Herein, we have examined distinctive structural and functional variations of cellular components during apoptotic cell death induced by a targeted theranostic nanoprobe, MMP-SQ@GNR@LAH-DOX, which acted as a SERS “on/off” probe in the presence of a MMP protease and executed synergistic photothermal chemotherapeutic responses continue to be long standing challenges from the previous decades.1,2 In spite of the fact that an assortment of strategies exist to screen the progress of treatment, many of them are unable to provide a clear picture of the structural consequences during therapy from a molecular perspective. A sensitive and reliable method capable of providing an in-depth understanding of the changes at the cellular level during therapy can provide new insights to optimize therapeutic efficacies. Recently, a surface-enhanced Raman scattering (SERS) technique has emerged as an ideal diagnostic fingerprinting, corresponding to the phosphodiester backbone of DNA. The impact of SERS in prognosis and diagnosis is expected to be ground breaking.

The integration of SERS image-guided diagnosis and treatment within a relatively simple nanoplatform is feasible through the multi-functional roles played by nanomaterials.7,8 Many noble metal nanoparticle (NP) based SERS substrates, namely, gold nanorod (GNR),9,10 gold nanocage11,12 and gold nanopopcorn,13 are excellent photothermal agents.14 Photothermal therapy (PTT) is a promising cancer treatment therapy wherein plasmonic nanoparticles convert NIR light to heat, leading to hyperthermia induced cell death. Enormous efforts have been carried out to analyze the cell death profile following photothermal treatment. Recently, El-Sayed and coworkers reported real time monitoring of molecular changes during cell death induced by PTT through SERS.15 Photothermal therapy for cancer treatment using gold NPs is currently under clinical trials.16 However, in case the of diffused cancerous cells, local photothermal cytotoxicity is not sufficient for complete tumor ablation. Therefore, the combination of various therapeutic approaches with different mechanisms is a promising strategy to improve the therapeutic efficiency.17,18 The spatio-temporal synchronization of PTT and chemotherapy19,20 is highly desirable for efficient cancer treatment with synergistic effect.21,22 The combination of PTT with chemotherapy was reported to reverse multidrug resistance and stimulate therapeutic efficacy in drug resistant cancers.23,24

Multimodal theranostic systems that can specifically target cancer cells with minimal adverse effects on normal cells and tissues require intelligent fabrication strategies with tumor specific ligands. Matrix metalloproteinases (MMP) are a family of zinc-dependent proteins highly expressed in many human cancers and are widely considered as promising targets for cancer diagnosis.25 Ahn and co-workers developed a fluorescence imaging probe for the simultaneous detection of MMP and activation of PTT. Recognition of MMPs could provide valuable information in cancer progression and various imaging modalities were developed for this purpose.26,27 Considering this, we sought to develop a targeted...
theranostic nanoprobe, designated as MMP-SQ@GNR@LAH-DOX, which can spot MMP expressing cancer cells selectively and execute targeted photothermal-chemotherapy, combining the photothermal effects of GNRs and site specific cytotoxic action of the anticancer agent doxorubicin (DOX). To the best of our knowledge, no SERS guided theranostic nanoprobe for the treatment of MMP expressing cancer cells has been reported to date. Herein, we have integrated multiple functions into a single nano platform and performed a meticulous investigation of molecular events induced by a targeted theranostic nanoprobe through the strong diagnostic platform provided by SERS. Schematic of the fabrication and mode of action of the targeted MMP detection probe for imaging and photothermal-chemotherapy is illustrated in Scheme 1.

The construction of the nanoprobe was initiated with the synthesis of a squaraine dye (SQ) as Raman reporter by the condensation reaction of a mixture of squaric acid and 2,3,3-trimethylindoline in a toluene-butanol eutectic mixture with continuous removal of water using a Dean Stark apparatus. A MMP peptide substrate with the representative amino acid sequence Pro-Leu-Gly-Leu-Ala-Gly-Cys was synthesized by solid phase peptide synthesis (SPPS) (Fig. S1†) which was further bonded to the SQ free amine terminal by amide coupling to generate a SQ attached MMP peptide substrate (MMP-SQ). To obtain a rapid response to the lower pH of acidic organelles, we introduced an acid labile hydrazone linker between the doxorubicinyl group and lipoic acid moiety, denoted as LAH-DOX. The detailed characterization of the intermediates of MMP-SQ and LAH-DOX are given in the ESI.† MMP-SQ and LAH-DOX were anchored onto the surface of the GNR via strong Au–S covalent interactions, generating MMP-SQ@GNR@LAH-DOX (Fig. 1A). The wide applications of GNRs in the biomedical field have been limited due to the existence of cytotoxic CTAB coated on their surfaces. We expected that a large portion of the CTAB would be exchanged with MMP-SQ and that this could decrease the cytotoxicity of the GNR. To prove this, cell viability tests for GNR and MMP-SQ@GNR were performed. MTT assay revealed that biocompatibility of GNR was significantly improved upon conjugation with MMP-SQ because CTAB bilayers, which are non-covalently adsorbed onto the GNR surface, were replaced by the MMP substrate (Fig. S2†). The morphology of GNR was examined with TEM (Fig. 1B). TEM images showed uniformly dispersed GNRs of aspect ratio of approximately 4 (length/width). By tuning the aspect ratio, the strong localized surface plasmon resonance (LSPR) was adjusted to the NIR region to minimize absorption by tissue and maximize laser (808 nm) absorption by GNR. It is expected that the thiol group on both ends of MMP-SQ can link two GNRs, which may lead to the aggregation of GNRs. However, this only happens at higher molar ratio of MMP-SQ than that of GNR (Fig. S6†). DLS and Zeta potential measurements were performed on gold nanorods, MMP-SQ@GNR and MMP-SQ@GNR@LAH-DOX (Fig. S7 and S8†). Both the longitudinal and transverse LSPR of the GNR were slightly red shifted after conjugation. The absorption band corresponding to MMP-SQ centered at 620 nm is consistent with the spectrum of MMP-SQ@GNR@LAH-DOX, which indicated the successful conjugation of MMP-SQ on the surface of GNR (Fig. 1C). The number of MMP-SQ and LAH-DOX attached per GNR were estimated to be approximately 18 × 10^2 and 22 × 10^2, respectively, using UV-visible spectroscopy.
Initial evaluation of SERS spectral features of MMP-SQ@GNR was carried out under a confocal Raman microscope at 633 nm laser wavelength (Fig. S5†). The SERS spectrum of MMP-SQ@GNR comprised peaks from the MMP peptide sequence and SQ. The shift in the position of 2°-amide NH deformation vibration of the MMP peptide substrate at 680 cm\(^{-1}\) to 590 cm\(^{-1}\) confirms the successful conjugation of the MMP peptide substrate with SQ. In addition, the peaks at 741, 1093, and 1474 cm\(^{-1}\) correspond to C-H out of plane bending, aromatic vibration of the polycyclic structure, and g(CH\(_3\)-C) anti-symmetric stretching, respectively. The peak that appeared at 1366 cm\(^{-1}\) resembled the typical stretching vibration of indole rings.\(^{29}\)

The MMP-SQ@GNR conjugate is believed to be a strong SERS “on/off” nanoprobe. The excellent SERS spectral features exhibited by MMP-SQ@GNR were found to decrease in intensity when it approached the target protease because the enzymatic recognition results in cleavage of the MMP peptide substrate, which ultimately detaches the Raman signature molecule (RSM) from the GNR surface, leading to the gradual decrease in SERS signal intensity. The incubation of MMP-SQ@GNR with activated MMP-2 enzymes (50 ng mL\(^{-1}\), 37 °C, 40 min) resulted in significant decrease in the SERS signal intensity, notably the peaks at 590 cm\(^{-1}\), along with all other characteristic spectral signatures of the squaraine dye (Fig. 2A). The decrease in the SERS intensity of MMP-SQ@GNR was induced by the specific decomposition of the peptide substrate because no remarkable change in SERS intensity was observed in the presence of the MMP-2 inhibitor (Fig. 2B). The enzyme selectivity of MMP-SQ@GNR was evaluated with various other MMP enzymes (MMP-7, -9) by obtaining SERS spectra in different time intervals after incubating with the respective enzymes (Fig. S10 and S11†).

After evaluating the sensitivity of the probe towards MMP, photothermal conversion efficiency of MMP-SQ@GNR@LAH-DOX was monitored by recording the temperature changes under NIR laser irradiation at 0.1 W cm\(^{-2}\) with time using a thermocouple-integrated multimeter. The temperature of MMP-SQ@GNR@LAH-DOX, MMP-SQ@GNR and GNR rapidly increased to 57 °C within 6 minutes of laser irradiation, while water alone showed almost no heating effect (Fig. 2C). Such an increased variation of temperature can lead to irreversible damage to tumor cells. Then, the cleavage of hydrazone bonds of the LAH-DOX moiety at lower pH was investigated. The quenched fluorescence of DOX due to the attachment of LAH-DOX on the GNR surface recovered gradually once it was detached from GNR.\(^{30}\) The release of DOX from MMP-SQ@GNR@LAH-DOX was monitored with time in acetate buffer (pH 5.0, 0.02 M), which showed a maximum release within 4 h of incubation. However, incubation of MMP-SQ@GNR@LAH-DOX in phosphate buffered saline (PBS) at pH 7.4 did not exhibit any significant fluorescence recovery even after 12 h of incubation (Fig. S9†). Furthermore, we incubated MMP-SQ@GNR@LAH-DOX in acetate buffer at pH 5.0 and obtained the fluorescence emission spectrum at different time intervals (Fig. 2D). These data demonstrate the release of DOX from MMP-SQ@GNR@LAH-DOX with response to acidic pH.

The cellular uptake and intracellular drug release efficiency of MMP-SQ@GNR@LAH-DOX was studied using fluorescence microscopy in the human fibrosarcoma cell line HT1080, which over-expresses MMPs (Fig. S13†). When MMP-SQ@GNR@LAH-DOX approaches the target cells, enzymatic recognition causes cleavage of the MMP peptide substrate, followed by hydrazone bond cleavage from the LAH-DOX conjugate, in the acidic tumor micro environment, resulting in DOX release. HT1080 cells incubated with the nanoprobe for 3 h, showed red fluorescence from doxorubicin mainly localized in the nucleus, which was confirmed by co-localization with Hoechst nuclear staining dye. The release of DOX at an acidic pH will exert cytotoxicity to the tumor cells while excluding normal cells, which will significantly decrease side effects.

Cytotoxic potential of MMP-SQ@GNR@LAH-DOX was evaluated in HT1080 cells by MTT assay (Fig. 3A). The cytotoxicity of CTAB coated GNR was reduced upon conjugation with MMP-SQ, as a large portion of the CTAB was replaced with MMP-SQ. A maximum cytotoxic effect was observed upon laser irradiation after 4 h of incubation with MMP-SQ@GNR@LAH-DOX. The superior cytotoxic effect observed under laser irradiation highlights the combined effects of PTT and chemotherapy with the MMP targeted nanoprobe. However, a much less cytotoxic effect was observed in the normal fibroblast cell 3T3-L1 incubated with the nanoprobe (Fig. S12†). These

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**Fig. 2** SERS spectra of MMP-SQ@GNR at different time intervals measured under confocal Raman microscope (A) in presence of MMP-2 enzyme and (B) in presence of MMP-2 inhibitor. (C) Temperature changes of GNR, MMP-SQ@GNR, and MMP-SQ@GNR@LAH-DOX irradiated with an 808 nm laser at a power density of 0.1 W cm\(^{-2}\) as a function of time. (D) Fluorescence emission spectra of MMP-SQ@GNR@LAH-DOX in acetate buffer (pH 5.0, 0.02 M) at different time intervals.
results indicated the targeting efficiency of MMP-SQ@GNR@LAH-DOX towards HT1080 cells. To investigate the cell death mechanism induced by our nanoprobe in cancer cells, various apoptotic assays were conducted. In comparison with the respective controls, acridine orange/ethidium bromide dual staining displayed a change in colour from green to yellow/red, which is associated with other apoptotic features such as the presence of apoptotic bodies, damaged cell membranes and nuclear condensation upon treatment (Fig. 3B). In concordance with the toxicity study, cells treated with the targeted theranostic nanoprobe after irradiation displayed pronounced indications of apoptosis. Morphological changes observed with staining were further confirmed by Annexin V-FITC assay, using flow cytometry. HT1080 cells were treated with MMP-SQ@GNR@LAH-DOX for 4 h with or without laser irradiation, and labelled with PI, as well as Annexin V. The percentage of apoptotic cells after drug treatment or negative control are represented in Fig. 3C. There was a significant (P < 0.001) increase in the Annexin V positivity with the combined effect of DOX and laser irradiation upon treatment with MMP-SQ@GNR@LAH-DOX. The results showed that MMP-SQ@GNR@LAH-DOX with laser irradiation more significantly induced apoptosis (63%) compared with those treated by laser alone (9%) or MMP-SQ@GNR@LAH-DOX alone (54%). The cytotoxic studies unravelled the superior effect of the targeted nanoprobe upon laser irradiation through the induction of apoptosis.

Raman spectral investigation of chemical modifications has proven to be a genuine alternative to detection of biological changes at the molecular level related to cell death.31 We have demonstrated SERS as a detection tool for identifying the minute changes in biochemical components of organelles inside cells subjected to photothermal-chemotherapy. The differential changes of the SERS spectral pattern of cells before and after therapy were analyzed (Fig. 4).

A closer inspection of the spectra revealed unique modifications in Raman features of several vibrations associated with cellular components. Significant spectral changes were observed at positions characteristic to nucleic acid and protein vibrations after therapy. The spectra revealed pronounced bands at 833, 994, 1145, 1274, and 1450–1650 cm\(^{-1}\), which were assigned to the O–P–O backbone of DNA, proteins, deoxynucleotides, C–H deformation of guanine and adenine and N7–H stretching of guanine and adenine, respectively.32 The molecular vibration most sensitive to cell death was the O–P–O stretching of the DNA at 833 cm\(^{-1}\). The 833 cm\(^{-1}\) O–P–O backbone vibration decreased, while the 1450–1650 cm\(^{-1}\) N7–H vibration of guanine and adenine significantly increased after therapy. The increase in guanine and adenine vibrations after therapy is associated with their increased exposure to the plasmonic field of the SERS substrate during cell death due to the detachment of DNA from histone proteins. We have carried out time and dose dependent SERS spectral profile evaluation to track the apoptosis mechanism (Fig. S15–S17†). The spectral profile of HT1080 cells incubated, with MMP-SQ@GNR@LAH-DOX, with and
without laser irradiation were analysed to investigate the effect of chemotherapy and combined photothermal chemotherapy. Chemotherapy was initiated after 4 hours of incubation, as reflected the gradual decrease of the peak at 838 cm\(^{-1}\), where within 10 minutes the intensity rapidly diminished and the peak was missing after photothermal chemotherapy. The intensity of the 1550–1650 cm\(^{-1}\) band increased considerably in all cases and the increase was very fast in the case of photothermal chemotherapy. It is known that an increase in Raman intensity often occurs when DNA bases become unstacked. These alterations suggest DNA double strand break.

To validate this assumption, DNA isolated from HT1080 cells before and after photothermal-chemotherapy was investigated for differential SERS spectral pattern. The spectra revealed pronounced bands at 700, 795, 838, 955, 1025, 1137, 1296, 1347, and 1405–1568 cm\(^{-1}\), which were assigned to the nucleic acids, adenine, cytosine, thymine, O–P–O backbone of DNA, proteins that remained in the DNA extract after isolation, deoxynucleotides, C–H deformation of guanine and adenine, N\(_7\)–H stretch of guanine and adenine, respectively (Fig. 5A). After therapy, the peak at 838 cm\(^{-1}\) for the O–P–O backbone vibrations decreased, whereas the 1405–1568 cm\(^{-1}\) N\(_7\)–H vibration of guanine and adenine significantly increased, which is in accordance with the result obtained from whole cell study.

Thus, it is evident that apoptotic DNA fragmentation occurred during photothermal-chemotherapy, which can be effectively monitored through SERS. DNA fragmentation during therapy was further confirmed by agarose gel electrophoresis (Fig. 5B). Our nanoprobe induced DNA ladder formation through photothermal chemotherapy. DNA laddering is a feature observed in an apoptotic event wherein the DNA is broken down into multiple fragments that can be viewed by agarose gel electrophoresis. Therefore, a conclusive agreement was established between SERS based monitoring for apoptotic DNA fragmentation and DNA ladder formation.

Conclusions

The current investigation revealed the application of the therapeutic nanoprobe MMP-SQ@GNR@LAH-DOX as an excellent cancer-targeted cytotoxic agent for photothermal-chemotherapy assisted by SERS fingerprinting. The cumulative impact of radiation and chemotherapeutic agent produced cytotoxicity through a programmed cell death mechanism in a targeted fashion. Molecular events during cell death, from the single whole cell scale to the DNA level, were investigated through the SERS platform. Although further perspectives are required in a complex multifaceted disease such as cancer, the current investigation reveals the potential of SERS based diagnosis as a future strategy for monitoring therapeutic efficiency.

Acknowledgements

AcSIR Ph.D. student, Ms Nisha N expresses thanks to the Council of Scientific and Industrial Research (CSIR), and Lakshmi V. Nair acknowledges SCTIMST for the Research Fellowships. Dr K. K. Maiti greatly acknowledges the Science and Engineering Research Board (SERB, no. SR/S1/OC-67/2012), CSIR, New Delhi [12th FYP project on Molecules to Materials to Devices (M2D) (CSC 0134) and Nanomaterials: Applications and Impact on Safety, Health and Environment (NanoSHE), (BSC 0112)] and Department of Biotechnology (DBT, no. BT/PR14698/NNT/28/832/2015) for financial assistance. R. S. Jayasree is thankful to ICMR for supporting the project on GNR (5/13/128/2011/NCD-III).

Notes and references

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