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SERS assisted with Fluorescence Microscopy for the Invitro analysis of Drug-treated Cancer cells

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ABSTRACT: Surface Enhanced Raman Spectroscopy (SERS), an emerging technique of Raman spectroscopy, can be used for the characterization of molecular vibrations in drug treated breast cancer cells. This paper deals with the integration of photonics and nanoplasmonics applications in to the field of cancer research. For the enhancement of Raman signals, Gold Nanorod (AuNR) with a peak absorption wavelength of 692 nm is used as the plasmonic substrate due to their tunable surface plasmon property. The biocompatible AuNR is obtained by replacing Cetyltrimethylammonium bromide (CTAB) with Thiol polyethylene glycol (PEG) on its surface. The physical and chemical properties of the conjugates was monitored through optical measurements to confirm its surface chemistry. The conjugates are then binded with Mitoxantrone (MXT) which is an anticancer drug and their successful binding is monitored through the longitudinal surface plasmon resonance (LSPR) peaks, fluorescence quenching and Raman spectrum. Further the resultant conjugate is then incubated in to the MDA-MB-231 breast cancer cell lines. The real time monitoring of molecular changes in cancer cells under the drug treatment is obtained by analyzing the spectra from SERS on assistance with Fluorescence Microscopy.

KEYWORDS: Breast cancer, Fluorescence, Gold nanorod, LSPR, Surface enhanced raman spectroscopy.

I. INTRODUCTION

Breast cancer is one of the most common deadly disease found in women. It has become a global health problem, where 12% of women worldwide are affected [1]. The current cancer therapeutic techniques such as chemotherapy, radiation therapy, photothermal therapy etc, has a lot of limitations such as non specific dispersion of anticancer drugs, insufficient delivery of drug concentrations to the target site, toxicity, limited ability to track and control the site specific therapeutic responses, side effects, and the development of drug resistance [2]. Therefore, new techniques are needed that could provide improved therapy monitoring and guiding patient treatment. These reasons has explored the invention of vibrational spectroscopic techniques such as SERS, which has emerged as a very promising spectroscopy method with single molecule sensitivity and plasmonic nanostructures, which can improve the capabilities of SERS towards single molecule detection limits. As a result this combinations of biophotonics application is the solution for the deadly disease in humans, such as breast cancer.

SERS is a laser based optical spectroscopic technique which can be used as a noninvasive and label-free analysis tool for measuring the inelastically scattered photon from the analyte. It can identify molecular vibrations and can achieve detailed fingerprint spectral information from the molecules that are in close vicinity with the plasmonic nanomaterial. SERS is an emerging technique of Raman Spectroscopy that enhance Raman signals by many orders of magnitude. The SERS process and the enhancement factor depends on laser excitation, spot size, Raman scattering, plasmon resonances, surface chemistry, SERS probes and SERS substrates. Its applications can be used to detect illegal drugs, explosives, pollutants in rivers, chemical/biological weapons and agents, art preservation, cancer diagnosis and therapy, detection of biomolecules etc. One of the main task of SERS for the detection of biomolecules in submicromolar concentrations is the synthesis of noble metal nanoparticles of different shapes and sizes to allow for fine-tuning of plasmonic properties which can lead to maximum optical field, and hence maximum Raman signal enhancement [3]. So, it is a big challenge for obtaining a surface modified nanoprobe concurrently with high-quality SERS enhancement,

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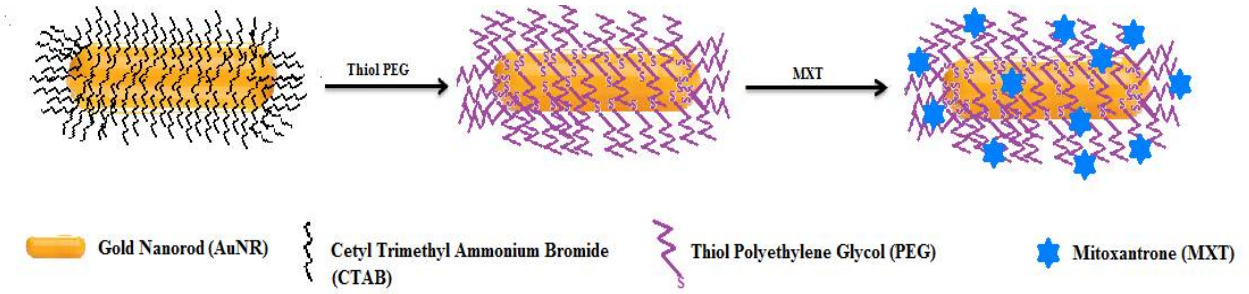
low toxicity and high biocompatibility. Hence, this technique is a tool that links both photonics and nanoplasmonics applications.

Cancer nanotechnology is a relatively novel interdisciplinary area of comprehensive research that combines the basic sciences, engineering and medicine. Drug delivery research is now advancing from the micro to the nanosize scale for cancer treatment which may reduce the adverse side effects of existing therapies [4]. Nanotechnology can provide rapid and sensitive detection of cancer related targets and can generate highly effective therapeutic agents. It can be used as an efficient targeted drug delivery and controlled release carriers over a prolonged period of time that offers the means to aim therapies directly and selectively at cancerous cells.

The key areas of this work includes formulating biocompatible drug delivery and controlled release carriers using Gold nanorod, that can effectively bind anticancer drugs. The effects of anticancer drug is investigated on the biomolecules of the MDA-MB-231 breast cancer cell using SERS assisted with Fluorescence microscopy. Fluorescence imaging is obtained for the monitoring of drug entry into the cells and then in situ SERS spectra of cell were obtained by a Raman spectrometer. Finally, the SERS spectral changes of cell that are caused by targeting small molecules can be obtained by comparing the normal and drug treated cancer cells.

II. NANOSYSTEM MODEL

The drug loaded Gold nanorod system design is shown in scheme 1. Here AuNR is prepared by assembling PEG around AuNRs with better biocompatibility and then it is conjugated with MXT. Scheme 1 shows the treatment process for cancer cells, where MDA-MB-231 cells were incubated with the AuNR-PEG-MXT nanoprobes for the in situ SERS detections. The entry of drug into the cell can be observed from fluorescence imaging. Then, in situ SERS spectra in one cell is measured and analysed to disclose the effects of MXT on native molecules in the cell.



Scheme1. Nanosystem design of AuNR-PEG MXT.

III. MATERIALS AND METHODS

Materials: Cetyltrimethylammonium bromide (CTAB), Chloroauric acid ($HAuCl_4.3H_2O$), lipoic acid (LA), ascorbic acid (AA), thiol PEG, Thiol Polyethylene Glycol (PEG) were purchased from Sigma, Bangalore, India. Sodium borohydride ($NaBH_4$),Silver Nitrate ($AgNO_3$), sodium hydroxide (NaOH) and hydrochloric acid (HCl) were obtained from Merck India Pvt. Dulbeccos Modified Eagles Medium (DMEM), penicillinstreptomycin and fetal bovine serum (FBS) were obtained from Himedia. All glassware was cleaned using aqua regia (1:3 ratio of HCl and HNO_3). The water used in all experiments was Millipore Milli Q grade (18 M3 cm).

Synthesis of Gold Nanorods: AuNRs were synthesized according to the well-established seed-mediated growth method [5]. In a flask, 132 mL of 0.1M CTAB was mixed with 120 mL of 1mM $HAuCl_4$, and then 2.4 mL of a 10 mM silver nitrate aqueous solution and 2.2 mL of 2 M hydrochloric acid were added to the flask. After gently mixing the solution, 1.92 mL of 0.1 M ascorbic acid was added. Under continuous stirring, a certain concentration of seed solution was added finally to initiate the growth of the AuNRs. The AuNRs were aged 5 h to ensure full growth at 28°C. The color of the solutions turned pink to violet within 10 min, depending upon the amount of seed present. After preparation,

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excess CTAB molecules were removed by centrifuging twice at 10000 rpm for 10 min, and then redispersed in distilled water.

Surface modification of AuNR-CTAB with Thiol PEG: In a typical synthesis, 5 mL of the as-prepared AuNRs solution was added into 10 mL of distilled water under magnetic stirring to ensure homogeneous mixing. Next, 1 mg of PEG-SH (Mw= 5000) was added to the AuNRs solution and stirred for 2 h to covalently modify the surface of the AuNRs with PEG. Finally, the resulting PEG coated AuNRs were collected by centrifugation at 10,000 rpm for 30 min and subsequently washed twice with distilled water [6].

Conjugation of AuNR-PEG with MXT: The anticancer drug Mitoxantrone was fabricated onto the surface of AuNR-PEG by a simple stirring method. In brief, 2mg MXT is diluted in 5 ml of water and 2 ml of the sample obtained is again diluted to 5ml and finally AuNR-PEG is added. The mixture was stirred overnight at room temperature in the dark. After stirring, the suspension was centrifuged at 10,000 rpm for 15 min to remove unconjugated MXT. The pellet of the AuNR-PEG-MXT conjugate was redispersed in 2 mL of Milli-Q water.

Cell culture: MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (ATCC; Manassas, USA). The MDA-MB-231 cells were grown in vented T25 culture flasks and were cultured in 20% of Dulbeccos Modified Eagles Medium (DMEM). The media were supplemented with 10% heat-inactivated FBS and 1% penicillinstreptomycin at 37 ^{0}C under a 5% CO_2 and 95% air humidified atmosphere [7].

Characterization: The size and surface morphology of as synthesized gold nanorods were analyzed using a high-resolution transmission electron microscope (Hitachi) operated at 200 kV. Absorption/SPR spectra of as-prepared AuNRs and the surface modified AuNRs were measured with a UV-visible spectrophotometer (Horiba Jobin Yvon) in the 200-900 nm wavelength range. The fluorescence emission spectra were recorded using a fluorescence spectroscopy (Horiba Fluorolog) at an excitation wavelength of 430 nm. Fluorescent microscopic examination was performed using a Olympus IX 83. Raman spectra of AuNR and cells were taken from Raman Spectroscopy (Witech Alpha 300 R), using 532 nm as laser source.

IV. RESULT AND DISCUSSIONS

Study Design: To obtain fingerprint spectral information of drug treated breast cancer cells by using SERS spectroscopy, a nanoprobe with strong SERS enhancement ability, good biocompatibility efficient drug delivery and control release is needed. In this study AuNR with surface function modification were designed and employed. The TEM image and the UV absorption spectrum of AuNRs are shown in Figure 1. Figure 1(a) is the TEM image of the AuNRs after chemical etching, and it shows that the prepared AuNRs are rod-like with a dimension of 50 nm and aspect ratio 3.4. It had exhibited two absorption bands: a weak short-wavelength band around 525 nm (TSPR-due to the transverse electronic oscillation) and a strong long-wavelength band around 692 nm (LSPR-due to the longitudinal oscillation of electrons) (Fig. 1(b)).

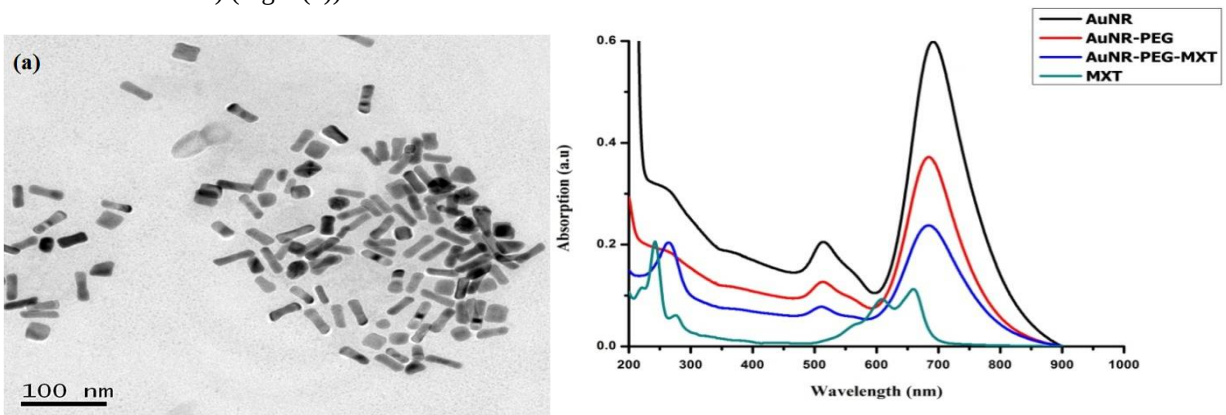

Fig. 1 (a) A TEM micrograph of AuNRs with aspect ratio 3.4; (b) Optical absorption spectra of AuNR, AuNR-PEG, MXT, AuNR-PEG-MXT.

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The existence of the cationic surfactant (*i.e.*, CTAB) which was used for capping and stabilizing the AuNRs during synthesis is replaced by the process of functionalization due to its high toxicity [8]. For this, the surface of AuNRs were modified with Thiol PEG to promote biocompatibility and acts as a linker between AuNR and MXT. The successful modifications of AuNRs with PEG and MXT were confirmed through the shift in wavelength observed via UV−visible spectroscopy (Fig. 1b). Comparing with the spectra of AuNRs, when CTAB coating over AuNRs were replaced by PEG, the LSPR band blue shifts to 683 nm whereas the TSPR band gets unchanged. On conjugation of MXT with AuNR-PEG, again a blue shift of LSPR band is observed to 684 nm and also one of the peak of drug is observed in the final conjugate at 260 nm. The LSPR shift can be due to the fact that the absorption of PEG and MXT increases the local refractive index around AuNRs [9].

The fluorescence emission spectrum of the surface modified AuNRs are observed via fluorescence spectroscopy (Fig. 2). At 430 nm excitation, two emission peaks of AuNR-PEG-MXT are observed at 634 nm and 675 nm. The emission spectrum of MXT is overlapped with UV-Visible absorption spectrum of AuNRs. This results in fluorescence quenching due to the spectral overlap of donors emission with acceptors absorption. The emission peak of free MXT with high intensity is observed at 679 nm which is quenched when conjugated with AuNR-PEG. This quenching of fluorescence can be an advantage for obtaining Raman spectrum, because it can hide Raman features as they are more intense than Raman scattering.

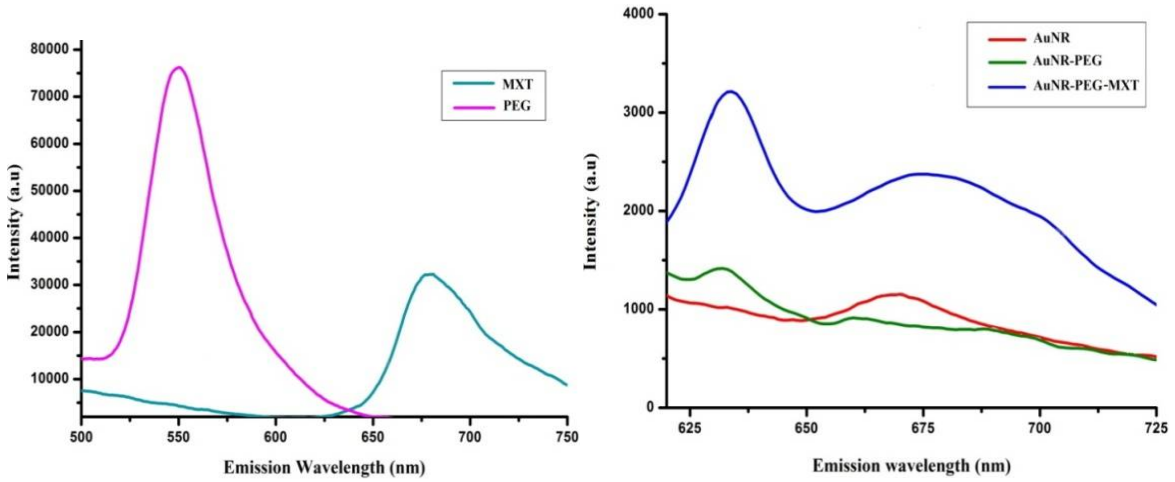

Fig. 2 (a) Fluorescence spectra of PEG and MXT (b) Fluorescence spectra of AuNR, AuNR-PEG, AuNR-PEG-MXT.

Differential interference contrast (DIC) microscopic images are taken to differentiate between live and dead cells. To investigate the cellular uptake of AuNR-PEG-MXT, fluorescence microscopic image was obtained which takes the advantage of the intrinsic fluorescence of MXT. Figure 3(a) and 3(b) is the DIC and fluorescence image of live MDA-MB-231 cells before incubation of AuNR-PEG-MXT. From the morphological variations of the cells in DIC image (Fig. 3(c)) taken after 4 hours of incubation time with AuNR-PEG-MXT, it can be stated that those cells are dead. 3(d) shows the fluorescence image of dead MDA-MB-231 cells. After 4 hours of incubation time, drug treated cells shows fluorescence emission, this is because when once cells are recognized by the entry of drug, they will start to radiate intensive fluorescence as MXT is a fluorescent drug which becomes “visible” under fluorescence imaging [10]. Since AuNR-PEG-MXT are diffused across the cell membrane by the process of receptor-mediated endocytosis.

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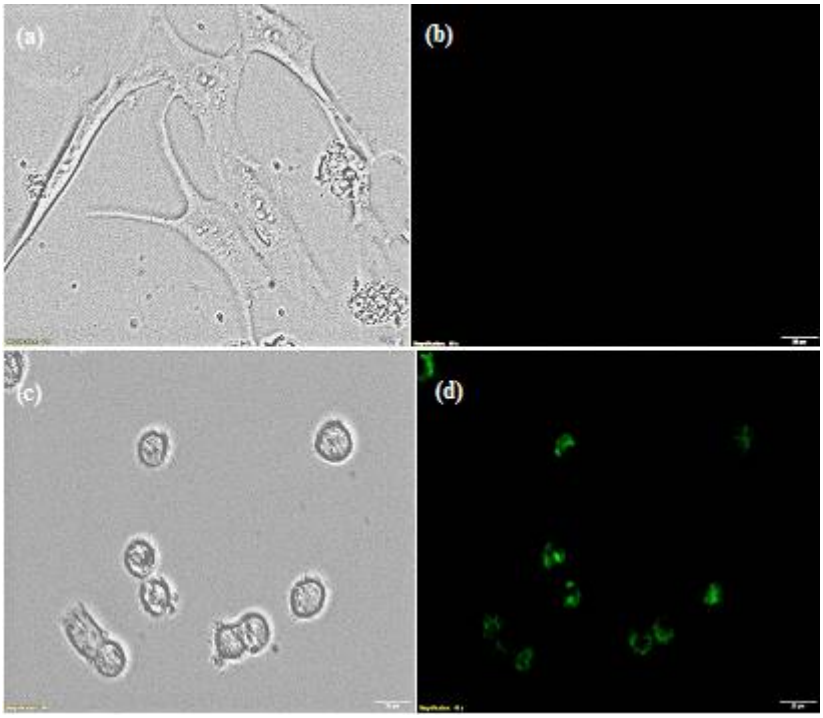

Fig. 3. (a) DIC image of live MDA-MB-231 cell; (b) Fluorescence image of live cell (c) DIC image of dead MDA-MB-231 cell treated with AuNR-PEG-MXT (d) Fluorescence image of dead cell.

The Raman spectrum of the AuNR-PEG-MXT nanosystem is shown in figure 4. Here, drug peak is observed at 1300 cm^{-1} which results in the successful binding of drug with AuNR-PEG [10].

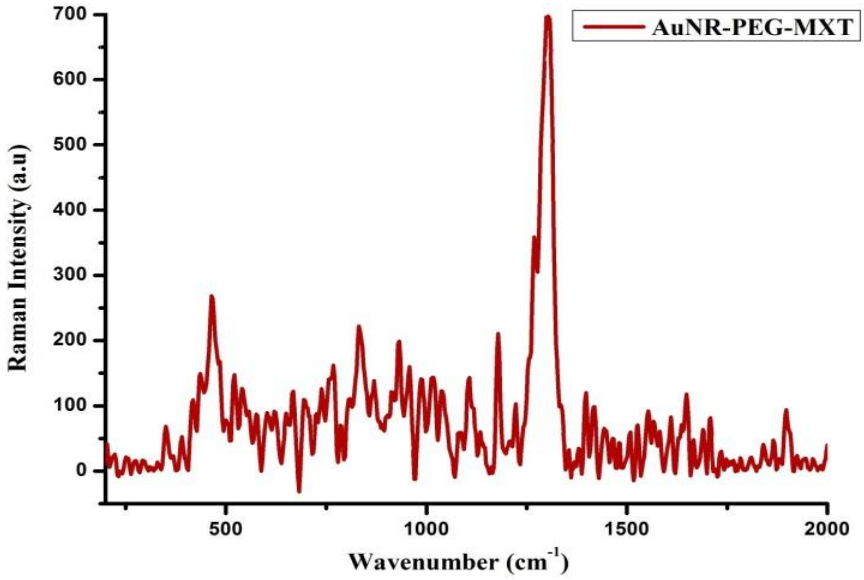

Fig. 4. Raman spectra of AuNR-PEG-MXT.

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Raman spectroscopy is used to find the molecular vibrations of biological macromolecules found in the cells such as DNA, proteins and lipids in the range 600-1800 cm^{-1}. The Raman spectra of healthy and drug treated cells were compared to identify the main spectral differences inorder to discriminate between healthy and dead cells (fig 5). The intensities of Raman peaks of dead cells corresponding to nucleic acids increases at 682 cm^{-1} (G, ring breath), 779 cm^{-1} (T, C=O out of plane bend), 805 cm^{-1} (deoxyribose), 1573 cm^{-1}(guanine and adenine) and decreases at 1075 cm^{-1} (phosphodioxy group PO^{2-}). In addition, the Raman peaks of proteins increases at 1605 cm^{-1} (Phe,Tyr) and decreases at 920 cm^{-1} (symmetric ring breathing mode of phenylalanine), 1294 cm^{-1} (Amide III), 1337 cm^{-1} (CH deformation), 1440 cm^{-1} (CH_2 deformation), 1650 cm^{-1} (Amide I) . The Raman peaks assosciated with lipids increases at 714 cm^{-1} (C-C-N+ symmetric stretching in phosphatidylcholine), 996 cm^{-1} (=CH bend in lipids) and decrease at 1314 cm^{-1} (CH_2 bending of phospholipids). These intensity fluctuations are related to the alterations in the secondary structures and degradation of proteins, the structural modification of lipids and the destruction of base-base stacking interactions in DNA upon drug treatment which will lead to further influence on the cell viability, metabolism, and growth. As a result, these spectral signatures makes it possible to use SERS as biosensors for indicating the cell death due to drug action [11] [12] [13] [14] [15].

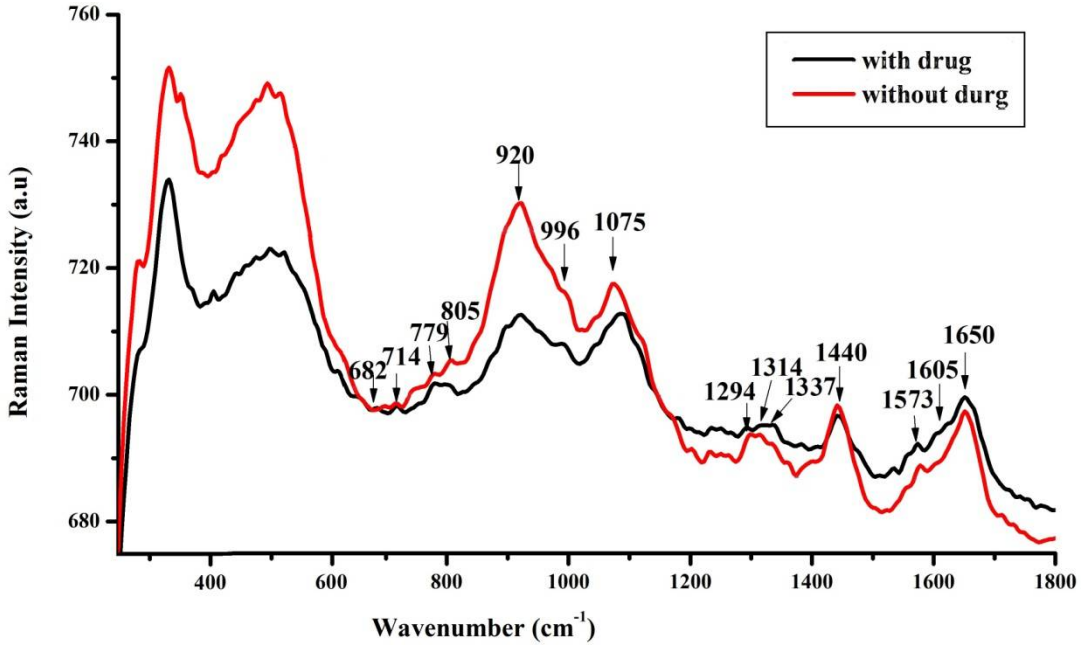

Fig. 5. Raman spectra of cell incubated with AuNR-PEG (live cell) and AuNR-PEG-MXT (dead cell).

V. CONCLUSION

The application of SERS techniques to biosensors provides quantitative analysis of small molecules such as nucleic acids, proteins, and lipids, on their interaction with plasmonic nanomaterials. This work has shown the anticancer activity of Mitoxantrone by analysing the difference in Raman active bands observed in the SERS spectrum of molecules before and after drug treatment within cells, on assistance with fluorescence microscopy. SERS enhancement are obtained from the molecules that are present in the electromagnetic field created by the plasmon coupling on the surface of biocompatible AuNRs which acts as a control release and drug delivery carrier. Thus SERS becomes an useful tool in the field of nanophotonics and spectroscopy which gives the detailed fingerprint analysis of both the plasmonic nanomaterial and the molecule interacting with it.

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BIOGRAPHY

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