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Unveiling NIR Aza-BODIPY Dyes as Raman Probes: SERS-Guided Selective Detection and Imaging of Human Cancer Cells

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Development of novel Raman reporters attracted immense attention in diagnostic research based on surface enhanced Raman scattering (SERS) techniques, a well established modality for ultrsensitive detection through molecular fingerprinting and imaging. Herein, for the first time, we report the unique and efficient Raman active features of the selected aza-BODIPY dyes **1-6**. These distinctive attribute could be extended at the molecular level detection through SERS upon adsorption onto the nano-roughened gold surface. Among the newly revealed Raman reporters, the amino substituted derivative, **4**, showed significantly high signal intensity at very low concentrations (*ca*. 0.4 μM for **4-Au**). Interestingly, an efficient nanoprobe has been constructed using gold nanoparticles as SERS substrate, and **4** as the Raman reporter (**4-Au@PEG**), which unexpectedly showed efficient recognition of three human cancer cells (lung: A549, cervical: HeLa, Fibrosarcoma: HT-1080) without any specific surface marker. We observed well reflected and resolved Raman mapping and characteristic signature peaks wherein, such recognition was not observed in a normal fibroblast (3T3L1) cells. In addition, to reconfirm the fact, SERS nanoprobe was conjugated

with a specific tumour targeting marker, EGFR (Epidermal Growth Factor Receptor), a well known targeted agent for Human Fibrosarcoma (HT1080). This nanoprobe efficiently targeted to the surface marker of HT 1080 cells, and thus demonstrating its use as an ultrasensitive Raman probe for the detection and targeted maging, leaving the normal cells unaffected.

1. Introduction

The sensitive detection of specific biomarkers for different oncological and non-oncological diseases has attracted immense attention in the medical diagnostic field.[1-5] The detection of multiple biomarkers from the complex biological matrices within the fastest possible time frame for efficient disease diagnosis is one of the challenges in current scenario.[6-7] Even though a vast variety of techniques exist for the detection and to monitor the progress of treatment, many of them failed to provide a vivid explanation of the structural consequence from its molecular perspectives during the treatment.[8-9] A sensitive method, which is capable of providing an in-depth understanding of the changes at cellular level during therapy, can provide new insights to optimize therapeutic efficiency.

Surface Enhanced Raman Spectroscopy (SERS) recently emerged as a well-established detection modality for the disease diagnosis that could improve upon the conventional methods.[10-13] It possesses single-molecule detection potentially accessible with noble-metal structures or substrates (typically a gold or silver nanoparticle). It is evolved as a versatile alternative to the common fluorescence techniques since it offers the potential to minimize photobleaching, peak overlapping, and low signal-to-noise ratio in complex biological systems.[14-16] In recent years, successful outcome have been noticed on cancer biomarker detection in patient's blood, saliva or serum samples or on target cells by means of SERS fingerprinting.[17,18] Such an instance, a label-free blood test method based on SERS was reported for nasopharyngeal, gastric and colorectal cancer detection.[19-21] The design, preparation and characterization of SERS-active substrates and Raman reporters, are one of

the most prominent evolutionary areas in Raman spectroscopy. In addition to the frequently used commercially available Raman reporters such as crystal violet (CV), malachite green isothiocyanate (MGITC), 4-mercaptobenzoic acid (4-MBA), Rhodamine 6G, diethylthiatri-carbocyanine (DTTC), there has been an increased interest in the development of novel Raman signature molecules for the diverse diagnostic and imaging applications.[22-25]

We have recently reported squaraine based Raman reporters for the construction of theranostic nanoprobe for photothermal-chemotherapy and an unique Raman probe by tetraphenyl ethylene (TPE) derivatives to detect the prostate specific antigen in LnCaP cell lines assisted by SERS fingerprinting.[26-28] Despite the diverse attempts made to discover new Raman reporter molecules, most of the reported ones suffer from several disadvantages such as low resolution, short wavelength absorption and high fluorescence background, which may mask the Raman signals. The aza-BODIPY dyes, one of the closest analogues to the widely used BODIPY dyes are characterized by their strong NIR absorption, high photostability and low fluorescence quantum yields.[29-35] These dyes are familiar for their excellent triplet excited state quantum yields and tuneable singlet oxygen generation efficiency.[36-38] The synthetic flexibility of these dyes make them attractive for diverse applications such as sensitizers for photooxygenation, photodynamic therapy as well as the probes for anions, cations and neutral molecules.[39-41]

However, no aza-BODIPY dyes are reported till date as Raman signature molecules may be due to their poor solubility in aqueous medium and difficulty to incorporate with nanomaterial substrate such as gold or silver nanoparticles. Considering these in mind, we have investigated the functionalized aza-BODIPY dyes **1-6** and unveiled their potential as effective Raman reporters for the first time in presence and absence of gold nanoparticles (Figure 1). Notably, the amino substituted aza-BODIPY, **4** showed surprisingly strong Raman signal, even in the absence of a metallic substrate, which turned out as a new generation Raman probe. Additionally, we have successfully demonstrated the potential of aza-BODIPY

dye, **4** to construct SERS nanotag for the selective detection and imaging of a set of human cancer cell lines, and the targeted SERS imaging of the human fibrosarcoma cell lines by tagging the monoclonal antibody against EGF receptor (EGFR).

<Insert Figure 1>

2. Results and Discussion

Synthesis of the halogenated aza-BODIPY derivatives **1-3** and the N-substituted derivatives **4-6** was achieved in good yields starting from their corresponding chalcones by following previously reported procedures.[36-41] The chalcones undergo nitromethane addition to get the nitro adduct, which upon reaction with ammonium acetate to form the aza-dipyrromethene followed by complexation with BF_3-OEt_2 to yield the target aza-BODIPY dyes (Schemes S1-S2, Supporting Information). The intermediates and target molecules were characterized by spectral and analytical evidences.[36-41] The halogenated derivatives exhibited strong absorption in the range of *ca*. 600-750 nm and fluorescence at *ca*. 700-850 nm, whereas the N-substituted dyes showed much larger red shifted absorption at *ca*. 700-800 nm (Figure 2A, Figure S1, Supporting Information). To investigate the efficiency of aza-BODIPY dyes as Raman probes, we have recorded the Raman spectra of the bare reporter molecules **1-6** in 1% DMSO-H_2O under a WI-Tec Raman microscope with a wavelength of 633 nm laser excitation and 20X objective. The single spectra were collected from the CCD detector with an integration time of 0.5 s and 10 accumulations. The aza-BODIPY derivatives **1-6** showed characteristic well resolved Raman spectra under similar experimental conditions with high signal to noise ratio (Figure 2B, Figures S2-S6, Supporting Information).

<Insert Figure 2>

The amino substituted aza-BODIPY, **4** exhibited well resolved intense Raman spectral profile with good signal to noise ratio and better hydrophilicity and photostability. Raman peaks obtained from the dye, **4** and corresponding peak assignments were tabulated (Table S2,

Supporting Information). The aza-BODIPY dye **4** exhibited a strong signal in the range of 1600-1610 cm^{-1} attributing to the C=N-C stretching frequency of the azomethine bonds. The lowest detectable limit of Raman intensity for the amino dye, **4** was determined from the concentration dependent experiments which gave a very low value of 4 μM (Figure 2C). Interestingly, the amino derivative, **4** showed enhanced Raman signal intensity, when compared to the most commonly used Raman reporters, such as Rhodamine B, Crystal Violet, which definitely mark the importance of the aza-BODIPY dyes as commercial Raman signatures for bio-imaging applications (Figure 2D).

As the aza-BODIPY dyes, showed remarkable Raman intensity upon irradiating with 633 nm, it is of our interest to examine the SERS enhancement of these dyes in presence of nanogold surface. The amino aza-BODIPY dye, **4** (30 μM) was mixed with as prepared Au-NPs (size *ca*. 40 nm) in 1:9 ratio (v/v) (See experimental section for the details of AuNP preparation). The mixture was incubated for 10 min to get maximum adsorption of the aza-BODIPY on Au-colloids through both chemisorption and physisorption. We observed that the dye, **4** binds strongly with the Au surface through the amino groups as well as through the electrostatic interactions. The formation of the gold colloid, **4-Au** was confirmed through the shift in the absorption spectra with respect to the parent aza-BODIPY dye as well as the colloidal Au solution (Figure S7, Supporting Information). The SERS enhancement has been investigated as described earlier, and reflected around *ca*. 10-fold increment in intensity as compared to normal Raman intensity observed by the bare molecule, **4** (Figure 3A). The SERS intensity was high enough when compared to the commercially available Raman reporters (Figure S8, Supporting Information), which reveals the efficiency of the dye **4** as a Raman signature molecule. The sensitivity of **4-Au** was determined from the concentration dependent SERS experiments and found to be as low as 0.4 μM (Figure 3B) which is higher than that of Rhodamine B (1 μM) and Malachite green (2.3 μM) under similar conditions (Table S1, Supporting Information).

<Insert Figure 3>

Further, we have constructed a straight forward biocompatible SERS nanotag with aza-BODIPY and colloidal nano gold to promote SERS mapping (imaging) and spectral demarcation of both cancer and normal cell lines. The biocompatibility of the colloids was achieved by the efficient encapsulation with PEG (**4-Au@PEG**) and examined their signal stability over days. We observed substantial stability of the nano-construct at 25 ^{o}C. The PEG encapsulation was carried out by modifying the previously described protocols.[42,43] The heterocyclic functional linker HS–PEG–COOH (M.W PEG: 3000 Da) was added to **4-Au** solution and then Au-colloid was exposed to excess of PEG-SH (M.W PEG: 5000 Da) for 15 min. Subsequently the aza-BODIPY reporter, **4** was efficiently incorporated into the thin PEG layer and confirmed by UV–Vis absorption spectroscopy, which showed a shift in absorption maxima *ca*. 4 nm compared with **4-Au** (Figure 4A). The HR-TEM analysis of **4-Au@PEG** showed a thin layer of PEG of thickness *ca*. 2–3 nm around gold nanoparticle surface with the increment in size from 43 nm to 46 nm after PEG encapsulation (Figure 4B). The DLS measurements also support the observation of TEM experiments for bare gold nanoparticles having diameter of *ca*. 40-45 nm. However, the PEG encapsulation leads to a much broader distribution of the particles, which may be due to the effect of surrounding aqueous medium (Figure S9, Supporting Information).

<Insert Figure 4>

The presence of Raman reporter molecule inside the PEGylated nanoconstruct was further validated by the consistent SERS signals with enhanced spectral intensity (Figure 4C). This was probably due to the better incubation of the molecule inside the PEG layers, which generate more hot spots. The time course SERS study was conducted to grade the stability of SERS intensity from the nano-construct and observed that SERS signal from the reporter

molecule remained stable over a period of one month (Figure 4D). It is expected that a strong interaction occurred between the aza-BODIPY core and PEGylated nanoparticle, with a superior molecular orientation of the Raman probe over the nanotag.

We have explored the targeted Raman imaging of PEGylated Raman probe, **4-Au@PEG** in cancer cell line and invented the specificity of the Raman probe in live cancer cells. *In vitro* cellular detection and their imaging capability has been investigated in three different cancer cell lines, HeLa (Human breast cancer), A549 (Human lung cancer) and HT1080 (Human fibrosarcoma) and a normal cell line, 3T3L1 as a control with the aid of a confocal Raman microscope. For cellular imaging, 20 μL of **4-Au@PEG** was added to cells in a chamber slide and was incubated at 37°C for 1 h. Single spectral analysis and mapping study was carried out after washing with PBS buffer to remove the unbound nanotags. SERS mapping was recorded by focusing the laser beam on the cell surface mapping area along X and Y directions. Figure 5A-C represents the bright field, Raman and cluster images of HeLa cell lines and Figure 5D-F and Figure 5G-I are the corresponding bright field, Raman and cluster images of A549 and HT1080 cancer cell lines. The cluster analysis performed based on characteristic Raman spectral scan on each cancer cell lines where it is presumed that the Raman nanotag's probable localization in the vicinity of the cell surface region. The localization of **4-Au@PEG** in the vicinity of cell surface is more evident from the three dimensional Raman and single spectral analysis, which gives the characteristic SERS peaks of the aza-BODIPY probe, **4-Au@PEG** (Figure S10, Supporting Information). Further, we have examined the potential localization of the Raman probe in normal 3T3L1 cells under similar conditions where **4-Au@PEG** was incubated as described previously. Interestingly, the Raman spectral and imaging pattern mostly resembled the presence of nanotags only in the buffer background and no such recognition has been observed in the cell surface or intracellular region (Figure S11, Supporting Information). Therefore, we unfolded an

interesting observation that showed the selective recognition of **4-Au@PEG** in different cancer cells evidenced the merit of the Raman signature of the aza-BODIPY probe, **4**.

<Insert Figure 5>

Finally, we have investigated and validated the selective recognition of the Raman probe **4-Au@PEG** for *in vitro* targeted cellular detection and imaging of human fibrosarcoma (HT1080) cancer cells. In this direction, **4-Au@PEG** has been conjugated with mouse monoclonal antibodies against epidermal growth factor receptor, EGFR which shows an over expression mostly in the cell surface of fibrosarcoma cancer cell lines. This antibody used to recognize the external epitope on respective antigens. Successful antibody conjugation was achieved by the well-documented EDC-NHS coupling strategy (See Experimental Section for details).[42] The heterofunctional PEG was covalently conjugated with the free amine terminal of the antibody, which was confirmed by UV-Vis absorption spectroscopy. The absorption spectra of antibody conjugated nanotag showed a protein absorption peak nearly around 280 nm, which is a clear indication of the antibody conjugation of the SERS nanotag **4-Au@PEG-EGFR** (Figure 4A). Further, we examined the recognition of HT1080 cancer line using antibody conjugated SERS nanotag, **4-Au@PEG-EGFR**. The experiment was carried out by incubating Raman tag with HT1080 cells in a chamber slide for 1 h and the SERS spectral pattern and mapping was recorded after washing with PBS to remove unbound nanotags. The presence of **4-Au@PEG-EGFR** accumulation in the cell surface was evidently observed through the single spectral analysis in which we observed the fingerprint SERS peaks of the probe (Figure S12, Supporting Information). Bright field images of the representative cells with the corresponding Raman image and color coded 3D Raman image are shown in Figure 6A-C, which represents the efficient recognition of the antibody conjugated aza-BODIPY probe, **4-Au@PEG-EGFR** on the surface of HT1080 cells. The cluster mapped image after the analysis of characteristic Raman image by two clusters (**a** & **b**)

are shown in Figure 6D. The spectra abstracted from the cluster image exhibited characteristic fingerprint SERS peaks of the nanotag from the cell surface (**a**) and not from the nucleus (**b**) which suggest their effective localization in the surface of HT1080 cells (Figures 6E). The histogram image (Figure 6F) shows the statistical representation of the most intense peaks coming from the Raman reporter (I_{1606}/I_{1342}) chemisorbed on the gold nanoparticle substrates. It is clearly observed the recognition of the targeted nanotag reflecting strong mapping images, wherein the **4-Au@PEG-EGFR** is mostly localized on the cell surface demonstrating the selectivity of the aza-BODIPY **4** based Raman probe for targeted recognition of cancer cells. Further, we have confirmed the effective binding of **4-Au@PEG-EGFR** in EGFR positve HT1080 in comparison with normal cell line 3T3L1 which has a minimum expression of EGFR. The comparative binding experiment was carried out by incubating the Raman probe **4-Au@PEG-EGFR** with mouse fibroblast 3T3L1 cells where EGFR expression is quite less[44] under the same experimental condition. Interestingly, there is no such recognition has been observed in the cell surface or intracellular region (Figure S13, Supporting Information) which proves that the probe is actually binding to EGFR receptors on the membrane surface.

<Insert Figure 6>

3. Conclusion

In summary, we have invented a series of NIR absorbing aza-BODIPY dyes with varying substitution at the peripheral phenyl rings as Raman probes with multiplexing signal pattern. The amino substituted derivative, **4** showed highest Raman signal intensity, with sensitivity of 4 μM, having excellent signal to noise ratio. Interestingly, the aza-BODIPY-gold nanoparticle conjugate, **4-Au** exhibited enhanced Raman signals with extremely high sensitivity reflecting the detection as low as 0.4 μM. Further we have identified the specific recognition of **4-Au@PEG** in different cancer cells such as HeLa, A549 and HT1080 over

normal 3T3L1 cells**.** An unique targeted SERS nanoprobe, **4-Au@PEG-EGFR** has been constructed in conjugation with target specific EGFR antibodies, which confirmed the specific recognition on HT1080 cell lines. The nanoprobe, **4-Au@PEG-EGFR** efficiently recognized the human fibrosarcoma cell lines, leaving the normal cells unaffected, thereby facilitating its ultrasensitive detection and targeted Raman mapping. Our results demonstrate the potential of the functionalized aza-BODIPYs as Raman reporters, which can be used as a marker bio-imaging and diagnostic detection modalities.

4. Experimental Section

Gold Nanoparticle Synthesis: For the synthesis 40 nm gold nanoparticles, water was heated until boiling, then added chloroauric acid solution ($HAuCl_4$) and boiled again for 10 min. After 10 min, sodium citrate solution was added, resulting in the colour change from pale yellow to dark purple. The colour change to red will take around 5 min and heating was stopped to let the solution to cool for 90 min to 25 ^{o}C. The whole reaction was accompanied with constant stirring by a Teflon coated magnetic stir bar at 400 rpm. The as formed gold nanoparticles were purified by centrifugation and characterized by the surface plasmon resonance through UV visible spectroscopy.

Transmission Electron Microscopy (TEM) Analysis: TEM analysis was performed on JEOL 100 kV high resolution transmission electron microscope (HRTEM). The samples in aqueous medium were drop casted on the top of carbon-coated Cu grid. The samples were dried by a vacuum pump under reduced pressure for 1 h at 25 ^{o}C. The accelerating voltage of the transmission electron microscope was 100 kV and the beam current was 65 A. Samples were imaged using a Hamamatsu ORCA CCD camera.

Dynamic Light Scattering (DLS) Analysis: The DLS studies were carried out on a Nano Zeta Sizer, Malvern instruments. The samples were prepared in water/PBS buffer at required

concentrations. The light scattering experiments were performed under low polydispersity index by using glass cuvettes. The hydrodynamic diameters and polydisperse indices of the samples were determined using a Malvern Zeta Nano-ZS system.

Antibody Conjugation and In Vitro Raman Imaging: The antibodies were purified by filtration using Amicon Ultra 3K centrifuge filters (Milipore) to remove sodium azide. The carboxylic groups of the PEG encapsulated nanotag were activated by EDC N-(3-(dimethylamino)-propyl)-N-ethylcarbodiimide (EDC, 25 mM) and N-hydroxysuccinimide (NHS, 25 mM). After 30 min incubation, excess of EDC and NHS were removed by 3 rounds of centrifugation (8000 rpm, 15 min), and re-suspended in PBS. The activated nanotag was incubated with antibody EGFR (25 mL, 200 mg/ mL) at 25 °C for 2 h. The antibody conjugated nanotag was stored at 4 °C overnight. Further unbound antibodies were removed by a five round of centrifugation at 10000 rpm for 5 min. The pellet containing the antibody conjugated nanotag was then again re-suspended in PBS and stored at 4 °C.

Evaluating the efficacy of a aza-BODIPY as a Raman reporter was done with the aid of a confocal Raman microscope (WI-Tec, Inc., Germany) with a laser beam directed to the sample through 20 X objective with a Peltier cooled CCD detector. For cellular imaging 20 µL (1mM) of 4-Au@PEG-EGFR was added to cancer and normal cells and was incubated at 37°C for 1 h. SERS mapping was recorded by focusing the laser beam on the cell surface selected at a position z = 0 µm using 0.5 as integration time, 150 x 150 as points per line and 50 × 50 µm mapping area along X and Y directions. The cellular mapping were acquired over a motorized scan stage. Raman images were subsequently subjected to cluster mapping, a statistical analysis where a set of similar objects are grouped for the better understanding about the distribution of the Raman probe molecule in different sub cellular region.[45]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Figure captions

Figure 1. A) Schematic representation of the targeted SERS imaging using aza-BODIPY attached to NPs. B) Structures of the halogenated (first row) and N-substituted (second row) aza-BODIPY derivatives **1-6** investigated.

Figure 2. A) Normalised absorption and fluorescence spectra of **4** in DMSO (10 µM). B) Characteristic Raman spectra of the aza-BODIPY derivative, **4** in 1% DMSO-H_2O mixture (10 µM). C) Concentration dependent Raman signals of the bare aza-BODIPY, **4** for the calculation of limit of detection D) Relative Raman signals of **4** with respect to the commercially available Raman reporters Rhodamine B (**Rh**) and Crystal violet (**CV**) at 10 µM.

Figure 3. A) Comparative Raman spectra for the derivative **4**, and **4-Au** in 1% DMSO-H_2O mixture (10 µM). B) Concentration dependent SERS spectrum of **4-Au** for the calculation of limit of detection.

Figure 4. A) Absorption spectra of aza-BODIPY-Au colloid (**4-Au**), **4-Au@PEG** and **4-Au@PEG-EGFR** in aqueous medium. B) TEM images of the AuNPs and **4-Au@PEG** colloids under similar conditions. C) Single spectral characterization of different constructs. D) Stability measurements of the construct **4-Au@PEG** for 5 weeks in 1% DMSO-H_2O mixture.

Figure 5. A) Bright field, B) Raman, C) cluster image of **4-Au@PEG** incubated with *HeLa* cells. D), E) and F) are the same in *A549* cells after the incubation of **4-Au@PEG**. G), H) and I) correspond to *HT1080* cells under similar experimental conditions.

Figure 6. A) Bright field image, B) Raman image, C) color coded 3D Raman image, D) cluster mapped Image of **4-Au@PEG-EGFR** incubation with HT1080 cell line, E) single spectra abstracted from the cluster mapped region a and b in the cluster mapped image, F) histogram corresponds to the most intense peak coming from the Raman reporter (I_{1606}/I_{1342}).

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We unfold the unique Raman activity of aza-BODIPY dyes at the molecular level as well as their SERS upon adsorption onto nano-roughened gold surface. The nanoprobe successfully target to HT1080 cell lines, thus facilitates its effective detection and Raman mapping.

Keywords: aza-BODIPY, SERS, cancer detection, cellular targeting, Raman mapping