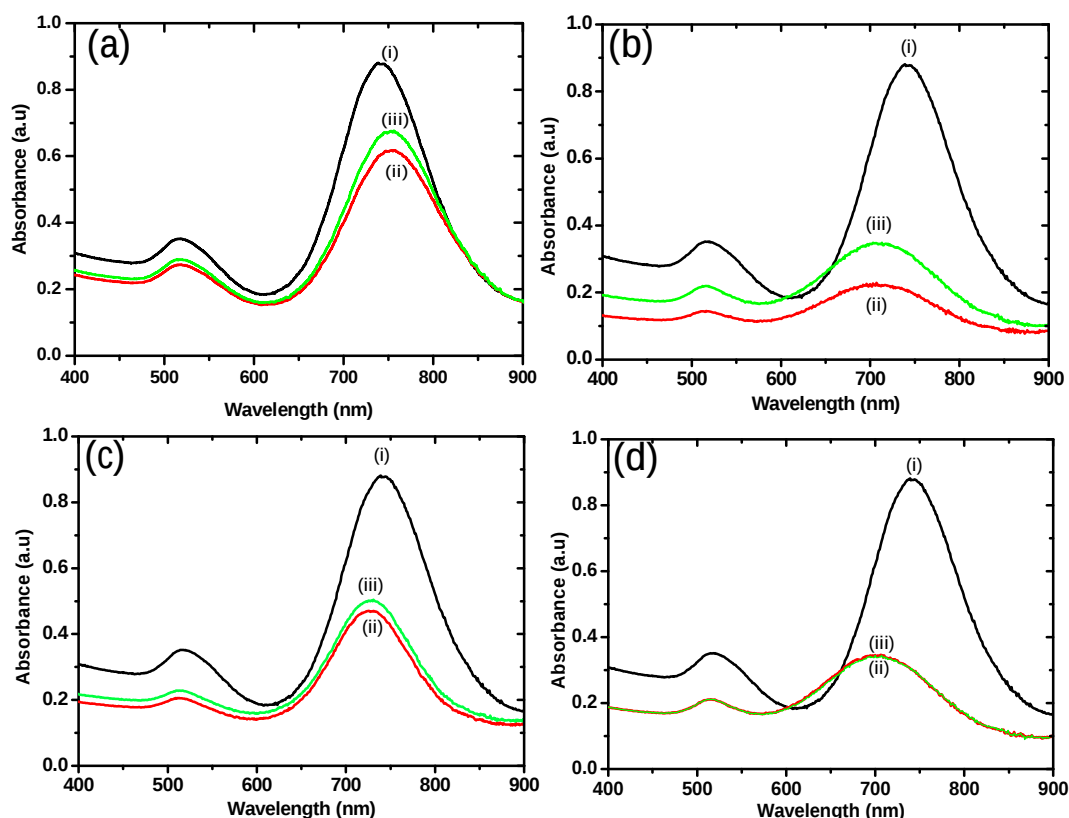


Figure 4.2: Absorption spectra of AuNRs capped by CTAB and four different Raman active alkanethiols; (a) $\text{HS}-(\text{CH}_2)_{11}\text{-NHCO-coumarin}$, (b) $\text{HS}-(\text{CH}_2)_{11}\text{-triphenylimidazole}$, (c) $\text{HS}-(\text{CH}_2)_{11}\text{-indole}$, and (d) $\text{HS}-(\text{CH}_2)_{11}\text{-hydroquinone}$.

TEM images of all synthesized AuNRs mixed monolayer protected clusters revealed the well-established tendency of alkanethiol-capped nanoparticles to form self-assembled ordered superlattices [44]. TEM images of CTAB capped gold nanorods with an aspect ratio of 3.9 are shown in Figure 4.3. The aspect ratio decreased from 3.9 to 3.2 after introduction of the 1% $\text{HS}-(\text{CH}_2)_{11}\text{-NHCO-coumarin}$. No significant change in the aspect

ratio was noted when 50 % HS-(CH₂)₁₁-NHCO-coumarin was added. The nanorods of 3.3 aspect ratios were obtained. There was no aggregation observed as more of HS-(CH₂)₁₁-NHCO-coumarin was added.

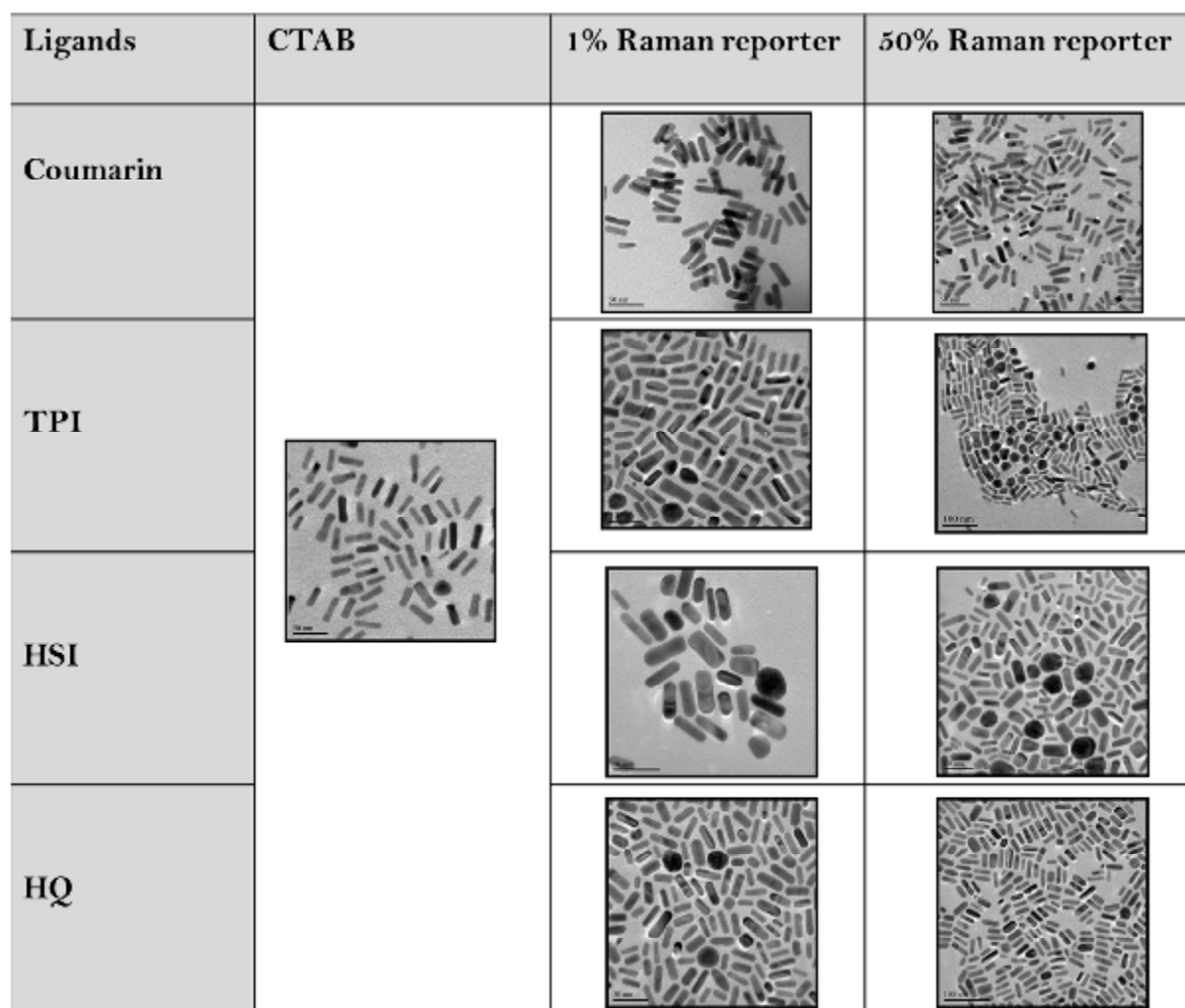


Figure 4.3: TEM images of CTAB stabilized AuNRs and, (1% and 50%) of each HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, and HS-(CH₂)₁₁-hydroquinone

The TEM images of different stoichiometric ratios (1% and 50%) of HS-(CH₂)₁₁-triphenylimidazole are also shown in Figure 4.3. Addition of a 1% stoichiometric ratio led to a decrease of an aspect ratio to 3.1. Few spherical-like gold nanoparticles were observed.

However, addition of a 50% stoichiometric ratio led to formation of more spherical particles. The change in AuNRs to spherical-like gold nanoparticles suggests that HS-(CH₂)₁₁-triphenylimidazole ligands displace CTAB ligands responsible for inhibiting the growth of the lattice plane that leads to the formation of spherical gold nanoparticles.

The TEM images of AuNRs made from different stoichiometric ratios (1 and 50%) of HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone are shown in Figure 4.3. The formation of spherical-like gold nanoparticles was still evident in all images and no significant change in the aspect ratio was observed when these different ratios were added.

The zeta potentials of the AuNRs capped with CTAB and different stoichiometric ratios of four different Raman active alkanethiols are shown in supplementary data (Figure S4.1 to Figure S4.4). The magnitude of the zeta potential determines the possible degree of stability of a colloidal system. The zeta potential results proved that there was no noticeable aggregation as the stoichiometric ratios of Raman active compounds were increased from 1% to 50 % except in HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone. The large positive and small negative charge values were associated with the high stability. The charges induce AuNRs repulsion, thus leading to stable gold nanorods without any aggregation as shown in Figure 4.3. Any charge close to zero indicates the possible attraction of particles which can lead to aggregation. CTAB is a weak acid of pH 5 to 7.5, known to be a positive charge bearing stabilizer, and as expected CTAB-capped nanorods showed a huge positive charge value of 59.3 mV as shown in Figure S4.1 (a) to Figure S4.4 (a). This value decreased to 32.9 and 11.9 mV as the stoichiometric ratio of HS-(CH₂)₁₁-NHCO-coumarin was increased from 1% to 50 % as shown in Figure S4.1 (b) and (c), respectively. The decrease in zeta potential confirmed the addition of HS-(CH₂)₁₁-NHCO-coumarin on the nanorod surface. The zeta potential results confirmed the noticeable dielectric change in surface plasmon resonance (SPR) peak of the

longitudinal section of rods which was observed in Figure 4.2(a). The zeta potentials of the AuNRs capped with CTAB and different stoichiometric ratios of HS-(CH₂)₁₁-triphenylimidazole are shown in Figure S4.2. The addition of 1% stoichiometric ratio led to a huge decrease of the potential to -34.1 mV as shown in Figure S4.2 (b). The higher ratio of 50% resulted to an increase of the zeta potential to -7.96 mV as shown in Figure S4.2 (c); this is attributed to an increase of the spherical like particles as observed in Figure 4.3 (e).

A similar trend was also observed with the potentials of higher stoichiometric ratios of 50% in both HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone. The huge decrease of Z-potential from 59.3 mV of CTAB capped AuNRs to -56.5 and -11.7 mV for 1% HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone as observed, as shown in Figure S4.3 (b) and Figure S4.4 (b), respectively. The increase of the potentials was observed when higher stoichiometric ratios of 50% HS-(CH₂)₁₁-indole (-2.01 mV) and HS-(CH₂)₁₁-hydroquinone (-4.22 mV) were introduced to surface of CTAB capped AuNRs as shown in Figure S4.3 (c) and Figure S4.4 (c), respectively.

Figure 4.4 shows the influence of -COOH group on the zeta potentials, an introduction of 50% HS-(CH₂)₁₁-PEG-COOH resulted to a decrease in potentials. The decrease in zeta potentials (weak positive) is attributed to an increase of acidic property of the AuNR solutions due to an additional thiol groups (weak acid) and also the carboxylic groups. A further increase of -COOH to 99% led to further decrease of zeta potentials of all Raman alkanethiols (HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone) except for HS-(CH₂)₁₁-NHCO-coumarin. The decrease of zeta potential suggests the deprotonation of the carboxylic group to form carboxylic ion which influences the negative zeta potential.

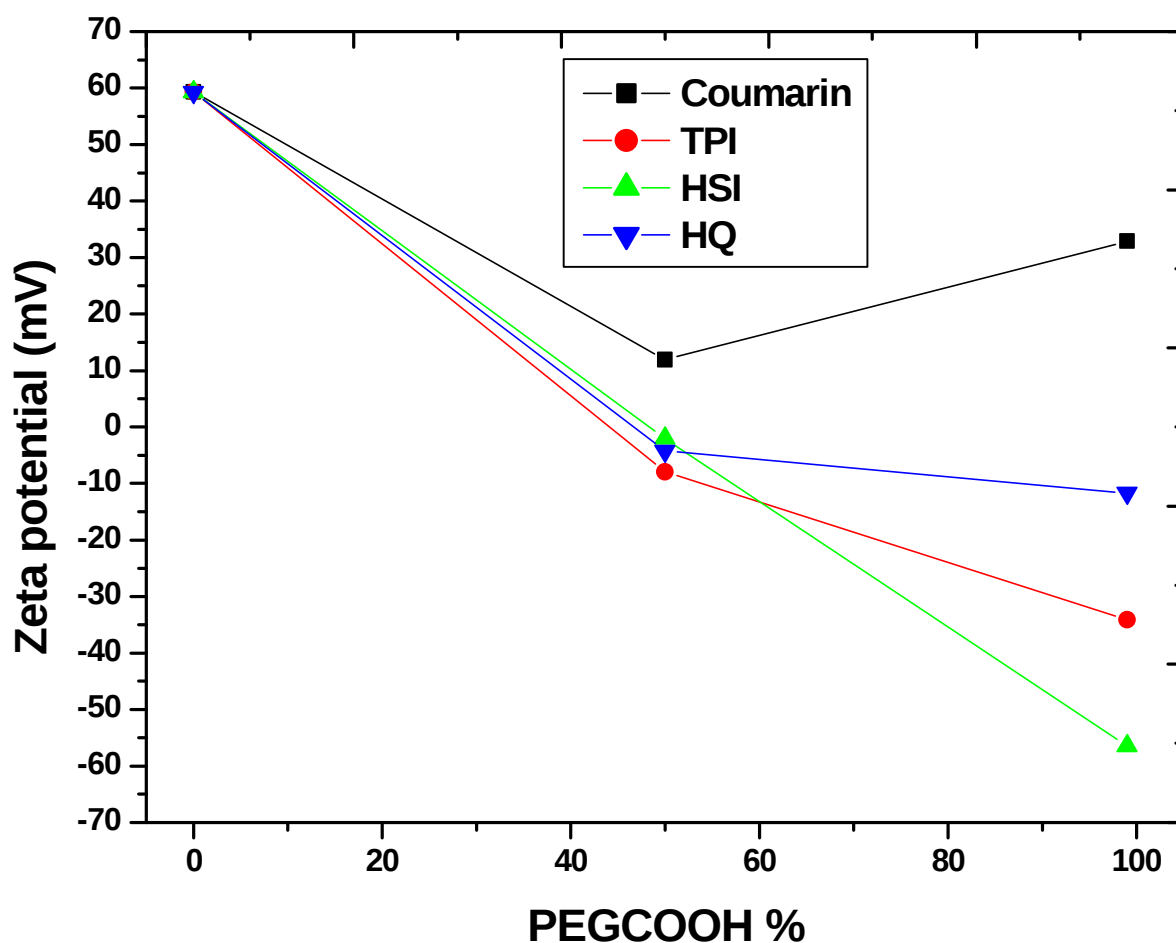


Figure 4.4: Zeta potential graphs of AuNRs against the –COOH percentages

Figures 4.5 to 4.8 show the Raman spectra of AuNRs that were used to study SERS activities of four different Raman active alkanethiols (HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone) in different stoichiometric ratios. The CTAB Raman spectrum is shown in all Figures 4.5(iv) to Figure 4.8(iv). Nikoobakht *et al.*, [45] reported that the only enhanced peak in the spectrum of CTAB-capped gold nanorods was the $\nu(\text{C-Br})$ vibrations at 174 cm⁻¹. The CTAB spectrum is dominated by strong symmetric stretching (C-H) and (N-H) vibrations in the region of 2900 to 3000 cm⁻¹. The asymmetric vibrations of (C-H) were also observed in the region of 1400 to 1450 cm⁻¹. The summary of all the Raman active alkanethiols adsorbed on the CTAB-capped AuNRs are shown in the supplementary data (Table S4.1 to Table S4.4).

The interaction of the thiol end present in all four alkanethiols with gold nanorod's surface, was confirmed by the disappearance of the $\nu(-S-H)$ vibration peak in the 2500 cm^{-1} region as shown in Figures 4.5(i) to 4.8(i).

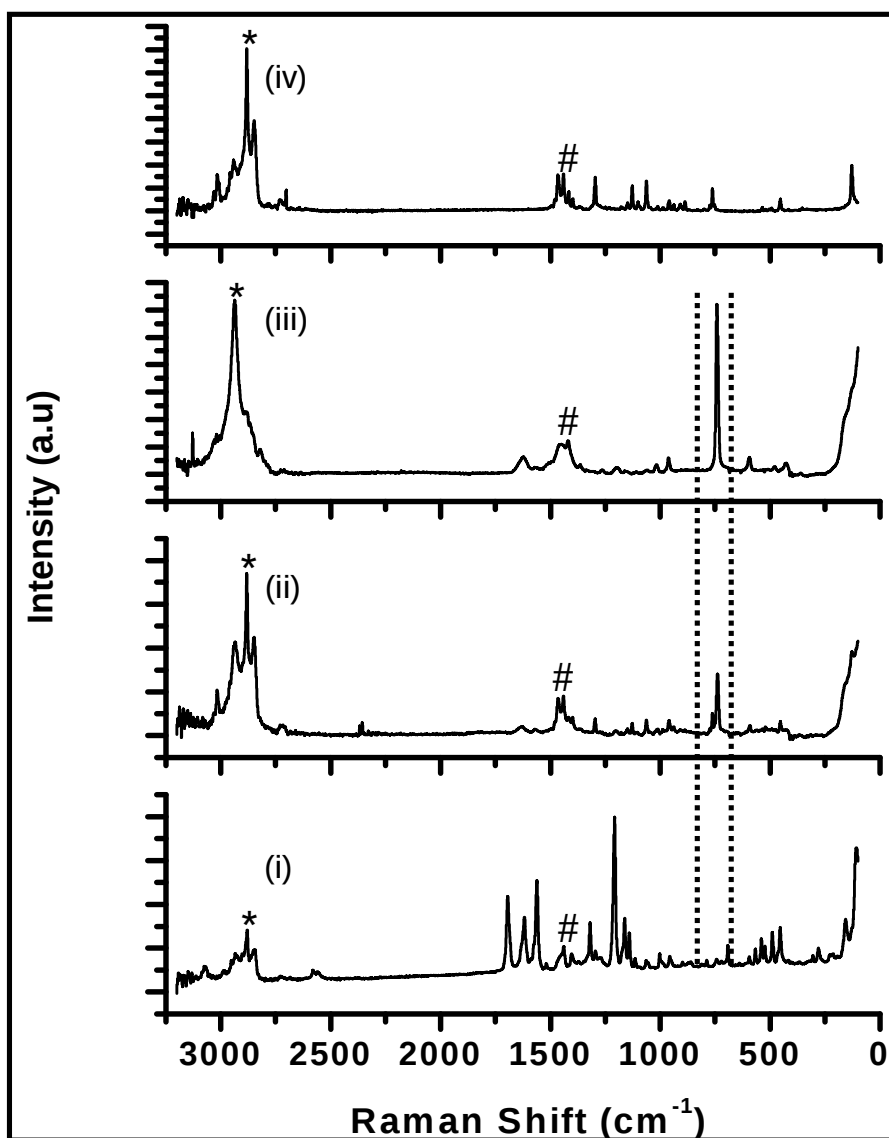


Figure 4.5: Raman spectra (i) unmodified HS-(CH₂)₁₁-NHCO-coumarin, (ii) 1% HS-(CH₂)₁₁-NHCO-coumarin, (iii) 50% HS-(CH₂)₁₁-NHCO-coumarin and (iv) unmodified CTAB. The * and # denotes the symmetric and asymmetric bands of C-H bonds.

The prepared mixed monolayer protected gold nanorods showed no Raman signal enhancements at this region; this is attributed to the high affinity of alkanethiols to the gold surface as suggested by the shoulder observed above 200 cm^{-1} region, corresponding to the Au-S vibration peak. SERS signals of the AuNRs with HS-(CH₂)₁₁-NHCO-coumarin shown in Figure 4.5(ii) and (iii) are much stronger than the unmodified HS-(CH₂)₁₁-NHCO-coumarin and CTAB capped nanorods, shown in Figure 4.5(i) and (iv) respectively. The spectrum of AuNRs capped with CTAB and 1% HS-(CH₂)₁₁-NHCO-coumarin in Figure 4.5(ii) and 50% HS-(CH₂)₁₁-NHCO-coumarin 4.5(iii) are dominated by a strong electromagnetic effect assigned to the $\nu(\text{C-S})$ vibrations at 739 cm^{-1} . This is indicative of the interaction of a thiol group with the nanorod's surface. This peak was found to increase with increasing stoichiometric ratio of HS-(CH₂)₁₁-NHCO-coumarin. A similar peak was also observed in the free CTAB spectrum and is attributed to $\nu(\text{C-Br})$ vibrations.

SERS spectra of different stoichiometric ratio of HS-(CH₂)₁₁-triphenylimidazole are shown in Figure 4.6(ii) and (iii). The noticeable feature was the smaller enhancements of the aromatic vibrations suggesting that the rings were not close to the surface of AuNRs. A clearer peak was observed for the $\nu(\text{C-S})$ vibration as the stoichiometric ratio was increased confirming the interaction of the thiol end with AuNRs surface.

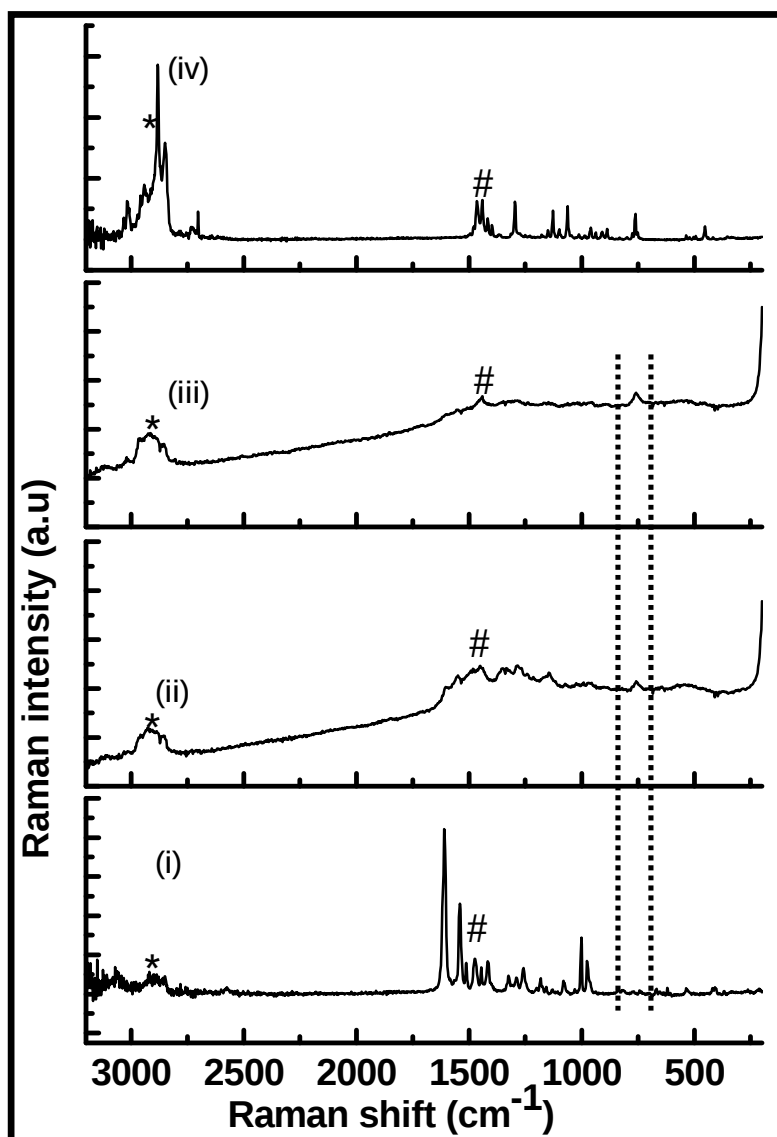


Figure 4.6: Raman spectra (i) unmodified HS-(CH₂)₁₁-triphenylimidazole, (ii) 1% HS-(CH₂)₁₁-triphenylimidazole, (iii) 50% HS-(CH₂)₁₁-triphenylimidazole and (iv) unmodified CTAB. The * and # denotes the symmetric and asymmetric bands of C-H bonds

The Raman spectra of HS-(CH₂)₁₁-indole are shown in Figures 4.7 (ii and iii). A higher stoichiometric ratios of HS-(CH₂)₁₁-indole (50%) showed a smaller enhancement of all vibrational peaks compared to 1% stoichiometric ratio suggesting a poor ability of HS-(CH₂)₁₁-indole in stabilizing the AuNRs, as shown in Figure 4.7 (iii). A lower

stoichiometric ratio showed a high enhancement in the aromatic region (1400 to 1500 cm^{-1}), and also the asymmetric and symmetric vibrations of (CH_2) in the region of 1400 to 1450 cm^{-1} (#) and 2900 to 3000 cm^{-1} (*), respectively, as observed in Figure 4.7 (ii). The enhancement for the (C-S) vibration and the disappearance of (-S-H) vibration observed confirmed the interaction of the thiol end with AuNRs surface.

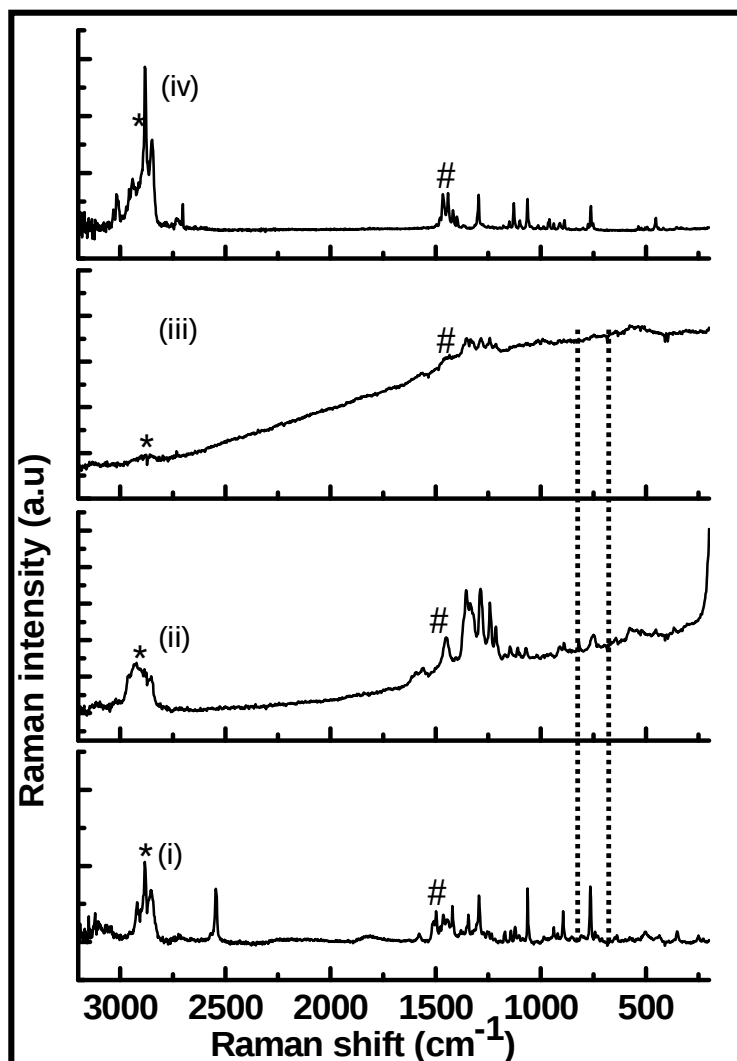


Figure 4.7: Raman spectra (i) unmodified HS-(CH_2)₁₁-indole, (ii) 1% HS-(CH_2)₁₁-indole, (iii) 50% HS-(CH_2)₁₁-indole and (iv) unmodified CTAB. The * and # denotes the symmetric and asymmetric bands of C-H bonds.

The Raman spectra of HS-(CH₂)₁₁-hydroquinone adsorbed on CTAB-capped AuNRs in different stoichiometric ratios are shown in Figure 4.8 (ii) and (iii). A summary of the selected peaks is given in Table S4.4. Similar features to the other three alkanethiols were also observed, together with the enhancement of asymmetric and symmetric vibrational peaks of (CH₂), and the disappearance of the (-S-H) vibration at 2500 cm⁻¹ enhancing the (C-S) vibration at 752 cm⁻¹.

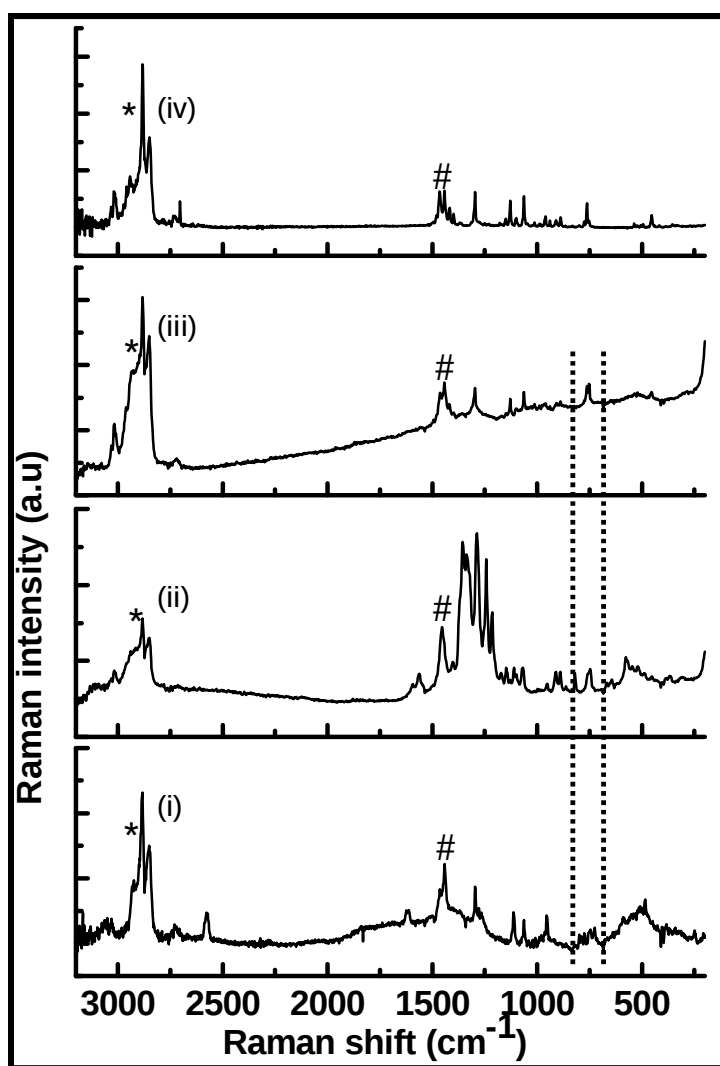


Figure 4.8: Raman spectra (i) unmodified HS-(CH₂)₁₁-hydroquinone, (ii) 1% HS-(CH₂)₁₁-hydroquinone, (iii) 50% HS-(CH₂)₁₁-hydroquinone and (iv) unmodified CTAB. The * and # denotes the symmetric and asymmetric bands of C-H bonds.

4.4 Conclusions

Mixed monolayer protected gold nanorods were successfully synthesized by the addition of stoichiometric ratios of Raman active alkanethiols, i.e.; HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, HS-(CH₂)₁₁-hydroquinone and HS-(CH₂)₁₁-PEG-COOH. The optical analysis of the synthesized mixed monolayer protected gold nanorods showed a slight red shift in the longitudinal peaks as the stoichiometric ratios (1% and 50%) of HS-(CH₂)₁₁-NHCO-coumarin were added onto CTAB-capped gold nanorods surface. The blue shift of the longitudinal peak was observed in optical analysis of HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone, and is attributed to the formation of spherical like gold nanoparticles, as confirmed by their TEM images. The TEM images for different stoichiometric ratios of all four alkanethiols showed no distinct change in the aspect ratio of nanorods. The stability of mixed monolayer protected gold nanorods was confirmed through Zeta potentials which revealed no aggregation of the as-prepared mixed monolayer protected gold nanorods. The Raman spectra of all alkanethiols adsorbed on the surface of CTAB-capped gold nanorods confirmed the interaction of alkanethiols via the thiol end through enhancement of the (C-S) vibration, and the presence of the (Au-S) vibration in the region 700 to 800 cm⁻¹ and 200 cm⁻¹, respectively.

4.5 References

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Chapter Five

Synthesis and functionalization of different silver nanoparticle sizes to mixed monolayer protected clusters with different Raman reporters

5.1 Introduction

Silver nanoparticles (AgNPs) have been one of the extensively studied substrates for SERS, and have demonstrated a high plasmonic effect compared to gold nanoparticles [1]. However, a synthetic method to a size-controlled and monodispersed AgNPs remains a challenge [2]. The most widely used synthesis methods of isotropic AgNPs are centred on the reduction of silver salts by sodium citrate [3], sodium borohydride [4], hydroxylamine hydrochloride [5], or polyvinylpyrrolidone [6]. The shortcoming of all these method is that, regardless of their individual advantages, they are incapable of giving size-controlled monodispersed AgNPs. Glycerol has been used in colloidal synthesis to increase the monodispersity of gold nanostars [7], and gold nanoplates through the hydroxyl groups which has led to a higher stability of the NPs against oxidation/ripening [8]. Recently, Steinigeweg and Schlücker [9] reported the use of various compounds bearing the hydroxyl groups including glycerol to produce monodispersed AgNPs. The method relied on the hydroxyl groups to control size and dispersity.

The chapter reports the functionalization of monodispersed of different AgNP size with four alkanethiols. The resulted AgMMPCs were further used to study the effect of AgNP size on the enhancement factor was studied using four Raman alkanethiols.

5.2 Experimental details

5.2.1 Chemicals

Glycerol, $C_3H_5(OH)_3$ was purchased from Associated chemical enterprises (PTY) LTD, South Africa, and all other chemical used were obtained as given in chapter 3

5.2.2 Instrumentation

The characterization techniques were used as given in chapter 3 and chapter 4..

5.2.3 Synthesis of Ag nanoparticles

Silver nanoparticles of different sizes were prepared by dissolving 10 mg of $AgNO_3$ into the mixture of 40 % glycerol-water. The solution was heated up to 95 °C, and then added to different volumes (0.25 ml; 0.5 ml and 0.75 ml) of 3% Sodium citrate to give AgNPs of different sizes.

5.2.4 Functionalization of AgNPs with alkanethiols

The resultant AgNPs were filtered, then in each 10 ml (all different AgNPs sizes) 200 μ l of different percentages of HS-(CH₂)₁₁-NHCO-coumarin and HS-(CH₂)₁₁-PEG-COOH (1 and 50 % respectively) were used to co-stabilize nanoparticles. The solutions were swirled at room temperature for three hours. For HS-(CH₂)₁₁-NHCO-coumarin 1%, 0.02 mg was dissolved in 1 mL methanol and then thoroughly mixed with HS-(CH₂)₁₁-PEG-COOH (1.98 mg) in 1 mL of methanol. For 50%, 1 mg of each alkanethiol was dissolved in methanol and thoroughly mixed. Similar procedure was followed for the preparation of AuMMPCs using the other three Raman active alkanethiols i.e.;(HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, HS-(CH₂)₁₁-hydroquinone).

5.2.5 Density Functional Theory (DFT) calculations and models

All calculations were carried out with Gaussian 03 programs as stated in section .3.2. For silver (Ag), the LANL2DZ set electron core potential simulates the 28 of the total 47 (Ag)

electrons. The remaining 19 electrons are described by an electron basis sets consisting of a (8s6p4d) set of primitive Gaussian type functions contracted to the [3s3p2d]. For C, H, N, S and O the LANL2DZ basis consists of a (10s5p) set contracted to the (3s2p) set, while for H a (4s) set contracted to the (2s) basis set was used.

5.3 Results and discussion

The synthesis of different AgNPs was achieved by variation of 3% sodium citrate volume used to reduce silver ions to silver atoms in the presence of a glycerol-water mixture as shown in equation (1). The synthesized AgNPs were functionalized with different stoichiometric ratios of four different alkanethiols (HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, HS-(CH₂)₁₁-hydroquinone) and HS-(CH₂)₁₁-PEG-COOH for further bioconjugation or attachment of biomarkers) as shown in equation (2). It has been reported that the interaction of sulphur atom of the alkanethiol chain with the metal surface nanoparticles tends to form self-assembled supper lattices [10], and similar observations were predicted for Raman active alkanethiols and HS-(CH₂)₁₁-PEG-COOH on silver nanoparticles as shown schematically in Figure 5.1 (equation 2). The self-assembled formation is very critical during this reaction as it allows the control on the substitution reaction of alkanethiols to give a uniform intensity enhancement.

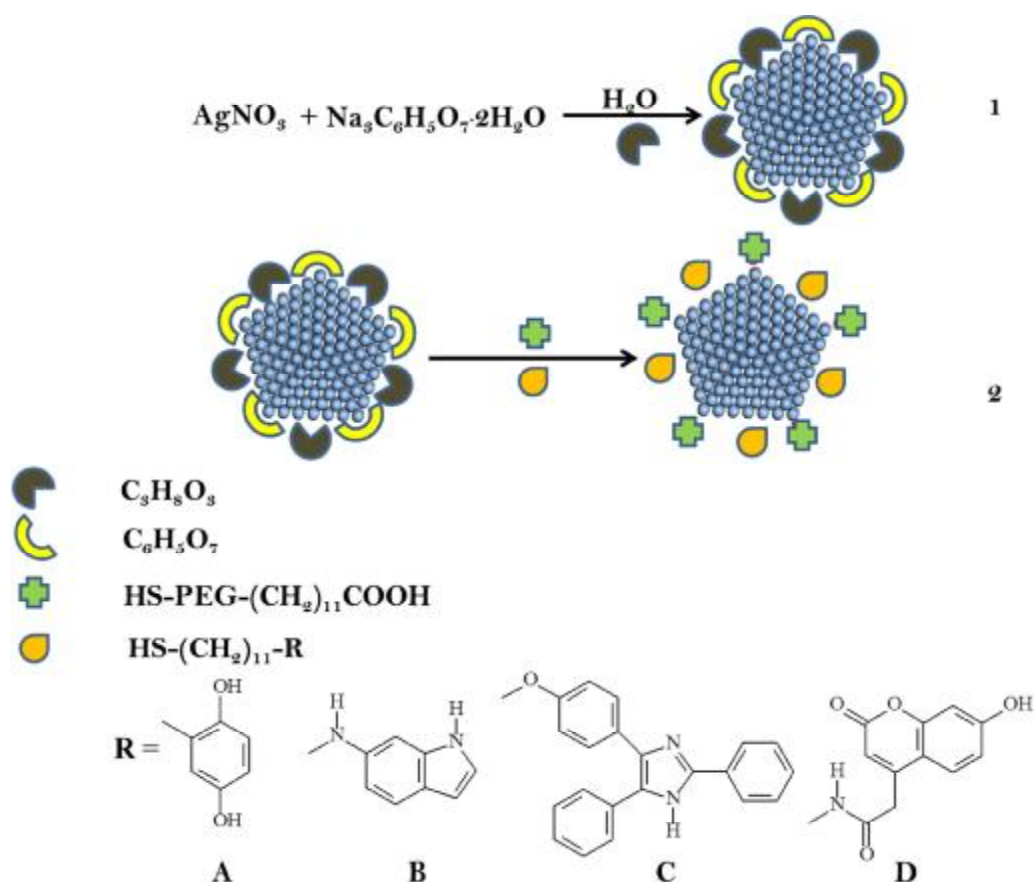


Figure 5.1: General synthetic scheme for the synthesis of AgMMPCs from co-stabilized citrate and glycerol-AgNPs using (a) HS-(CH₂)₁₁-hydroquinone (b) HS-(CH₂)₁₁-indole, (c) HS-(CH₂)₁₁-triphenylimidazole and (d) HS-(CH₂)₁₁-NHCO-coumarin.

A summary of the SPR bands of all the different AgNPs functionalized with four different Raman active alkanethiols in different stoichiometric ratios are shown in Table 5.1. The AgNPs sizes are classified as 16, 30 and 40 nm throughout the section, the calculated average diameter sizes are shown under the particle size distribution section. The observed SPR bands in Table 5.1 were found to be between 400-500 nm, depending on the AgNP size, and similar to the results reported by Lin *et al.* [11]. The absorption spectral data for the samples are presented in Table 5.1 and the spectra are shown in the supplementary data in Figures S5.1 to S5.4. All SPR bands for the different sizes of AgNPs were red-shifted as the different stoichiometric ratios of alkanethiols were changed; this is clearly shown in

Figures S5.1 to S5.4. The SPR bands obtained from all the AgMMPCs using the four different alkanethiols (HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, HS-(CH₂)₁₁-hydroquinone), revealed a slight blue-shift as the AgNP size increased from 16 nm to 40 nm. This suggests that the increase in size of AgNP is associated with a decrease in surface area which prevents the adsorption of more ligands in the larger AgNPs than in smaller AgNPs.

Table 5.1: Summary of SPR bands of AgNPs and AgMMPCs

Sample		SPR (nm)
	Ag16nm	412
	Ag30nm	415
	Ag40nm	413
Coumarin	Ag16 1%	425
	Ag16 50%	425
	Ag30 1%	426
	Ag30 50%	428
	Ag40 1%	422
	Ag40 50%	422
TPI	Ag16 1%	417
	Ag16 50%	418
	Ag30 1%	414
	Ag30 50%	415
	Ag40 1%	421
	Ag40 50%	406
HSI	Ag16 1%	421
	Ag16 50%	421
	Ag30 1%	410
	Ag30 50%	411
	Ag40 1%	420
	Ag40 50%	420
HQ	Ag16 1%	424
	Ag16 50%	432
	Ag30 1%	410
	Ag30 50%	412
	Ag40 1%	410
	Ag40 50%	411
SPR= surface plasmon resonance		

TEM images were used to calculate the average diameter sizes of AgNPs. Figure 5.2 presents the particle distribution histograms of the citrate and glycerol co-stabilized AgNPs and gives the average diameter sizes 16; 30, and 40 nm. These AgNPs were functionalized with different Raman active alkanethiols to form AgMMPCs. The TEM images of AgMMPCs functionalized with 1% and 50% HS-(CH₂)₁₁-NHCO-coumarin are shown in Figure 5.3.

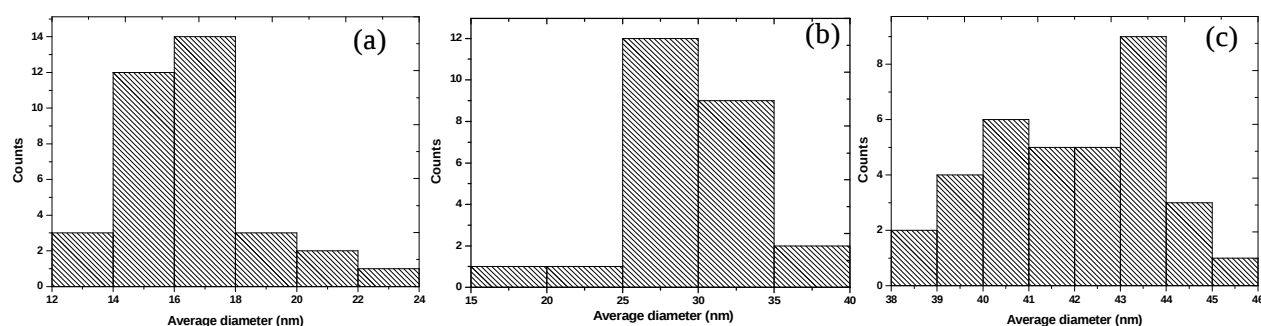


Figure 5.2: Particle distribution histograms of citrate stabilised AgNPs with average size of (a) 16 nm, (b) 30 nm, and (c) 40 nm.

The TEM images reveal some traces of rod-like particles which are more prevalent in smaller AgNPs sizes (16 to 30 nm) as shown in Figure 5.3. The polydispersity of the particles is attributed to a slower nucleation rate due to the use a smaller amount of the reducing agent (sodium citrate), resulting to a slower growth rate of the nanoparticles. The steady growth rate will lead to the aggregation of smaller particles leading to the formation of an elongated rod-like particle. The polydispersity of particles was also confirmed by the tailoring of the SPR bands as observed in Figure S5.1 (a) for the 16 nm AgNPs. Figure 5.3 show TEM images of citrate/glycerol-AgNPs of 40 nm, 1%, and 50% HS-(CH₂)₁₁-NHCO-coumarin stoichiometric ratios, respectively. The TEM images for AgNPs 30 and 40 nm with their corresponding 1 and 50 % stoichiometric ratios show a good dispersity with no rod-like particles observed as on the use of a higher volume of the reducing agent which

resulted in a faster growth rate. However, similar rod-like particles were also observed for AgMMPCs prepared with different stoichiometric ratios of HS-(CH₂)₁₁-triphenylimidazole with different AgNPs sizes as shown in Figure 5.4. .

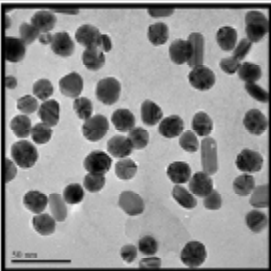
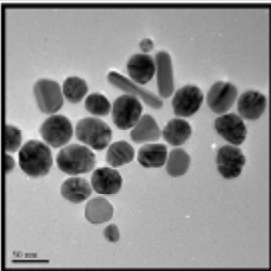
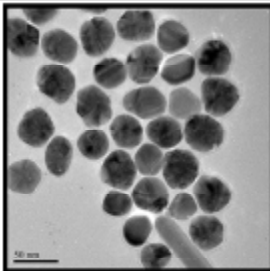
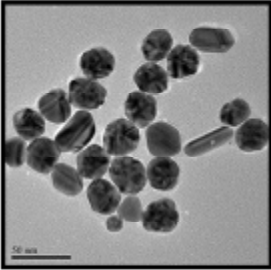
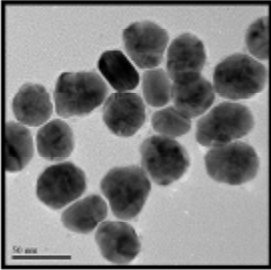
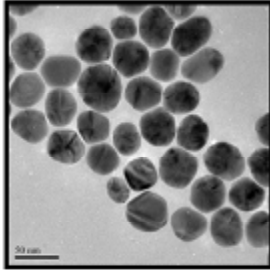
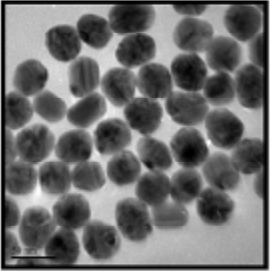
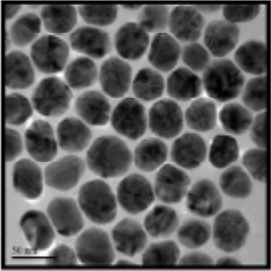
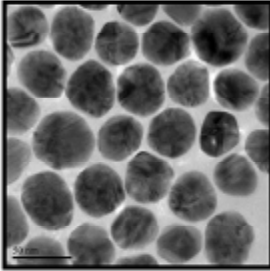
Ligands	Citrate	1% Raman reporter	50% Raman reporter
Sizes 14 nm			
30 nm			
40 nm			

Figure 5.3: TEM images of AgMMPCs of different stoichiometric ratios of HS-(CH₂)₁₁-NHCO-coumarin prepared with different AgNPs sizes

The TEM images of the other two alkanethiols i.e.; HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone are shown in the supplementary data in Figure S5.5 and S5.6, respectively. The hydroxyl group in glycerol stabilized the prepared different AgNPs sizes, this was established by Xia *et al.*, where they reported higher stability of nanoparticles in the presence of glycerol with hydroxyl groups [12]. The displacement of glycerol and citrate from the AgNPs with the different stoichiometric ratios of Raman active alkanethiol and HS-(CH₂)₁₁-PEG-COOH can temper with the stability of AgNPs. It was therefore necessary to evaluate the stability of the as-prepared AgNPs using a Malvern ZetaSizer. The zeta potentials obtained from AgNPs and AgMMPCs are presented in Table 5.2, and their corresponding Zeta graphs are shown in the supplementary data (Figure S5.7 to S5.18). AgNPs revealed negative values which were found to approach zero as different stoichiometric ratios of these different alkanethiols were introduced to the silver surface.

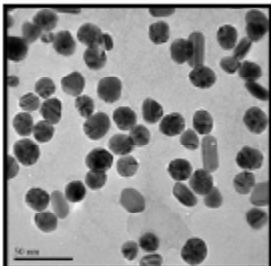
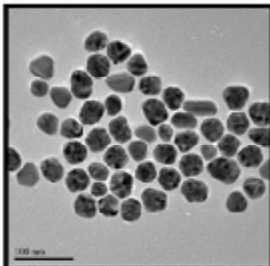
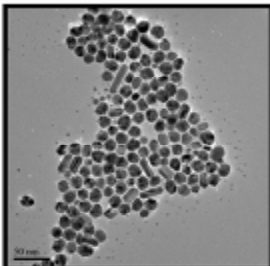
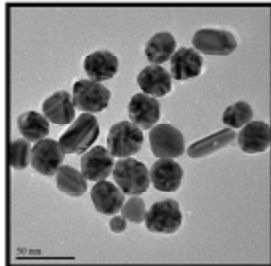
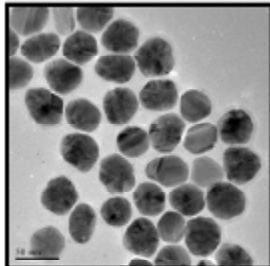
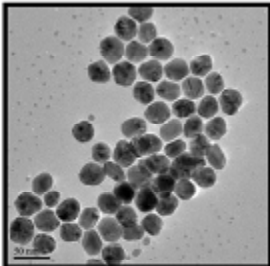
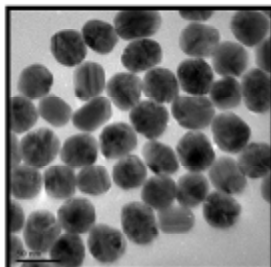
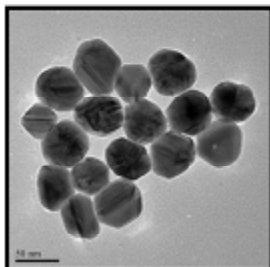
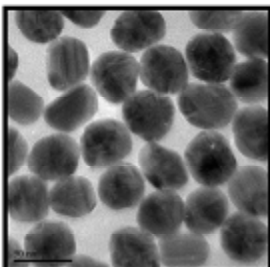
Ligands	Citrate	1% Raman reporter	50% Raman reporter
Sizes 14 nm			
30 nm			
40 nm			

Figure 5.4: TEM images of AgMMPCs of different stoichiometric ratios of HS-(CH₂)₁₁-triphenylimidazole prepared with different AgNPs sizes.

Table 5.2: Values of Zeta potentials of AgNPs and AgMMPCs

Sample		Zeta potential (mV)
	Ag16nm	-32.7
	Ag30nm	-27.4
	Ag40nm	-25.4
Coumarin	Ag16 1%	-2.45
	Ag16 50%	-3.07
	Ag30 1%	-2
	Ag30 50%	-2.38
	Ag40 1%	-2.54
	Ag40 50%	-2.07
TPI	Ag16 1%	-2.04
	Ag16 50%	-1.88
	Ag30 1%	-1.88
	Ag30 50%	-1.97
	Ag40 1%	-1.99
	Ag40 50%	-1.55
HSI	Ag16 1%	-2.19
	Ag16 50%	-2.54
	Ag30 1%	-1.82
	Ag30 50%	-1.82
	Ag40 1%	-2.66
	Ag40 50%	-2.04
HQ	Ag16 1%	-27
	Ag16 50%	-26.7
	Ag30 1%	-10.6
	Ag30 50%	-11
	Ag40 1%	-16
	Ag40 50%	-15.4

The influence of the carboxylic acid group on the zeta potential of AuMMPCs was investigated, and graphs are shown in Figure 5.5. The citrate/glycerol stabilized AgNPs displayed strong negative for all sizes. The potentials were observed to increase as a 50% mixture of alkanethiols with HS-(CH₂)₁₁-PEG-COOH. The resulted weak negative values were associated with the increasing acidic property on AgNP solution. A further increase of -COOH percentage showed no significant change of zeta potential.

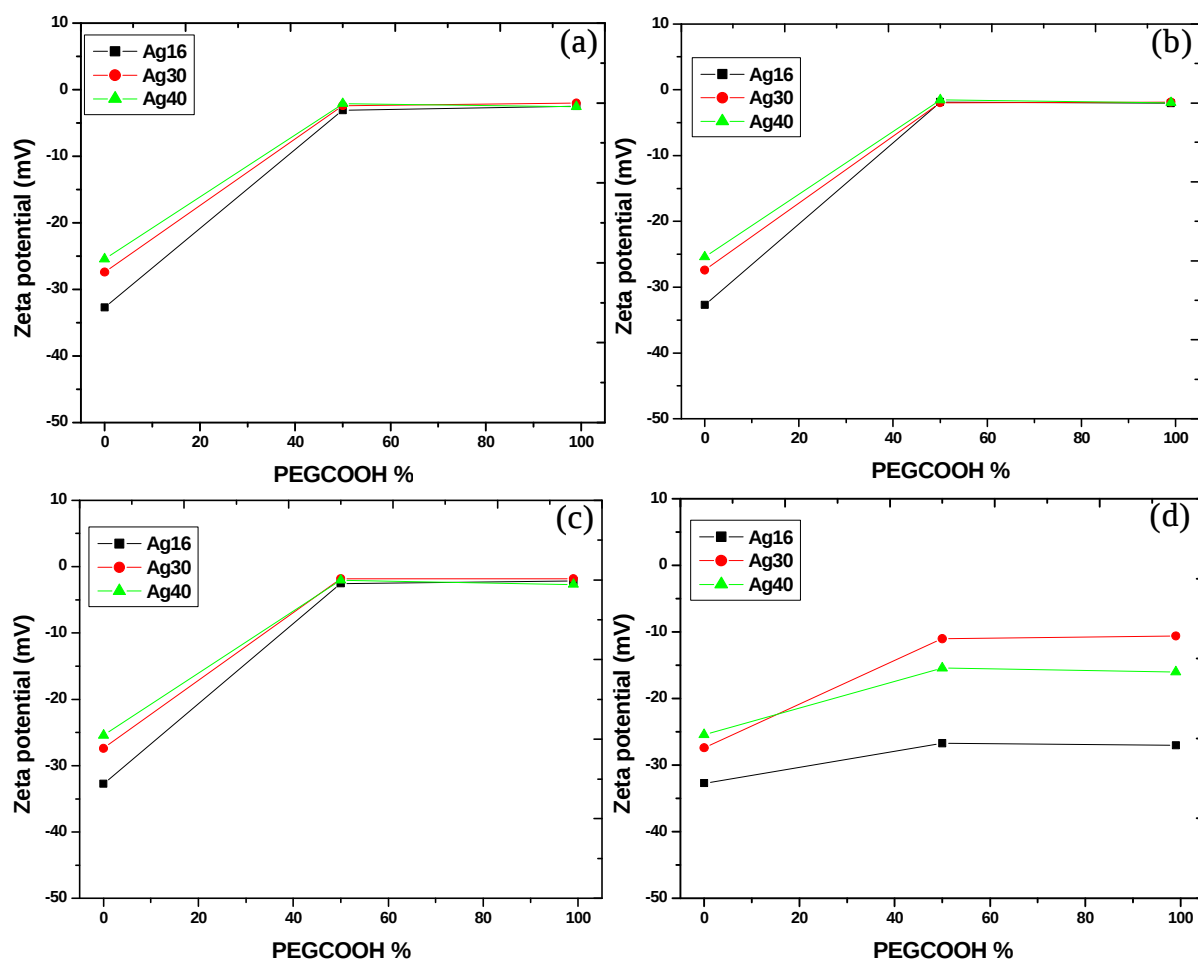


Figure 5.5: Zeta potential graphs of AgMMPCs of (a) HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole and (d) HS-(CH₂)₁₁-hydroquinone against the –COOH percentages

The theoretical spectra of different alkanethiols adsorbed on silver nanoparticle's surface were obtained by using the Gaussian 03 program and were correlated to experimental Raman spectra. The optimised geometries of four different alkanethiols adsorbed on silver nanoparticle were used to obtain the theoretical Raman spectra and are shown in Figure 5.6. The adsorption of Ag-S-(CH₂)₁₁-NHCO-coumarin is represented by Figure 5.6 (a), Ag-S-(CH₂)₁₁-triphenylimidazole in (b), Ag-HS-(CH₂)₁₁-indole by (c) and Ag-S-(CH₂)₁₁-hydroquinone (d). The optimised geometries of free or unmodified alkanethiols are shown in the supplementary data as Figure S3.19.

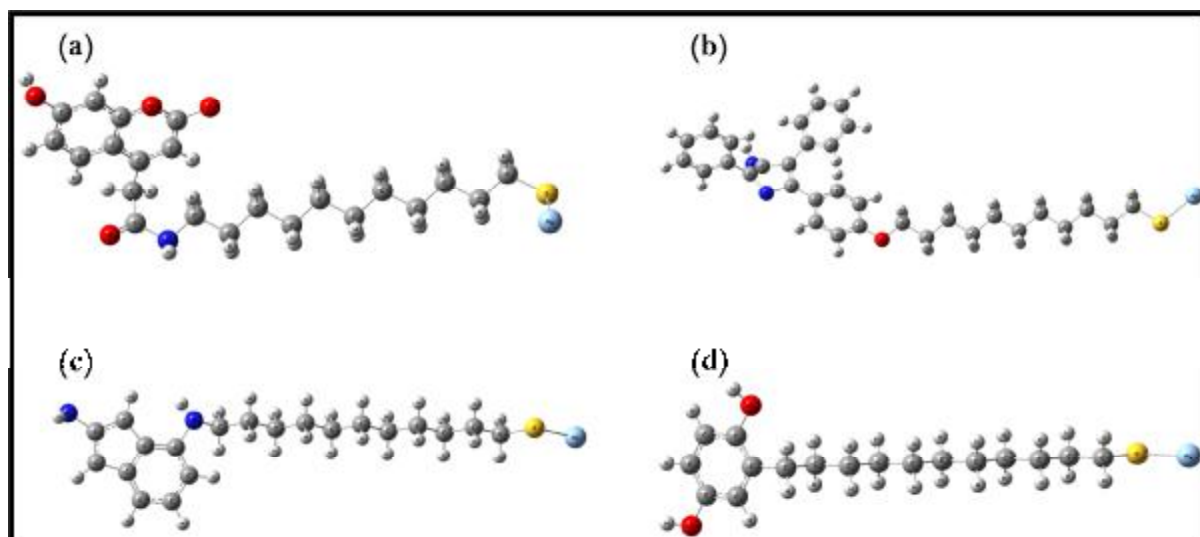


Figure 5.6: Optimized geometries of (a) Ag-S-(CH₂)₁₁-NHCO-coumarin, (b) Ag-S-(CH₂)₁₁-triphenylimidazole, (c) Ag-S-(CH₂)₁₁-indole and (d) Ag-S-(CH₂)₁₁-hydroquinone

The theoretical spectra of for alkanethiols adsorbed on silver nanoparticle's surface with their corresponding theoretical spectra of free alkanethiols are shown in Figure 6.7 {(a) present the calculated HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole and (d) HS-(CH₂)₁₁-hydroquinone}. The calculated Ag-alkanethiol spectra are presented as (ii) in Figure 6.7 (a) to (d). All the theoretical spectra showed the disappearing of ν (-S-H) vibrations observed in the region $\sim 2600\text{ cm}^{-1}$ upon the interaction with silver surface. This result in the new vibrational peak seen in the region of 200 cm^{-1} associated with ν (Ag-S).

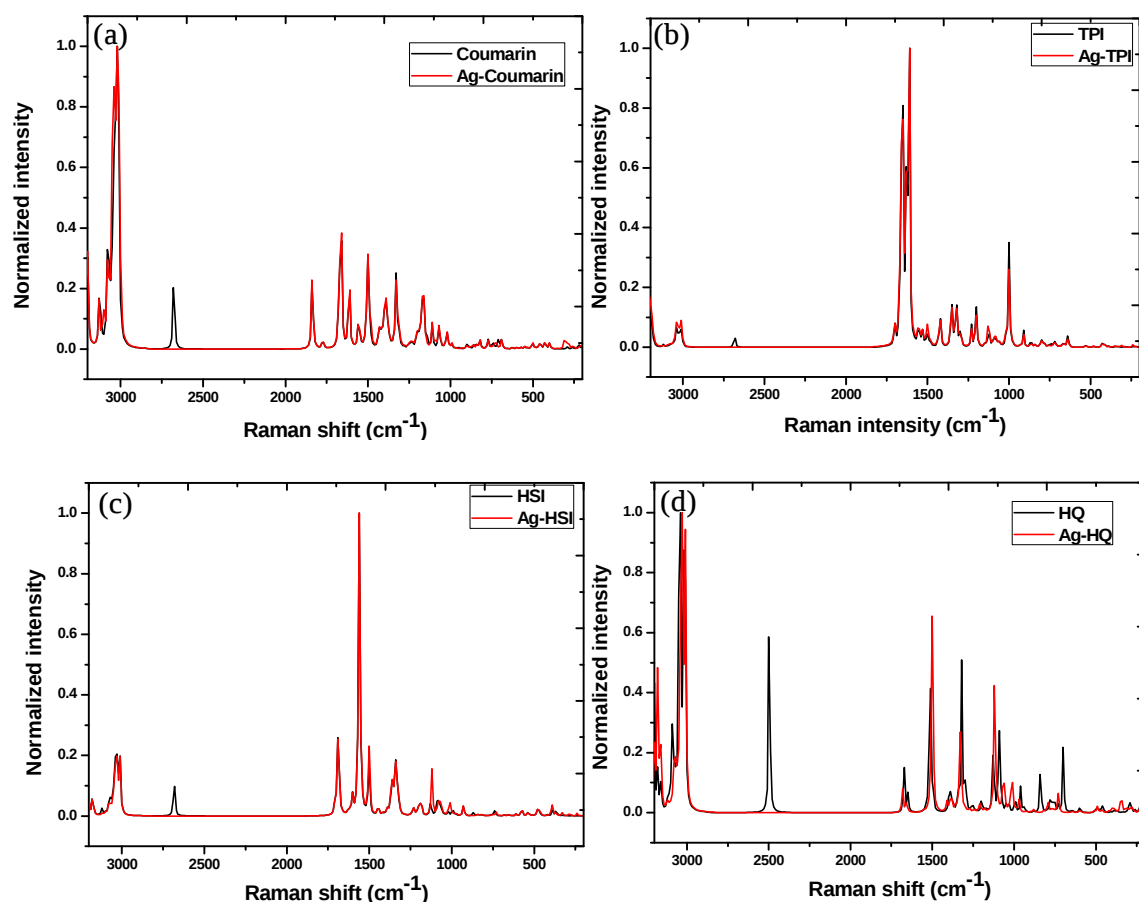


Figure 5.7: DFT calculated Raman spectra of AgMMPCs of (a) HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole and (d) HS-(CH₂)₁₁-hydroquinone.

The summary of all AgMMPCs Raman peaks prepared from the four Raman active compounds are shown in the supplementary data as Table S 5.1 to S5.4. The SERS spectra of AgMMPCs prepared from different stoichiometric ratios of HS-(CH₂)₁₁-NHCO-coumarin in three different sizes of AgNPs are shown in Figure 5.8, { (a) 16 nm, (b) 30 nm and (c) 40 nm}. In Figure 5.8 (a) to (c), (i) represents free- HS-(CH₂)₁₁-NHCO-coumarin, while (ii) and (iii) are the spectra of the AgNPs with different stoichiometric ratios of HS-(CH₂)₁₁-NHCO-coumarin (1% and 50%) respectively. In all spectra, the disappearing of the S-H vibrations at 2570 and 2590 cm⁻¹ and the emergence of an enhanced peak at 234 cm⁻¹

(assigned to an Ag-S vibration) is confirmed. This reveals the interaction of HS-(CH₂)₁₁-NHCO-coumarin through the thiol end. This assignment of ν (Ag-S) at 234 cm⁻¹ is in agreement with a previously reported peak found at 235 cm⁻¹ [13].

Another important feature observed was the weakening intensity of ν (C-S) at 694 cm⁻¹ which was found to be red-shifted to 680 cm⁻¹. The weakening of ν (C-S) can be explained by the increasing double bond character of ν (C-S) which resulted in strong vibrational peaks observed between 1000 and 1200 cm⁻¹ due ν (C=S). The SERS spectra of all AgMMPCs also displayed a strong enhancement of the symmetric and asymmetric vibrational bands of ν (C-H) in the region of 2900 to 3000 cm⁻¹ (*) and 1400 to 1500 cm⁻¹ (#), respectively.

A summary of selected vibrational peaks is shown in Table S5.1. SERS spectra of AgMMPCs using different stoichiometric ratios of HS-(CH₂)₁₁-triphenylimidazole are shown in Figure 5.9. Similar features were observed as in the SERS spectra of HS-(CH₂)₁₁-NHCO-coumarin, except a stronger enhancement of the ν (C-H) of the symmetric stretch compared to the asymmetric stretching vibration of ν (C-H). The SERS spectra and the table of the selected vibrational peaks for AgMMPCs functionalized with different stoichiometric ratios using HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone are shown in the supplementary data, (Figures S5.19 and S5.20, Tables S5.3 and S5.4).

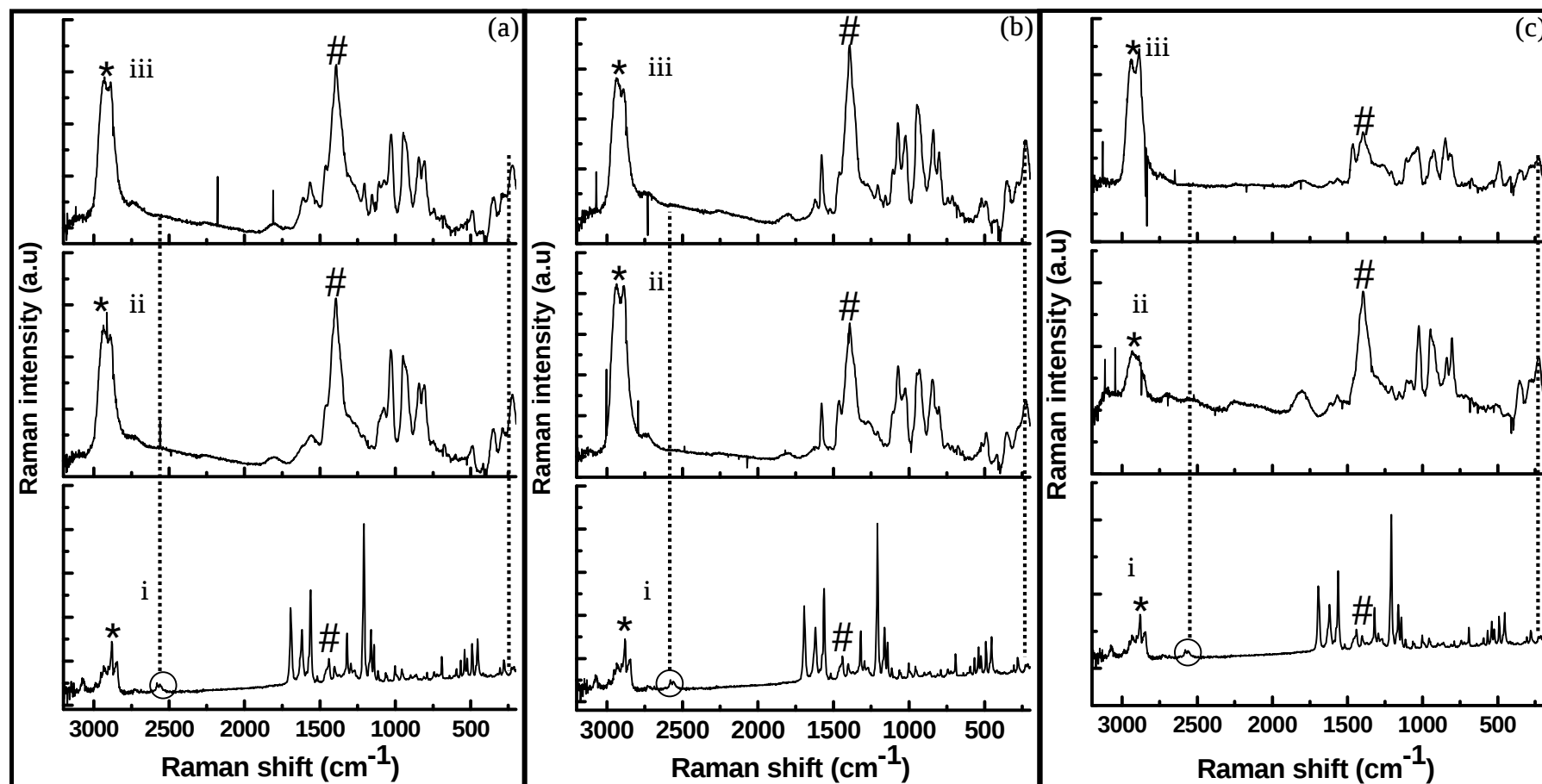


Figure 5.8: Raman spectra of AgMMPCs of different stoichiometric ratios of HS-(CH₂)₁₁-NHCO-coumarin prepared with different AgNPs sizes (a) 16 nm, (b) 30 nm, and (c) 40 nm. The circled peak is the vibrational band of S-H, (* and #) denotes the symmetric and asymmetric bands of C-H bonds.

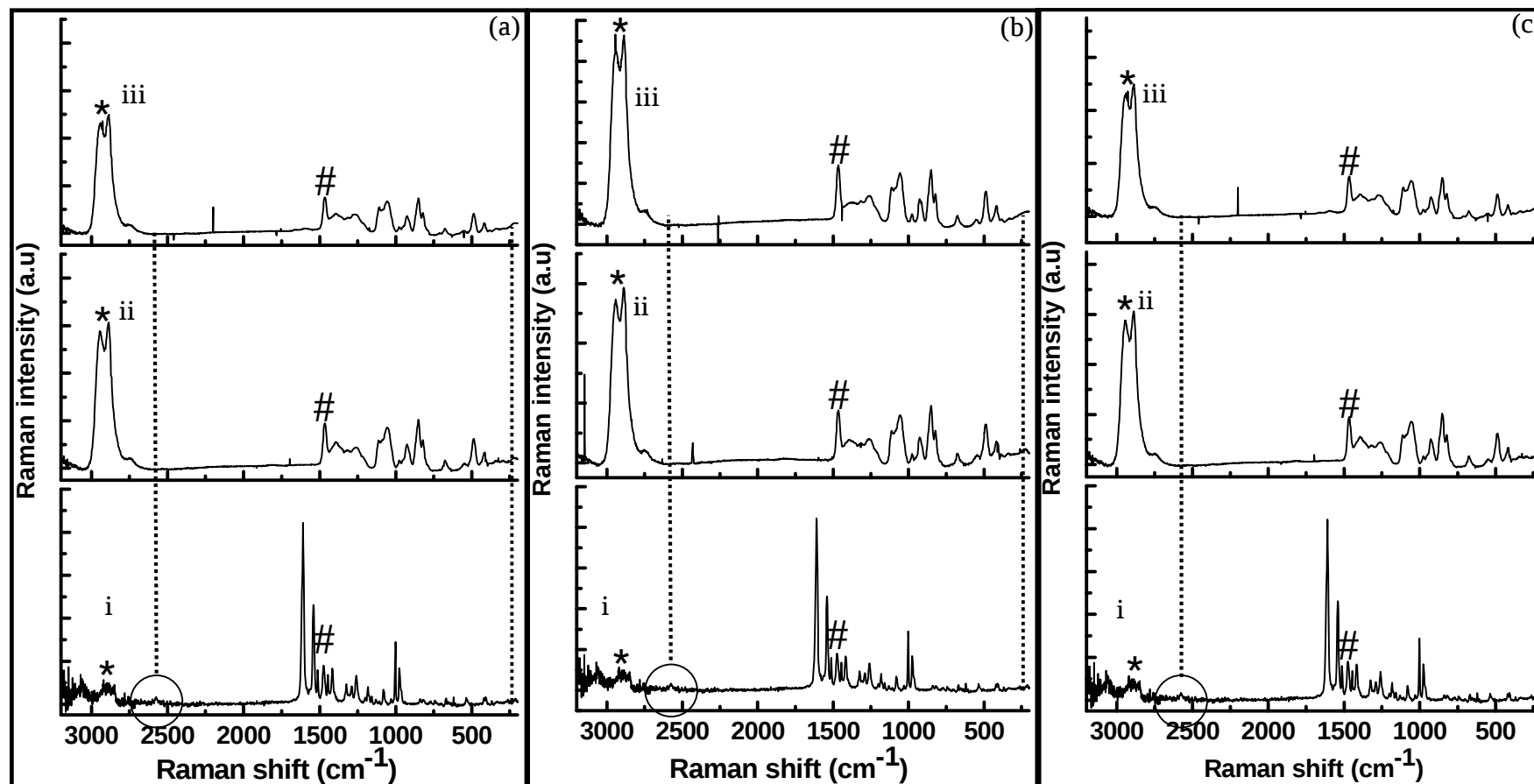


Figure 5.9: Raman spectra of AgMMPCs of different stoichiometric ratios of HS-(CH₂)₁₁-triphenylimidazole prepared with different AgNPs sizes (a) 16 nm, (b) 30 nm, and (c) 40 nm. The circled peak is the vibrational band of S-H, (* and #) denotes the symmetric and asymmetric bands of C-H bonds.

The enhancement factors of all the alkanethiols adsorbed on different AgNPs sizes were calculated using equation 4, shown in chapter three. The intensities of the C-H vibrations in the region 2900 - 3100 cm^{-1} were used to calculate the EF's. The bar graphs of the calculated EF's are shown in Figure 5.10, {(a) HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole and (d) HS-(CH₂)₁₁-hydroquinone}. The EF graphs are consistent with the improvement of the enhancement as higher stoichiometric ratios of alkanethiols were added. This is further supported by the calculated EF's which were higher for all 50% ratios compared to 1% ratios. A similar trend of decreasing EF values was observed on increasing the size of the AgNPs. These results were similar to the results obtained for AuNPs given in chapter three.

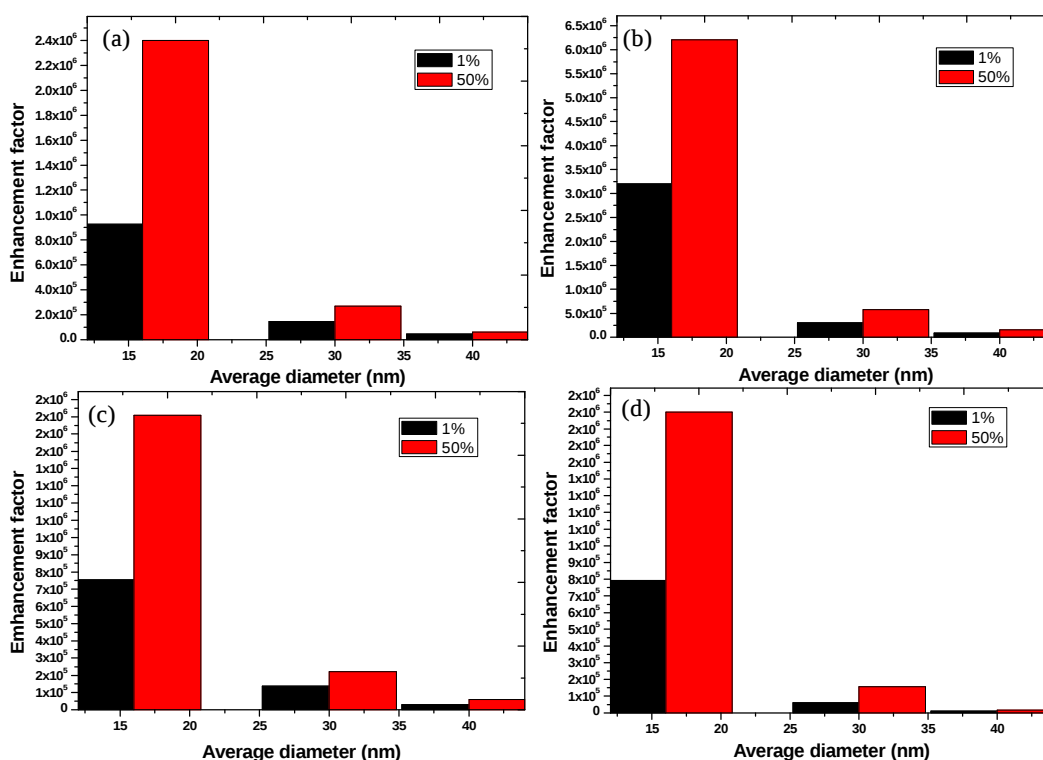


Figure 5.10: Bar graphs correlating the calculated EF's of all alkanethiols (a) HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole and (d) HS-(CH₂)₁₁-hydroquinone adsorbed on different AgNPs sizes

The calculated EF's of the alkanethiols adsorbed on 16 nm AgNPs was found to be in descending order: 6.2×10^6 for HS-(CH₂)₁₁-triphenylimidazole, 2.4×10^6 for HS-(CH₂)₁₁-NHCO-coumarin, 1.7×10^6 for HS-(CH₂)₁₁-hydroquinone, and 1.8×10^6 for HS-(CH₂)₁₁-indole. The high EF for alkanethiols with more phenyl rings show the promotion of electron delocalization, making the phenyl rings to more Raman active yielding to more intensity enhancement.

5.4 Conclusions

The highly stable AgMMPCs made from different AgNP sizes (16, 30 and 40 nm) functionalized with different stoichiometric ratios (1% and 50%) of alkanethiols (HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, HS-(CH₂)₁₁-hydroquinone) and HS-(CH₂)₁₁-PEG-COOH) were successfully prepared. The SPR band shift to higher wavelength as AgNP increased from 16 to 30 and 40 nm, was observed using UV-vis spectroscopy. TEM images revealed no aggregation; only the formation of self-assembled superlattices were observed and the stability confirmed by Zetasizer measurements. The Raman activities showed an improvement in the vibrational peak intensities as the stoichiometric ratios were increased from 1% to 50%. The SERS spectra of all the Raman reporters employed confirmed that the interaction was via the thiol-end. The calculated EF's revealed that HS-(CH₂)₁₁-triphenylimidazole was the best Raman reporter, followed by HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-hydroquinone and HS-(CH₂)₁₁-indole. The calculated EF's were observed to decrease with an increase in AgNP size.

5.5 References

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Chapter Six

Design and development of gold and silver nanoparticles SERS immunoassay for the detection of malaria and tuberculosis biomarkers

6.1 Introduction

The ever increasing rapid growth of nanotechnology and nanoscience has opened up novel opportunities in applications that were unimaginable in the past decades, ranging from improving energy, health to water challenges. Biomedical applications in particular have witnessed development in chemical sensing [1-3], and imaging [4,5] to cancer treatment [6,7] and targeted drug delivery [8,9]. The use of nanomaterials in biological applications takes an advantage of the association of biomolecules with the surface of nanomaterials. SERS has emerged as an ultra-sensitive vibrational technique for the detection of molecules on or close to the surface of a plasmonic nanostructure [10]. The detection of a single-molecule via SERS has attracted much attention for numerous applications [3, 11]. A particular interest has been focused on applications of molecular sensors operating via SERS for biochemical studies (selective and sensitive detection of proteins [12], clinical diagnosis [4,5] (imaging); and environmental monitoring [13]. SERS immunoassays based on antigen-antibody interaction have been reported for different biomarkers as shown in Chapter 2, Table 2.2. The successful application of SERS immunoassays requires SERS-labelled biomolecules (antigens and antibodies) with a high binding affinity to the corresponding target and the SERS-active probe. Although many biomarkers have been reported for most diseases perceived as a world health threat, less work has focused on using SERS-based immunoassays for life-threatening diseases like malaria and tuberculosis, which are more prevalent to developing countries. Malaria, TB and HIV/AIDS form the major public health challenges of the developing world. Therefore any attempt to early

diagnosis of these diseases can never be overstated. According to the World Health Organization (WHO) report [14], malaria is caused by five species of parasite that affect humans, and all of these species belong to the genus *Plasmodium*; *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. Amongst these, *P. falciparum* and *P. vivax* are the most prevalent ones [14]. TB is an infectious disease caused by the *Mycobacterium tuberculosis* [15].

The use of nanoparticles in biological applications requires the conjugation of bio-moieties to enhance their properties and to make them biocompatible. A range of bio-moieties can be conjugated to nanoparticles i.e.; low molecular weight ligands (folic acid, thiamine, dimercaptosuccinic acid), peptides (synthetic and natural), proteins (antibodies, transferrin, BSA, cytokines, lectins, thrombin, fibrinogen), polysaccharides, polyunsaturated fatty acids, DNA, plasmids, and siRNA [16]. Although most of these bio-moieties show proven selectivity and ability of crossing biological membranes when conjugated to nanomaterials, antibodies are mostly preferred due to some added advantages including;

- Ø specific immunogenicity
- Ø less susceptibility to enzymatic degradation
- Ø improved cellular uptake and stability

6.2 Structure of antibodies and their function

Antibodies (immunoglobins) are host proteins that are produced by the immune system in response to foreign molecules that enter the body. These foreign molecules are antigens and their recognition by an immune system stimulates the selective secretion of antibodies that specifically bind them. The antibody is made up of one or more glycoproteins, with a Y-shaped structure (Figure 6.1) of bifunctional molecules with two identical domains for

antigen recognition (Fab fragment) and two identical domains with effector functions (Fc fragment).

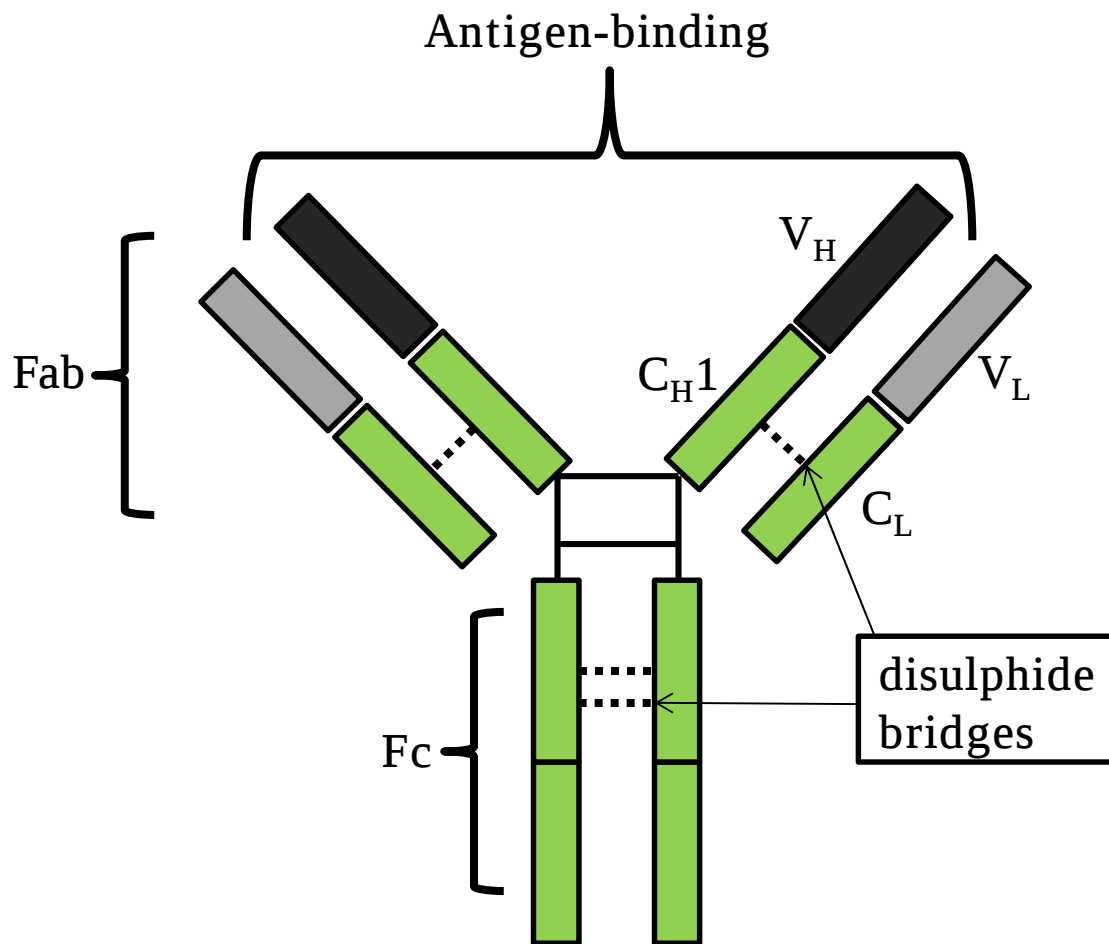


Figure 6.1: General structure of an immunoglobulin [17]

The highly specific antigen-binding region varies among the antibodies. Antibodies have two identical light chains of 24-25 kD (κ or λ), and two identical heavy chains of 55-70 kD (γ , δ , α , μ , or ϵ) bound by disulphide bridges [18-21]. The type of immunoglobulin produced depends on the heavy chains, and vertebrate animals have five classes of immunoglobins (IgG, IgE, IgD, IgA and IgM) each with a distinct functionality. The immunoglobins also possess intracatenary disulphide bridges that provide stability [22]. The light chain (L) is made up of two domains of approximately 100 residues known as variable domain (V_L) at its amino terminal, and the constant domain (C_L). The heavy chain (H) consist of a variable

domain (V_L) and three to four constant domains (C_{H1} , C_{H2} , C_{H3} , C_{H4}), depending on the class of immunoglobulin [23]. The V regions of H and L chains comprise of the antigen-binding sites of immunoglobulin molecules [24,25].

6.2 Experimental details

6.2.1 Materials and Chemicals

Recombinant *Plasmodium Vivax Lactate Dehydrogenase* (Pv-LDH) and *mycobacterium tuberculosis* 38 kDA, were purchased from CTK Biotech (USA), *monoclonal anti-Plasmodium Lactate Dehydrogenase* (anti-pLDH) pan-malaria, and *monoclonal anti-pLDH vivax-specific* clone obtained were from Vista Diagnostics International (USA). The polyclonal anti-Protein A from rabbit, Protein A, glycerol (molecular biology, $\geq 99\%$), phosphate-buffered saline (PBS) sachets and Bovine Serum Albumin (BSA), were all purchased from Sigma Aldrich (South Africa). The nitrocellulose Whatman AE 98 fast membranes were purchased from Diagnostic Consulting Network (DCN USA). All other chemical used were obtained as given in chapter 3

6.2.2 Instrumentation

The ESE Quanti reader lateral flow studio was used to determine the lines' intensities and designed to do the analysis of test strips with dimension of 5 x 100 mm (b x l). The Biodot XYZ Series dispensing system was used to immobilise proteins at a rate of 1 μ L/cm and the conjugate at 10 μ L/cm. All other characterization techniques are given in chapter 3.

6.2.3 Synthesis and conjugation of AuNPs and AgNPs

The synthetic procedure followed is outlined in chapters three and five, for AuNPs (14 nm) and AgNPs (16 nm), respectively. The pH of both AuNPs and AgNPs was adjusted to be between 7 to 8 before adding 100 μ L of monoclonal anti-pLDH pan-malaria and or Protein A. The mixture was swirled for 30 minutes before 100 μ L of each Raman active alkanethiol,

(HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, HS-(CH₂)₁₁-hydroquinone) was introduced. Swirling was then continued at room temperature for an hour. The resultant conjugates were purified through centrifugation (12000 rpm, 10 min and 10°C).

6.2.4 Preparation of immunochromatographic strips

Different concentrations (0.5; 1.0 and 1.5 mg/mL) of recombinant Pv-LDH and mycobacterium tuberculosis 38 kDA in phosphate buffered saline (PBS) were used as the capture antigen on the immunochromatographic tests nitrocellulose membranes. The different concentrations of antigens were dispensed onto the nitrocellulose membranes as the test lines using the Biodot XYZ Series dispensing system. After drying the membranes for 30 minutes at 37 °C, they were blocked with a membrane blocking buffer (MBB) that consisted of 1% sucrose, 1% BSA, 2.5% PVP and 0.1 M NaH₂PO₄. The membranes were then dried for another 30 minutes at 37°C. Drops of AuNPs and AgNPs with all alkanethiols were added at the tip of the immunochromatographic strip and allowed to migrate across the membranes.

6.2.5 Coating of substrates with gold film

In order prevent competitive binding that could be induced on the substrate for SERS-based immunoassay, gold film which facilitate an easy attachment of biomarkers and can be blocked by BSA was coated. Different substrates (glass slide, silicon wafer, aluminium foil and steel (Raman holders) were coated with 100 nm gold film. The substrates were placed inside the vacuum chamber, and flushed with (20 kPa) argon for 5 seconds. The sputter coater was set to 150 seconds, and the current used was 40 mA. After flushing with argon, the vacuum pressure of 10⁻³ was restored by leaking some of the gas in the chamber. The coating was noted after a cloud of gold atoms was sputtered onto the substrates.

6.2.6 Preparation of SERS-based immunoassays

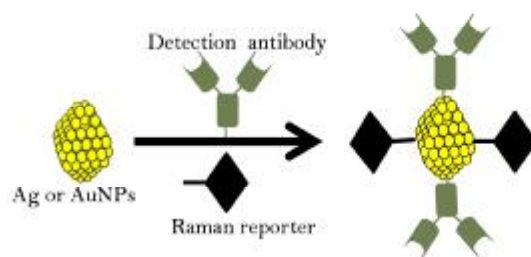
The preparation steps followed on developing the SERS-based immunoassay are shown in Figure 6.2. On the gold-coated steel substrates, 10 μL of monoclonal anti-pLDH vivax-specific clone and anti-Protein A antibodies were immobilised, then dried for 30 minutes at 37 °C. After the antibodies were immobilised, the nonspecific binding sites were blocked using 10 % BSA, and dried for 30 minutes at 37 °C. After blocking, 10 μL of recombinant Pv-LDH and or mycobacterium tuberculosis 38 kDA (1 mg/mL) were added, followed by the incubation for 30 minutes at 37 °C. After incubation, AuNP and AgNP conjugates of different alkanethiols were added, and dried for 30 minutes at 37 °C. The unbound conjugates were washed off with 0.1 M PBS buffer.

6.3 Results and discussion

This project is part of an ongoing research project to use metal nanoparticles based SERS immunoassay using Raman reporters which are fundamental probes to enable the development of a system that has uniform and strongly enhanced signals. In this regard, our focus was on evaluating the sensitivity of four different alkanethiols as Raman reporters for the detection of *recombinant Pv-LDH* (malaria) and *mycobacterium tuberculosis 38 kDA* (tuberculosis). The steps followed for the development of SERS-based immunoassay are shown in Figure 6.2. AuNPs and AgNPs of small sizes (14 and 16 nm) were used in the development. These sizes were selected because they have revealed high enhancement factors with Raman active alkanethiols as discussed in chapters three and five. The 50% stoichiometric ratios were also preferred in the development of the immunoassays as improvement on the Raman peaks and an increase in the EF's were observed in the previous chapters when compared to 1% stoichiometric ratios. The conjugation of the antibodies was carried out using the direct or electrostatic attachment on the surface of metal nanoparticles. The reaction was performed using mild conditions followed by the

adsorption of the alkanethiols, as shown Figure 6.2 (A). The immobilization of a capture antibody is shown in Figure 6.2 (B). A range of substrates were investigated, including gold-coated glass slide, gold-coated silicon wafer, gold-coated aluminium foil and the gold-coated steel. The images of these substrates are shown in the supplementary data, Figure S6.1. As shown in Figure 6.2 (B), a gold-coated steel substrate was preferred over the others. The silicon wafer and aluminium foil substrates were not used because of their delicate nature. The peeling of the gold film was observed during incubation and the glass slide substrate was not used to avoid the interference of silicate vibrational bands. To prevent competitive binding that could be induced by the active substrate of the SERS-based immunoassay gold-coated steel, the substrate was blocked with BSA, followed by the addition of the antigen, and washing off of the non-binding antigen with PBS {Figure 6.2 (C)}. Prior to the addition of the conjugates, an immunochromatographic test was performed. The general principle of an immunochromatographic test strip with a control and test line is shown in Figure 6.3(A).

A. Target conjugate preparation



B. Capture substrate preparation



C. Assay procedure

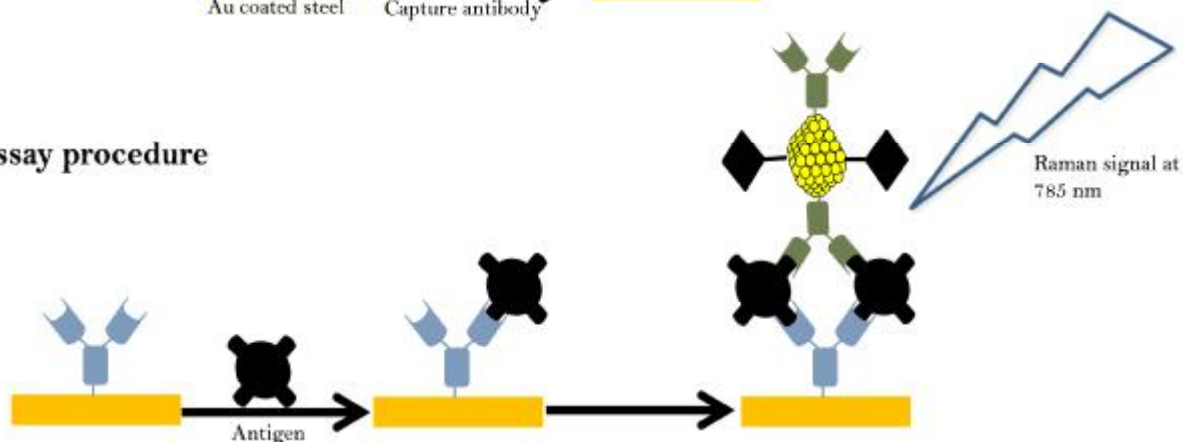


Figure 6.2: Schematic design of SERS-based immunoassays

The analyte of interest, in the sample to be tested, binds to the detection conjugate and the complex migrates along the membrane to the test line, where a capture protein (usually an antigen) is immobilized, shown in Figure 6.3(B). The analyte, coupled to the detection conjugate, binds to the antigen at the test line and results in the display of a coloured line as observed in Figure 6.3(C). The antibody therefore serves as an anchor that links the detection conjugate to the capture antigen on the test line

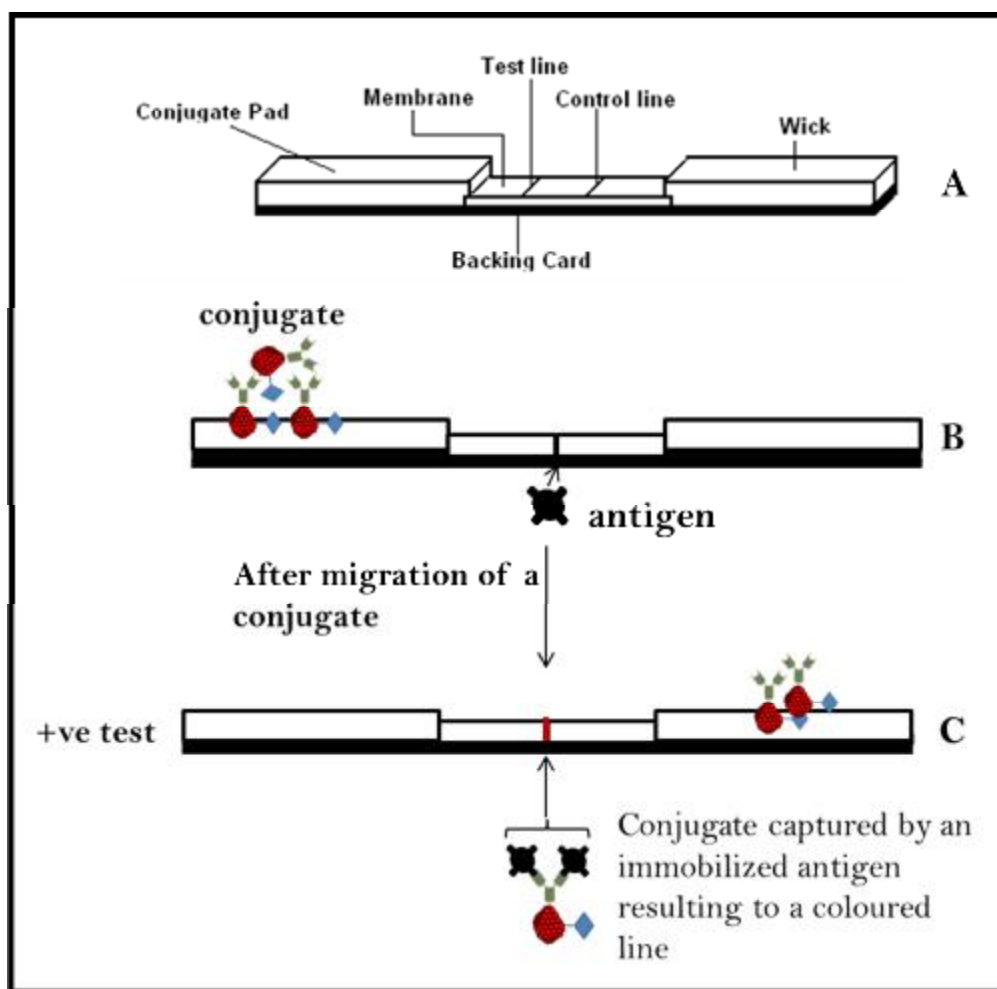


Figure 6.3: Diagram showing (A) the standard immunochromatographic test, (B) the process followed on testing the interaction of prepared conjugates and an antigen, and (C) resulting coloured line after the capture by an immobilized antigen.

After the confirmation of antigen-antibody interaction on an immunochromatographic test, the conjugates were added on the gold-coated steel substrate with antigen and analysed using Raman spectroscopy.

6.3.1 Malaria detection (*Plasmodium vivax*)

The immunochromatographic test results of PAN-conjugated AuNPs and AgNPs with 50% stoichiometric ratios of all Raman active alkanethiols i.e.; HS-(CH₂)₁₁-NHCO-coumarin(C),

HS-(CH₂)₁₁-triphenylimidazole (TPI), HS-(CH₂)₁₁-indole (HSI) and HS-(CH₂)₁₁-hydroquinone (HQ) are shown in Figure 6.4 and 6.5, respectively.

The rows from top to bottom represent different concentration of recombinant Pv-LDH antigen immobilised on the test strips, 0.5; 1.0 and 1.5 mg/ml. When the analyte of interest is present in the tested sample, the detection conjugate will bind to the analyte; this will lead to the formation of a coloured line from the antigen-antibody complex. In both Figures 6.4 and 6.5, an increase in antigen concentration from 0.5 to 1.0 mg/ml revealed a clearer and more intense line. An increase to 1.5 mg/ml showed no distinct difference compared to 1.0 mg/ml sample.

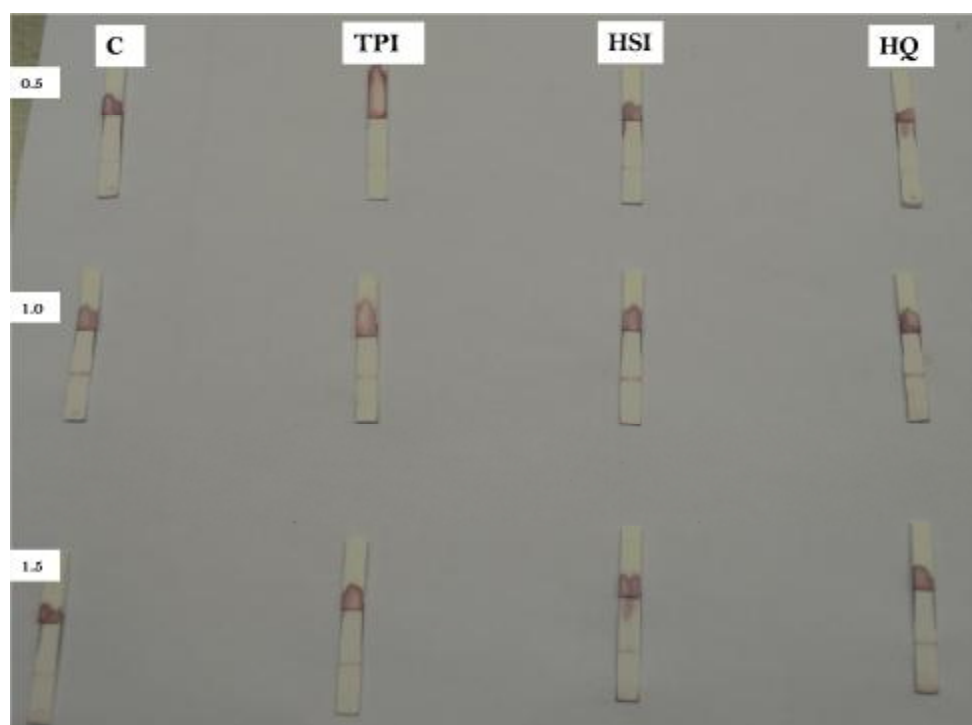


Figure 6.4: Images of the test strips showing the effect of the antigen concentration on the intensity of the test control lines using AuNPs.

An increase of line intensity from 0.5 to 1.0 mg/ml, with an increase of an antigen concentration was confirmed by the strip-reader. The graphs from the strip-reader of

AuNPs and AgNPs conjugates are shown in the supplementary data as Figures S6.2 and S6.3, respectively.

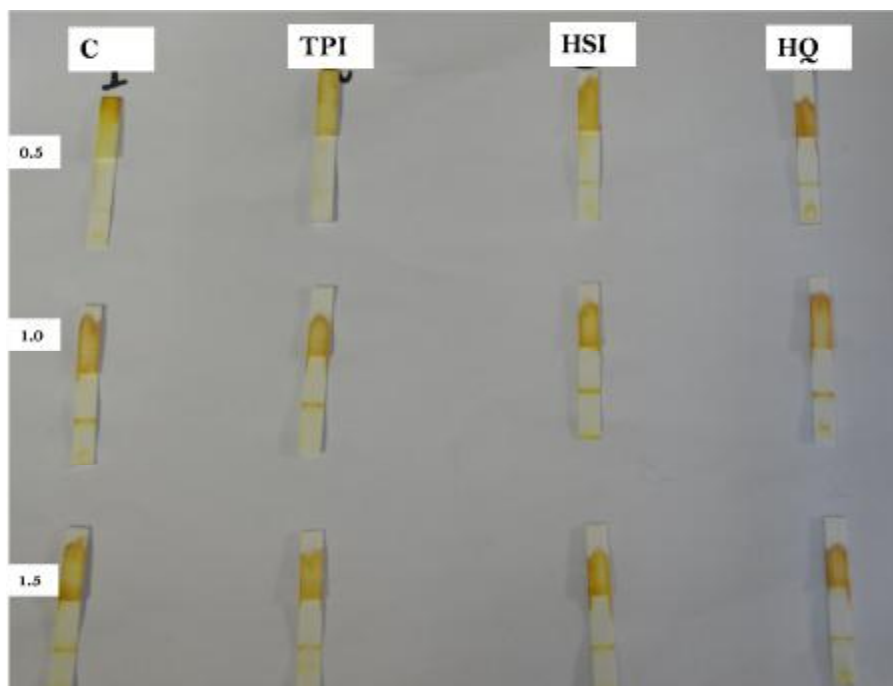


Figure 6.5: Images of the test strips showing the effect of the antigen concentration on the intensity of the test control lines using AgNPs.

In Figures S6.2 and S6.3, the 1.0 mg/ml plot showed higher intensities for most of Raman active alkanethiols, and this concentration was chosen to be immobilised on gold-coated steel substrate.

The optical stability of Au and Ag-conjugates were evaluated using flocculation test assay of varying NaCl concentrations. The SPR bands were measured using UV-vis spectroscopy. The SPR bands of AuNP PAN-conjugates with different alkanethiols are shown in Figure 6.6. The unmodified or unconjugated AuNPs under various electrolytic concentrations (NaCl) are presented in Figure 6.6 (a). An increase in NaCl concentration from 0.2 M to 1.0 M revealed a shift of an SPR bands from 512 nm to 521 nm showing signs of instability or agglomeration at these various concentrations, due to a change in

ionic strength induced by these NaCl concentrations. AuNP PAN-conjugates with four different alkanethiols are shown in Figure 6.6 (b) to (e) the conjugates showed stability during the flocculation test, this was an indication that the conjugation was successful on metal nanoparticles, the conjugate were stable to be used as Raman probes.

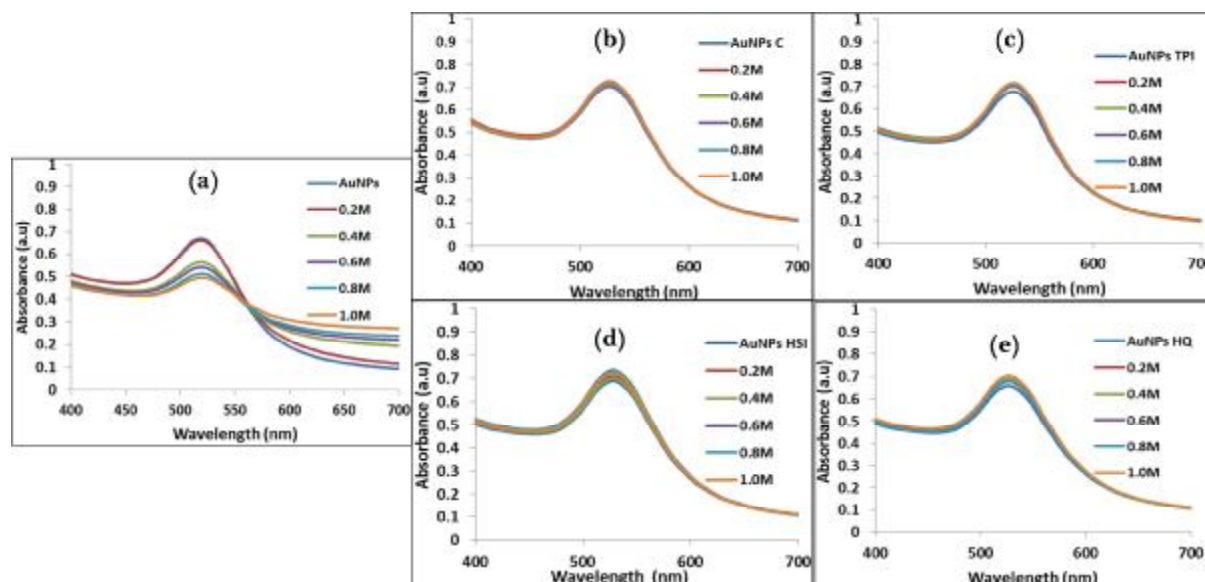


Figure 6.6: SPR measurements under different concentrations of NaCl of (a) un conjugated AuNPs, (b) to (e) AuNPs PAN-conjugates with different alkanethiols (b) HS-(CH₂)₁₁-NHCO-coumarin, (c) HS-(CH₂)₁₁-triphenylimidazole, (d) HS-(CH₂)₁₁-indole, and (e) HS-(CH₂)₁₁-hydroquinone

All spectra (Figure 6.6 (b) to (e)) revealed no shift of the SPR band as the concentration of NaCl was increased from 0.2 to 1.0 M. The stability of these AuNPs PAN-conjugates was also confirmed with a flocculation test shown in Figure 6.8 (a). The change in colour from wine red to blue was only observed for the un conjugated AuNPs. All AuNP PAN-conjugates remain wine red during the flocculation studies with different concentrations of NaCl. The SPR bands of AgNPs shown in Figure 6.7 (a) revealed a similar shift of the SPR band (411 nm) as observed for the AuNPs. The AgNP PAN-conjugates shown in Figures 6.7 (b) to (d) also revealed no signs of instability as no SPR shifts were observed.

This was also confirmed by a lack of colour change as the concentration of NaCl was increased 10 fold, as shown in Figure 6.8 (b).

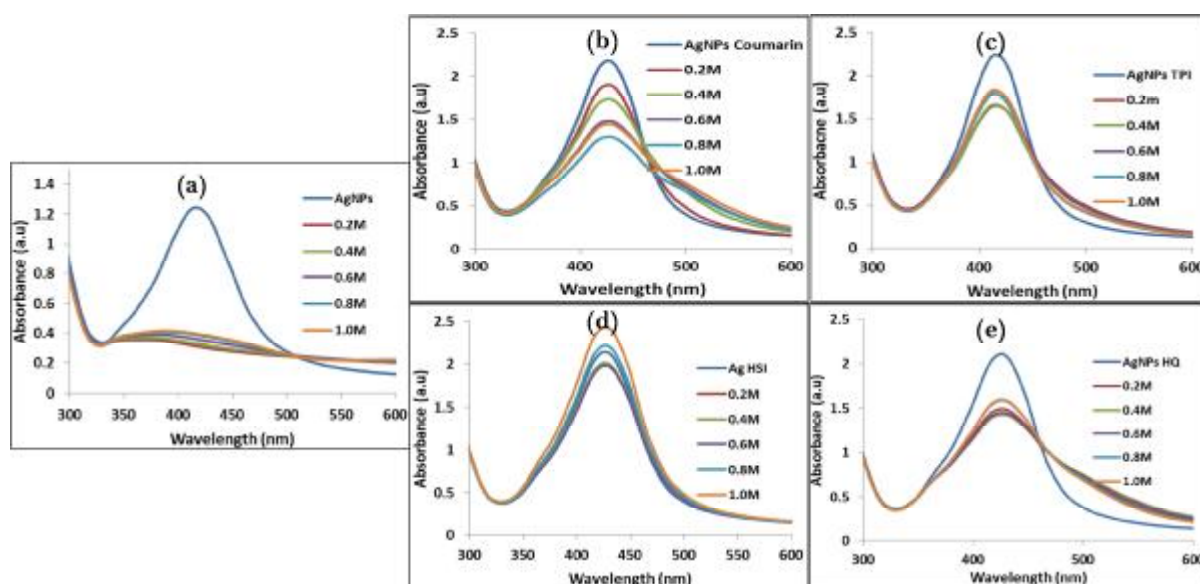


Figure 6.7: SPR measurements under different concentrations of NaCl of (a) unconjugated AgNPs, (b) to (e) AgNPs PAN-conjugates with different alkanethiols (b) HS-(CH₂)₁₁-NHCO-coumarin, (c) HS-(CH₂)₁₁-triphenylimidazole, (d) HS-(CH₂)₁₁-indole, and (e) HS-(CH₂)₁₁-hydroquinone

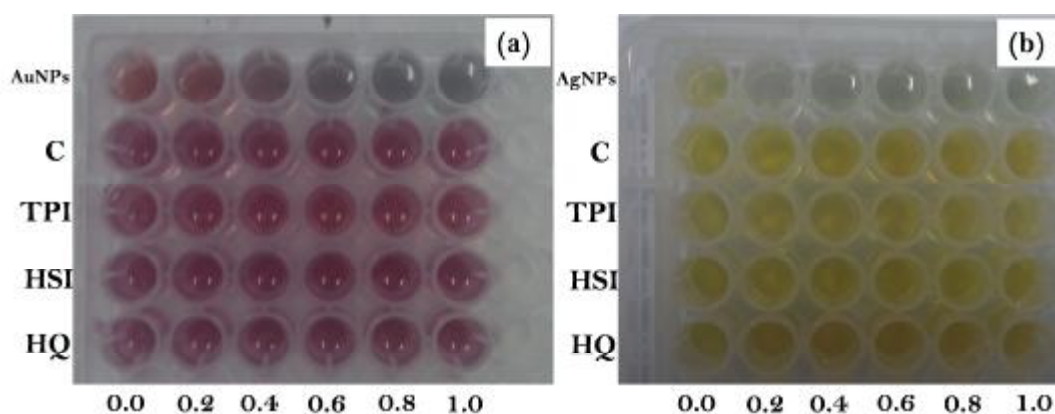


Figure 6.8: Images for flocculation of (a) AuNPs PAN-conjugates and (b) AgNPs PAN-conjugates under different concentrations of NaCl.

The TEM images of AuNP PAN-conjugates with different alkanethiols also revealed no aggregation as shown in Figure 6.9. The conjugates were found to be well dispersed, and no formation of self-assembled superlattices was observed.

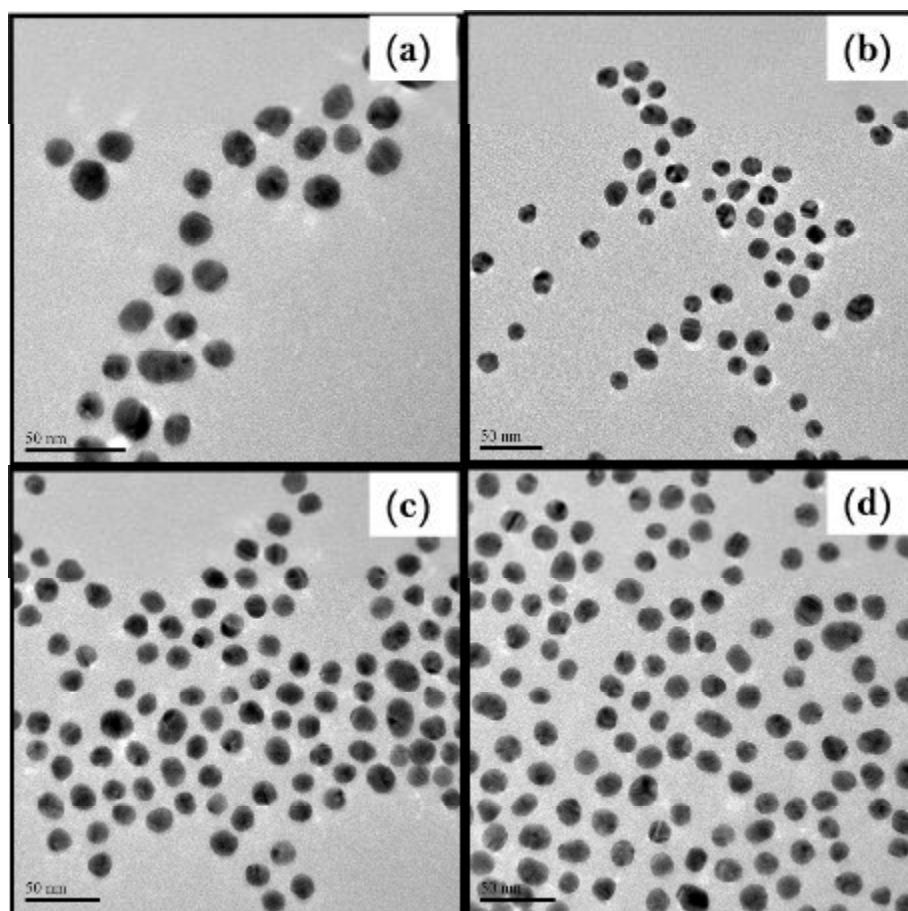


Figure 6.9: TEM images of AuNPs PAN-conjugates with alkanethiols (a) HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole, and (d) HS-(CH₂)₁₁-hydroquinone

The TEM images of AgNP PAN-conjugates with four alkanethiols are shown in Figure 6.10, (a) HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole, and (d) HS-(CH₂)₁₁-hydroquinone. The presence of rod-like nanoparticles was observed due to a steady growth rate at smaller volume of the reducing agent as discussed

in chapter 5. The images show no sign of aggregation, revealing the good capping ability of the alkanethiols.

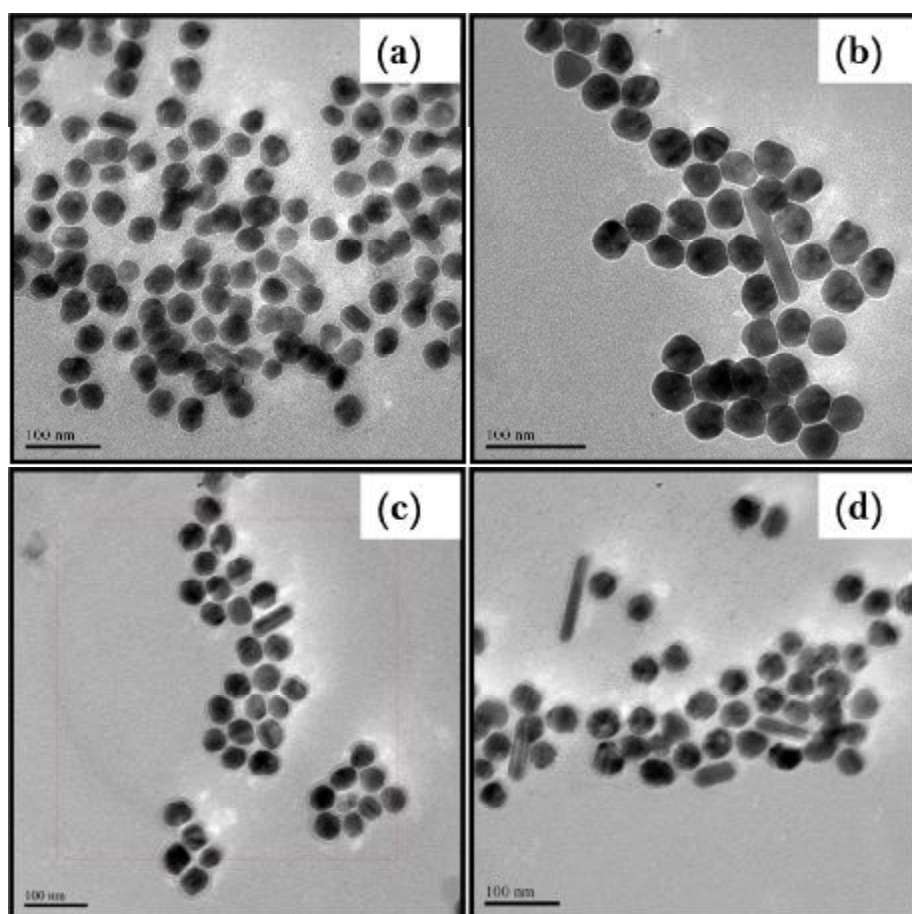


Figure 6.10: TEM images of AgNPs PAN-conjugates with alkanethiols (a) HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole, and (d) HS-(CH₂)₁₁-hydroquinone

Gold-coated steel substrates were chosen as suitable scaffolds to allow nanomaterial bioconjugates to interact with the analyte of choice, and the interaction was evaluated using Raman spectroscopy. SERS spectra of AuNPs PAN-conjugate with HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole are shown in Figure 6.11 (a), and (b), respectively. In Figure 6.11 (a) and (b) Raman spectra are presented, of (i) unmodified alkanethiols, (ii) free gold-coated steel substrates, (iii) gold-coated steel with anti-PLDH

antibody, (iv) the capturing of Pv-LDH antigen, and (v) the conjugate of the two alkanethiols as (v). The SERS spectra of the interaction of the conjugate of HS-(CH₂)₁₁-NHCO-coumarin, and HS-(CH₂)₁₁-triphenylimidazole with an antigen captured by the PLDH antibody are presented as (vi) in Figure 6.11 (a) and (b), respectively. Both SERS spectra of HS-(CH₂)₁₁-NHCO-coumarin, and HS-(CH₂)₁₁-triphenylimidazole revealed similar vibrational peaks compared to their unmodified Raman spectra in the region between 1100 cm⁻¹ to 1600 cm⁻¹. The region is dominated by $\nu(\text{CC})$ aromatic ring peaks at 1568 cm⁻¹, and the asymmetric $\delta(\text{CH}_2)$ vibrational bands observed at 1452 and 1394 cm⁻¹ for the HS-(CH₂)₁₁-NHCO-coumarin based immunoassay. Similar vibrational bands were observed for the HS-(CH₂)₁₁-triphenylimidazole based SERS immunoassay, with the $\nu(\text{CC})$ aromatic ring peak at 1600 cm⁻¹ region, and asymmetric $\delta(\text{CH}_2)$ bands at 1400 cm⁻¹. The presence of these peaks in Figure 6.11 (a) and (b) is the confirmation of the interaction between an antibody and the antigen, as these peaks are not observed in Figure 6.11 (iv) for both (a) and (b). The SERS spectra of HS-(CH₂)₁₁-indole, and HS-(CH₂)₁₁-hydroquinone are shown in the supplementary data as Figure S6.4 (a) and (b). Both SERS spectra in Figure S6.4 (a) and (b) showed similar features of retaining the vibrational bands in the region of 1100 to 1600 cm⁻¹. The difference noticed was the relative intensities of these bands, and smaller peaks were observed compared to the SERS spectra of HS-(CH₂)₁₁-NHCO-coumarin, and HS-(CH₂)₁₁-triphenylimidazole. SERS spectra of all four alkanethiols also showed the vibrational bands of $\nu(\text{CC})$ aliphatic between 800 and 1100 cm⁻¹ region. The SERS spectra of all alkanethiols also showed the enhancement of the vibrational peaks in the region of about ~ 1020 cm⁻¹. The difference in relative intensities and the enhancement of $\nu(\text{CC})$ aliphatic chain vibration is associated with the alkanethiol and the concentration of an antigen used.

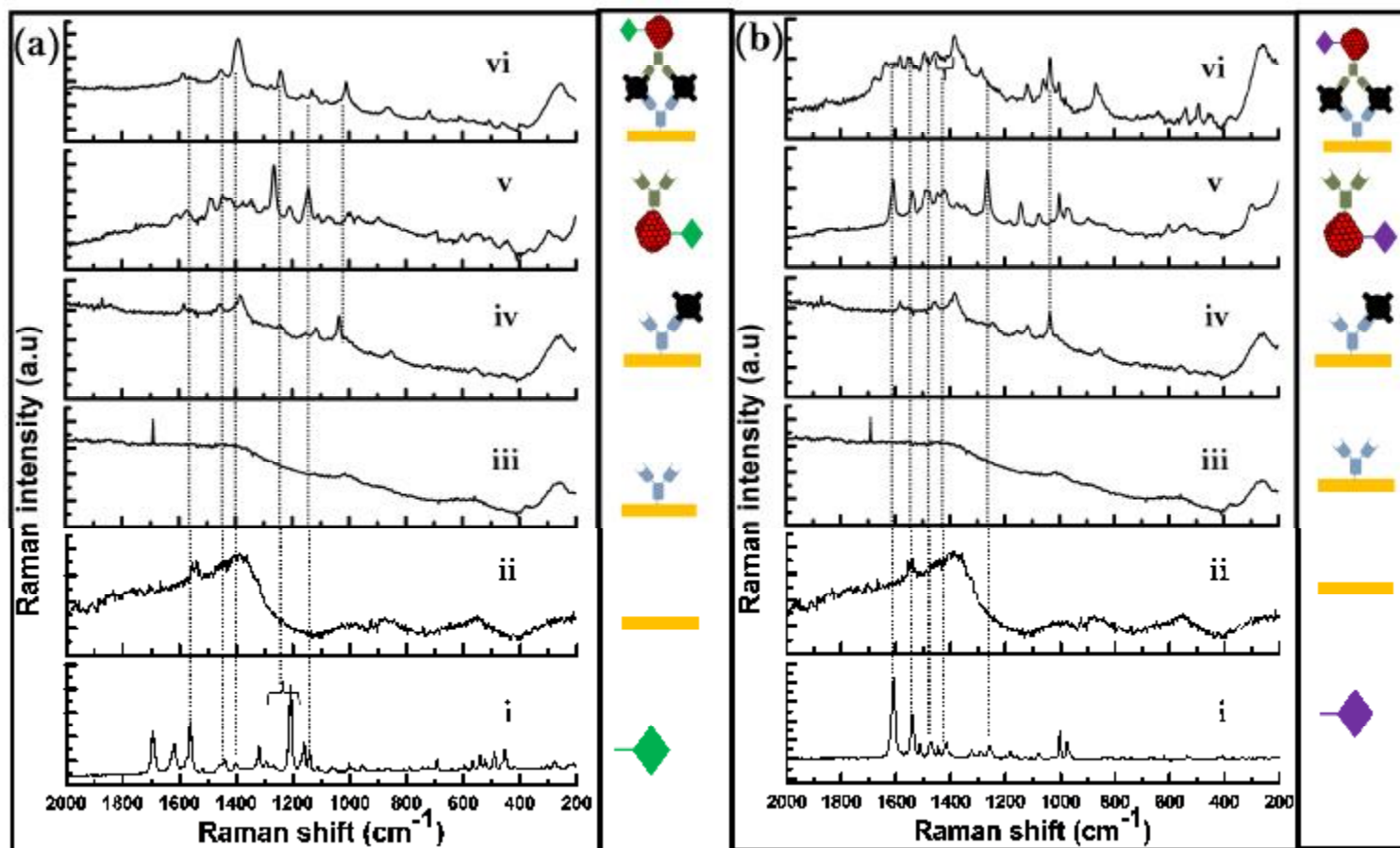


Figure 6.11: SERS immunoassay spectra, (a) AuNPs PAN-HS-(CH₂)₁₁-NHCO-coumarin + recombinant Pv-LDH antigen and (b) AuNPs PAN-HS-(CH₂)₁₁-triphenylimidazole + recombinant Pv-LDH antigen.

The intensity of the $\nu(\text{CC})$ aliphatic vibration at 1020 cm^{-1} was found to be higher for HS-(CH₂)₁₁-indole followed by HS-(CH₂)₁₁-hydroquinone. HS-(CH₂)₁₁-NHCO-coumarin and the HS-(CH₂)₁₁-triphenylimidazole showed the smallest intensity as shown in Figure 6.12. The intensity of the $\nu(\text{CC})$ aliphatic band was observed to be higher for the less sterically hindered alkanethiols. This is attributed to their ability to use the thiol head group to adsorb onto the AuNP surface with a PAN antibody, compared to bulky HS-(CH₂)₁₁-NHCO-coumarin and HS-(CH₂)₁₁-triphenylimidazole. Lesser intensities were observed for the bulky alkanethiols since they promote electron delocalization thereby making phenyl rings to be more Raman active. This can be confirmed by the observation of peaks in the $\nu(\text{CC})$ aromatic region for bulky Raman reporters {Figure 6.11 (a) and (b)}, than less bulky reporters (Figure S6.4).

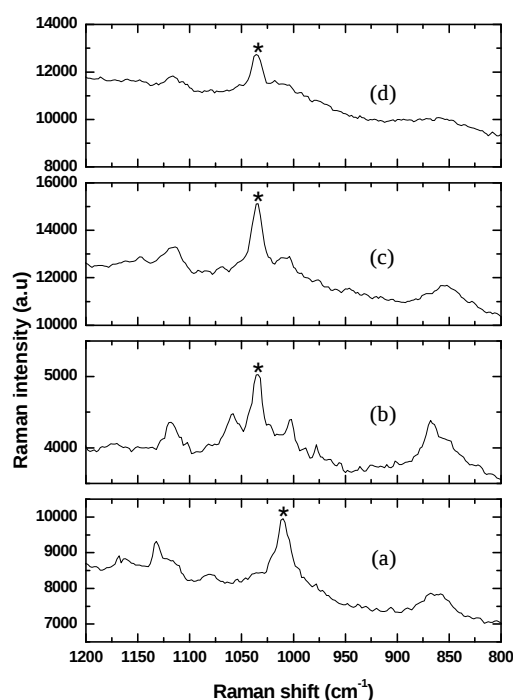


Figure 6.12: SERS immunoassay spectra showing the $\nu(\text{CC})$ aliphatic chain vibration for, (a) HS-(CH₂)₁₁-NHCO-coumarin (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole, and (d) HS-(CH₂)₁₁-hydroquinone adsorbed on AuNPs. (* denotes $\nu(\text{CC})$ aliphatic vibration peak)

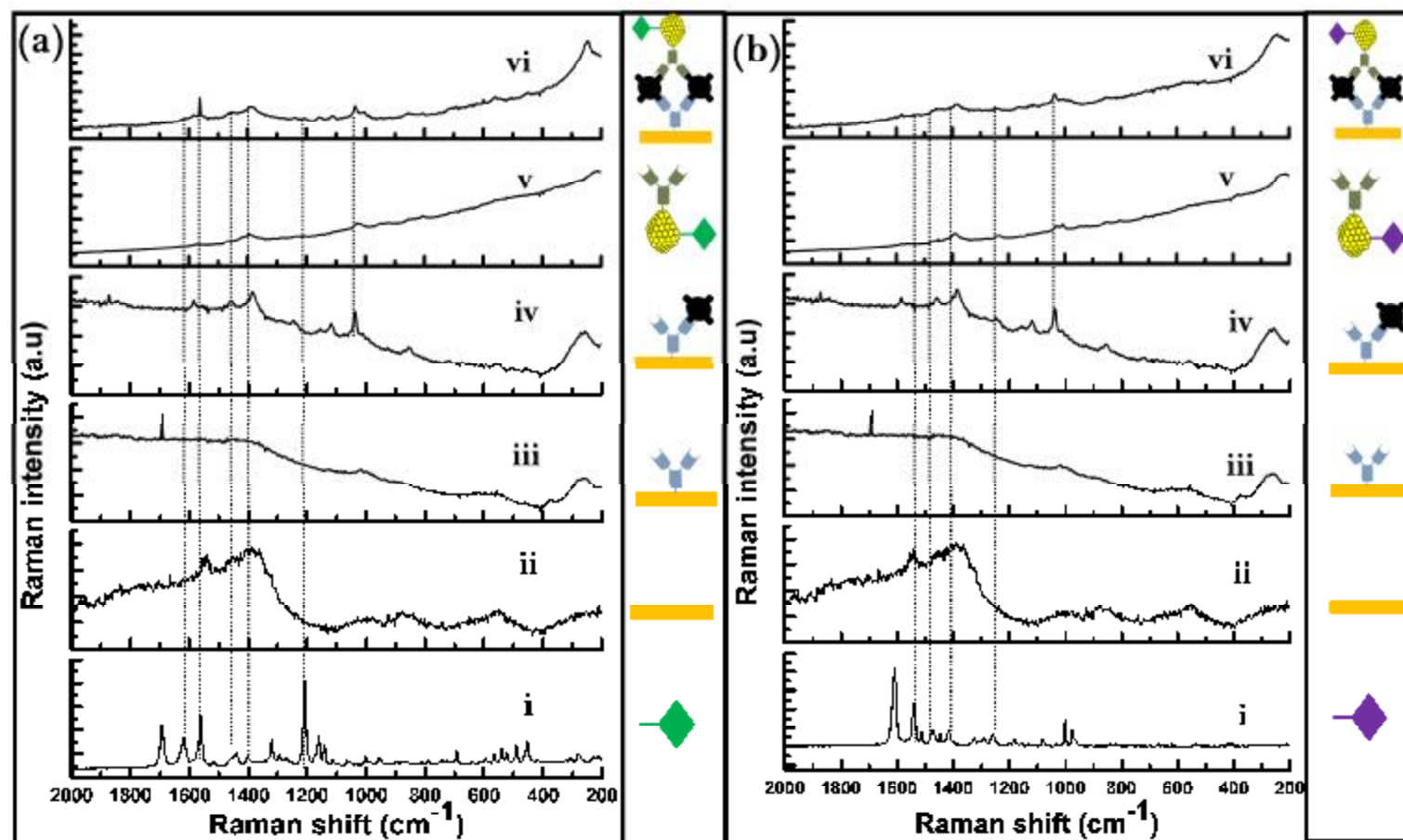


Figure 6.13: SERS immunoassay spectra, (a) AgNPs PAN-HS-(CH₂)₁₁-NHCO-coumarin + recombinant Pv-LDH antigen and (b) AgNPs PAN-HS-(CH₂)₁₁-triphenylimidazole + recombinant Pv-LDH antigen

The SERS spectra of AgNP PAN-conjugate with HS-(CH₂)₁₁-NHCO-coumarin, and HS-(CH₂)₁₁-triphenylimidazole are shown in Figure 6.13 (a), and (b), respectively. The vibrational peaks with smaller intensities for AuNP PAN-conjugates were observed in the region of 1100 cm⁻¹ to 1600 cm⁻¹. The SERS spectra of AgNP conjugates with HS-(CH₂)₁₁-indole, and HS-(CH₂)₁₁-hydroquinone are shown in the supplementary data as Figure S6.5 (a) and (b). All SERS spectra in Figure S6.5 (a) and (b) showed a vibrational peak in the region 200 to 300 cm⁻¹, associated with an Ag-S interaction. The spectra also revealed the dependence of the $\nu(\text{CC})$ aliphatic chain vibration at ~ 1020 cm⁻¹ with the alkanethiol used as a reporter. The descending order of the intensity was found to be HS-(CH₂)₁₁-indole, HS-(CH₂)₁₁-hydroquinone, HS-(CH₂)₁₁-NHCO-coumarin, and HS-(CH₂)₁₁-triphenylimidazole as shown in Figure 6.14.

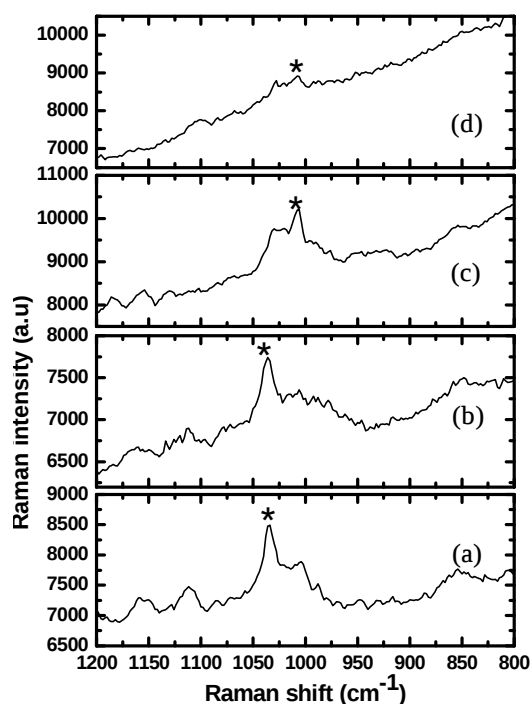


Figure 6.14: SERS immunoassay spectra showing the $\nu(\text{CC})$ aliphatic chain vibration for, (a) HS-(CH₂)₁₁-NHCO-coumarin (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole, and (d) HS-(CH₂)₁₁-hydroquinone adsorbed on AgNPs. (* denotes $\nu(\text{CC})$ aliphatic vibration peak).

6.3.2 Tuberculosis detection (*Mycobacterium tuberculosis*)

The development of an *in vitro* SERS based diagnostic assay for tuberculosis was designed. AuNPs and AgNPs were firstly conjugated to Protein A at mild condition before introducing the alkanethiols {HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, and HS-(CH₂)₁₁-hydroquinone}. All conjugates were also subjected to immunochromatographic tests. The images of the test strips immobilised with different concentration of *mycobacterium tuberculosis* 38 kDA for both AuNPs and AgNPs with all four different alkanethiols are shown in Figures 6.15 and 6.16, respectively. The concentrations for the immobilised antigen used were 0.5; 1.0 and 1.5 mg/ml. The positive test lines were only clearly visible or intense for 1.0 mg/ml antigen concentration for both AuNP and AgNP conjugates with alkanethiols. The intensities of these lines were confirmed using a strip-reader. The plots are shown in the supplementary data as Figures S6.6 and S6.7 for AuNPs and AgNPs conjugates respectively. The plots in Figures S6.6 and S6.7 revealed that the sample with 1.0 mg/ml concentration had a high intensity. The stability of AuNP and AgNP-Protein A conjugates was evaluated under various electrolytic concentrations (NaCl). The images of these conjugates are shown in Figure 6.17. The change in colour was only observed for the unconjugated metal nanoparticles when exposed to 0.2 M to 1.0 M NaCl. The stability of Protein A conjugates with AuNPs and AgNPs was also confirmed by the SPR measurements carried out using a UV-vis spectrophotometer. The optical spectra are shown in the supplementary data as Figures S6.8 and S6.9. In Figures S6.8 and S6.9, there were shifts of the SPR bands which were observed for unconjugated AuNPs and AgNPs under different NaCl concentration.

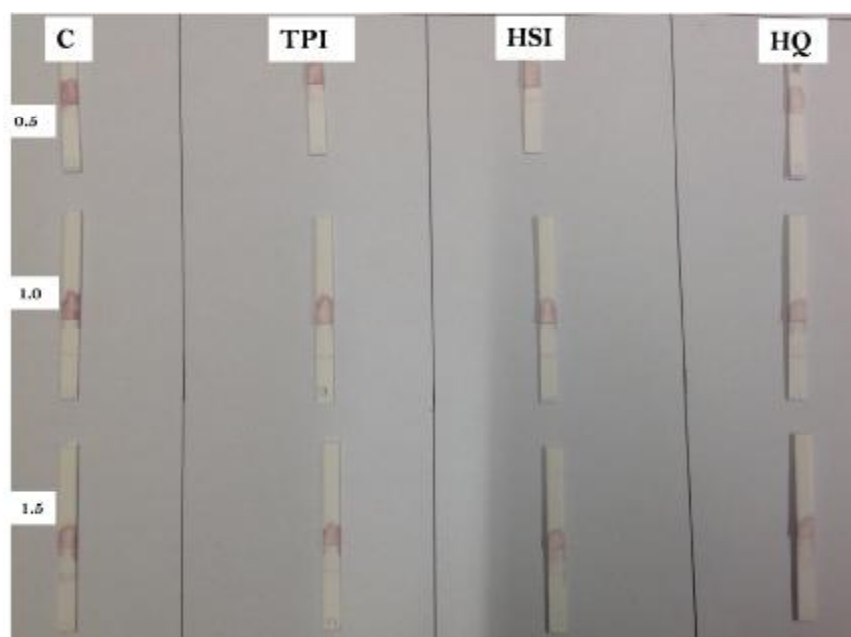


Figure 6.15: Images of the test strips showing the effect of the antigen concentration on the intensity of the test control lines using AuNPs

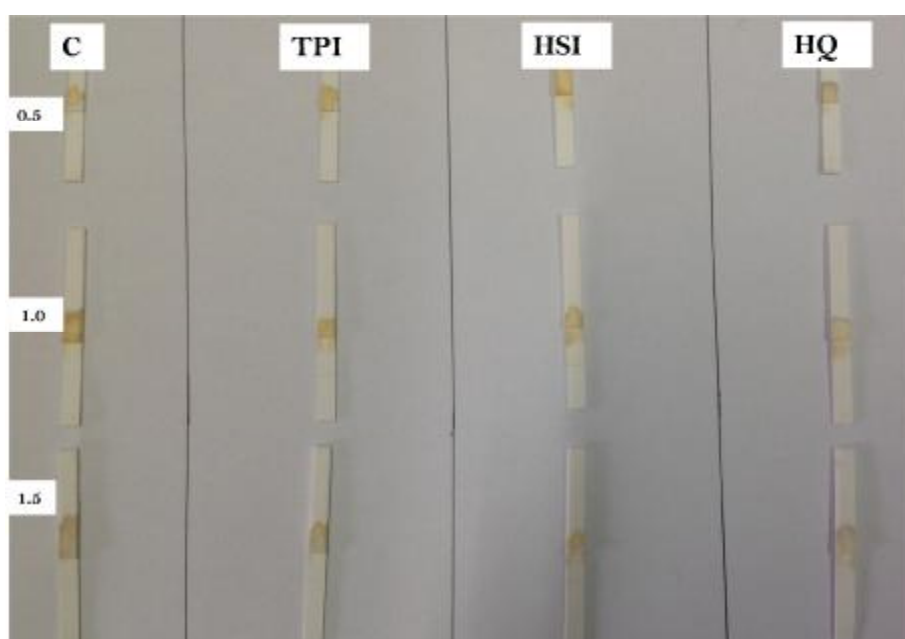


Figure 6.16: Images of the test strips showing the effect of the antigen concentration on the intensity of the test control lines using AgNPs

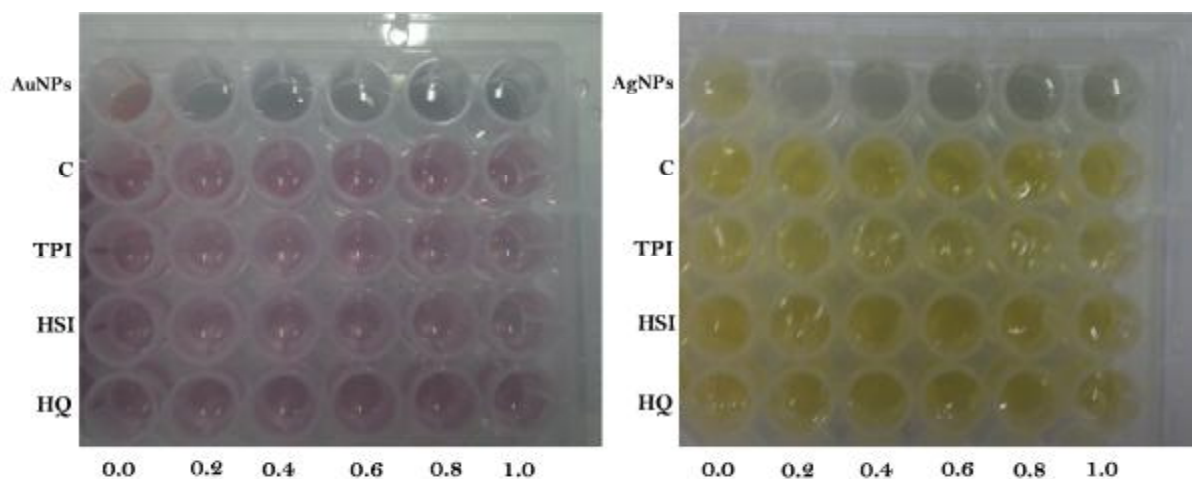


Figure 6.17: Images for flocculation of (a) AuNPs PAN-conjugates and (b) AgNPs PAN-conjugates under different concentrations of NaCl

The TEM images of Protein A conjugates of AuNPs and AgNPs with four alkanethiols are shown in the supplementary data as Figures S6.10 and S6.11, respectively. In Figures S6.10 and S6.11, no signs of aggregation were observed. The SERS spectra of HS-(CH₂)₁₁-NHCO-coumarin and HS-(CH₂)₁₁-triphenylimidazole alkanethiols adsorbed on both AuNPs and AgNPs are shown in Figures 6.18 and 6.19. Similar trends in vibrational bands that were observed in previous SERS immunoassays for the detection of *Plasmodium vivax* were observed in the *mycobacterium tuberculosis* detection. The SERS spectra of HS-(CH₂)₁₁-indole and HS-(CH₂)₁₁-hydroquinone immunoassays on both AuNPs and AgNPs are shown in the supplementary data as Figures S6.12 and S6.13, respectively. These spectra revealed the formation of antigen antibody complexes as per the previous malaria SERS assays. The bulky alkanethiols (HS-(CH₂)₁₁-NHCO-coumarin, and HS-(CH₂)₁₁-triphenylimidazole) revealed more vibrational peaks in the region of 1100 to 1600 cm⁻¹ compared to the less bulky alkanethiols (HS-(CH₂)₁₁-indole, and HS-(CH₂)₁₁-hydroquinone). The SERS spectra were also observed to consist of an enhanced vibrational band at the region of ~1120 cm⁻¹, and a metal to sulphur interaction band in the region 200 to 300 cm⁻¹.

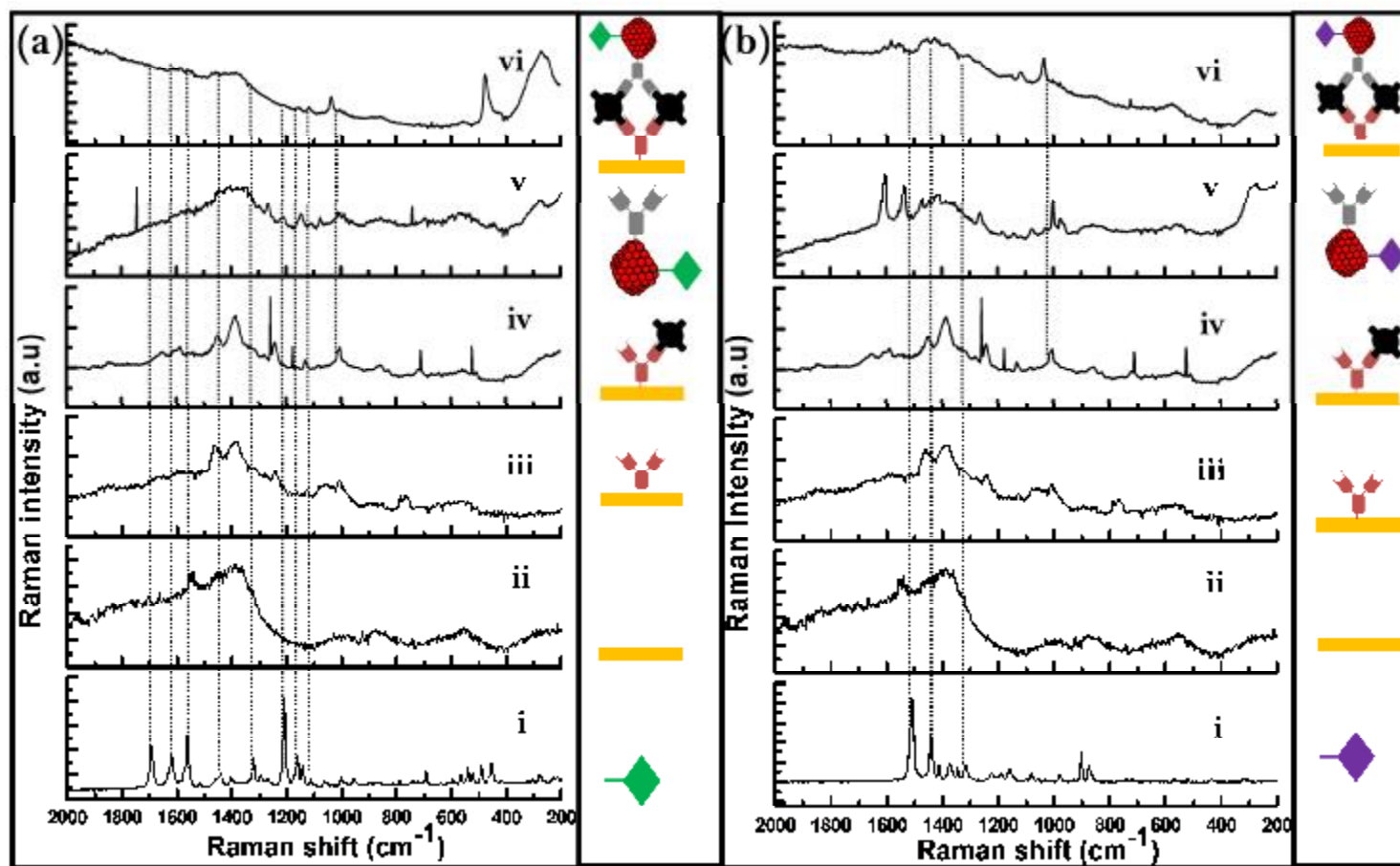


Figure 6.18: SERS immunoassay spectra, (a) AuNPs Protein A-HS-(CH₂)₁₁-NHCO-coumarin + mycobacterium tuberculosis 38 kDA antigen and (b) AuNPs Protein A-HS-(CH₂)₁₁-triphenylimidazole + mycobacterium tuberculosis 38 kDA

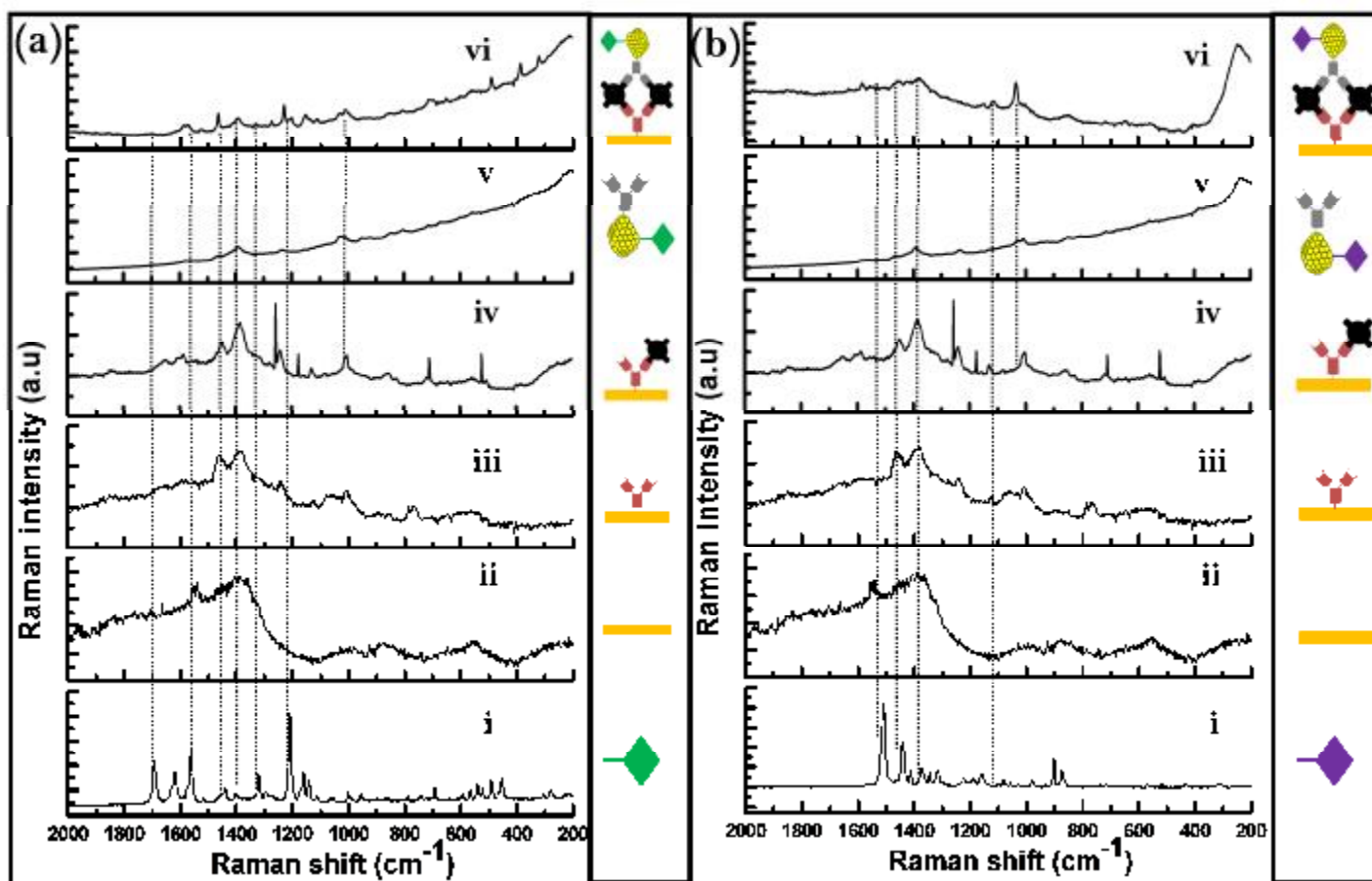


Figure 6.19: SERS immunoassay spectra, (a) AgNPs Protein A-HS-(CH₂)₁₁-NHCO-coumarin + mycobacterium tuberculosis 38 kDA antigen and (b) AgNPs Protein A-HS-(CH₂)₁₁-triphenylimidazole + mycobacterium tuberculosis 38 kDA antigen

The relative intensities of $\nu(\text{CC})$ aliphatic vibration in the region 1020 cm^{-1} was compared for alkanethiols adsorbed on AuNPs and AgNPs, as shown in their SERS spectra in Figures 6.20 and 6.21. For alkanethiols adsorbed on AuNPs, the highest intensity of $\nu(\text{CC})$ aliphatic was observed on bulky reporters ($\text{HS}-(\text{CH}_2)_{11}\text{-NHCO-coumarin}$, and $\text{HS}-(\text{CH}_2)_{11}\text{-triphenylimidazole}$), followed by $\text{HS}-(\text{CH}_2)_{11}\text{-hydroquinone}$, and the least intensity was shown by $\text{HS}-(\text{CH}_2)_{11}\text{-indole}$. The intensity of $\nu(\text{CC})$ aliphatic chain for the same alkanethiols adsorbed on AgNPs depicted a different trend; $\text{HS}-(\text{CH}_2)_{11}\text{-triphenylimidazole} > \text{HS}-(\text{CH}_2)_{11}\text{-indole} > \text{HS}-(\text{CH}_2)_{11}\text{-hydroquinone} > \text{HS}-(\text{CH}_2)_{11}\text{-NHCO-coumarin}$.

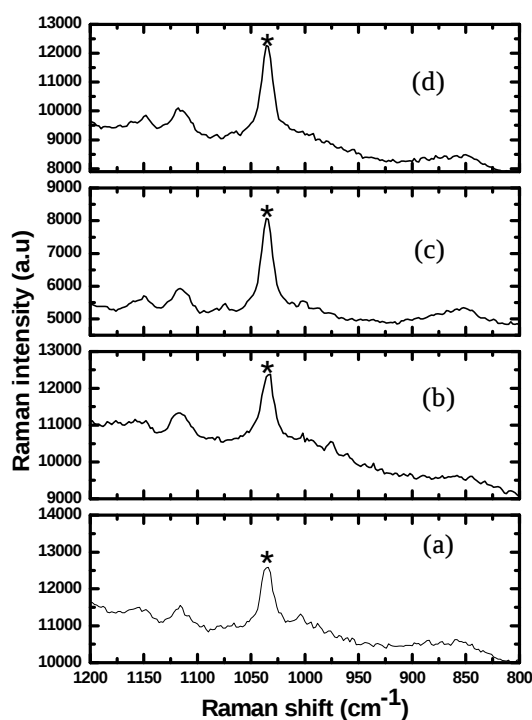


Figure 6.20: SERS immunoassay spectra showing the $\nu(\text{CC})$ aliphatic vibration for, (a) $\text{HS}-(\text{CH}_2)_{11}\text{-NHCO-coumarin}$ (b) $\text{HS}-(\text{CH}_2)_{11}\text{-triphenylimidazole}$, (c) $\text{HS}-(\text{CH}_2)_{11}\text{-indole}$, and (d) $\text{HS}-(\text{CH}_2)_{11}\text{-hydroquinone}$ adsorbed on AuNPs. (* denotes $\nu(\text{CC})$ aliphatic vibration peak)

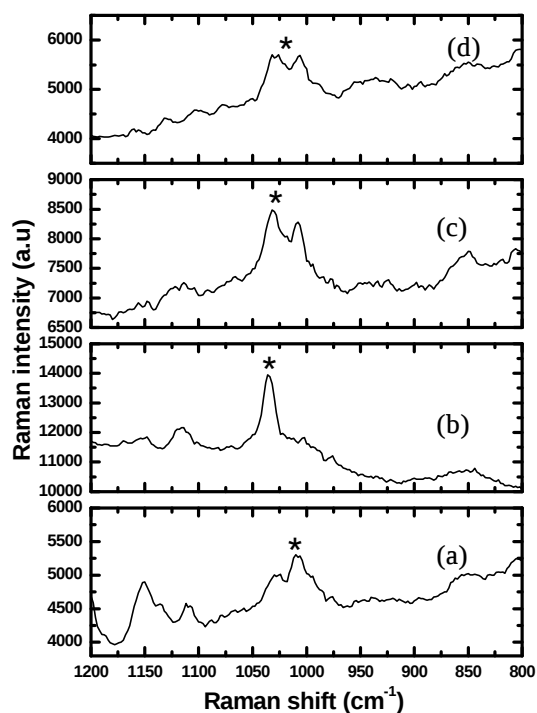


Figure 6.21: SERS immunoassay spectra showing the $\nu(\text{CC})$ aliphatic vibration for, (a) HS-(CH₂)₁₁-NHCO-coumarin (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole, and (d) HS-(CH₂)₁₁-hydroquinone adsorbed on AgNPs. (* denotes $\nu(\text{CC})$ aliphatic vibration peak).

6.4 Conclusions

The metal (Au and Ag) nanoparticle conjugates of monoclonal anti-pLDH pan-malaria and Protein A with four different alkanethiols were used to capture 1 mg/mL of recombinant Pv-LDH and or mycobacterium tuberculosis 38 kDA, respectively. The capture of the antigens with the conjugates was confirmed by an immunochromatographic test and Raman spectroscopy. The presence of the coloured line on the test revealed the interaction of the immobilised antigens and antibodies on the conjugates. The presence of the alkanethiol vibrational peaks present on the conjugates, after washing the unbound species with PBS, on the SERS spectra confirmed the capture of the antigen. The intensity of a vibrational

peak at 1020 cm⁻¹ was observed to be dependent on the alkanethiols used, and on the captured concentration of an antigen.

6.5 References

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Chapter Seven

Conclusions and Recommendations

7.1 Conclusions

7.1.1 Synthesis and characterisation of different sizes and shape of Au and Ag nanoparticles

Metal nanoparticles (spherical and rods) were synthesised and then characterised using a combination of spectroscopic techniques to determine their size and morphology dependant properties. Metal nanoparticles (AuNPs and AgNPs) depicted SPR peak that shifted to higher wavelength as their size increased. Successful AuNR synthesis was confirmed by its unique double absorption peaks corresponding to a transverse surface plasmon appearing around 500 nm, and a longitudinal surface plasmon occurring above 700 nm. The average diameter and the aspect ratio for the AuNRs were measured and calculated from TEM images. No sign of aggregation was observed.

7.1.2 Functionalization and characterization of metal nanoparticles and gold nanorods with four Raman reporters and PEGs to form MMPCs.

The as-synthesized nanomaterials were successfully functionalized with different stoichiometric ratios of HS-(CH₂)₁₁-PEG-COOH and alkanethiols (Raman reporters), i.e; HS-(CH₂)₁₁-NHCO-coumarin, HS-(CH₂)₁₁-triphenylimidazole, HS-(CH₂)₁₁-indole, HS-(CH₂)₁₁-hydroquinone to mixed monolayer protected clusters. The stability of different metal nanoparticles was attained by firstly introducing HS-(CH₂)₁₁-PEG-OH, before the addition of stoichiometric ratios of HS-(CH₂)₁₁-PEG-COOH and alkanethiols. The resulting MMPCs were characterized by a combination of spectroscopic methods. The notable change was the formation of self-assembled ordered superlattices, which is a well-known tendency for thiol-capped nanoparticles. The MMPCs were further evaluated for their Raman activities, and their enhancement factors were calculated. The calculated EFs

were found to be higher for smaller sized metal nanoparticles (14 nm for AuNPs and 16 nm AgNPs) compared to 30 and 40 nm metal nanoparticles. Higher EFs were observed for HS-(CH₂)₁₁-NHCO-coumarin adsorbed on AuNPs, while AgNPs showed a higher EF value for HS-(CH₂)₁₁-triphenylimidazole, compared to other alkanethiols used.

7.1. 3 Conjugation of the MMPCs and their evaluation as SERS probes for detection of malaria and tuberculosis biomarkers

Smaller sizes of AuNP (14 nm) and AgNP (16 nm) were further conjugated to malaria and tuberculosis antibodies for the detection of their respective antigens. The conjugation was pioneered by a direct conjugation procedure rather than the failed attempts on using the EDC coupling through activation of –COOH end of HS-(CH₂)₁₁-PEG-COOH. The capture of the conjugates was confirmed by an immunochromatographic test, which displayed coloured lines on the test strips with the immobilised antigens. The SERS immunoassay results revealed the vibrational peaks corresponding to the Raman reporters used.

7.2 Recommendations

- The designed SERS immunoassay can be improved by varying the concentration of an antigen, by comparing the intensity enhancement of a vibrational peak at 1020 cm⁻¹. A plot of relative intensity and the concentration of an antigen would give the limit of detection of the malaria and tuberculosis biomarkers.
- The detection can also be improved by employing extremely stable and monodispersed smaller sized metal nanoparticles than used in this study. The use of metal nanorods rather than metal nanoparticles, and a different substrate rather than gold-coated steel for the SERS immunoassay can open a door for improvement of the sensitivity.

- The designed SERS assay can be used in veterinary applications as a laboratory based detection tool for diseases such as Rift Valley Fever, Rabies and Foot & Mouth disease.

Appendix

Supplementary material

Chapter three

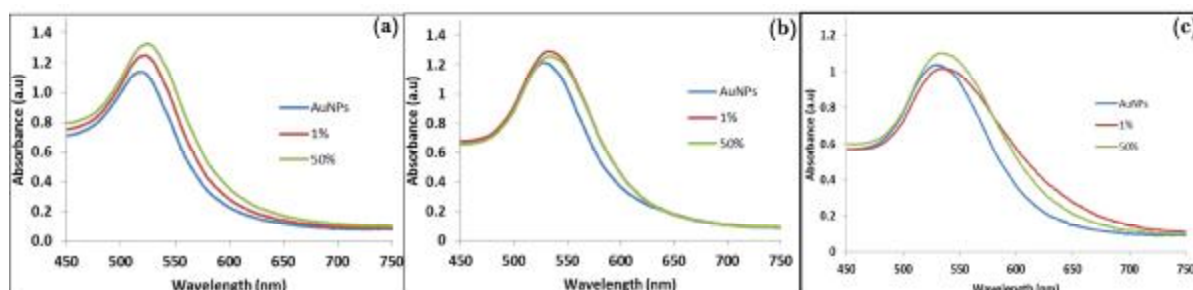


Figure S3.1: Absorption spectra of different AuNPs sizes with their corresponding AuMMPCs in different stoichiometric ratios of HS-(CH₂)₁₁-NHCO-Coumarin (a) 14 nm, (b) 30 nm and (c) 40 nm.

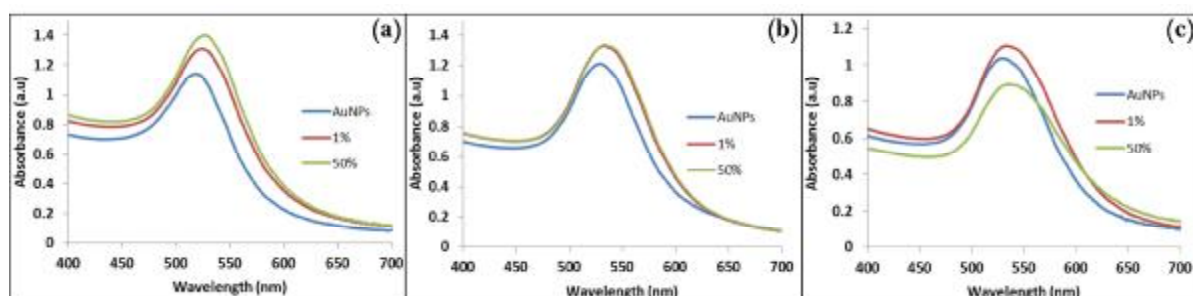


Figure S3.2: Absorption spectra of different AuNPs sizes with their corresponding AuMMPCs in different stoichiometric ratios of HS-(CH₂)₁₁-triphenylimidazole (a) 14 nm, (b) 30 nm and (c) 40 nm.

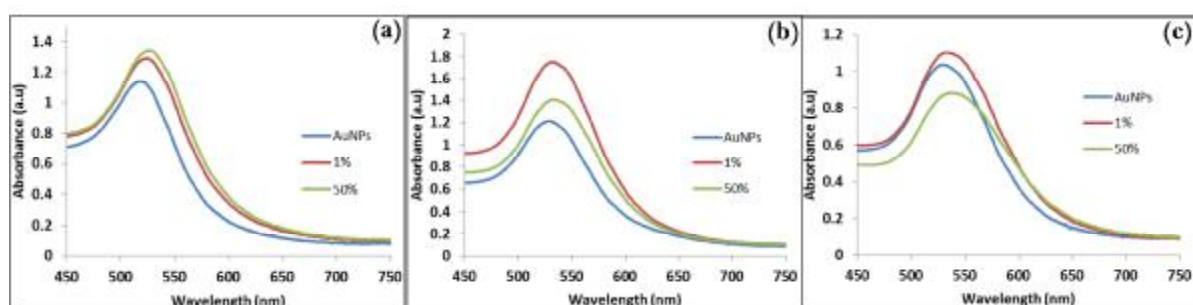


Figure S3.3: Absorption spectra of different AuNPs sizes with their corresponding AuMMPCs in different stoichiometric ratios of HS-(CH₂)₁₁-indole (a) 14 nm, (b) 30 nm and (c) 40 nm.

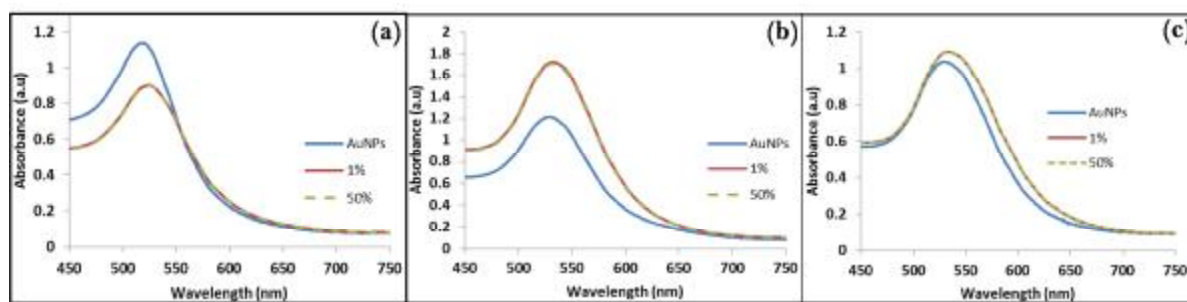


Figure S3.4: Absorption spectra of different AuNPs sizes with their corresponding AuMMPCs in different stoichiometric ratios of HS-(CH₂)₁₁-hydroquinone (a) 14 nm, (b) 30 nm and (c) 40 nm.

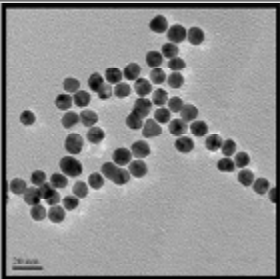
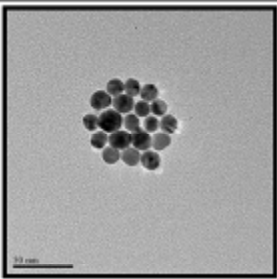
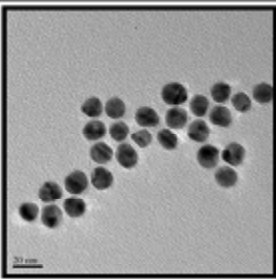
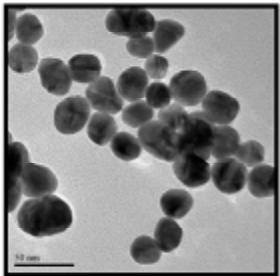
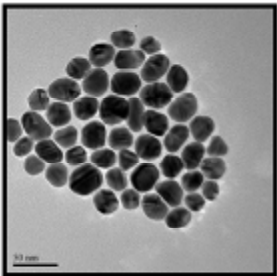
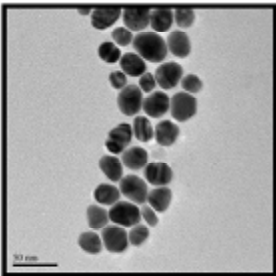
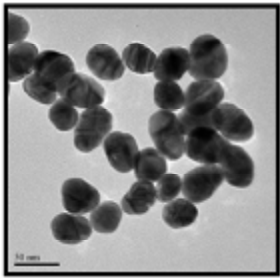
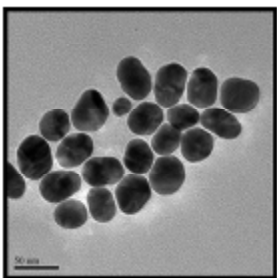
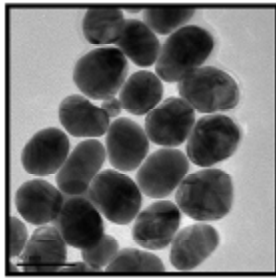
Ligands	Citrate	1% Raman reporter	50% Raman reporter
Sizes 14 nm			
30 nm			
40 nm			

Figure S3.5: TEM images of different AuNPs sizes and corresponding AuMMPCs of 1% and 50% of HS-(CH₂)₁₁-indole.

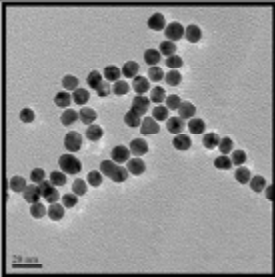
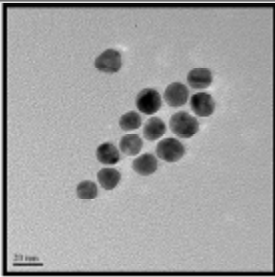
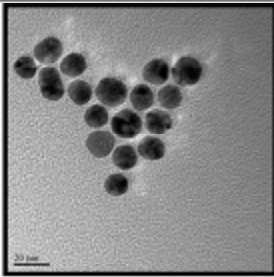
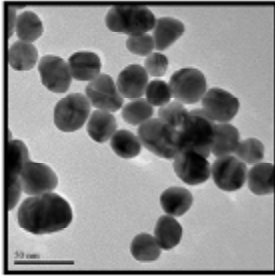
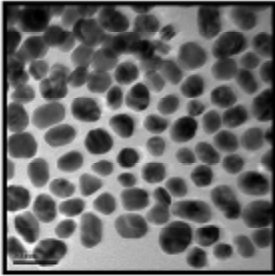
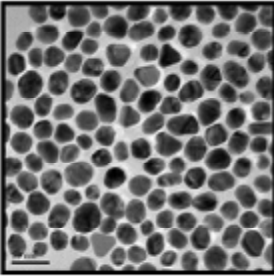
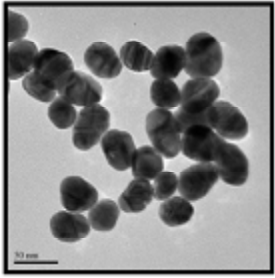
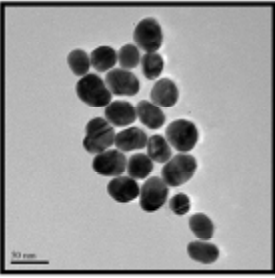
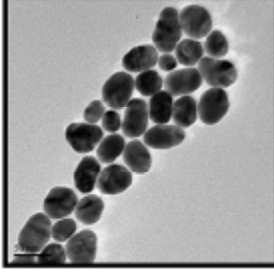
Ligands	Citrate	1% Raman reporter	50% Raman reporter
Sizes 14 nm			
30 nm			
40 nm			

Figure S3.6: TEM images of different AuNPs sizes and corresponding AuMMPCs of 1% and 50% of HS-(CH₂)₁₁-hydroquinone.

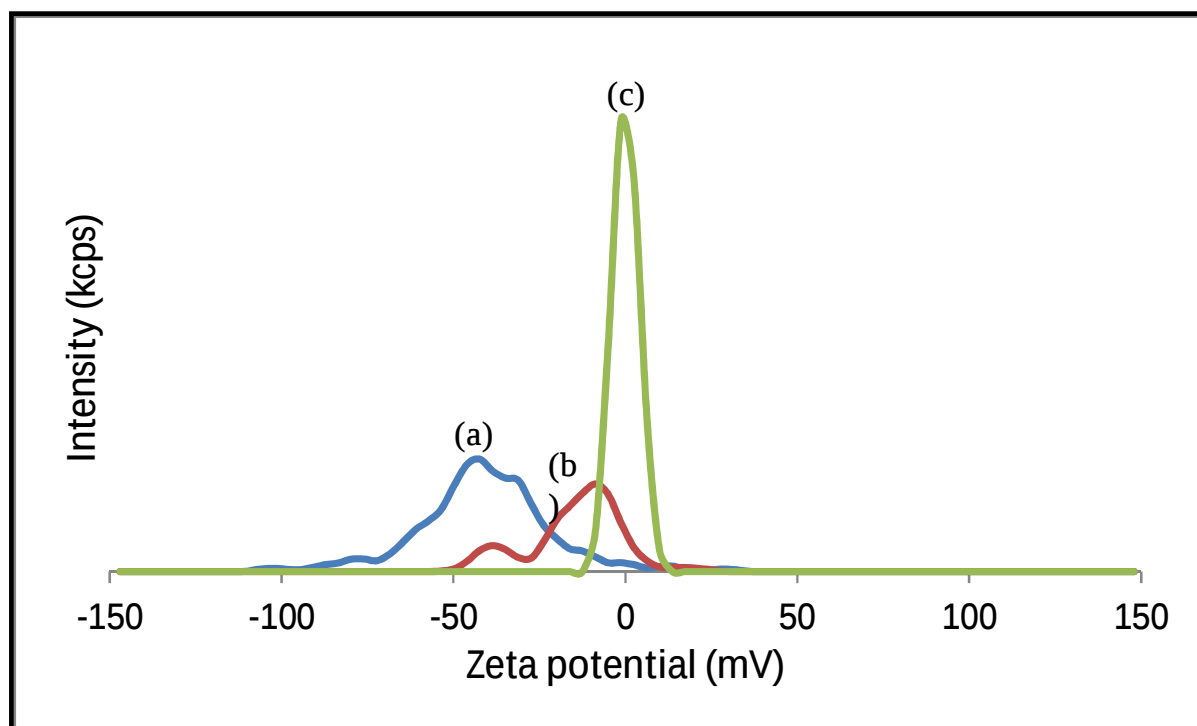


Figure S3.7: Zeta potentials of AuNPs 14nm stabilized by (a) Citrate (b) 1% HS-(CH₂)₁₁-NHCO-coumarin (c) 50% HS-(CH₂)₁₁-NHCO-coumarin.

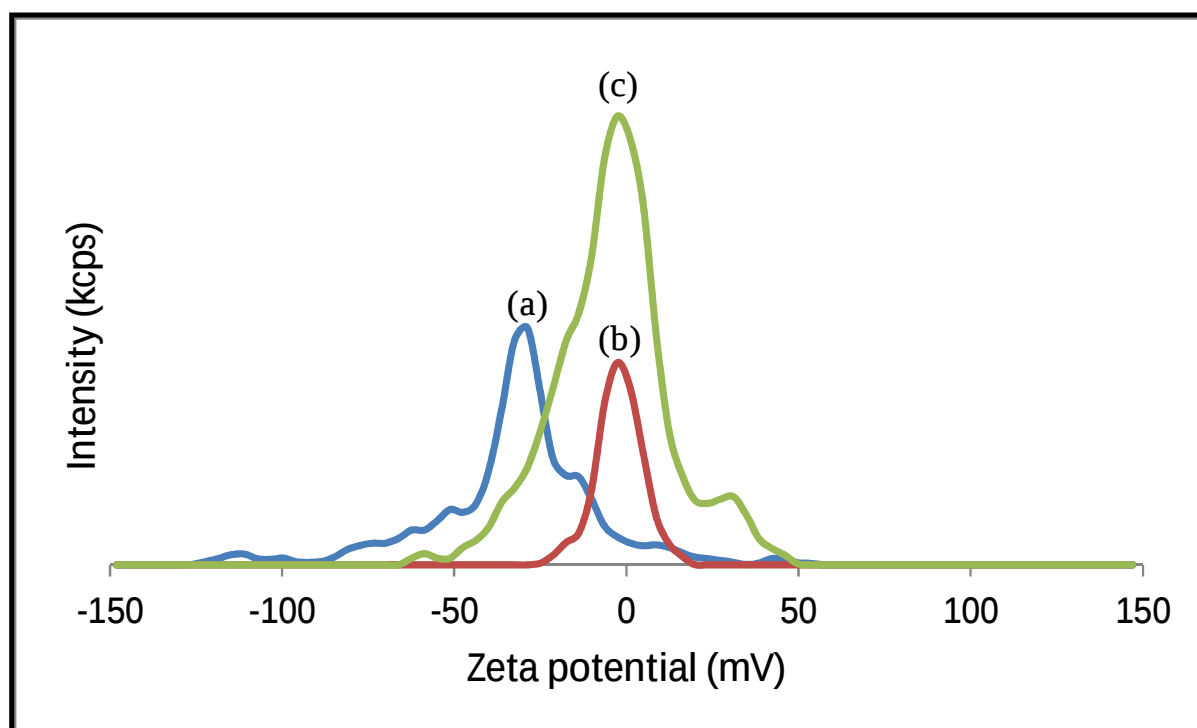


Figure S3.8: Zeta potentials of AuNPs 30 nm stabilized by (a) Citrate (b) 1% HS-(CH₂)₁₁-NHCO-coumarin (c) 50% HS-(CH₂)₁₁-NHCO-coumarin.

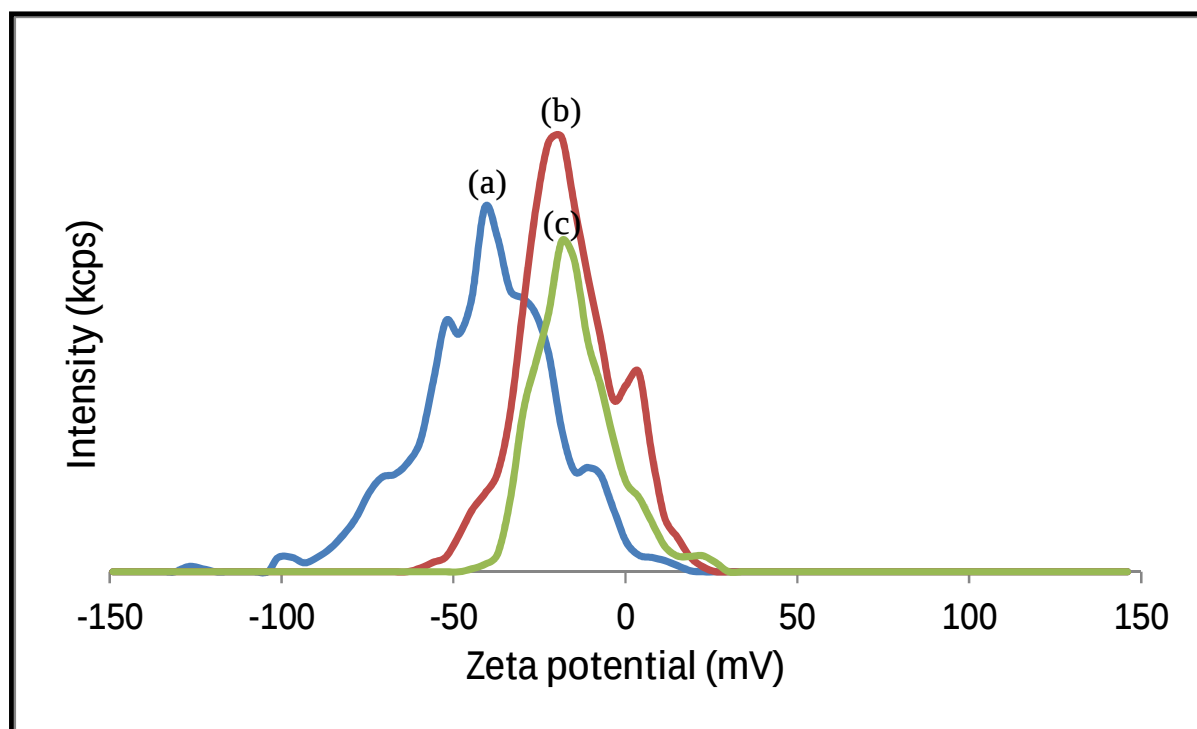


Figure S3.9: Zeta potentials of AuNPs 40 nm stabilized by (a) Citrate (b) 1% HS-(CH₂)₁₁-NHCO-coumarin (c) 50% HS-(CH₂)₁₁-NHCO-coumarin.

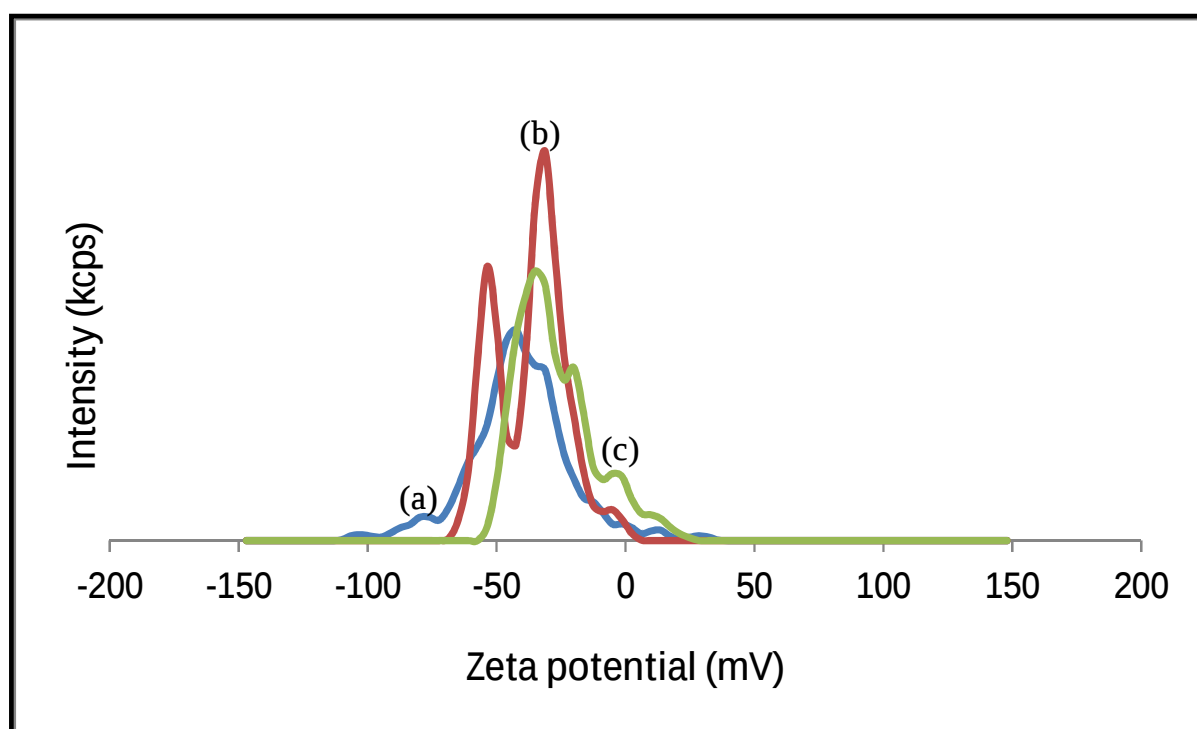


Figure S3.10: Zeta potentials of AuNPs 14 nm stabilized (a) Citrate (b) 1% HS-(CH₂)₁₁-triphenylimidazole and (c) 50% HS-(CH₂)₁₁-triphenylimidazole.

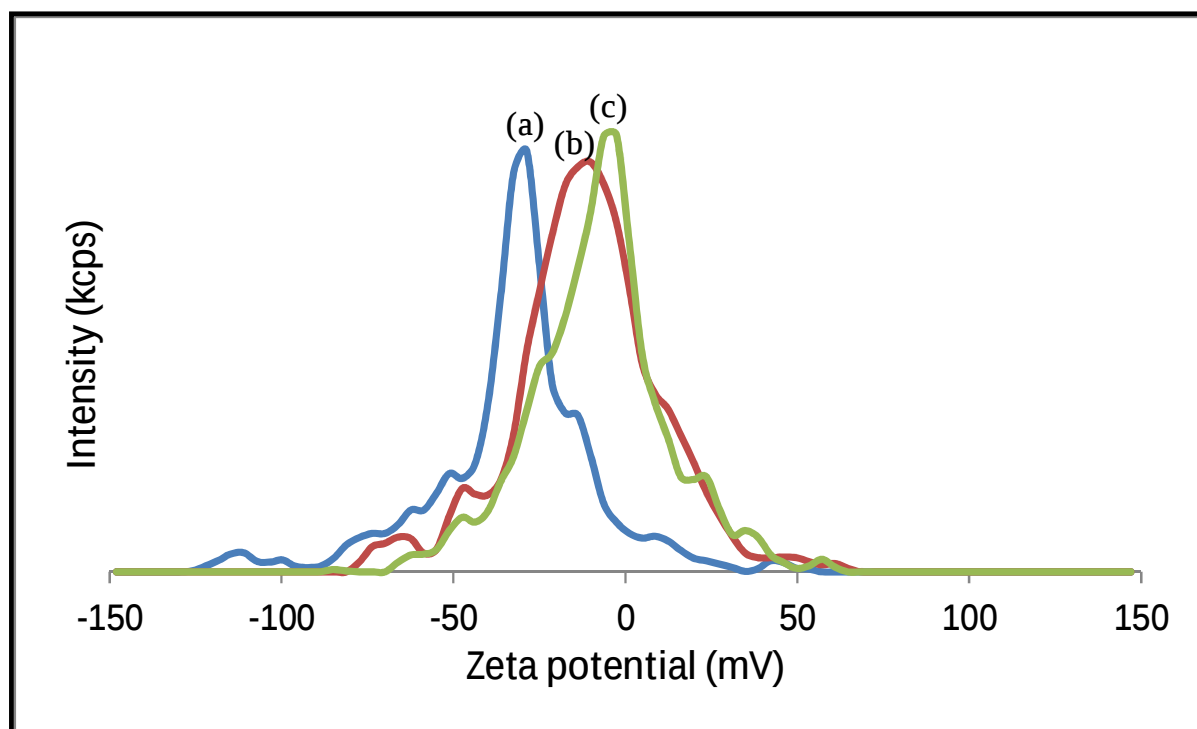


Figure S3.11: Zeta potentials of AuNPs 30 nm stabilized (a) Citrate (b) 1% HS-(CH₂)₁₁-triphenylimidazole and (c) 50% HS-(CH₂)₁₁-triphenylimidazole.

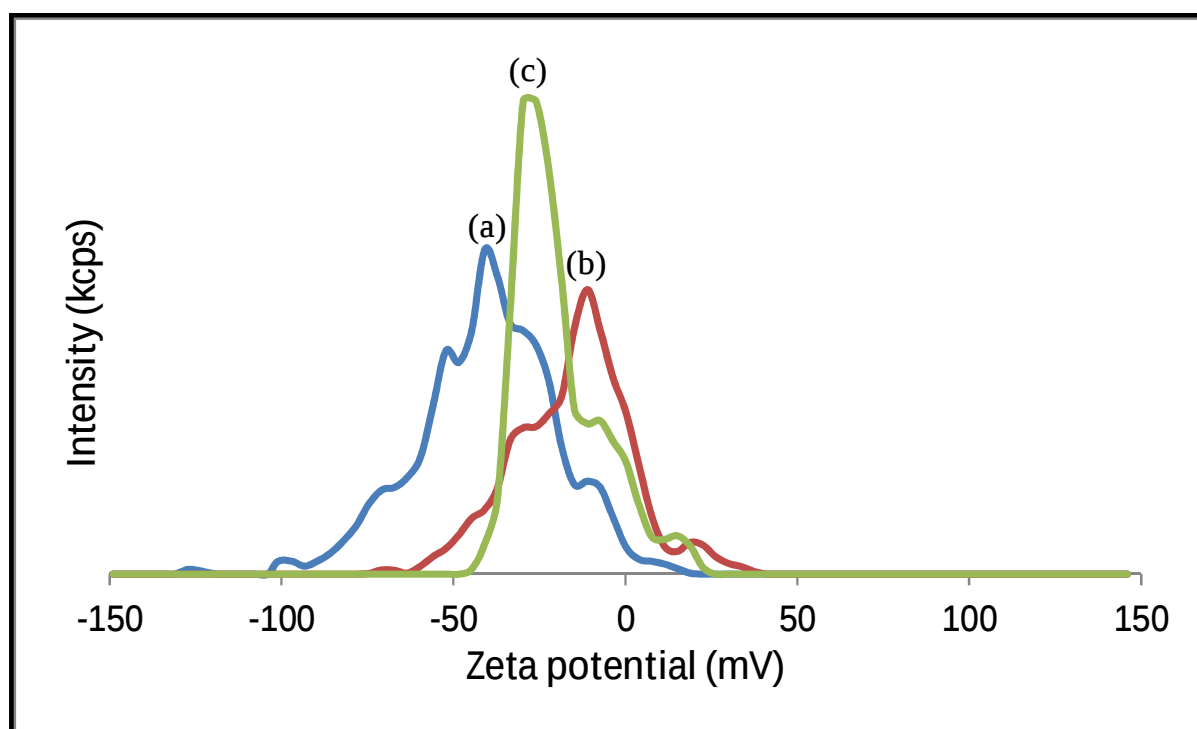


Figure S3.12: Zeta potentials of AuNPs 40 nm stabilized (a) Citrate (b) 1% HS-(CH₂)₁₁-triphenylimidazole and (c) 50% HS-(CH₂)₁₁-triphenylimidazole.

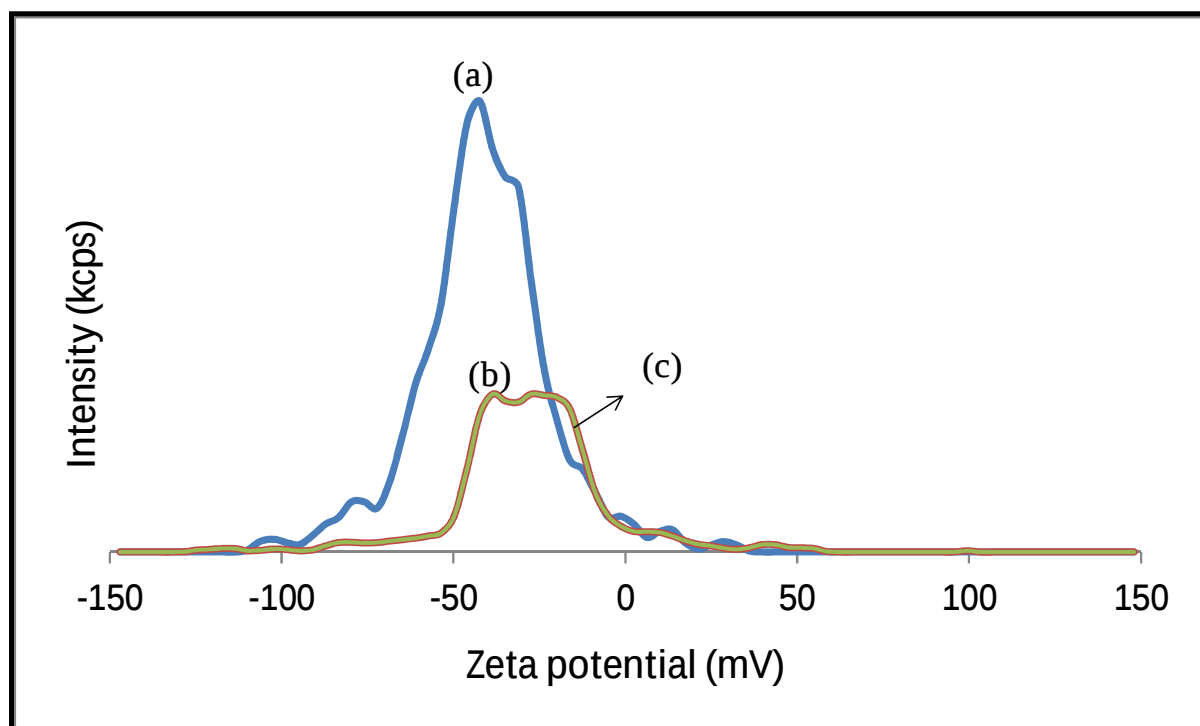


Figure S3.13: Zeta potentials of AuNPs 14 nm stabilized by (a) Citrate, (b) 1% HS-(CH₂)₁₁-indole and (c) 50% HS-(CH₂)₁₁-indole.

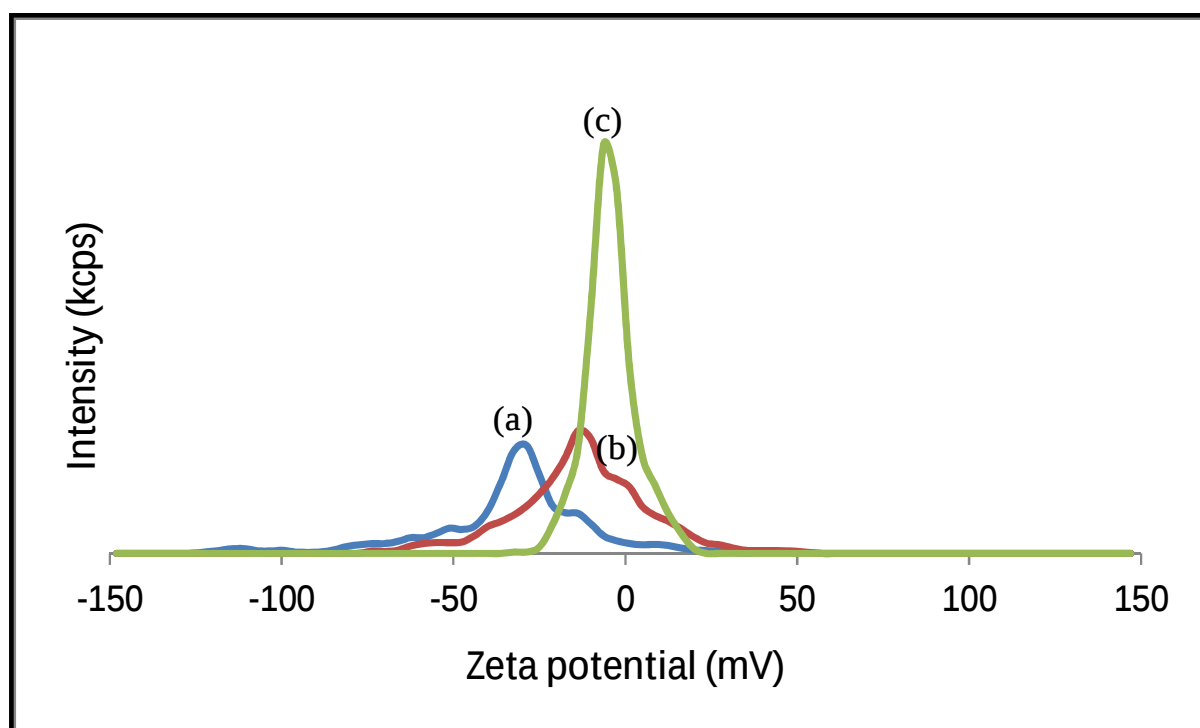


Figure S3.14: Zeta potentials of AuNPs 30 nm stabilized by (a) Citrate, (b) 1% HS-(CH₂)₁₁-indole and (c) 50% HS-(CH₂)₁₁-indole.

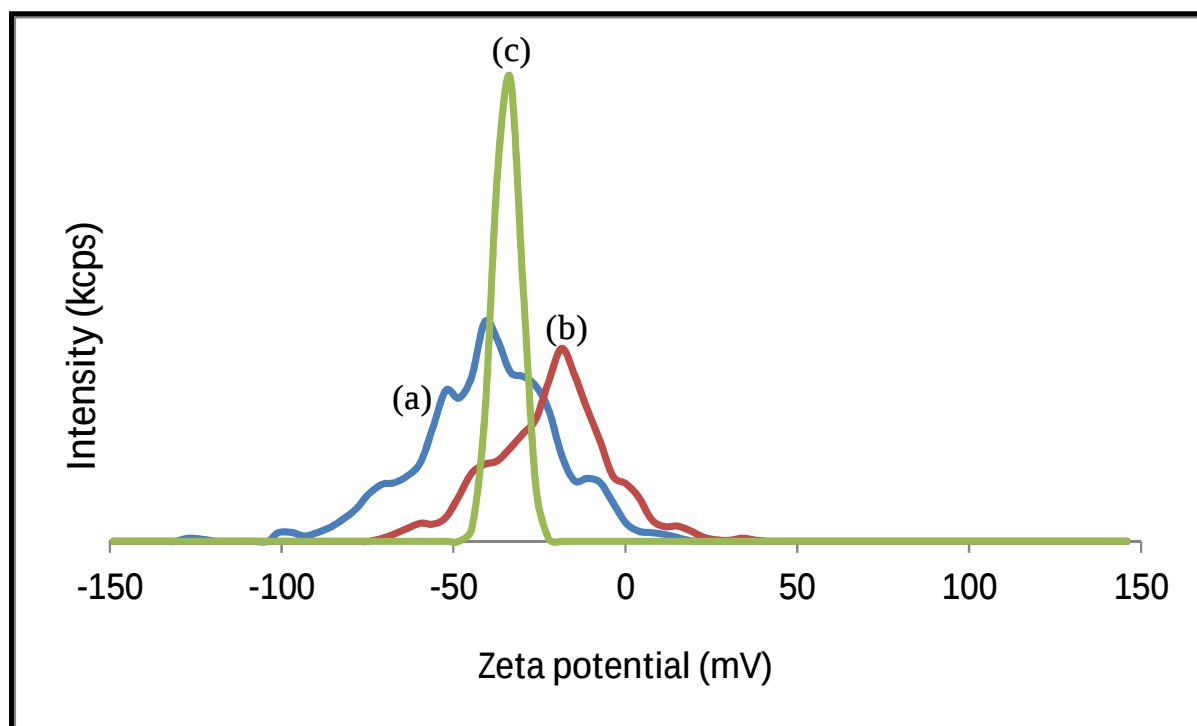


Figure S3.15: Zeta potentials of AuNPs 40 nm stabilized by (a) Citrate, (b) 1% HS-(CH₂)₁₁-indole and (c) 50% HS-(CH₂)₁₁-indole.

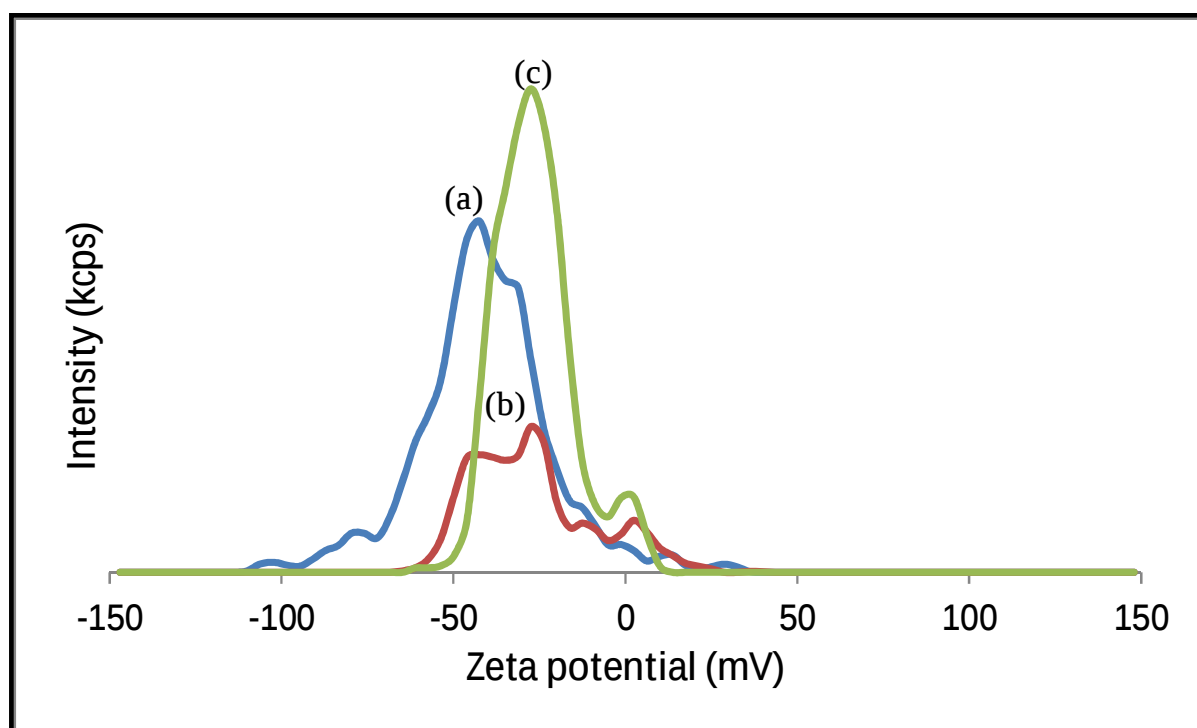


Figure S3.16: Zeta potentials of AuNPs 14 nm stabilized by (a) Citrate (b) 1%HS-(CH₂)₁₁-hydroquinone and (c) 50% HS-(CH₂)₁₁-hydroquinone.

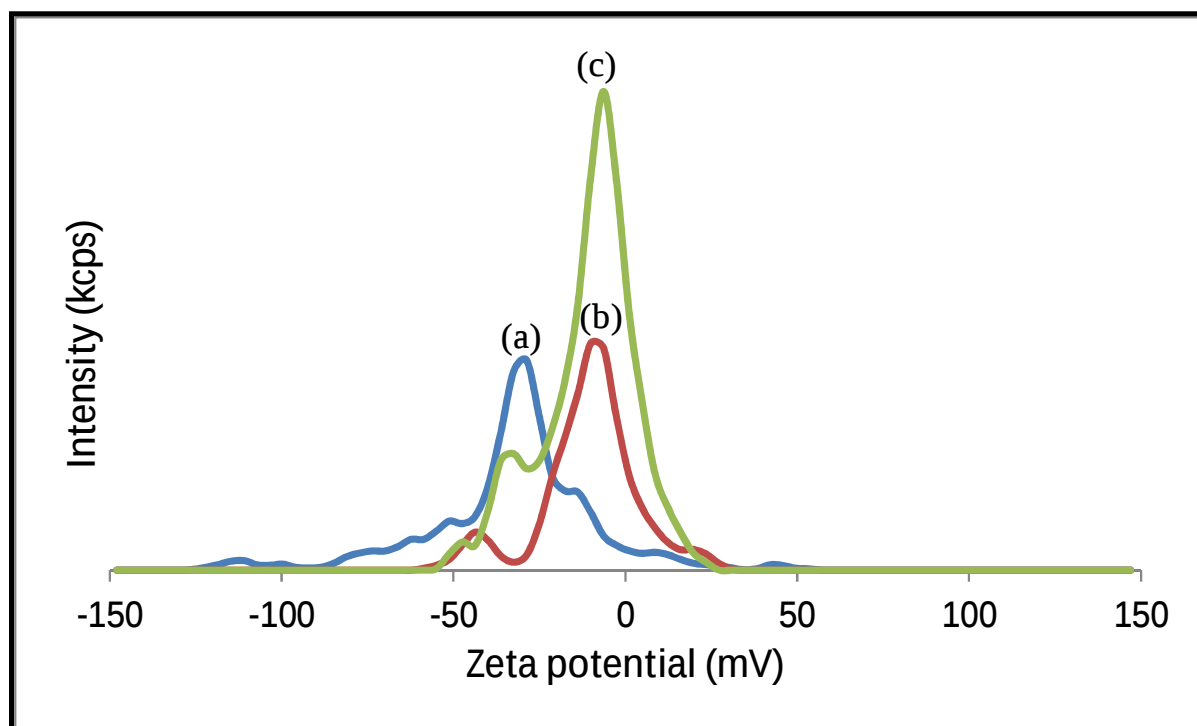


Figure S3.17: Zeta potentials of AuNPs 30 nm stabilized by (a) Citrate (b) 1%HS-(CH₂)₁₁-hydroquinone and (c) 50% HS-(CH₂)₁₁-hydroquinone.

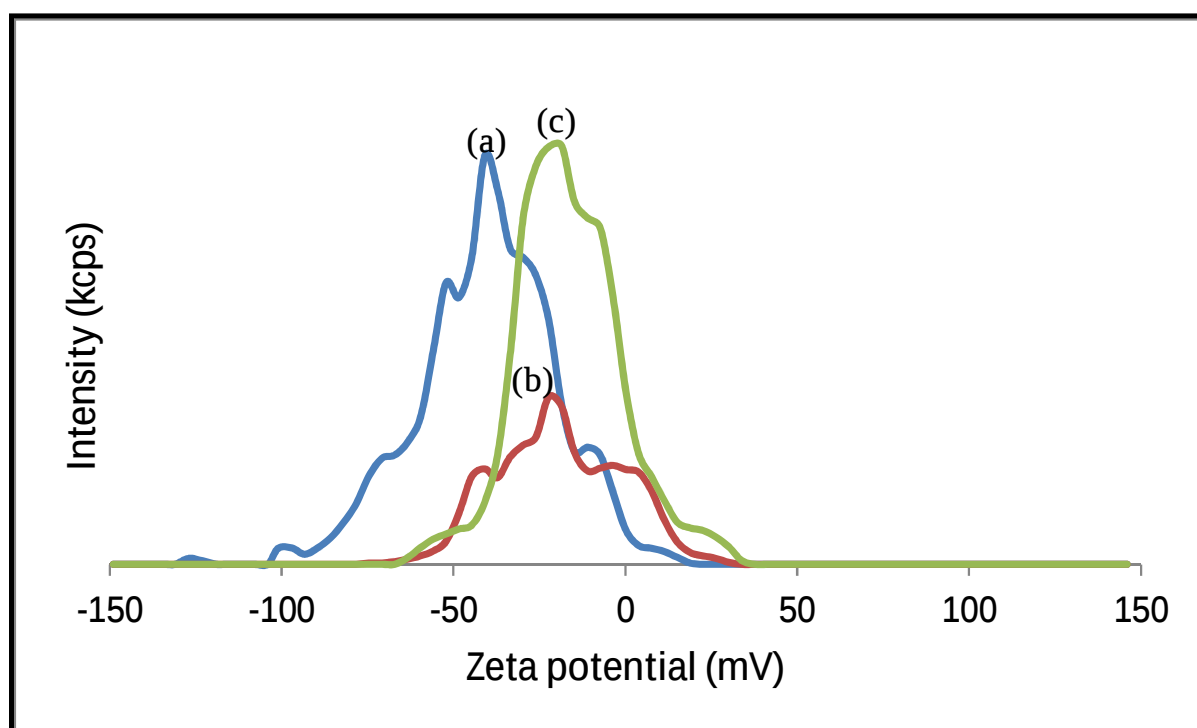


Figure S3.18: Zeta potentials of AuNPs 40 nm stabilized by (a) Citrate (b) 1%HS-(CH₂)₁₁-hydroquinone and (c) 50% HS-(CH₂)₁₁-hydroquinone.

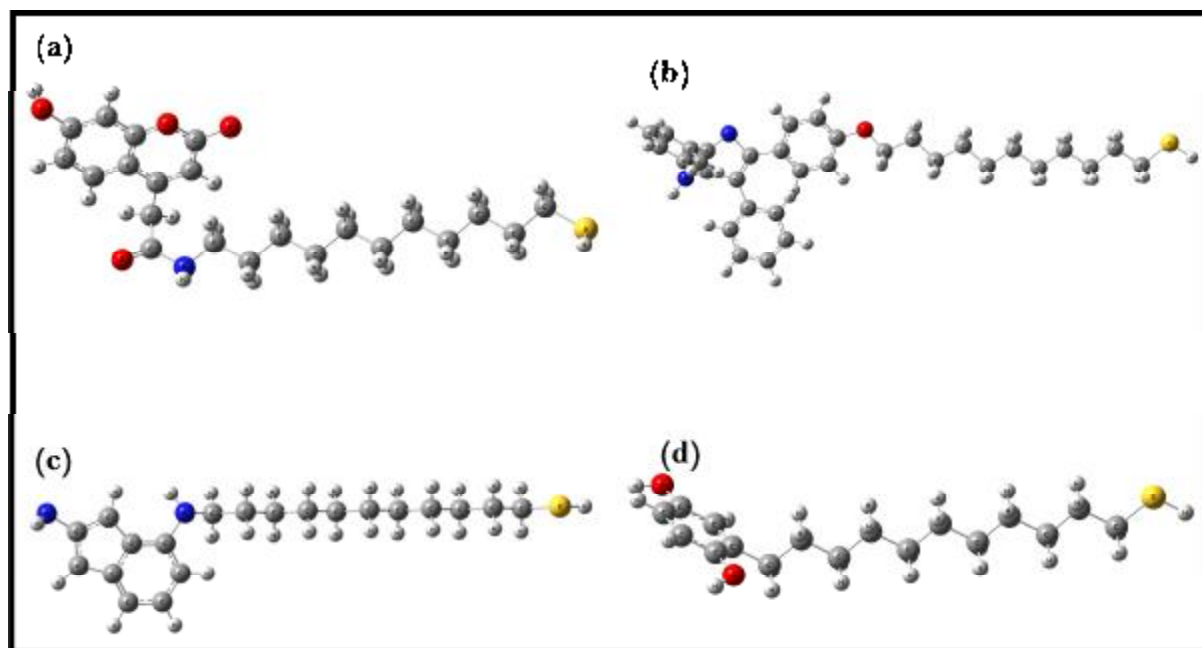


Figure S3.19: Optimized geometries of (a) HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole and (d) HS-(CH₂)₁₁-hydroquinone

Table S3.1: Summary of the peak assignments of HS-(CH₂)₁₁-NHCO-coumarin different stoichiometric ratios adsorbed on different AuNPs

Band assignment	$\nu(\text{Au-S})$	$\nu(\text{C-S})$	$\nu(\text{CC})$ aliph	$\delta(\text{CH}_2)$ asym	$\nu(\text{CC})$ arom rings	$\nu(-\text{S-H})$
HS-Coumarin	no peak	739 (small)	814, 864, 962	1410	1564, 1622	2570, 2590
Au 14 nm 1% HS-Coumarin	266 (strong)	798 (shoulder)	944, 1028, 1074, 1050 992, 1064, 1110, 1138, 1156,	1394	1588	no peak
Au 14 nm 50% HS-Coumarin	298	716	1296	1396, 1432 1400	1572, 1606	no peak
Au 30 nm 1% HS-Coumarin	264 (strong) 283	714 (shoulder)	952, 1024, 1149, 1200, 1298 844, 898, 956, 1063, 1150, 1200,	(broad) 1420	1585	no peak
Au 30 nm 50% HS-Coumarin	(shoulder)	763 (shoulder) 714 (shoulder),	1276 859, 995, 1063, 1111, 1156,	(strong)	1566, 1611	no peak
Au 40 nm 1% HS-Coumarin	251	801 720 (shoulder),	1160, 1295	1484	1563, 1622	no peak
Au 40 nm 50% HS-Coumarin	291	798	859, 995, 1110, 1158, 1160, 1295	1432	1566, 1614	no peak

HS-Coumarin = HS-(CH₂)₁₁-NHCO-coumarin, Au = Gold, Aliph = aliphatic, arom = aromatic, asym=asymmetric

Table S3.2: Summary of the peak assignments of HS-(CH₂)₁₁-triphenylimidazole different stoichiometric ratios adsorbed on different AuNPs

Band assignment	$\nu(\text{Au-S})$	$\nu(\text{C-S})$	$\nu(\text{CC})$ aliph	$\delta(\text{CH}_2)$ asym	$\nu(\text{CC})$ arom rings	$\nu(\text{-S-H})$
HS-Coumarin	no peak	740	796, 844	1416, 1470	1564, 1622	2572
Au 14 nm 1% TPI	274(broad) 294	(small) 780	870, 962, 976, 1002, 1066, 1198 (broad)	1404 (broad)	1552, 1608	no peak
Au 14 nm 50% TPI	(broad)	(broad) 770	862, 9888, 1020, 1114, 1122	1378 (shoulder) 1374 (broad), 1468	1552, 1608	no peak
Au 30 nm 1% TPI	272	(broad)	806, 978, 1002, 1072, 1136	(shoulder)	1554, 1606	no peak
Au 30 nm 50% TPI	278	722, 744 736	820, 992, 1068, 1112, 1164	1422	1538, 1612	no peak
Au 40 nm 1% TPI	236	(small) 736	834, 962, 1050, 1186	1428, 1471	1556, 1604	no peak
Au 40 nm 50% TPI	244	(small)	876, 896, 962, 980, 1002, 1186	1428, 1474	1556, 1604	peak

TPI = HS-(CH₂)₁₁-Triphenylimidazole, Au = Gold, Aliph = aliphatic, arom = aromatic, asym=asymmetric

Table S3.3: Summary of the peak assignments of HS-(CH₂)₁₁-indole different stoichiometric ratios adsorbed on different AuNPs

Band assignment	$\nu(\text{Au-S})$	$\nu(\text{C-S})$	$\nu(\text{CC})$ aliph	$\delta(\text{CH}_2)$ asym	$\nu(\text{CC})$ arom rings	$\nu(\text{-S-H})$
HSI	no peak	766	894, 938	1418, 1490	1578	2544
Au 14 nm 1% HSI	234	714(broad), 748	848, 894, 992, 1068, 1106, 1186, 1234	1358 (shoulder), 1436	1530	no peak
Au 14 nm 50% HSI	238	714 (broad), 758	866, 904, 986, 1010, 1058, 1112, 1230	1358, 1442	1574, 1622	no peak
Au 30 nm 50% HSI	288 (shoulder)	712	996, 1024, 1062, 1168, 1282(shoulder)	1342(shoulder), 1482	1604, 1662	no peak
Au 30 nm 50% HSI	282	714	840, 1014, 1068, 1114, 1294	1352, 1440	1594(shoulder) r)	no peak
Au 40 nm 1% HSI	256	794 (shoulder)	1034, 1052, 1100, 1276(shoulder)	1352	1532	no peak
Au 40 nm 50% HSI	240	784	1052, 1120, 1146, 1200(shoulder)	1358, 1434	1544	no peak

HSI= HS-(CH₂)₁₁-indole , Au= Gold, Aliph = aliphatic, arom = aromatic, asym=asymmetric

Table S3.4: Summary of the peak assignments of HS-(CH₂)₁₁-hydroquinone different stoichiometric ratios adsorbed on different AuNPs

Band assignment	$\nu(\text{Au-S})$	$\nu(\text{C-S})$	$\nu(\text{CC})$ aliph	$\delta(\text{CH}_2)$ asym	$\nu(\text{CC})$ arom rings	$\nu(\text{-S-H})$
HQ	no peak	730	794	1294	1444	2586
Au 14 nm 1% HQ	278	732 (small)	804, 878, 978, 1016, 1066, 1106	1394 (broad)	1674	no peak
Au 14 nm 50% HQ	280	724	806, 884, 976, 1006, 1060, 1116	1434	1666	no peak
Au 30 nm 1% HQ	276 (broad)	714	878, 1000, 1026, 1110, 1294	1350, 1434	1594	no peak
Au 30 nm 50% HQ	282	710 (broad)	850, 978, 1012, 1044, 1112, 1292	1352, 1430	1598	no peak
Au 40 nm 1% HQ	294	714	856, 976, 1006, 1058, 1108, 1294	1434	1610	no peak
Au 40 nm 50% HQ	272	718	856, 960, 1000, 1054, 1104, 1294	1436	1602	no peak

HQ = HS-(CH₂)₁₁-hydroquinone, Au = Gold, Aliph = aliphatic, arom = aromatic, asym=asymmetric

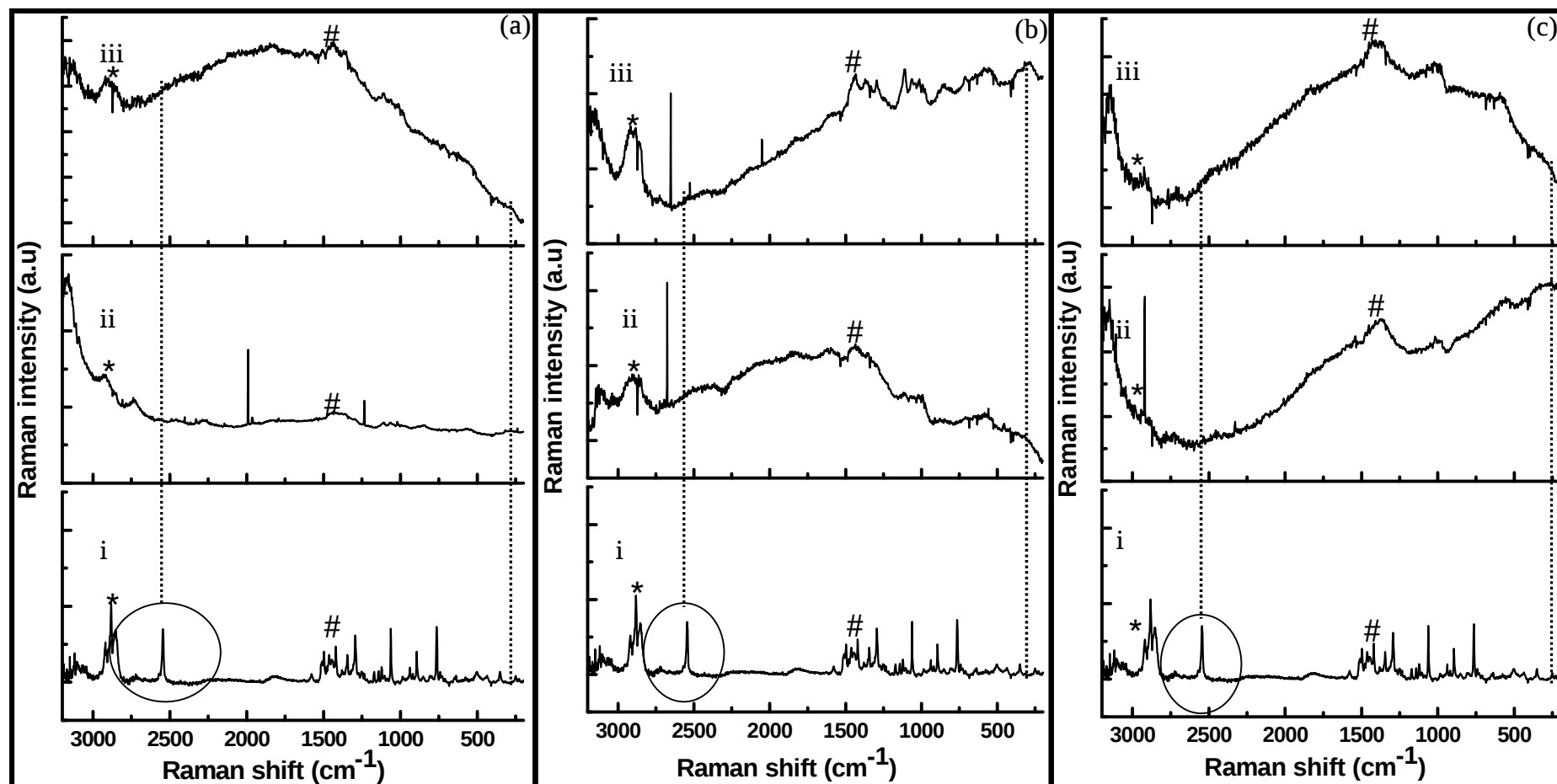


Figure S3.20: Raman spectra of AuMMPCs of different stoichiometric ratios of HS-(CH₂)₁₁-indole prepared with different AuNPs sizes (a) 14 nm, (b) 30 nm, and (c) 40 nm. The circled peak is the vibrational band of S-H, (* and #) denotes the symmetric and asymmetric bands of C-H bonds

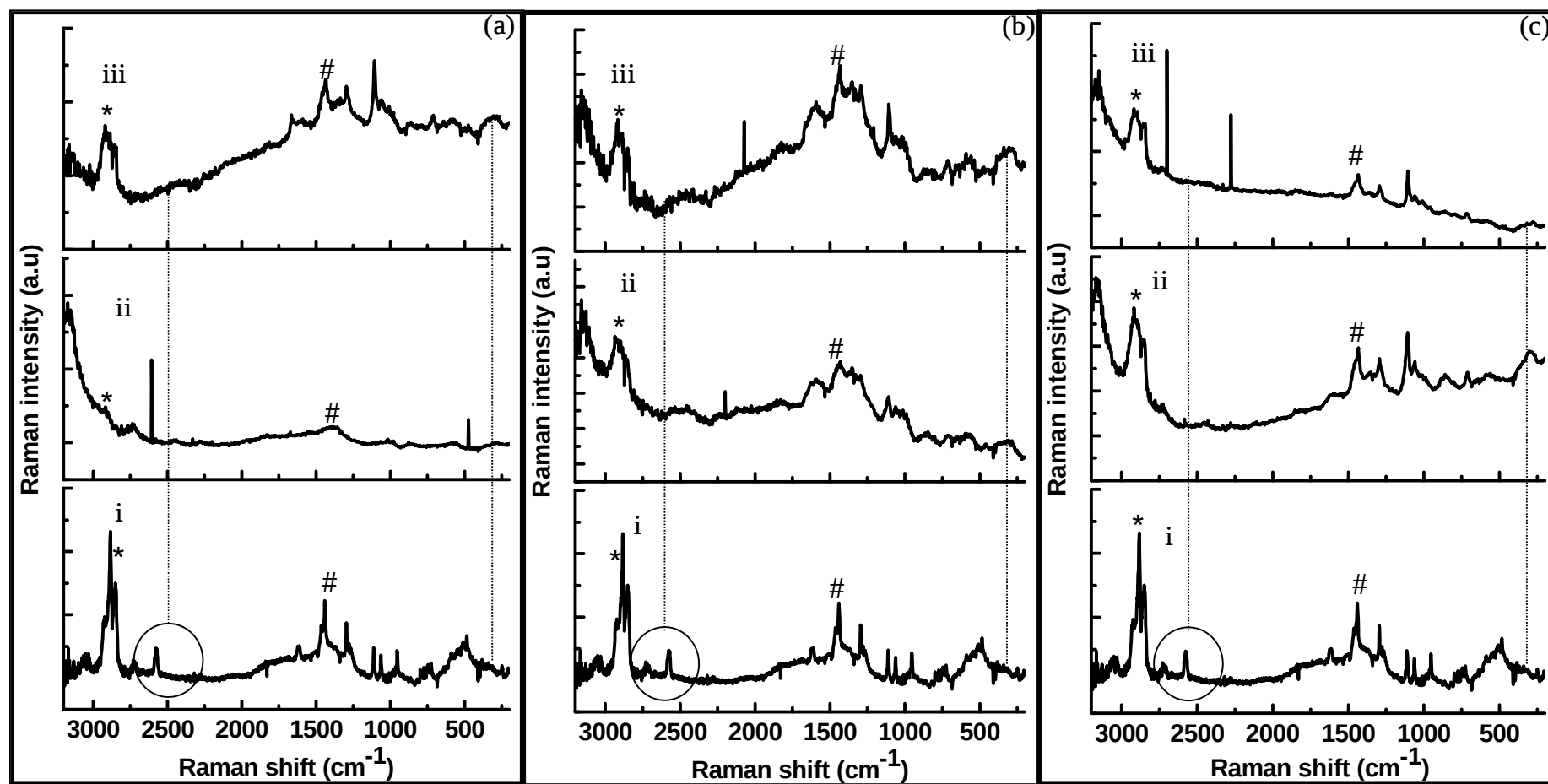


Figure S3.21: Raman spectra of AuMMPCs of different stoichiometric ratios of HS-(CH₂)₁₁-hydroquinone prepared with different AuNPs sizes (a) 14 nm, (b) 30 nm, and (c) 40 nm. The circled peak is the vibrational band of S-H, (* and #) denotes the symmetric and asymmetric bands of C-H bonds.

Chapter four

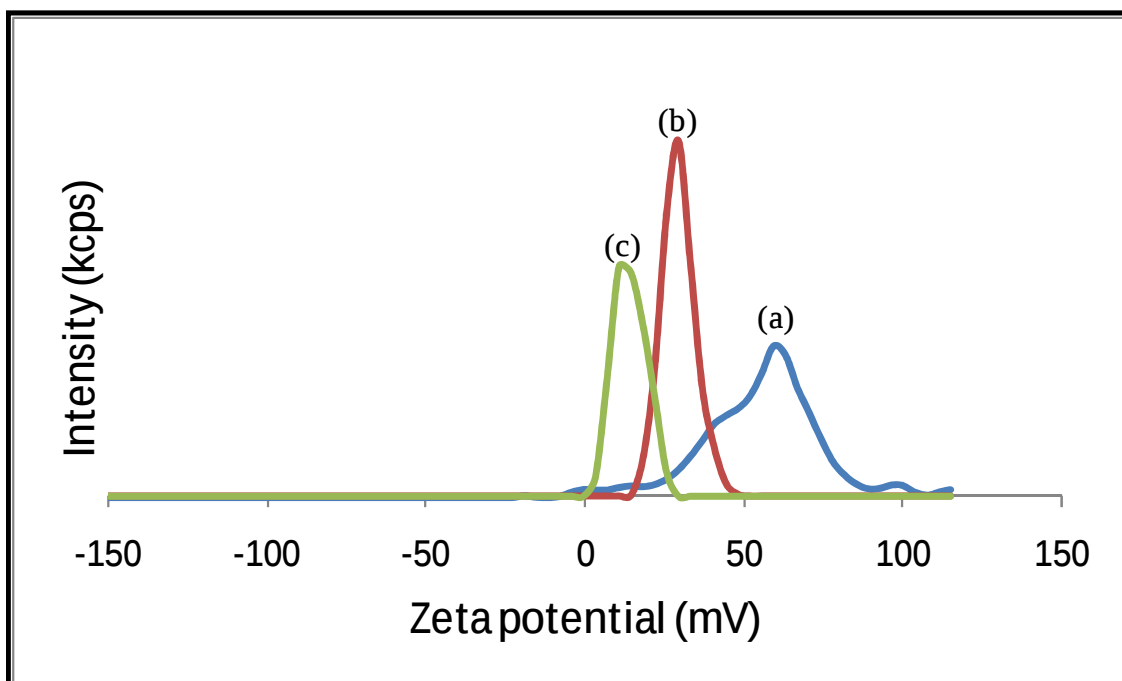


Figure S4.1: Zeta potentials of AuNRs capped by (a) CTAB, (b) 1% HS-(CH₂)₁₁-NHCO-Coumarin and (c) 50% HS-(CH₂)₁₁-NHCO-Coumarin

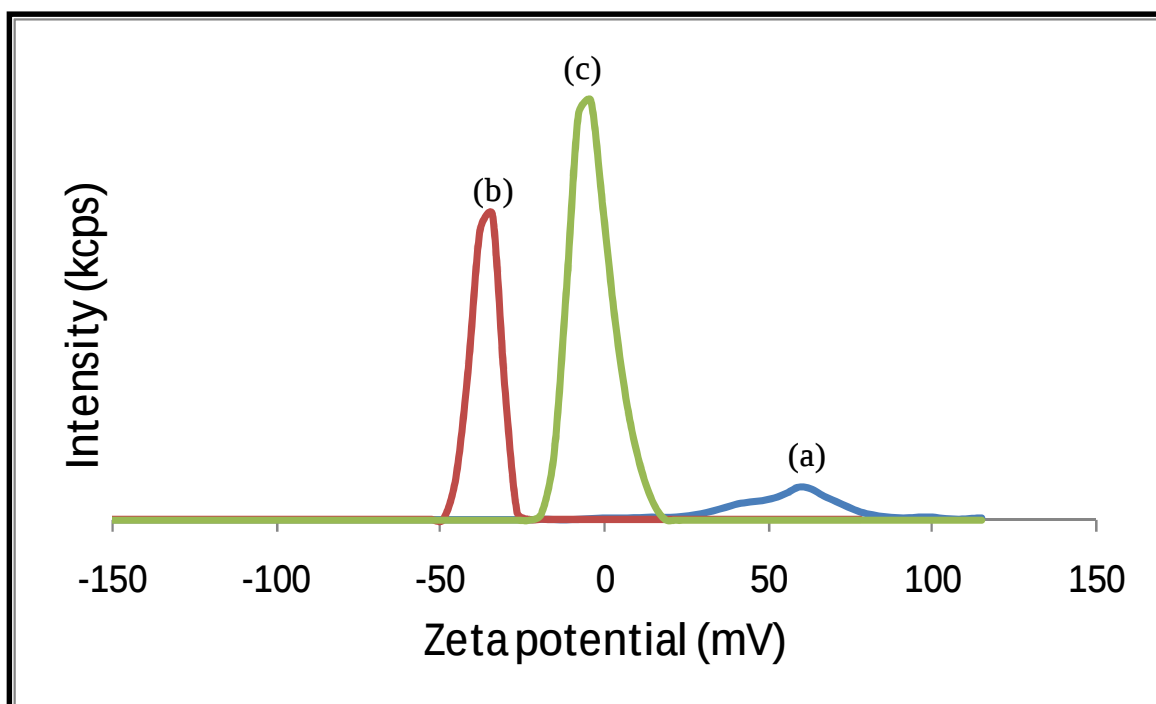


Figure S4.2: Zeta potentials of AuNRs capped by (a) CTAB, (b) 1% HS-(CH₂)₁₁-triphenylimidazole and (c) 50% HS-(CH₂)₁₁-triphenylimidazole

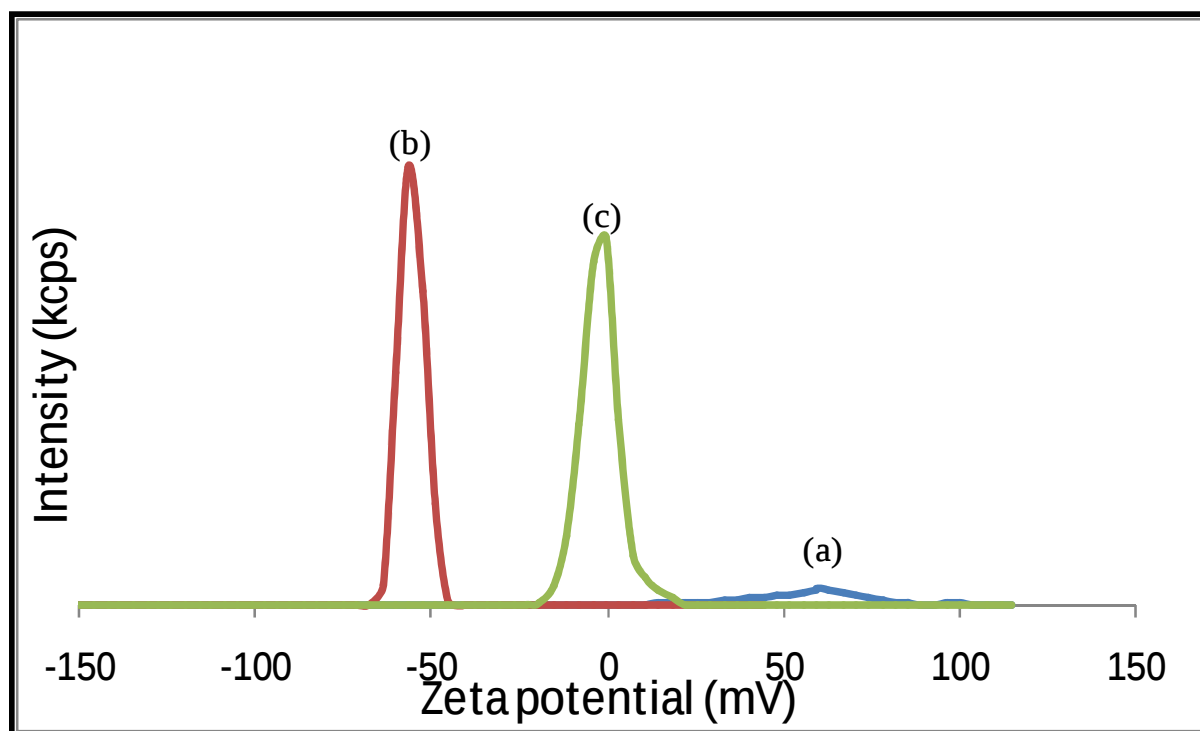


Figure S4.3: Zeta potentials of AuNRs capped by (a) CTAB, (b) 1% HS-(CH₂)₁₁-indole and (c) 50% HS-(CH₂)₁₁-indole.

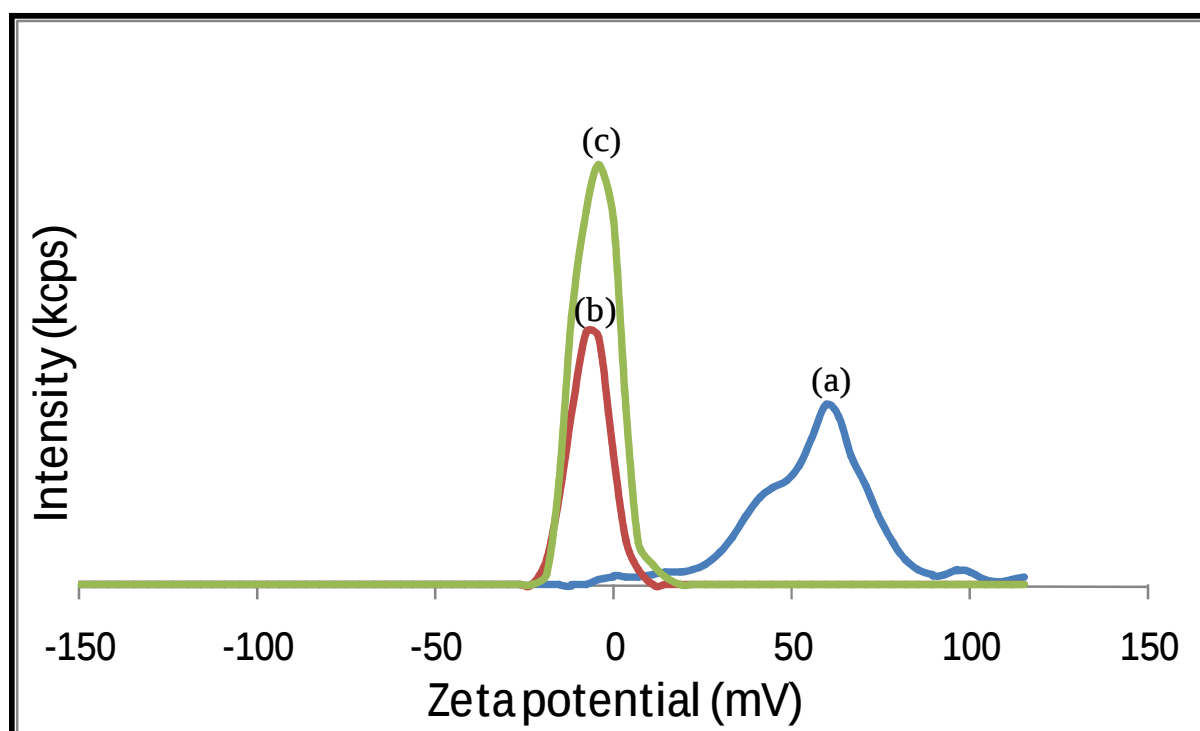


Figure S4.4: Zeta potentials of AuNRs capped by (a) CTAB, (b) 1% HS-(CH₂)₁₁-hydroquinone and (c) CTAB with 50% HS-(CH₂)₁₁-hydroquinone.

Table S4.1: Summary of the peak assignments of the HS-(CH₂)₁₁-NHCO-coumarin different stoichiometric ratios adsorbed on CTAB-capped AuNRs

Band Assignment	HS-Coumarin	1% HS-Coumarin on AuNRs	50% HS-Coumarin on AuNRs
	Peak frequency (cm ⁻¹)		
ν (Au-S)	no peak	236(shoulder)	236 (shoulder)
ν (C-S)	739 (small)	739 (strong)	739 (very strong)
ν (CC) aliph rings	814, 864, 962	964	982 (broad)
δ (CH ₂) asym	1410	1400 (shoulder),1438	1396
ν (CC) arom rings	1564, 1622	1570, 1650	1628
ν (-S-H)	2570, 2590	no peak	no peak

HS-Coumarin = HS-(CH₂)₁₁-NHCO-coumarin, AuNRs = Gold nanorods, Aliph = aliphatic, arom = aromatic, asym=asymmetric

Table S4.2: Summary of the peak assignments of the HS-(CH₂)₁₁-triphenylimidazole different stoichiometric ratios adsorbed on CTAB-capped AuNRs

Band Assignment	TPI	1% TPI on AuNRs	50% TPI on AuNRs
	Peak frequency (cm ⁻¹)		
$\nu(\text{Au-S})$	no peak	258(shoulder)	258 (shoulder)
$\nu(\text{C-S})$	740	752	752
$\nu(\text{CC})$ aliph rings	796, 844	892	898
$\delta(\text{CH}_2)$ asym	1416, 1470	1448	1450
$\nu(\text{CC})$ arom rings	1564, 1622	1558, 1622(shoulder)	1548, 1608(shoulder)
$\nu(\text{-S-H})$	2572	no peak	no peak

TPI = HS-(CH₂)₁₁-Triphenylimidazole, AuNRs = Gold nanorods, Aliph = aliphatic, arom = aromatic, asym=asymmetric

Table S4.3: Summary of the peak assignments of the HS-(CH₂)₁₁-indole different stoichiometric ratios adsorbed on CTAB-capped AuNRs

Band Assignment	HSI	1% HSI on AuNRs	50% HSI on AuNRs
	Peak frequency (cm ⁻¹)		
$\nu(\text{Au-S})$	no peak	266	266
$\nu(\text{C-S})$	766	752	752
$\nu(\text{CC})$ aliph rings	894, 938	890	890
$\delta(\text{CH}_2)$ asym	1418, 1490	1396, 1450	1434
$\nu(\text{CC})$ arom rings	1578	1558, 1590	1568
$\nu(\text{-S-H})$	2544	no peak	no peak

HSI= HS-(CH₂)₁₁-indole , AuNRs = Gold nanorods, Aliph = aliphatic, arom = aromatic, asym=asymmetric

Table S4.4: Summary of the peak assignments of the HS-(CH₂)₁₁-hydroquinone different stoichiometric ratios adsorbed on CTAB-capped AuNRs

Band Assignment	HQ	1% HQ on AuNRs	50% HQ on AuNRs
	Peak frequency (cm ⁻¹)		
$\nu(\text{Au-S})$	no peak	290	290
$\nu(\text{C-S})$	730	752	752
$\nu(\text{CC})$ aliph rings	794	890	816, 890
$\delta(\text{CH}_2)$ asym	1294	1296	1284, 1354
$\nu(\text{CC})$ arom rings	1444	1468	1452
$\nu(\text{-S-H})$	2586	no peak	no peak

HQ = HS-(CH₂)₁₁-hydroquinone , AuNRs = Gold nanorods, Aliph = aliphatic, arom = aromatic, asym=asymmetric

Chapter five

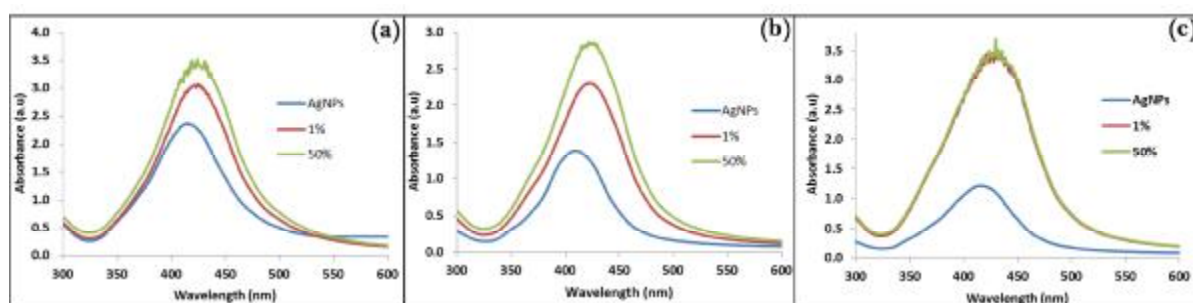


Figure S5.1: Absorption spectra of different AgNPs sizes with their corresponding AgMMPCs in different stoichiometric ratios of HS-(CH₂)₁₁-NHCO-Coumarin (a) 16 nm, (b) 30 nm and (c) 40 nm.

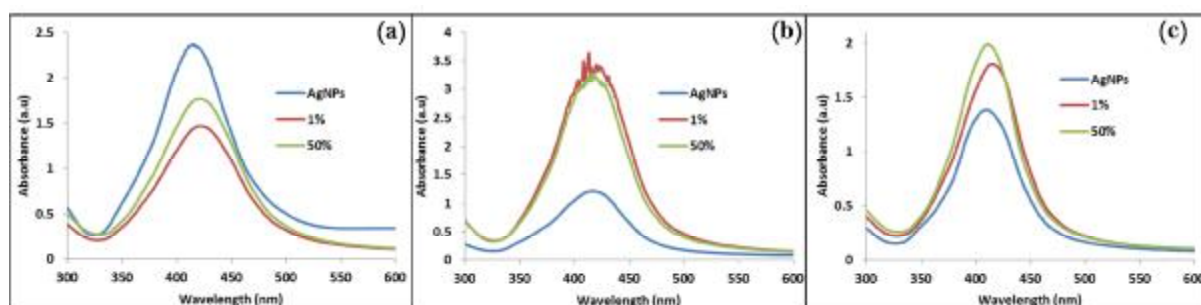


Figure S5.2: Absorption spectra of different AgNPs sizes with their corresponding AgMMPCs in different stoichiometric ratios of HS-(CH₂)₁₁-triphenylimidazole (a) 16 nm, (b) 30 nm and (c) 40 nm.

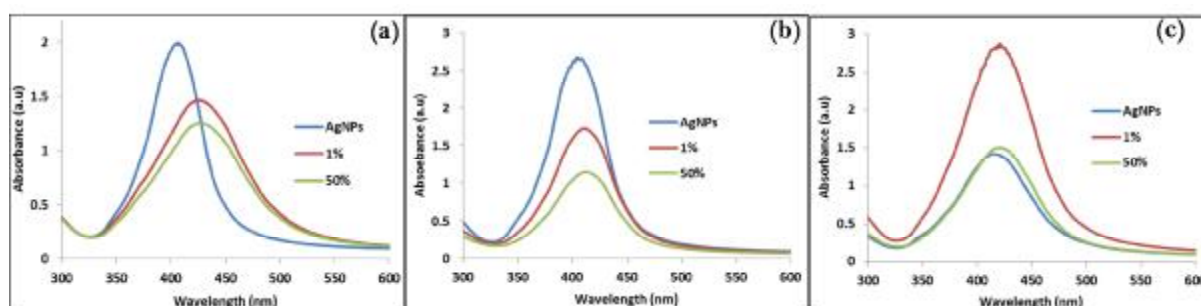


Figure S5.3: Absorption spectra of different AuNPs sizes with their corresponding AgMMPCs in different stoichiometric ratios of HS-(CH₂)₁₁-indole (a) 16 nm, (b) 30 nm and (c) 40 nm.

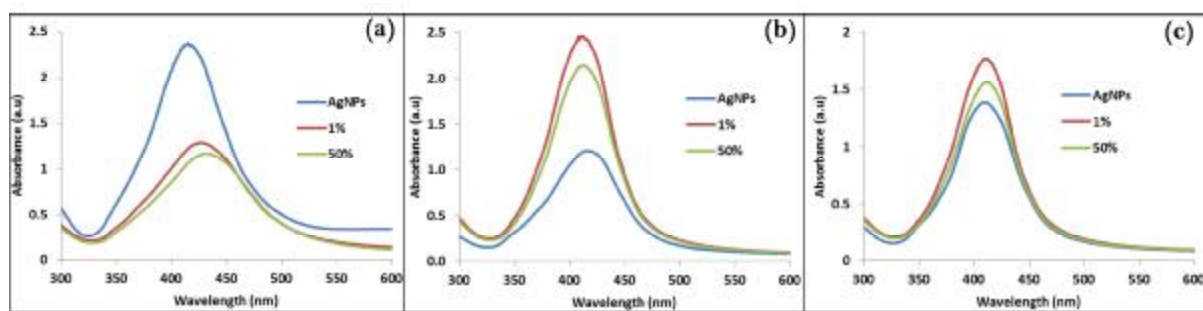


Figure S5.4: Absorption spectra of different AgNPs sizes with their corresponding AgMMPCs in different stoichiometric ratios of HS-(CH₂)₁₁-hydroquinone (a) 16 nm, (b) 30 nm and (c) 40 nm.

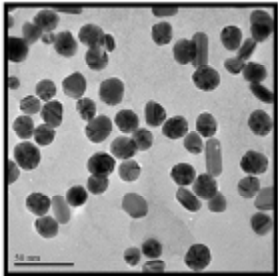
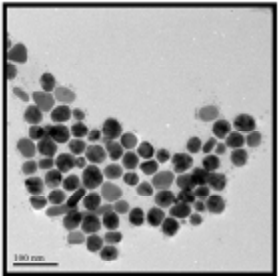
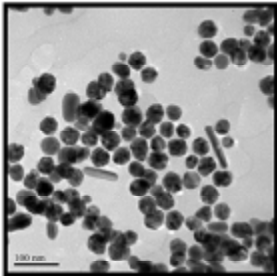
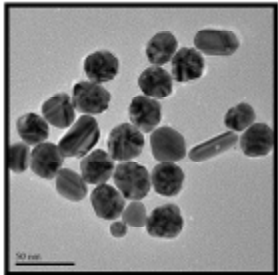
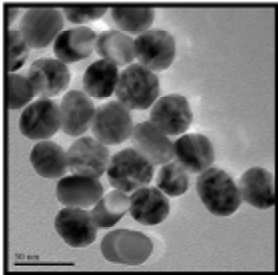
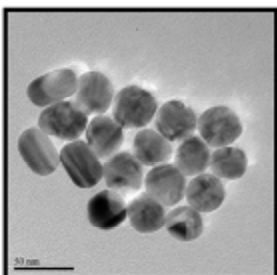
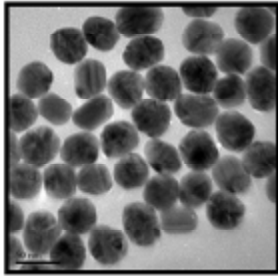
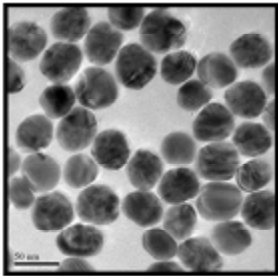
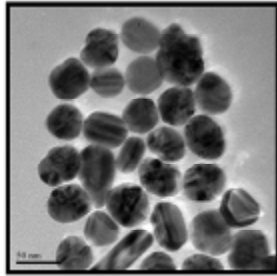
Ligands	Citrate	1% Raman reporter	50% Raman reporter
Sizes 14 nm			
30 nm			
40 nm			

Figure S5.5: TEM images of AgNPs with their AgMMPCs of different stoichiometric ratios of HS-(CH₂)₁₁-indole.

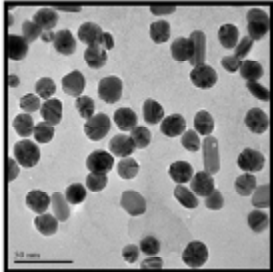
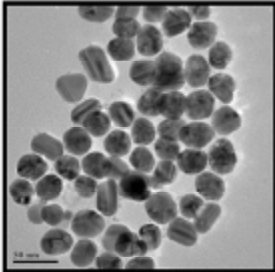
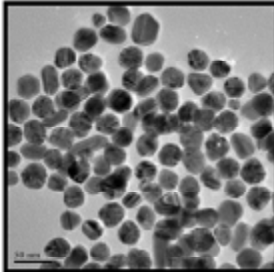
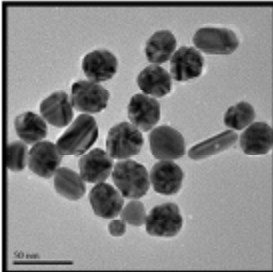
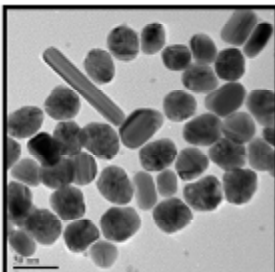
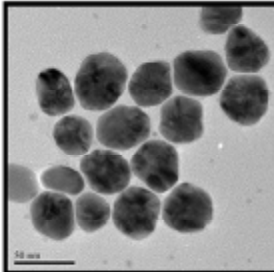
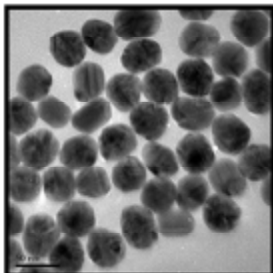
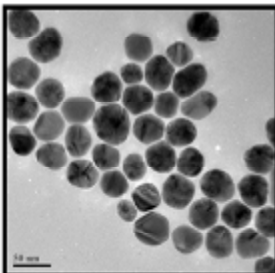
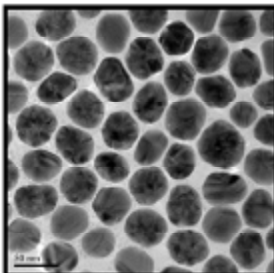
Ligands	Citrate	1% Raman reporter	50% Raman reporter
Sizes 14 nm			
30 nm			
40 nm			

Figure S5.6: TEM images of AgNPs with corresponding AgMMPCs of different stoichiometric ratios of HS-(CH₂)₁₁-hydroquinone.

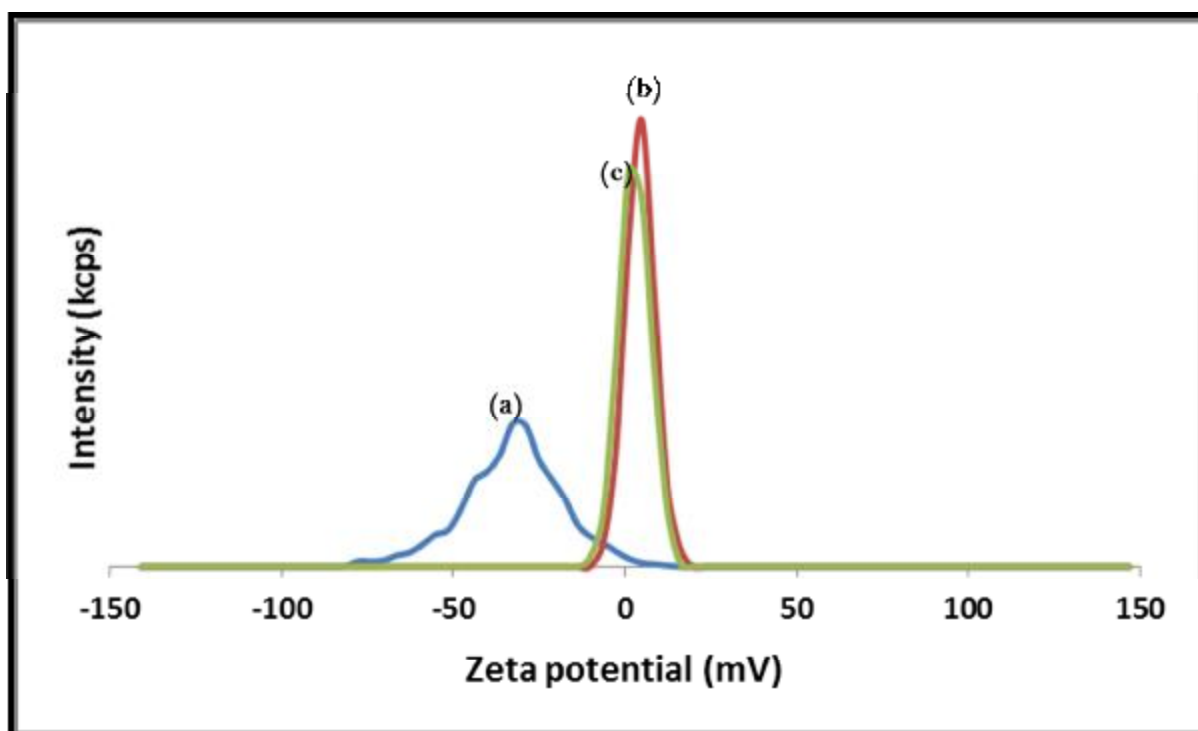


Figure S5.7: Zeta potentials of AgNPs 16nm stabilized by (a) Citrate (b) 1% HS-(CH₂)₁₁-NHCO-coumarin (c) 50% HS-(CH₂)₁₁-NHCO-coumarin.

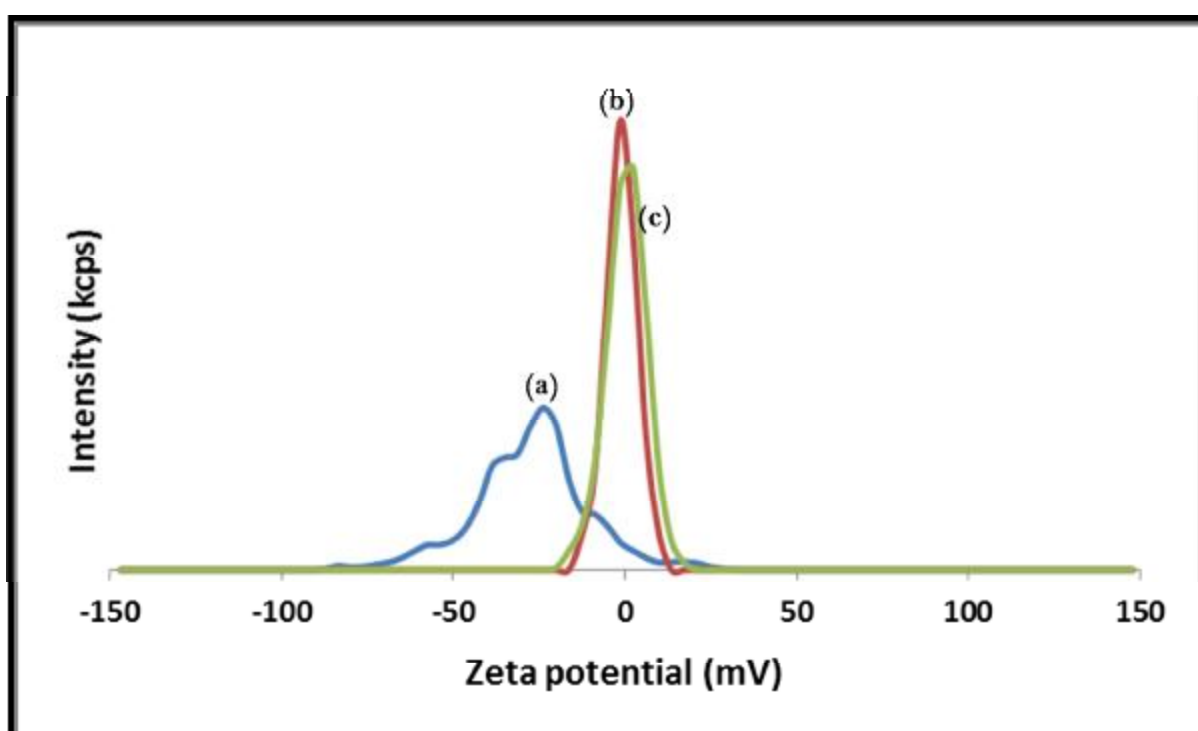


Figure S5.8: Zeta potentials of AgNPs 30 nm stabilized by (a) Citrate (b) 1% HS-(CH₂)₁₁-NHCO-coumarin (c) 50% HS-(CH₂)₁₁-NHCO-coumarin.

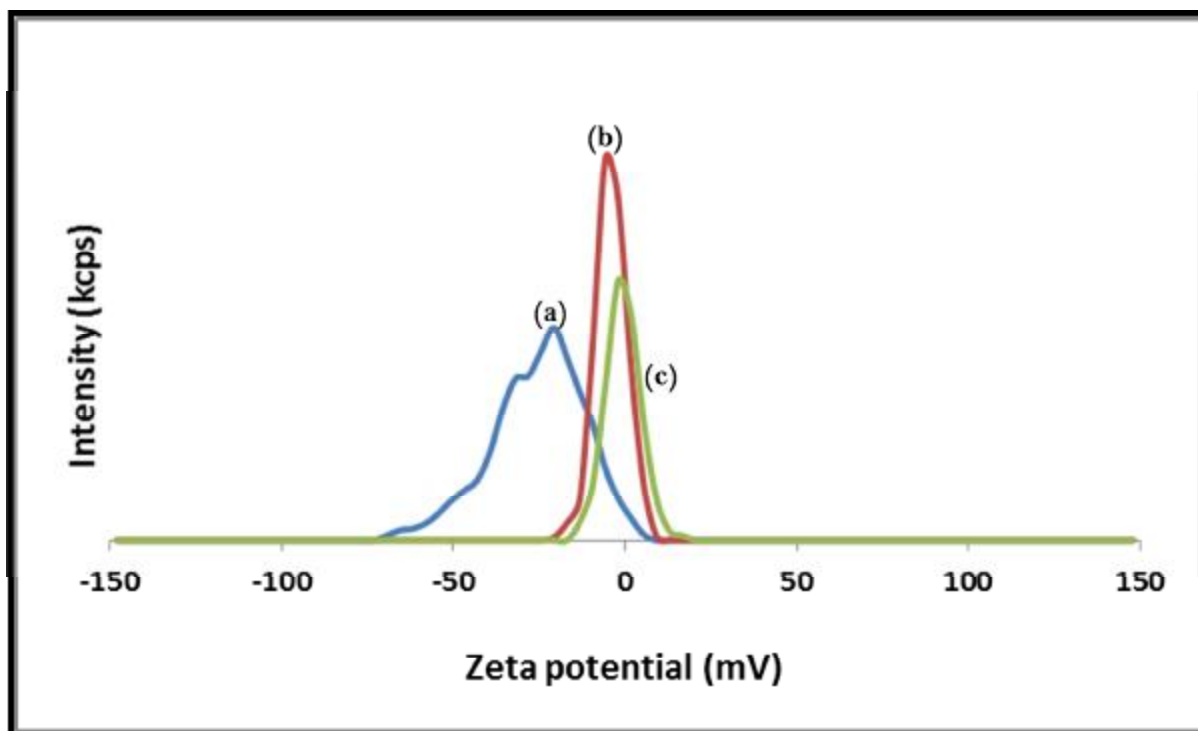


Figure S5.9: Zeta potentials of AgNPs 40 nm stabilized by (a) Citrate (b) 1% HS-(CH₂)₁₁-NHCO-coumarin (c) 50% HS-(CH₂)₁₁-NHCO-coumarin.

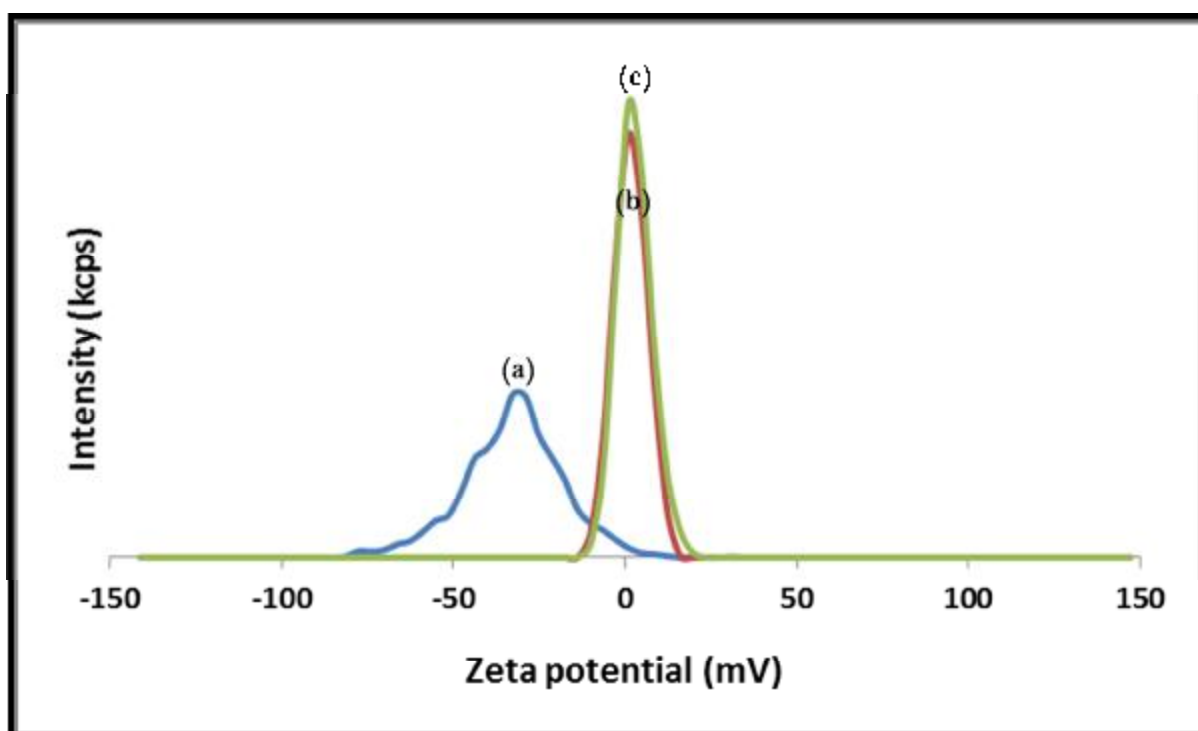


Figure S5.10: Zeta potentials of AgNPs 16 nm stabilized (a) Citrate (b) 1% HS-(CH₂)₁₁-triphenylimidazole and (c) 50% HS-(CH₂)₁₁-triphenylimidazole.

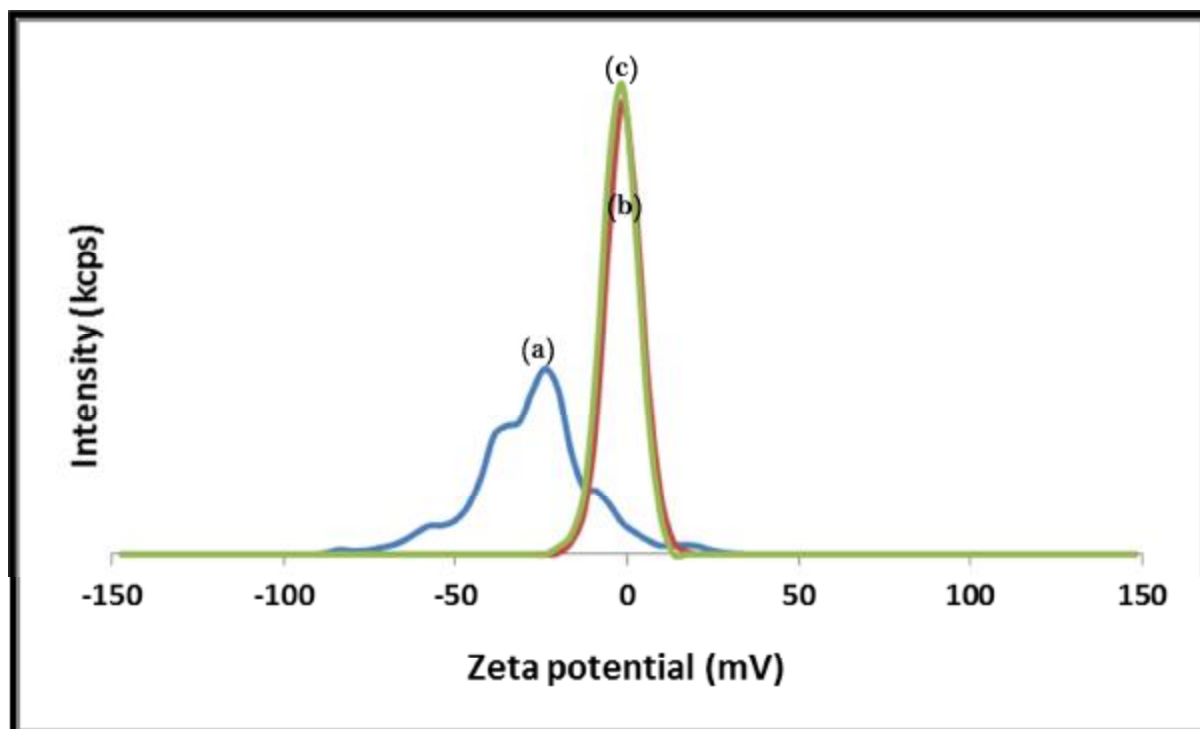


Figure S5.11: Zeta potentials of AgNPs 30 nm stabilized (a) Citrate (b) 1% HS-(CH₂)₁₁-triphenylimidazole and (c) 50% HS-(CH₂)₁₁-triphenylimidazole.

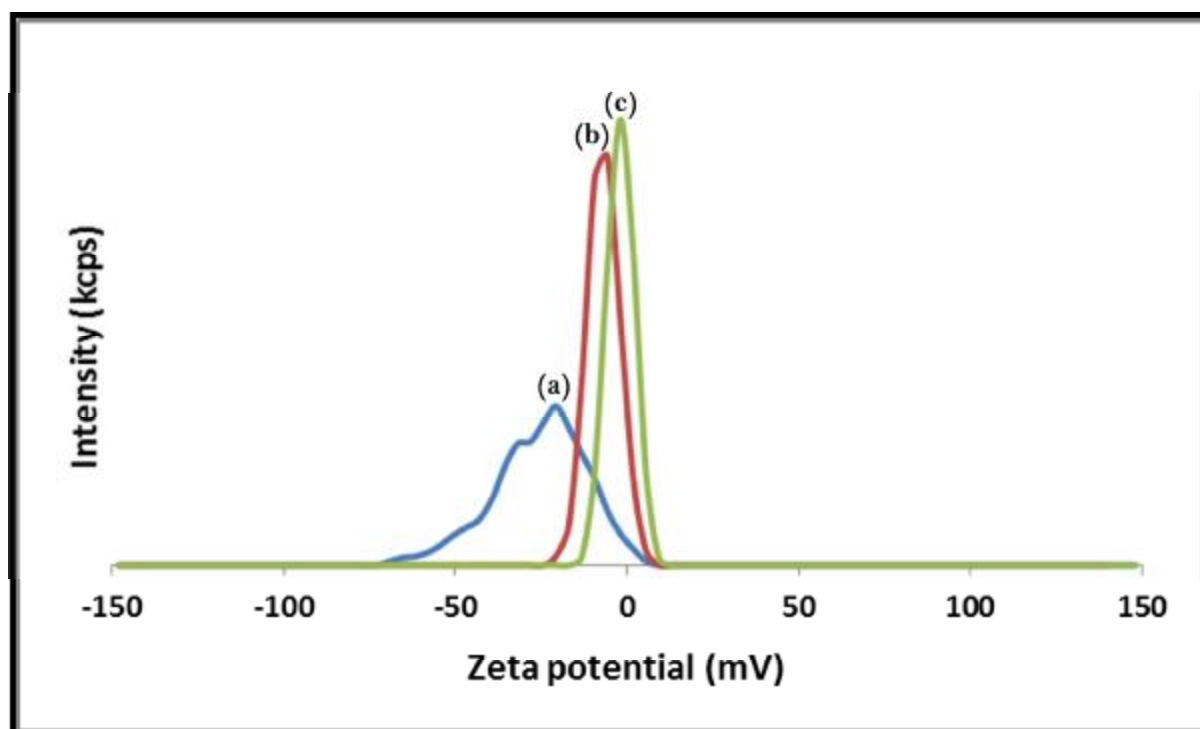


Figure S5.12: Zeta potentials of AgNPs 40 nm stabilized (a) Citrate (b) 1% HS-(CH₂)₁₁-triphenylimidazole and (c) 50% HS-(CH₂)₁₁-triphenylimidazole.

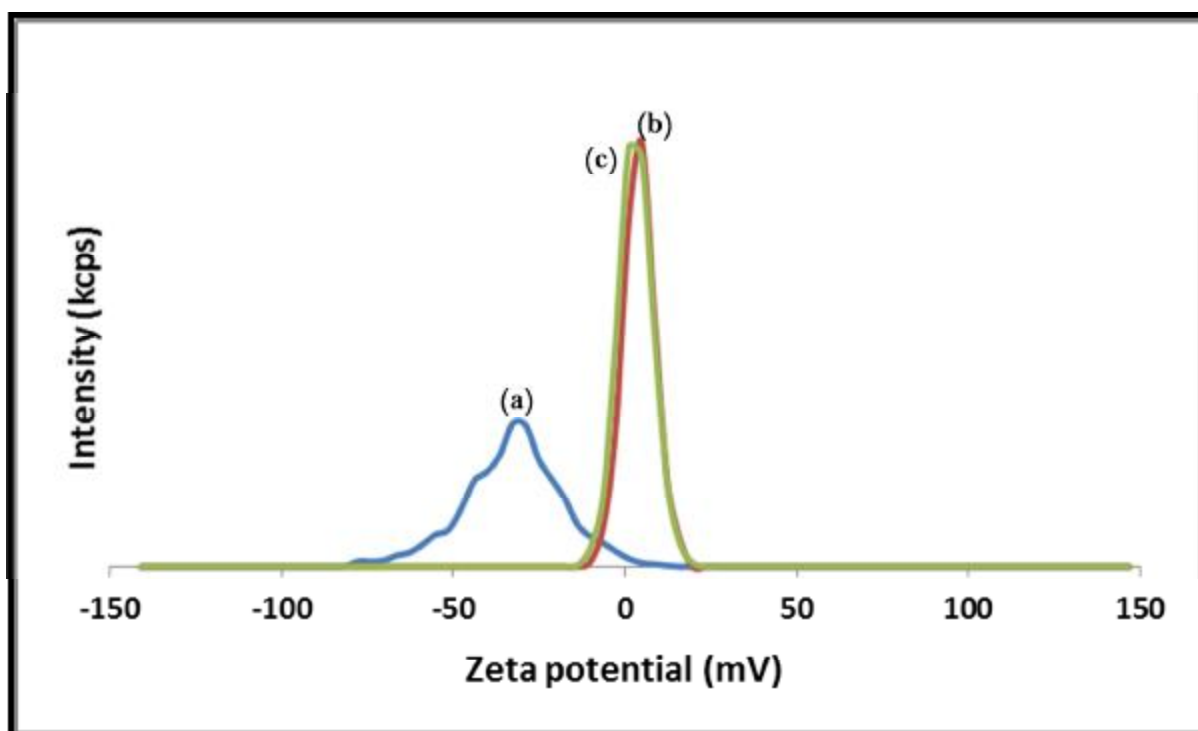


Figure S5.13: Zeta potentials of AgNPs 16 nm stabilized by (a) Citrate, (b) 1% HS-(CH₂)₁₁-indole and (c) 50% HS-(CH₂)₁₁-indole.

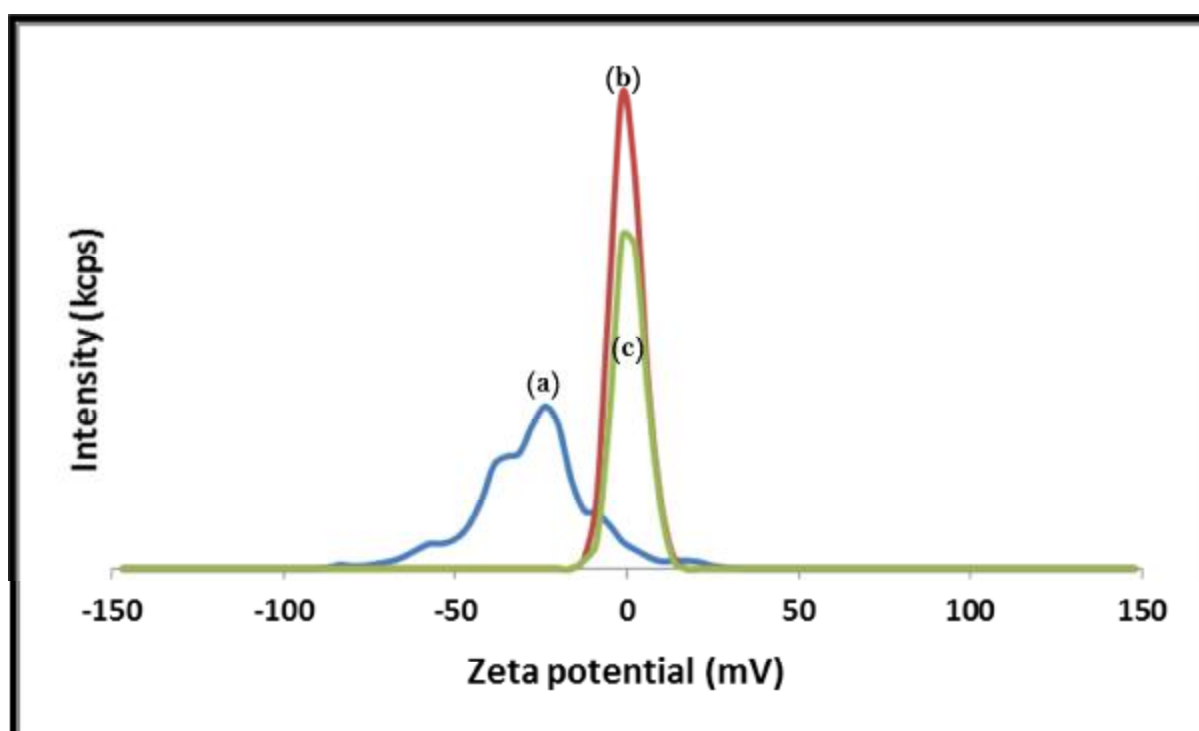


Figure S5.14: Zeta potentials of AgNPs 30 nm stabilized by (a) Citrate, (b) 1% HS-(CH₂)₁₁-indole and (c) 50% HS-(CH₂)₁₁-indole.

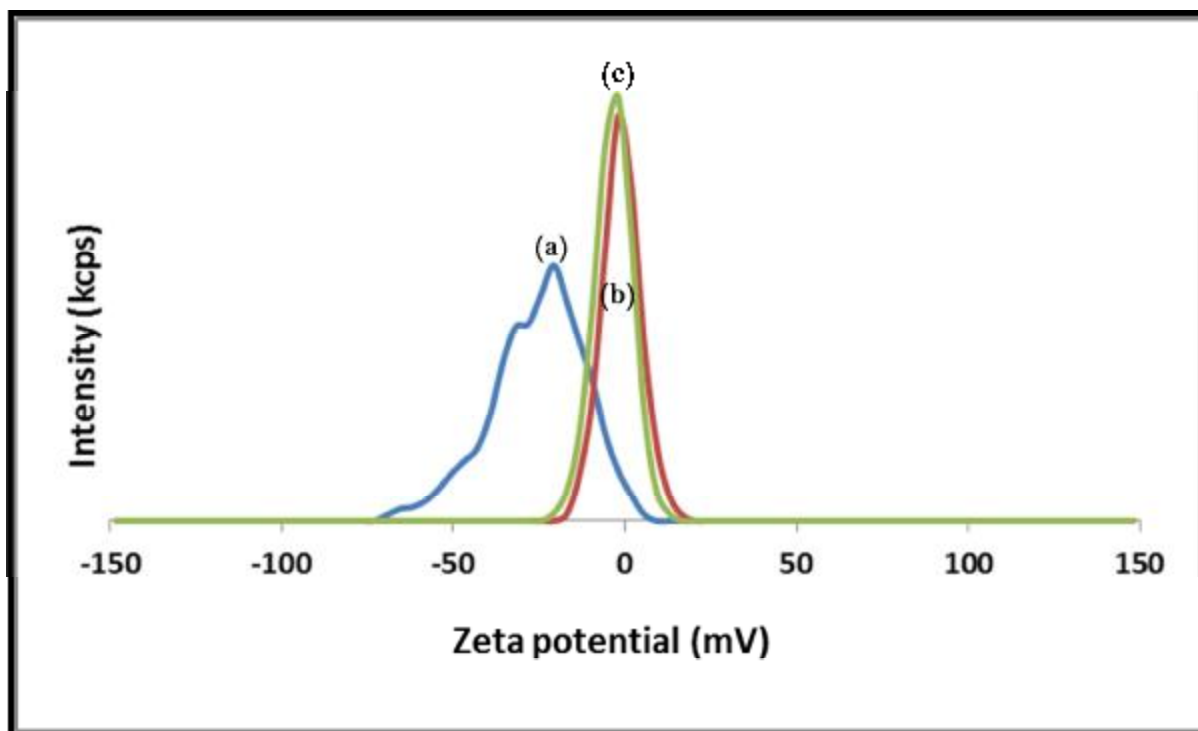


Figure S5.15: Zeta potentials of AgNPs 40 nm stabilized by (a) Citrate, (b) 1% HS-(CH₂)₁₁-indole and (c) 50% HS-(CH₂)₁₁-indole.

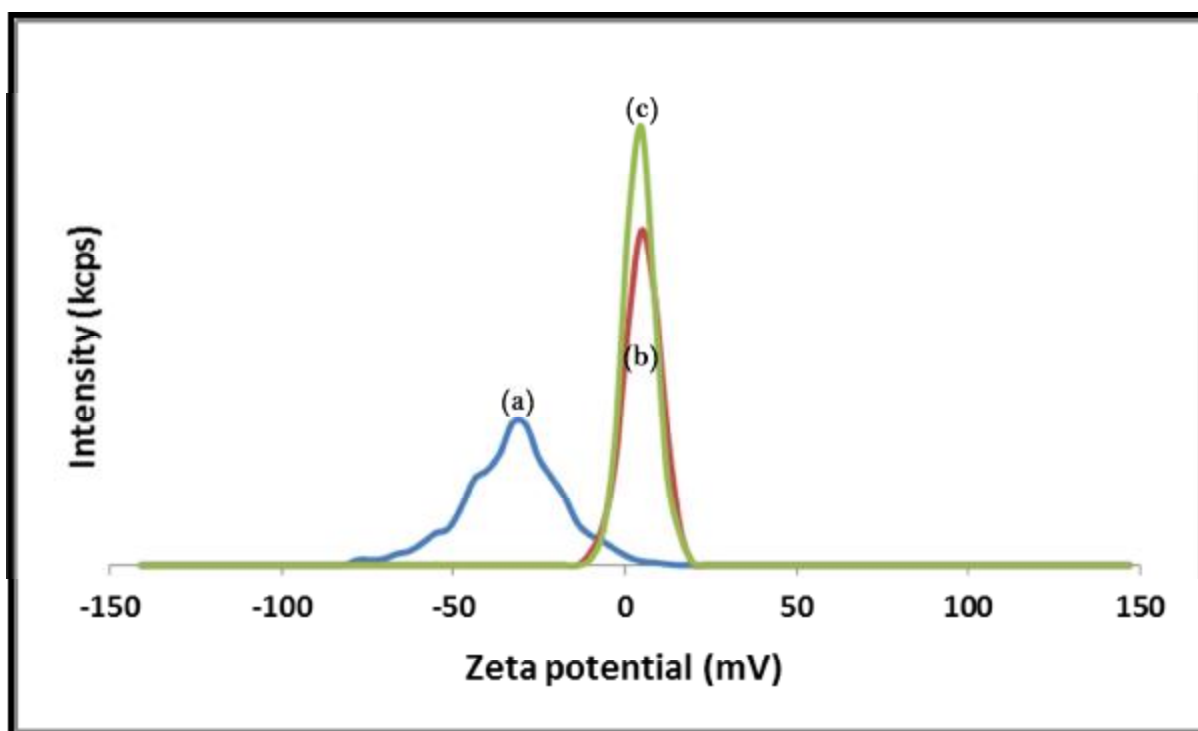


Figure S5.16: Zeta potentials of AgNPs 16 nm stabilized by (a) Citrate (b) 1% HS-(CH₂)₁₁-hydroquinone and (c) 50% HS-(CH₂)₁₁-hydroquinone.

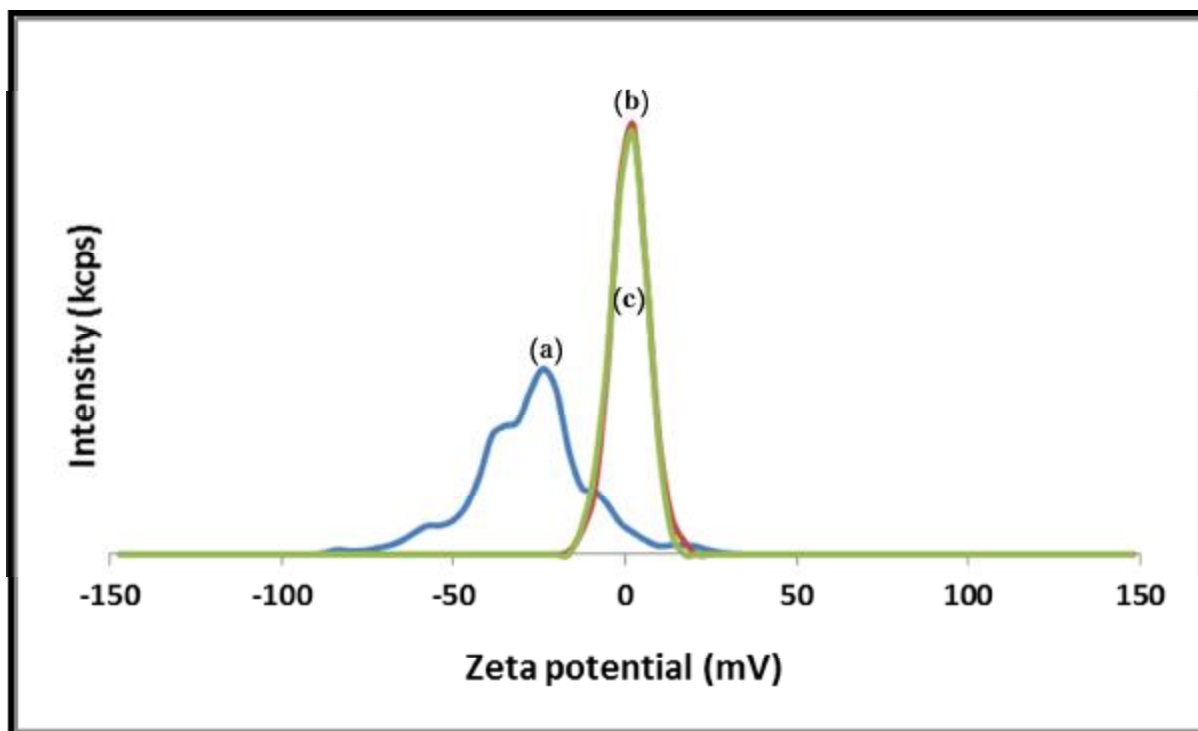


Figure S5.17: Zeta potentials of AgNPs 30 nm stabilized by (a) Citrate (b) 1%HS-(CH₂)₁₁-hydroquinone and (c) 50% HS-(CH₂)₁₁-hydroquinone.

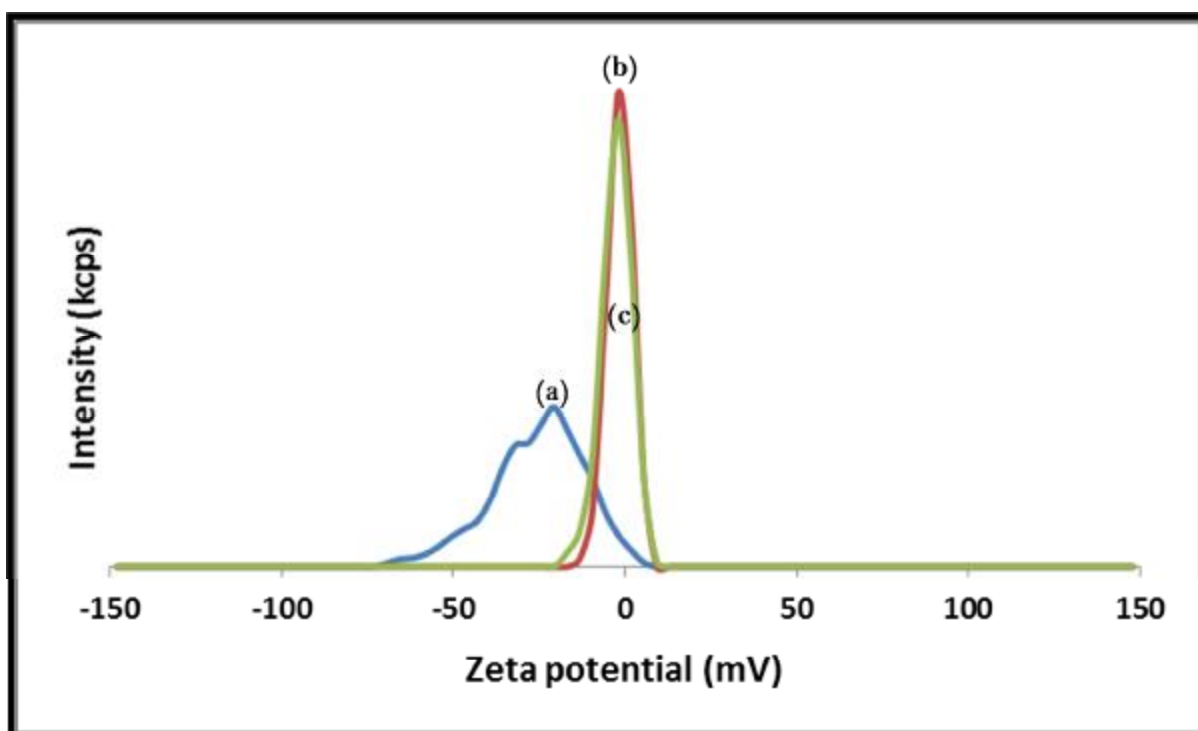


Figure S5.18: Zeta potentials of AgNPs 40 nm stabilized by (a) Citrate (b) 1%HS-(CH₂)₁₁-hydroquinone and (c) 50% HS-(CH₂)₁₁-hydroquinone.

Table S5.1: Summary of the peak assignments of HS-(CH₂)₁₁-NHCO-coumarin different stoichiometric ratios adsorbed on different AgNPs

Band assignment	$\nu(\text{Ag-S})$	$\nu(\text{C-S})$	$\nu(\text{CC})$ aliph	$\delta(\text{CH}_2)$ asym	$\nu(\text{CC})$ arom rings	$\nu(\text{-S-H})$
HS-Coumarin	no peak	739 (small)	814, 864, 962	1410	1564, 1622	2570, 2590
Ag 16 nm 1% HS-Coumarin	234	674 (broad), 732, 778	850, 952, 1092	1400	1582	no peak
Ag 16 nm 50% HS-Coumarin	236	674 (broad), 732 (shoulder)	854, 952, 1034	1398, 1394,	1574, 1624	no peak
Ag 30 nm 1% HS-Coumarin	238	688 (broad)	850, 950, 1074, 1258	1464	1582, 1606	no peak
Ag 30 nm 50% HS-Coumarin	238	688 (broad), 738 (shoulder)	846, 952, 1018, 1070, 1200	1396, 1400,	1584, 1626	no peak
Ag 40 nm 1% HS-Coumarin	232	674 (broad), 782	838, 954, 1028, 1100	1484, 1404,	1562, 1610, 1586, 1612,	no peak
Ag 40 nm 50% HS-Coumarin	250	674 (broad)	856, 944, 1070	1474	1664	no peak

HS-Coumarin = HS-(CH₂)₁₁-NHCO-coumarin, Ag = Silver, Aliph = aliphatic, arom = aromatic, asym=asymmetric

Table S5.2: Summary of the peak assignments of HS-(CH₂)₁₁-triphenylimidazole different stoichiometric ratios adsorbed on different AgNPs

Band assignment	$\nu(\text{Ag-S})$	$\nu(\text{C-S})$	$\nu(\text{CC})$ aliph	$\delta(\text{CH}_2)$ asym	$\nu(\text{CC})$ arom rings	$\nu(\text{-S-H})$
HS-Coumarin	no peak	740	796, 844	1416, 1470	1564, 1622	2572
Ag 16 nm 1% TPI	234	680	860, 932, 1066, 1116, 1248	1382, 1460	1554 (small), 1600 (small)	no peak
Ag 16 nm 50% TPI	222	675	854, 922, 1046, 1110, 1256	1398, 1458	1552, 1608	no peak
Ag 30 nm 1% TPI	244	688	858, 932, 1076, 1118, 1284	1416, 1458	1554, 1606	no peak
Ag 30 nm 50% TPI	226 (shoulder)	672	844, 920, 1044, 1112, 1260	1390, 1460	1538, 1612	no peak
Ag 40 nm 1% TPI	230 (broad)	680	822, 852, 926, 976, 1058, 1114, 1258	1314, 1392,1470	1556, 1604	no peak
Ag 40 nm 50% TPI	238 (broad)	670	824, 858, 930, 1046, 1114, 1260	1360, 1458	1556, 1604	no peak

TPI = HS-(CH₂)₁₁-Triphenylimidazole, Ag = Silver, Aliph = aliphatic, arom = aromatic, asym=asymmetric

Table S5.3: Summary of the peak assignments of HS-(CH₂)₁₁-indole different stoichiometric ratios adsorbed on different AgNPs

Band assignment	$\nu(\text{Ag-S})$	$\nu(\text{C-S})$	$\nu(\text{CC})$ aliph	$\delta(\text{CH}_2)$ asym	$\nu(\text{CC})$ arom rings	$\nu(\text{-S-H})$
HS-Coumarin	no peak	740	796, 844	1416, 1470	1564, 1622	2572
Ag 16 nm 1% TPI	234	680	860, 932, 1066, 1116, 1248	1382, 1460	1554 (small), 1600 (small)	no peak
Ag 16 nm 50% TPI	222	675	854, 922, 1046, 1110, 1256	1398, 1458	1552, 1608	no peak
Ag 30 nm 1% TPI	244	688	858, 932, 1076, 1118, 1284	1416, 1458	1554, 1606	no peak
Ag 30 nm 50% TPI	226 (shoulder)	672	844, 920, 1044, 1112, 1260	1390, 1460	1538, 1612	no peak
Ag 40 nm 1% TPI	230 (broad)	680	822, 852, 926, 976, 1058, 1114, 1258	1314, 1392, 1470	1556, 1604	no peak
Ag 40 nm 50% TPI	238 (broad)	670	824, 858, 930, 1046, 1114, 1260	1360, 1458	1556, 1604	no peak

TPI = HS-(CH₂)₁₁-Triphenylimidazole, Ag = Silver, Aliph = aliphatic, arom = aromatic, asym=asymmetric

Table S5.4: Summary of the peak assignments of HS-(CH₂)₁₁-hydroquinone different stoichiometric ratios adsorbed on different AgNPs

Band assignment	$\nu(\text{Ag-S})$	$\nu(\text{C-S})$	$\nu(\text{CC})$ aliph	$\delta(\text{CH}_2)$ asym	$\nu(\text{CC})$ arom rings	$\nu(-\text{S-H})$
HQ	no peak 240	730	794	1294	1444	2586
Ag 16 nm 1% HQ	(shoulder) 240	672	808, 834, 940, 960, 1032, 1126, 1228	1334, 1416, 1452	1504, (small), 1542 (small)	no peak
Ag 16 nm50% HQ	(shoulder) 226	670	822, 856, 920, 978, 1048, 1116, 1252	1338, 1396, 1468	1506, (small), 1536 (small)	no peak
Ag 30 nm 1% HQ	(shoulder) 242	674	822, 862, 938, 996, 1074, 1122, 1278	1308, 1396, 1492	1506, (small), 1592 (small)	no peak
Ag 30 nm50% HQ	(shoulder)	674	822, 852, 926, 974, 1056, 1112, 1258 850, 926, 1014, 1076, 1130, 1162,	1318, 1382, 1464	1504, (small), 1598 (small)	no peak
Ag 40 nm 1% HQ	234	670	1236, 1274	1314,1390 1310, 1338,	1562, 1592, 1652	no peak
Ag 40 nm 50% HQ	234	670	858, 930, 1010, 1074, 1130, 1232, 1276	1452	1558, 1592, 1622	no peak

HQ = HS-(CH₂)₁₁-hydroquinone, Ag = Silver, Aliph = aliphatic, arom = aromatic, asym=asymmetric

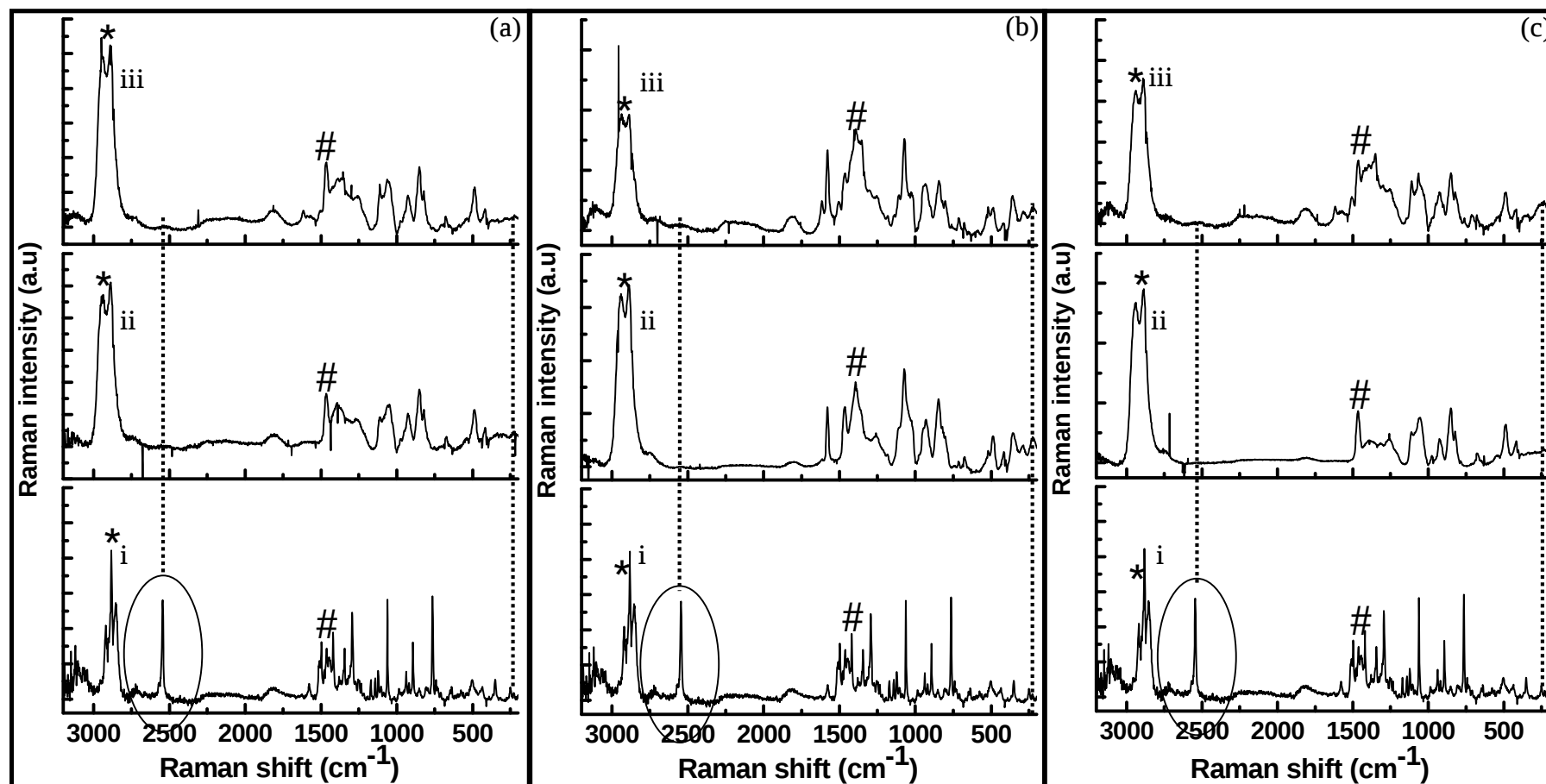


Figure S5.19: Raman spectra of AgMMPCs of different stoichiometric ratios of HS-(CH₂)₁₁-indole prepared with different AgNPs sizes (a) 16 nm, (b) 30 nm, and (c) 40 nm. The circled peak is the vibrational band of S-H, (* and #) denotes the symmetric and asymmetric bands of C-H bonds

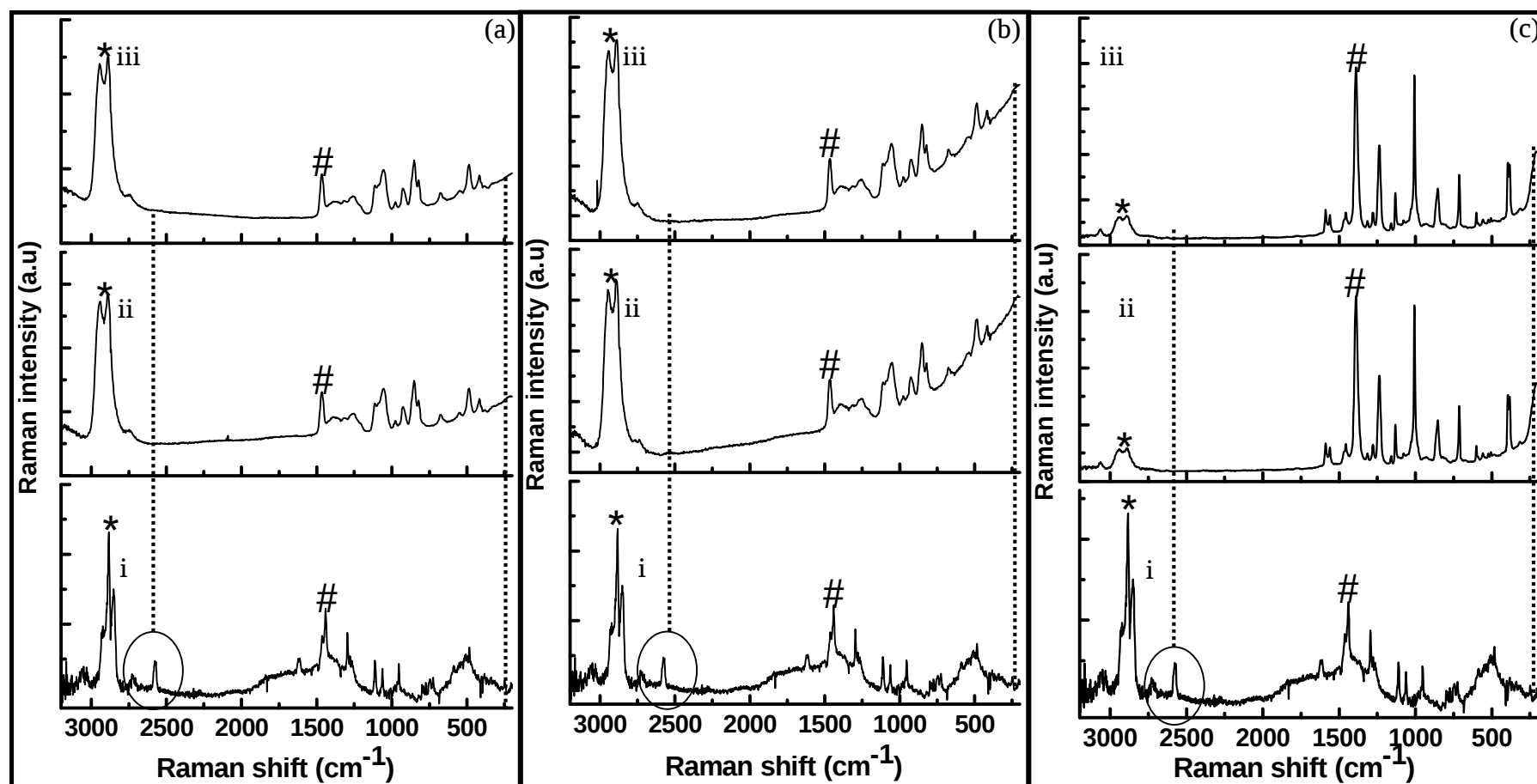


Figure S5.20: Raman spectra of AgMMPCs of different stoichiometric ratios of HS-(CH₂)₁₁-hydroquinone prepared with different AgNPs sizes (a) 16 nm, (b) 30 nm, and (c) 40 nm. The circled peak is the vibrational band of S-H, (* and #) denotes the symmetric and asymmetric bands of C-H bonds

Chapter six

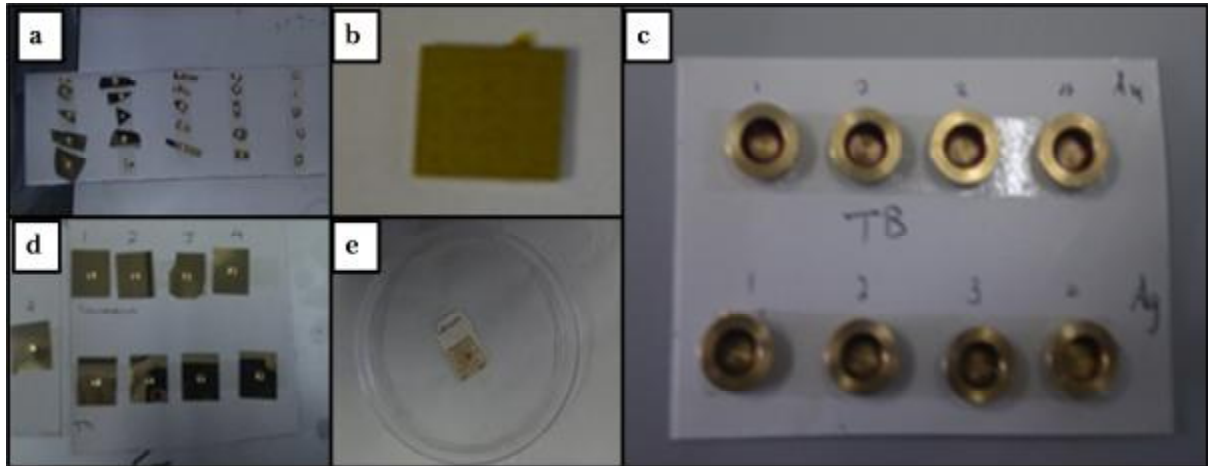


Figure S6.1: The images of gold coated substrates

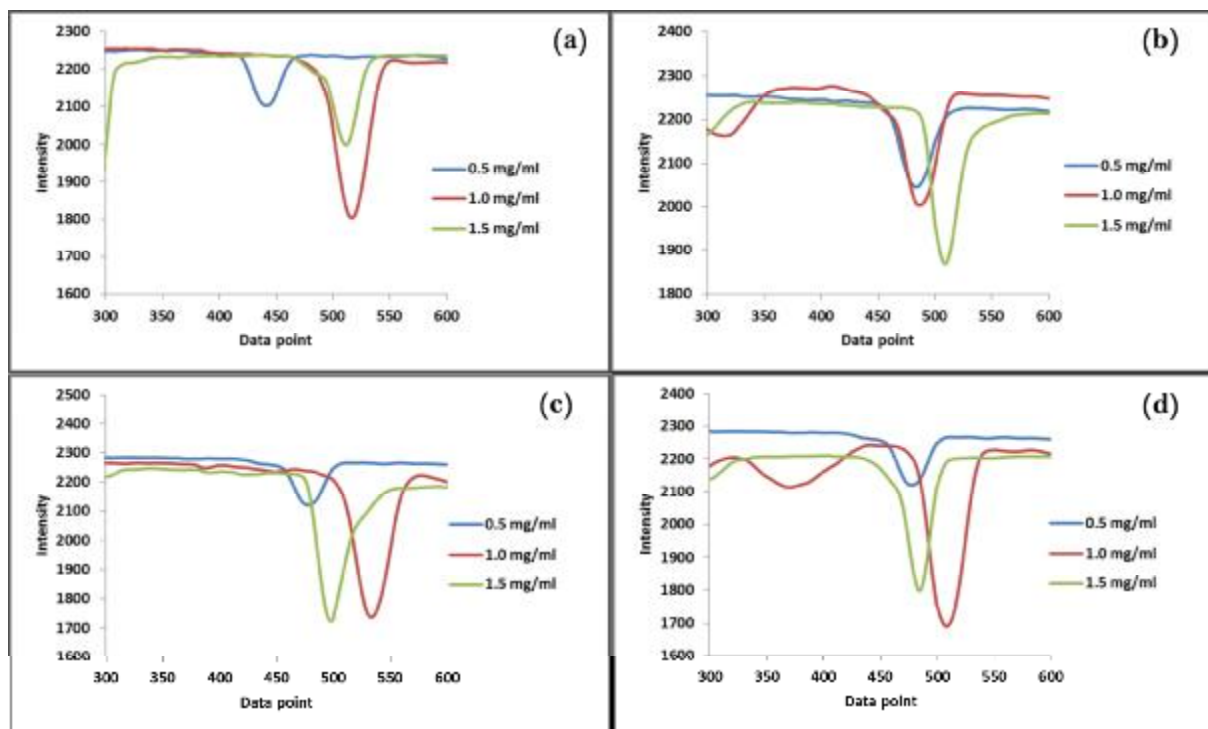


Figure S6.2: Graphs showing the change in the intensities of the test line on an immunochromatographic test with respect to change in concentration of recombinant Pv-LDH antigen with AuNPs PAN conjugates.

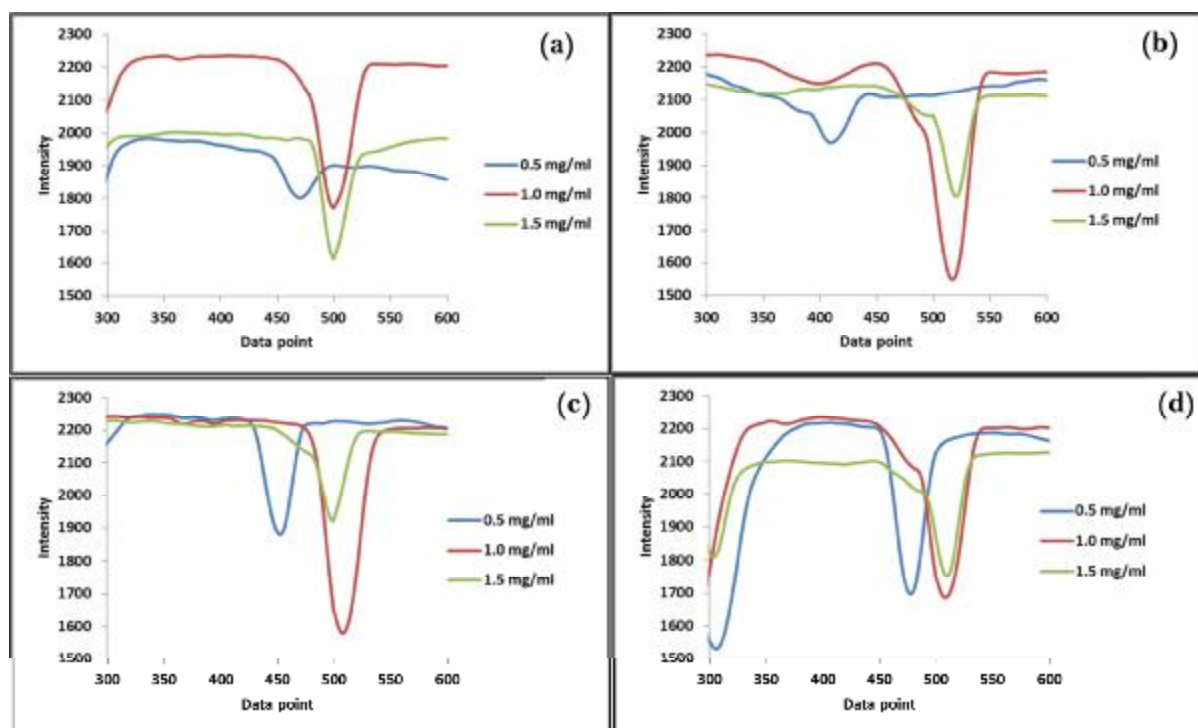


Figure S6.3: Graphs showing the change in the intensities of the test line on an immunochromatographic test with respect to change in concentration of recombinant Pv-LDH antigen with AgNPs PAN conjugates

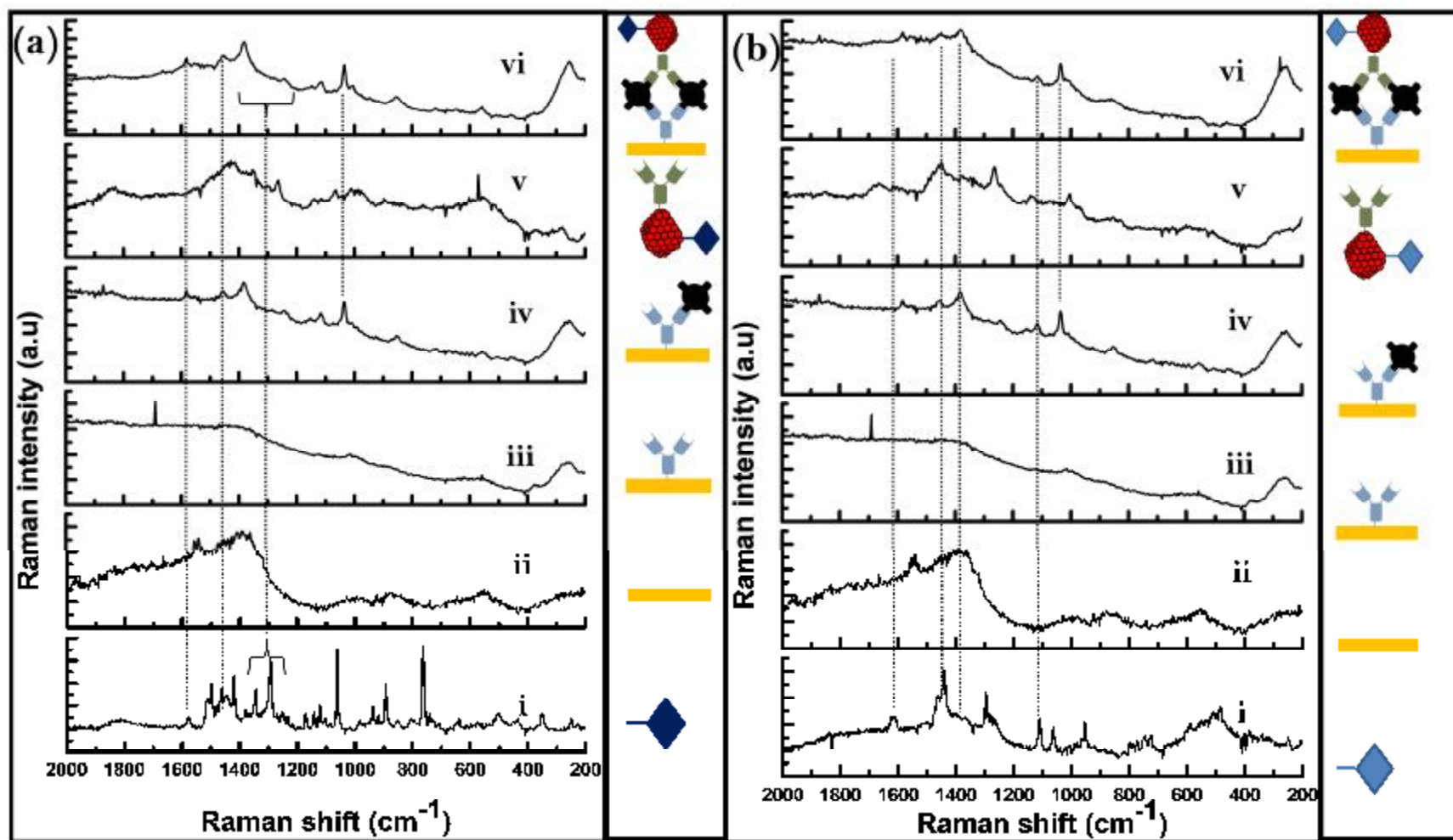


Figure S6.4: SERS immunoassay spectra, (a) AuNPs PAN- HS-(CH₂)₁₁-indole + recombinant Pv-LDH antigen and (b) AuNPs PAN- HS-(CH₂)₁₁-hydroquinone + recombinant Pv-LDH antigen

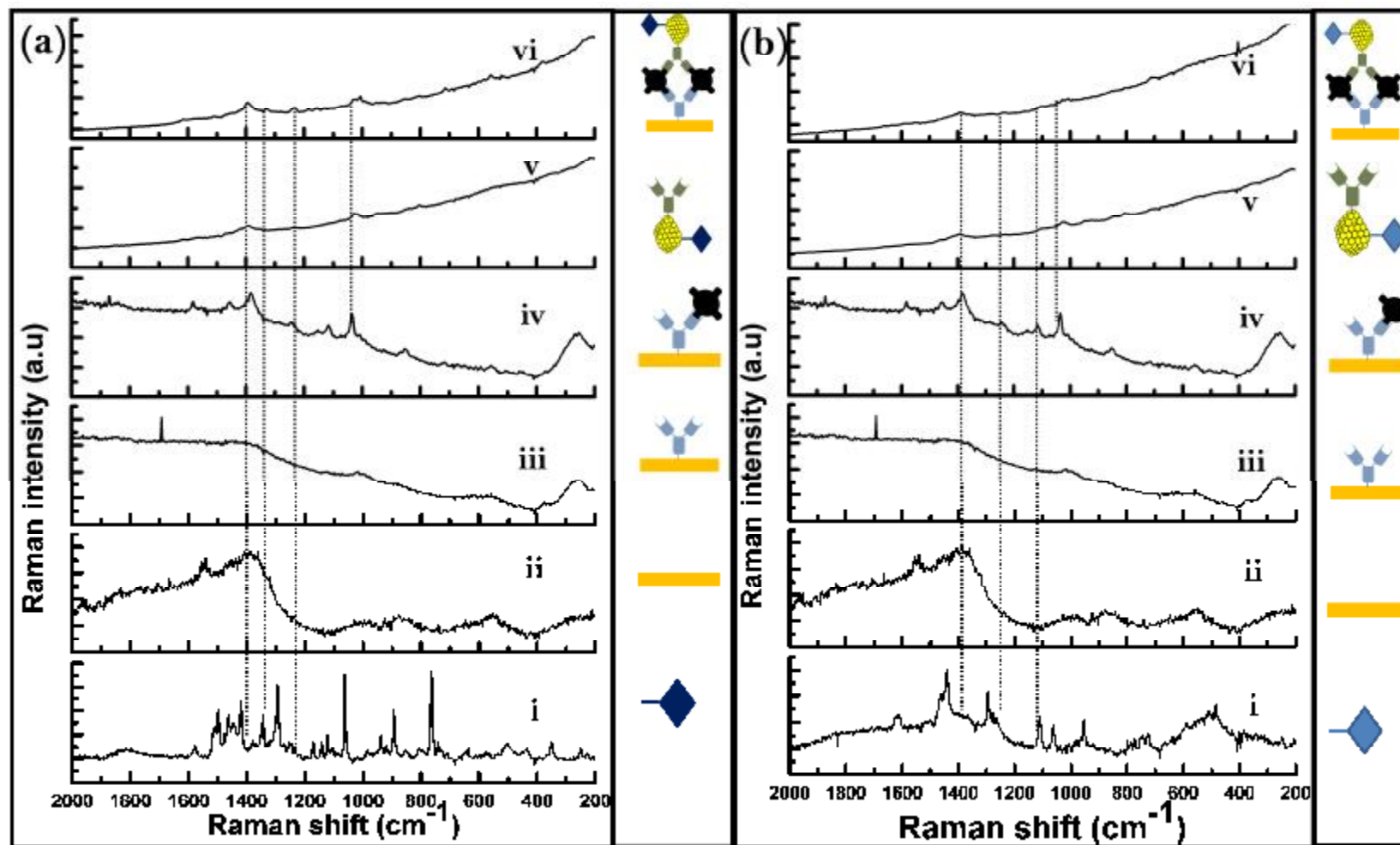


Figure S6.5: SERS immunoassay spectra, (a) AgNPs PAN- HS-(CH₂)₁₁-indole + recombinant Pv-LDH antigen and (b) AgNPs PAN- HS-(CH₂)₁₁-hydroquinone + recombinant Pv-LDH antigen

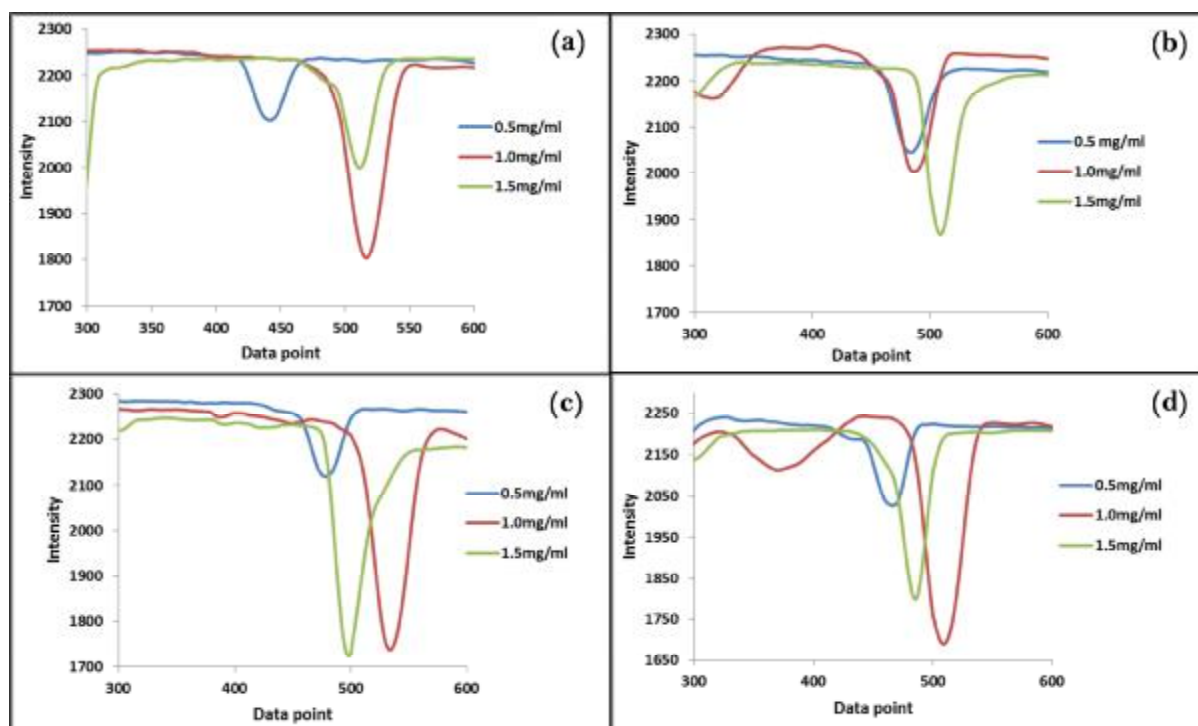


Figure S6.6: Graphs showing the change in the intensities of the test line on an immunochromatographic test with respect to change in concentration of recombinant mycobacterium tuberculosis 38 kDA antigen with AuNPs Protein A conjugates

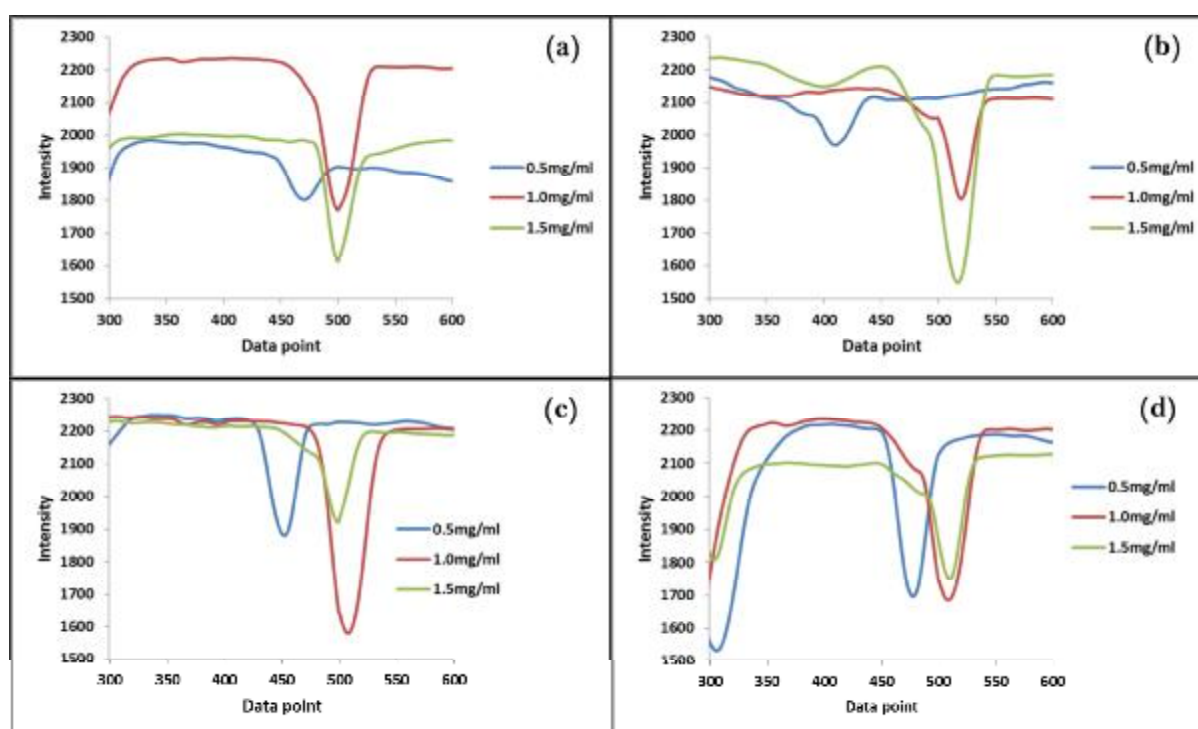


Figure S6.7: Graphs showing the change in the intensities of the test line on an immunochromatographic test with respect to change in concentration of recombinant mycobacterium tuberculosis 38 kDA antigen with AuNPs Protein A conjugates

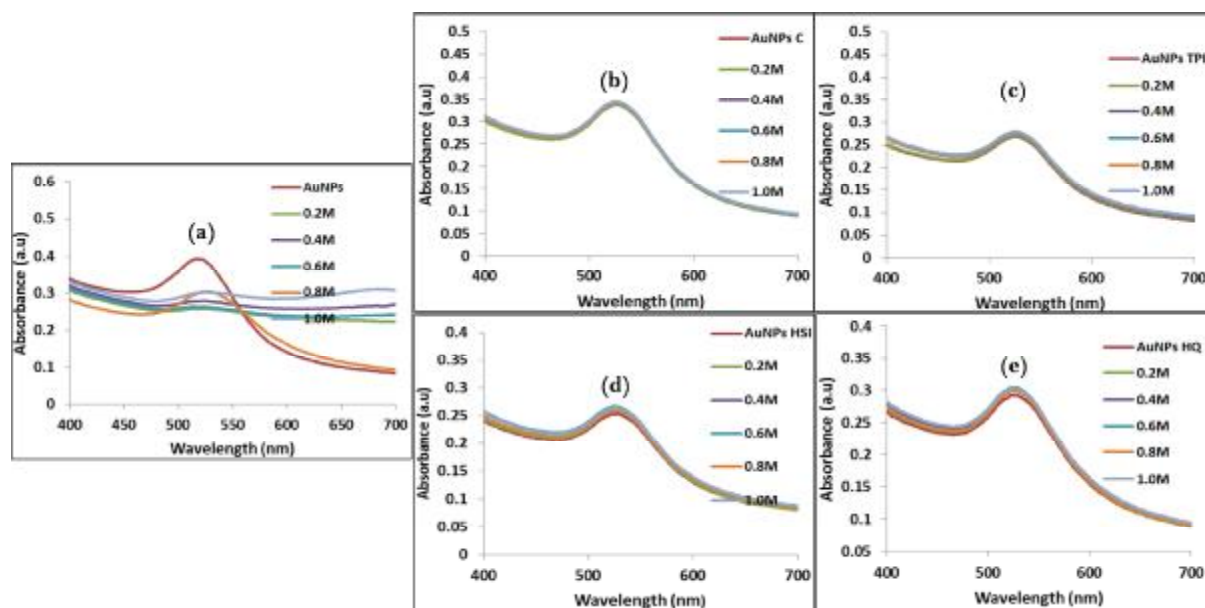


Figure S6.8: SPR measurements under different concentrations of NaCl of (a) unconjugated AuNPs, (b) to (e) AuNPs Protein A conjugates with different alkanethiols (b) HS-(CH₂)₁₁-NHCO-coumarin, (c) HS-(CH₂)₁₁-triphenylimidazole, (d) HS-(CH₂)₁₁-indole, and (e) HS-(CH₂)₁₁-hydroquinone

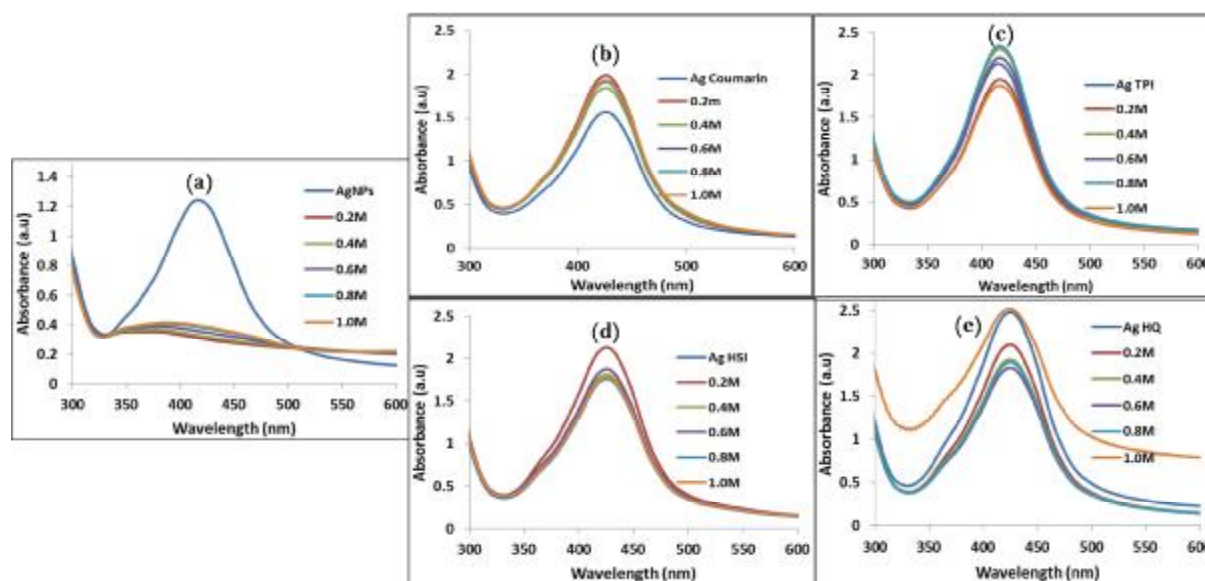


Figure S6.9: SPR measurements under different concentrations of NaCl of (a) unconjugated AgNPs, (b) to (e) AgNPs Protein A conjugates with different alkanethiols (b) HS-(CH₂)₁₁-NHCO-coumarin, (c) HS-(CH₂)₁₁-triphenylimidazole, (d) HS-(CH₂)₁₁-indole, and (e) HS-(CH₂)₁₁-hydroquinone

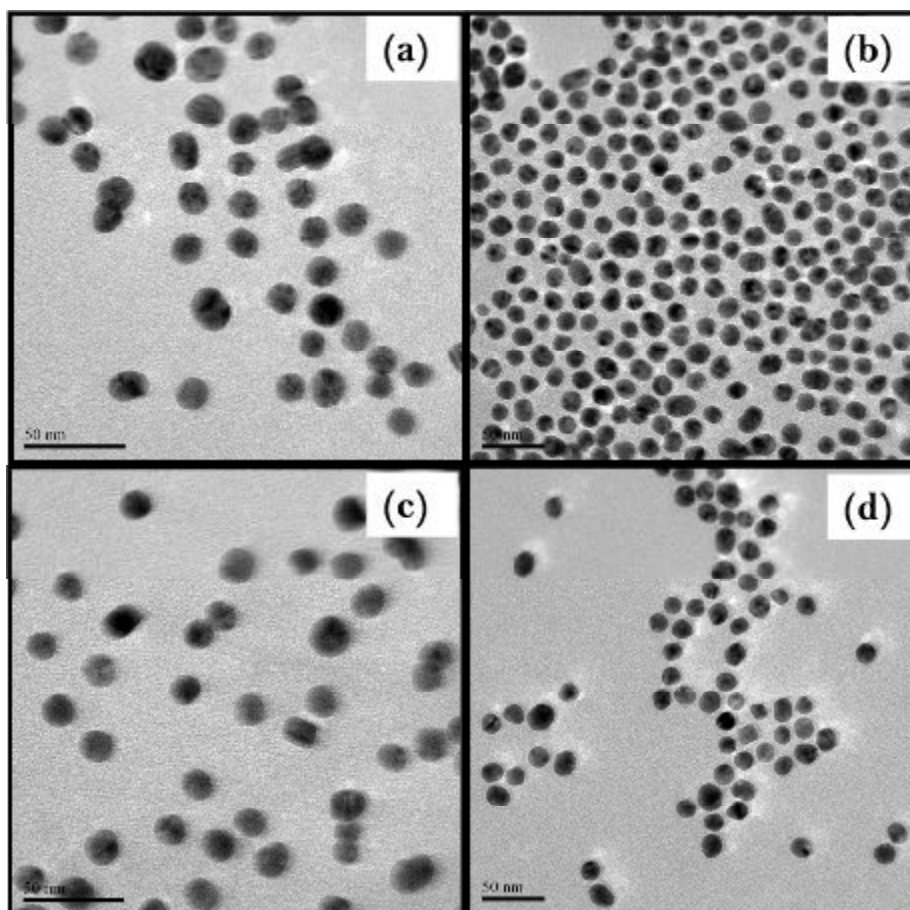


Figure S6.10: TEM images of AuNPs Protein A conjugates with alkanethiols (a) HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole, and (d) HS-(CH₂)₁₁-hydroquinone

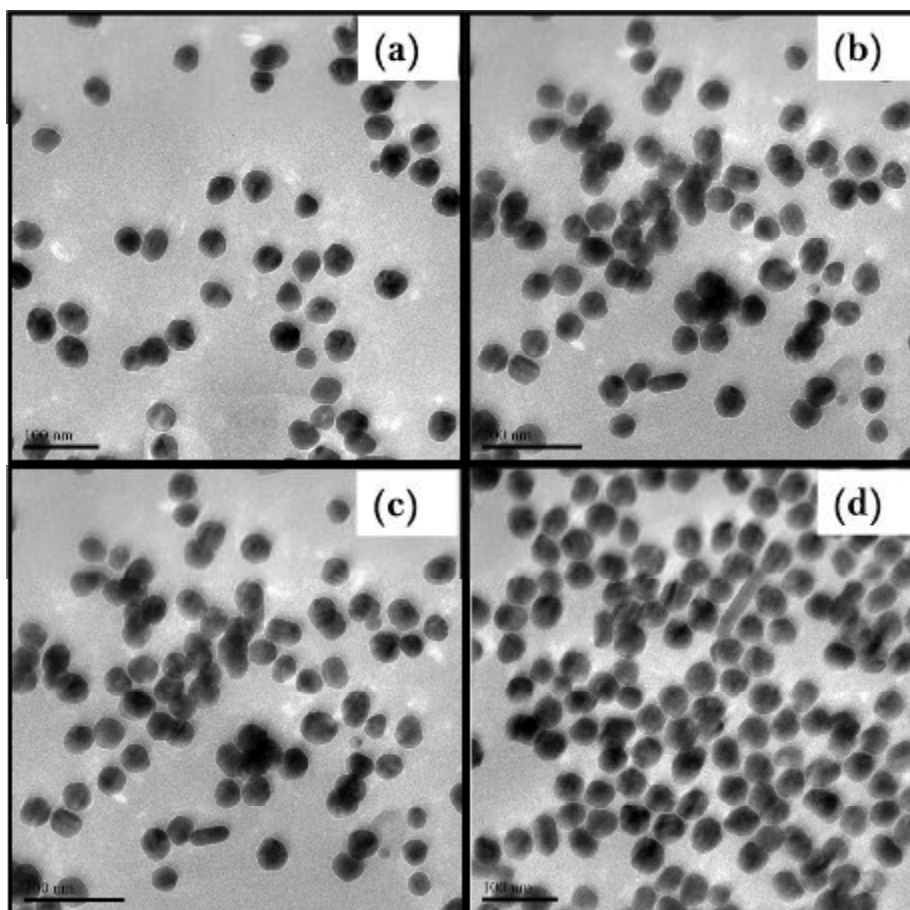


Figure S6.11: TEM images of AgNPs Protein A conjugates with alkanethiols (a) HS-(CH₂)₁₁-NHCO-coumarin, (b) HS-(CH₂)₁₁-triphenylimidazole, (c) HS-(CH₂)₁₁-indole, and (d) HS-(CH₂)₁₁-hydroquinone

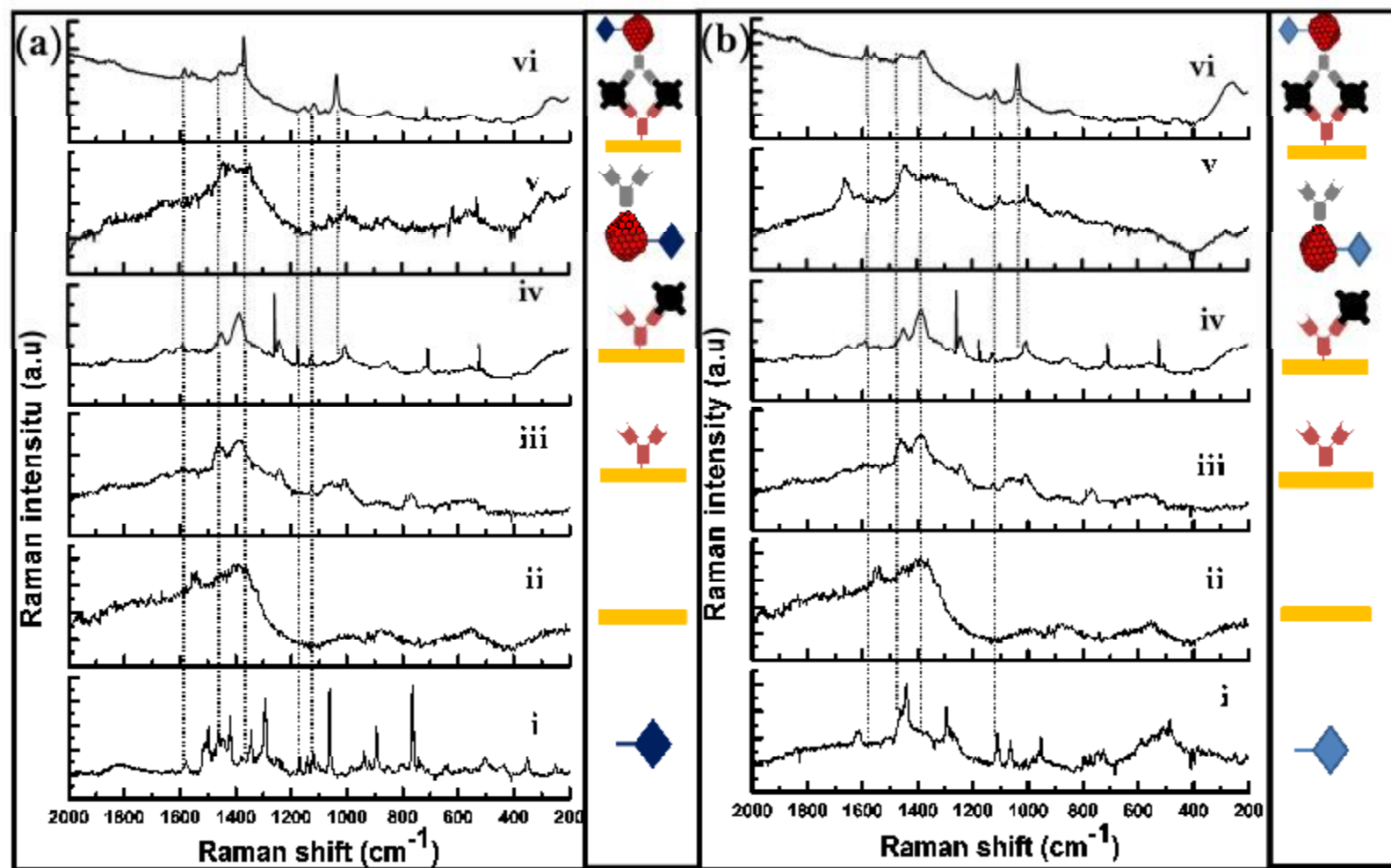


Figure S6.12: SERS immunoassay spectra, (a) AuNPs Protein A- HS-(CH₂)₁₁-indole + mycobacterium tuberculosis 38 kDA antigen and (b) AuNPs Protein A-HS-(CH₂)₁₁-hydroquinone + mycobacterium tuberculosis 38 kDA antigen

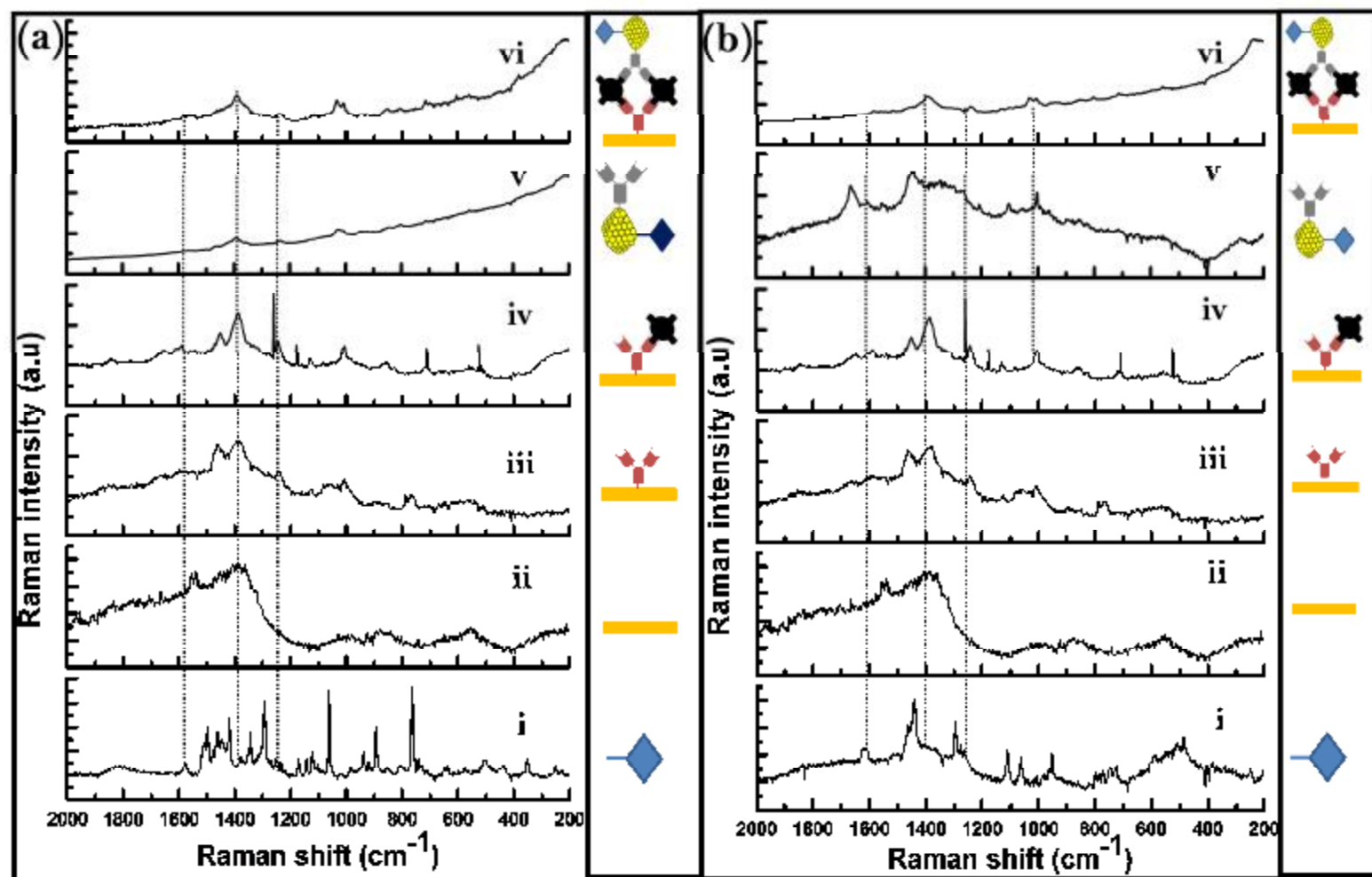


Figure S6.13: SERS immunoassay spectra, (a) AgNPs Protein A- HS-(CH_2)₁₁-indole + mycobacterium tuberculosis 38 kDA antigen and (b) AgNPs Protein A-HS-(CH_2)₁₁-hydroquinone + mycobacterium tuberculosis 38 kDA antigen