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A green and facile approach for synthesizing imine to develop optical biosensor for wide range detection of bilirubin in human biofluids

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ABSTRACT

Bilirubin, a key biomarker for the jaundice and its clinical diagnosis needs a better analytical tool. A novel and simple fluorescent platform based on (2,2′-((1E,1′E)-((6-bromopyridine-2,3-diyl) bis(azanylylidene)) bis(-methanylylidene diphenol) (BAMD) was designed. BAMD showed a remarkable fluorescent intensity with a very good quantum yield of 0.85 and lifetime of 870 ps. Hence, it was applied for the determination of bilirubin using both colorimetric and fluorimetric techniques in physiological and basic pH. Under optimized experimental conditions, the probe detects bilirubin selectively in the presence of other interfering biomolecules and metal ions. The linear range of detection is 1 pM–500 μM at pH=7.4 and LOD is 2.8 and 3.3 pM at pH=7.4 and 9.0, respectively, which were reported so far. The probe detects the bilirubin through FRET mechanism. The practical application of the probe was successfully tested in the human blood and urine samples. Based on all above advantages, this simple idea can be applied to design a simple clinical diagnostic tool for jaundice.

1. Introduction

During the enzymatic reaction of heme oxygenase enzyme on heme system, the precursor biliverdin is produced and then it is further processed by biliverdin reductase to covert the final bilirubin. Generally bilirubin is classified into three major categories as direct, (conjugate with glucuronic acid), indirect (not conjugated with glucuronic acid) and total bilirubin (direct and indirect bilirubin) (Cheifetz et al., 2010). Once it is conjugated in the liver, bilirubin is excreted into the bile and transported through the gut with food and further broken down, contributing to the color of stool (Thor and Ruud, 2010). It is a real threat full factor among humans to diagnosis the jaundice because of its acute fatal. The normal level of bilirubin in human blood is < 25 μmol (< 1.2 mg/dL) and in jaundice condition the level is increased to > 50 μmol/L (> 2.5 mg), which finally leads liver disorder (Silbernagl and Despopoulos, 2009). Considering these serious health issues, an accurate detection of bilirubin concentration in human serum is clinically becoming more important. Earlier, some methods include enzymatic assay using bilirubin oxidase (Morimoto et al., 2000) and vanadate oxidation assays (Ameri et al., 2011) had been used to detect the bilirubin in human serum. Currently, most available clinical methods are measuring the conjugated (direct) and total bilirubin using a classic spectrophotometric method based on the endpoint diazo reactions. However, it has some drawbacks like less stability, degradation, decoloration etc., (Rand and di Pasqua, 1962).

After a keen review of literatures reported, it is clearly understood that only a very few biosensors have been reported for the detection of bilirubin so far. Various electrochemical biosensors with different biocompatible platforms containing nanomaterials and bilirubin oxidase (BOx) have been used for the detection of bilirubin (Kannan et al., 2011; Feng et al., 2013; Batra et al., 2013;). For example, by utilizing BOx enzyme on gold micro electrode, a simple and selective sensor has also been reported (Jagriti et al., 2015). Recently, a non-enzymatic bilirubin (BR) sensor has also been developed based on spin coated reduced graphene oxide (RGO)–poly styrene sulfonate (PSS) composite film on a glassy carbon electrode (Balamurugan and Sheela, 2015).

Fluorimetric is also a powerful analytical tool to detect various toxic metal ions and biomolecules owing to its operational simplicity and very high sensitivity with a wide analyte concentration range. (Komatsu et al., 2007; Nolan and Lippard, 2007; Yoon et al., 2005; Kwon et al., 2005; Wang et al., 2006; De Silva et al., 1997; Miao et al., 2016; Xiao et al., 2013). A fluorimetric biosensor based on Au nanoparticles-conjugated HSA protein was reported for the sensing of bilirubin in human serum (Santhosh et al., 2014). In this study, the quenching emission profile of human serum albumin (HSA) stabilized gold nanoclusters was observed upon interaction with bilirubin. But for

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the first time, another research group has reported fluorescent biosensor using water soluble polyfluorene polymer for the bilirubin detection with the limit of detection in nanomolar range (150 nM) in human blood plasma (Senthilkumar and Asha, 2015).

In general, imine based fluorescent molecules show a very good response towards various metal ions (Soojin et al., 2012). But, biomolecule concern, only a very few reports are available especially for amino acids since from the last decades (Ellen and Timothy, 2003). Further, no imine based fluorescent sensor is reported so far for most important biomolecules like dopamine, ascorbic acid, creatinine and bilirubin. Generally, most of our disease causing pathogens and biomolecules could have a tendency to bind with hydroxyl groups of glucose unit present in our body (Cuihua et al., 2006). Generally, these disease relating pathogens or biomolecules contains functional groups like amide, primary or secondary amines etc. and they are having a tendency to form hydrogen bonding with the hydroxyl group which is present in the carbohydrates. Similar type of intermolecular hydrogen bonding is also possible between organic molecules containing –OH and amines groups (Ravi et al., 2015; Oscar et al., 2011; Leo and Kline, 1950; Ambler, 1929; Reem and Moustafa, 2010; Gary and Ronald, 1980; Atsushi et al., 1996). We have synthesized a simple new imine 2,2′-((1E,1′E)-((6-bromopyridine-2,3-diyl)Bis (azanylylidene)) bis(-methanylylidene)) diphenol based fluorescent molecule and applied for the determination of bilirubin since it has hytroxy groups. The structure and photophysical studies of the synthesized molecule were done using various analytical techniques. Compare to the previous reports, our synthetic scheme is very simple, cost effective, less time consuming (10 min) and also followed a green synthetic route. We have designed a fluorescent biosensor based on the above BAMD moiety for the determination of direct bilirubin at two different pHs (7.4 and 9.0) and it was found that the biosensor was sensitive and selective towards bilirubin in the presence of other interfering biomolecules and metal ions. The detection limit and linear range of the biosensor towards bilirubin is very good comparing to earlier reports.

2. Materials and methods

2,3 - Diamino-5-Bromo Pyridine, Salicyaldehyde, Glacial Acetic acid, HPLC grade ethanol, Bilirubin, Uric Acid, Urea, Creatinine, Creatine, L-Dopamine, Ascorbic Acid, D-Glucose, and various acetate salts of metal ions (Zn^{2+}, Cd^{2+} Ca^{2+}, Ag^{+}, Na^{+}, K^{+}, Fe^{2+} Fe^{3+}, Co^{2+}, and Cu^{2+}) were purchased from Sigma Aldrich and used as such. The human serum was isolated from the blood of the students at Department of Chemistry, School of Biotechnology, Madurai Kamaraj University, Madurai.

^{1}H and ^{13}C NMR analysis was done using $CDCl_3$ and DMSO-d_6 as solvents containing a trace quantity of tetramethylsilane (TMS) as internal standard using Bruker- 300 MHz spectrometer and chemical shifts were reported in ppm at 25 °C. Mass spectra and UV spectra were recorded. Using ESI ionization in MS-LCMS mass spectrometer and SHIMADZU single beam UV–vis spectrophotometer, respectively. Fluorescent measurements were done using Cary Eclipse spectrophotometer having a 450 W xenon lamp. Fluorescence lifetime decays were counted using FLS 980 setup from EDINBURGH INSTRUMENTS U.K, with a λmax=435 nm) having a full width at half-maximum of 89 ps as a sample excitation source. The 5 nm and 2.5 nm were maintained as excitation and emission slit widths, respectively, throughout the experiments.

3. Synthesis and characterization of 2,2′-((1E,1′E)-((6-bromopyridine-2,3-diyl) bis(azanylylidene)) bis(methanylylidene)) diphenol (BAMD)

Usually irradiation with high intensity sound or ultrasound, acoustic cavitations occurs and results the implosive collapse of bubbles. These bubbles have temperature around 5000 K and pressure of roughly 1000 atm that are actually not achieved by normal or conventional synthetic method which requires more reaction time. So herein, we have used sonochemical method for the synthesis of BAMD. Briefly, 2,3 –diamino-5 bromo pyridine (1.0 g, 5 mmol, 1 equiv.)] was dissolved in 10 mL of absolute ethanol (solution) and mixed with solution (II) which was prepared by dissolving salicyaldehyde (1.3 g, 10 mmol, 2 equiv) in 5 mL of ethanol solution and then 0.5 mL of glacial acetic acid was added into the mixture. The mixture becomes slightly brown color and it was sonicated for 10 min and a dark-yellow colored solution was obtained. The resulting solution was allowed to cool at room temperature and a yellow product (III) was formed (Fig. S1.). The crude product was washed twice with ethanol and diethyl ether and dried over $MgSO_4$ under vacuum. The completion of product formation was confirmed by Thin Layer Chromatography (appearance of single spot). The yield was found to be 1.97 g (98%). We have performed the same reaction under normal reflux condition for 3 h and obtained only 92% yield. So, to the best of our knowledge, this method is first fast method for the synthesis of imine. Meanwhile, we have also compared the yield of the molecules with imine molecules prepared by sonochemical method reported previously. Only 90% yield was obtained at 15 min previously. (Christabel and Cheng-Wei, 2009; Thomas et al., 2009; Anjali et al., 2013; Cintas and Luche, 1999; Mason, 1997; Cravotto and Cintas, 2006). In order to confirm the reliability of the method, the reaction was repeated for 3 times and obtained the same results.

The NMR data for the synthesized imine are given below. ^{1}H NMR (300 MHz, $CDCl_3$, 298): δ=13.20 (s, 1 2 H), 12.57 (s, 2 H), 9.45 (s, 1 H), 8.61 (s, 1 H), 7.68 (d, 1 H), 7.50 (m, 7 H) ppm.^{13}C NMR (300 MHz, $CDCl_3$, 298 K): δ =166.68 (C8 and C8′), 162.59 (C13 and C13′), 147.55 (C6) 140.64(C5), 134.84(C2), 134.08(C3), 133.12(C4), 119.80(C9 and C9′), 119.63(C10 and C10′), 119.20(C11 and C11′), 118.25(C12 and C12′), 118.07(C14 and C14′). $C_{19}H_{14}BrN_3O_2$ (calculated mass 396): observed mass $[M + H]^{-}$ =395 [Fig. S2a, S2b & S3].

4. Results and discussion

4.1. Photo physical properties of BAMD molecule

The normalized UV–Vis absorption spectra of BAMD was recorded in phosphate buffer at pH=7.4. The absorption spectra shows two absorbance maxima at 297 and 435 nm corresponding to π-π* and n-π* transitions, respectively (Fig. S4a). Similarly, the fluorescent property of BAMD molecule was studied at 435 nm as excitation wavelength at neutral pH (7.4). The BAMD exhibited a sharp fluorescence peak at 550 nm with very good fluorescence intensity (Fig. S4b). Then, effect of pH on fluorescent property of the BAMD was also studied in different buffer solutions (from pH 2–12.5). When the pH was adjusted from 2 to 6, there was a gradual increment in fluorescent intensity up to pH (7.4). But, on further increase from 7.4 to 12.5, the fluorescent intensity decreased along with blue shift (Fig. S6). The maximum fluorescent intensity was obtained at pH=7.4. Hence, all spectroscopic measurements were carried out in phosphate buffer (pH 7.4). This phenomenon may be due to that at low pHs, BAMD may be existing in protonated form which leads to inhibition of electronic charge transfer among the molecule and results a poor increment in fluorescent intensity. But under neutral medium, the molecule emits very high intensity due to the availability of lone pair electrons of hydroxyl group of BAMD for complete electron delocalization. Meanwhile, in basic medium, deprotonation may lead to the internal charge transfer process among the molecule and it is clearly supported by observing a remarkable blue shift along the decrement in intensity and results in green fluorescent color (Masayasu et al., 2008; Lin et al., 2009; Ellairaja et al., 2015).

The quantum yield of BAMD was calculated using the data obtained from on the UV and Emission under optimized condition, as 0.85 (λ_{ex}=435 nm). In this study, Rhodamine 6 G was used as a standard in