

**SURFACE ENHANCED RAMAN SCATTERING-
ELECTROCHEMICAL METHODS FOR
CHARACTERIZATION OF SELECTED THERAPEUTIC
DRUGS: SPECTROSCOPIC AND DFT STUDY**

BY

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I would like to dedicate this thesis to those people who are working for the welfare of
human beings

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LIST OF ABBREVIATIONS

NRS	:	Normal Raman Spectra
SERS	:	Surface Enhanced Raman Scattering
EC-SERS	:	Electrochemical- Surface Enhanced Raman Scattering
LSPR	:	Localized Surface Plasmon Resonance
EM	:	Electromagnetic Enhancement
CHEM	:	Chemical Enhancement
CT	:	Charge Transfer
AgNPs	:	Silver Nanoparticles
AuNPs	:	Gold Nanoparticles
CNS	:	Carbon Nano Sphere
CNPs	:	Circular Nano Plates
HRG	:	Highly Reduced Graphene
DFT	:	Density Functional Theory
TEM	:	Transmission Electron Microscopy
FTIR	:	Fourier-transform Infrared Spectroscopy
EDS	:	Energy Dispersive X-ray Spectroscopy
XPS	:	X-ray Photoelectron Spectroscopy

CV	:	Cyclic Voltammetry
EIS	:	Electrochemical Impedance Spectroscopy
LOD	:	Limit of Detection
LDR	:	Linear Dynamic Range
VA	:	Valeric Acid
FTO	:	Fluorine doped Tin Oxide
RSD	:	Relative Standard Deviation
HC	:	Hydroxycarbamide
NMR	:	Nuclear Magnetic Resonance
MMPU	:	1-methoxy-1-methyl-3-phenylurea
SeINOE	:	Selective Nuclear Overhauser Effect
EF	:	Enhancement Factor
PCM	:	Polarizable Continuum Model
SDD	:	Stuttgart/Dresden basis set

ABSTRACT

Full Name : [Muhammad Haroon]
Thesis Title : [Surface Enhanced Raman Scattering-Electrochemical Methods for Characterization of Selected Therapeutic Drugs: Spectroscopic and DFT Study]
Major Field : [Chemistry]
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The use of spectroanalytical approaches for detecting low concentrations of organic compounds in a variety of media is an active direction of research that intersects with vital medical, environmental and energy sectors. In this study, Raman and electrochemical techniques were employed for the qualitative and quantitative analysis of selected widely used therapeutic drugs and organic herbicides, namely procaine, sulindac, velaric acid, hydroxycarbamide, linuron and 1-methoxy-1-methyl-3-phenylurea. The main objective was to develop sensitive analytical protocols for the sensing and characterization of these bioactive compounds. Various nanostructured plasmonic materials were developed and characterized by using Raman spectroscopy, Fourier-transform infrared spectroscopy (FT-IR), UV-vis absorption, transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS) and energy dispersive X-ray spectroscopy (EDX). The electrochemical activity of the developed nanomaterials was investigated using electrochemical cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). These nanomaterials were implemented to enhance the Raman scattering light and enable us to apply the surface-enhanced Raman scattering (SERS) spectroscopic method for low concentration detection and characterization. SERS is a sensitive technique that provides fingerprint information of the compounds under study. Raman signals are

enhanced when an analyte molecule is appropriately adsorbed on the surface of nanostructured materials, and the observed enhancement results from either electromagnetic or charge transfer mechanisms, or the combination of both. It has been noticed that SERS signals were further improved by incorporating electrochemistry. Furthermore, structural and electronic properties of studied therapeutic systems were theoretically predicted using density functional theory (DFT) and were correlated with our spectroscopic observations.

To begin this research, the SERS based approach was employed to establish a method for quantification of valeric acid (VA). Spherically structured Ag-NPs were utilized for the trace detection of VA in aqueous media, and a wide dynamic range with a lower detection limit (1×10^{-10} M) could be achieved. The vibrational modes in the free form VA molecules and with the Ag-NPs interactions responsible for the SERS enhancement were analyzed via density functional theory (DFT) calculations.

Next, the identification and quantification of sulindac using the surface-enhanced Raman scattering (SERS) technique and employing silver nanoparticles (AgNPs) substrates led to an unprecedented sensitivity of this drug of a wide dynamic range (1×10^{-2} M to 1×10^{-9} M) and a low detection limit (1×10^{-10} M). The SERS peaks resulting from the solvent DMSO molecules and the target sulindac drug molecules were carefully analyzed and properly interpreted. Molecular modeling results for the interaction of sulindac molecules with silver nanoparticles were correlated with the experimental findings. Significant enhancements, due to charge transfer processes, in the Raman bands at 1111, 1653, 1607 and 1340 cm^{-1} corresponding to the -S=O stretching, -C=O stretching, -C=C- stretching and ring stretching vibrations were observed.

In the next part of the research which involved an intensive amount of work, SERS spectroscopy was implemented for the determination of procaine in aqueous media by utilizing gold nanoparticles (AuNPs) as an active substrate. The intensity enhancement obtained for a number of Raman peaks was employed to develop a reliable quantitative determination approach for procaine in aqueous solutions. Such an approach exhibited a high sensitivity and reproducibility with a reasonably wide linear dynamic range. The limit of detection of procaine achieved was up to 1×10^{-10} M. This has been outstanding compared with other methods such as chromatography and electrochemical methods. We continued these efforts by developing an electrochemically modulated sensing approach for the quantitative detection of procaine using in-situ SERS. This integrated approach is known as electrochemical-SERS or simply EC-SERS. A fluorine-doped tin oxide electrode modified using silver carbon nanosphere (AgCNS-FTO) was designed for this purpose, electrochemically evaluated, and used as an efficient substrate. The investigation of the electrochemical interaction of procaine molecules towards the modified electrode revealed that upon the optimum electrochemical pre-concentration potential (-0.1 V) and time (400 s), significant enhancements in the intensity of key Raman peaks corresponding to the NH₂ bending, C=C stretching, CH₂ twisting and C-N stretching modes could be observed. The reported approach enabled a remarkable sensitivity and selectivity towards procaine, resulting in an unprecedented detection limit down to the level of 10^{-13} M. The reproducibility and stability of the modified FTO electrode were ensured over a duration of one month, and a low RSD of 2.4% was achieved.

In the next effort, we synthesized palladium-based highly reduced graphene oxide (Pd-HRG) with localized surface plasmon resonance (LSPR) properties as an effective SERS

substrate. Its utility in the highly sensitive detection of procaine was tested. LSPR excitations are critical towards achieving sizable spectral enhancements of the Raman scattered light. The Pd-HRG nanocomposite showed a remarkable LSPR response with a Raman enhancement factor of 8.7×10^2 . The Pd-HRG is employed to modify fluorine doped tin oxide electrode (Pd-HRG/FTO), resulted with an enhancement factor of 7.5×10^4 corresponding to the EC-SERS technique with reproducible and reliable result even in real urine sample.

Moreover, the anti-cancer drug hydroxycarbamide (HC) was analyzed and characterized with EC-SERS approach using gold-coated copper oxide (Au-CuOx) circular nanoplates (CNPs). Several prominent Raman peaks associated with HC and ascribed by the NCN bending, CN stretching, NO stretching, CO stretching and NH₂ wagging vibrational modes showed substantial enhancements upon the electrochemically induced interaction between the SERS substrate and the drug molecules. A limit of detection of one-tenth of nanomolar concentration was achieved under the optimum conditions.

The SERS investigations were extended to cover some bioactive compounds used in the herbicides industry. Here, the monitoring of organic herbicides linuron and 1-methoxy-1-methyl-3-phenylurea (MMPU) were carried out by employing nuclear magnetic resonance (NMR) and Raman spectroscopic techniques supported with DFT to investigate the conformational behavior and electronic aspects of linuron

In conclusion, spectroscopic and electro-spectroscopic techniques, namely SERS and EC-SERS, have been shown to be a sensitive analytical approach for the quantitative analysis of certain therapeutic drugs and other bioactive compounds used as herbicides and anti-

cancer drugs. This work has potential and can lead to the development and design of sustainable sets of detective devices with affordable cost. Such devices can be easily applied in point-of-care stations in hospitals and medical sectors, as well as QA labs in environmental agencies and industry.

ملخص الرسالة

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عنوان الرسالة: تطوير طرق تحليل كهروطيفية باستخدام تقنية رامان ذات السطح المحسن مدعمة بالخصائص

الكهروكيميائية لتوصيف عدد من المركبات الدوائية: دراسة طيفية مع حسابات نظرية كثافة الدالة (DFT)

التخصص: كيمياء

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يعد استخدام مناهج التحليل الطيفي والكهروطيفي للكشف عن التراكيز المنخفضة للمركبات العضوية مجالاً بحثياً نشطاً. في هذه الدراسة، تم استخدام مطيافية رامان ذات السطح المحسن مدعمة بالتقنيات الكهروكيميائية للقيام بالتحليل النوعي والكمي لبعض المركبات العضوية المختارة والتي تستعمل في صناعة الأدوية وكذلك مبيدات الأعشاب ، وهي بروكايبين ، سولينداك ، حمض الفيلاريك ، هيدروكسي كارباميد ، لينورون و 1-ميثوكسي-1-ميثيل-3-فينيل يوريا. كان الهدف الرئيسي هو تطوير بروتوكولات تحليلية حساسة لاستشعار وتوصيف هذه المركبات النشطة حيويًا. تم تحضير عدد من المواد البلازمية ذات البنية النانوية التي تنتج تحسيناً ملموساً حين تطبيق مطياف الرامان. تم توصيف هذه المواد النانوية بأدوات مختلفة كالتحليل الطيفي للأشعة تحت الحمراء (FTIR) ، وامتصاص الأشعة فوق البنفسجية ، والفحص المجهر الإلكتروني النفقي (TEM) ، والتحليل الطيفي للإلكترون بالأشعة السينية (XPS) والتحليل الطيفي للأشعة السينية المشتتة للطاقة (EDX). تم فحص النشاط الكهروكيميائي للمواد باستخدام مقياس الجهد الدوري الكهروكيميائي (CV) والتحليل الطيفي الكهروكيميائي (EIS). تم استخدام هذه المواد النانوية رامان ذات السطح المحسن (SERS) للكشف والتوصيف منخفض التركيز. SERS هي تقنية حساسة وتوفر معلومات البصمة الجزيئية للمركبات. في SERS ، يتم تحسين إشارة Raman عندما يمتص جزيء التحليل على سطح المواد الهيكلية النانوية ويكون التحسين ناتجاً عن آليات نقل الشحنات الكهرومغناطيسية. تم تحسين إشارة SERS بشكل أكبر من خلال دمج التقنيات الكهروكيميائية، وتم حساب الخصائص الجزيئية والالكترونية للأنظمة المدروسة نظرياً باستخدام نظرية كثافة الدالة (DFT) ، ولقد تم توثيق جميع النتائج وبالتفصيل في وحدات هذا البحث.

استنتجنا من هذا البحث أن التقنيات الطيفية الكهروكيميائية المستخدمة، أعني SERS و EC-SERS ، أثبتت أنها نهج سلس وفعال للتحليل الكمي للأدوية العلاجية والمركبات النشطة حيويًا ، وإن هناك مجال كبير لتعزيز قدرتها وفعاليتها بصورة أكبر، ونعتقد انها تمثل تقنيات واعدة للمستقبل لتطوير أجهزة قياسات حساسة سهلة الاستخدام ومنخفضة التكلفة، يمكن ان توظف في المستشفيات ومحطات

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Spectroscopy has played an increasing role in bio and agrochemical analysis because it can provide structural information of bioactive molecules. The light received by objects is governed by the fundamental principles of electromagnetic radiation propagation and interaction with matter developed many decades ago. Different techniques including Fourier transform infrared (FTIR) spectroscopy, X-rays based computed tomography, nuclear magnetic resonance (NMR) spectroscopy and Raman spectroscopy, have been used to determine biomarkers in the human body [1–5]. There are certain advantages and limitations associated with these techniques. For examples, Nuclear magnetic resonance (NMR) spectroscopy can be useful to identify the tumors which are life threatening and thus requires a radical treatment [6]. NMR spectroscopy is less sensitive as compared to other techniques for biochemical detection [7]. FT-IR spectroscopy is generally employed for locally confined and invasive techniques through their characteristic peaks. Infrared bands associated with water molecules cause some spectral interference and hence represent a source of complexity to analyze the output [8,9]. The light scattering spectroscopy has demonstrated unique capabilities for the characterization of bioactive compounds. The employment of nanoscience in the light scattering spectroscopy increased its sensitivity and has been used as molecular sensors. The capabilities of nanomaterials for enhancing the Raman signals allows for single molecule level sensitivity and identification by using the effect of localized surface plasmon resonance. The light

scattering spectroscopy has established as potential diagnostic tool for the sensitive detection of bioactive molecules in biological samples.

1.1 BIOACTIVE MOLECULES

Bioactive molecules such as drugs and herbicides are considered crucial due to their deep impact on human life. Drug and herbicides characterizations and their quantitative analysis are the essential tasks in the pharmaceutical and agriculture industry to ensure an implementation of a reliable means of quality control in the analytical labs [10–13].

Procaine, for example, is among the most common local anaesthetics in neural therapy and is being used for the treatment of obesity, depression and even for cancer [14,15]. It stops the pain sensation for the specified part of the body by stopping the transfer of nerves from the peripheral system to central nervous system. Procaine also possesses some serious adverse effects such as allergic reactions, neurological and cardiac toxicity [16–18]. Sulindac, a common non-steroidal anti-inflammatory drug (NSAID) approved by the United States Agency for Food and Drug Administration (FDA) in 1978 [19], is used in the treatment of inflammatory infections, such as joint stiffness, shoulder tendonitis, gouty arthritis and arthritis of the spine [20]. A number of reports on the use of sulindac in the treatment of lung tumorigenesis, colorectal cancer, skin cancer, and prostate cancer have also been documented [21–25]. However, despite its tumorigenic and anti-inflammatory activities, the long-term exposure or overdose of sulindac increases the risk of heart attack [26], stroke or intestinal bleeding which can be fatal [27,28]. Hydroxycarbamide (HC), derivative of urea, is a non-alkylating antineoplastic agent used in the treatment of a wide range of tumors, including breast, gastric and intestinal cancers, as well as bladder and colorectal cancer, in addition to melanoma, hepatocellular carcinoma and

myeloproliferative diseases [29–31]. Its most accepted action mechanism is to inhibit the synthesis of DNA by affecting the cell cycle in a reversible manner [30]. However, the adverse countereffects of HC are obvious due to its high toxicity towards normal cells in addition to the rapid impairment of spermatogenesis in males. Furthermore, the HC drug faces a low dissolution capacity problem and low efficacy in vivo tests [32,33]. These detrimental impacts could be reduced by developing a sensitive platform which has the potential of monitoring trace HC existence. Valeric acid (VA) is found in *Valeriana officianilis* which has been used for the treatment of neurological disorders [34]. It is considered as an example of renewable lignocellulose derived platform compound which has lately attracted substantial attention [35]. Its production from lignocellulose derived from levulinic acid and g-valerolactone was established to be effective, and robust [36]. VA is an intermediate in the conversion of lignocellulose to valeric acid esters, which are a class of cellulosic biofuels [37]. It has a structure similar to that of neurotransmitter gamma-Aminobutyric acid (γ -Aminobutyric acid) (GABA) [38].

In the agriculture field, the utilization of organic herbicides to improve the quality of plantation crops has become inevitable in the modern life [39,40]. However, such an activity has become a serious source of various toxins which are released to the environment and hence pose a big menacing to the aquatic ecosystem. Among the widely used compounds that control the weed in agricultural and nonagricultural fields are the phenylurea herbicides [41–43], which have recently gained a global attention due to their widespread on the land and ground water as well as their long-term persistence in the environment with a high toxicity [44–46]. The detection of substituted phenylurea derivatives has been performed using a variety of analytical techniques.

Phenylurea (linuron and 1-methoxy-1-methyl-3-phenylurea) commonly used to control the growth of grassy and broad leaf weeds in the cultivation of various types of crop plants, especially cereals and vegetables [47,48]. During the process of absorption through the plant roots and being transported to the leaves, linuron disrupts the process of photosynthesis by inhibiting the photo-dependent electron transportation, leading to the accumulation of reactive oxygen species in plant cells [49–51]. Linuron-based herbicides have attracted considerable attention due to their high biotoxicity and carcinogenic properties towards non-target aquatic organisms and mammals, as well as their widespread in soil, ground, and surface water as they slowly degrade [52–54]. They pose a serious toxic impact on the environment [55–57]. 1-Methoxy-1-methyl-3-phenylurea (MMPU) can bind with proteins in plants inhibiting the photo-driven electron transfer and adversely affect the photosynthesis in plant cells. The structural formulas of therapeutic drugs and herbicides are given in Fig 1. 1.

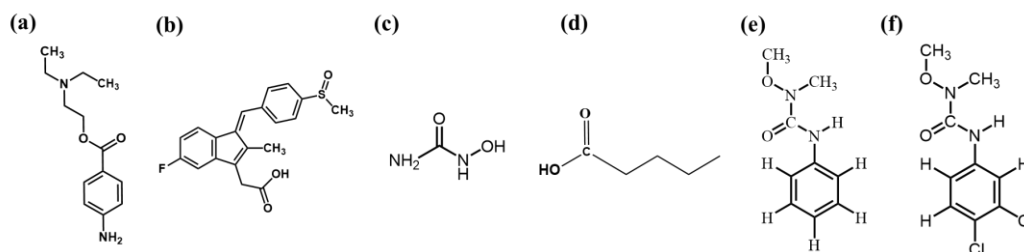


Figure 1. 1 Structural formulas of bioactive molecules; (a) Procaine, (b) Sulindac, (c) Hydroxycarbamide, (d) Valeric Acid, (e) 1-Methoxy-1-Methyl-3-Phenylurea and (f) Linuron.

The high level of these organic based drugs and herbicides were detected in industrial pharmaceutical wastewater, agricultural drain-water and effluents from wastewater treatment plants leading to contamination of the aquatic environment [58–60]. In addition to the pharmacological effects and therapeutic properties, these organic based drugs can be lethal at high intake dose causing potentially toxic effects on aquatic organisms. Due to the

potential side effects, there is a critical need to investigate efficient monitoring protocol for these bioactive molecules to avoid any undesirable results. There is a lot of efforts made to develop a sensitive analytical approach for the therapeutic drugs monitoring such as spectroscopic, chromatographic, titrimetric and electrochemical techniques [61–68]. Among all the analytical techniques, Raman spectroscopy is an attractive alternative for the sensing of organic based drug and herbicides because of their strong molecular specificity and linear relationship of the signal intensity to the analyte concentrations

1.2 RAMAN SPECTROSCOPY

Raman scattering is a vibrational spectroscopic technique which is based on scattering of radiations produced by a gas, solid or liquid samples involving high energy photons for emission and excitation [69]. The main advantage of Raman scattering technique is the ability to yield molecular fingerprint spectrum for every distinct analyte, which provides information of vibrational energies of different functional groups in the analyte. In Raman scattering phenomenon, a photon from the laser light excites the electron of the target molecules to a virtual energy level, causing vibrations in molecule as illustrated in Fig. 1.2. Upon the relaxation process, the excited state of molecules ends up and produce a photon with different wavelength. The relaxing of molecule possesses three possible different energy scenarios of scattered photon; (a) Rayleigh scattering, (b) Stoke scattering and (c) Anti-Stokes scattering.

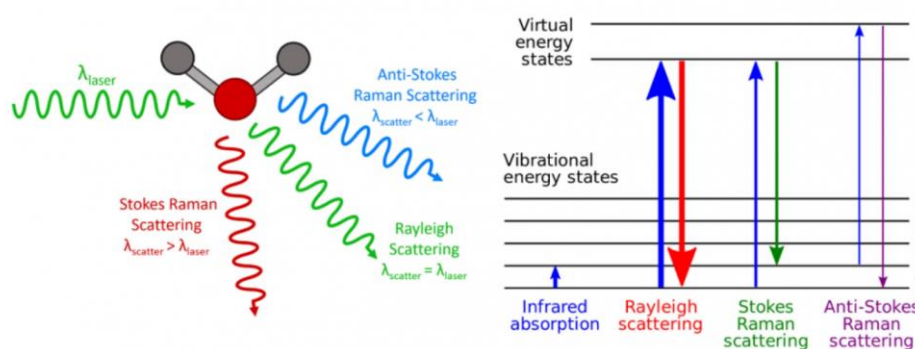


Figure 1. 2 Raman effect and electronic transition in the analyte molecules showing Rayleigh, strokes and anti-strokes Raman scattering.

However, this technique lacks merit for highly sensitive detections due to inherently weak Raman signals.

1.3 SURFACE ENHANCED RAMAN SCATTERING (SERS) SPECTROSCOPY

In order to maximize the sensitivity and enhancement of the Raman scattered lights, gold and silver-based nanomaterials have been utilized. The technique developed by combination of Raman spectroscopy and nanotechnology is known as surface-enhanced Raman scattering (SERS) spectroscopy [70–73]. The discovery that the Raman intensity can be enhanced greatly for the analyte molecule which is adsorbed on a surface of nanostructured metals, especially Au and Ag, motivated enormously the interest in this technique as shown in Fig 1.3. The significant enhanced sensitivity of SERS technique is one of its main advantages over conventional Raman spectroscopy.

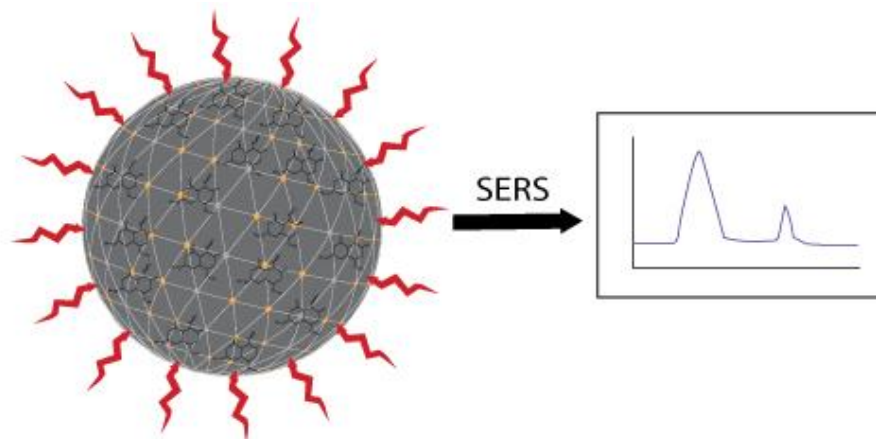


Figure 1. 3 Schematic representation of the adsorption of analyte molecules on the metal nanoparticles loaded on nanomaterials leading to enhanced SERS signals.

The enhancement of Raman signal is the consequences of plasmonic effect of metallic nanoparticles at nanometric scale, which is contributed to the localized surface plasmon resonance (LSPR) of the nanomaterials. The LSPR is caused by the collective oscillation of the electrons when an electromagnetic radiation interacts with metallic nanoparticles as depicted in Fig 1.4.

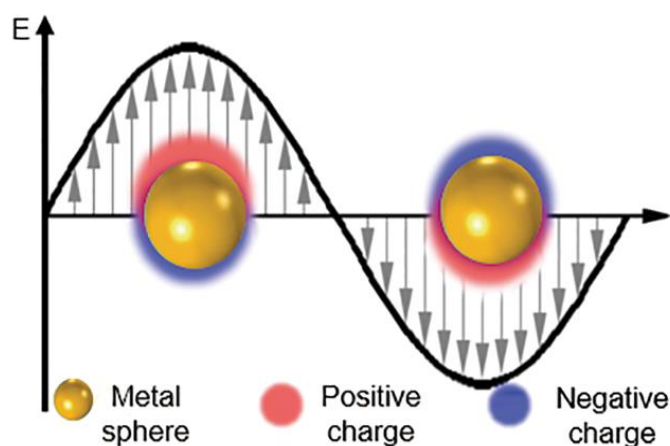


Figure 1. 4 Surface plasmon resonance of a dipolar mode induced by incident electromagnetic light on a metallic sphere.

1.4 ENHANCEMENT MECHANISM

The enhancement effect of the SERS approach is contributed by two widely accepted mechanisms: chemical enhancement and electromagnetic enhancement [74,75] illustrated

in Fig 1.5a. The electromagnetic field contributed more to the SERS enhancement in most Raman systems. Chemical enhancement plays a vital role in the characterization of the chemical species related with chemical properties of substrate and surface species. The contribution of both mechanisms can be presented by the following equation (1.1) [76,77].

$$I_{SERS} \propto G_{EM} \sum_{\rho,\sigma} \left| (\alpha_{\rho\sigma})_{nm} \right|^2 \quad (1.1)$$

Where, Enhancement factor (G_{EM}) results from the electromagnetic field of light scattered from the surface of the substrate. The sum term of $(\alpha_{\rho\sigma})_{nm}$ is the frequency dependent polarizability contributing to the chemical enhancement which is due to the chemical sorption interaction and photon motivated charge transfer between the metal surface and the analyte resulting in shift of the frequency and relative intensity of Raman signals. The latter form of enhancement is important for the characterization of SERS spectra. Both enhancement mechanisms can be affected by varying the applied potential on the electrode.

1.4.1 Electromagnetic Enhancement

In chemical enhancements, the target molecule is adsorbed on the surface of nanomaterials. As a result, the Raman spectra of complex are modified to those of corresponding isolated molecules. The charge transfer could occur in three different ways depending on the nature of substrate and adsorbed molecules as shown in Fig 1.5b. (i) electrons from the valance band of nanomaterial (metal/metal oxide) to its conduction band by taking energy of the incident light and then injected to adsorbed molecules. (ii) electrons from the HOMO of analyte molecules to its LUMO and then injected to the conduction band of nanomaterial. (iii) valence electrons of nanomaterial to surface state energy levels and then injected into LUMO of adsorbed molecules.

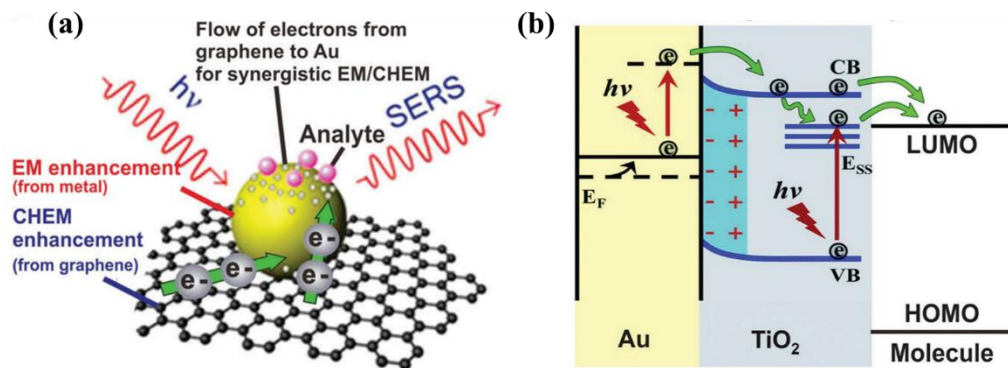


Figure 1. 5 (a) SERS enhancement including electromagnetic and chemical enhancements using graphene/metal hybrid substrate, (b) Chemical enhancement including charge transfer (CT) using Metal/semiconductor substrate

1.4.2 Electromagnetic Enhancement

In electromagnetic enhancement, when the analyte molecule is close to plasmonic materials or between the plasmonic nanoparticles (hotspots), The Raman intensity is increased because of LSPR as shown in Fig 1.4.

1.5 ELECTROCHEMICAL-SURFACE ENHANCED RAMAN SCATTERING (EC-SERS) SPECTROSCOPY

SERS coupled with electrochemistry for the mechanistic and dynamic investigation of analyte molecules absorbed on the electrochemical interfaces of metallic electrode at a given applied potential has been established. Electrochemical surface-enhanced Raman scattering (EC-SERS) started attracting the attention as an attractive analytical tool in the detection of various type of therapeutic medicines by increasing the number of adsorbed analyte molecules on the electrode surface to observe possible structural changes and electrochemical stability at different applied potentials [78]. In this method, the potential is applied to create electrostatic attractions between the target molecules and the SERS substrate in order to achieve maximum enhancement of Raman intensity. The EC-SERS technique, which can be implemented for both identification and characterization, normally consists of electrolyte and metal nanostructured electrodes. Therefore, an important and

complicated region, an electrochemical double layer is developed in between. Such a technique has attracted more attention lately due to its unique physical aspects. Several efforts made to collect useful information for exhibiting the reaction mechanism or the adsorption configuration from electrochemical point of view were reported [79,80].

When incident light falls on the surface of the nano-structured electrode to induce a SERS phenomenon, an electric field is developed across the region of electrochemical double-layer which influences the interaction and surface orientation of the analyte with the nanostructured electrode. The second most important parameter of EC-SERS is the electrode potential, and the Raman signal is strongly associated with the electrode potential. Surface charge density and polarity will be altered by adjusting the electrode potential which leads to a modification of the adsorption orientation and coverage of analyte molecule resulting with the change in SERS intensity [81]. The two factors that could be accounted for to better understand the EC-SERS phenomenon are: (i) change in the binding interaction of the molecule with the surface of the electrode leading to a alteration in the electronic structure of the analyte molecule, and (ii) the direction of the charge transfer between metals to molecule which depends on electronic properties of the adsorbate molecule, electrode materials and the excitation wavelength as well. The results of many reports revealed that both factors should be considered for understanding of the complex potential dependency [82,83].

The electromagnetic field and chemical SERS enhancement in electrochemical systems might be influenced by the applied potential. The charge density of electrode surface can be monitored by varying the applied potential resulting in a shift in the SPR frequency. The redshift in the plasmon resonance band was observed by applying a positive potential,

while applying a negative potential would lead to a rise in the plasmon resonance band. This phenomenon can be expressed by the charge density dependent plasmon frequency (ω_p) in the following equation (1.2) [84].

$$\omega_p = \sqrt{4\pi n e^2 / m \epsilon_0} \quad (1.2)$$

where, m , e , n and ϵ_0 are respectively, the mass, charge, number of free electrons and vacuum permittivity. It is obvious that a change in the value of n results in a shift of the plasmon frequency. The SERS intensity is closely associated with electrode potential which can be explained by photon motivated charge transfer from molecule to metal utilizing the concept of energy states. If the applied potential is not sufficient to induce the photon motivated charge transfer states on the surface with a given excitation energy, the change in the SERS intensity could be explained by the bonding effect. When the potential is increased, the excitation energy matches the required charge transfer energy which leads to an enhancement in the SERS signal of relevant vibrational modes [85,86]. It is obvious from the previous SERS reports using Ag, Cu and Au that a necessary, but insufficient, surface roughness is the basic requirement for a desirable surface enhancement. With the development of nanotechnology and nanoscience, the SERS substrates have also been expanded from massive metal electrodes and metals colloidal sols to template fabricated substrates and nanoparticles assembled electrodes for larger enhancement. Many methods have been reported in literature for the fabrication of SERS substrate suitable for EC-SERS [87]. The Raman enhancement can be influenced by varying the applied electrode potential because the Fermi level of metal and dielectric constant of interfacial electrolyte strongly associated with the chemical enhancement mechanism in an EC-SERS system. When a laser light falls on a electrode surface to initiate the SERS process, an optical electric field

is established in the electrochemical double layer regions. It influences effectively the interaction between metal and analyte molecules, orientation of adsorbate molecules to the structure of double layer. The change in electrode potential cause change in the surface charge density and polarity. As shown in Fig 1.6, when the potential of electrode is more positive than the potential of zero charge, the surface will be positively charges while electrode potential is moved to negative, the surface will be negatively charges which influences the orientation and interaction of analyte molecules [88].

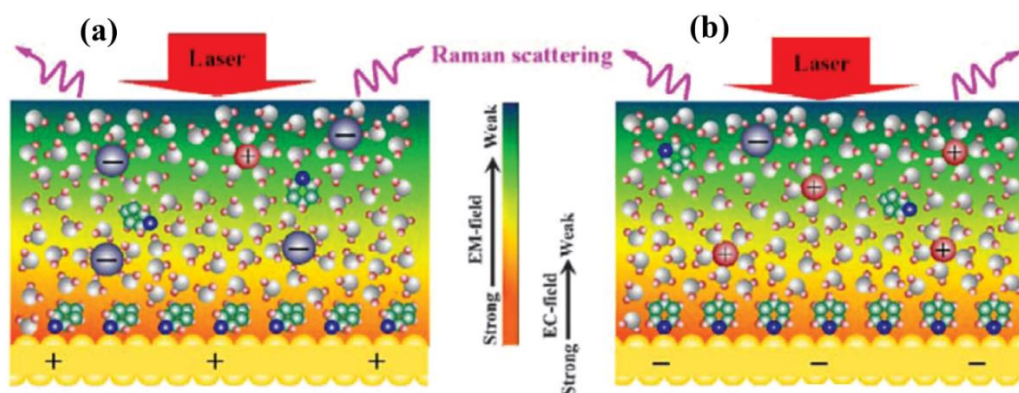


Figure 1. 6 Electrochemical interfaces indicating SERS with electrochemical field and electric field at the electrode potentials (a) positive or (b) negative to the potential of zero charge.

The SERS substrate can be regenerated by varying the applied potential after the completion of molecular sensing. EC-SERS is also promoting adsorption of low affinity target molecules to SERS active substrate in order to enhance the performance of biosensors [89]. Several studies on EC-SERS applications to detect some organic molecules have been reported. Lee and his co-workers designed an EC-SERS setup for the quantification of molecules such as dopamine, isoproterenol, catecholamine, and epinine using silver as working electrode at pH 7.2 [90]. Screen printed electrodes modified with multi-layered silver and gold nanoparticles deposited on carbon was used for sensitive sensing of uric acid biomarker [91]. Later, the EC-SERS method was demonstrated for the

clinical quantification of dobutamine using AuFON as SERS substrate. The therapy drug dobutamine does not have detectable normal Raman scattering signals in the clinical range [92]. Greene et al., developed EC-SERS approach for the sensing of biomarker 6-thiouric acid using drop casting method for the modification of commercially available screen-printed electrode with Ag-nanoparticles [93]. Insight into the electronic structure and bond lengths in di-nickel and tri-nickel complexes by the advantages of EC-SERS based electrochemical control to change the oxidation state of nickel centers were reported [94]. Xiaowei et al., characterized ligand binding on the surface of PbS quantum dots, reporting potential-dependent changes in the chelation state of the oleic acid ligands interacting with surface lead atoms [95]. Another EC-SERS study was conducted to investigate the sorption behaviour of phenolic acids on silver, gold and copper substrate and the effect of applied potentials and metals used [96]. Besides the reported modified carbonaceous and metallic electrode, semi-conductors such as fluorine-doped tin oxide (FTO) have shown excellent physiochemical properties such as transparency, conductivity and stability. Many reports have been published for the sensorial application of non-modified and modified FTO electrodes. For instance, Zhao and his co-workers successfully designed a zinc oxide nanocluster modified FTO electrode for the detection of H₂S gas [97]. Esmaeeli et al developed methods for the sensing of glucose by using copper oxide polyaniline nanofiber modified fluorine doped tin oxide (CuO/PANI-NF/FTO) electrode [98]. The different synthesis and electrode fabrication approaches were applied to achieve reliable and uniform SERS substrate for significant Raman enhancement

CHAPTER 2

EXPERIMENTAL AND COMPUTATIONAL

METHODOLOGY

2.1 EXPERIMENTAL

In this section of PhD dissertation, the experimental procedures including substrate fabrication and set up adopted for the development of the sensitive detection of various organic based drugs and herbicides using normal Raman spectroscopy, Surface enhanced Raman scattering (SERS) and electrochemical surface enhanced Raman scattering (EC-SERS) spectroscopies are included. The analytical techniques used for the characterization of the designed nanostructured materials and fabricated electrodes are discussed.

2.1.1 Synthesis of Nanostructured SERS Substrates

The silver nanoparticles were synthesized by the reduction method [99], where 10.0 mL of 3.0×10^{-2} M sodium hydroxide and 10.0 ml of 1.5×10^{-2} M hydroxylamine hydrochloride were mixed and poured into the silver nitrate solution (180 mL of 1.11×10^{-3} M) under stirring and heating. The prepared mixture was again agitated for 30 min. The prepared silver colloids were kept at 5 °C before the utilization for SERS analysis.

Gold nanoparticles (AuNPs) were prepared by the reacting gold (III) chloride with sodium citrate. Approximately 100 mL of a 0.1 M aqueous HAuCl_4 was boiled on a hot plate with stirring, and 10 mL of 0.5 M sodium citrate solution was added into the boiling solution of

gold to obtain a gray-blue solution which turned red-violet after 2 min. Thereafter, the solution was heated for 10 min and allowed to cool down to room temperature [34, 35].

Carbon nanosphere (CNS) was synthesized by a modified hydrothermal synthesis method [100] as follows: 0.5 g of glucose was dissolved in 30 mL of water to form a clear solution with the aid of stirring for 35 min. At the end of 35 min, the solution was transferred into a Teflon sealed autoclave (40 mL) and heated at 180 °C for 3 h. After the completion of the reaction, the autoclave was allowed to cool in the air naturally. The suspension was centrifuged at 8000 rpm for 15 min and the product was washed and dispersed in DI water. The centrifugation and rinsing cycles were repeated three times to obtain high purity CNS. Moreover, a 50-mg dosage of the CNS was dispersed in 15 mL deionized water with ultrasonication to obtain a suspension. A 10- μ L portion of 1.5 mM AgNO₃ was cast to the suspension drop by drop under stirring at room temperature. After the adsorption of silver ions on the surface of CNS for around 1 h, the suspension was added to 15 mL of a sodium citrate solution with a concentration 0.5 mM and stirred for a further 30 min. Finally, the dispersion was transferred to the Teflon sealed autoclave (40 mL) and heated at 110 °C for 1 h without stirring. The final product was first washed with acetone to remove the excess sodium citrate and then washed multiple times with deionized water. The high purity Ag decorated nanomaterial was obtained and stored in the refrigerator.

The CuO_x nanoplates were synthesized by modifying the existing Cu₂O sphere method reported by Pang et al. [101]. In this modified method, PVP was used as a shape directing and capping agent, and hydrazine hydrate was used as a reducing agent into the alkaline copper ion solution in 2-propanol. Typically, 1.0 mM solution of Cu(NO₃)₂·3H₂O was prepared in 120 mL 2-propanol solvent and then 0.5g of PVP was added. A 1mM-NaOH

solution was added and stirred for 30 minutes, and colour has changed from blue to green. After, 4.0 mL of $\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}$ (35%) was added dropwise in the above reaction mixture, and the color of the reaction mixture turned into dark brown. The dark brown copper oxide nanoplates were coated with gold using a wet chemical process. That is 3 mL of 0.6 mM aqueous solution of HAuCl_4 was added, the reaction mixture was stirred for 20 minutes, then 3mL of 5mM $\text{HAuCl}_4\cdot 3\text{H}_2\text{O}$ was added dropwise while stirring for a further 30-minute duration. Black precipitates formed and were centrifuged. The collected black product was washed with DI water and ethanol. The product was dried at room temperature for 12 hours, then for 2 hours in oven at 40°C .

2.1.2 Characterization of Synthesized Nanomaterials

Synthesized nanomaterials were characterized to comprehend their optical, morphological and structural properties. Spectroscopic evaluation involves Uv-Vis and FTIR analysis. Whereas SEM and TEM were used to study the morphology of the nanomaterials which include shape and size of the particles. Elemental compositions were analyzed using X-ray photoelectron spectroscopy (XPS) and energy dispersive X-ray spectroscopy (EDS). The electrochemical activity of the materials was investigated using electrochemical cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS).

2.1.3 Fabrication of Electrodes

Transparent glass substrates coated with conductive layer fluorine-doped tin oxide on one side (FTO Ref. 43705) having a thickness of 2.2 mm, a dimension of 5 x 5 cm, and a surface resistivity of 7 ohm/sq were purchased from SOLARONIX Switzerland. The glass substrates were cut into 1.2 x 1.2 cm square pieces to fit the size of the electrochemical-SERS cell. Prior to the deposition of AgCNS, The FTO substrates were thoroughly cleaned

by subjecting to 50 °C ultrasonic treatment [99] first in acetone, then in isopropyl alcohol followed by deionized water with a duration 15 min. for each solvent. The washed substrates were subsequently dried in an oven at 50 °C for 10 min. The suspension of the nanosphere material was prepared by dispersing 10 mg in 5 mL deionized water and sonicated for 10 min. The deposition of AgCNS on the FTO substrates was carried out by the drop-casting method [100]. An AgCNS suspension was uniformly drop-casted on the conductive surface of the substrates and dried in an oven at 60 °C for 20 min as depicted in Fig 2.1.

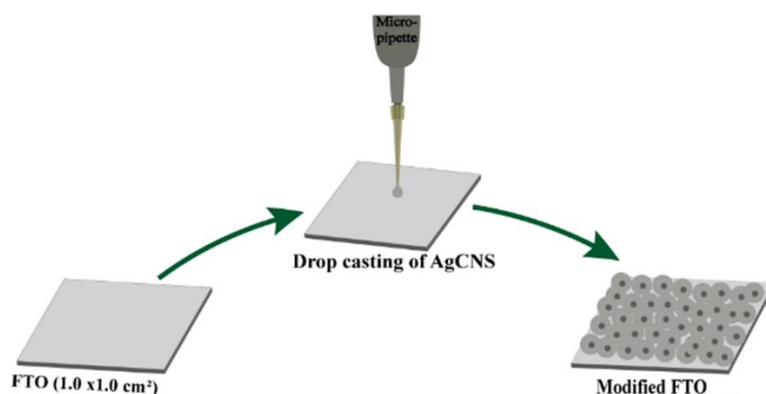


Figure 2. 1 Electrode fabrication using drop casting technique

2.1.4 Design and Development of a Spectro-electrochemical Cell

The core components of EC-SERS analysis are Spectro-electrochemical cell and experimental setup. The spectro-electrochemical cell configuration designed for the EC-SERS analysis is shown in Fig. 2.2. The cell is made of a plexiglass block with the help of the mechanical department of KFUPM. It is circular in shape with 4.5 cm diameter and 2.5 cm height and inner sample holder cavity of the cell is 1.5 cm cube as given in Fig 2.3a. There are three holes of different sizes in the cell to insert the three electrodes to develop a closed circuit for electrochemical analysis. It consists of three electrodes: SERS substrate

as working electrode, Pt-wire as counter electrode and Ag/AgCl as reference electrode. These three electrodes are assembled in good relative geometrical positions to conduct efficient and accurate electrochemical and Raman measurements.

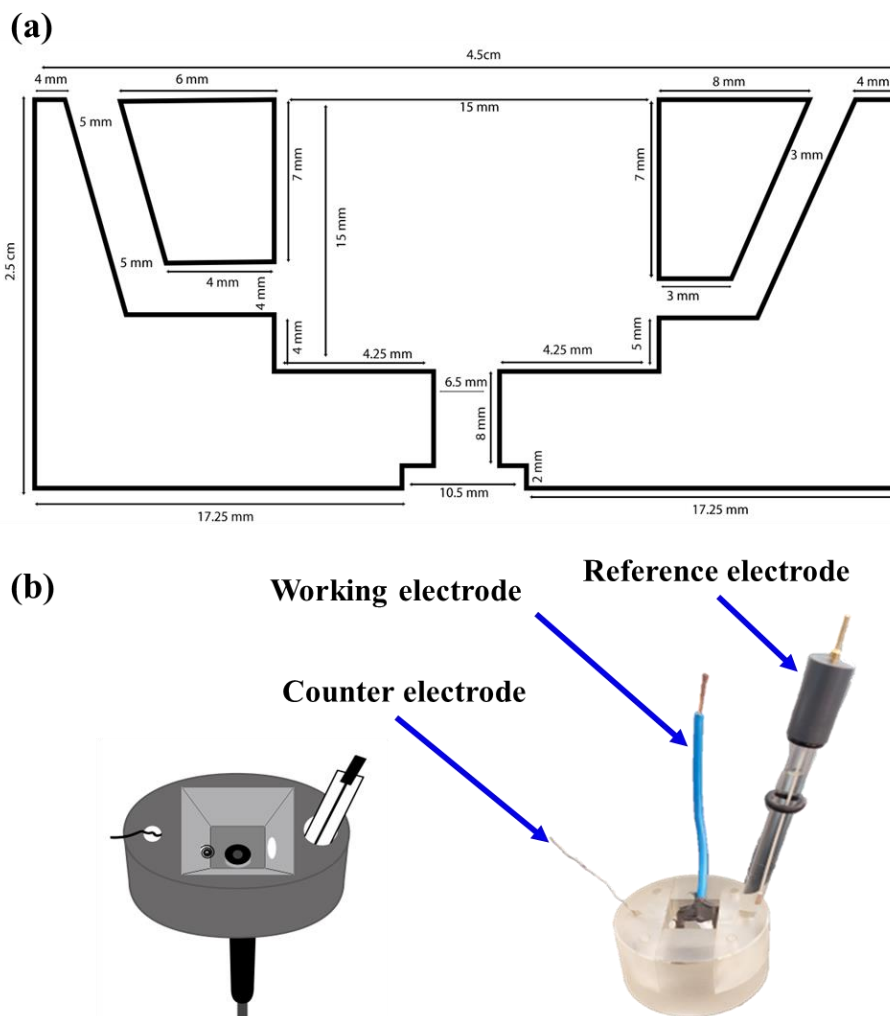


Figure 2. 2 (a) The cross-sectional view and (b) 3D model of Spectro-electrochemical cell

A typical experimental set up used in our lab for EC-SERS analysis is presented in Fig. 2.3. The experimental set consists of Spectro-electrochemical cell, CHI potentiostat, three electrodes system and HORIBA LabRAM HR evolution Raman spectrophotometer. The electrochemical stripping voltammetry was used for the application of electrical potential.

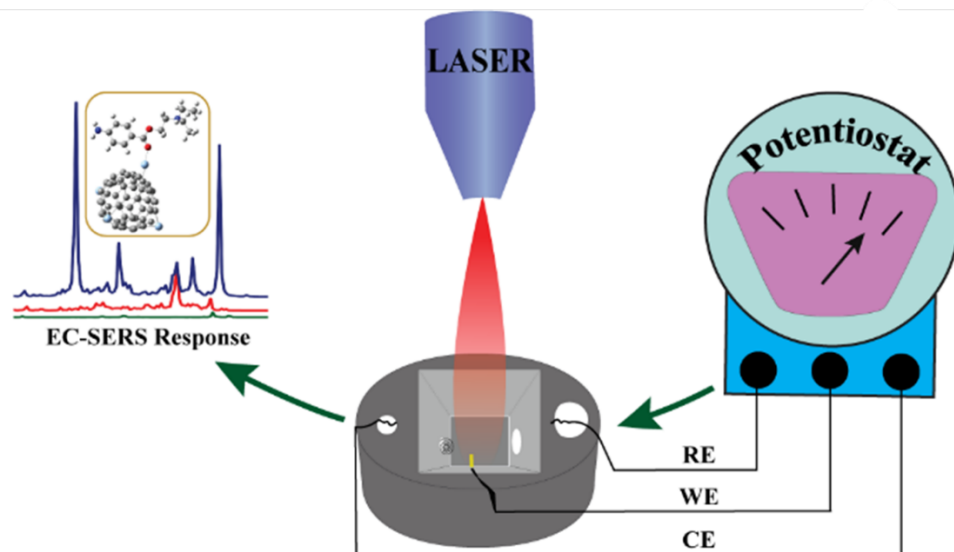


Figure 2. 3: A typical EC-SERS experimental set used in the lab for measurements

2.1.5 SERS and electrochemical SERS measurements

The LabRAM HR evolution spectrophotometer equipped with an internal He-Ne laser 17 mW and two different excitation wavelength sources (633 and 532 nm) and thermoelectrically cooled CCD detector was used to collect normal Raman, SERS and EC-SERS spectra. The Raman spectra were recorded by varying laser power, the acquisition time, a number of scans and three different objective lenses of a microscope such as 10X, 50X and 100X. The SERS spectra were collected under the same conditions as used for normal Raman spectra and the ratio of nanomaterial suspension and analytes were varied to achieve the optimum signal. The EC-SERS measurements were conducted using a typical three-electrode system. The spectra were collected at open circuit potentials followed by a cathodic sweep or anodic sweep of potential using electrochemical stripping voltammetry. All electrolyte solutions were purged with a high purity argon gas (99.999%) prior to measurements.

2.2 COMPUTATION

Quantum chemical calculations were conducted using GAUSSIAN 09 [101] with the adoption of the Becke 3-Parameter (Exchange), Lee, Yang and Parr (B3LYP) functional introduced by Becke [102] and Lee et al [103], and various basis sets for the non-metal atoms and metallic systems. These basis sets were selected due to their reliability to produce reasonable results that assent to experimental data of moderate size organic systems at reasonable costs [104–106]. DFT computation helps to gain insights on the interactions between the substrate and analyte molecules and thus understand the mechanism of SERS enhancements as a result of these interactions. Further, the aqueous solution corrections were computed via the Polarizable Continuum Model (PCM) developed by Tomasi [107]. Frequency calculations were conducted to ascertain the nature of convergence of analyte molecule and substrate complexes. The same was used to predict the vibrational assignments of the wavenumbers for the compounds understudied. To ensure a good agreement with the Raman experimental results, the theoretical wavenumbers were scaled by a suitable factor as mentioned in the literature [108].

2.3 RESEARCH OBJECTIVES

The mechanistical approach of SERS technique makes it capable of finding basic structural information of molecules adsorbed on the surface of metal nanomaterials including adsorption mode, surface orientation and conformation. Therefore, SERS technique is a powerful analytical means for the characterization of the adsorbed analyte and assessing the orientation changes and interface processes of analyte molecule in the vicinity of metal nanoparticles [109–112]. We reported several spectroscopic and analytical studies of SERS method and its applications for several drugs, such as 2-thiouracil [113], Sulindac[114],

clotrimazole [115], procaine [116,117] and valeric acid [118]. In all these studies, gold and/or silver-based nanomaterials were employed as substrates for the SERS analysis. Competitive detection limits of these compounds could be achieved, and analysis of the vibrational modes involved in the interaction and prediction of the adsorption scenarios were proposed based on quantum chemical assessments.

The specific objectives of this work are as follows:

1. Synthesis and characterization of SERS substrates composing metal nanoparticles deposited on carbonaceous materials.
2. Fabrication of electrodes using synthesized nanomaterials and carrying out relevant characterization tests.
3. Investigation of the EC-SERS efficiency towards procaine, procainamide and Sulindac samples and optimization of electrochemical and spectroscopic parameters.
4. Implementation of DFT calculation to provide meaningful assignments of the SERS spectra.

CHAPTER 3

Characterization of Valeric Acid Molecules Using Substrate of Silver Nanoparticles with SERS

3.1 INTRODUCTION

Valeric acid (VA), called also pentanoic acid is a compound extracted from *Valeriana wallichii* [119]. It is a straight chain alkyl carboxylic acid with a chemical formula of $C_5H_{10}O_2$ and has a melting point of $34.5^{\circ}C$. Valeric acid (VA) is considered as an example of renewable lignocellulose derived platform compound which has lately attracted substantial attention [120]. Its production from lignocellulose derived from levulinic acid and γ -valerolactone was established to be effective, and robust [121]. VA is an intermediate in the conversion of lignocellulose to valeric acid esters, which are a class of cellulosic biofuels [122]. It has a structure similar to that of neurotransmitter gamma-Aminobutyric acid (γ -Aminobutyric acid) (GABA) [123].

Surface-enhanced Raman spectroscopy (SERS) is identified as a powerful technique with improved sensitivity with several bands which could be used for evolving analysis methods for various analyses [124]. SERS is commonly used for aromatic acids like nitrobenzoic acid, aminobenzoic acid, benzene tricarboxylic acid, mercaptobenzoic acid, hydroxybenzoic acid, 2-naphthoic acid, pyrazinoic acid, 2-thiouracil and methimazole is analyze by using silver and gold nanoparticles [125–132]. However, there are only a few reports on its application for the aliphatic acids. Moskovits and Suh were identified as the

pioneer researchers to study the use of silver colloids for the SERS experiments on some of the aliphatic acids. In addition, SERS technique was extended to the investigation of the adsorption of carboxylic acids and amino acids containing molecules onto the surface of different metals such as silver, copper, and gold. In this application, the nanoparticle substrate was required to enhance the Raman band signal[128].

Particles like gold and silver are normally used as a substrate for the signal enhancement, but their drawback is agglomeration which in turn decreases the SERS signals. However, this problem has been addressed using alumina and carbon nanostructures such as graphene as a spreader to compact and stabilize the particles and prevent agglomeration. Good dispersal would afford efficient spreading of the nanoparticles [133,134]. In line with this, the nanoparticles' decoration of graphene nanosheets characterized by the high surface area, has been found promising for SERS applications [135]. Furthermore, graphene loaded with metal particles was reported for the SERS detection of many analysts ranging from protein to rhodamine as well as drug compounds with high selectivity and sensitivity [136]. For quantitative determination and elucidation, 2-Thiouracil was used to enhance the surface of Raman spectroscopy while employing silver nanoparticles seeded on graphene nanosheet as a substrate [137]. Various reports have shown high enhanced SERS spectra resulting from the use of the different monolayer graphene silver composite [138]. In addition, graphene-copper composites, as well as gold nanoparticles loaded graphene [139,140] have been documented as efficient substrates for SERS experiments.

The aim of this work was to establish a procedure for the characterization and trace analysis of valeric acid using synthesized spherical silver nanoparticles with SERS. To

understand the mechanism of interactions, DFT study was conducted to assign the obtained bands.

3.2 EXPERIMENTAL

3.2.1 Materials and Synthesis

Valeric acid, hydroxylamine hydrochloride (HAH) and sodium hydroxide were obtained from Sigma-Aldrich. Silver nitrate was obtained from Fisher. There was no further purification of the reagents. The silver nanoparticles were prepared by reduction procedure. For the synthesis, 10 ml of 1.5×10^{-2} M solution HAH was introduced to 10.0 mL of 3.0×10^{-2} M sodium hydroxide. After that, the components were introduced to 180 mL of 1.11×10^{-3} M silver nitrate solution under agitation. The mixture was further stirred for about 30 min without heating. The resulting silver colloids were first stored in a refrigerator prior to its use for SERS analysis.

3.2.2 Characterization

The synthesized Ag-NPs were characterized by the use of Fourier-transform infrared spectroscopy (FTIR), UV-vis spectroscopy and Transmission electron microscopy. FTIR spectrum was obtained by Nicolet 6700 FT-IR spectrometer coupled to helium-Neon laser operated at 0.5W for excitation. With a KBr beam splitter, the DTGS KBr detector was used to collect the spectrum in the range between 400 and 4000 cm^{-1} with resolution of 2 cm^{-1} . The UV spectrum was collected using UV–Visible spectrophotometer, Agilent Technologies, with standard quartz cuvette. The TEM image was recorded by JEM -2100F Field Emission Electron Microscope; JEOL - USA at acceleration voltage of 200.0 kV.

3.2.3 Sample preparation and SERS measurements

Aqueous solutions with various concentrations of valeric acid were prepared for the SERS measurement. Then, the spectra of VA were measured by the use of Lab Ram HP Evolution Raman spectrometer coupled with internal He-Ne 17 mW red laser at excitation wavelength of 633 nm. 50% of its power was used on the cuvette (with 1 cm radius, 1cm height) containing VA solution with Ag-NPs solution with equal amounts. A 10x objective was operated in order for focusing the laser beam on the target. The microscope was coupled confocally to a 600 mm focal length spectrograph with two switchable grating systems. The data acquisition time was the 50s with 64 accumulations of collection for each SERS spectrum.

3.3 COMPUTATION

All the DFT study was accomplished using GAUSSIAN 09. The B3LYP method developed by Becke [141,142] and Lee et al [143] was adopted with a basis set of 6-311++G(d,p) for the non-metal while Stuttgart/Dresden (SDD) basis set was utilized for the silver atoms. The choice of this method together with the basis set was based on their reliability established in the literature in terms of providing good agreement with the experimental data of medium size organic molecules at a reasonable cost [144]. The aqueous solution was simulated using Polarizable Continuum Model (PCM) introduced by Tomasi and co-workers [145] while the frequency calculation was carried out using the same basis sets, with the help of Gaussian software to check the convergence nature of valeric acid-Ag₄ complexes and also for the vibrational assignments of the theoretical wavenumbers computed for these complexes. In order to have better assent with the Raman experimental data, the calculated wavenumbers were scaled down by a factor of 0.95 [146]

while the Raman intensities were computed using expression described in the literature [147].

3.4 RESULT AND DISCUSSION

3.4.1 Results of the Characterization

Following the preparation of silver colloid via a reduction process, the UV-visible characterization of the colloid is essential as it reveals its average particle size, shape, the state of agglomeration as well as its surface property [148]. As such, the UV-visible of the prepared colloid was recorded and the absorption maximum (λ_{max}) was determined as 420 nm. This value indicates that the colloidal particles are active in the visible light, thus, it could be used for SERS measurements. The morphology of the Ag-NPs was characterized by TEM. The image depicted in Fig 3.1 indicates that the nanoparticles have a spherical shape with a size of around 20 nm.

Another important characterization worth conducting on the silver nanoparticles is the infrared measurement which is believed to give insight into vibrational properties of molecules at the molecular level. The infrared spectrum obtained for the hydroxyl amine reduced silver colloid showed a broad band between 3200-3700 cm^{-1} which is ascribed to OH stretching vibrational mode. Another medium broad band was found between 2100-2200 cm^{-1} while a medium sharp band corresponding to NH_2 scissoring was observed at 1653 cm^{-1} . A weak broad band which could be associated with NH_2 wagging mode was found between 740-755 cm^{-1} . On the other hand, the Raman spectra of the nanoparticles presented in Fig 3.2 showed three small bands between 150-285 cm^{-1} . This allows using

the silver nanoparticles in the range above 300 to 4000 cm^{-1} without significant interferences with the bands from VA.

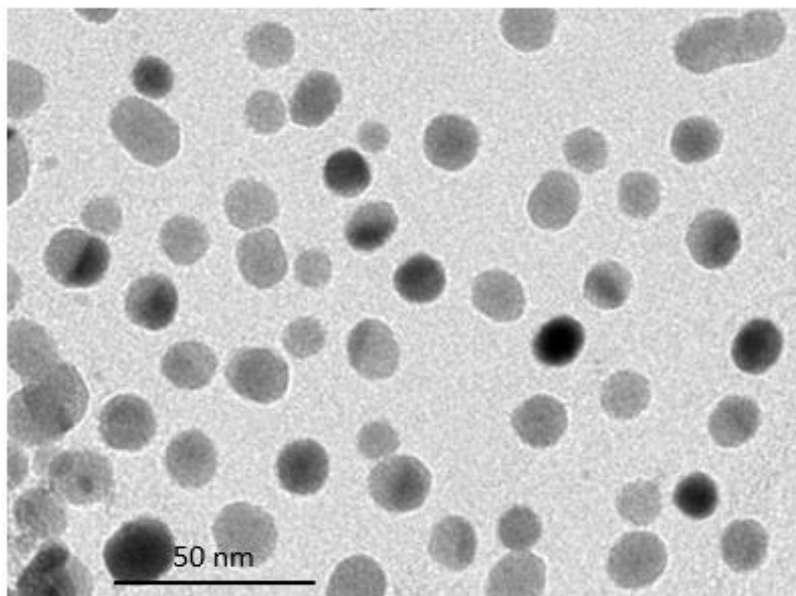


Figure 3. 1 TEM image of the prepared silver nanoparticles

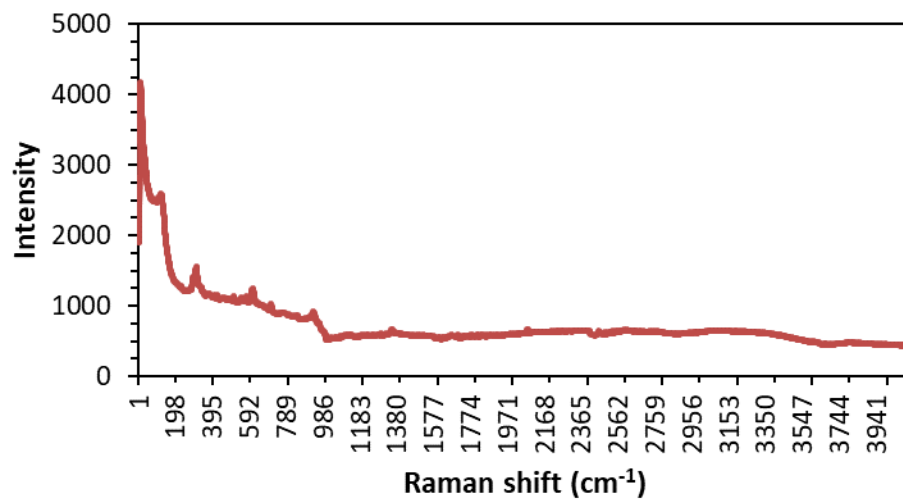


Figure 3. 2 Raman spectrum of the prepared silver nanoparticles

3.4.2 Valeric acid Detection

Spectroscopic technique and chromatography are important tools usually employed in the detection of a very low concentration of targets. However, the band enhancement brought

about by SERS has given spectroscopic technique an edge as the target could be detected at 10^{-10} M level. Based on this, SERS method was employed to quantitatively detect VA at different concentrations using hydroxylamine-reduced silver colloid (HRSC) as substrate. The Raman spectrum of pure VA presented in Fig 3.3 is characterized with a lot of strong bands.

Upon the SERS experiment, the bands found between 1074 cm^{-1} and around 1000 cm^{-1} (Fig 3.4), corresponding to C-O and C-C stretching respectively were identified as the most enhanced and symmetric bands. As such, they were used for the qualitative detection of the VA in solution. The result presented in Fig 3.5 shows that the intensity of SERS spectra enhanced with the presence of substrate. This indicates a direct relationship between the SERS intensity and the amount of VA in solution. Within the dynamic range for the vibrational wavenumber at band 1002 cm^{-1} , the minimum concentration of VA observed was 10^{-7} M. Moreover, the calibration curve at this frequency was established by plotting SERS intensities against the logarithmic concentration of valeric acid in aqueous medium. The resultant curve gave a linear relationship to the dynamic range of 1×10^{-7} to 1×10^{-2} M (Fig 3.5 b) with 0.995 correlation coefficient (R^2). The physical detection limit was found to be 1×10^{-10} M. However, this range does not fit within the linearity dynamic range of the calibration curve.

The enhancement obtained by SERS using Ag as the substrate can be explained by the interaction between Ag and VA molecules. There are two enhancement mechanisms, i.e. electromagnetic mechanism, and chemical mechanism. The electromagnetic enhancement of the incident and scattered fields where the scattering occurs in the enhanced local optical fields of the silver substrate. The chemical or electronic enhancement occurs when the

incident light excites charge transfer resonances between the silver and VA molecule. The interaction between VA molecules and the Ag substrate, Fig 3.6, displays large Raman process at a cross-section of interaction compared to the Raman cross section of VA without substrate.

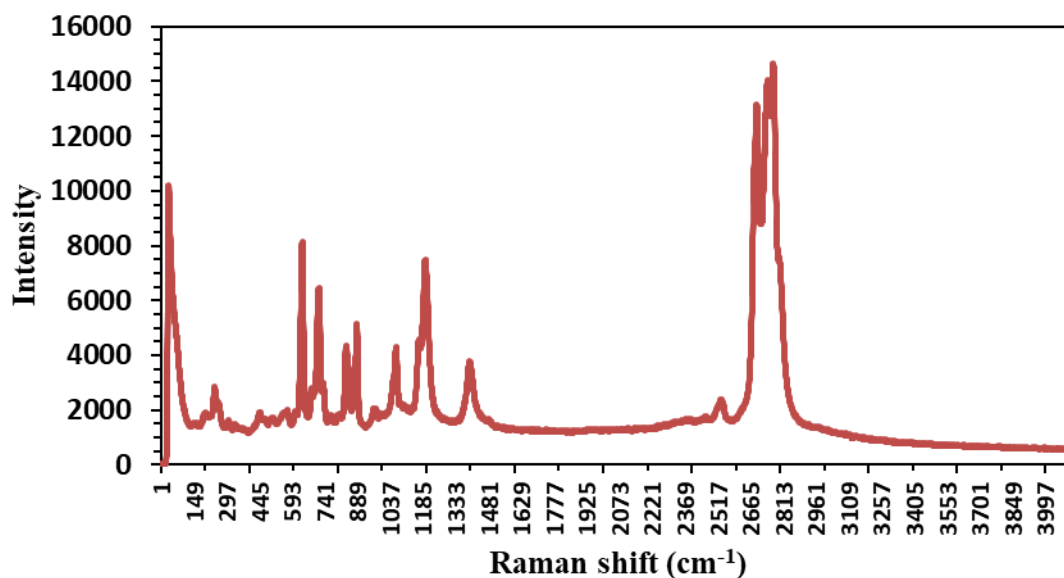


Figure 3. 3 Raman spectra of pure solid Valeric acid

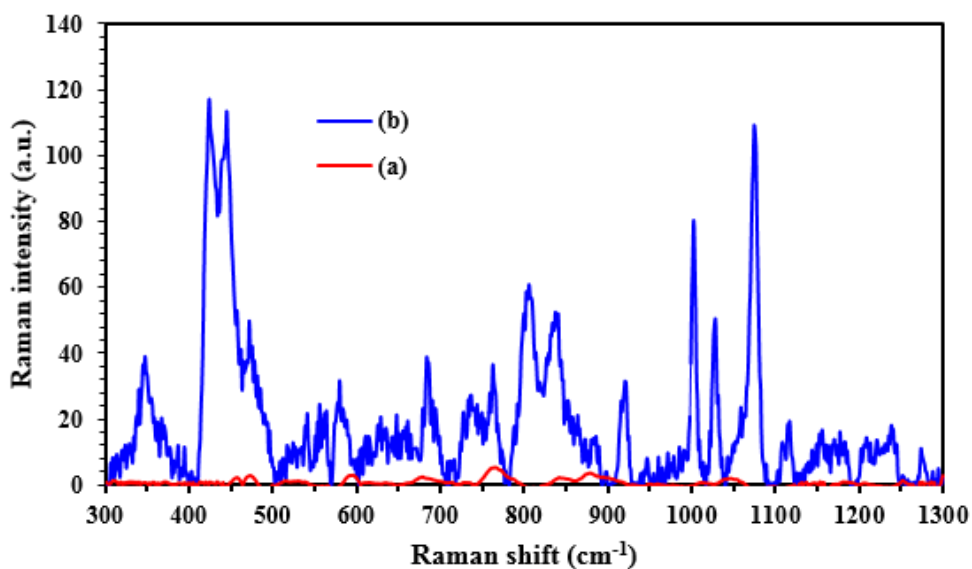


Figure 3. 4 Raman spectra of Ag nanoparticles (a) and SERS of VA solution 0.1 M (b)

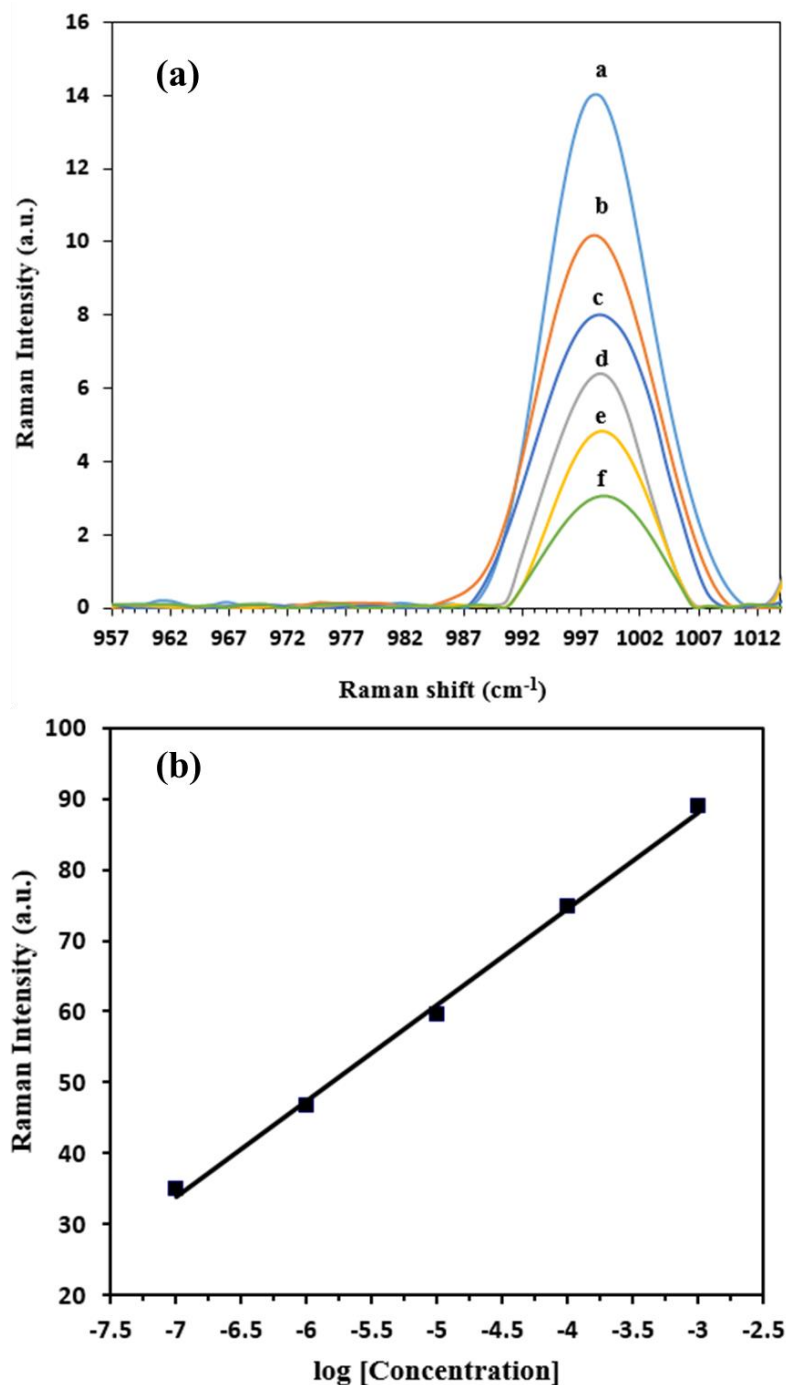


Figure 3. 5 (a) SERS spectra of Valeric acid at different concentrations (a 10^{-2} , b 10^{-3} , c 10^{-4} , d 10^{-5} , e 10^{-6} , f 10^{-7}). Red Laser $\lambda = 633\text{nm}$, Lase power; 50%, acquisition time; 50s, accumulation; 64, objective; 10 $\times$, and range of spectra; 950-1110 cm^{-1} . (b) Calibration curve of the band at 1020 cm^{-1} . Conditions: Laser $\lambda = 633\text{nm}$, Lase power; 50%, acquisition time; 50s, accumulation; 64, objective; 10 $\times$, and range of spectra; 957-115 cm^{-1} .

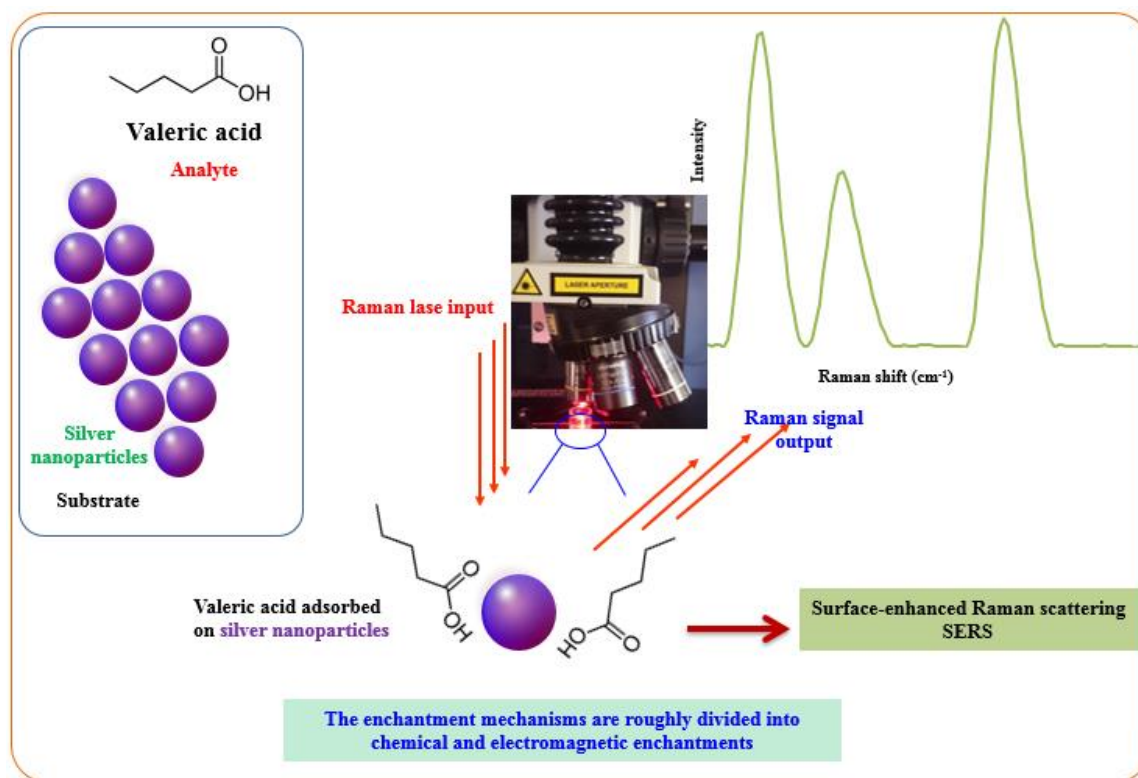


Figure 3. 6 Schematic illustration of SERS procedure for the detection of VA

3.4.3 DFT investigation of Raman and SERS spectra

The detailed vibrational assignment is essential in mastering vibrational properties of molecules and this requires inspection of vibrational modes associated with different frequencies identified in the spectrum. On the other hand, the resultant spectrum from SERS experiment is usually characterized with Raman line shifts as well as changes in intensities when compared with free molecule's Raman. In line with these, vibrational assignments were provided for the valeric acid and valeric acid-Ag₄ complex spectra within the wavenumber range of 300 to 1300 cm⁻¹ with the aid of Gauss View [143]. In order to effectively assign the vibrational modes, theoretical wavenumbers were computed at the B3LYP/6-311++G(d,p) and they were found to coherently agree with the experimental data as highlighted in Table 3.1 and presented in Fig 3.7. The Raman line 1266 cm⁻¹ is assigned to CH₂ twisting vibrational mode and this assignment is in good agreement with

wavenumbers reported for CH₂ twisting [149]. In addition, the Raman bands observed at 1240 cm⁻¹ and 1190 cm⁻¹, but predicted at 1256 cm⁻¹ and 1212 cm⁻¹ can be associated with the CH₂ wagging modes which are normally found in the range of 1305 – 1195 cm⁻¹ [150,151]. On the other hands, the Raman bands noticed at 1114 cm⁻¹ and 1059 cm⁻¹, but predicted at 1097 cm⁻¹ and 1052 cm⁻¹ correspondingly, can be attributed to vibrational modes associated with C-O stretching. The wavenumber 1114 cm⁻¹ is within the characteristic frequencies range (1180 – 1080 cm⁻¹) reported for C-O stretching vibrations of monomeric carboxylic acid [152] whereas the lower wavenumber 1059 cm⁻¹ together with its predicted values is coherent with the 1063 cm⁻¹ reported for valeric acid C-O stretching vibration by Badawi et al [149]. Moreover, the wavenumbers associated with C-C stretching modes were noted at 997 cm⁻¹, 931 cm⁻¹, and 833 cm⁻¹, and predicted at 1016 cm⁻¹, 931 cm⁻¹, and 828 cm⁻¹ respectively. These assignments assent with the 1200 – 800 cm⁻¹ wavenumbers range, for which the C-C stretching vibrational modes are expected to be found [153,154]. Since the wavenumber is a function of bond strength, the higher stretching wavenumber numbers predicted for C-O over that of C-C stretching modes could be attributed to stronger C-O bond strength as established by Silverstein et al [154]. In addition, the Raman band observed at 748 cm⁻¹ (compared to 769 cm⁻¹ from DFT) could be assigned to the CH₂ rocking mode [149]. However, the bending vibrational modes connected with carboxylic OH were identified at 630 cm⁻¹ (614 cm⁻¹ from DFT), 590 cm⁻¹ (599 cm⁻¹ from DFT) and 446 cm⁻¹ (492 cm⁻¹ from DFT) and these assignments are in consonant with the wavenumbers reported for the OH bending modes in carboxylic acid [155,156]. Furthermore, the Raman bands identified at 428 cm⁻¹ and 356 cm⁻¹ could be

attributed to C-C-C bending vibrational mode. This is in line with wavenumbers established for C-C-C bending mode [157,158].

It is apparent that the SERS experiment involves interaction between Ag-cluster and valeric acid which in turn alters the bands positions as well as their intensities in the resulting SERS spectrum. In order to gain insight into the mode of interactions occurring between Ag-cluster and valeric, molecular modeling was carried out via DFT calculation. Rhombus shape consisting of four silver atoms which have been established as the most stable geometry [159] was used to simulate the Ag-cluster. The choice of this geometry was also based on the fact that the electron cloud garnered by these four Ag atoms makes this configuration more susceptible to ionization process [160]. Moreover, the two most probable modes of interactions predicted for the Ag-valeric acid complex are presented in Fig 3.7. They are the feasible modes of interactions in the sense that the reactivity of valeric acid is predicated upon the -COOH ; its functional group consisting of C=O and OH . As such, Ag_4 interacted with these groups and the energy of interaction was found to be higher for O=C (Table 3.2). Besides, the negative values recorded for these energies indicate that the process is exergonic. Moreover, this interaction was confirmed by analyzing the changes in Mulliken charges upon interaction and the result presented in Table 3.3 reveals that electrons were transferred from Ag_4 to valeric acid in the Ag-OH interaction whereas, an opposite trend was found for that of Ag-O=C coupled with Ag-HO . Consequently, the spectrum (Fig 3.7c) resulting from the interaction between the Ag_4 and hydroxyl oxygen was observed to have bands pattern as well as values that are in better agreement with the SERS spectrum. Moreover, upon interaction, the intensities of valeric acid bands were observed to generally increase with the most enhancements occurring at bands 1074

cm^{-1} , 1002 cm^{-1} and 445 cm^{-1} corresponding to C-O stretching, C-C stretching and C-C-C bending vibrational modes respectively in both SERS and theoretical spectra (Fig 3.7). These observed enhancements are in line with the Castro and co-workers report on the SERS study of tartaric and malic acids using silver colloids [161]. Furthermore, the intensities enhancement recorded in SERS spectrum is due roughened silver surface which resonantly creates highly plasmonic light field upon laser excitation and this, in turn enhances the light scattering of valeric acid lying close to the plasmonic field and thus, resulting to the enhanced Raman signals. Another effect of the interaction noticed in the SERS spectrum are the shifts in wavenumbers which could be attributed to electrons transfer brought about by this interaction as revealed by the Mulliken charge analysis (Table 3.3). The transfer of electrons is expected to affect the bond strength, an important parameter in the determination of wavenumber. Consequently, the vibrational modes associated with the hydroxyl oxygen (ν_4 , ν_5 , ν_{10} and ν_{12}) were predicted to redshift from 1097 cm^{-1} , 1052 cm^{-1} , 614 cm^{-1} and 492 cm^{-1} to 1087 cm^{-1} , 1038 cm^{-1} , 571 cm^{-1} and 468 cm^{-1} respectively. This is inconsonant with the downward shift observed in the SERS spectrum. The red shift recorded could be attributed to the electron transfer as well as the vibrational constraint caused by the silver atom. On the other hand, the Raman lines 1266 cm^{-1} , 1190 cm^{-1} and 748 cm^{-1} corresponding to CH_2 twisting, wagging and rocking vibrational modes respectively were observed to blue shift to 1274 cm^{-1} , 1209 cm^{-1} and 763 cm^{-1} accordingly. In addition, the comparison of theoretical wavenumbers listed in Table 3.1 suggests that the resultant SERS spectrum might involve a contribution from double interactions (d) depicted in Fig 3.7. The C-C stretching vibrations predicted for this

interaction at 1013 cm^{-1} and 844 cm^{-1} are in better agreement with 1002 cm^{-1} and 834 cm^{-1} observed respectively in SERS.

Table 3. 1 Assignment of vibrational modes of both calculated and experimental Raman spectra of valeric acid and valeric acid-Ag in a 1300-600 cm⁻¹ range

Description	Observed (cm ⁻¹)		Calculated (cm ⁻¹)		
	Raman	SERS	Valeric acid ^b	Valeric acid-Ag cluster	
				Ag – OH interaction	Ag–O=C and Ag–HO interactions
v ₁ CH ₂ twist	1266 vw	1274 w	1266 (58)	1266 (70)	1264 (3)
v ₂ CH ₂ wag	1240 vw	1238 w	1256 (8)	1246 (26)	1222 (100)
v ₃ CH ₂ wag	1190 w	1209 w	1212 (7)	1214 (7)	-
v ₄ C-O str.	1114 m	1074 s	1097 (11)	1087 (91)	1131 (86)
v ₅ C-O str.	1059 m	1027 m	1052 (20)	1038 (73)	1066 (17)
v ₆ C-C str. (Consecutive CC & CC antisym. str.)	997 vw	1002 s	1016 (38)	1016 (100)	1013 (3)
v ₇ C-C str. (Alternating CC & CC antisym. str.)	931 vw	920 m	977 (13)	978 (26)	979 (0)
v ₈ C-C str. (OC-C str.)	833 s	834 m	828 (17)	868 (71)	844 (30)
v ₉ CH ₂ rock	748 vw	763 m	769 (0)	770 (0)	767 (0)
v ₁₀ OH bend (out-of-plane)	630 vw	556 w	614 (4)	571 (12)	680 (62)
v ₁₁ O=C-OH bend	590 vw	580 w	599 (56)	602 (48)	625 (58)
v ₁₂ OH bend (out-of-plane)	446 vw	425 s	492 (26)	468 (25)	533 (8)
v ₁₃ C-C-C bend	428 vw	445 s	476 (4)	486 (91)	490 (69)
v ₁₄ C-C-C bend	356 vw	345 m	315 (100)	322 (78)	322 (72)

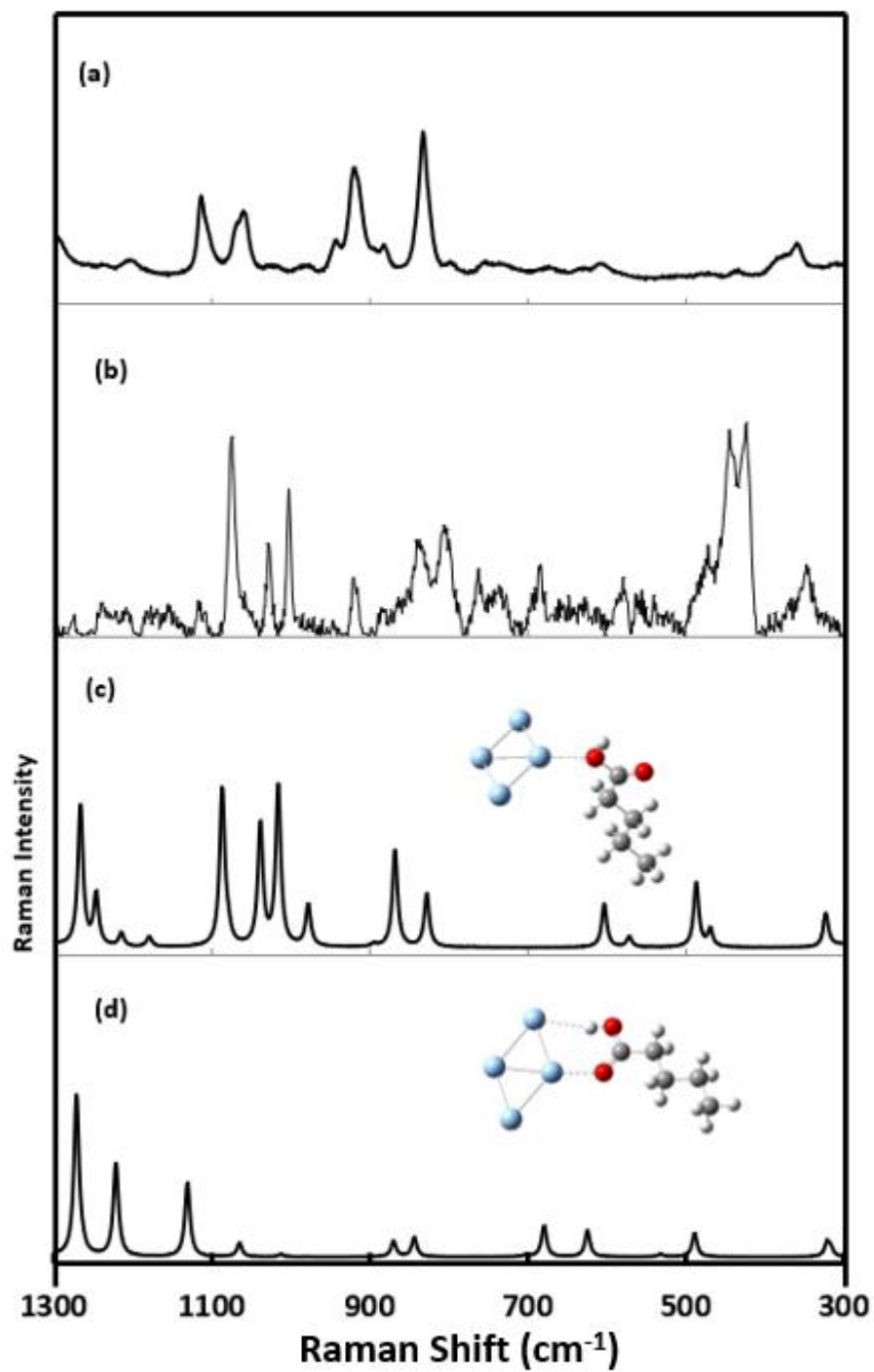


Figure 3. 7 Raman spectra; (a) Valeric acid spectrum, (b) SERS, (c) Calculated spectrum for Ag-hydroxyl oxygen interaction (d) Calculated spectrum for double interaction involving Ag-carbonyl oxygen Ag-hydroxyl hydrogen interactions

Table 3. 2 Energy of interaction

Interaction	Energy (kcal/mol)
Ag – OH	-7.61
Ag–O=C and Ag–HO	-12.78

Table 3. 3 The sum of Mulliken charges in the system

Compound	Free	Upon interaction	
		Ag – OH	Ag–O=C and Ag–HO
Ag4	0	0.012	-0.123
Valeric acid	0	-0.012	0.123

CHAPTER 4

SERS-based trace-level quantification of sulindac: spectroscopic and molecular modeling evaluation

4.1 INTRODUCTION

Sulindac, a common non-steroidal anti-inflammatory drug (NSAID) approved by the United States Agency for Food and Drug Administration (FDA) in 1978 [19], is used in the treatment of inflammatory infections, such as joint stiffness, shoulder tendonitis, gouty arthritis and arthritis of the spine [20]. Several reports on the use of sulindac in the treatment of lung tumorigenesis, colorectal cancer, skin cancer, and prostate cancer have also been documented [21–25]. However, despite its tumorigenic and anti-inflammatory activities, the long-term exposure or overdose of sulindac increases the risk of heart attack [26], stroke or intestinal bleeding which can be fatal [27,28]. There is, therefore, a need to develop a convenient and accurate means of its detection in pharmaceutical formulations [162,163]. To date, a number of analytical techniques, such as high-performance liquid chromatography [164], gas chromatography/mass spectrometry [165], reverse-phase liquid chromatography [166], electrochemical sensing [20], and ultra-performance liquid chromatography [167] have been developed for the detection and quantification of sulindac. While these techniques were proven to be highly sensitive, in most of the cases they are time-intensive, require complicated derivatization processes and consume an ample amount of organic solvents. Therefore, the development of alternative highly

sensitive techniques, which are at the same time convenient and quick for the quantification of sulindac, is indeed essential.

Surface-enhanced Raman-scattering (SERS) spectroscopy is a potential analytical approach that has attracted attention over the years due to its distinctive spectroscopic fingerprints, high sensitivity, non-destructive data acquisition and rapid response [168–170]. This technique has reportedly shown a significant amplification in Raman signals by factors up to 10^6 [171], making it an attractive choice for the detection of trace concentration of biomolecules [172,173], pharmaceutical applications [174] and in food analysis [175]. Besides providing unique molecular fingerprints of molecules, SERS has the tendency to achieve ultra-trace detection limits and is suitable for on-site detection, contrary to other analytical methods. Commonly used substrates in SERS applications are silver nanoparticles (AgNP) and gold nanoparticles (AuNP) due to their stability and high electromagnetic and chemical signal amplification [176–178]. The electromagnetic signal enhancement occurs due to surface plasmon resonance of the nanoparticles, which under irradiation by the incident light leads to charge transfer between the metal nanoparticles and the target molecules with ultimate Raman intensity improvement [179]. Chemical enhancements, on the other hand, occur mainly due to interactions between the nanoparticles and the target molecules such as the formation of complexes with a semi-covalent type of bonding. To study the nature of the various interactions of these substrates with target analytes leading to the signal amplification phenomenon, quantum chemical techniques can be utilized [180–182].

In the present study, a sensitive, selective and rapid SERS based analytical approach was developed for the qualitative and quantitative investigation of the non-steroid anti-

inflammatory drug sulindac by utilizing silver nanoparticles (AgNP) as a SERS substrate and carrying out quantum chemical calculations. The physical aspects of the possible sulindac interaction with AgNP were rationalized at the molecular level using density functional theory (DFT) calculations, and conclusive assignments of the active SERS modes were conducted.

4.2 METHODOLOGY

4.2.1 Experimental

Sulindac, sodium hydroxide and hydroxylamine hydrochloride were bought from Sigma-Aldrich, while silver nitrate was obtained from Fisher. The silver nanoparticles were synthesized by the reduction method [183], where 10.0 mL of 3.0×10^{-2} M sodium hydroxide and 10.0 mL of 1.5×10^{-2} M hydroxylamine hydrochloride were mixed and poured into the silver nitrate solution (180 mL of 1.11×10^{-3} M) under stirring and heating. The prepared mixture was again agitated for 30 min. The prepared silver colloids were kept at 5 °C before the utilization for SERS analysis. A 1.0 M solution of sulindac was prepared in DMSO, and lower concentrations were prepared by successive dilutions.

4.3 COMPUTATION

All DFT calculations were accomplished using GAUSSIAN 09 [101] with the adoption of the hybrid B3LYP functional introduced by Becke [102] and Lee et al [103]. The basis set def2svp was utilized for non-metals, while the Stuttgart/Dresden (SDD) basis set was adopted for silver atoms. These basis sets were selected due to their ability to assent to experimental results of moderate size organic systems at a reasonable cost. Frequency calculations were conducted on the same basis sets to ascertain the nature of convergence of sulindac-silver complexes and to provide rational assignments of the associated

vibrational modes[184–187]. The effect of DMSO was accounted for using the Polarizable Continuum Model (PCM). Molecular-level interaction studies of sulindac and the metal nanoparticles were conducted, and the magnitudes of interaction energies were computed using the following equations (4.1 & 4.2):

$$E_{binding} = E_{complex} - (E_{sulindac} + E_{metal}) \quad (4.1)$$

$$E_{interaction} = -E_{binding} \quad (4.2)$$

Where, $E_{complex}$, E_{metal} and $E_{sulindac}$ denote the free energies of silver-sulindac complex, silver cluster, and isolated sulindac molecule, respectively

4.4 SPECTROSCOPIC CHARACTERIZATION

The infrared spectrum of the synthesized silver nanoparticle was collected in the range 500-3800 cm^{-1} (Fig 4.1) on a Nicolet 6700 FT-IR spectrometer. The prominent bands observed at the wavelengths 1700 and 1108 cm^{-1} correspond to C-C and C-N stretching, respectively. These bands provide a proof of the stabilization of the silver nanoparticles (AgNPs) and are in agreement with the reported silver nanoparticles spectrum [188]. The AgNPs in DMSO Raman spectra were recorded in the range of 975-2500 cm^{-1} as depicted in Fig 4.2 and exhibit three prominent peaks at 1010, 1410 and 1375 cm^{-1} which correspond to the solvent DMSO. The morphology of the prepared silver nanoparticles was characterized by the field-emission scanning electron microscopy (FESEM). The SEM and TEM image depicted in Fig 4.3 reveals spherical shape nanoparticles with almost a uniform nano-sized particle distribution. The obtained particle size of AgNPs is in the range between 40 to 60 nm. For informative interpretation of the Raman and SERS spectral characteristics of sulindac, vibrational assignments of the key peaks were carried out. When compared to the Raman spectrum of the pure drug sample, the SERS spectrum has

undergone a number of shifts and changes in the intensities in some Raman bands as presented in Fig 4.4.

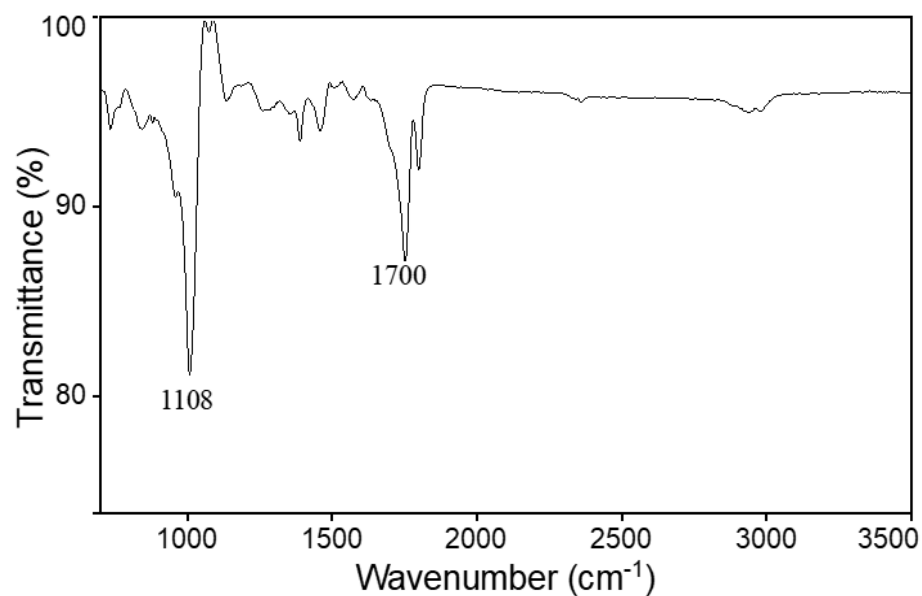


Figure 4. 1 The FT-IR spectrum of AgNPs.

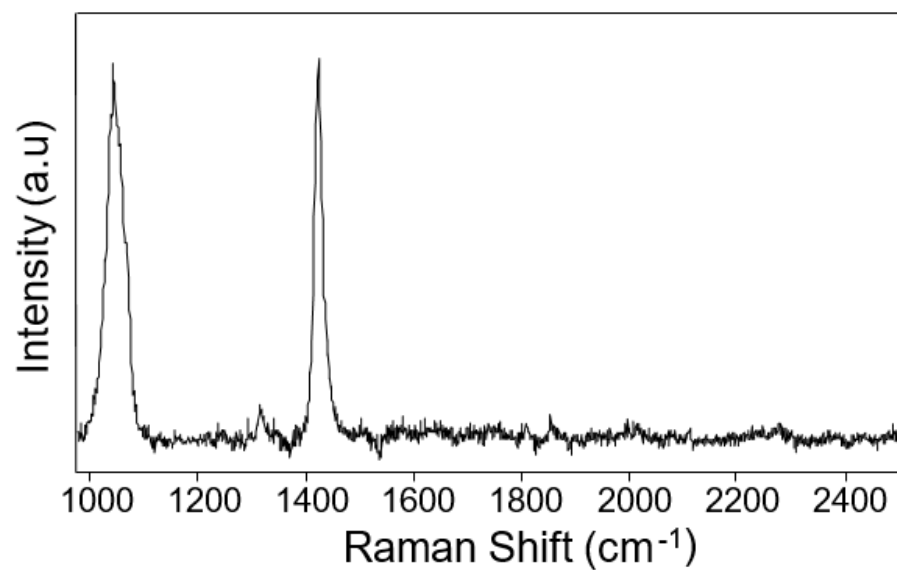


Figure 4. 2 The Raman Spectrum of synthesized AgNPs dissolved in DMSO

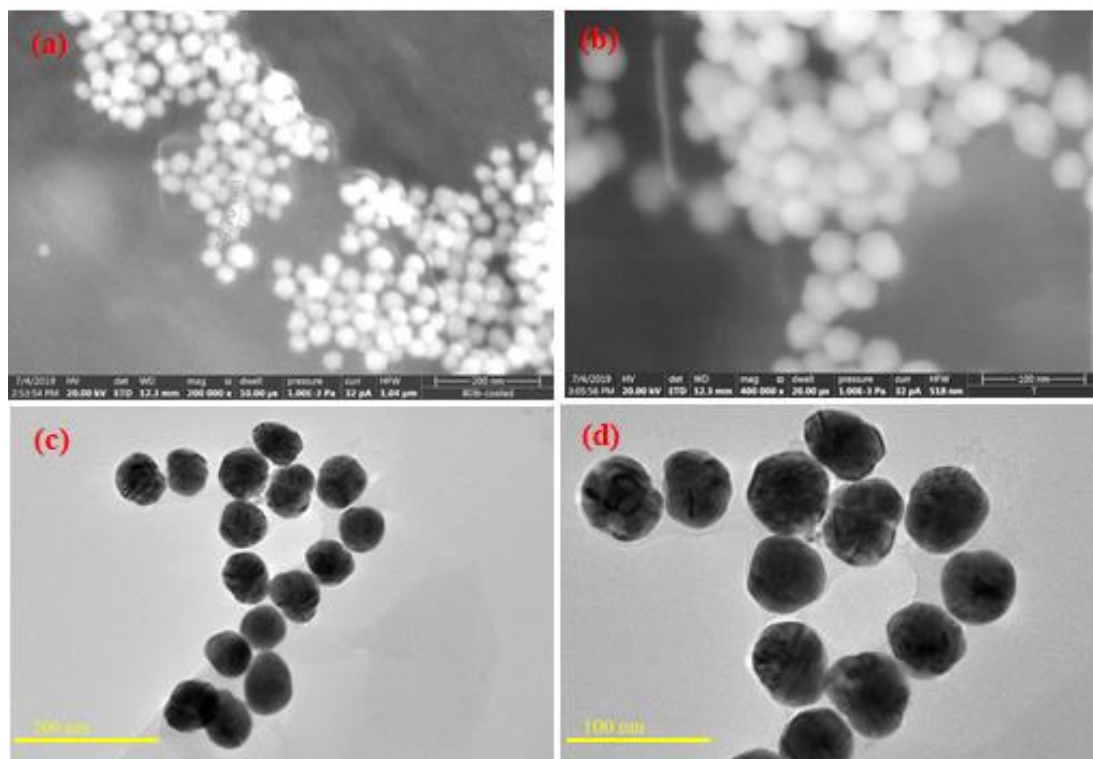


Figure 4. 3 FESEM (a,b) and TEM (c,d) images of the prepared silver nanoparticles (AgNPs) with different magnification

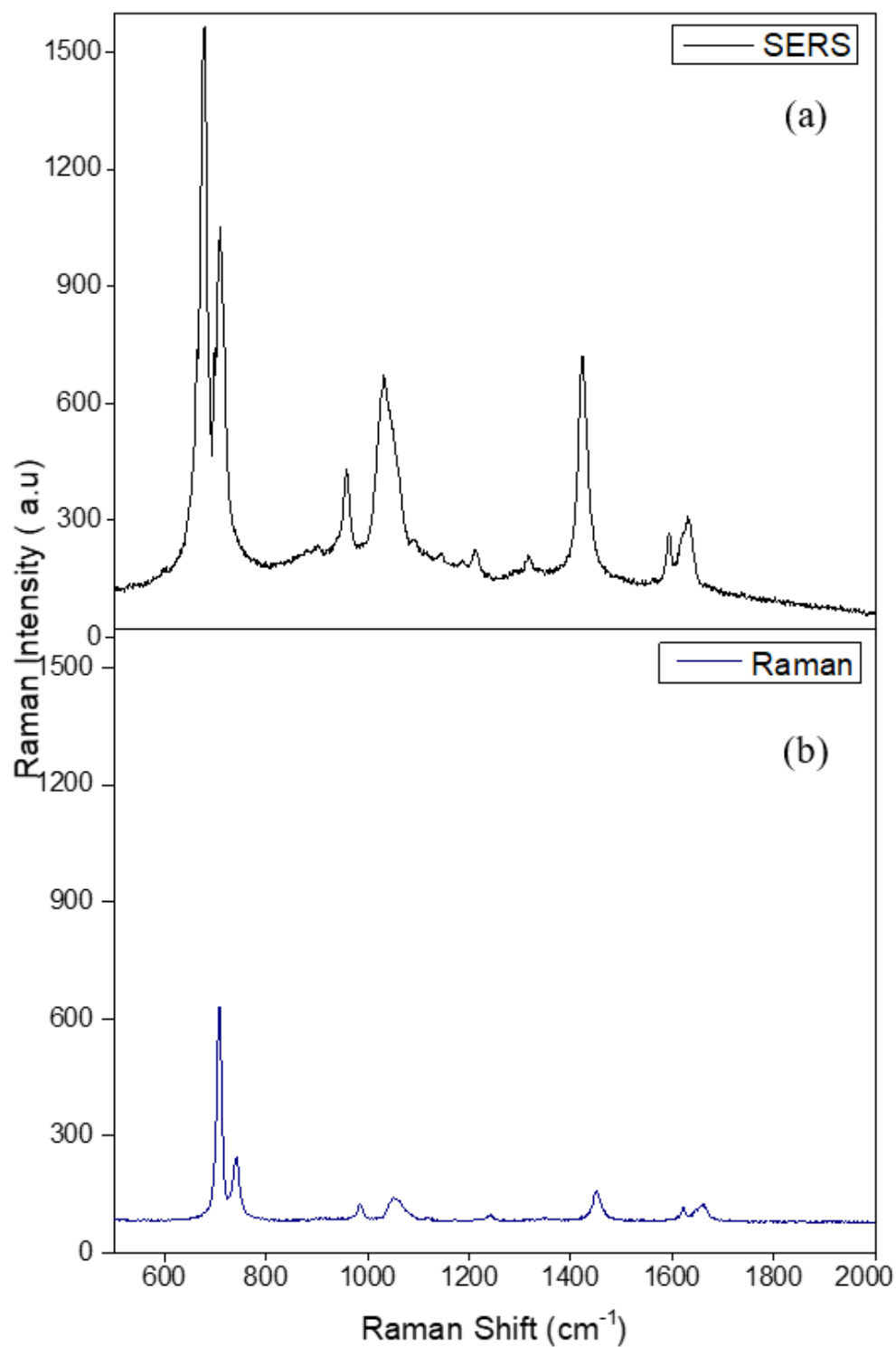


Figure 4. 4 Experimental (a) SERS Spectrum and (b) Raman Spectrum of a 0.1 M solution of sulindac (laser λ = 633nm, Laser power; 25%, acquisition time; 10s, accumulation; 10, and objective; 10 $\times$).

4.5 RESULTS AND DISCUSSION

To gain insights into the type of molecular-level interactions between the molecules of sulindac and AgNps, a simple modeling approach was constructed using a rhombus-shaped Ag_4 cluster as reported in our previous studies [189]. Two possible interaction modes, the $\text{HO}\cdots\text{Ag}\cdots\text{OC}$, and the $\text{Ag}\cdots\text{S}=\text{O}$ were identified and fully optimized as presented in Fig 4.5. It is established that the rhombus shaped Ag_4 cluster is the most stable geometry among all silver clusters as previously reported in molecular simulation investigations. The electron cloud sustained by the rhombus shaped Ag_4 makes its geometry more susceptible for the ionization process due to higher binding energies per atom relative to clusters with three silver atoms [190,191]. In addition, the use of a four-atom silver cluster to simulate interactions is computationally less expensive compared to clusters of a macroscopic size yet achieves results in good agreement with experimental data. The resulting theoretical Raman spectrum describing the $\text{OH}\cdots\text{Ag}\cdots\text{OC}$ interaction was found to be in better agreement with the experimental SERS spectrum in the range $975\text{-}2000\text{ cm}^{-1}$ as shown in Fig 4.6. Significant enhancements were observed in the Raman intensities of the bands centered at 1607 , 1653 , 1253 and 1111 cm^{-1} corresponding to $\text{C}=\text{C}$ stretching, $\text{C}=\text{O}$ stretching, CH_2 wagging and $\text{S}=\text{O}$ stretching (Table 4.1) as a result of the high plasmonic light field resonance upon laser excitation of the roughened silver surface. The localized plasmonic light field on the silver surface further excites the $\text{C}=\text{C}$, $\text{C}=\text{O}$, $\text{C}-\text{H}$ and $\text{S}=\text{O}$ vibrational modes in sulindac molecule leading to the enhancement in their Raman signals. The signal amplification in SERS has been reported to depend on a number of factors among which is the orientation of the molecule on the surface of the substrates, i.e. the interaction of specific functional groups in the molecule with the metal atoms [175]. The

calculated energies of the two modes (Table 4.2) further revealed that the OH $\cdots$ Ag $\cdots$ OC interaction was more feasible corresponding to coordination distances of 2.30 Å (for Ag $\cdots$ OH) and of 2.45 Å (for Ag $\cdots$ CO), compared to the Ag $\cdots$ S=O interaction. This could be attributed to the minimal repulsive effects enjoyed in this mode of interaction as well as the steric effects around the S=O functional group which prevents a proper overlap between the metal atoms and the S=O group. Furthermore, the functional groups are important in order to envisage the reactive spots, and as such the -COOH and -SO moieties of sulindac are considered to interact with Ag-cluster. Fig 4.7b shows the electrostatic potential map of sulindac in which red cloud around -COOH and -SO represents high electron density. The atomic charges of sulindac as depicted in (Fig 4.7a) tend to be more negative on the surroundings of the -SO and -COOH moieties showing an appreciable electron density. The functional group -COOH is predicted to be more favorable for such an interaction with metals because of its low ESP and high electron density as compared to the -SO, in addition to the minimal repulsive effects already highlighted. The changes in the sum of Mulliken charges upon the interaction of the metal cluster with sulindac (Table 4.2) further reveal that a significant electron transfer takes place in both modes of interaction from sulindac to the metal atoms. The calculated interaction energies, coordination distances and the resulting spectra further confirmed that the OH-Ag-OC scenario is the most favorable mode of interaction of AgNPs with sulindac molecules.

To facilitate the understanding of the intra- and inter-molecular interactions taking place between the molecules of sulindac and AgNPs, vibrational assignments were carried out on sulindac and SERS spectra in the range 975-2000 cm⁻¹ with the help of Gaussview 5.0 [192] visualization capability. To assign the vibrational modes effectively, theoretical

Raman frequencies were computed at B3LYP/def2svp level. and the results were found to be consistent with experimental data as shown in Table 1. The stretching vibrational mode associated with the C=O group was observed at 1638 cm^{-1} , though predicted at 1635 cm^{-1} from DFT in accordance with wavenumbers reported for the C=O stretching mode [193]. In addition, uncoupled C=C stretching vibrational mode was observed at 1598 cm^{-1} although predicted at 1600 cm^{-1} which falls within the established range of $1625\text{--}1400\text{ cm}^{-1}$ [194]. The ring stretching vibration was further observed at 1565 cm^{-1} and it was predicted at 1570 cm^{-1} in accordance with reported values in the range of $1500\text{--}1600\text{ cm}^{-1}$ [195–197]. The characteristic Raman band observed at 1325 cm^{-1} (1324 cm^{-1} from DFT) was assigned to the CH₂ wagging mode which usually occurs in the $1330\text{--}1195\text{ cm}^{-1}$ range [194,198]. Others are the C-F stretch, the C-H deformation, C-C stretch and S=O stretch bands observed at 1290, 1210, 1142 and 1091 cm^{-1} , (predicted from DFT at 1285, 1220, 1153 and 1096 cm^{-1}), respectively in accordance with established values in the literature [194,199–202].

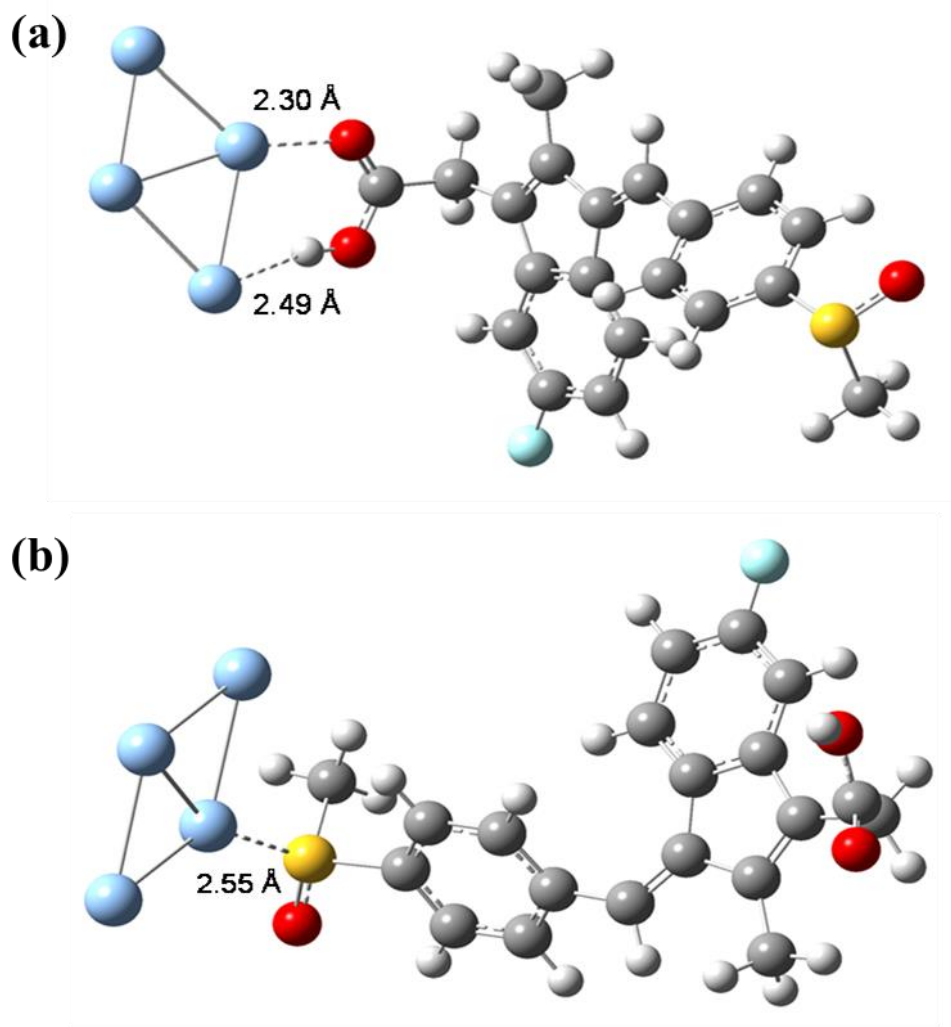


Figure 4. 5 Optimized interaction of Ag_4 cluster with (a) -HO and -OC and (b) -S=O of sulindac molecules.

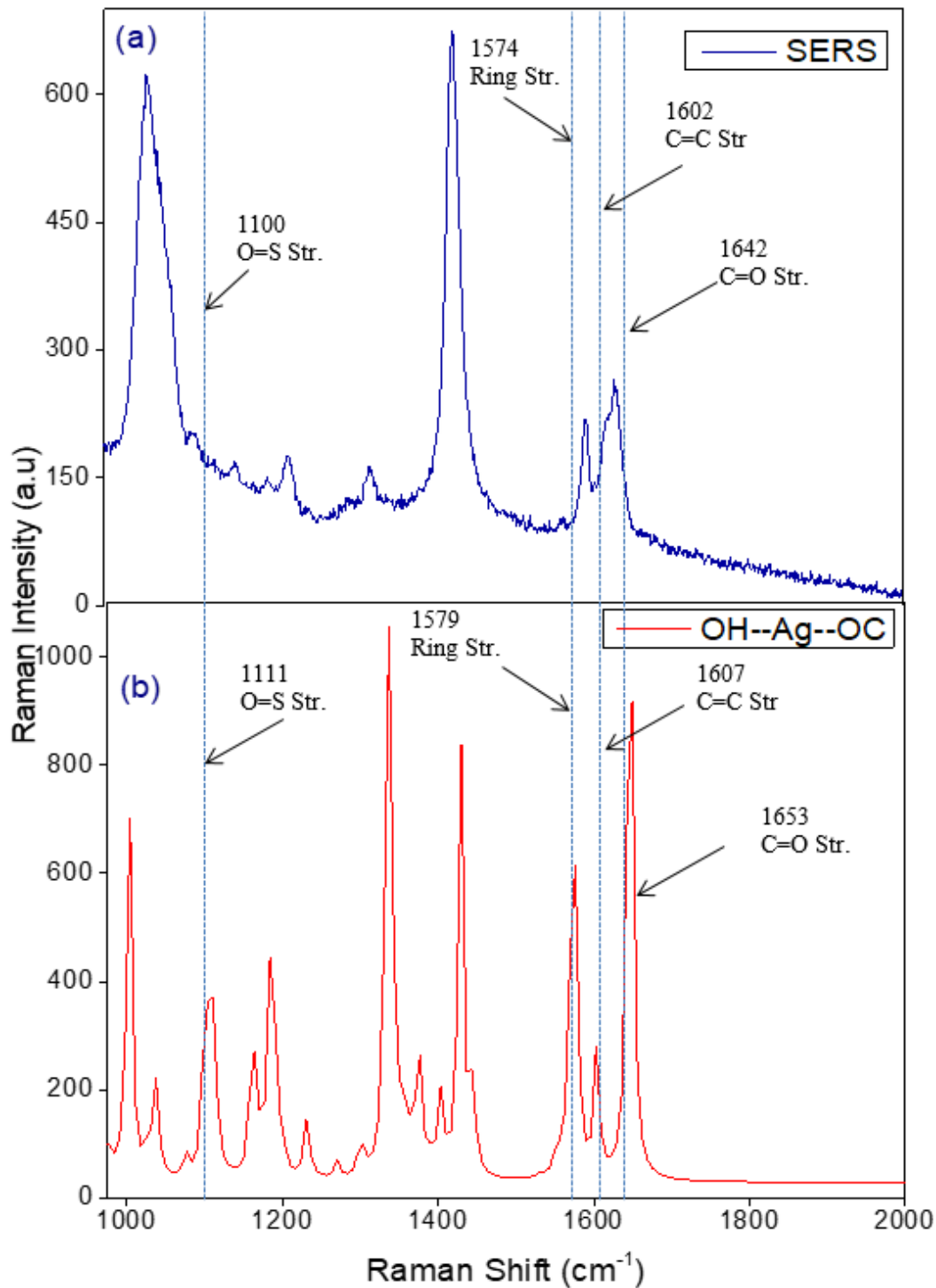


Figure 4. 6 Comparison of the (a) experimental SERS Spectrum and (b) the calculated Raman spectrum involving the sulindac molecule interaction with Ag_4 cluster through the $\text{OH}\cdots\text{Ag}_4\cdots\text{OC}$ moiety.

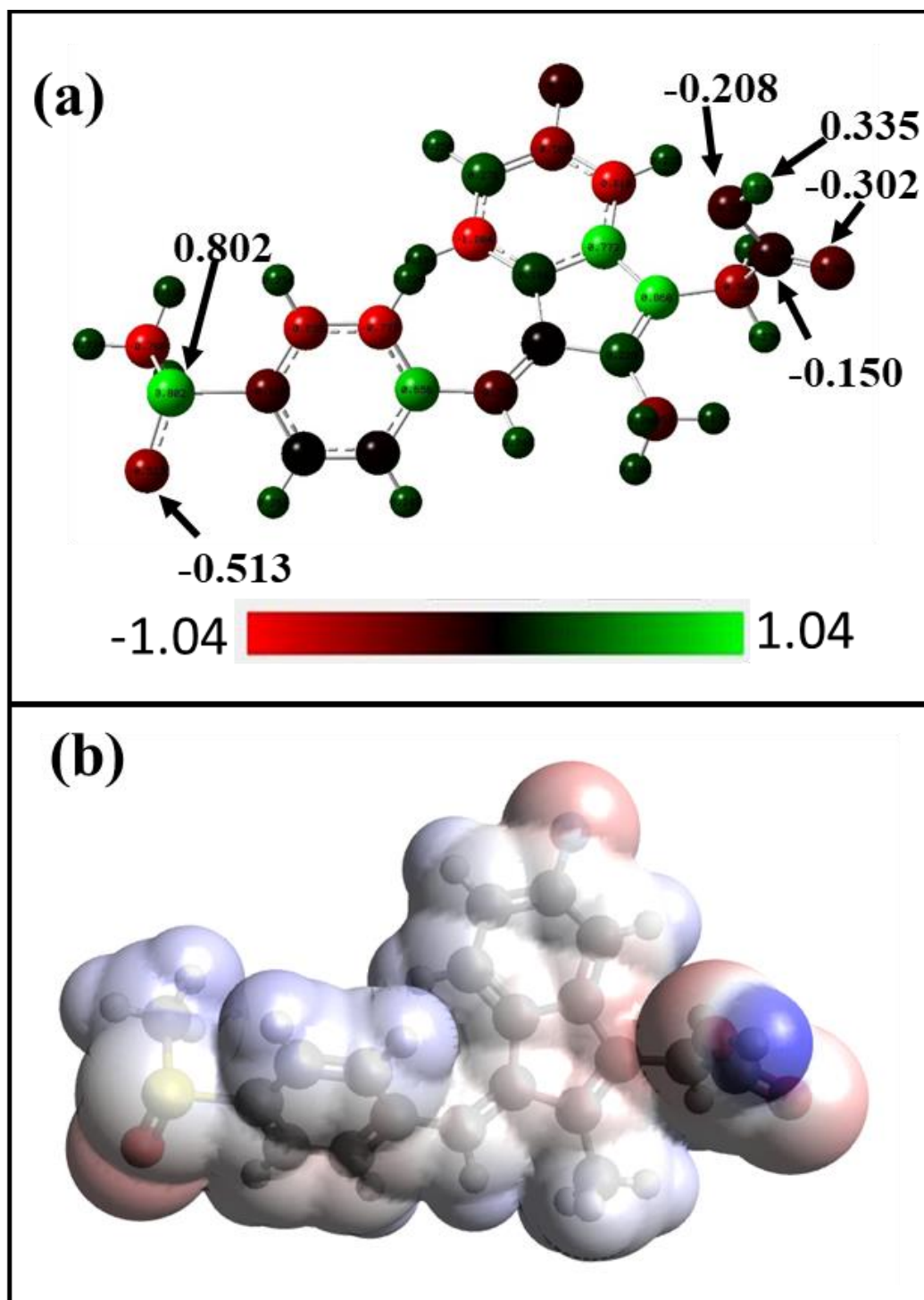


Figure 4. 7 (a) Calculated Mulliken atomic charges of sulindac molecules with color range and (b) Molecular electrostatic Potential map of sulindac drug.

Analyses of the calculated Raman spectra of Ag₄-sulindac interactions revealed that the Raman bands associated with the Ag₄-S=O interactions (ν_1 , ν_2 , ν_4 , ν_7 and ν_8) and corresponding to the C=O stretch, C=C stretch, CH₂ wag, C-C stretch, and S=O stretch were predicted to redshift from 1635, 1600, 1324, 1153 and 1096 cm⁻¹ to 1685, 1608, 1326, 1178 and 1127 cm⁻¹. The observed redshifts are attributed to the electron transfer and the vibrational constraint brought by the silver atoms. Furthermore, the bands at 1285 and 1220 cm⁻¹ corresponding to C-F stretching and C-H deformation, respectively were predicted to undergo a blue shift to 1237 cm⁻¹ and 1200 cm⁻¹ accordingly. In addition, the several Raman bands associated with the OH—Ag₄—O=C interactions were predicted to undergo a redshift from 1635, 1600, 1570, 1324, 1285, 1153 and 1096 cm⁻¹ to 1653, 1607, 1579, 1340, 1306, 1163 and 1111 cm⁻¹, respectively.

Table 4. 1 Assignment of vibrational modes of both calculated and experimental Raman and SERS spectra of sulindac and sulindac-Ag in 2000-975 cm⁻¹ range

Description	Observed (cm ⁻¹)		Calculated (cm ⁻¹)		
	Raman ^a	SERS ^a	Sulindac ^b	Sulindac-Ag cluster	
				Ag – SO interaction ^b	Ag–O=C and Ag–HO interactions ^b
v ₁ C=O str.	1638 s	1642 vs	1635 (100)	1695 (100)	1653 (92)
v ₂ C=C str.	1598 s	1602 s	1600 (11)	1608 (5)	1607 (22)
v ₃ Ring str.	1565 w	1574 w	1570 (23)	1572 (76)	1579 (85)
v ₄ CH ₂ wag	1325 w	1323 m	1224 (13)	1224 (61)	1340 (100)
v ₅ C-F str.	1290 vw	1294 m	1285 (18)	1237 (8)	1306 (8)
v ₆ C-H def.	1210 vw	1240 vw	1220 (9)	1200 (55)	1186 (37)
v ₇ C-C str.	1142 w	1146 w	1153 (50)	1178 (3)	1163 (16)
v ₈ O=S str.	1091 m	1100 w	1096 (10)	1127 (69)	1111 (30)

^a Observed Raman intensities; s : strong, vw : very strong, m : medium, w : weak, vw : very weak

^b Calculated relative Raman intensities are given in parentheses.

Table 4. 2 Interaction Energy, Binding Distance and the Change in the sum of Mulliken Charges for the two types of interactions.

Interaction Type	Energy (kcal/mol)	Binding Distance ($\text{\AA}$)	Mulliken Charges	
			Free	Complex
OH—Ag—O=C	-12.24	OH—Ag:2.49, Ag—CO: 2.30	Ag-Cluster: 0.000 Sulindac: 0.000	Ag ₄ -Cluster: -0.090 Sulindac: 0.090
Ag—S=O	-12.05	2.55	0.000	Ag ₄ -Cluster: -0.139 Sulindac: 0.139

4.5.1 SERS Measurements

The method proposed in this study was further utilized for the detection and quantification of sulindac. The SERS band at 1642 cm^{-1} was selected to achieve the lowest LOD and a wide linear dynamic range. In order to improve sensitivity of the proposed analytical method, The suspension of different AgNPs contents such as 1 mg, 3 mg, 5 mg and 7 mg in 10 mL DMSO were prepared and their effect on the SERS response were observed at 0.1M Sulindac solution with the ratio of 1:1. The SERS intensity increases with the increase of AgNPs contents from 1 mg to 5 mg. With further increase of AgNPs content, no significant change was observed on SERS response. Thus, in our following experiments, 5mg AgNPs content was chosen as optimal content. The ratio of 5mg of AgNPs in 10 ml of DMSO suspension and 0.1 M sulindac solution in DMSO was optimized 1:1 to achieve maximum SERS peak height. Under optimized experimental parameters, Different concentration solutions of sulindac in DMSO were examined, and a concentration-intensity calibration curve ($10^{-2} - 10^{-9}\text{ M}$) was plotted by using the intensity of the SERS band at 1642 cm^{-1} (1638 cm^{-1} from Raman) as depicted in Fig 4.8. A corresponding increase in the

intensity of SERS band with an increase in sulindac concentration was observed which suggests that signal enhancement depends on the population of sulindac molecules, which was further promoted by the action of AgNPs. The proposed method exhibits good linearity within the log concentration region with the coefficient of determination, R^2 of 0.9996, a high signal to noise ratio based on visual observation, a wide dynamic range (1×10^{-2} M to 1×10^{-9} M) and a low limit of detection (1×10^{-10} M). When compared to other previously reported analytical methods such as HPLC, GC-MS, and potentiometry for the quantification of sulindac (Table 4.3), the proposed method has a competently lower limit of detection and a wide dynamic range and hence could serve as an alternative approach in the detection of sulindac.

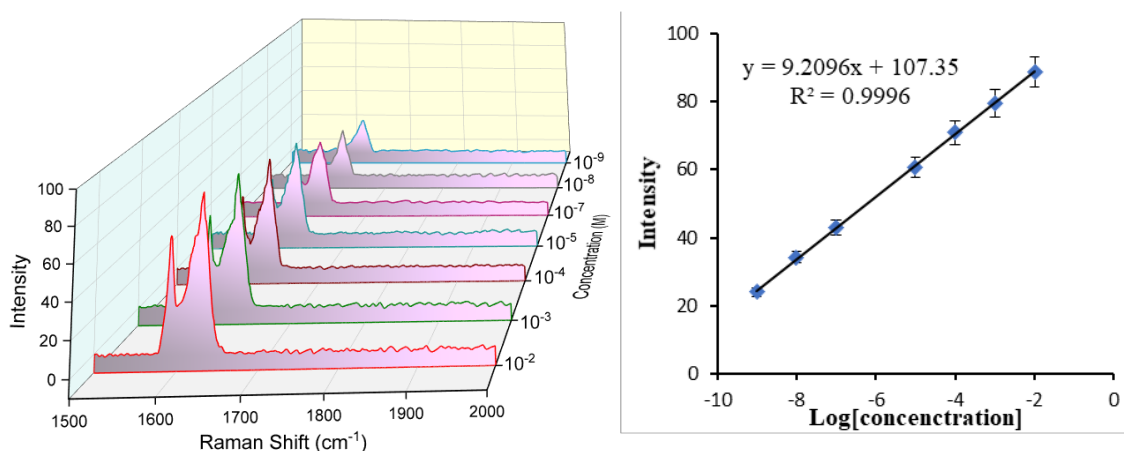


Figure 4. 8 3D graph (Left side) and Calibration curve (Right side) of SERS intensity at 1642 cm^{-1} at different concentration solutions of Sulindac (10^{-2} - 10^{-9}) using Sulindac (laser excitation $\lambda = 633 \text{ nm}$, Laser power; 25%, acquisition time; 10s, accumulation; 10, and objective; 10x

4.5.2 Interference effect

The effect of possible interfering species was examined on the detection of sulindac. 0.1 molar solutions of the interfering species such as valeric acid, ascorbic acid, ciprofloxacin, and clotrimazole were prepared in 0.001M solution of sulindac and SERS band at 1642

cm^{-1} was selected to study the interference effect on the developed methods of sulindac. No significant effect was observed on the intensity of selected SERS band of sulindac as shown in (Fig 4.9) which showed the proposed method of sulindac is selective and good anti-interfering capability in the presence of ten times higher concentrated interfering drugs.

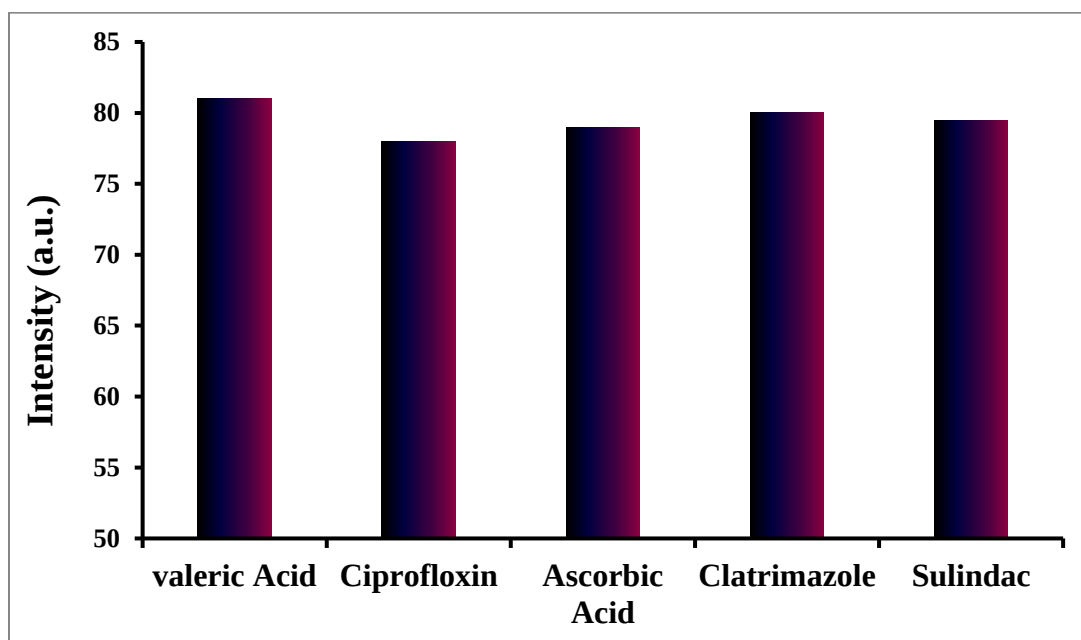


Figure 4. 9 Interfering Study on the SERS response of sulindac in the presence of interfering species

Table 4. 3 Comparison of the current developed method and reported methods

Method	LOD	LOQ	LDR	Ref
HPLC	0.1380µg/mL	101.4µg/mL	0.5-100.0 µg/mL.	[164]
UPLC	-----	LLOQ=0.062µg/mL	0.814–104.120 µg/mL.	[167]
RP-HPLC	10 ng/ml	50 ng/ml.	50-1000 ng/ml	[166]
Potentiometry	$1.4 \times 10^{-5} \text{ mol L}^{-1}$	-----	5×10^{-5} – $1 \times 10^{-2} \text{ mol L}^{-1}$	[20]
GC-MS	1.7ng/L	5ng/L	5-500 ng/L	[165]
SERS	10^{-10}M	10^{-9}M	$10^{-2} - 10^{-9} \text{ M}$	This work

CHAPTER 5

Sensitive SERS Detection and Characterization of Procaine in Aqueous Media by Reduced Gold Nanoparticles

5.1 INTRODUCTION

Procaine is one of the most widely used local anesthetic in neural therapy [203–205]. Several other medical uses of procaine for the treatment of ailments such as obesity, depression, inflammation, and even cancer have also been reported [14,15]. Its biological action has been extensively studied for decades [16–18,206]. It is less active when in contact with skin and mucous membranes but shows a substantial effect when used by infiltration. Using it as a depot from where benzylpenicillin is slowly released into the stream of blood requires intramuscular monitoring. It usually stops the reversible conduction of impulse along nerve axons and membrane which use sodium channels as a basic source of potential generation. This action by procaine is clinically employed to stop the sensing of pain at a specific part of the body by inhibiting the nerve transmission from the peripheral nerves to the central nervous system [16–18]. It migrates into tissues in an unmodified form of molecules and acts intracellularly as cation [206]. Procaine normally exhibits action for a moment and has some side effects ranging from cardiac to neurological toxicity. It can even show allergenic reactions in some cases [207]. Hence, the determination of trace quantities procaine in aqueous media is needed to check its

concentration during pharmaceutical preparations for quality assurance as well as obtaining optimum therapeutic concentrations. In the same vein, its detection would provide information essential for the investigation of its binding mechanism towards biomolecules and DNA [208].

Due to the need of developing a convenient and accurate technique for the detection of procaine, a number of analytical spectroscopic methods have been reported [12-23]. These include fluorimetry [61], voltammetry [62,63], ultraviolet-visible spectrophotometry [64–66], high performance liquid chromatography (HPLC) [67,68], chemiluminescence [67,209] and gas chromatography [210]. For instance, Pasekova et al. [67] utilized the sequential injection analysis (SIA) method with chemiluminescence detection (CD) where the emission occurred, when procaine sample was oxidized using permanganate in a solution of sulfuric acid along with different chemiluminescence enhancers such as glutaraldehyde, rhodamine, and formic acid. Differential pulse voltammetry (DPV) using pumice modified carbon paste electrode in 1.25×10^{-3} mol L⁻¹ buffer solution of KH₂PO₄ and Na₂HPO₄ was also employed to detect procaine [211]. With this method, the concentration range of procaine between 9×10^{-7} to 2.6×10^{-5} mol L⁻¹ exhibited a good linear relationship with a detection limit of 5×10^{-8} mol L⁻¹. Moreover, Mouyed et al. [212] developed batch and flow injection spectrophotometric approach for the detection of procaine and Beer's law was obeyed in the range of 1-40 and 5-400 µg/mL concentrations. On the other hand, illicit drugs have been successfully detected by HPLC in conjunction with SERS with the aid of silver halide as matrix stabilizer and SERS active surface[213]. As a quest for providing alternative sensitive and reliable techniques for procaine detection,

we explored the workability of Raman spectroscopy to put forward a more competitive detection limit.

The molecular vibrational modes of the targeted drug molecule can be probed and intensively analyzed via Raman technique [214–216]. However, the detection by the standard Raman method is usually characterized with low sensitivity and requires high concentration for solutions to produce appreciable signals [217,218]. These shortcomings can be effectively overcome with the use of modified nanoparticles either gold (AuNPs) and silver (AgNPs) that brings about Raman signal enhancement; an analytical method that is widely known as the surface-enhanced Raman scattering (SERS) spectroscopy [219–225]. AuNP and AgNP substrates can drastically improve the sensitivity of Raman scattered light [226]. Due to the realization of the essence of nanoparticles applications, several synthetic techniques have been developed to prepare nanoparticles with a large surface area that can function in solid [227,228] and aqueous [229,230] media.

In this work, we explored the SERS technique for the detection and quantification of procaine using gold nanoparticles as a SERS substrate. The interaction of AuNPs substrate with the procaine, the analyte resulted in enhanced Raman signals. However, the mode of interactions between the AuNPs and procaine were investigated via DFT calculations. Above all, the proposed method was simple, economical and gave a lower limit of detection (LOD) than previously reported methods

5.2 EXPERIMENTAL

5.2.1 Chemicals and materials

Procaine hydrochloride (purity $\geq 97\%$) was purchased from Sigma Aldrich while gold (III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, purity $\geq 49\%$ Au premise) was obtained from Acros Organics BVBA. Trisodium citrate dehydrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$, purity $\geq 99\%$) was procured from Fisher Scientific Company. Arrangements were set up with ultrapure water obtained from a water cleansing framework (Ultra ClearTM Lab Systems, Siemens Water Technologies USA).

5.2.2 Synthesis and Characterization

Gold nanoparticles (AuNPs) were prepared by the reacting gold (III) chloride with sodium citrate. Approximately 100 mL of a 0.1 M aqueous HAuCl_4 was boiled on a hot plate with stirring, and 10 mL of 0.5 M sodium citrate solution was added into the boiling solution of gold to obtain a gray-blue solution which turned red-violet after 2 min. Thereafter, the solution was heated for 10 min and allowed to cool down to room temperature [34, 35]. For the procaine solution, 1 molar aqueous solution was first prepared from the procaine hydrochloride, after which the lower concentrations were prepared via the dilution method and were later used for the SERS study.

The gold nanomaterials were characterized by Thermo ScientificTM Quattro S, scanning electron microscope (SEM). The FT-IR spectrum of AuNPs was recorded using Perkin Elmer Fourier-transform infrared spectroscopy spectrophotometer. On the other hand, the SERS study was carried out using HORIBA Scientific LabRAM HR Evolution Raman spectrometer. Following the preliminary investigation, a He-Ne laser source working at 17

mW and 633 nm excitation wavelengths with 50% laser power was chosen over that of 532 nm because it gave a higher signal to noise ratio. The spectra were collected for a 1:1 volume ratio of procaine and the gold nanoparticle solutions. A 10x objective lens was used to focus the laser into the sample solution while the microscope was coupled with a 600.0 mm focal length spectrograph equipped with two switchable gratings. An acquisition time of 10 sec with 100 accumulations was set for the SERS spectra collection of the samples placed in a cylindrical quartz cuvette with dimensions of 1 cm radius and 1 cm height

5.3 COMPUTATION

All DFT calculations were accomplished using GAUSSIAN 09 [101] with the adoption of the Becke 3-Parameter (Exchange), Lee, Yang and Parr (B3LYP) process introduced by Becke [102] and Lee et al [103], and a basis set of 6-311++G(d,p) for a non-metal atom. For the gold atom, the calculations were performed using the Stuttgart/Dresden (SDD) basis set. These basis sets were selected due to their ability to produce reasonable results that assent to experimental results of moderate size organic systems at reasonable costs [104–106]. On the other hand, the aqueous solutions corrections were computed via the Polarizable Continuum Model (PCM) developed by Tomasi [107]. The frequency calculations were undertaken by the same basis set, with the aid of Gaussian software to ascertain the nature of convergence of procaine-Au₄ complexes. The same was used to predict the vibrational assignments of the wavenumbers for the compounds understudied. To ensure good agreement with the Raman experimental results, the theoretical wavenumbers were adjusted by a factor of 0.985 [108] whereas, the Raman intensity was determined according to the method established in the literature [106,108]

5.4 RESULT AND DISCUSSION

5.4.1 Characterization of the gold nanomaterials

The gold nanomaterials were characterized by SEM. As shown in Fig 5.1, the SEM images of gold nanoparticles indicate a uniform shape of the nanoparticles with average size of around 40 nm. Particles with nanoscale provide a large surface area for accumulating more procaine molecules. This allows the laser beam to irradiate on more procaine molecules and thus yield high Raman spectra. AuNPs were also characterized using FTIR and Raman. The vibrational modes associated with the AuNPs could be seen in the infrared and Raman spectra presented in Fig 5.2 and Fig 5.3 respectively. The broad band observed between 3600-3400 cm^{-1} in the infrared spectrum can be attributed to -OH stretching vibrational mode whereas, the sharp band situated at 1625 cm^{-1} could be assigned to COO^- anti-symmetric stretching mode [25]. In addition, the shoulder bands appearing at 755 cm^{-1} and 735 cm^{-1} could be ascribed to COO deformation and CH_2 rocking vibrational modes respectively [26]. The recorded FT-IR spectrum assent to the previously reported ones [231,232]. On the part of Raman spectrum (Fig 5.3) only a broad Raman peak corresponding to -OH stretching vibrational mode was observed between 3500-3400 cm^{-1} while the other peaks identified in the infrared spectrum were Raman inactive. Thus, any Raman line observed before 3400 cm^{-1} during the SERS measurements is due to only procaine.

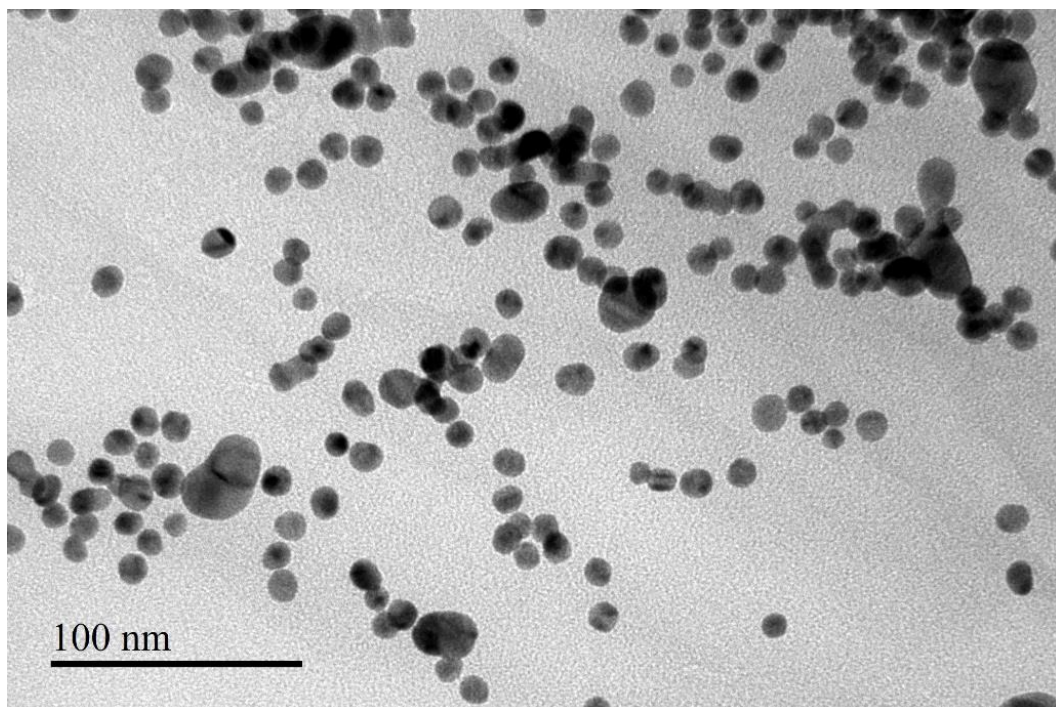


Figure 5. 1 TEM images of the gold NPs with different magnifications

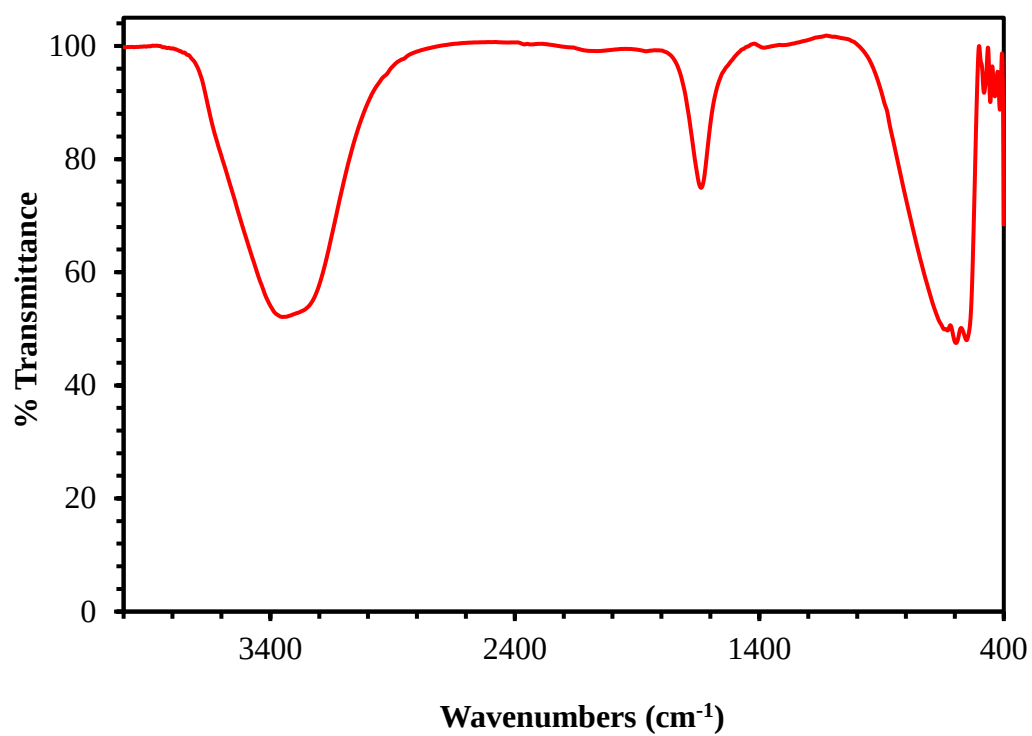


Figure 5. 2 FT-IR spectrum of AuNPs.

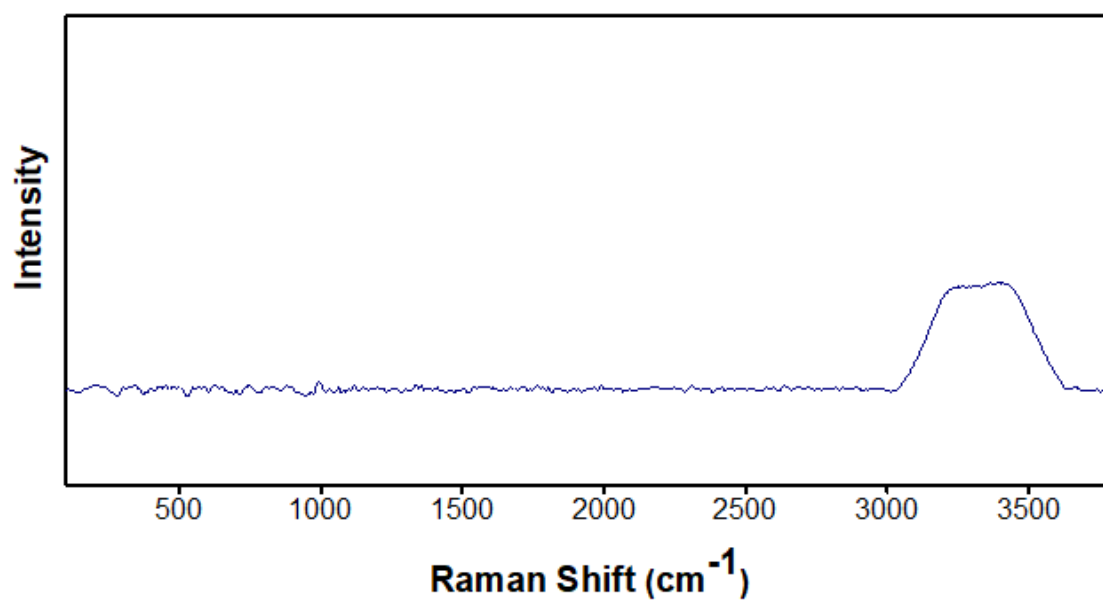


Figure 5. 3 The Raman spectrum of AuNPs ($\lambda_{\text{laser}} = 633 \text{ nm}$, Lase power of 50%, with acquisition time; 10s, accumulation; 100, and objective of 10 $\times$).

5.4.2 SERS detection of procaine

The SERS spectra of all the samples were recorded by utilizing LabRAM HR Evolution spectrophotometer which was equipped with internal He-Ne laser 17 mW at excitation wavelength 633 nm and thermo-electrically cooled CCD detector. The laser 633 nm was used for the excitation because it gave a higher signal to noise ratio over 532 nm counterpart. The optimum conditions arrived at, for the collection of SERS data were 1:1 volume ratio for procaine and AuNPs, 10 seconds acquisition time with 100 accumulations and 50 % laser power using a 10x objective microscope. The Raman spectrum collected for solid procaine at the wavenumber range of 50 - 4000 cm^{-1} is depicted in Fig 5.4, whereas Fig 5.5 represents a comparison between the Raman spectrum of 0.1 M procaine solution and that of the SERS spectrum, for the same concentration of procaine mixed with AuNPs in the frequency range of interest. Moreover, the SERS spectra of different molar concentrations of procaine and gold substrate solutions are given in Fig 5.6. The intensity of SERS peaks is noticed to increase with the logarithmic concentration of procaine, thus indicating that the intensity is linearly related to the logarithmic concentrations of procaine solutions. Raman intensity is a function of the population of the molecules, and this can further be aided by the action of nanoparticles interacting with the analyte in SERS experiment. Fig 5.5 shows peaks' enhancement in SERS spectrum of 0.1 M solution of procaine when compared with the Raman of the same solution. The enhancement mechanisms in the Raman signals with the use of gold nanoparticles can be roughly divided into chemical and electromagnetic enhancements [34, 39]. The change in the concentration of procaine solution would change the number of procaine molecules on the nanoparticles, thus resulting into the change in the Raman intensity as recorded in Fig 5.6. The spectra

were recorded in the range of 1000 - 1700 cm^{-1} where the intensity was observed to be decreasing with the dilution of procaine solution. Although a number of significant or moderate enhancements were noticed in the Raman peaks, the highest was recorded at 1608 cm^{-1} in SERS spectra and this was eventually chosen for a further quantitative determination of procaine. The different shape and position of the peaks observed could be due to the different type of interactions existing between the procaine and the nanoparticles. The SERS response as portrayed in Fig 5.7, shows a linear correlation with a coefficient (R^2) of 0.9971 for the plot of the intensity against logarithmic concentration of procaine in the range of 10^{-3} to 10^{-8} M. Besides, the residual plot of the data presented in Fig 5.8, shows that the data points are evenly distributed and that the residual sum of squares is 0.04. As a mean of establishing how effective, the SERS method adopted in this work can compete with those previously reported for the quantitative detection of procaine. Table 5.1 presents a comparison of important parameters such as linear dynamic ranges, coefficients, and limit of detections of various methods. The result clearly shows that the SERS-based approach developed for the trace determination of procaine possesses a lower limit of detection based on visual evaluation and signal-to-noise ratio [233] as well as a wider dynamic range than other methods.

Table 5. 1 Linear dynamic range (LDR) and limit of detection (LOD) of different previously reported analytical approaches compared to SERS for the trace determination of procaine.

Method	LDR	LOD	R^2	Ref.
DPV	9.0×10^{-7} to 2.6×10^{-5} M	5.0×10^{-8} M	0.995	[211]
CV	5×10^{-6} to 2×10^{-4} M	0.5×10^{-6} M	0.999	[208]
SIA-CL	1.8×10^{-6} to 1.8×10^{-4} M	1.1×10^{-6} M	0.9998	[234]
HPLC	0.05 to 5.0 μ g/mL	–	≥ 0.998	[235]
LC/MS/MS	10.0 to 100.0 ng/mL	0.100 ng/mL	0.9954	[236]
UV-Vis	1.50×10^{-7} to 4.15×10^{-6} M	3.80×10^{-8} M	0.9783	[237]
SERS	1×10^{-3} to 1×10^{-8} M	1×10^{-10} M	0.9971	This work

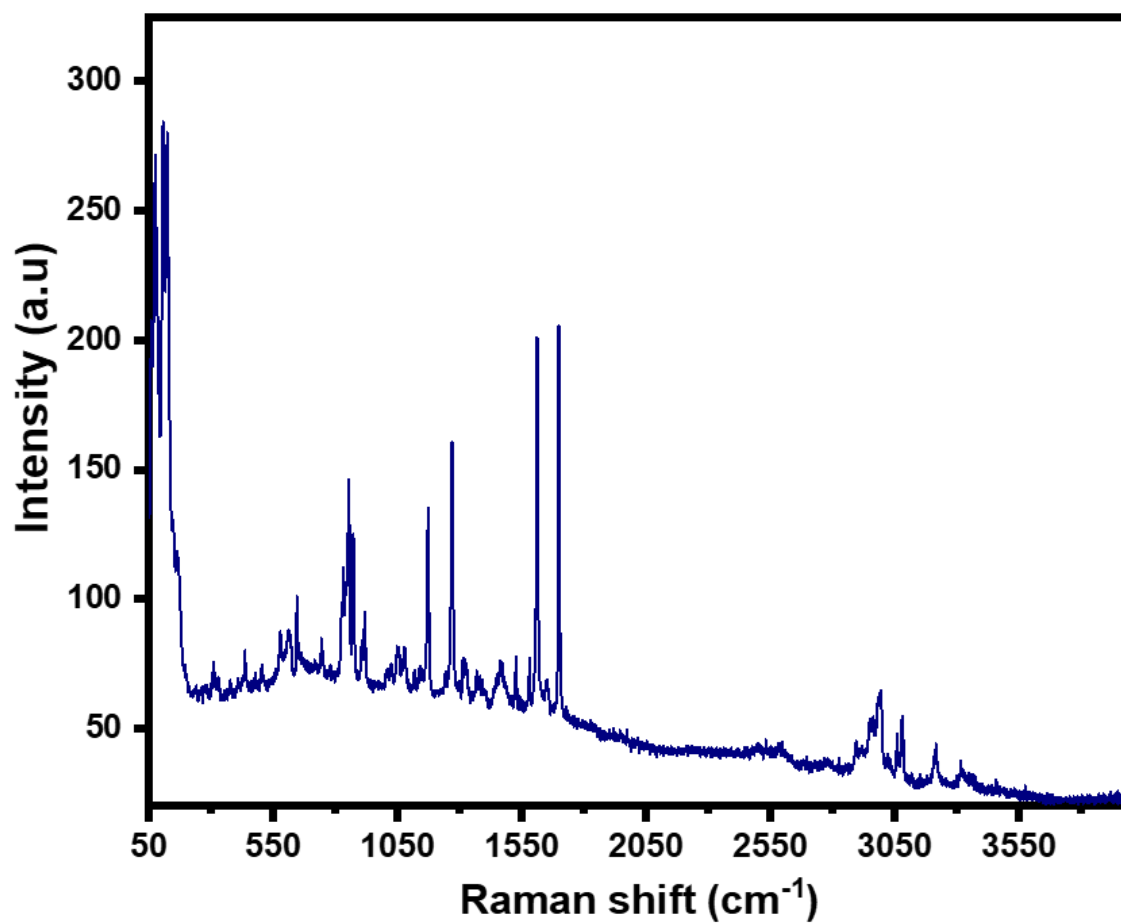


Figure 5. 4 Raman spectrum of solid procaine (laser λ = 633nm, Lase power; 50%, acquisition time; 10s, accumulation; 100, and objective; 10 $\times$).

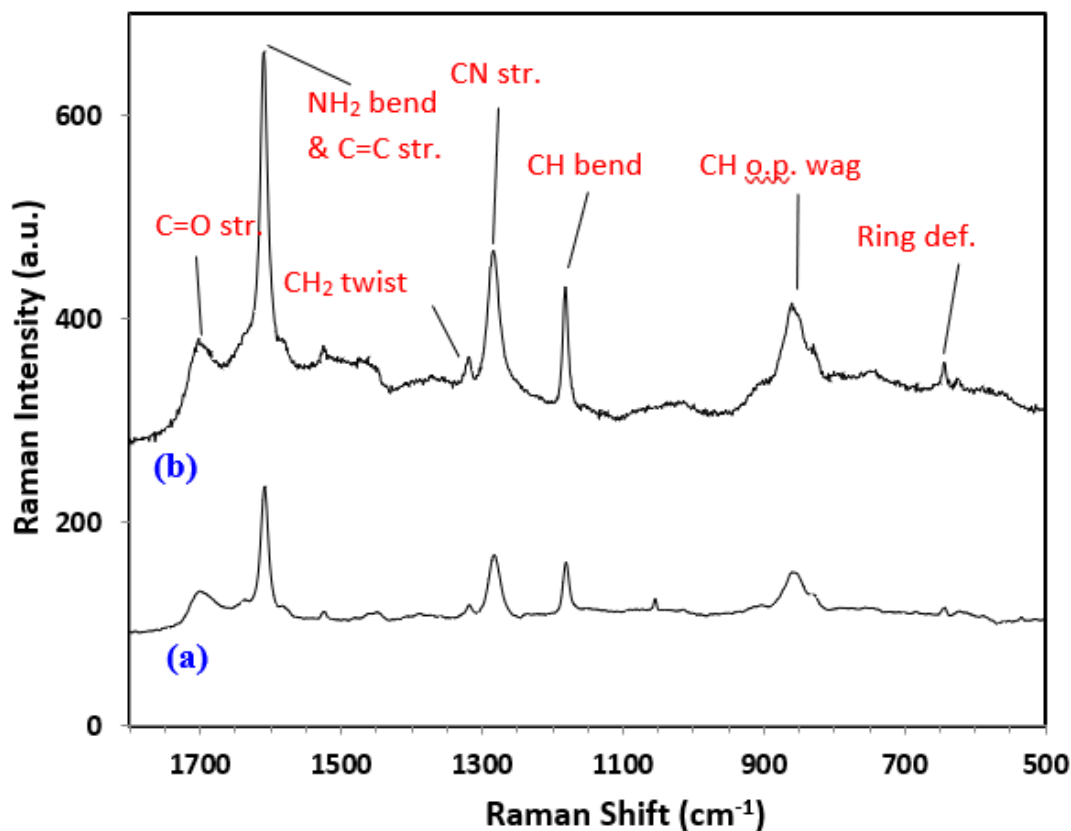


Figure 5. 5 A comparison between the Raman spectrum of 0.1 M procaine solution (a) and the SERS spectrum of 0.1 M procaine solution with AuNP (b) in the region 500-1800 cm^{-1} .

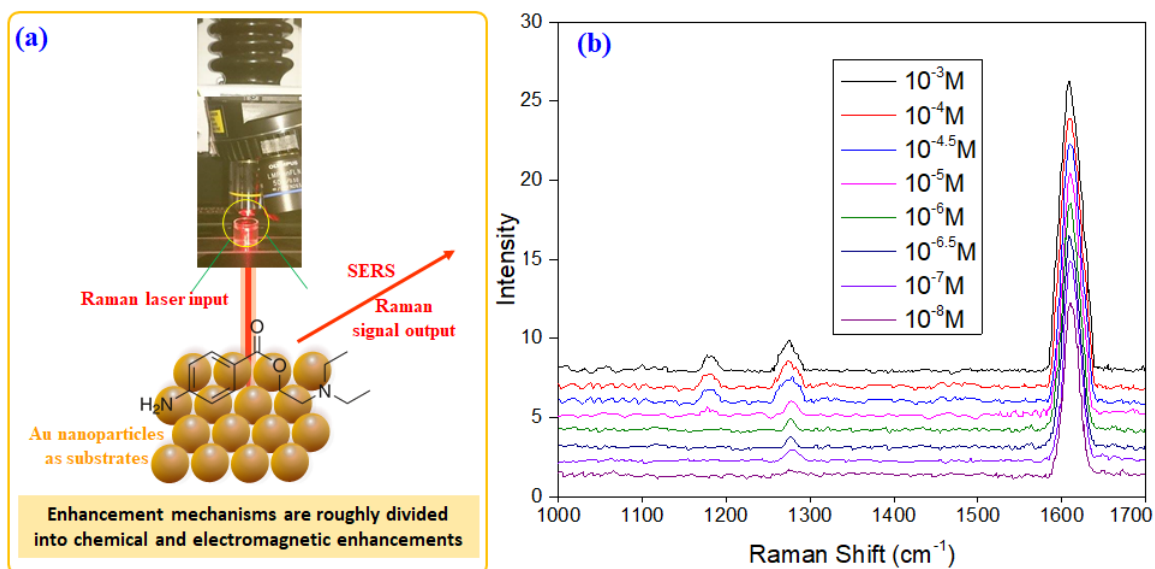


Figure 5. 6 SERS spectra of procaine at different concentrations (10^{-3} – 10^{-8} M). Note that the procaine concentrations, 10^{-9} M and 10^{-10} M, were not well fitted in the linear working dynamic range of the calibration, so were not used.

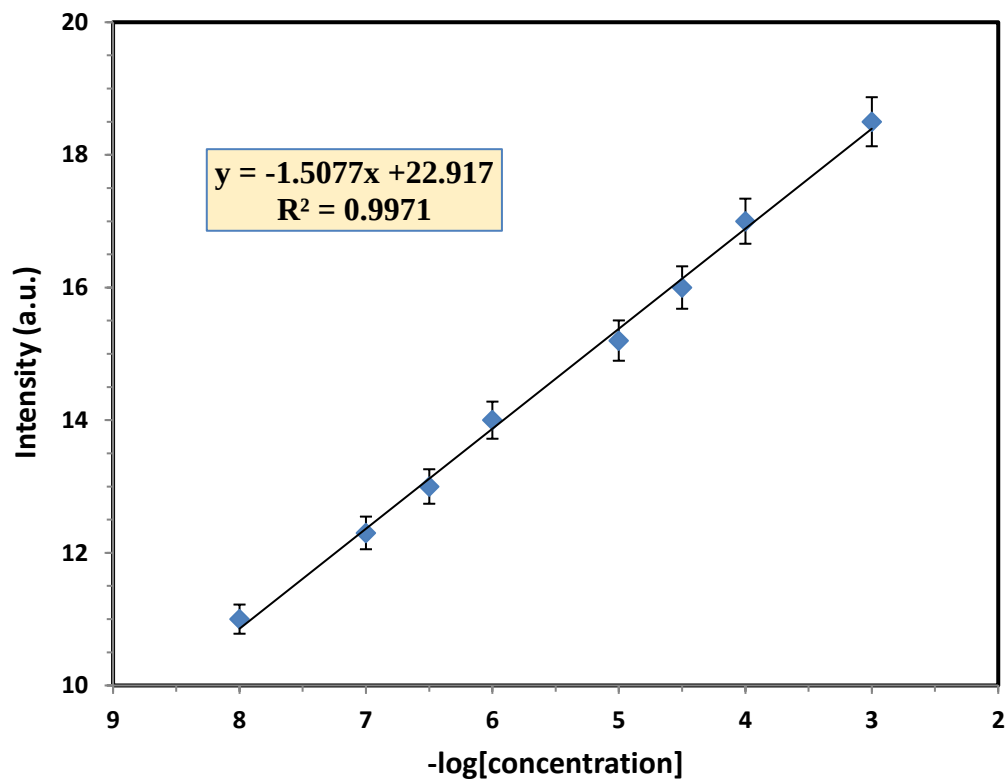


Figure 5. 7 Calibration curve of the band at 1608 cm^{-1} of different procaine concentrations ($10^{-3} - 10^{-8}\text{ M}$) at laser $\lambda = 633\text{ nm}$, Laser power; 50%, acquisition time; 10s, accumulation; 100, objective; 10 $\times$, and range of spectra; $1000\text{--}1700\text{ cm}^{-1}$

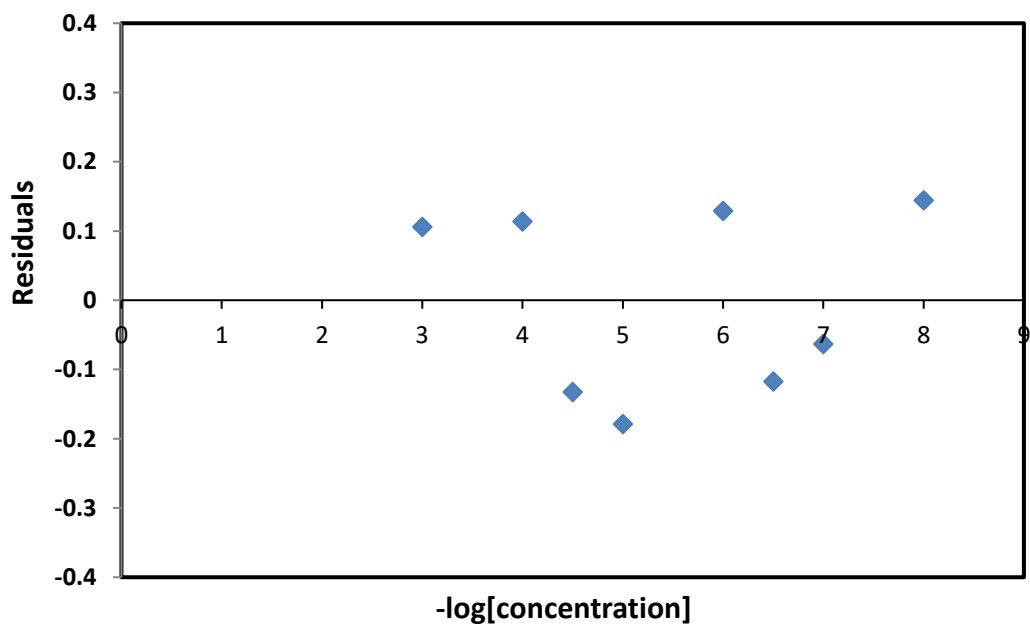


Figure 5. 8 Residual representation of data points

5.4.3 Repeatability and reproducibility

The repeatability of the proposed method was evaluated by performing six SERS experiments for the quantification of procaine. The relative standard deviation (RSD) for the SERS response towards the 10^{-5} M solution of procaine was 1.5 %. The reproducibility of SERS method was also investigated by performing SERS measurement of 10^{-5} M solution of procaine by preparing AuNPs and Relative Standard Deviation (RSD) was calculated for the SERS response and was found less than 2%. The results revealed that the quantification of the procaine can be carried out with good repeatability and reproducibility.

5.4.4 Vibrational assignments of SERS spectra

The mastery of vibrational properties of molecules is predicated upon detailed assignments in which vibrational modes and various wavenumbers observed in the spectrum are meaningfully interlinked. Furthermore, the spectrum resulting from SERS experiments normally shows shifts in the Raman lines together with intensity changes when compared with the conventional Raman spectrum of the same molecule. As a mean of getting insight into how the vibrational frequencies were affected upon interacting with the gold substrate in the SERS experiment, vibrational assignments were provided for the procaine and the resulting procaine-Au₄ complex spectra within the region between 500 and 1700 cm^{-1} . This task was effectively undertaken using the GaussView [238] for visualization while the DFT harmonic frequency calculation was carried via the B3LYP/6-311++G(d,p) approach. The calculated wavenumbers were found to be in consonance with the results obtained experimentally as listed in Table 5.2 and depicted in Fig 5.9. The stretching vibrational

mode associated with the C=O group was identified at 1696 cm⁻¹ but predicted at 1673 cm⁻¹ and this is in accord with wavenumbers reported for the C=O stretching mode [239]. On the other hand, the wavenumbers observed at 1633 cm⁻¹ and 1608 cm⁻¹ (calculated as 1630 cm⁻¹ and 1609 cm⁻¹) have been best expressed as NH₂ bending coupled with C=C stretching modes. In addition, uncoupled C=C stretching vibrational modes was observed at 1578 cm⁻¹ and predicted as 1573 cm⁻¹. The former assignments are within the 1650 – 1580 cm⁻¹ bending range established for NH₂ [239,240] whereas the latter is in agreement with the wavenumber reported for the C=C stretching mode [241]. Moreover, the CH bending modes were found at 1522 cm⁻¹ (1519 cm⁻¹ from DFT) and 1179 cm⁻¹ (1170 cm⁻¹ based on DFT), and the wavenumbers are within the 1600 – 1000 cm⁻¹ region reported for aromatic CH in-plane bending vibrational modes[242]. Furthermore, the wavenumber associated with CH₂ twisting was recorded at 1317 cm⁻¹ (1318 cm⁻¹ based on DFT) while the one for CN stretching was noticed at 1281 cm⁻¹ (1302 cm⁻¹ from DFT). The assignments assent to the wavenumber established for CH₂ twisting [239], and that for CN stretching [241]. However, the wavenumber recorded at 853 cm⁻¹ is assigned to CH out-of-plane wagging mode in accordance with the literature [241,242] whereas those identified at 825, 642 and 612 cm⁻¹ could be allied with the vibrational modes associated with ring deformation [241].

It is reported in our previous works that the alteration of bands, and the intensities in the SERS, is due to the interaction between the analyte and substrate used for the SERS experiment. In order to justify this assertion, a molecular quantum-chemical calculation was carried out to investigate the nature of interaction occurring between the Au-cluster and procaine. A gold cluster consisting of four atoms in rhombus geometry, identified as the most stable form [243] was employed in the cluster's simulation. Besides, the

preference for this geometry is predicated upon the electron cloud amassing ability of the four Au atoms which in turn increases the configuration ionization process tendency [105,244]. Furthermore, this type of cluster has been found to give results that assent to the experimental SERS data when used in the modeling of interactions between nanoparticles substrate and the analyte [27-29]. On the other hand, the use of a cluster of more than four atoms is considered computationally costly because gold is a 5d transition metal characterized with huge wavefunctions that require large basis set. In principle, molecules use their function groups for chemical activities and as such procaine is expected to interact with Au₄ via its –NH₂ and –COO– moieties. Consequently, molecular electrostatic potential (MEP) calculation was carried out to predict the distribution of electron density over the free procaine. The red cloud surrounding the –COO moiety in the MEP result presented in Fig 5.10 is an indication of low electrostatic potential but high electron density whereas the blue cloud for the –NH₂ signals high electrostatic potential [28]. Thus, the mode of interaction could involve the transfer of electrons between the procaine and Au₄, and this could be investigated via Mulliken charge analysis. The negative values predicted for the Mulliken charges of Au₄ in both Au₄-ester and Au₄-NH₂ interactions as highlighted in Table 5.3 suggests that the interaction involves the transfer of an electron from procaine to Au₄. Hence, Au₄-ester interaction is more probable, and this is corroborated by the better agreement between the pattern of the peaks of its resultant calculated spectrum and that of SERS as portrayed in Fig 5.9. However, the fair agreement between the predicted wavenumbers resulting from Au₄-NH₂ interaction and that of SERS, as listed in Table 5.2 is an indication that it could be an alternative interaction occurring in the system.

One of the effects of the interaction between the substrate and the analyte in the SERS experiment is the change in band intensities. This could be noticed in Fig 5.5 where enhancements were recorded at SERS bands 1608 cm^{-1} , 1313 cm^{-1} , 1281 cm^{-1} and 1181 cm^{-1} corresponding to coupled NH_2 bending and $\text{C}=\text{C}$ stretching, CH_2 twisting, CN stretching and CH bending modes respectively. This enhancement could be attributed to highly plasmonic light field resonantly produced by the rough gold surface upon laser excitation, which results in the increased light scattering of procaine molecules residing within the plasmonic field. This phenomenon eventually leads to an enhanced Raman signal. Another consequence of interaction is the band shift normally recorded in SERS spectra due to the transfer of electrons which affects bond strength. Red shifts were observed for the wavenumbers associated with the rocking type of the CH bending mode ($1522 - 1512\text{ cm}^{-1}$) and the CH_2 twisting mode ($1317 - 1313\text{ cm}^{-1}$). Such an observation agrees with the downward shift in wavenumbers, $1519 - 1516\text{ cm}^{-1}$ and $1318 - 1313\text{ cm}^{-1}$ predicted for CH bending and CH_2 twisting vibrational modes in $\text{Au}_4\text{-NH}_2$ interaction. Blue shifts were recorded for the wavenumbers attributed to a wagging type of CH bending mode ($1179 - 1181\text{ cm}^{-1}$) and an out-of-plane CH wagging mode ($853 - 856\text{ cm}^{-1}$). Moreover, this result is in good consonant with the upward shift in calculated wavenumbers, $1170 - 1175\text{ cm}^{-1}$ and $844 - 856\text{ cm}^{-1}$ (Table 5.2) as obtained for the CH bending and out-of-plane wagging modes, respectively, in the $\text{Au}_4\text{-NH}_2$ interaction.

Table 5. 2. Assignments of vibrational modes for experimental and calculated Raman and SERS spectra of the procaine molecule and procaine-Au clusters in the 1700 – 500 cm⁻¹ region.

Description	Observed (cm ⁻¹)		Calculated ^b (cm ⁻¹)		
	Raman ^a	SERS ^a	Procaine	Procaine-Au cluster	
				Au ₄ -NH ₂ interaction	Au ₄ -ester interaction
v ₁ C=O str.	1696 w	1693 w	1673 (42)	1689 (24)	1700 (64)
v ₂ NH ₂ bend, C=C str.	1633 vw	1631 w(h)	1630 (10)	1630 (2)	1630(10)
v ₃ NH ₂ bend , C=C str.	1608 vs	1608 vs	1609 (100)	1616 (100)	1607 (100)
v ₄ C=C str.	1578 vw	1578 w(h)	1573 (5)	1601 (2)	1570 (4)
v ₅ CH bend (rock)	1522 vw	1512 w	1519 (3)	1516 (1)	1519 (2)
v ₆ CH ₂ twist	1317 w	1313 w	1318 (1)	1313 (1)	1288 (1)
v ₇ CN str.	1281 m	1281 m	1302 (5)	1275 (27)	1309 (9)
v ₈ CH bend (wag)	1179 m	1181 m	1170 (33)	1175 (23)	1167 (40)
v ₉ CH out-of-plane wag	853 m	856 m	844 (0)	855 (4)	839 (2)
v ₁₀ Ring breath	825 m(h)	828 w(h)	818 (15)	809 (2)	815 (4)
v ₁₁ Ring def.	642 w	642 w	640 (4)	645 (4)	639 (5)
v ₁₂ Ring def.	612 vw	618 w	620 (0)	619 (7)	618 (1)

^a Observed Raman intensities; vs: very strong; s: strong; m: medium; w: weak, h: hunch.

^b Calculated relative Raman intensities are given in parentheses.

Table 5. 3 Analysis of total Mulliken charges in the system

Compound	Free	Upon interaction	
		Au₄-ester	Au₄-NH₂
Au ₄	0	-0.142	-0.05
Procaine	0	0.142	0.05

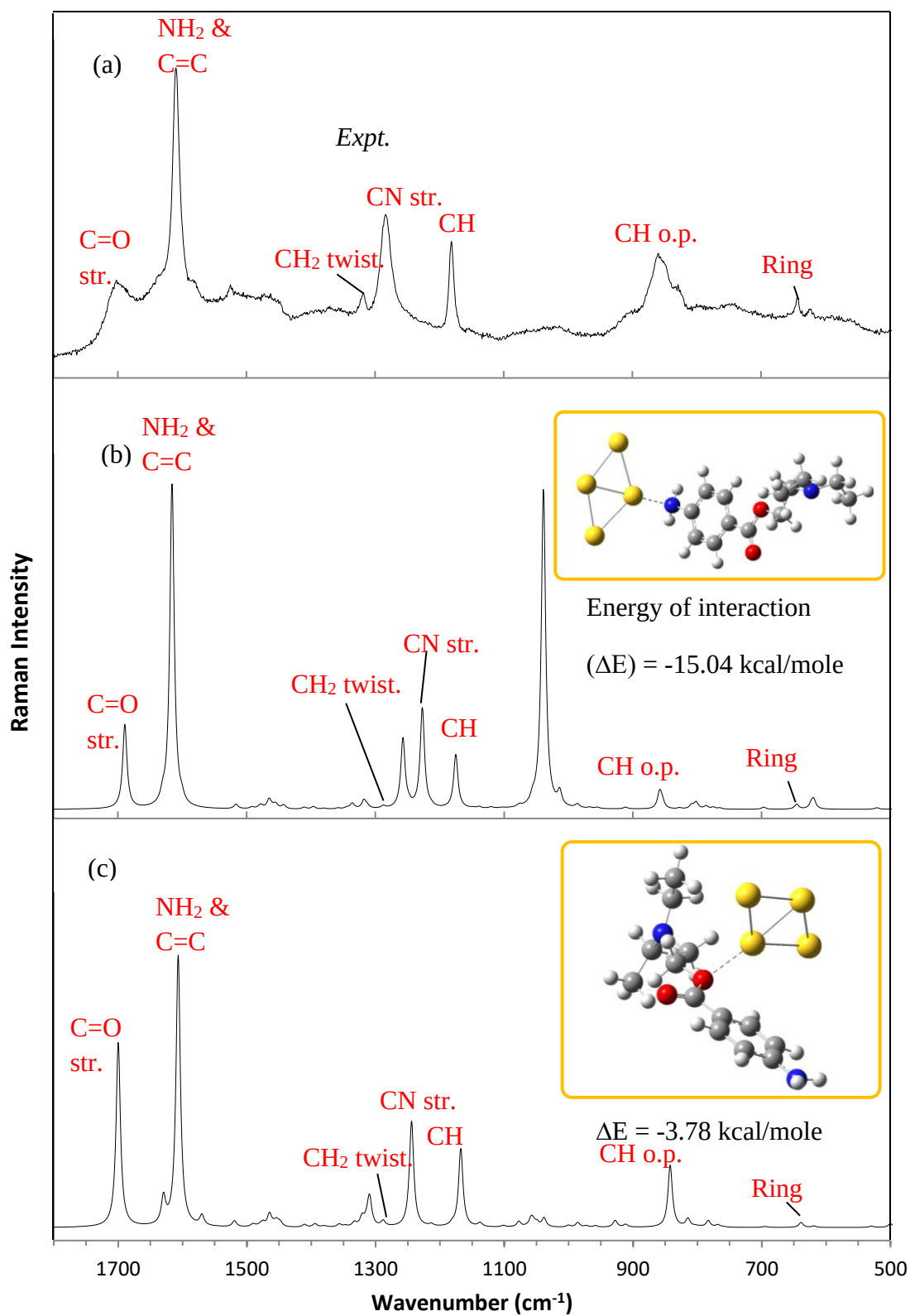


Figure 5. 9 Raman spectra; (a) SERS, (b) calculated spectrum for Au-NH₂ interaction, and (c) calculated spectrum for Au-ester interaction.

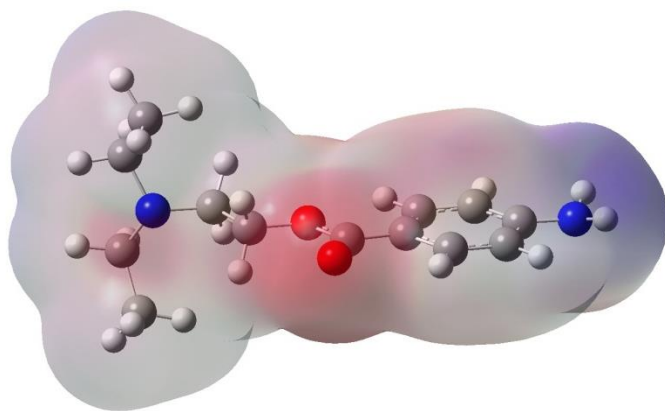


Figure 5. 10 Molecular electrostatic potential of procaine mapped at the electronic isosurface of 0.02 a.u

CHAPTER 6

Electrochemically modulated SERS detection of procaine using FTO electrodes modified with silver-decorated carbon nanospheres

6.1 INTRODUCTION

Procaine is a widely used organic-based drug indicated for the production of regional and local anesthesia particularly in neural therapy and oral surgery. It is used to reduce bleeding by its advantage of constricting blood vessels [204,205,245,246]. It was also found useful in the medical treatments of inflammation, obesity, cancer, and depression [14,15]. It possesses a wide scope of biological effects as it has been studied for decades [16–18,206]. On the other hand, procaine-containing drugs exhibit momentary actions with some unfavorable side effects, such as cardiac and neurological toxicity. In some extreme cases, it was reported that it even exhibits allergenic reactions [207]. Thus, continuous monitoring of patients administered with procaine-containing treatment is important in order to detect any excessive presence of the drugs in the body fluids [208]. For the sake of the convenient and accurate detection of procaine contents, a number of analytical and spectroscopic techniques, such as UV-vis spectrophotometry, chemiluminescence, voltammetry, gas chromatography (GC), high-performance liquid chromatography (HPLC), and surface-enhanced Raman scattering (SERS), were successfully implemented [61–68,209,210,247].

Of these techniques, SERS spectroscopy has attracted more attention due to its high surface selectivity and compatibility with aqueous environments. By carefully inspecting the vibrational modes of the targeted drug upon the interaction with the SERS substrates, useful structure-activity insights which are important to understand the drug action could be accomplished. The aforementioned advantages make the SERS an attractive choice in pharmaceutical applications, food analysis, and pesticides detection [214,215].

Regardless of its sensitive detection, SERS enhancements can be improved even further [248] when combined with other techniques [249–251]. Among such integrations is the coupling of electrochemical phenomena with SERS measurements (EC-SERS) which resulted in significantly more enhancement of the key Raman lines. While the signal enhancement in the conventional SERS is attributed to the electromagnetic (EM) attenuation, the EC-SERS technique is susceptible to the chemical enhancement (CE) effect as well. Both EM and CE phenomena facilitate the chemisorption interaction and charge transfer between the substrate and the targeted molecule [252]. The application of an electrical potential allows better mobility of the surface charges of the molecules and, hence, increases the adsorption on the substrate. It has been shown that EC-SERS is a useful approach in various areas, such as tracking reaction dynamics [253], assessment of drugs and their metabolites [254], and the quantification of electrode materials [255]. It has been applied very recently to probe single molecules through the integration with the tip-enhanced Raman spectroscopy (EC-TERS) [256].

In our previous reports, we explored the use of SERS for the sensitive quantification of widely used medications such as procaine [257,258], sulindac [259], clotrimazole [260], ketoconazole [261], valeric acid [262], methimazole [263] and thiouracil [264], using a

variety of nanomaterial-based substrates. In the present report, we have developed an *in situ* surface-enhanced Raman spectroscopic (EC-SERS) approach for the quantitative detection of procaine on silver-decorated carbon nanosphere (AgCNS) deposited on a fluorine-doped tin oxide (FTO) electrode. Structural properties and the binding mechanism between AgCNS and procaine molecules were explored using quantum chemical techniques. In-silico molecular docking simulation was also carried out to understand the anti-inflammatory mechanism of procaine action towards the chemokine receptor CXCR4

6.2 EXPERIMENTAL

6.2.1 Materials

Silver Nitrate (CAS Number: 7761-88-8, Purity > 99%), (D-(+)-Glucose (CAS Number 50-99-7, Purity > 99.5%),), Sodium citrate (CAS Number: 6132-04-3, Purity > 99%),), Procaine (CAS Number: 51-05-8 Purity > 97%), Phosphate Buffer solution (pH=7), Potassium chloride (CAS Number: 7447-40-7 Purity > 99%) and Potassium ferricyanide (CAS Number: 13746-66-2 Purity > 99%) were purchased from Sigma-Aldrich and used without any further purification. The ultrapure water for solution preparation was obtained from a water purification system (Ultra Clear™ Lab Water Systems, Siemens Water technologies USA)

6.2.2 Synthesis and Characterization of silver decorated carbon nanosphere

Carbon nanosphere (CNS) was synthesized by a modified hydrothermal synthesis method [265] as follows: 0.5 g of glucose was dissolved in 30 mL of water to form a clear solution with the aid of stirring for 35 min. At the end of 35 min, the solution was transferred into a Teflon sealed autoclave (40 mL) and heated at 180 °C for 3 h. After the completion of

the reaction, the autoclave was allowed to cool in the air naturally. The suspension was centrifuged at 8000 rpm for 15 min and the product was washed and dispersed in DI water. The centrifugation and rinsing cycles were repeated three times to obtain high purity CNS. Moreover, a 50-mg dosage of the CNS was dispersed in 15 mL deionized water with ultrasonication to obtain a suspension. A 10- μ L portion of 1.5 mM AgNO₃ was cast to the suspension drop by drop under stirring at room temperature. After the adsorption of silver ions on the surface of CNS for around 1 h, the suspension was added to 15 mL of a sodium citrate solution with a concentration 0.5 mM and stirred for a further 30 min. Finally, the dispersion was transferred to the Teflon sealed autoclave (40 mL) and heated at 110 °C for 1 h without stirring. The final product was first washed with acetone to remove the excess sodium citrate and then washed multiple times with deionized water. The high purity Ag decorated nanomaterial was obtained and stored in the refrigerator. In this work, silver particles were loaded onto the CNS in order to prevent agglomeration and thus a better dispersion of Ag particles could be achieved, which is a key factor for successful SERS analysis. The synthesized AgCNS was characterized with the aid of a scanning electron microscope (SEM) and energy-dispersive X-ray spectroscopy (EDX).

6.2.3 Functionalization and characterization of FTO glass substrates

Transparent glass substrates coated with conductive layer fluorine-doped tin oxide on one side (FTO Ref. 43705) having a thickness of 2.2 mm, a dimension of 5 x 5 cm, and a surface resistivity of 7 ohm/sq were purchased from SOLARONIX Switzerland. The glass substrates were cut into 1.2 x 1.2 cm square pieces to fit the size of the electrochemical-SERS cell. Prior to the deposition of AgCNS, The FTO substrates were thoroughly cleaned by subjecting to 50 °C ultrasonic treatment [99] first in acetone, then in isopropyl alcohol

followed by deionized water with a duration 15 min. for each solvent. The washed substrates were subsequently dried in an oven at 50 °C for 10 min. The suspension of the nanosphere material was prepared by dispersing 10 mg in 5 mL deionized water and sonicated for 10 min. The deposition of AgCNS on the FTO substrates was carried out by the drop-casting method [100]. A 40- μ L AgCNS suspension was uniformly drop-casted on the conductive surface of the substrates and dried in an oven at 60 °C for 20 min. The fabrication steps of the active EC-SERS substrate, pre-concentration, and detection of procaine drug are illustrated in Fig 6.1. The fabricated FTO was characterized using SEM and EDX and X-ray photoelectron spectroscopy (XPS) techniques, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The SEM micrographs were recorded using Tescan Lyra 3 field-emission scanning electron microscopy (FESEM) equipped with an EDX detector. The X-ray photoelectron spectroscopy (XPS) analysis was conducted using the X-ray photoelectron spectrometer (Thermo Scientific ESCALAB 250 Xi). Electrochemical experiments were conducted using Gamry workstation. A single compartment electrochemical cell consisting of three electrodes, modified/non-modified FTO electrode as a working electrode, saturated Ag/AgCl as a reference electrode and Pt wire as a counter electrode. The electrochemical behavior of the functionalized and non-functionalized electrodes was examined using electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) in 5.0 mM of potassium ferricyanide ($K_3[Fe(CN)_6]$) containing 1.0 M KCl supporting electrolyte solution as electrochemical probe. The EIS measurements were conducted at open circuit potential (E_{ocp}) in the frequency range 100000-0.1 Hz at 10 mV r.m.s amplitude.

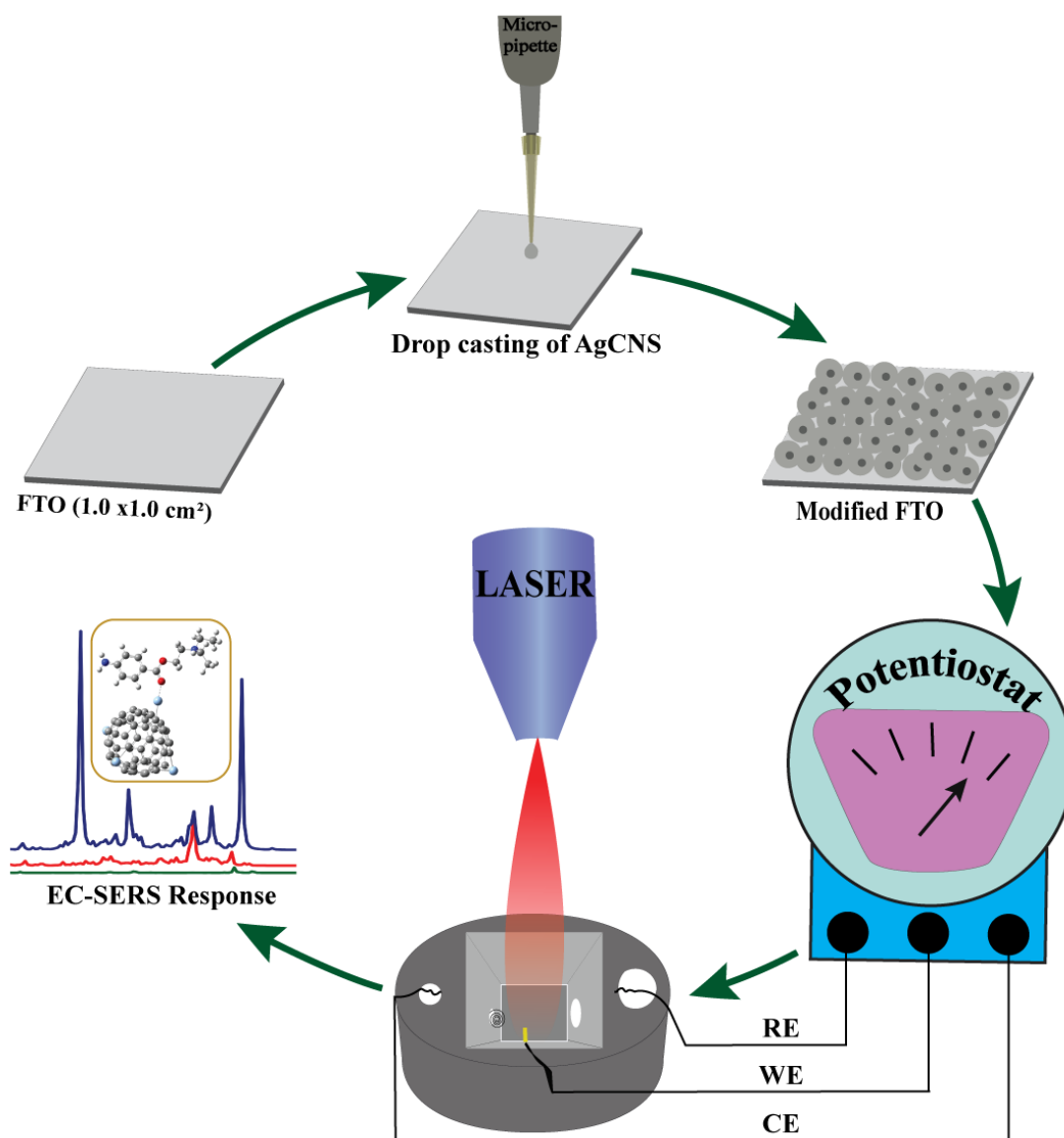


Figure 6. 1 Fabrication process of FTO, electrochemical pre-concentration and EC-SERS detection of procaine

6.2.4 SERS and electrochemical SERS measurements

The LabRAM HR evolution spectrophotometer equipped with an internal He-Ne laser 17 mW at excitation wavelength 633 nm and thermo-electrically cooled CCD detector was used to collect Raman and SERS spectra. The Raman spectra were recorded at 25% laser power, the acquisition time of 10 sec, a number of scans of 10 with 50× lens of a microscope. The SERS spectra were collected under the same conditions in a suspension

containing AgCNS and procaine in the ratio 1:1. The EC-SERS measurements were conducted using a typical three-electrode system as described above. The spectra were collected at open circuit potentials followed by a cathodic sweep from -0.05 to -0.25 V at a scan rate of 80 mV and sample intervals of 0.001 V. All electrolyte solutions were purged with a high purity argon gas (99.999%) prior to measurements.

6.3 COMPUTATION

Quantum chemical calculations were conducted using GAUSSIAN 09 [101] with the adoption of the Becke 3-Parameter (Exchange), Lee, Yang and Parr (B3LYP) functional introduced by Becke [102] and Lee et al [103], and a basis set of 6-31G* for the non-metal atoms. For the metal atoms, the calculations were performed using the Stuttgart/Dresden (SDD) basis set. These basis sets were selected due to their reliability to produce reasonable results that assent to experimental data of moderate size organic systems at reasonable costs [104–106]. DFT computation helps to gain insights on the interactions between the substrate and procaine molecules and thus understand the mechanism of SERS enhancements as a result of these interactions. Further, the aqueous solution corrections were computed via the Polarizable Continuum Model (PCM) developed by Tomasi [107]. Frequency calculations were conducted to ascertain the nature of convergence of procaine-AgCNS complexes. The same was used to predict the vibrational assignments of the wavenumbers for the compounds understudied. To ensure a good agreement with the Raman experimental results, the theoretical wavenumbers were scaled by a factor of 0.98 [108].

6.4 RESULTS AND DISCUSSION

6.4.1 AgCNS material and FTO electrode characterization

The morphology and elemental composition of the synthesized AgCNS indicate the formation of spherical-shaped carbon nanomaterials with uniform particles size distribution with an average size of 255 nm, as shown in the FESEM micrograph (Fig 6.2a). The elemental mapping image (Fig 6.2b) shows a uniform distribution of silver nanoparticles on the surface of the carbon nanospheres. The successful modification of the surface FTO electrode using AgCNS as a SERS active substrate was elucidated by FESEM and EDX techniques. After the deposition of AgCNS, the dispersed features of the nanomaterial are clearly shown in Fig 6.2c & 6.2d revealing the successful immobilization and uniform distribution of the nanomaterials on the electrode surface. The presence of target elements was confirmed by recording the EDX elemental spectrum as depicted in Fig 6.2e. The diffraction pattern of the AgCNS, FTO, and AgCNS modified FTO are presented in Fig 6.2f. The diffraction peak at 20.3° represents a hexagonal graphitic phase of the carbon atoms while the centered peaks at 29.2° (200) and 30.3° (004) (Fig 6.2f(ii)) are attributed to the presence of metallic silver on the nanosphere material and on the AgCNS modified FTO surface. All other peaks in the AgCNS-FTO are characteristic of the FTO glass and are similar to the fresh FTO pattern (Fig 6.2f(iii)), confirming the successful functionalization of the FTO electrode with AgCNS material.

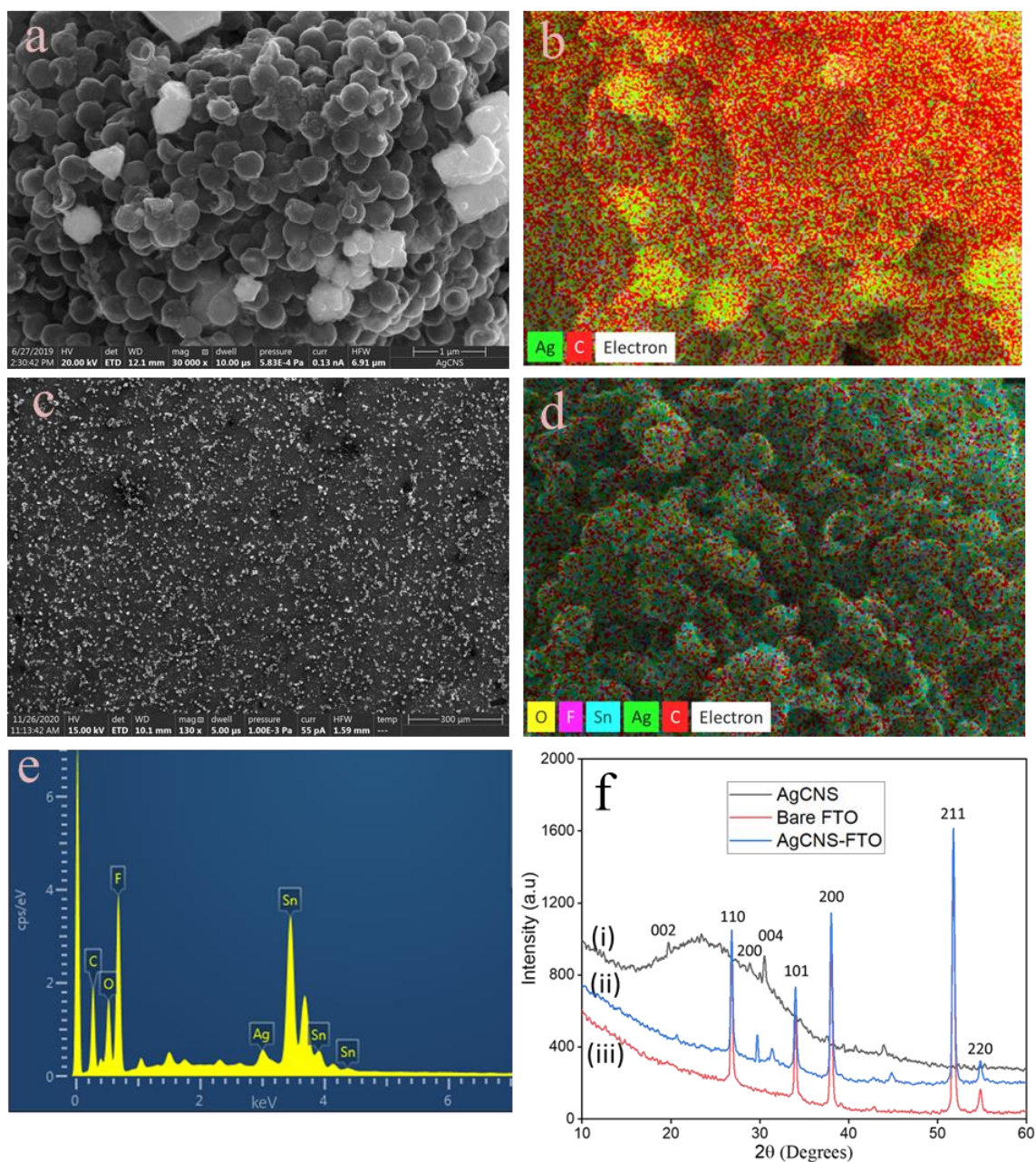


Figure 6. 2 (a) FESEM image of synthesized AgCNS, (b) elemental mapping of AgCNS, (c) FESEM image of AgCNS-FTO, (d) elemental mapping of AgCNS-FTO surface, (e) EDX elemental analysis spectrum of AgCNS-FTO and (f) XRD patterns of (i) AgCNS, (ii) AgCNS-FTO, and (iii) FTO.

The XPS spectra of AgCNS modified FTO (Fig 6.3) indicates the presence of carbon, silver, oxygen and tin. The appearance of tin peaks is due to their presence on the fluorine-

doped tin oxide (FTO). The deconvoluted spectra of Ag(3d) gave two peaks at 367.5 eV and 373.5 eV assigned to the Ag(3d_{3/2}) and Ag(3d_{5/2}) core levels, respectively. These peaks showed a slight shift towards lower binding energies compared to those in the AgCNS, indicating the successful fabrication of the FTO. The deconvoluted spectra of tin (Fig 6.3b) revealed three peaks at 486.9 eV, 495.3 eV and 496.8 eV corresponding to the Sn(3d_{3/2}), Sn(3d_{5/2}) and elemental tin, respectively. The deconvoluted spectrum of O(1s) reveals a single peak at 531.5 eV. The C(1s) spectrum (Fig 6.3d) exhibits shifting of the C-C and C=O peaks to lower binding energy as compared to AgCNS materials.

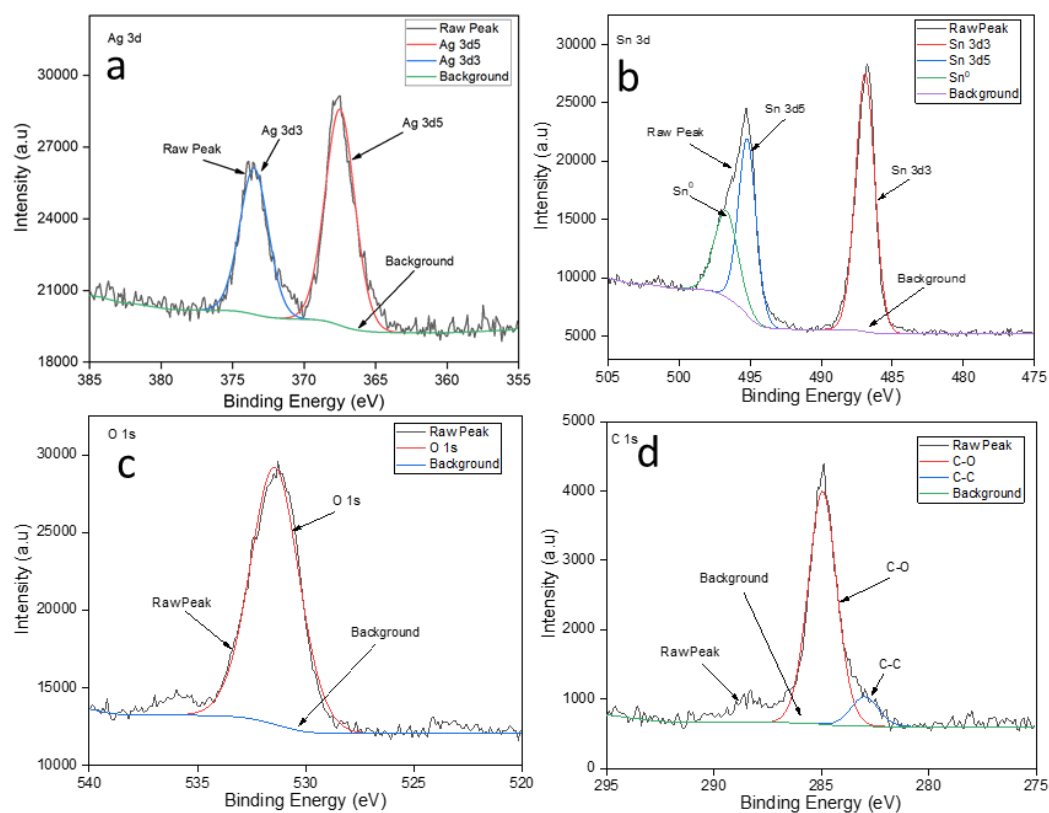


Figure 6. 3 (a) XPS of AgCNS-FTO (b-e) deconvoluted spectra representing the Ag(3d), Sn(3d), O(1s) and C(1s), respectively.

The electrochemical activity of the bare and functionalized surfaces of the FTO electrodes was investigated and presented in Fig 6.4. The Nyquist measurements (Fig 6.4a) resulted in a semicircle at the high-frequency region in both the bare and modified electrodes

corresponding to a charge transfer resistance, R_{ct} . The linear portion at the low-frequency region indicates a mass transport that can be defined by the Warburg resistance, W , and represents the diffusion of electroactive species through the bulk of the solution to the surface of the electrodes. The Randle's circuit elements presented in Fig 6.4 show that the AgCNS-FTO electrode has a Warburg resistance of $0.878\text{ m}\Omega$, which is almost 2.8 times greater than the bare FTO ($W = 0.314\text{ m}\Omega$). Furthermore, AgCNS-FTO exhibited an R_{ct} value of $0.715\text{ k}\Omega$ which is significantly lower compared to the bare analogue ($1.394\text{ k}\Omega$). The decrease in the capacitive loop on the AgCNS-FTO electrode corresponds to a lower resistance to charge migration and a consequent lower overpotential for effective mass transport of the species on the modified electrode.

The CV responses of bare FTO and AgCNS-FTO in $5.0\text{ mM K}_3[\text{Fe}(\text{CN})_6]$ containing 1.0 M KCl supporting electrolyte solution and at a scan rate of 80 mV/s are illustrated in Fig. 32b (the inset represents the blank measurements in 1.0 M KCl supporting electrolyte). The CV measurement were carried out in a potential window ($-0.3 - +0.9\text{ V}$) and sweep from -0.3 to 0.9 direction. The oxidation peak representing the oxidation of Ag to its ion resulting in the formation of AgCl can be seen at a potential of 0.1 V , with a weak reduction peak appearing at -0.25 V . This indicates the fast oxidation of Ag ions to AgCl and the slow quasi-reversible reduction to Ag(0) on the electrodes. An evident change in both the anodic and cathodic peaks for the redox couple $[\text{Fe}(\text{CN})_6]^{3-/4-}$ could be observed on the AgCNS-FTO. Both peaks were enhanced relative to the bare FTO, and an exceedingly narrow potential separation between the signals was observed resulting in a quasi-reversible behavior. The peak current ratio (I_{pa}/I_{pc}) of the bare FTO and AgCNS-FTO electrodes were estimated at 0.85 and 0.88 , respectively. This suggests that a substantial amount of the

redox species was oxidized and reduced on the AgCNS-FTO electrode. The peak separation of the modified and the bare electrodes were found to be 596.6 and 487.6 V, respectively suggesting the free movement of electrons through the double layer interface on the AgCNS-FTO electrode as compared to bare FTO electrode. The CV results were consistent with the findings of EIS measurements and imply that the functionalized electrode maintains a high conductivity sufficient for an effective charge transfer. Using Randles Sevcik's equation, the electroactive surface areas were estimated at 0.22 cm² for the bare and 0.44 cm² for the modified FTO electrodes, implying more area of the modified electrode is accessible for the redox ions.

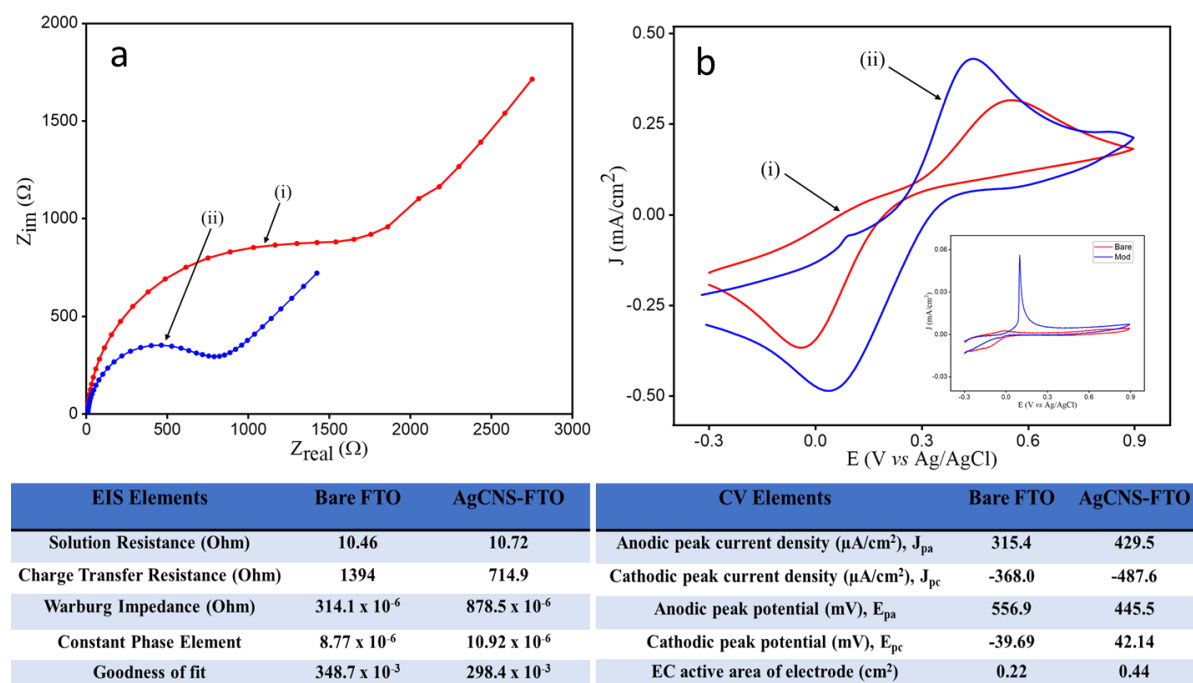


Figure 6. 4 (a) Nyquist plots and (b) cyclic voltammetry curves characterizing (i) bare FTO and (ii) AgCNS-FTO electrodes, in 5 mM of K₃[Fe(CN)₆] containing 1.0 M KCl supporting electrolyte. The inset in b represents the CV of the bare and the modified electrodes in 1.0 M KCl supporting electrolyte. The tables show the respective EIS and CV parameters of the bare and modified FTO electrodes.

6.4.2 Electrochemical activity of procaine

The stability of the AgCNS nanomaterials on the surface of the FTO electrode was measured by conducting multiple CV scans (50 cycles) at a scan rate of 80 mV/s. No significant decline in the anodic and cathodic peak currents (Fig 6.5a) was noticed. The feasibility of the electroreduction of procaine on the AgCNS-FTO electrode in 0.1 M PBS was analyzed by cyclic voltammetry and compared to the bare FTO electrode. The results (Fig 6.5b) revealed that the reduction of procaine on the surface of AgCNS-FTO occurred at -0.12 V vs. Ag/AgCl relative to the weak reduction peak at -0.06 V on the bare FTO. The CV responses of the bare FTO and the AgCNS-FTO in 0.1 M PBS are shown in Fig 6.5b (i) and (ii), respectively. The nanomaterials on the surface of AgCNS-FTO possibly availed a large surface area, providing sufficient reaction sites for the reduction of procaine ions. When compared to the bare FTO, the AgCNS-FTO electrode exhibited higher reduction currents as a result of the high electroactivity of the electrode.

To maximize the utilization of AgCNS-FTO electrode for the EC-SERS detection of procaine ions, the pH effect in the range from 4 to 8 on the electrochemical response of 0.1 M procaine in 0.1 M PBS was investigated and presented in Fig 6.5c. The reduction peak of procaine increases with the increase in pH reaching its optimum value at pH 7 before dying out. This is attributed to the lack of solubility of the procaine ions at pH values greater than 7. A corresponding positive shift in the cathodic peak potentials was observed in the pH range of 5 to 6, suggesting the lowering of energy needed for the reduction of procaine ions. An optimum pH value of 7 in 0.1 M PBS was, therefore, affirmed and chosen for the EC-SERS measurements.

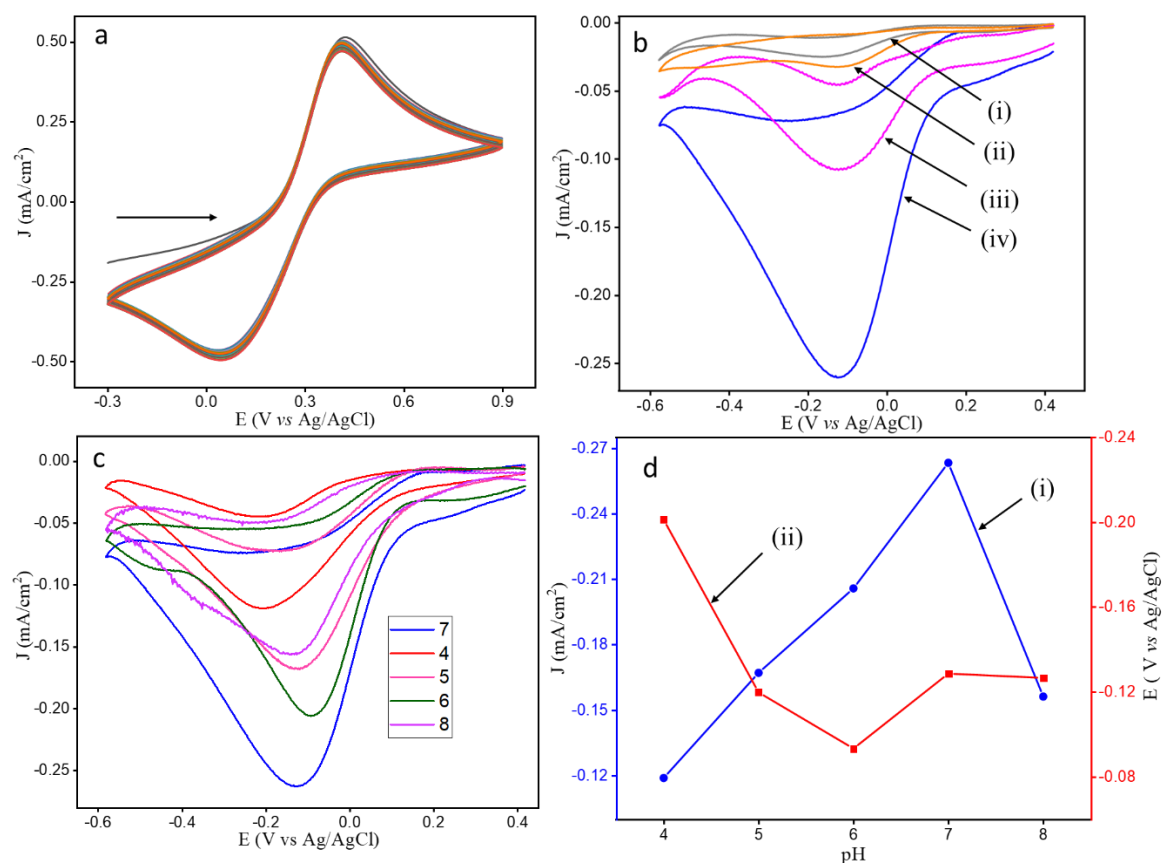


Figure 6. 5 (a) CV responses over the 50 cycles at AgCNS-FTO electrode using 80 mV/s scan rate in 5 mM potassium ferricyanide containing 1M KCl supporting electrolyte, (b) CV responses for 0.1 M PBS (i & ii) and 0.1 M procaine (iii & iv) at bare FTO (i & ii) and AgCNS-FTO (iii & iv) electrodes, (c) CV responses of 0.1 M procaine with respect to different pH values in the range of 4-8 at AgCNS-FTO and (d) Plots of cathodic peak (i) current density and (ii) potential changes with pH range 4-8 using CV in 0.1 M procaine at AgCNS-FTO electrode,

6.4.3 EC-SERS electrochemical pre-concentration and trace detection of procaine

In-situ EC-SERS measurements were conducted in a typical three-electrode system as explained earlier. To obtain the maximum possible Raman response, the volume of suspension of AgCNS drop cast on the FTO as well as electrochemical parameters such as the pre-concentration potential and pre-concentration time were optimized and plotted as a function of the Raman intensity of the band at 1611 cm^{-1} . The maximum enhancements were obtained at 40 μL suspension and at -0.1 V vs. Ag/AgCl and 400 sec., respectively. At more positive reduction potentials, the surface of the AgCNS-FTO electrode was not sufficiently negative to attract the positively charged procaine ions, yielding weak signals. By the application of increasingly negative potential, the surface charges were made gradually more negative and hence more molecules were reduced resulting in a significant enhancement. An optimum pre-concentration potential of -0.1 V was reached and adopted for further EC-SERS experiments. In a similar manner, the pre-concentration time was optimized to be 400 sec. While lower limits of detection are achievable at a longer pre-concentration time, too long pre-concentration often results in the fouling of the electrode surface and leads to a decline in the analytical performance. Therefore, an optimum pre-concentration time of 400 sec was adopted. Other spectroscopic parameters such as Raman acquisition time, number of accumulations and laser power were also optimized at 10 sec, 10 accumulations, and 25% laser power at excitation wavelength of 633 nm, respectively.

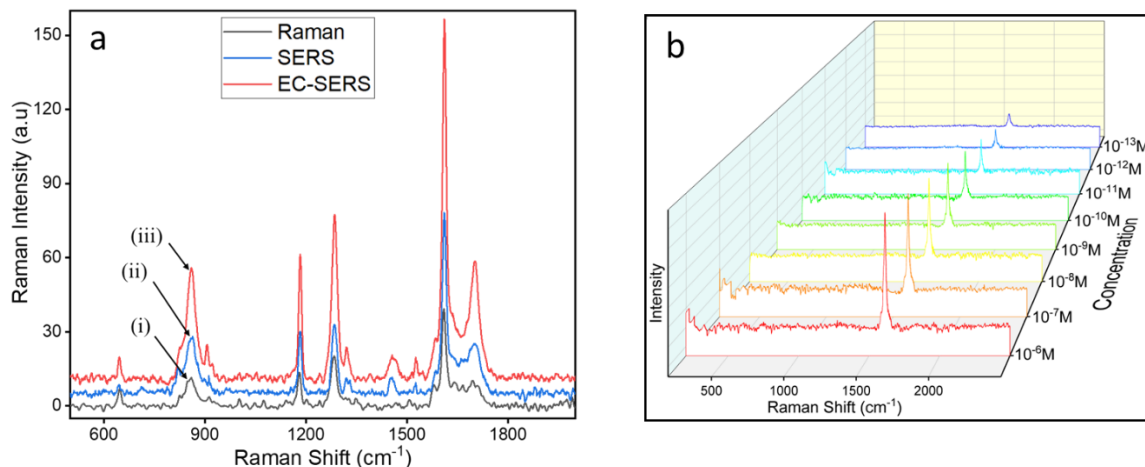


Figure 6. 6 (a) (i) Normal Raman spectrum of 0.1 M procaine solution, (ii) SERS spectrum with solution ratio 1:1 of AgCNS suspension and 0.1 M procaine solution and (iii) EC-SERS spectrum at AgCNS-FTO electrode and (b) 3D representation of EC-SERS measurements on different concentrations of procaine solution.

The normal “non-enhanced” Raman, SERS and EC-SERS spectra of 0.1 M procaine solution with pH 7 under the optimum electrochemical conditions are depicted in Fig 6.6a. While most of the Raman peaks were found to moderately enhance in intensity in the EC-SERS spectrum, a significant enhancement was observed at the Raman band at 1611 cm⁻¹ corresponding to the NH₂ bending mode and was further selected for quantitative purposes. At the optimized conditions, EC-SERS spectra of different concentrations of procaine solution were collected in the range 200 – 2500 cm⁻¹ as presented in Fig 6.6b. It is observed that the EC-SERS intensity increases as a function of molecular population and yielded remarkable linearity within the dynamic range of 10⁻⁶ – 10⁻¹² M, an excellent limit of detection of 10⁻¹³ M (S/N = 3) [233] and a correlation coefficient, R^2 , of 0.9994. When compared to other previously reported methods for the quantification of procaine (Table 6.1), the *in situ* EC-SERS method exhibited a superior sensitivity, a wider dynamic range, a lower detection limit and has the potential to serve as a promising tool for possible and fast quantification of procaine.

Table 6. 1: Comparison of reported methods from literature and the developed method in this work for the trace quantification of procaine.

Method	LDR	LOD	R^2	Ref.
DPV	9.0×10^{-7} – 2.6×10^{-5} M	5.0×10^{-8} M	0.995	[211]
CV	5×10^{-6} – 2×10^{-4} M	0.5×10^{-6} M	0.999	[208]
SIA-CL	1.8×10^{-6} – 1.8×10^{-4} M	1.1×10^{-6} M	0.9998	[234]
HPLC	0.05–5.0 μ g/ml	-----	≥ 0.998	[235]
LC/MS/MS	10.0–100.0 ng/ml	0.100 ng/mL	0.9954	[236]
UV-Vis	1.50×10^{-7} – 4.15×10^{-6} M	3.80×10^{-8} M	0.9783	[237]
SERS	1×10^{-3} – 1×10^{-8} M	1×10^{-10} M	0.9971	[247]
EC-SERS	1×10^{-6} – 1×10^{-12} M	1×10^{-13} M	0.9994	This Work

The selectivity of the proposed *in situ* EC-SERS method towards procaine in the presence of other structural analogues was further investigated. The possible interferents studied were ciprofloxacin, valeric acid, sulindac and ascorbic acid at 1000 times the concentration of the procaine solution (1 μ M). The results revealed that the tested interfering species displayed negligible interference and that the Raman intensity of the procaine analyte was maintained above 90% of its original value as presented in Fig 6.7. This suggests that the proposed *in situ* EC-SERS method by virtue of its high sensitivity and selectivity demonstrates the potential to serve as a quantitative method for the detection of procaine in practical samples. The repeatability test of the proposed method was carried out on five AgCNS modified FTO electrodes in a batch for the detection of 0.1 M procaine solution under the optimum conditions. The five set of measurements results with similar results with a relative standard deviation, RSD, of 1.5%. The reproducibility of the proposed method was also investigated by repeating the five set of measurements at AgCNS-FTO prepared in different batches after storing the electrodes for 30 days. The Raman signal

intensities in 0.1 M procaine solution did not show a significant decrease from the original values, yielding an RSD of 2.4%. This confirms the sufficient reproducibility and stability of the proposed method for the quantification of procaine.

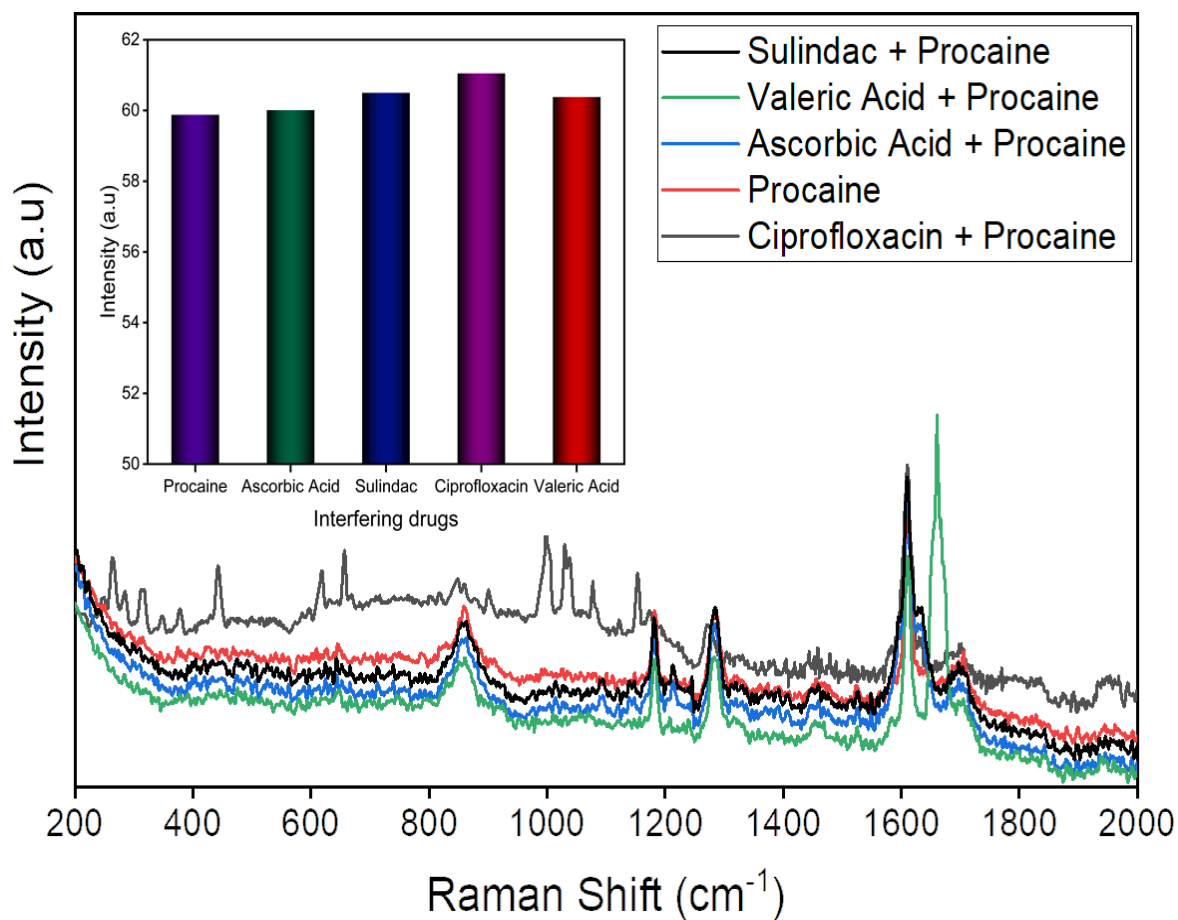


Figure 6. 7 Interference studies of the *in-situ* EC-SERS approach for the quantification of procaine in the presence of 1000 times concentration of sulindac, valeric acid, ascorbic acid and ciprofloxacin, The inset is the plot of EC-SERS response at 1611 cm^{-1} in the presence of interfering species.

6.4.4 Molecular interaction on the AgCNS surface

Surface Raman enhancement experiments involve the interaction of the AgCNS with the procaine at the molecular level. A comprehensive interpretation of the vibrational properties of the procaine molecule upon interaction with the AgCNS thus provides an insight into the adsorption modes of the drug molecules. Molecular modeling simulations using the density functional theory (DFT) approach were conducted to gain more understanding of the nature of the interaction. Two feasible interaction modes; AgCNS-NH₂ and AgCNS-O=C were identified, and the simulated Raman spectra resulting from these interactions are presented in Fig 6.8. The resulting spectra were found to be in good

agreement with the experimental SERS and EC-SERS spectra. Moreover, interaction energies of -13.73 and -14.55 kcal/mol and binding distances of 2.16 and 2.25 Å were predicted for the (AgCNS)-O=C and (AgCNS)-NH₂ interaction modes, respectively, as presented in Table 8. The higher energy obtained for the latter interaction arises due to the minimum steric effect obtainable in this mode which allows the proper overlap of the electronic orbitals of the interacting atoms. The direction of flow of electrons was analyzed by the change in Mulliken charges which indicates that electrons flow in the direction from the procaine molecules to the AgCNS surface (Table 6.2) in agreement with the electronic charge distribution. Comparing the atomic charges on the carbonyl oxygen (-0.547) and the amino nitrogen (-0.804) further revealed the preference of the AgCNS to bind through the (AgCNS)-NH₂ interaction mode resulting in a significant enhancement in the Raman bands which is comparable to the experimental SERS.

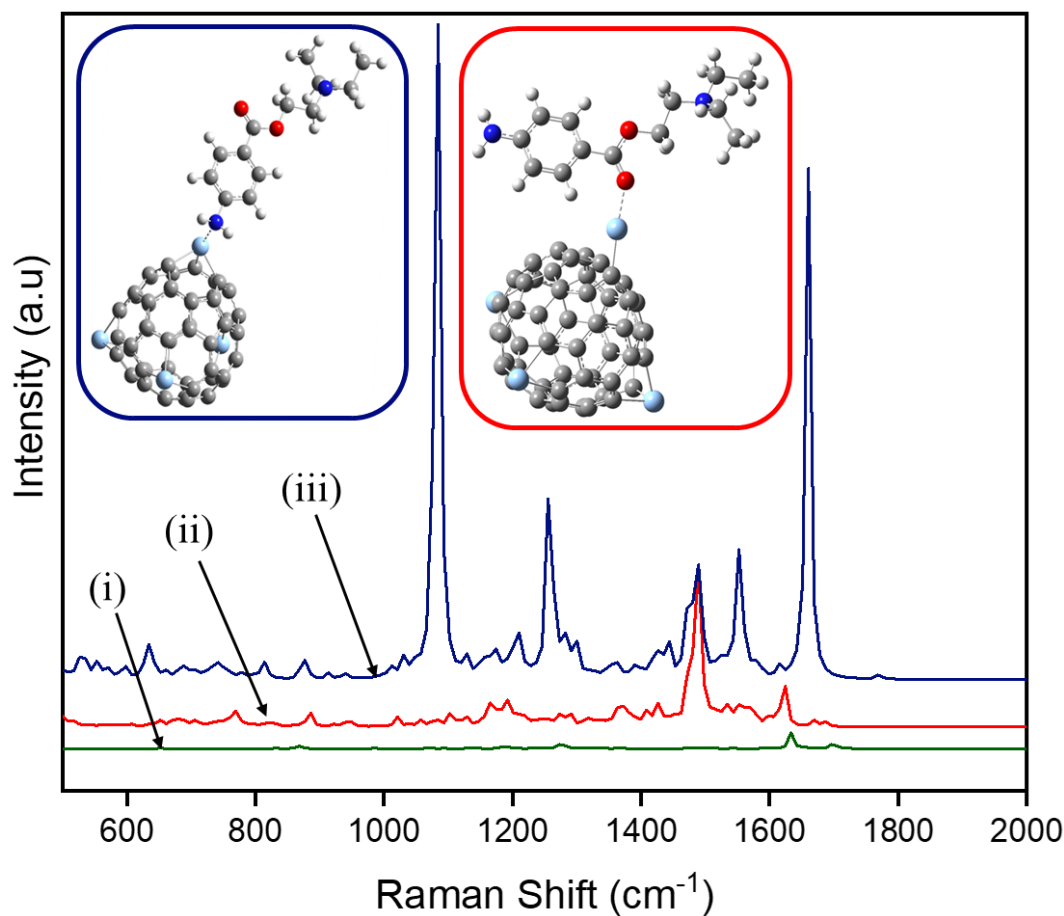


Figure 6. 8 Calculated Raman spectrum of (i) procaine and (ii & iii) upon interaction between procaine and AgCNS via the C=O functional group and NH₂ functional group of procaine, respectively (as shown in insets).

Table 6. 2 Interaction energies, binding distances and the change in the sum of Mulliken charges for the two interaction modes.

Interaction Type	Energy (kcal/mol)	B. Distance (Å ⁰)	Free	Mulliken Charges	
				Complex	
AgCNS-O=C	-13.73	2.16	AgCNS: 4.000	AgCNS:	3.711
				Procaine:	0.289
AgCNS-NH ₂	-14.55	2.25	Procaine: 0.000	AgCNS:	3.646
				Procaine	: 0.354

A detailed understanding of the underlying inter- and intra-molecular processes following the interactions of AgCNS nanomaterials with the procaine molecules is essential for a better interpretation of the Raman spectra, the SERS spectra and the corresponding

enhancement observed in the EC-SERS spectra. To do this, vibrational modes were computed at B3LYP/6-31G* and were found to be in good agreement with the experimental results as shown in Table 6.3 . The stretching vibrational mode associated with the -C=O functional group appeared at 1695 cm^{-1} though predicted at 1696 cm^{-1} in accordance with the wavenumbers reported for the -C=O stretching mode [239]. The band at 1610 cm^{-1} (calculated at 1633 cm^{-1}) has been best expressed as the -NH₂ bending coupled with -C=C- stretching modes, while the uncoupled -C=C- stretching vibrational mode was observed at 1505 cm^{-1} and predicted as 1540 cm^{-1} . The former assignments are within the 1650 – 1580 cm^{-1} range of vibrational bending established for NH₂ functional group [239,240], whereas the latter is in agreement with the wavenumber reported for the -C=C- stretching mode [241]. Furthermore, the -CH bending vibration was observed at 1468 cm^{-1} (1489 cm^{-1} from DFT) which falls within the 1600 – 1000 cm^{-1} region reported for aromatic -CH in-plane bending vibrational modes[242]. The Raman band associated with the -CH₂ twisting mode was observed at 1348 cm^{-1} (1363 cm^{-1} calculated from DFT) while the one for -CN stretching appeared at 1286 cm^{-1} (1373 cm^{-1} from DFT) in consonant with the vibrational wavenumbers established for -CH₂ twisting [61], and that for -CN stretching [241]. Lastly, the wavenumber observed at 913 cm^{-1} was assigned to the -CH out-of-plane wagging vibration in accordance with the literature [63,64], whereas the ring deformation vibrational modes appeared at 859 and 648 cm^{-1} [241].

Of the effects of substrate-analyte interactions in SERS and EC-SERS experiments is the enhancement in Raman band intensities and the shift either to lower or higher wavenumbers. As observed from Fig 6.8 the most prominent enhancements were recorded at EC-SERS bands 1611 cm^{-1} , 1322 cm^{-1} , 1285 cm^{-1} and 1184 cm^{-1} corresponding to

coupled -NH₂ bending and -C=C- stretching, -CH₂ twisting, -CN stretching and -CH bending modes respectively. This enhancement could be attributed to the highly plasmonic light field resonance produced on the surface of AgCNS upon laser excitation which resulted in the increased light scattering of procaine molecules residing within. This phenomenon eventually led to an enhancement in the Raman signals. Additional noticeable effects in the SERS spectra are the red shifts associated with the -C=O vibrational stretching, the -C=C- stretching and the -NH₂ bending mode. Blue shifts were observed at the bands attributed to the -CH bending, -CH₂ twisting, -CN stretching and an out-of-plane -CH wagging mode as presented in Table 6.3. The DFT-based quantum chemical calculations and vibrational assignments provides an overview of the processes resulting in the enhancement in Raman signals and predicted the most probable interaction modes of the AgCNS nanomaterials with the procaine molecules.

Table 6. 3 Assignments of vibrational modes for experimental and calculated Raman, SERS and EC-SERS spectra of the procaine molecule and procaine-AgCNS in the 2000 – 500 cm⁻¹ regions.

Description	Observed ^a (cm ⁻¹)		Calculated ^b (cm ⁻¹)			
	Raman ^a	SERS ^a	EC-SERS	PRCN	Procaine-AgCNS	
					AgCNS-NH ₂	AgCNS-O=C
ν_1 C=O str.	1695 w	1699 m	1702 m	1696 (42)	1768 (0)	1687 (4)
ν_2 NH ₂ bend,C=C str.	1610 vs	1611 vs	1611vs	1633 (100)	1660 (80)	1624 (40)
ν_3 C=C str.	1505 vw	1525 w	1526w	1540 (5)	1552 (25)	1534 (14)
ν_4 CH bend	1468 vw	1456 w	1460w	1489 (3)	1489 (22)	1489 (100)
ν_5 CH ₂ twist	1348 w	1320 w	1322 m	1363 (1)	1363 (1)	1372 (15)
ν_6 CN str.	1286 s	1284 s	1285 s	1273 (45)	1255 (37)	1273 (9)
ν_7 CH out-of-plane wag	1181 m	1184 s	1184 s	1192 (33)	1084(100)	1102 (40)
ν_8 CH bend (wag)	913 w	912 m	907 w	915(0)	1030 (4)	1021 (2)
ν_9 Ring breath	859 m(h)	864 w(h)	860 m	868 (15)	877 (6)	886 (17)
ν_{10} Ring def.	648 m	645 w	646 w	652 (0)	634 (7)	652 (3)

^a Observed Raman intensities; vs: very strong; s: strong; m: medium; w: weak, h: hunch.

^b Calculated relative Raman intensities are given in parentheses. PRCN= Procain

6.4.5 Molecular docking of procaine

Through the action of the enzyme pseudocholinesterase, procaine is metabolized in the plasma via hydrolysis into para-aminobenzoic acid (PABA), which is later excreted into the urine by the kidneys[266]. Although not globally utilized, procaine exhibits anti-inflammatory properties and has the tendency to inhibit the secretion of prostaglandins which are produced by the enzyme cyclooxygenase (COX) within the body. While these chemicals are responsible for accelerating healing processes, they promote the inflammation of body organs resulting in pain and fever. The use of anti-inflammatory drugs, therefore, blocks the COX enzymes and inhibits the secretion of prostaglandins thereby reducing ongoing inflammation, fever and pain during the healing process. Despite its potential as an anti-inflammatory drug, the mode of action of procaine is not well established. Hence, we conducted molecular docking in the present study to provide insights into the pharmacological actions of the drug procaine as an anti-inflammatory agent and to evaluate the possibility of the detection of procaine in clinical samples without the interference of the human body enzymes. Molecular docking analysis was carried out on the crystal structure of the chemokine receptor, CXCR4 (an inflammatory protein), PDB ID: 4RWS [267] using the AutoDock Vina molecular docking software [268]. For an effective binding of the ligand to the receptor, molecules of water were removed from the original crystal structure of the protein. The docking was conducted in a grid box of dimension $95 \times 10 \times 53$ and a spacing of 0.375. The number of iterations was set to 8 as no significant difference was observed in the binding modes by varying the value up to 64. At the end of the docking, the ligand was optimized to yield a binding energy of -5.7 kcal/mol for the pose with the highest affinity (Fig 6.9a). Favorable interactions between

the ligand and the receptor such as the pi-sigma, pi-pi and the pi-alkyl were found indicating a strong binding between procaine and the receptor (Fig 6.9b). The binding modes and the molecular level interactions between procaine and the CXCR4 chemokine receptor protein suggests the anti-inflammatory tendency of procaine and revealed the possibility of procaine quantification by the proposed *in-situ* EC-SERS approach without interference from the enzymes in the human body. The proposed approach provides a new perspective on the application of the drug procaine in anti-inflammatory applications and could be extended to other structural analogues.

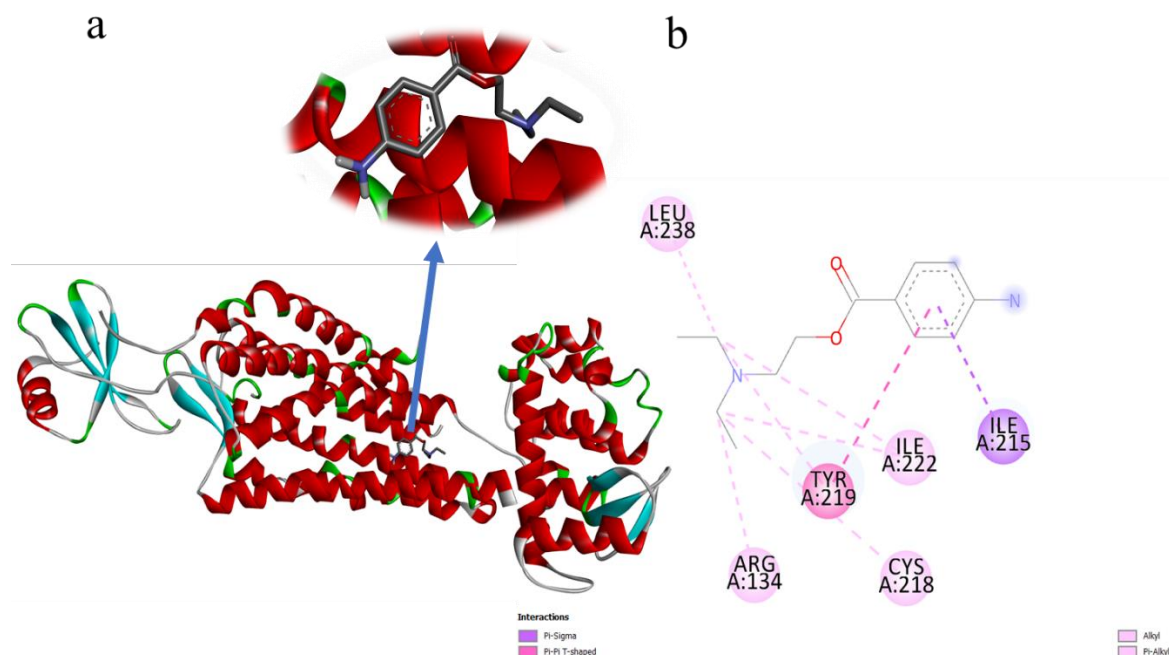


Figure 6. 9 (a) Docked conformation of the most active pose of procaine on the chemokine receptor, PDB ID: 4RWS, and (b) the molecular interactions of procaine with the active amino acids on the receptor.

CHAPTER 7

SERS and EC-SERS detection of local anesthetic procaine using Pd loaded highly reduced graphene oxide nanocomposite substrate.

7.1 INTRODUCTION

Surface-enhanced Raman scattering (SERS) spectroscopy has gained a tremendous interest from the research community owing to its highly sensitive detection of analyte trace concentrations for materials and medicine related applications [269]. The sensitivity of SERS originates mainly from the electromagnetic enhancement that amplifies Raman scattered emission signals of a given analyte [270]. Nanoparticles with doses from commonly coinage metals [271], in particular silver and gold, which can display localized surface plasmon resonance (LSPR) serve as attractive SERS substrates. On the other hand, the electrochemical (EC) phenomenon is a powerful analytical means to detect the target of interest by employing modified electrode systems that monitor the redox potentials of the molecules to be characterized. The combination of electrochemical techniques and SERS (EC-SERS) can offer multiple transducers on one platform that allow added advantages of higher sensitivity and specificity [272]. In case of EC-SERS, an applied voltage to a SERS-active electrode enables the analyte to get closer to the surface or change the orientation of the adsorbed molecules, which in turn leads to a measurable change in

the signal intensities [273]. Therefore, the development of metallic nanoparticles based dual functional substrates that contain optical and electrical properties for EC-SERS sensing has gained a widespread attention in recent years. Consequently, dual functional substrates including Au-nanostructured microarrays on polystyrene beads [274], Au-coated silicon nanowires [275], Ag-SiO₂ nanocomposite core shell [276] and Ag-nanowires on coffee filter [277] have been developed. EC-SERS has increasingly found utility for the ultrahigh sensitive detection of biomolecules in biological samples [278–282]. Nevertheless, further advances in the development of SERS techniques necessitate the realization of new methods that would allow fabrication of flexible and tunable plasmonic nanostructures materials [283–285]. In continuation to our ongoing search for the SERS and EC-SERS based ultrasensitive detection of therapeutic drug molecules [286–288], we report herewith the synthesis of palladium-based highly reduced graphene oxide (Pd-HRG) as a potential dual SERS and EC-SERS substrate and its utility in the detection of trace concentrations of procaine. The Pd nanoparticles, acting as a plasmonic substrate, would generate a sufficient electromagnetic field which results with an appreciable enhancement of the Raman scattered photons, while the HRG would help improving the signals by reducing the background noise. Procaine is a local anesthetic that is widely used in neural therapy and dental procedures [203–205] and as a remedy in obesity, depression, infections, and cancer [14,15]. Overdoses and long term exposure of the drug causes side effects, such as cardiac toxicity, neurotoxicity and some allergic reaction [207]. A number of analytical techniques including high performance liquid chromatography (HPLC) [289], gas chromatography (GC) [290], differential pulse voltammetry (DPV) [291], chemiluminescence (CL) [292], have been employed in the

detection of procaine. Although these methods exhibit a high selectivity and sensitivity, they are often accompanied with intensive and complicated laborious analysis [293]. Furthermore, although the SERS approach has been effectively used for the trace concentration detection of procaine [286,287,294], these reports utilized only silver or gold nanoparticles as substrates. Through this communication, the synthesis of Pd-HRG and its use as a coating material onto fluorine doped tin oxide (FTO) electrodes in the deployment of SERS and EC-SERS platforms, respectively, for the detection of procaine were investigated. A quantification method for the ultralow concentrations of procaine through coupling SERS with electrochemical techniques has also been proposed. Density functional theory (DFT) calculations were performed to provide insights into the nature of interactions between the target drug molecules and the nanostructured substrate.

7.2 METHODOLOGY

7.2.1 Chemicals and Materials

Sodium Nitrate (CAS Number: 7631-99-4, Purity > 99.0%), graphite powder (CAS Number 7782-42-5, Purity $\geq$ 99.9%), Sulfuric acid (CAS Number: 7664-93-9, Purity $\geq$ 97%), potassium permanganate (CAS Number: 7722-64-7, Purity $\geq$ 99%), procaine (CAS Number: 51-05-8 Purity $\geq$ 97%), sodium tetrachloropalladate (II) (CAS Number: 13820-53-6, Purity $\geq$ 99.5 %), phosphate Buffer (pH=7.2) were bought from Sigma-Aldrich and used as received. The ultrapure water from a water purification system (Ultra Clear™ Lab Water Systems, Siemens Water technologies USA) were utilized for the preparation of solutions.

7.2.2 Synthesis of Pd-HRG nanocomposite

The HRG was synthesized using a modified hummers method, as previously reported by our group [295,296]. Briefly, NaNO_3 (300mg) was mixed with graphite powder (300mg) in a round-bottom flask and conc. H_2SO_4 was added. The mixture was stirred on an ice bath for 10 minutes followed by the addition of KMnO_4 (2g). After 30 minutes, a color change from black to dark green was observed, and the temperature of reaction mixture was then increased to 37°C . The produced graphene oxide (GRO) was then further reduced using hydrazine hydrate to achieve HRG. The Pd-HRG nanocomposite was prepared by adding a solution of Na_2PdCl_4 in ethanol to a suspension of HRG in ethanol under ultrasonication treatment for 1 hour. The resultant Pd-HRG nanocomposite was washed and dried.

7.2.3 Fabrication of the functionalized FTO electrode

Transparent and conductive FTO substrate was sequentially cleaned in acetone, isopropanol followed by deionized water using ultrasonic bath and for 20 min each. The well cleaned transparent and conductive substrate was functionalized with Pd-HRG suspension via a drop drawing method. The Pd-HRG suspension was drop-casted onto the FTO glass, and then the functionalized FTO substrate was dried. The detailed functionalized procedure is given in Supplementary Information.

7.2.4 SERS and EC-SERS Measurements

For the SERS measurement, the Pd-HRG suspension was prepared by dispersing Pd-HRG nanocomposite (10 mg) in deionized water (10 mL) under sonication at 40°C for 30 minutes. Thereafter, 200 μL of Pd-HRG of as prepared suspension and 200 μL of 0.1 M procaine solution were mixed in a cuvette and the mixture was allowed to stay for 5 min in

order to maximize procaine and Pd-HRG interactions. Normal Raman and surface enhanced Raman scattering (SERS) spectra were recorded using LabRAM HR evolution spectrophotometer, which was equipped with He-Ne laser having power 17 mW and exciting wavelength 633 nm and CCD detector. The normal and SERS spectra were recorded using acquisition time 20 sec, 50% laser power, and number of accumulations 6. The sample was focused with 50x lens of microscope. The ratio of procaine drug solution and Pd-HRG suspension was set 1:1 for collecting the SERS spectra. EC-SERS measurements were conducted using CHI 1232A potentiostat and LabRAM HR evolution spectrophotometer. A conventional three-electrode system comprising of the functionalized FTO as a working electrode, Pt wire as a counter electrode and Ag|AgCl as a reference electrode was used for all EC-SERS experiments. Electrochemical parameters of scan rate 100 mV, sample interval 0.001 V and cathodic sweep were employed for collecting the EC-SERS spectra. The overall illustration of methodological approach, including synthesis of Pd-HRG, functionalization of FTO coated glass substrate, interaction phenomenon between Pd-HR and drug molecules, and SERS and EC-SERS analysis, is depicted in Fig 7.1.

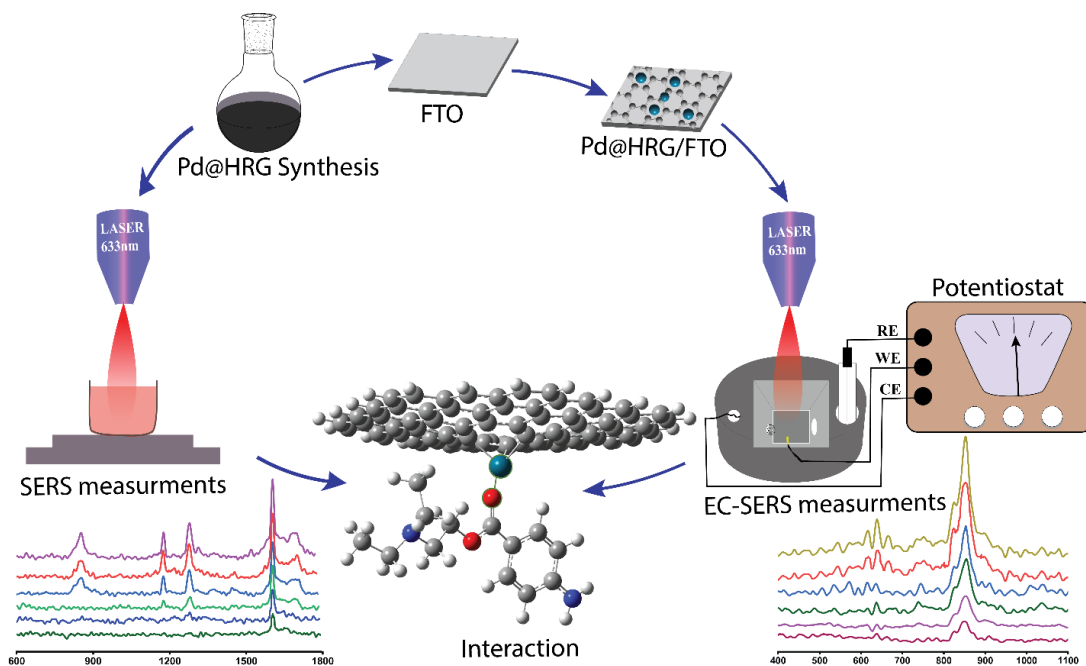


Figure 7. 1 Illustration of overall analysis methodology of procaine drug molecule using SERS and EC-SERS measurements.

7.2.5 Characterization techniques

The morphology of the synthesized Pd-HRG nanocomposite and elemental composition were monitored using Field-emission scanning electron microscopy FE-SEM (Tescan Lyra-3) operated at 20 kV. The energy-dispersive X-ray spectroscopy (EDX) analysis of the samples were performed using Lyra 3 integrated in the FESEM using LINK INCA program system. The phase purity of the samples was confirmed using powder X-ray diffraction pattern (XRD) recorded using Rigaku miniflex II X-ray diffractometer in step scan mode between 20 and 90° 2 θ (scan rate 0.02° 2 θ s⁻¹) using Cu K α radiation (λ = 1.5414 Å).

7.3 COMPUTATION

All the quantum chemical calculations including geometrical optimization of the HRG, Pd-HRG and their interactions were carried out with the aid of Gaussian09 program [297]. A

monolayer graphene sheet consisting of 27 hexagons and 73 carbon atoms was considered as HRG model. The hydrogen atoms were added to the edge of carbon atoms of graphene sheet to avoid unsaturated boundary effect and optimized geometry. The Pd-HRG was modeled by substituting a carbon atom of graphene sheet with a single Pd atom. The geometry optimization and calculations involving energies of the compounds involved in the study was obtained at same theory level, using DFT with the B3LYP functional and basis sets 6-31G(d,p) for non-metals and SDD for palladium atoms. The frequency calculations did not reveal any imaginary frequency, which suggests a complete optimization of geometries. The binding energies of interaction between procaine and Pd-HRG were calculated, using well established methods [298–300].

7.4 RESULT AND DISCUSSION

7.4.1 Characterization of Pd-HRG nanocomposites and Pd-HRG/FTO electrode

The synthesized Pd-HRG and functionalized FTO electrode were extensively characterized using Raman, XRD, FESEM and EDX techniques. The phase purity and crystallinity of the as synthesized HRG and Pd-HRG nanocomposite were determined using XRD analysis (Fig 7.2a). The graphitic powder shows a highly intensive reflection at $2\theta = 26.6^\circ$ corresponding to the (002) lattice plane of graphene layers stacked in graphite [301]. However, after oxidation, different functionalities containing oxygen atom were introduced between the layers of graphite, which subsequently led to increase in the interlayer distance and thus shifted the reflection in X-ray diffractogram to a lower Bragg angle of $2\theta = 12.5^\circ$ [302]. Upon reduction of GRO to HRG, the Bragg angle at $2\theta = 12.5^\circ$

vanished and a new reflection at an angle $2\theta = 24.5^\circ$ appeared, indicating a loss of a long-range order. The Pd-HRG nanocomposite, in addition to HRG reflections, shows two new reflections (2θ) centered at 36.8° and 45.4° related to the (111) and (200) crystalline planes of a face centered cubic (FCC) structure of Pd nanoparticles [290].

The Raman spectra of graphene-based materials exhibited two principal G and D-bands. The relative ratio of these bands dependent upon the overall sp^2 character, and the presence of defects caused by the synthesis procedure. The stacked Raman spectra of graphite powder, GRO and HRG are presented in Fig 7.2b. The Raman spectrum of graphite powder (black line) shows a moderately intense G band but a very small D band that are centered at 1589 cm^{-1} and 1350 cm^{-1} , respectively. The G band after oxidation in GRO turned narrower and slightly red shifted close to 10 cm^{-1} . After the reduction of GRO to form HRG, the intensity of D band increased, and thus the relative D/G band intensity ratio was also increased. This suggested the formation of more sp^2 domains on the material surface as compared to graphene oxide, which confirms the reduction of GRO into HRG. A similar spectral pattern was obtained after the deposition of Pd nanoparticles, suggesting the intact structure of graphene during the synthesis of the Pd nanoparticles [303].

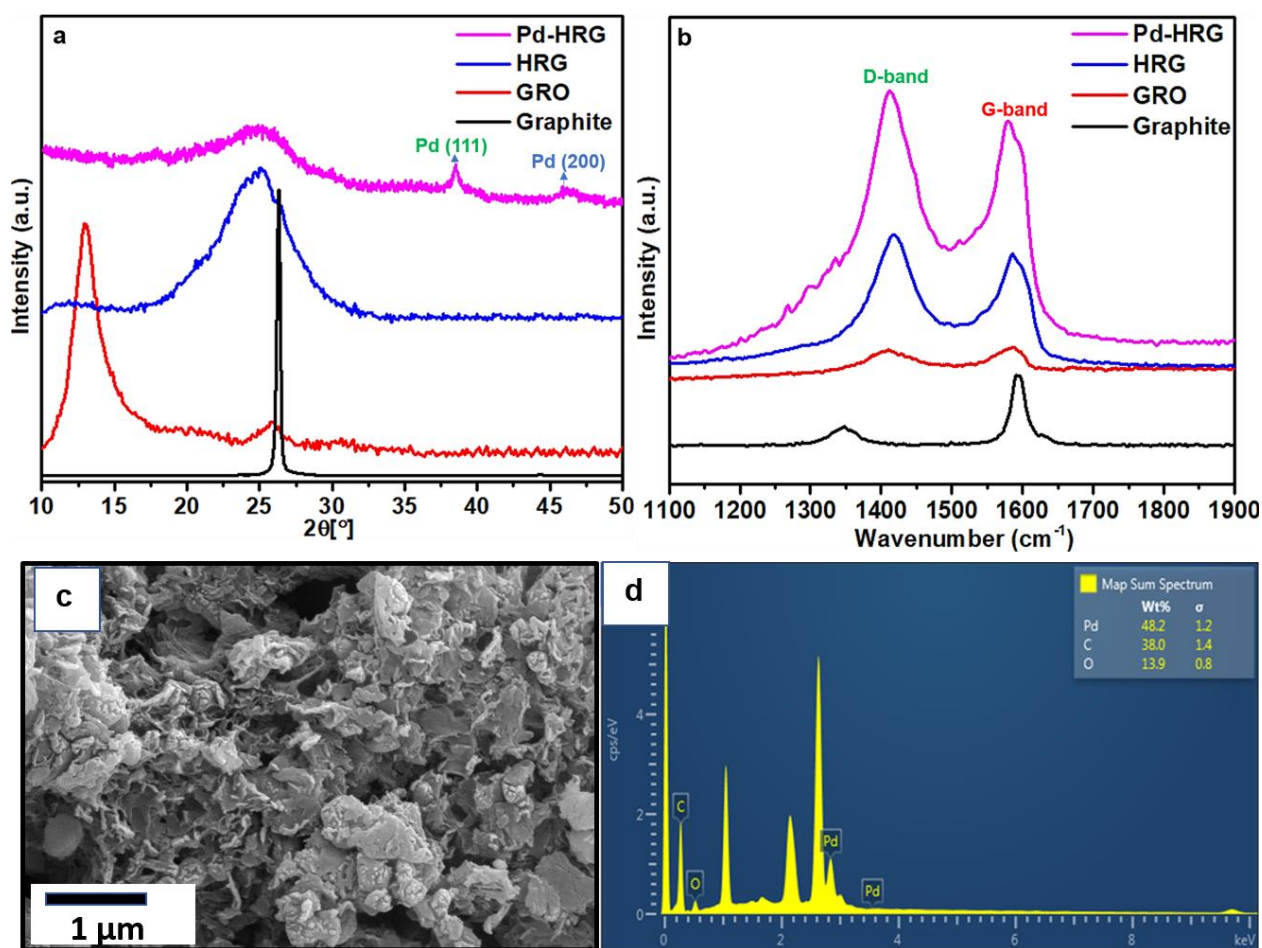


Figure 7. 2 (a) XRD diffractograms of HRG (red line), graphite powder (black line), GRO (blue line) and Pd-HRG (violet line), (b) Raman spectra of graphite powder (black line), GRO (red line), HRG (blue line) and Pd-HRG (violet line), (c) FESEM images at 1 μ m and 500 nm scales of the as prepared Pd-HRG sheet-like structures, and (d) the EDX elemental composition spectrum of the Pd-HRG nanocomposite.

Further, FESEM micrographs (Fig 7.2c) show the morphology of the synthesized Pd-HRG nanocomposite. Although the EDX spectrum (Fig 7.2d) confirms the presence of significant amount of Pd inside the composite but unfortunately the size of the Pd nanoparticles is beyond the resolution limits of microscope. However, the SEM image

shown in Fig 7.2c indicates the curled sheets of HRG. The Pd-HRG nanocomposites were characterized using TEM. The TE micrograph confirms a few layers thickness of HRG. These sheets are uniformly coated with Pd nanoparticles as published earlier [31]. The electrical conductivity of Pd-HRG fabricated EC-SERS electrode onto FTO substrate (Pd-HRG/FTO) was assessed using electrochemical impedance spectroscopy (EIS). The Nyquist plots of bare and functionalized FTO substrates (Fig. 7.3b) indicates two semi-circles in functionalized FTO, which indicates the deposition of the nanocomposite onto the electrode. The small semi-circle refers to the lower charge transfer resistance and higher electrical conductivity as compared to the bare FTO. This was further confirmed by the presence of D band at 1333 cm^{-1} and G band at 1590 cm^{-1} in the Raman spectrum of Pd-HRG functionalized FTO (Fig 7.3a). The FESEM image (Fig 7.3c) of the functionalized electrode indicates the uniform fabrication of Pd-HRG onto the FTO surface., Likewise, the EDX spectrum (Fig 7.3d) illustrates the elemental composition of FTO fabricated electrode.

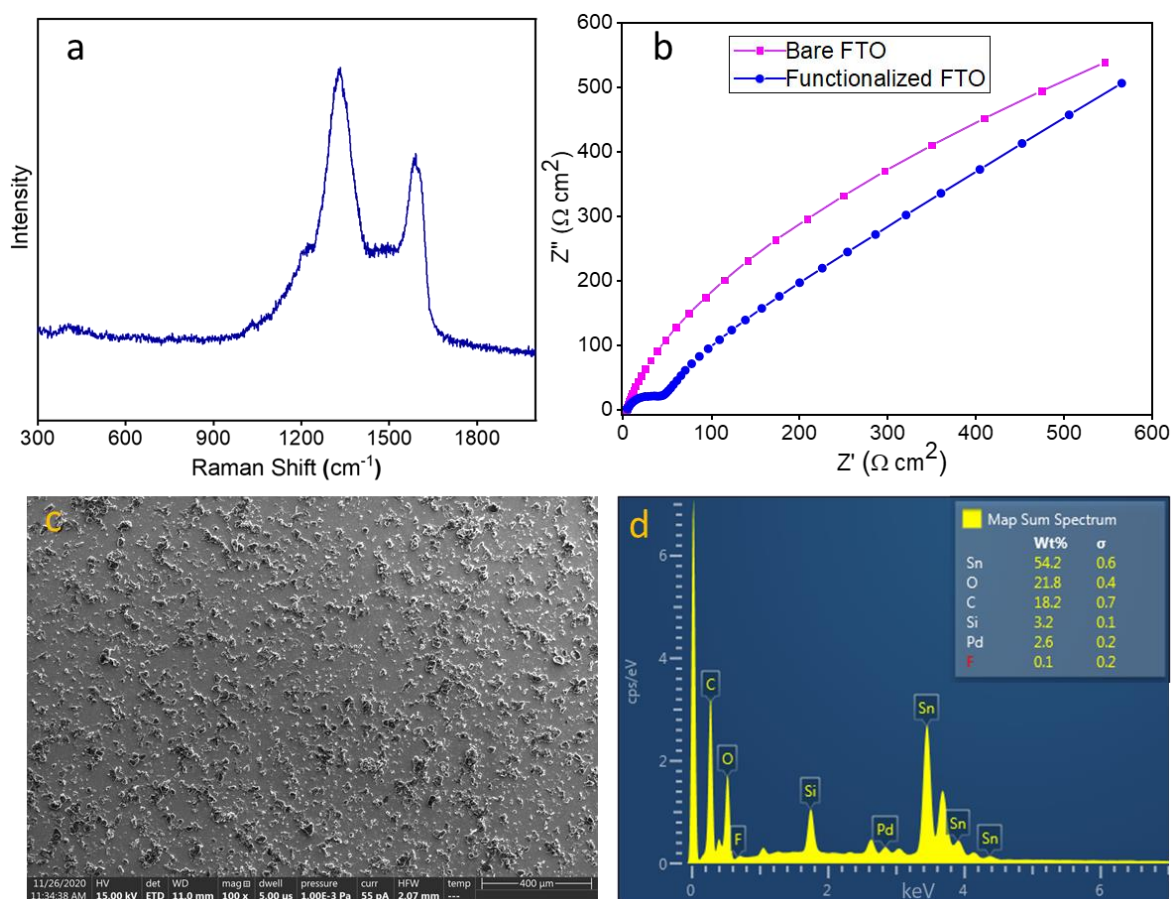


Figure 7. 3 (a) Raman spectrum of Pd-HRG functionalized FTO, (b) EIS of bare and Pd-HRG functionalized FTO, (c) FESEM image of the Pd-HRG functionalized FTO, and (d) EDX spectrum of the Pd-HRG functionalized FTO.

7.4.2 SERS and EC-SERS based detection

The apparent augmentation in the electrical and plasmonic activity of the fabricated palladium nanocomposite was the main motivation to investigate their spectroscopic behavior as potential SERS substrates. Although the catalytic activity of Pd-based materials is well documented [303,304], their LSPR properties for the sensing of therapeutic drug have not been explored. We have now studied Raman, SERS and EC-SERS analyses of procaine and assessed its interaction with the Pd-HRG fabricated substrate. The Raman spectra of 0.1 M procaine solution with and without the SERS and EC-SERS substrates were optimized and collected in the range 400-2000 cm^{-1} , as described

in Fig 7.4. The laser excitation wavelength (λ_{ex}) of 633 nm was maintained, while the sample was focused with a 50x microscopic lens. A circular cuvette with a diameter of 2 cm and depth 1 cm was used to hold the solution under the Raman microscope.

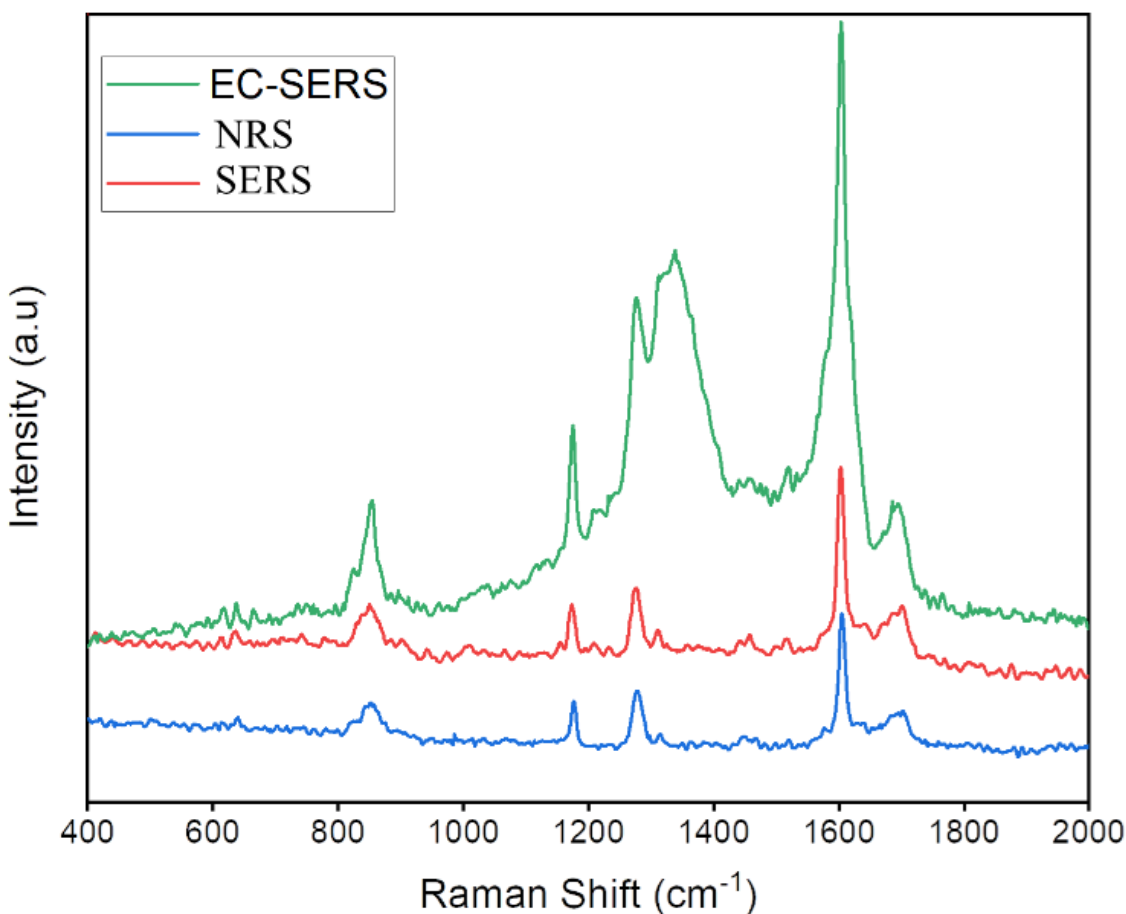


Figure 7. 4 Raman spectra collected for 0.1 M procaine solutions using different techniques. The normal Raman spectrum (blue) was recorded at $\lambda_{\text{ex}} = 633$ nm, $t_{\text{acq}} = 20$ sec, $P_{\text{ex}} = 8.5$ mW, 50 $\times$ LWD objective, the SERS spectrum (red) was recorded at $\lambda_{\text{ex}} = 633$ nm, $t_{\text{acq}} = 20$ sec, $P_{\text{ex}} = 8.5$ mW, 50 $\times$ LWD objective with 1:1 volume ratio of procaine solution and Pd-HRG suspension, and the EC-SERS spectrum (green) was recorded at $\lambda_{\text{ex}} = 633$ nm, $t_{\text{acq}} = 20$ sec, $P_{\text{ex}} = 8.5$ mW, 50 $\times$ LWD objective, potential = -0.1V and pre-concentration time=400 sec.

The 0.1 M procaine / Pd-HRG solution SERS spectrum (Fig 7.4) was collected at 50% laser power, and the concentration dependent SERS spectra of procaine solutions of a wide range of concentrations adsorbed onto the surface of Pd-HRG nanocomposite is depicted in Fig 7.5a. Focusing on the most prominent Raman peak appearing at 1610 cm^{-1} , the linear dynamic range of the SERS intensity response vs. different procaine logarithmic

concentration solution was established and observed within the range of 10^{-2} - 10^{-7} M, with a correlation coefficient $R^2 = 0.9936$ and limit of detection of 10^{-8} M on the basis of $S/N = 3$ (Fig 7.5b). In addition to the enhancement of the peak intensity described above, the interaction of procaine molecules with the Pd-HRG nanocomposite resulted in the frequency shifts in Raman band positions. The Raman bands at 490, 640, 858, 1181 and 1518 cm^{-1} were red-shifted to 493, 646, 860, 1184 and 1528 cm^{-1} , respectively. On the other hand, bands at 913, 1073, 1316, 1468, 1611 and 1701 cm^{-1} were blue-shifted to 902, 1069, 1313, 1460, 1610, 1701 cm^{-1} , respectively (Table 7.2).

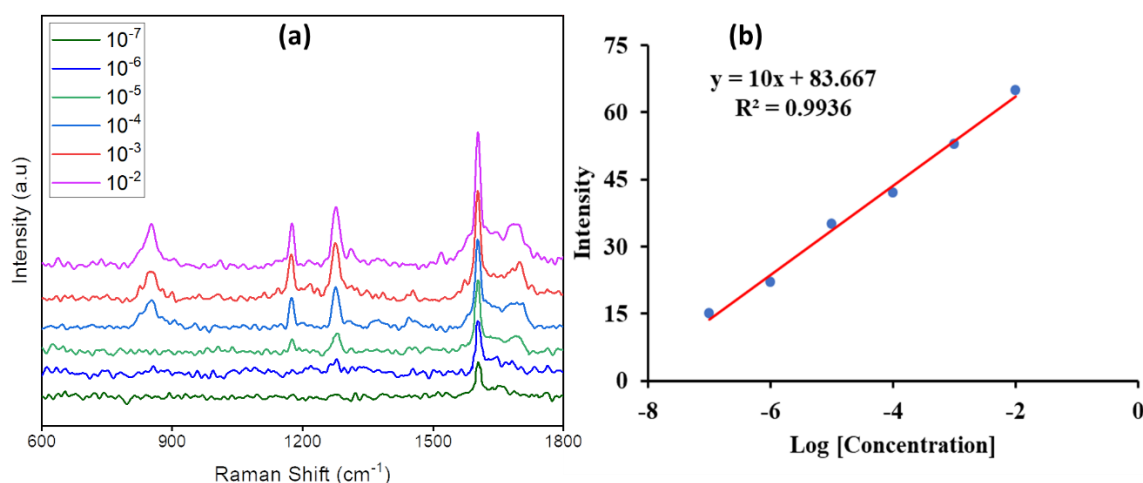


Figure 7. 5 (a) SERS spectra of different procaine concentration solutions with Pd-HRG substrate at $\lambda_{\text{ex}} = 633\text{ nm}$, $t_{\text{acq}} = 20\text{ sec}$, $P_{\text{ex}} = 8.5\text{ mW}$, $50\times$ LWD objective with 1:1 volume ratio of procaine solution and Pd-HRG suspension and (b) The linear response of SERS response vs different concentration of procaine solutions at band 1610 cm^{-1} .

For the integration of electrochemical technique in the Raman measurements, a three-electrode system was developed in an EC-SERS cell with a dimension of $1.5\text{ cm} \times 1\text{ cm} \times 1\text{ cm}$. The functionalized FTO substrate was used as a working electrode which shows D and G bands of graphene. These graphene bands gave higher background signals within the range of 1000 to 1700 cm^{-1} of the EC-SERS spectrum. The Raman band located at 858

cm^{-1} was, therefore, selected to investigate EC-SERS analytical performance of the Pd-HRG functionalized FTO substrate and compared it with the SERS method. It was observed that EC-SERS method showed a substantial enhancement in the Raman signals of procaine with Pd-HRG nanocomposite, irrespective of its background noise. The pre-concentration of the procaine analyte on the Pd-HRG surface was induced with the help of linear stripping voltammetry. The pre-concentration potential (Fig 7.6a) was optimized by sweeping the cathodic potential from 0.05 to 0.20 V, and a strong Raman enhancement was achieved at 0.1 V. The surface of electrode was not enough negative to attract the negatively charged procaine ions producing a weak Raman signal. On increasing the more negative potential, the more procaine molecules are reduced on the surface of electrode and optimum potential (-0.1 V) was achieved and further potential increase does not affect the Raman signal significantly. However, selecting a more negative potential results in background hydrogen evolution which interferes with the signal of procaine. The optimized pre-concentration time (Fig 7.6b) was also examined by sweeping the time between 200 sec to 800 sec. The optimum SERS response was achieved at 600 sec. Remarkable enhancements in the Raman intensity induced by electrochemical optimization were observed in several Raman bands.

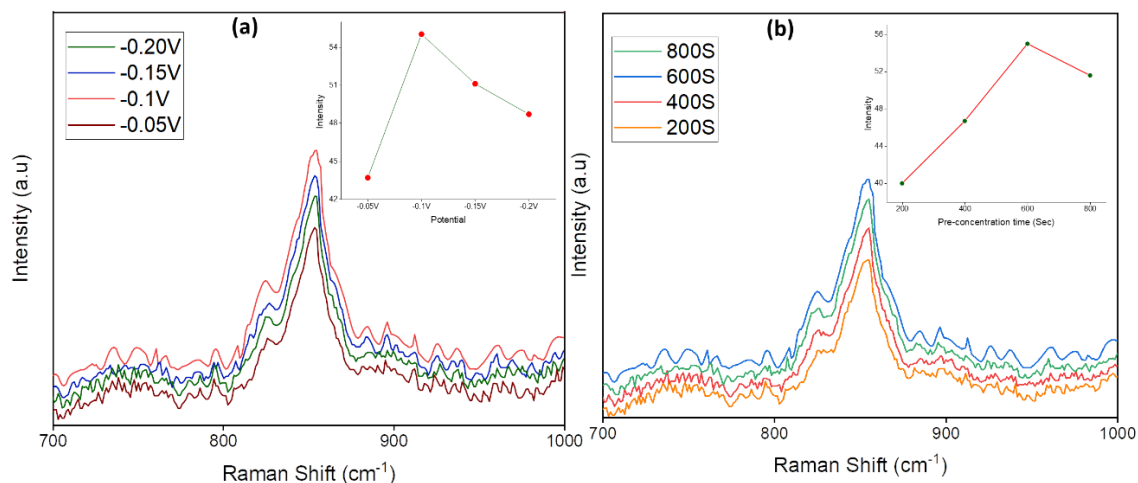


Figure 7. 6 (a) Optimization of EC-SERS response for pre-concentration potentials at band 858 cm⁻¹, (b) Optimization of EC-SERS response of pre-concentration time at band 858 cm⁻¹.

It is pertinent to mention that to achieve a desirable signal-to-noise ratio in the Raman response due to fluorescence could be challenging. Therefore, the SERS substrate that has a low background of fluorescence signal is preferred, which can withstand these fluctuations. Two-dimensional materials have been reported in improving the signal to noise ratios [305,306]. Among them, graphene is prominent as it can quench the fluorescence, which can ultimately improve the efficacy of the analyte detection [307]. Such substrates can be employed to form an electrochemical double layer at the surface of the substrate, which in turn enables modulating electrical interaction between the analytes and substrates.

In addition, the combining effect of applying the electrical potential and the interaction of procaine with Pd-HRG nanocomposite was observed in the change in Raman line positions. The Raman peaks at 495, 642, 863, 1186, 1287, 1526 and 1708 cm⁻¹ were red shifted from their original positions of 490, 640, 858, 1181, 1285, 1518 and 1705 cm⁻¹. Similarly, peaks

at 908, 1070, 1313, 1462 and 1609 cm^{-1} were blue shifted from 913, 1733, 1316, 1468 and 1611 cm^{-1} , respectively (Table 7.2). A linear dynamic range of 10^{-1} - 10^{-6} M was observed for the EC-SERS response with a $R^2 = 0.9953$ correlation coefficient and a limit of detection of 10^{-7} M on the basis of $S/N = 3$ (Fig 7.7).

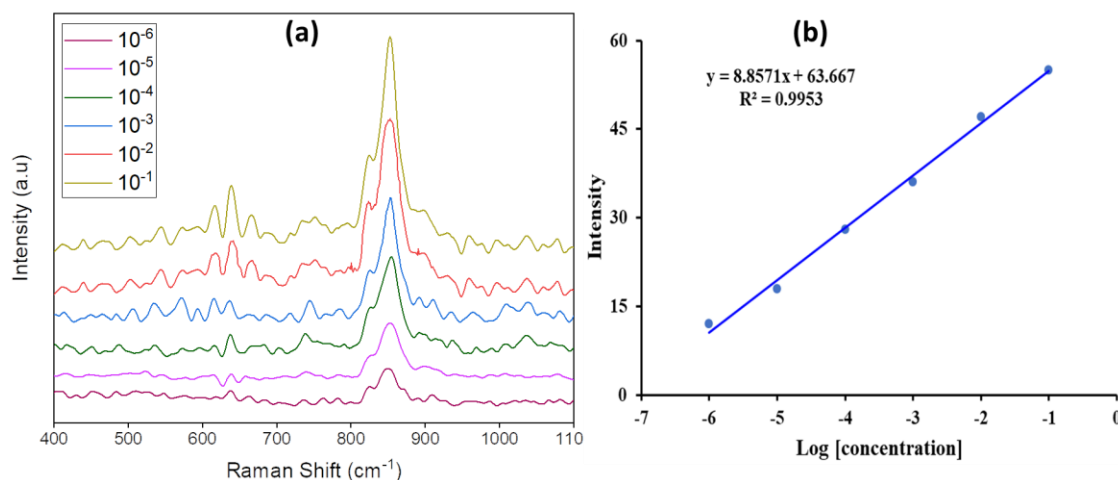


Figure 7. 7 (a) EC-SERS spectra of different procaine concentration soln on Pd-HRG modified FTO electrode recorded at $\lambda_{\text{ex}} = 633$ nm, $t_{\text{acq}} = 20$ sec, $P_{\text{ex}} = 8.5$ mW, $50\times$ LWD objective, potential = -0.1V and pre-concentration time = 400 sec. (b) The linear response of EC-SERS response vs different concentration of procaine solutions at band 858 cm^{-1} .

In order to present the best comparison between the SERS and EC-SERS performance using Pd-HRG nanocomposite, the Raman peak of procaine drug at 858 cm^{-1} was selected. That Raman peak is located outside the spectral range of background signals observed in the EC-SERS analysis. The two key parameters, enhancement factor (EF) and limit of detection (LOD), were compared for SERS and EC-SERS. The Raman enhancement factor was calculated using a well-established approach [308], and detailed explanation can be found in the supporting information. The limit of detection (LOD) was evaluated on the basis of $S/N = 3$ [309]. The EF and LOD for SERS were found to be 8.7×10^2 and 10^{-4} M, respectively, compared to 7.5×10^4 and 10^{-7} M for the EC-SERS method. The value of EF and LOD confirmed that the incorporation of the electrochemical pre-concentration factors

provides a significant enhancement and a lower detection limit. The proposed Pd-HRG nanocomposite-based SERS substrate exhibited a promising candidate for the use in characterization and analysis of organic based therapeutic drugs.

7.4.3 Stability and Reproducibility

To assess Pd-HRG nanostructured substrate stability in terms of analytical performance, Raman band of 0.1 M procaine at 858 cm^{-1} was chosen and 28 days stability of EC-SERS signal was tested as given Fig 7.8a. It is noticed that over the 28-day period, 5.73% decrease in analytical EC-SERS signal is dropped which indicates that EC-SERS Pd-HRG substrate is good stable substrate of EC-SERS analysis.

The Reproducibility of proposed analytical method based on EC-SERS was evaluated by adopting all the preparation steps (different batches and days) such as preparation of conductive surface electrode, its fabrication and EC-SERS analysis except the synthesis of Pd-HRG. The SERS substrate prepared in different batches was examined by conducting EC-SERS measurements of 0.1 M procaine solution and Raman band (858 cm^{-1}). EC-SERS measurements of Pd-HRD substrate presents reproducible results with 2.45% RSD as illustrated in Fig 7.8b.

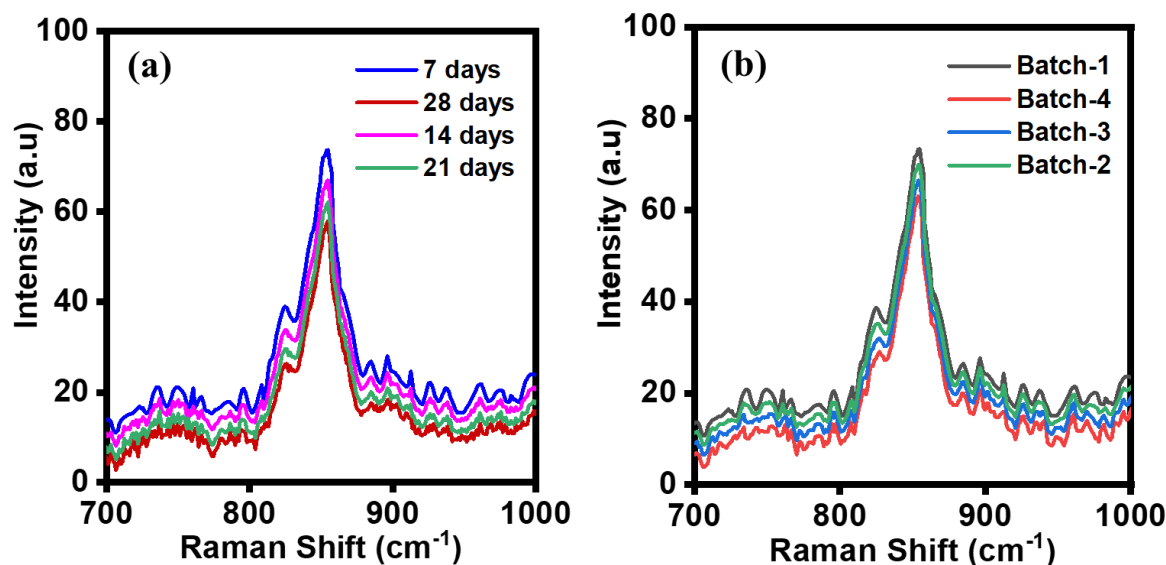


Figure 7. 8 EC-SERS measurements of 0.1 M procaine solution at Raman band 858 cm^{-1} for (a) Pd-HRG stability for 28 days and (b) reproducibility of developed methods.

7.4.4 Analysis of real urine sample

The application of the proposed method was evaluated by adding procaine in urine sample. A urine sample of a healthy man was collected, centrifuged, and filtered by using $0.2\text{ }\mu\text{m}$ pore size syringe filter. The 1mM solution of procaine solution was spiked in filtered urine sample and subjected to EC-SERS measurements as indicated in Fig 7.9. The urine SERS spectrum consist of three peaks at 665 , 742 and 1002 cm^{-1} which are attributed to the $\text{O}=\text{C}-\text{N}$, creatinine structure and $\text{C}-\text{N}$ stretching respectively [310,311]. The procaine drug was added to the urine to prepare 1mM solution and subjected to electrochemical SERS analysis. A new peak appeared at 563 cm^{-1} that indicates procaine. The proposed SERS method produced an average procaine recovery of $94.4\% \pm 6.25\%$. The procaine recovery values indicate that EC-SERS method presented highly reliable results to meet the requirements of practical application.

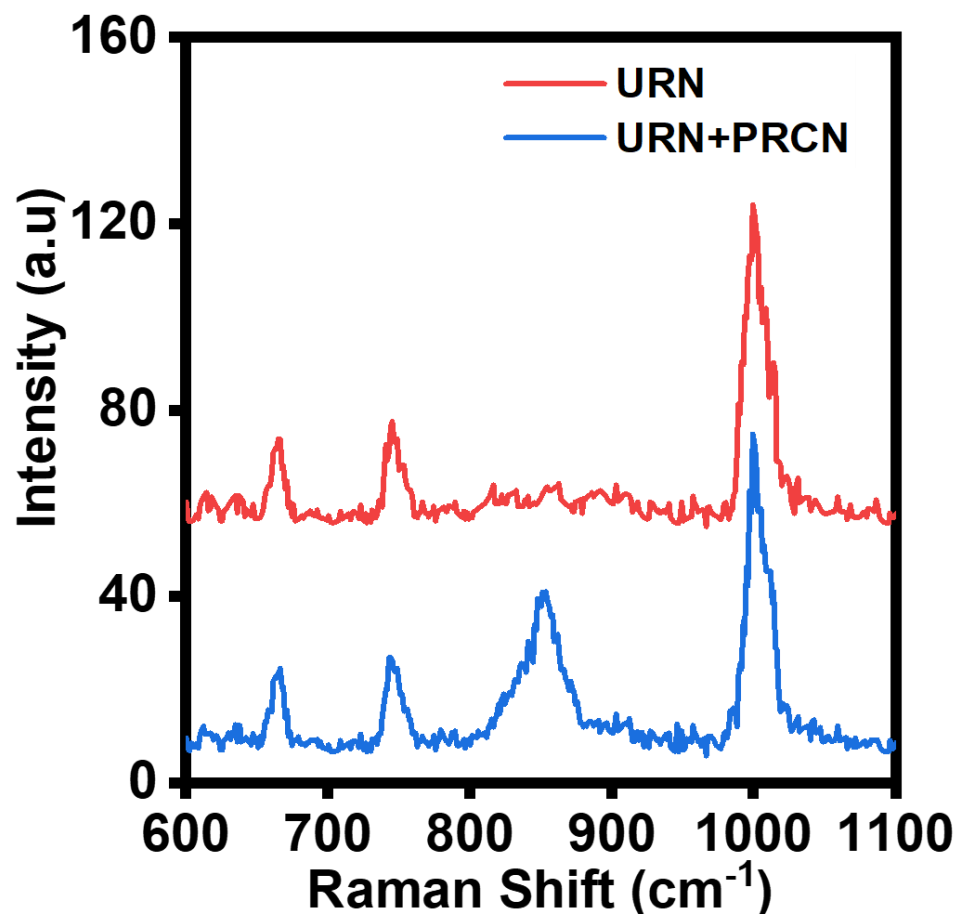


Figure 7.9 Figure 46: EC-SERS measurements of urine (red) and procaine added urine (blue).

7.4.5 Molecular modeling study

Further insight into the possible adsorption modes of procaine with Pd-HRG nanocomposite was made with density-functional theory (DFT) calculations. The modes of interaction were based on the computed molecular electrostatic potential map (MESP) of the procaine molecule. The interacting models of graphene-Pd...NH₂ and graphene-Pd...O=C were presented in Fig 7.10. The associated binding energies, binding distances, and the Mulliken charges of these two possible interactions are listed in Table 7.1. The

Pd...O=C and Pd...NH₂ distances were calculated as 2.276 and 2.339 Å, respectively, whereas the computed binding energy values of the corresponding interactions were found to be -4.897 and -5.230 kcal/mol. These values suggested that the interaction between the Pd-HRG and procaine is mainly via the NH₂ moiety, which played a major role in the enhancement of Raman peaks. Charge transfer analysis between the analyte molecule and graphene nanocomposite supported the chemical enhancement mechanism of SERS.

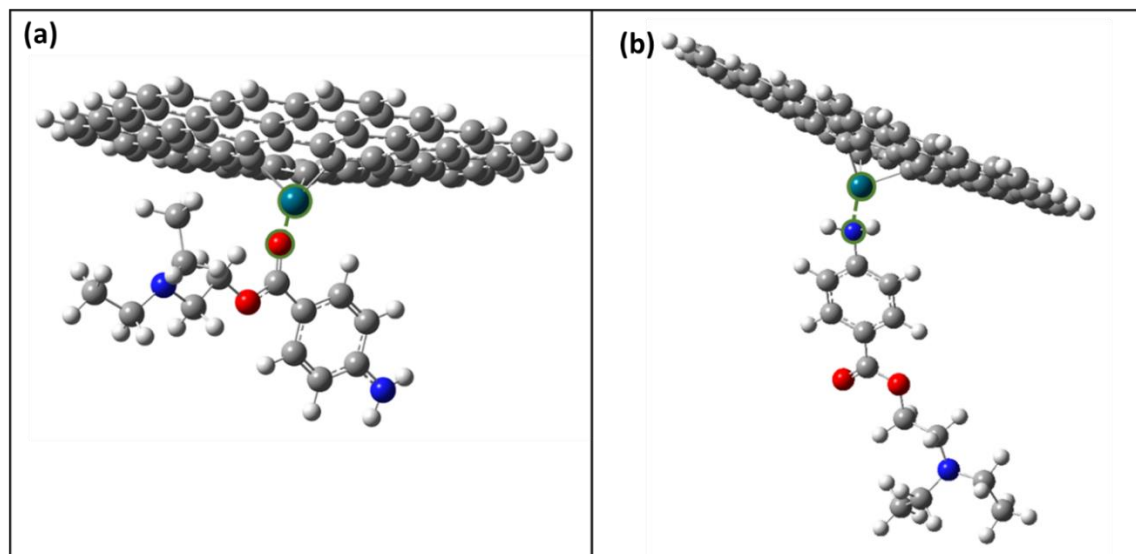


Figure 7. 10 DFT-modelled interactions of a procaine molecule via (a) HRG-Pd...C=O mode and (b) HRG-Pd...NH₂ mode.

Table 7. 1 Binding distances, binding energies and the change in the sum of Mulliken charges for the two possible interactions calculated at the B3LYP/6-31G(d,p) level of theory.

Interaction types	Binding Distance (Å)	Binding Energy (kcal/mol)	Mulliken Charges	
			Free	Upon interaction
C=O...Pd-HRG	2.276	-4.897	Pd-HRG: 0.0000	Pd-HRG: -0.2332
				Procaine: 0.2332
H ₂ N...Pd-HRG	2.339	-5.230	Procaine: 0.0000	Pd-HRG: -0.1907
				Procaine: 0.1907

The conclusive vibrational assignments of Raman, SERS and EC-SERS bands were carried out based on DFT results. Prominent Raman peaks of procaine were observed at 490, 640, 858, 913, 1073, 1181, 1285, 1316, 1468, 1518, 1611 and 1705 cm^{-1} which were attributed to NH_2 wagging, CH bending, ring deformation, CH bending, C-O stretching, CH bending, CH_2 twisting, CH rocking, CH wagging, ring stretching, HN_2 bending and C=O stretching, respectively. The Shifting of Raman bands was also observed in SERS and EC-SERS spectral profiles (Table 7.2) instigated by the interaction between procaine molecule and Pd-HRG nanocomposite and electrical potential effect, respectively.

Table 7. 2 Assignments of the vibrational modes in the SERS and EC-SERS as compared with the normal Raman (NR) spectra for procaine.

Sr. No.	EC-SERS	SERS	NR	Assignments
1	495	493	490	NH ₂ wagging
2	642	646	640	CH bending
3	863	860	858	Ring Deformation
4	908	902	913	CH bending
5	1070	1069	1073	C-O stretching
6	1186	1184	1181	CH bending
7	1287	1285	1285	CH ₂ twisting
8	1313	1313	1316	CH rocking
9	1462	1460	1468	CH wagging
10	1526	1528	1518	Ring stretching
11	1609	1610	1611	NH ₂ bending
12	1708	1701	1705	C=O stretching

CHAPTER 8

The role of gold-loaded copper oxide circular nanoplates in the formulation of sensitive EC-SERS substrates for the detection of anti-cancer drugs: Spectro-electro and DFT studies

8.1 INTRODUCTION

Hydroxycarbamide (HC), a derivative of urea, is a non-alkylating antineoplastic agent used in the treatment of a wide range of tumours, including breast, gastric and intestinal cancers, as well as bladder and colorectal cancer, in addition to melanoma, hepatocellular carcinoma and myeloproliferative diseases [29–31]. Its most accepted action mechanism is to inhibit the synthesis of DNA by affecting the cell cycle in a reversible manner [30]. However, the adverse countereffects of HC are obvious due to its high toxicity towards normal cells in addition to the rapid impairment of spermatogenesis in males. Furthermore, the HC drug faces a low dissolution capacity problem and low efficacy in vivo tests [32,33]. These detrimental impacts could be reduced by developing a sensitive platform which has the potential of monitoring trace HC existence. For such reasons, a wide variety of analytical methods have been established and are being used for qualitative and quantitative detection of the drug. The previously used colorimetric methods to characterize HC contents were later supplanted by separation and spectroscopy techniques,

such as high-performance liquid chromatography (HPLC), LC-MS/MS, gas chromatography with mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) and others [312–314]. Since the structure of hydroxycarbamide comprises electrostatically active groups, electrochemical methods could be an attractive approach for its trace level determination [315]. Electrochemical sensors could be modified with specific coating of the working electrode to develop a built-in selectivity toward target molecules of interest [316].

Although Raman spectroscopy is a well-established detection tool for different types of biofluids and known for its non-destructive, reagent-free and rapid analysis advantages [317–320], its effective utilization is sometimes hindered due to the weak nature of its scattered light. The detailed Raman fingerprints of target analytes [321–323] can be made more measurable using the surface enhanced Raman scattering (SERS) module, in which the intensities of normal peaks are several-fold improved with short acquisition time by simply employing a nanostructured metal surface. The signal enhancement occurred as a consequence of the interaction between the laser beam and the electrons of the metallic surface through activating the surface plasmons [183,287,294,324,325]. For this reason, the synthesis of nanostructured materials with tuned photoelectric properties is a crucial step in the successful development of SERS sensing platforms [116,326]. Another important parameter to be screened is the composition of the nanomaterial substrates leading to an adequate SERS and electrochemical-SERS response [327].

Desired optical properties can be also achieved by combining two different plasmonic metals in the bimetallic nanostructured material [328]. Semiconductor oxide materials, such as TiO_2 , CuO_2 and ZnO , were shown to contribute to the Raman enhancement factor

(EF) up to 10^6 [329]. It has been recently found that semiconductors with transition metals, mainly Au and Ag, exhibit a strong Raman response by inducing the charge transfer at their interface [330]. A significant effort has been made towards the design of semiconductor-noble metal combinations (e.g. ZnO/Ag, TiO₂/Ag and CuO/Ag) for the improvement of SERS activity [331–333]. Copper oxide is a well-known semiconductor with a narrow band gap and is used in diverse practical areas due to its unique electrical and optical characteristics [334]. The gold coating on copper oxide provides chemical stability and strong plasmonic output. An ultrasensitive analytical protocol is still required to gain the desired enhancement in the Raman signals corresponding to a lower limit of detection.

In this work, a simple, quick one-pot synthesis method was developed to design gold coated copper oxide (Au-CuO_x) circular nanoplates (CNPs) which were then employed for the electrochemically induced Raman spectroscopic detection of the anti-cancer drug hydroxycarbamide (HC). As pointed out earlier, the morphology of the nanostructured substrate is crucial in terms of SERS analysis and can provide hot spots that stimulate the charge transfer driven Raman enhancement. The degree of contribution of the charge transfer to Raman signal enhancement can be also calculated. First-principles molecular modelling was implemented to establish a fundamental insight on the evolution of the SERS phenomenon associated with the interaction and the possible role of the electron transfer between the HC molecules and CNPs.

8.2 METHODOLOGY

8.2.1 Chemical and reagent

Hydroxycarbamide (NH_2CONHOH , Purity 98%), copper nitrate ($\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$, Purity > 99.8 %), gold chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, Purity > 99.8 %), phosphate buffer solution (pH=7), and potassium chloride (KCl, Purity > 99%) were obtained from Sigma-Aldrich. Fluorine doped tin oxide (FTO) conductive glass was purchased from SOLARONIX Switzerland, while sodium hydroxide (NaOH, Purity > 99.6 %), 2-propanol (Purity > 99.5%), ethanol (Purity > 99 %), polyvinylpyrrolidone (PVP, MW= 40000 g/mol), potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$, Purity > 99%) were purchased from Merck. Hydrazine hydrate ($\text{H}_4\text{N}_2 \cdot \text{H}_2\text{O}$, 35%) and Sodium borohydride (NaBH_4 , Purity 98%) were obtained from Fisher Scientific. All chemical reagents were used as received without further purification.

8.2.2 Synthesis of Au coated copper oxide CNPs

The CuO_x nanoplates were synthesized by modifying the existing Cu_2O sphere method reported by Pang et al. [335]. In this modified method, PVP was used as a shape directing and capping agent, and hydrazine hydrate was used as a reducing agent into the alkaline copper ion solution in 2-propanol. Typically, 1.0 mM solution of $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ was prepared in 120 mL 2-propanol solvent and then 0.5g of PVP was added. A 1mM-NaOH solution was added and stirred for 30 minutes, and colour has changed from blue to green. After, 4.0 mL of $\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$ (35%) was added dropwise in the above reaction mixture, and the color of the reaction mixture turned into dark brown. The dark brown copper oxide nanoplates were coated with gold using a wet chemical process. That is 3 mL of 0.6 mM

aqueous solution of HAuCl_4 was added, the reaction mixture was stirred for 20 minutes, then 3 mL of 5 mM $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was added dropwise while stirring for a further 30-minute duration. Black precipitates formed and were centrifuged. The collected black product was washed with DI water and ethanol. The product was dried at room temperature for 12 hours, then for 2 hours in oven at 40°C .

8.2.3 Preparation of the electrochemically active FTO electrode

The FTO coated glass having a surface resistivity of 7 ohm/sq. was cut into 1 cm^2 square shaped pieces to make it fit in the electrochemical-SERS (EC-SERS) compartment cell. The glass substrate was thoroughly cleaned with acetone, isopropanol and deionized water, put in an ultrasonic treatment at 40°C , and dried at 50°C for 20 minutes prior to the fabrication of Au-CuO_x nanoplates [99]. For the fabrication of the FTO substrate, a suspension of Au-CuO_x CNPs was prepared by dispersing 12 mg nanomaterials in 5 ml deionized water and subjected to ultrasonication for 20 minutes. A drop casting method [100] was used to fabricate the well-cleaned FTO substrate by casting 50 μL suspension of nanoplate materials uniformly on the conductive surface and then dried it in air. The fabricated FTO substrates were used for electrochemical characterization and EC-SERS analysis.

8.2.4 CNPs characterization and spectra acquisition

The Au-CuO_x CNPs were characterized by several spectroscopic and imaging techniques. The samples for FE-SEM analysis were prepared using a conductive carbon tap on a special conductive sample holder. The aqueous solution of nanospheres was drop-casted on the carbon tap and allowed the samples to dry in air. Fourier transform infrared (FT-IR) spectra were acquired using Thermo Scientific NICOLET 6700 spectrometer at a resolution of 4

cm⁻¹ with 64 scans. The sample pellets for the ir analysis were prepared by mixing the sample with KBr. The X-ray diffraction (XRD) analysis was performed on Rigaku Ultima IV diffractometer using copper K- α radiation source. The X-ray radiation was generated at a power 40 kW and 30 mA, and the scanning speed was set at 1.0 degree/min. The recorded diffractograms were interpreted using HighScore software. The X-ray photoelectron (XPS) spectra were collected using Scientific ESCALAB 250X spectrometer equipped with a monochromatic magnesium K- α source and helium discharge lamp. The samples were prepared by depositing aliquots of the sample solution on the clean glass slide at room temperature and then air-dried. The electrochemical characterization of the working Au-CuO_x/FTO electrode was carried out by using Gamry workstation three-electrode system. The collected electrochemical spectra were processed using Gamry Echem Analyst. Raman analysis was conducted using 633 nm excitation source LabRAM HR evolution microscope fitted with an electrical temperature-controlled CCD camera and He-Ne laser having power 17 mW with two auto switched diffraction gratings 600 and 1800. Raman and SERS spectra were recorded at 20 sec acquisition time, 50% laser power, and number of scans of 8, while 50x lens of microscope was used to focus the sample. The ratio of 1:1 of drug and nanomaterials was managed to collect SERS spectra. The EC-SERS spectra were acquired using the three-electrode portable workstation system and under the same Raman spectrophotometric conditions.

8.3 QUANTUM AND CHEMICAL CALCULATION

All calculations were carried out using Gaussian 09 program [101] in order to establish some understanding behind the enhancement mechanism. The density functional theory (DFT) approach was employed with the hybrid B3LYP functional and the 6-311g+(d,p)

basis set for H, O, C, N atoms and SDD for metal atoms. Such a cost-effective theoretical level was chosen to produce reasonable results compared to experimental findings [336,337]. The geometry of HC was optimized, and its harmonic frequencies were computed. As Au-CuO_x represents a mixture of Cu(I) and Cu(II) based materials, two stable models of Au-CuO and Au-Cu₂O nanoclusters were constructed and fully optimized. The frequency calculations were performed to confirm the optimization convergence and to assign the vibrational modes of wavenumbers for the target drug molecule. Frequency calculation was also performed with *C_s* symmetry constraint in order to identify the more likely totally and non-totally symmetric modes which correspond to *A'* and *A''* irreducible representations, respectively, of the drug molecule. Furthermore, the adsorption of HC on the Au-CuO_x models was investigated at the same level of theory, and the relevant adsorption energies and magnitude of charge transfer were obtained.

8.4 RESULT AND DISCUSSION

A morphological analysis of the gold coated copper oxide (Au-CuO_x) was conducted (Fig. 8.1). The synthesized materials possessed a circular nanoplate (CNP) like structure as revealed from the SEM images collected at different magnifications (Fig 8.1b). The phase composition and types of crystallinities of Au-CuO_x were explored using the XRD analysis, and the diffractogram of the prepared circular nanoplates is depicted in Fig 8.1c. The characteristic diffraction peaks of gold were located at 44.5°, 64.6° and 77.6°, representing the Miller indices (200), (220) and (311), respectively. The diffraction peak of CuO (*111*) appeared at 36.3°, while those of the AuCu corresponding to (*111*) and (*311*) facets were observed at 38.2° and 81.8°, respectively. The Cu₂O peaks with Miller indices (*210*) and (*400*) were located at 29.5° and 61.2°. The XRD band associated to copper was located at

73.6° diffraction angle. Some XRD peaks of CuO (111, 200) at 35.7° and 41.6 ° and Cu₂O at 76.8° were superimposed under the Au diffraction peaks, which is evident of the gold being successfully coated over the copper oxide. Further, the infrared bands of metal oxide intra-atomic vibrations generally appear in the region below 1000 cm⁻¹. The formation of Au-CuO_x was confirmed by the stretching vibration of Cu-O absorbing at 584 cm⁻¹, while the absorption band at 514 cm⁻¹ could be attributed to the Au-O vibration (Fig 8.1d).

More idea about the chemical composition and oxidation states of Cu, Au and O present in the synthesized circular nanoplates could be obtained from the XPS data (Fig 8.2). The Cu 2p spectrum shows two peaks at 933.2 and 953.3 eV attributed to the p_{3/2} and p_{1/2} states, respectively. The broadness of these two peaks hints the presence of both Cu⁺ and Cu²⁺ states in the synthesized nanomaterials [338,339]. The triplet satellites peaks observed at 942, 945, 963 eV binding energies are ascribed to the CuO form [340,341]. The spectrum of Au 4f (Fig 8.2b) contains two peaks observed at 84.9 and 88.1 eV, which can be attributed to Au 4f_{7/2} and Au 4f_{5/2} states, respectively. These are the characteristics of the gold nanoparticles in the metallic state with zero oxidation state Au⁰ [342]. The O 1s spectrum (Fig 8.2c) is composed of two different types of O species which are attributed to lattice oxygen atoms in the Cu₂O/CuO metal oxide combination.

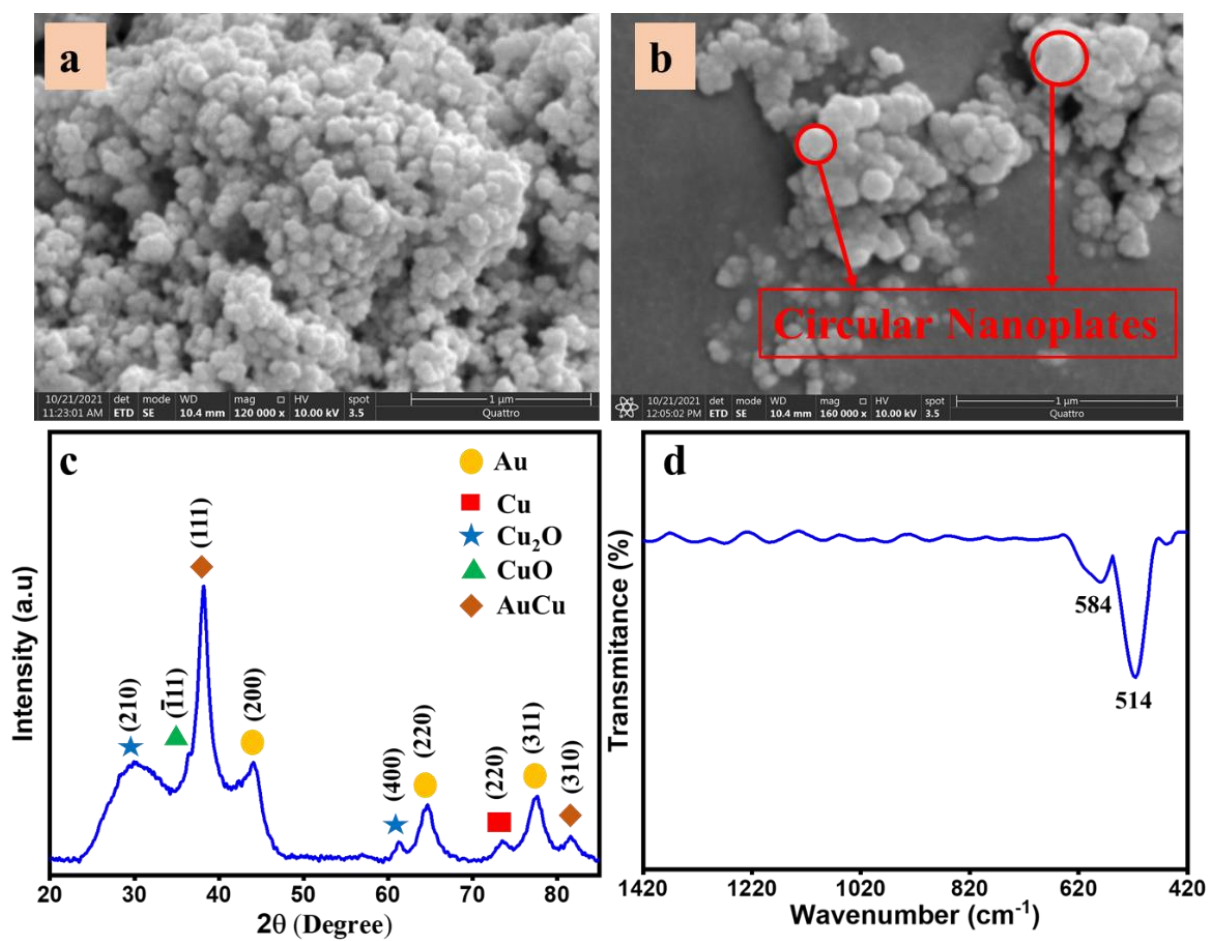


Figure 8. 1 Characterization of the prepared gold coated CuO_x nanoplates; (a & b) depict the SEM images at different magnifications, (c) shows the x-ray diffraction pattern, while (d) shows the FT-infrared spectrum of the nanomaterials.

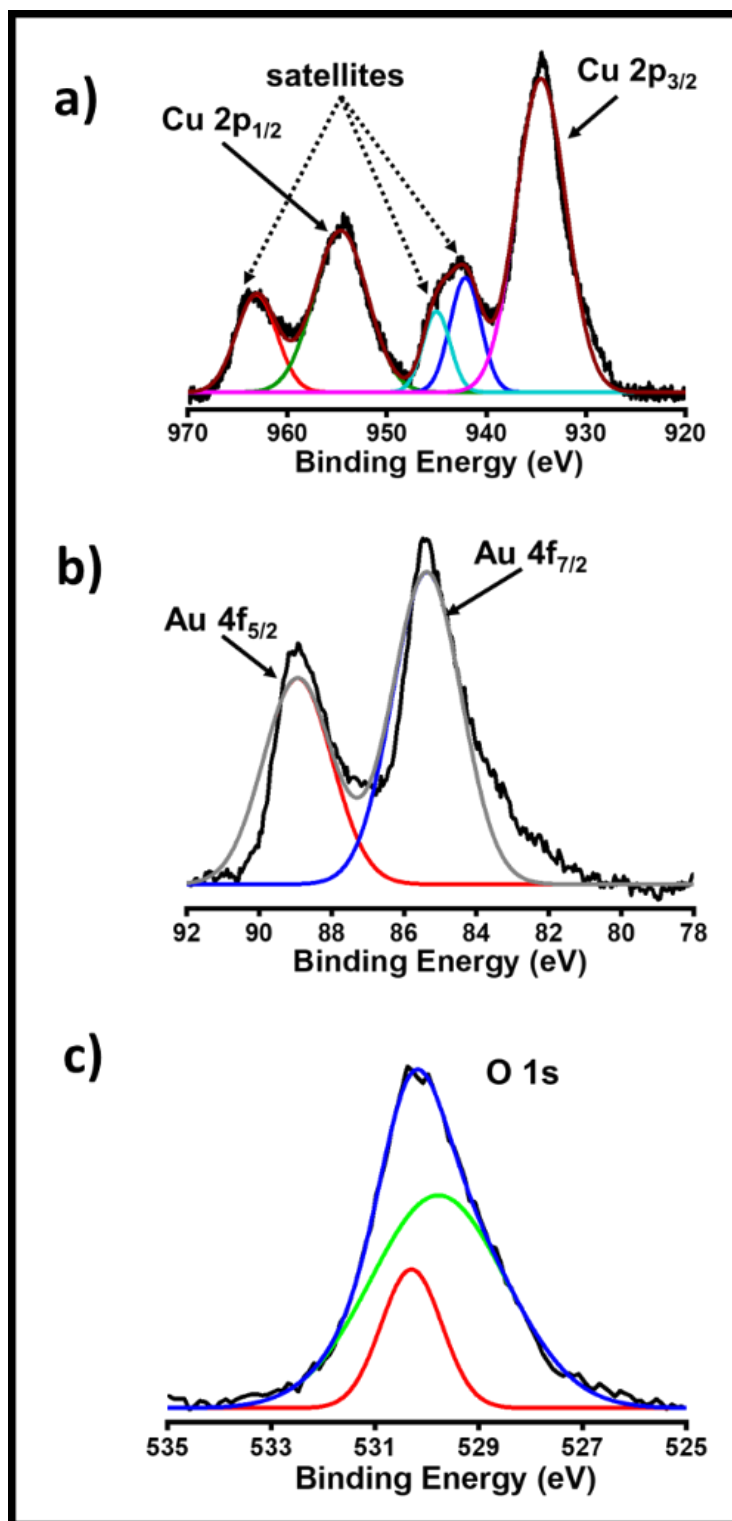


Figure 8. 2 XPS spectra and their deconvoluted components describing the (a) Cu 2p, (b) Au 4f, and (c) O 1s levels in the Au-coated CuO_x nanoplates.

The electrochemical activity of the fabricated FTO was examined using a single-compartment electrochemical cell containing three electrodes: Pt wire as a counter electrode, Ag/AgCl as a reference electrode, and FTO substrate as a working electrode. The cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were employed to compare the fabricated and non-fabricated glass in a phosphate buffer solution containing 5.0 mM potassium ferricyanide as an electrochemical probe and 1.0 M potassium chloride as a supporting electrolyte. The EIS measurements were performed at an applied potential of 0.42 V in the frequency range of 100000-0.1 Hz at 10 mV rms amplitude. The cyclic voltammograms (Fig 8.3a) were recorded using bare and functionalized substrates as working electrodes in the presence of $K_3[Fe(CN)_6]$ redox probe at 80 mV/sec scans rates. The increase in anodic and cathodic peak currents and the slight decrease in the peak potential separation were both noticed in the case of the modified electrode, which indicates that an electron transfer activity was favorable at the proposed fabricated FTO electrode. The EIS spectra (Fig 8.3b) recorded in the redox probe showed a faster electron transfer process for the CNPs/FTO with a charge transfer resistance (R_{ct}) of 1.1 k Ω , while the bare substrate encountered a lower magnitude of charge transfer with R_{ct} value of 6.2 k Ω . It confirms that Au-coated CuO_x nanoplates employed for the fabrication of the electrode significantly increased the electrochemical activity, reinforcing the CV findings.

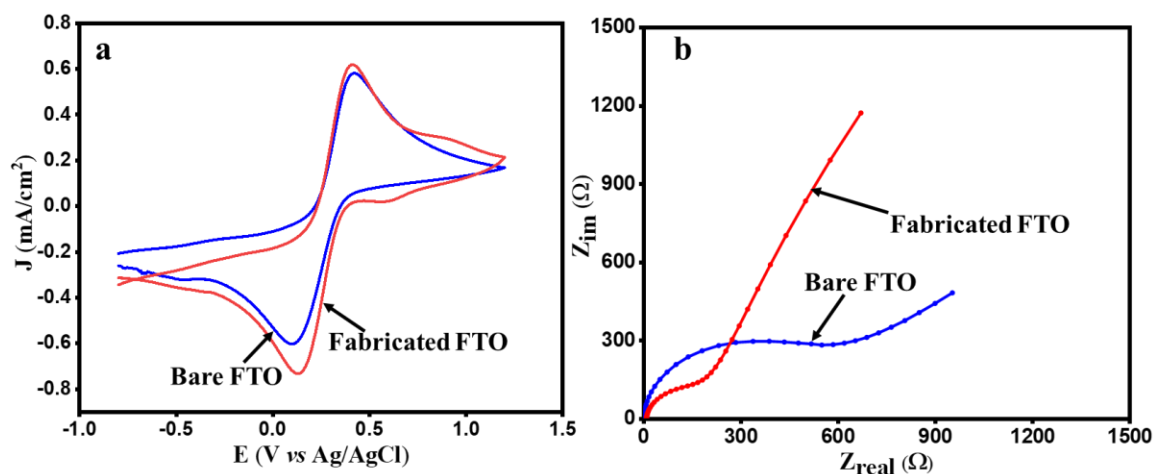


Figure 8. 3 (a) Cyclic voltammograms, and (b) electrochemical impedance spectra obtained for the bare FTO (blue) and the Au-CuO_x CNPs/FTO (red) electrodes in a phosphate buffer solution containing 5.0 mM K₃[Fe(CN)₆] and 1.0 M KCl.

The electrochemical-SERS (EC-SERS) investigation was carried out in a cell containing a three-electrode system. The in-situ electrochemical preconcentration of the anti-cancer drug hydroxycarbamide (HC) on the SERS substrate was optimized in terms of electrical potential and time to obtain best possible response at the Raman peak centered at 977 cm⁻¹. The oxidation of HC was reported at 0.9 V vs. Ag/AgCl [343,344], therefore, the preconcentration potential for HC was optimized by selecting 0.8, 0.9, 1.0 and 1.1V potential. The Raman optimum response (Fig 8.4a) was achieved at 0.9 V which is consistent with the literatures [345,346]. The preconcentration time at the optimum potential was further optimized (Fig 8.4b), and the maximum Raman enhancement was achieved at 250 sec. Optimum preconcentration conditions were employed for the EC-SERS analysis of HC.

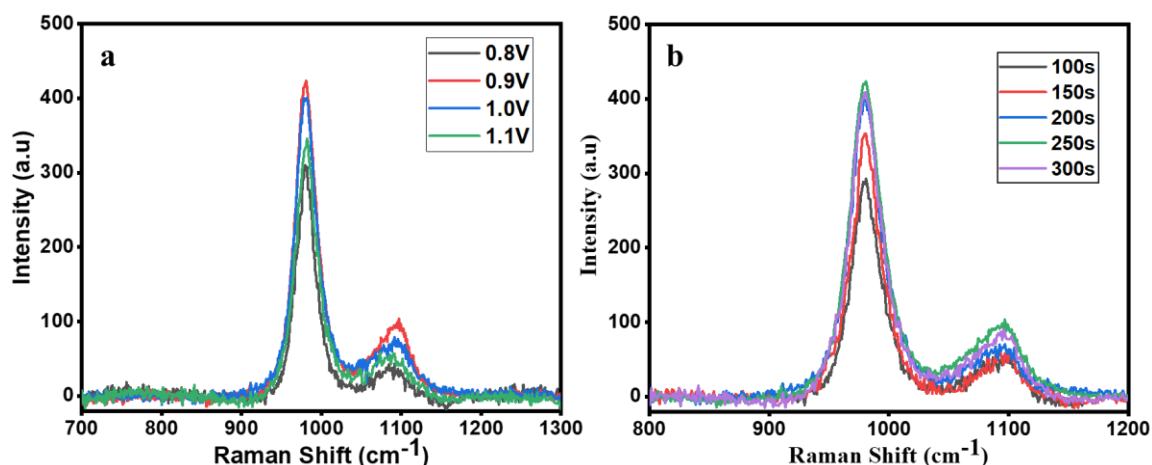


Figure 8. 4 Raman spectra of in-situ electrochemical preconcentrated solutions of 0.1 M HC drug showing the optimum (a) potential and (b) time, using electrochemical stripping voltammetry in a phosphate buffer.

The Raman, SERS and electrochemical-SERS spectra of 0.1 M HC in standard phosphate buffer solution (pH=7) were recorded at the optimum spectroscopic and electrochemical conditions as illustrated in Fig 8.5a. The enhancement in Raman signals was observed after employing nanoparticles substrates with 1:1 volume ratio of Au-CuO_x CNPs and anti-cancer HC drug. Moreover, a considerable enhancement was noticed upon applying the electrochemical preconcentration method using the Au-CuO_x/FTO electrode as an EC-SERS active substrate. The prominent Raman line at 977 cm⁻¹ attributed to the C-N stretching vibration was selected for the HC concentration-dependent response. The EC-SERS responses were screened at the optimum condition in the drug concentration region 0.1 M-10⁻⁸ M. A spectral 400 – 2000 cm⁻¹ range was recorded and shown in Fig 8.5b. The Raman intensity corresponding to the C-N stretching mode seems to correlate well with the HC species in aqueous solution. A wide linear dynamic range of 0.1 M – 10 nM was achieved, as presented in the inset of Fig 8.5b. The EC-SERS protocol proposed in this work yielded a competitive low limit of detection of 1.0 nM, as compared to other previously reported chromatographic and electrochemical techniques (Table 8.1), with a correlation coefficient value $R^2 = 0.9954$. The Raman enhancement factor (EF) which is a

key parameter reflecting the magnitude of intensity enhancement capability by the nanomaterial was calculated following a well-established method. EF of the process was found to be 1.43×10^7 , which implies that Au-CuO_x CNPs can be considered a promising substrate for SERS and EC-SERS application.

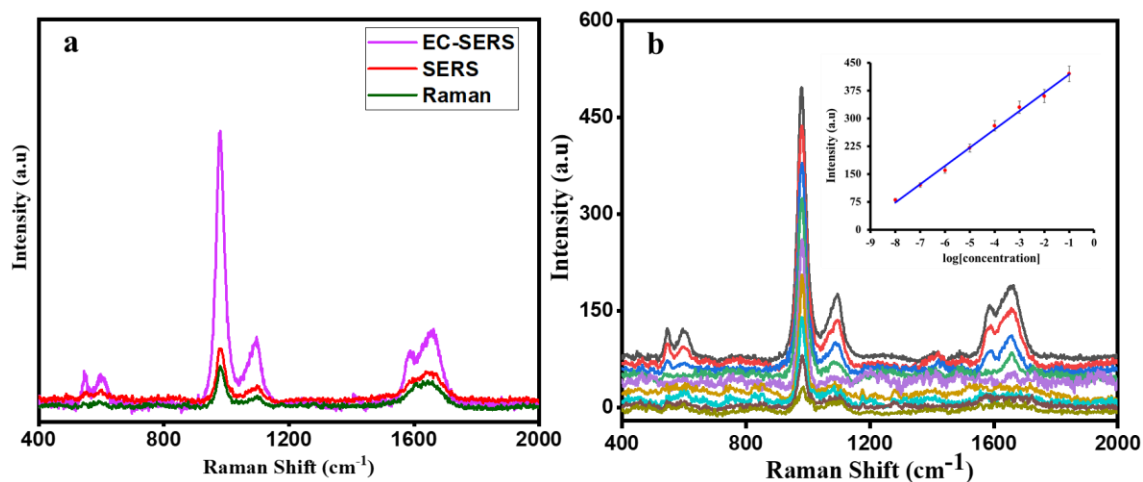


Figure 8. 5 (a) Normal Raman (green), SERS (red) and EC-SERS (pink) spectra using 0.1 M HC solution. (b) EC-SERS response of different HC concentrations (0.1-10⁻⁹ M) in phosphate buffer (pH=7). Inset shows the EC-SERS linear response with the logarithmic concentration of HC. Optimized Raman parameters are 6 scans, 20 sec, 50% laser power with 633 nm excitation source.

Table 8. 1 Comparison of the developed EC-SERS method in this work using Au-CuO_x CNPs with previously reported analytical methods for HC quantifications.

Analytical method	LDR	LOD	Materials	Ref
HPLC-UV	5 to 400 μ M	5 μ M	-----	[347]
GC-MS	0.313–500 μ g/ml	0.078 μ g/ml	-----	[348]
HPLC-EC	1.0–300.0 μ M	0.3 μ M	-----	[349]
EC-DPV	8.0-180.0 mg/L	0.160 mg/L	nano-TiO ₂ /CPE	[350]
EC-DPV	0.05 μ M to 1.0 mM	23.0 nM	Ag@Pt/MWCNTs/CPE	[351]
EC-DPV	50–500 μ M).	22.0 nM	MoS ₂ -Au-Electrodes	[352]
EC-SERS	0.1M-10 nM	1.0 nM	Au-CuO _x nanoplates	This work

The transfer of charges (CT) and surface plasmon resonance (SPR) are crucial in understanding the Raman enhancement process. In order to estimate contributions from CT and SPR in the EC-SERS enhancement of HC Raman peaks, the concept of “degree of CT ρ_{CT} ” introduced by Lombardi et al. [353] was employed using the following equation:

$$\rho_{CT(k)} = \frac{I_{(CT)}^k - I_{(SPR)}^k}{I_{(CT)}^k - I_{(SPR)}^0} \quad (8.1)$$

In Eq. (8.1), k is an index that identifies the individual molecular lines in the Raman spectrum. Two Raman peaks from the EC-SERS spectra were chosen for better understanding the nature of enhancement facilitated by the HC $\cdots$ Au-CuO_x complex. The Raman peak corresponding to a totally symmetric is associated with the surface plasmon response (SPR) effect, and its intensity is denoted as I^0 (SPR). For a totally symmetric line, I^k (SPR) = I^0 (SPR). The second selected peak, on the other hand, is regarded as non-totally symmetric, and its intensity is presented by I^k (CT) whose intensity is enhanced by the contribution of the CT resonance. The I^k (SPR) value is normally small or zero. Then, eq. (8.1) can be modified in the following form [354]:

$$\rho_{CT} = \frac{R}{1+R} \quad (8.2)$$

Here, R is the intensity ratio of the non-totally symmetric mode to the totally symmetric mode in the Raman spectrum. To suitably determine the non-totally symmetric and totally symmetric vibrations, the optimized C_s structure of HC was used to generate the vibrational frequencies in the layout of A' and A'' modes, where the formers describe the totally symmetric while the latter can be regarded as non-totally symmetric. The most prominent totally and non-totally symmetric lines, hence, are identified at 545 cm⁻¹ (476 cm⁻¹ from

DFT) and 977 cm⁻¹ (926 cm⁻¹ from DFT), respectively, in the SERS and EC-SERS spectra. These lines were chosen because they are comparatively intense and well separated from each other. It can be concluded from Eq. (8.1) that when $\rho_{CT} \rightarrow 0$, there is a negligible or zero contribution due to charge transfer, while when $\rho_{CT} \rightarrow 1$, charge transfer contribution dominates. Further, the contribution from CT and SPR will be about equal when $\rho_{CT} = 0.5$ [353]. The values of ρ revealed from the EC-SERS and SERS spectra are 0.12 and 0.30, respectively. In both cases the SPR phenomenon is the major contributor to the SERS and EC-SERS enhancements caused by the drug/CNPs complex. Moreover, it can be noticed that the plasmonic resonance properties of the material was improved by three-fold upon the incorporation of the electrochemical module [355,356], which indicates the influence of the electrochemical factor in accomplishing more sensitive, highly effective plasmonic SERS biosensors.

Although the charge transfer is not the major contributor to the enhanced Raman signals, the dependability of the electron transfer on the oxidation state of the copper metal would help in better understanding the properties of the CNP material. Hence, the interaction of drug molecules on the nanostructured surface was assessed by calculating the amount of charge transfer. Interaction of two chemical systems having different electronegativities results with an electron flow from a system with a lower electronegativity to a system with a higher electronegativity until chemical potentials of both systems become even [357]. The electron fraction (ΔN) transferred from HC molecule to the nanostructured material was evaluated accordingly using the following equation (8.3):

$$\Delta N = \frac{[\chi_{Au-CuOx} - \chi_{HC}]}{2[\eta_{Au-CuOx} + \eta_{HC}]} \quad (8.3)$$

where $\eta_{\text{Au-CuO}_x}$, η_{HC} , $\chi_{\text{Au-CuO}_x}$ and χ_{HC} denote absolute values of hardness and electronegativities, respectively, for the Au-loaded copper oxide and drug molecules [358]. The hardness and electronegativity values were computed [359] for the optimized Au-CuO, Au-Cu₂O and HC configurations (Table 8.2). The fraction of transferred electrons from HC molecule to Au-CuO and Au-Cu₂O were found to be 0.069 and 0.131, respectively. In the Au-CuO_x-HC system, it is anticipated that Au-Cu₂O contributes twice as much to the electron transfer as compared to the Cu(II) based material. The positive magnitude of ΔN implied that in both cases the drug molecule acts as an electron donor. These results support the assertion that adsorption of HC on the nanostructured surface occurs based on electron donor-acceptor interaction [360].

Table 8. 2 Electronegativity, hardness, and electron fraction of anti-cancer drug, Au-Cu₂O and Au-CuO

Compound	Electronegativity (χ) eV	Hardness (η) eV	Electron Fraction (ΔN)
HC	3.740	3.279	-----
Au-Cu ₂ O	4.959	1.385	0.131
Au-CuO	4.413	1.572	0.069

Prior to exploring the nature of interaction of HC with the nanomaterial, the electrostatic potential maps associated with the HC molecule and the Au-CuO_x clusters were computed (Fig 8.6). ESP analysis provides information on the electrophilicity and nucleophilicity of the adsorption system. Three potential local negative sites were found on the surface of the adsorbed molecule. These are located at oxygen atoms of both the carbonyl and hydroxyl groups, and the nitrogen atom of the amino moiety. The four positive local regions, on the

other hand, were predicted in the vicinities of H atoms of amino and hydroxyl groups. The computed electrostatic map of Au-CuO_x complex is depicted in Fig 8.6(b & c), in which Au and Cu transition metals were predicted to behave as electropositive electron receptors, while oxygens were confirmed to represent the electronegative regions. The detailed view of the ESP maps proposed potential interaction sites between the nanomaterial and the adsorbate drug molecule, in support to the general trend depicted above by the calculated electron transfer.

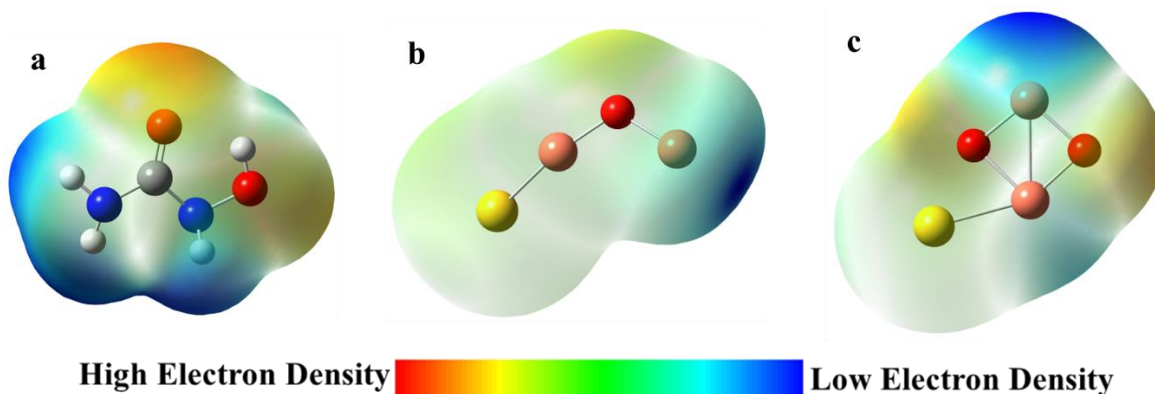


Figure 8. 6 Electrostatic potential (ESP) maps of (a) hydroxycarbamide (HC), (b) Au-Cu₂O and (c) Au-CuO nanoclusters with isovalue = 0.02. Red colour indicates electron-rich regions, while blue indicates electron-deficient regions.

Based on the hotspots revealed from the molecular ESP maps, possible drug...substrate interaction modes were identified, and the respective optimized geometries were obtained as illustrated in Fig 8.7. The adsorption energies associated to the complexation of Au-CuO_x with HC drug in the modes of (a) Cu₂O-Au...O=C, (b) Cu₂O-Au...NH₂, (c) NH...CuO-Au...OH, and (d) H₂N...CuO-Au...O=C were computed to be -10.2, -11.6, -7.7 and -11.5 kcal/mol, respectively, while their corresponding binding distances are predicted as 2.45, 2.49, 2.01 and 2.29 Å, respectively. The negative interaction values and the moderately short intermolecular distances indicate an energetically favourable adsorption of drug molecules on the surface of metallic nanomaterials. In the mode of

interaction of HC with Au-CuO, H-bonding occurring between the H atom of HC drug and O atom of Au-CuO along with the metallic interaction was observed and expected to play a vital role in achieving the desirable enhancement level in Raman signals [361]. In the $\text{NH}\cdots\text{CuO-Au}\cdots\text{OH}$ interaction mode, the drug molecule interacts with the bimetallic system via copper which hints to a possible role of Cu metal in the adsorption process. It was observed that the geometry of the Au-CuO cluster is distorted due to H-bonding between the amino group hydrogen and nanomaterial oxygen in the double interaction $\text{H}_2\text{N}\cdots\text{CuO-Au}\cdots\text{O=C}$ model. It can be thus concluded that the adsorption of drug molecules on the surface of the bimetallic nanomaterials occurs via both metals (i.e. Au and Cu) and facilitated with H-bonding, and therefore both metals exhibit a pivotal role in the plasmonic Raman enhancement.

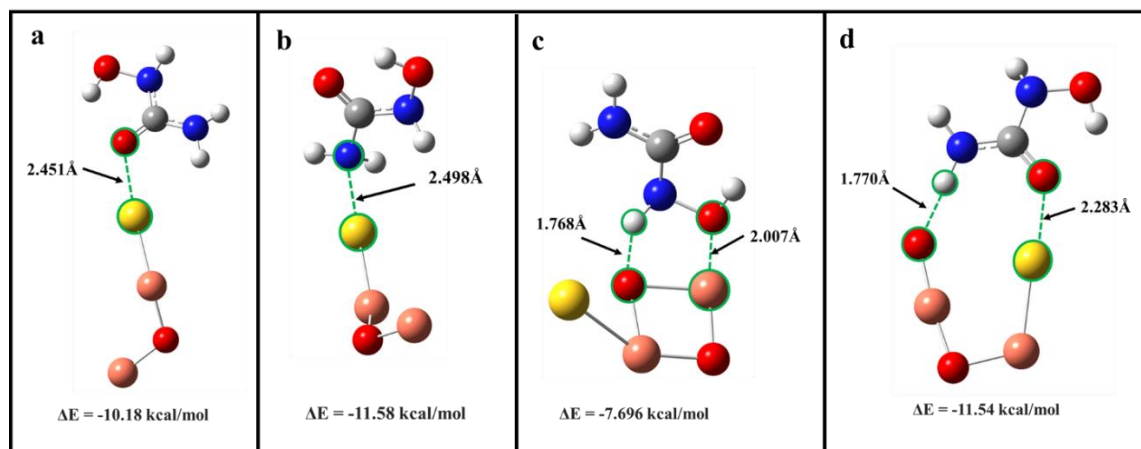


Figure 8. 7 Optimized configurations, calculated bond distances and adsorption energies for the interaction models of (a) $\text{Cu}_2\text{O-Au}\cdots\text{O=C}$, (b) $\text{Cu}_2\text{O-Au}\cdots\text{NH}_2$, (c) $\text{NH}\cdots\text{CuO-Au}\cdots\text{OH}$, and (d) $\text{H}_2\text{N}\cdots\text{CuO-Au}\cdots\text{O=C}$ computed at the DFT/B3LYP level with basis sets 6-311g(d,p) for non-metallic atoms and SDD for metallic atoms.

The experimental and theoretical Raman spectral profiles of HC were compared as presented in Fig 8.8. The comparison provided a good basis to assign key SERS peaks originated from the most involved vibrational modes which led to the electromagnetic field-based interaction. The experimental SERS spectra show six prominent Raman peaks

located at 545 cm^{-1} , 599 cm^{-1} , 977 cm^{-1} , 1095 cm^{-1} , 1587 cm^{-1} and 1661 cm^{-1} ascribed by the NCN bending, CN stretching, NO stretching, H_2N wagging, H_2N scissoring and CO stretching, respectively. The simulated SERS spectra that are most consistent with the experimental one (Fig 8.8) are those resulted from the interaction of the drug molecules via single $\text{Cu}_2\text{O-Au}\cdots\text{NH}_2$ and double $\text{H}_2\text{N}\cdots\text{CuO-Au}\cdots\text{O=C}$ mode complexes.

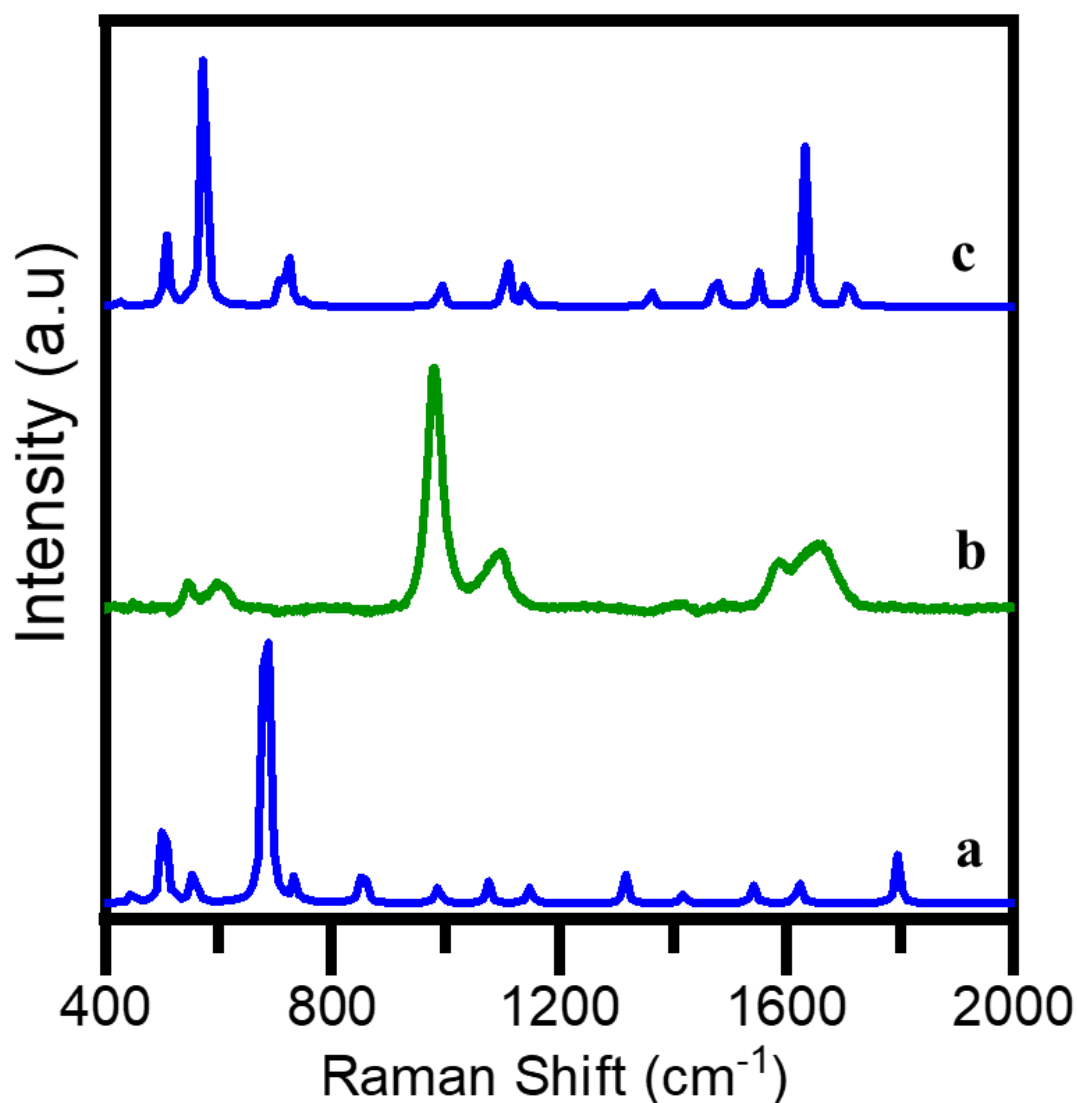


Figure 8. 8 Simulated SERS spectra of (a) $\text{Cu}_2\text{O-Au}\cdots\text{NH}_2$ and (c) $\text{H}_2\text{N}\cdots\text{CuO-Au}\cdots\text{O=C}$ complexes, compared with (b) the experimental EC-SERS spectrum (0.1 M solution of HC) at the optimum conditions.

CHAPTER 9

CONCLUSION AND PERSPECTIVE

9.1 CONCLUSION

The successful development of Surface enhanced Raman scattering (SERS) and electrochemical-SERS methods by developing various plasmonic SERS active substrates for the sensing and characterization of targeted drugs and organic herbicides were achieved. The research outcomes provided notable innovation in the spectro-electroanalytical area that give us lower limit of detection and wide linear dynamic range with reliable results. The DFT study provided meaningful information to understand the SERS-electrochemical phenomenon.

A spectroscopic and quantum chemical combined approach for the quantification of the non-steroidal anti-inflammatory sulindac drug was successfully carried out to study the interaction of SERS substrate with the drug molecules. Significant enhancements in intensities were obtained at the Raman bands at 1111 cm^{-1} , 1653 cm^{-1} , 1607 cm^{-1} , and 1340 cm^{-1} corresponding to -S=O stretching, -C=O stretching, -C=C- stretching and ring stretching vibrations. Possible scenarios of sulindac-AgNPs interactions leading to SERS enhancements were evaluated using density functional theory (DFT). The current approach exhibited competitive sensitivity, selectivity, good linearity ($R^2=0.9996$), a wide dynamic range (1×10^{-2} M to 1×10^{-9} M) and a low detection limit (1×10^{-10} M). It hence displays a high potential for the detection of sulindac and other drugs with similar structural properties.

Surface enhanced Raman scattering (SERS) were used for the quantitative evolution of procaine at various concentrations using reduced gold nanoparticles (AuNPs) as substrates. The SERS analysis showed reliable and excellent enhancement in the peaks of procaine. Procaine molecules are predicted from DFT calculations to fairly chemisorb on the surface of AuNPs. Its limit of detection of 1×10^{-10} M was accomplished with a value of correlation coefficient (R^2) of 0.9971 and excellent reproducibility being achieved.

A Spectroscopic approach reinforced with the electrochemistry phenomenon for efficient detection of procaine using silver-decorated carbon nanosphere (AgCNS) deposited on a fluorine-doped tin oxide (FTO) electrode was proposed and thoroughly evaluated. The electrochemical performance of the modified electrode (AgCNS-FTO) was examined using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). A significant enhancement in the Raman intensities of the bands centered at 1611, 1322, 1285 and 1184 cm^{-1} more likely corresponding to the NH_2 bending, $\text{C}=\text{C}$ stretching, CH_2 twisting and $\text{C}-\text{N}$ linkage stretching vibrational modes were observed upon the pre-concentration at the optimum conditions of -0.1 V and 400 sec along with the laser excitation of the procaine molecules. The proper incorporation of the electrochemical sensation in the Spectro-analytical measurements enabled a remarkable sensitivity and selectivity towards procaine molecules with a wide dynamic range (10^{-6} - 10^{-12} M), a high reproducibility, and stability over the duration of one month. It was possible to reach a very low detection limit of 10^{-13} M.

Spectro-electrochemical analysis based on SERS and EC-SERS techniques was successfully developed using palladium nanoparticles loaded on highly reduced graphene oxide (Pd-HRG) substrate for the trace concentration detection of procaine. The

synthesized Pd nanocomposite exhibited an adequate localized surface plasmon resonance (LSPR) response and promising Raman scattering properties. The analytical performance showed a wide combined SERS/EC-SERS linear dynamic range and a limit of detection of 10^{-8} M. First-principles calculations mimicking the behavior of the drug molecules on the Pd-HRG surface suggested the primary amine moiety being the most prominent site of the SERS interaction. The investigated palladium-based graphene oxide showed a great potential to fabricate effective non-silver and non-gold nanostructured substrates for sensitive SERS and EC-SERC measurements of therapeutic drugs.

Circular nanoplates (CNPs) of Au-loaded copper oxide with enhanced SPR characteristics were synthesized in a single-step procedure and investigated for possible Raman enhancement in an EC-SERS platform. Electrochemical stripping voltammetry was incorporated with SERS, and the synergic effect for the detection of the anti-cancer drug hydroxycarbamide (HC) was tested by fabricating the CNPs on a conductive glass support. A notably low limit of detection and a wide dynamic working range were successfully achieved by applying the electrochemically induced SERS technique. The effect of electrochemical potential and exposure time on the Raman signal were optimized. A highly linear response of the Raman intensity with the logarithmic HC concentration in the range of 0.1 M - 10^{-8} M was recorded. The limit of detection was shown to be in the order 1.0 nM , lower than previously reported analytical methods. It can be concluded that EC-based pre-concentration methods when properly integrated with the SERS technique would enhance the optical properties of the material substrate and can avail a reliable quantitative detection of organic-based drugs with a fast-throughput approach.

The herbicides including linuron and 1-methoxy-1-methyl-3-phenylurea (MMPU) were successfully investigated by applying NMR and Raman experimental techniques supported with computational DFT calculations. The Selective Nuclear Overhauser effects (SelNOE) have been thoroughly assessed to understand the conformational behavior of the target molecule in solution. The SERS based analytical methods were developed for the sensitive detection of phenyl urea herbicides.

9.2 PERSPECTIVES

Plasmonic nanostructured materials especially Ag, Au and CuOx were explored for the qualitative and quantitative analysis of therapeutic drugs and organic based herbicides using Raman and electrochemical techniques. The DFT molecular modelling was applied to understand the mechanism of Raman enhancement. The chemical enhancement mechanism was improved by functionalizing the Plasmonic material on the carbonaceous nanomaterial.

In future, a cost effective and efficient SERS substrate needs to be developed using less costly metal and their plasmonic effect should be explored such as alkali metal and other transition metals. To achieve maximum enhancement in Raman signals, new surface modification strategies need to be explored by functionalizing electron rich material with plasmonic materials. The environmentally friendly SERS substrate based on organic materials is a need of the hours which could be degradable and good for human health.

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- ❖ **Muhammad Haroon**, Abdulaziz A. Al-Saadi*. Nanoplates of gold-decorated copper oxide with enhanced plasmon sers performance for the detection of anti-cancer drug hydroxycarbamide. (Submitted, KFUPM Ref # 2022-153)

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