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Copper and nickel doped carbon dots for rapid and sensitive fluorescent turn-off detection of bilirubin

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Carbon dots doped with metals and non-metals have gained much popularity due to the enhancement in their optical and electronic properties. In this study, polyethyleneimine-functionalized transition metal (nickel or copper) doped carbon dots (CD, NiCD and CuCD) were synthesized through hydrothermal method. The carbon dots exhibited a blue fluorescence at 470 nm when excited at 350 nm. The as-synthesized carbon dots were utilised for the fluorimetric detection of bilirubin in the range 0.5 μM – 280 μM , with CuCD exhibiting the highest sensitivity of 155.38 a.u./log μM in the concentration range 0.5 to 10 μM and 84.01 a.u./log μM in the concentration range 10 to 280 μM . CuCD also exhibited the lowest limit of detection of 0.0907 μM and the lowest limit of quantification of 0.3023 μM . All the carbon dots showed negligible interference in the presence of biomolecules and metal ions present in human serum implying the remarkable selectivity of the method to bilirubin detection. Further, the carbon dots were successfully tested for their real-time application in human serum using bilirubin-spiked serum samples.

Keywords Carbon dots, Copper-doped carbon dots, Nickel-doped carbon dots, Bilirubin, Fluorescence quenching, Biosensor

Carbon dots (C-dots) have recently gained enormous attention in the field of biosensors. C-dots are a type of zero-dimensional carbon nanomaterial having a size of less than 10 nm which exhibit unique properties such as a stable fluorescence, electrical conductivity, low cytotoxicity, and good biocompatibility^{1,2}. The unique fluorescence property of C-dots has been exploited for the development of highly sensitive probes for the detection of various analytes. The physicochemical properties of carbon dots have been enhanced by doping with heteroatoms such as nitrogen, phosphorous, sulphur, fluorine etc. and metals such as Cu, Zn, Mg, Au, Ag etc³. The enhancement in properties of carbon dots is significant upon doping with metals in comparison to the commonly used heteroatoms, as metals are better electron donors⁴. Thus, the introduction of metals as dopants in C-dots could change their electronic structure, modulating the HOMO-LUMO energy gap and the optical properties³. In this context, metal-doped carbon dots have been employed for the detection of bilirubin.

Bilirubin (BR) is an important liver function biomarker produced by the catabolic breakdown of heme from senescent red blood cells. In tissues, bilirubin is transported by its tight binding with serum albumin forming water-insoluble unconjugated (indirect) bilirubin which is transported to the liver to form a conjugate with glucuronic acid to produce the water-soluble conjugated (direct) bilirubin. The conjugated bilirubin is channelized for its excretion through the kidneys and gut⁵. Hyperbilirubinemia is a medical disorder characterized by elevated levels of bilirubin in the bloodstream, which can indicate the potential for various health issues. The reference levels for total bilirubin are 1–12 μM , with a mean conjugated bilirubin level of 3 μM ⁶. A bilirubin level of 25 μM in neonates is considered dangerous and requires monitoring. Serum bilirubin is also a dependable indicator for detecting rejection in individuals receiving liver transplants. Thus, the prompt diagnosis and treatment of hyperbilirubinemia plays a major role in improving the lives of patients.

The most widely used conventional methods for the detection of bilirubin in serum are spectrophotometry based diazo method. In the diazo method, the soluble conjugated bilirubin reacts directly with diazotized sulphanilic acid produced by the reaction between sulfanilic acid and sodium nitrite in acidic medium to form

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azobilirubin which absorbs light at 550 nm, whereas the unconjugated bilirubin reacts with the diazo reagent only indirectly in the presence of an accelerator such as alcohol for its solubilization^{7,8}. Various other techniques for detecting bilirubin include chromatography, electrophoresis, electrochemistry, spectrophotometry, transcutaneous bilirubinometry, urine test strips, smartphone applications etc⁹.

Paper-based colorimetric assays suitable for point-of-care testing have been developed for the detection of various biomarkers as well as analytes in food and water^{10–13}. Paper analytical devices for bilirubin have been developed by utilizing the reducing nature of bilirubin leading to the formation of gold nanoparticles from chloroauric acid as the colorimetric reagent and the capability of bilirubin to form a chelate complex with copper(II) ions^{14,15}. An electrochemical detection of bilirubin based on voltammetry technique using screen-printed carbon electrode has been reported¹⁶. Various non-enzymatic bilirubin sensors based on nanomaterials such as graphene oxide/PANI, ceria nanocubes etc. have been reported^{17,18}.

Among the spectrophotometric techniques used, fluorescence-based biosensing approaches have garnered significant interest in recent times because of their sensitivity, simplicity, and specificity¹⁹. Ascorbic acid functionalised copper nanoparticles have been used as a turn-off fluorescent sensor for bilirubin by static quenching and IFE mechanisms²⁰. N-doped carbon quantum dots synthesized from citric acid as carbon source and nitric acid as nitrogen source have been used for the detection of bilirubin by FRET²¹. Fluorescence tests with carbon dots produced from different carbon sources have become increasingly prominent in recent times for detecting various molecules and the reports on the detection of bilirubin using carbon dots and other fluorescent nanomaterials are emerging^{20,22–27}.

Metal doped carbon dots represent a promising alternative to traditional methods of bilirubin detection such as spectrophotometry and paper-based assays. Unlike, spectrophotometry, which often displays limited sensitivity and interference from other biological molecules, the metal doped C-dots offer superior selectivity and sensitivity to detect bilirubin at much lower concentrations. The metal-doped carbon dots when incorporated in to paper-based assays which are portable, make an excellent candidate for highly sensitive detection of bilirubin while maintaining the cost-effectiveness and ease-of-use. Rapid response times, good photostability, and adaptability to be easily integrated into advanced sensing platforms further elevates their potential as an efficient and versatile tool for bilirubin detection, particularly in point-of-care and resource-limited settings. This work aims to demonstrate the unique capabilities of metal-doped C-dots as a highly efficient solution for clinical and diagnostic applications of bilirubin detection.

In this work, a single-step, one-pot hydrothermal method was used to synthesize fluorescent carbon dots to detect bilirubin. Polyethyleneimine was used as a precursor for the synthesis of nitrogen-doped carbon dots. To enhance the fluorescence properties and sensitivity of the polyethyleneimine-derived carbon dots, metal ions such as nickel and copper were introduced during the synthesis to yield nitrogen and metal-co-doped C-dots. In this work, copper and nickel doped C-dots were synthesized from Cu²⁺-PEI and Ni²⁺-PEI complexes as the precursors. Complexes of polyethyleneimine with copper was prepared by A. S. Ozerin et al. by mixing 0.001 M aqueous CuSO₄ solution and 0.01 M aqueous PEI solution with vigorous stirring resulting in a final solution pH of 8.2 and Cu²⁺/PEI molar ratio of 0.1²⁹. Hence, we chose to prepare the precursor metal-PEI complex in the same molar ratio to prepare copper-doped C-dots, as it was observed that after the formation of complex of PEI with Cu²⁺ ions, there are no more functional groups on PEI available for further complexation with metal ions ensuring an efficient complexation of the metal with PEI^{28,29}. To study the effect of doping different transition metals on carbon dots for sensing bilirubin, the same procedure was followed for the preparation of Ni²⁺-PEI complex and subjecting it to hydrothermal method of synthesis to produce nickel-doped C-dots.

The novelty of using copper- and nickel-doped carbon dots for bilirubin detection lies in the unique ability of copper and nickel to interact with bilirubin to form coordination complexes^{30,31}. The introduction of metal centres, copper and nickel enhances the binding affinity and specificity of the assay for bilirubin, significantly improving the detection performance of the doped carbon dots^{32–35}. The synthesized C-dots were highly selective to bilirubin compared to other biomolecules and metal ions present in human serum.

Experimental

Chemicals

Bilirubin conjugate, dopamine, uric acid, creatinine, urea, L-ascorbic acid, glucose, glycine, L-alanine, L-cystine, L-aspartic acid, Polyethyleneimine branched (PEI), vitamin B12, and human serum were procured from Sigma Aldrich (www.sigmaaldrich.com). Sodium chloride (NaCl), potassium chloride (KCl), zinc sulphate heptahydrate (ZnSO₄·7H₂O), calcium chloride dihydrate (CaCl₂·2H₂O), aluminium chloride (AlCl₃), cobalt chloride hexahydrate (CoCl₂·6H₂O), magnesium chloride hexahydrate (MgCl₂·6H₂O), ferric chloride (FeCl₃), ferrous chloride (FeCl₂), nickel nitrate (Ni(NO₃)₂), nickel sulphate hexahydrate (NiSO₄·6H₂O) and copper sulphate pentahydrate (CuSO₄·5H₂O) were obtained from Actylis Lab Solutions (www.actylislab.com). Mini Dialysis Kit, 1 kDa from GE Healthcare has been used for purification of the carbon dots. All aqueous solutions required for experiments were prepared using MilliQ water and direct light exposure was avoided while handling bilirubin.

Instrumentation

The UV-visible absorption spectra were obtained using a UV-Visible spectrophotometer (Shimadzu, Japan) and the PL spectra were recorded using a Cary Eclipse Fluorescence spectrophotometer (Agilent, USA) in microplate reader mode. The FTIR spectra were recorded using FTIR Spectrophotometer Alpha II (Bruker, US). XRD analysis was carried out using Malvern Panalytical AERIS Benchtop X-ray diffractometer and Energy-dispersive X-ray spectroscopy (EDX) was carried out in GEMINI 300, Carl Zeiss, Germany. JOEL JEM 2100 High-Resolution Transmission Emission Microscope (HRTEM) was employed to obtain the HRTEM images of carbon dots. The Zeta Potential analysis was carried out using Malvern Panalytical Zetasizer instrument.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) test was conducted using 7800 ICP-MS instruments from Agilent Technologies was used to confirm the presence of nickel and copper in C-dots. Fluorescence lifetime and quantum yield studies were performed in Edinburg Instruments FLS 1000.

Method of data analysis

The sensing method was evaluated by monitoring the changes in fluorescence emission intensity (PL intensity) of the carbon dots with the addition of bilirubin or corresponding analytes. Regression analysis was performed to evaluate the relationship between the PL intensity and logarithm of concentration of bilirubin, to determine the key analytical parameters such as linearity, limit of detection (LOD), and limit of quantification (LOQ). Statistical analysis parameters such as mean, standard deviation (SD) and standard deviation of mean (SDoM) were calculated for triplicate measurements at each analyte concentration to ensure the reproducibility and accuracy of the method. Calibration curves plotted were employed to determine the sensitivity, LOD and LOQ of the developed sensor. The coefficient of determination (R^2) value was calculated to validate the goodness of fit of the calibration curves, further supporting the reliability of the analytical method. Additionally, any anomalies or deviations were critically assessed to minimize systematic errors.

Synthesis of carbon dots

The C-dots were synthesized by a one-step hydrothermal method. Polyethyleneimine-derived C-dots were synthesized using a 30 mL solution of 0.02 M polyethyleneimine (PEI) as the precursor. For the synthesis of transition metal-doped carbon dots, a 30 mL PEI-metal complex (PEI-M^{2+}) was prepared by mixing aqueous solutions of copper sulphate or nickel sulphate with PEI in a molar ratio of 0.1 followed by stirring for 1 hour and ultrasonication for 15 minutes³⁶. The polyethyleneimine-copper complex (PEI-Cu^{2+}) exhibited a deep blue colour whereas the polyethyleneimine-nickel complex (PEI-Ni^{2+}) exhibited a pale pink colour. The precursor solutions were then transferred to a Teflon-lined autoclave and kept in the muffle furnace at a temperature of 200 °C for 8 h. After the complete cooling of the reaction mixture to room temperature, the obtained carbon dots were centrifuged at 10,000 rpm for 10 min to remove any unreacted impurities in the solution as precipitate. The C-dots were further subjected to syringe filtration using a 0.2 μm filter and dialysis using the Mini Dialysis Kit containing a dialysis membrane with a Molecular Weight Cut-Off (MWCO) of 1 kDa for 3 h as per the procedure mentioned in the kit. The C-dots suspension was stored at 4 °C in a refrigerator for further utilization.

Detection of bilirubin using the synthesized carbon dots

Different concentrations of bilirubin were added to the C-dots in an equal proportion and tested in a microplate reader. The selectivity of the C-dots to bilirubin was evaluated by adding other potential interference biomolecules and metal ions present in human serum. To explore the feasibility of the proposed method for the detection of bilirubin in complex biological samples, human serum spiked with different concentrations of bilirubin was also tested.

Results and discussion

Characterization of carbon dots

The optical and electronic properties of the aqueous solution of C-dots - CD, NiCD, and CuCD were investigated from the UV-Vis absorbance and fluorescence spectrophotometric studies conducted at ambient temperature. As shown in Fig. 1A-C, the absorbance spectra revealed two peaks at around 269 nm and 321 nm for CD, 270 nm and 320 nm for NiCD, and 270 nm and 325 nm characteristic for CuCD which can be attributed to the $\pi\text{-}\pi^*$ transition of aromatic sp^2 C=C bond and $\text{n-}\pi^*$ transition of C=O bond and heteroatom doping^{37,38}. The insets of Fig. 1A-C show the images of CD, NiCD, and CuCD in visible and UV illumination. All three carbon dots exhibited blue fluorescence under UV illumination at 365 nm (inset Fig. 1A-C). The fluorescence emission intensity (PL intensity) of carbon dots increased with metal doping as evidenced by Fig. 1A-C with the maximum intensity for CuCD compared to NiCD and CD.

The maximum wavelength of excitation of the carbon dots was determined by recording the fluorescence emission spectra at different excitation wavelengths from 300 to 360 nm as shown in Fig. S1 (Supplementary

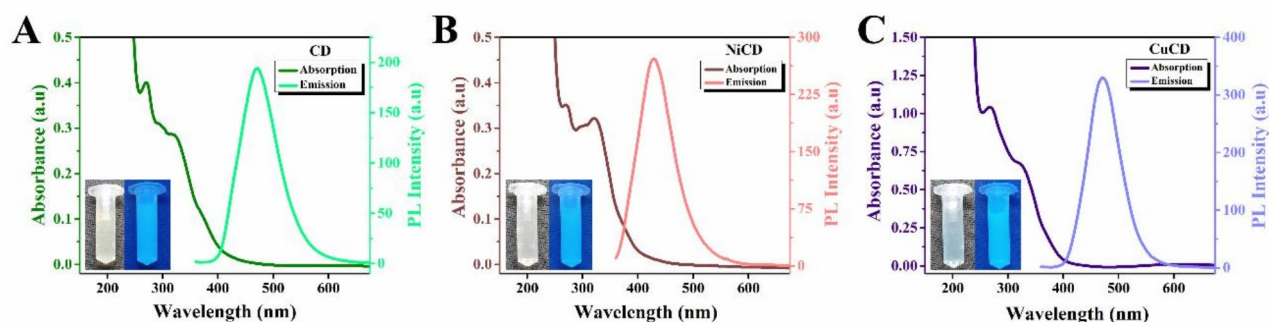


Fig. 1. UV-Vis absorption and PL spectra of (A) CD, (B) NiCD, and (C) CuCD at excitation wavelength 350 nm.

Material). A strong PL emission was observed at 470 nm corresponding to an excitation wavelength of 350 nm for CD, NiCD, and CuCD. As the excitation wavelength was changed from 300 to 350 nm, a redshift was exhibited with an increase in the PL emission intensity. However, a further increase in the excitation wavelength led to a reduction in the PL emission intensity. This excitation-dependent emission behaviour of the carbon dots could be attributed to the quantum confinement, surface defects or trap states with different energy levels^{39–42}.

The morphological characterization including the particle size of carbon dots was carried out using HR-TEM analysis and the obtained results are depicted in Fig. 2A–C. The HR-TEM micrographs of the three carbon dots CD, NiCD and CuCD exhibited a spherical morphology with average particle sizes of about 4 nm, 6 nm and 5.8 nm respectively as depicted in Fig. 2D–F. The interplanar spacing (d-spacing) of the C-dots CD, NiCD and CuCD are 0.217 nm, 0.24 nm and 0.211 nm respectively and are depicted in the insets of Fig. 2A–C. The doping of copper and nickel in the C-dots were confirmed by ICP-MS (Table S1 Supplementary Material) and EDX analysis (Fig. S2 Supplementary Material).

The XRD analysis of the carbon dots CD, NiCD, and CuCD exhibited broad diffraction peaks at 2θ of 20.15°, 20.09°, and 20.03° respectively as shown in Fig. 3A due to the existence of graphitic carbon peaks of carbon dots⁴². The diffraction peaks centred at around $2\theta = 20^\circ$ is attributed to the turbostratic carbon phase and the broadness of the peaks due to amorphous nature of carbon in carbon dots^{43,44}.

The FT-IR spectra of C-dots revealed peaks corresponding to the major functional groups present in the carbon dots (Fig. 3B). The broad peaks at 3353 cm^{-1} and 3286 cm^{-1} for CD, 3357 cm^{-1} and 3281 cm^{-1} for NiCD, and 3348 cm^{-1} and 3290 cm^{-1} for CuCD correspond to the stretching vibrations of hydroxyl (-OH) and amine (-NH₂) groups on the surface of carbon dots. The vibrational stretching frequency of C=O occurs at 1620 cm^{-1} for CD, 1625 cm^{-1} for NiCD, and 1629 cm^{-1} for CuCD⁴⁵. The peaks at 2949 cm^{-1} and 2833 cm^{-1} for CD, 2944 cm^{-1} and 2818 cm^{-1} for NiCD and 2939 cm^{-1} and 2823 cm^{-1} for CuCD are ascribed to the stretching vibrations of C-H. The distinguishable peaks at 1457 cm^{-1} and 1321 cm^{-1} of CD, 1461 cm^{-1} and 1316 cm^{-1} of NiCD, and 1456 cm^{-1} and 1307 cm^{-1} of CuCD can be assigned to C=C and C-N stretching vibrations⁴⁵. The peaks at 1119 cm^{-1} and 1056 cm^{-1} for CD, 1124 cm^{-1} and 1057 cm^{-1} for NiCD and 1124 cm^{-1} and 1052 cm^{-1} for CuCD correspond to C-O and C-O-C stretching vibrations^{38,45}.

Fluorescence detection of bilirubin using carbon dots

The fluorescence-based sensing performance of the synthesized carbon dots CD, NiCD and CuCD towards the detection of bilirubin was studied by adding different concentrations of laboratory bilirubin samples ranging from 0.5 to 280 μM to the carbon dots. The PL emission intensity of carbon dots was quenched with the addition of bilirubin, accompanied by a red shift (about 60 nm) in the emission spectra with the increase in the concentration of bilirubin as depicted in Fig. 4A–C for CD, CuCD and NiCD⁴⁶. A closer examination of absorption spectrum of bilirubin and the emission spectra of carbon dots reveals a considerable spectral overlap resulting in a decrease in the PL intensity which can be attributed to inner filter effect (IFE)⁴⁶. IFE is a non-irradiation energy conversion resulting from the absorption of emission or excitation of the fluorophore by the absorber or quencher in the detection system^{47,48}. This spectral overlap causes a quenching of the short-wavelength range of emission spectra with an increase in the concentration of bilirubin resulting in a red-shift in the emission spectra⁴⁶. IFE mediated concentration dependent red-shifts (CDRS) has been used as an analytical tool for the detection of various analytes.

The decrease in PL emission intensity of C-dots in the presence of bilirubin in the concentration range 0.5–280 μM is shown in Fig. 4A–C and the corresponding calibration plots depicted in Fig. 4D–F revealed a dual linear response of the PL emission intensity with the log of concentration of bilirubin. The non-linearity of PL response with increase in bilirubin concentration may be attributed to the IFE and red-shifting of emission maxima due to re-absorption effects⁴⁹. The sensitivities of C-dots have been tested and a comparison of their sensitivities resulted in an observation of the highest sensitivity for CuCD having 155.38 a.u./log μM in the concentration range 0.5 to 10 μM and 84.01 a.u./log μM in the range 10 to 280 μM compared to CD and NiCD as represented in Table S2 (Supplementary Material). The images of vials containing the carbon dots with increasing concentrations of bilirubin, and the quenching of fluorescence of carbon dots with increasing concentrations of bilirubin have been represented in Fig. 4G–I.

The fluorescent C-dots thus developed for the sensing of bilirubin were compared to analyse the most appropriate and sensitive C-dot for bilirubin detection. The limit of detection (LOD), limit of quantification (LOQ) and sensitivities of the developed carbon dots-based bilirubin sensors were calculated and depicted in Table S2 (Supplementary Material) using Eqs. 1 and 2.

The limit of detection (LOD) was calculated using the formula,

$$LOD = \frac{3 SD}{m} \quad (1)$$

And, the limit of quantification (LOQ) was calculated using the formula,

$$LOQ = \frac{10 SD}{m} \quad (2)$$

where, SD is the standard deviation of the lowest concentration detected and m is the slope of the calibration plot.

From the results depicted in Table S2, it is clear that CuCD exhibits the lowest detection limit of 0.0907 μM and the lowest limit of quantification of 0.3023 μM compared to CD and NiCD. The sensitivity of CuCD was also higher than CD and NiCD in the dual linear ranges 155.38 a.u./log μM (0.5–10 μM) and 84.01 a.u./log μM

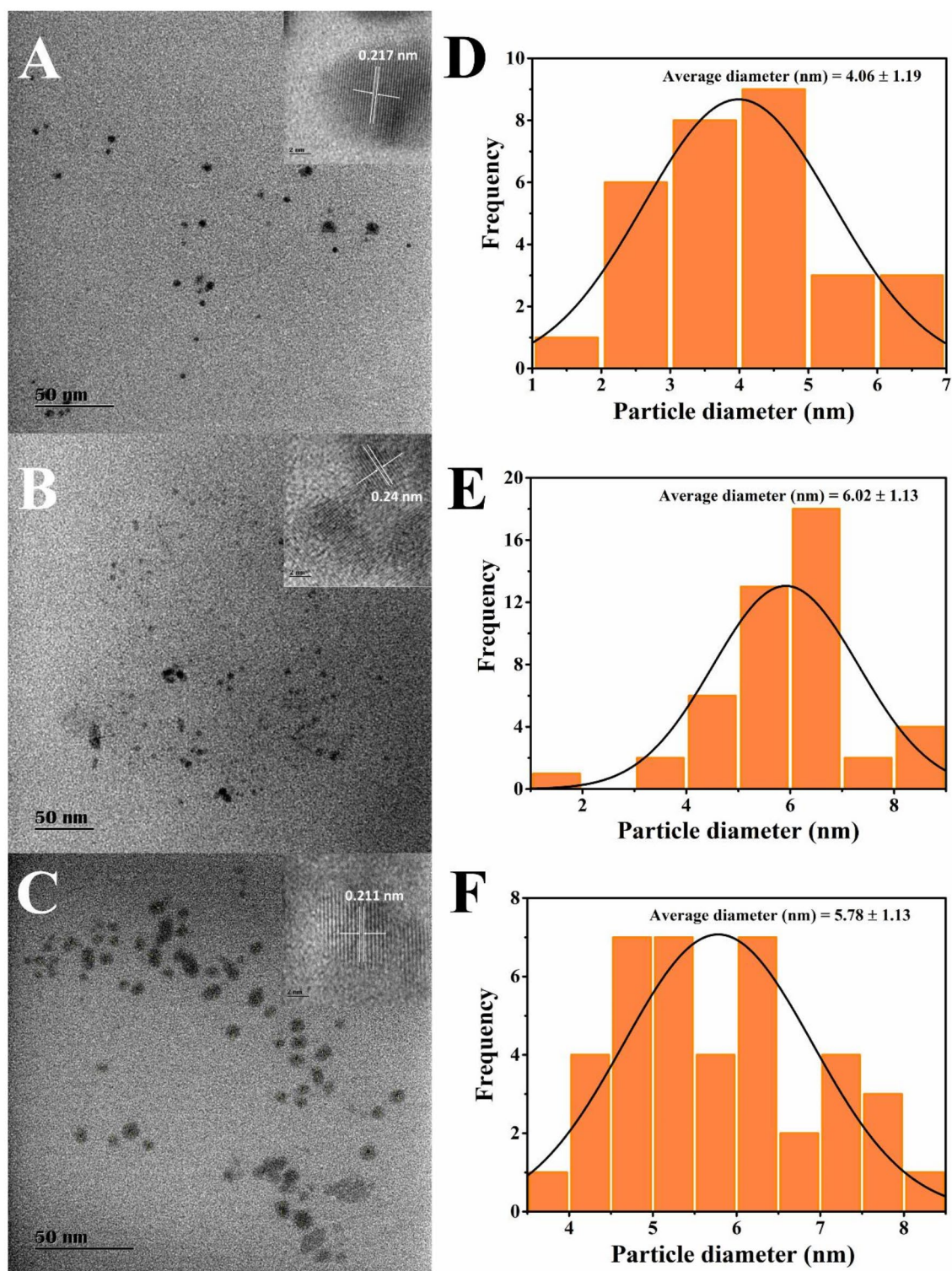


Fig. 2. HR-TEM images of (A) CD, (B) NiCD, and (C) CuCD and the particle size distribution histograms of (D) CD, (E) NiCD and (F) CuCD. (The insets depict the interplanar spacing (d-spacing) of the corresponding C-dots).

(10–280 μ M). Since CuCD has demonstrated excellent sensing parameters, it can therefore be concluded that CuCD can successfully be used for even low levels of bilirubin detection with exceptional sensitivity.

Some of the reported fluorescent materials and mechanisms for the detection of bilirubin have been compared based on the concentration ranges, LOD and LOQ with the proposed sensor and has been represented in Table 1.

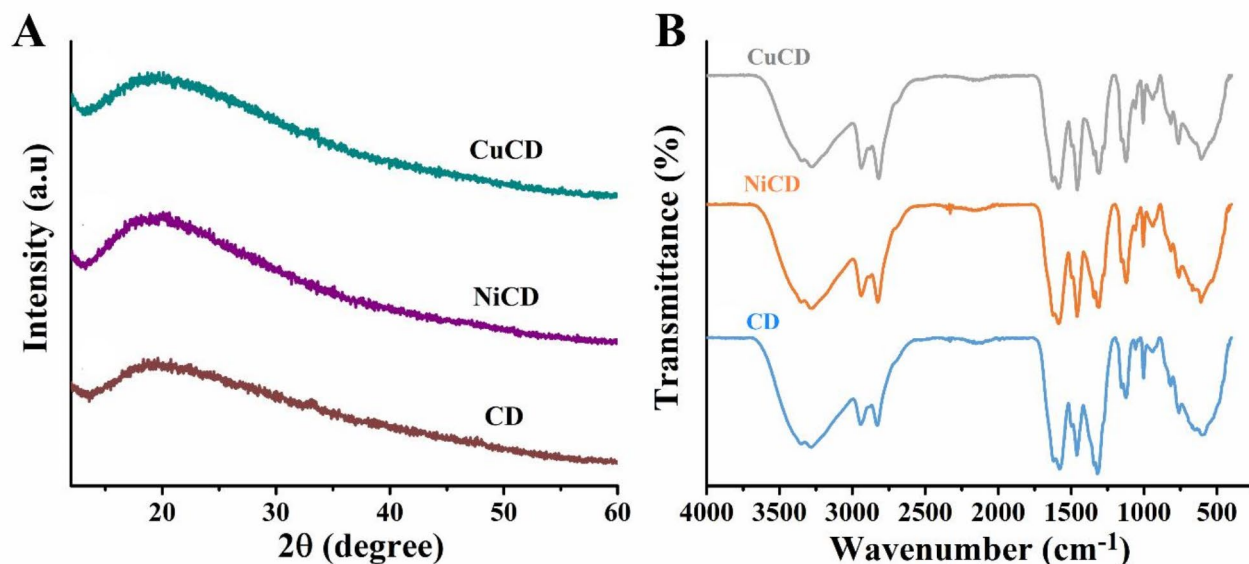


Fig. 3. (A) XRD and (B) FT-IR spectra of CD, NiCD and CuCD.

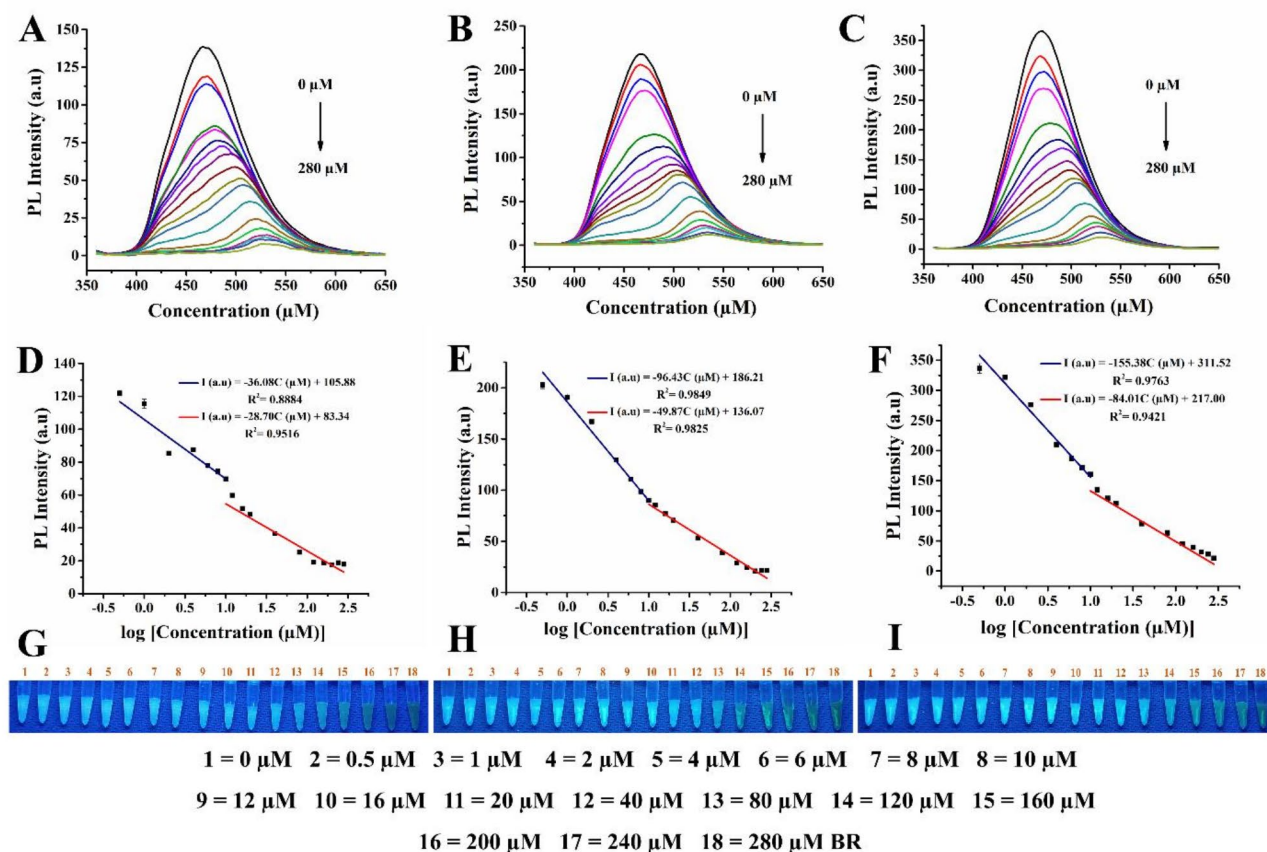


Fig. 4. PL emission spectra of the carbon dots (A) CD (B) NiCD and (C) CuCD with increasing concentration of bilirubin from 0.5–280 μM ($\lambda_{\text{ex}} = 350 \text{ nm}$). Dual linear plots of log of concentration of bilirubin (μM) with PL intensity of carbon dots (D) CD (E) NiCD and (F) CuCD in the ranges 0.5–10 μM and 10–280 μM. Photographs depicting the quenching of fluorescence of carbon dots (G) CD (H) NiCD and (I) CuCD with increasing concentrations of bilirubin under UV illumination.

Material	Concentration range	LOD	LOQ	Detection method	Reference
N-CQDs	1.35–14.20 μM	4.86 μM	-	Fluorescence quenching (FRET)	21
NCDs	8.319–161.290 μM	0.32 μM	1.08 μM	Fluorescence quenching (IFE)	27
Benzylamine capped CH ₃ NH ₃ PbBr ₃ perovskite quantum dots	0.71–24.21 μM	0.42 μM	-	Fluorescence quenching (FRET)	50
Copper nanoparticles protected by ascorbic acid (AACuNPs)	1.00 × 10 ⁻⁵ M – 5.00 × 10 ⁻⁷ M	0.248 μM	-	Fluorescence quenching (Static quenching assisted by IFE)	20
CuCD	0.5–280 μM	0.0907 μM	0.3023 μM	Fluorescence quenching (Static quenching and IFE)	This work

Table 1. Comparison of some of the reported fluorescent methods for bilirubin detection with the proposed sensor.

It is evident that the proposed work has produced comparable analytical results with a very low detection limit and a wide concentration range compared to the existing methods reported in literature.

Plausible mechanism of fluorescence quenching of carbon dots by bilirubin

The various possible mechanisms of quenching fluorescent materials include static quenching, dynamic quenching, inner filter effect (IFE), photoinduced electron transfer (PET), fluorescence resonance energy transfer (FRET) and so on⁴⁸. The static quenching mechanism is based on the formation of a non-fluorescent ground-state complex between the carbon dots and the quencher, in which the complex returns to the ground state almost immediately after the absorption of light without the emission of photons. To determine the quenching mechanism, Stern-Volmer equation (Eq. 3) was used to plot graphs with F_0/F vs. Concentration of BR (in μM).

$$\frac{F_0}{F} = 1 + K_a [Q] \tag{3}$$

where F_0 and F are the fluorescence intensities observed in the absence and presence of the quencher, respectively, K_a is the association constant of the complex and $[Q]$ is the concentration of the quencher. The plot of F_0/F vs. Concentration of BR exhibits a linear relationship in case of pure static quenching mechanism and an upward curvature in case if both static and dynamic quenching occur in the sample. However, the Stern-Volmer plots of F_0/F vs. Concentration of BR obtained in this study exhibited non-linear curve with a slight downward curvature (as depicted in Fig. S3 Supplementary Material), ruling out the possibility of pure static quenching as well as dynamic quenching mechanisms. Hence, the trend observed could be attributed to a system having accessible and inaccessible fluorophores or surface groups which is responsible for the quenching of fluorescence in the presence of the quencher (bilirubin)^{51,52}. The fluorescence lifetimes of carbon dots were also measured to acquire more insights into the quenching mechanism involved. The average lifetimes of CD before and after the addition of bilirubin were 4.569 ns and 4.580 ns, the average lifetime of NiCD before and after the addition of bilirubin were 4.881 ns and 4.776 ns, and the average lifetime of CuCD before and after the addition of bilirubin were 4.740 ns and 4.667 ns. The average lifetimes of all the carbon dots with and without the addition of bilirubin have remained almost unchanged suggesting a static quenching mechanism (Fig. S4 Supplementary Material).

The quenching effect may also be due to IFE, because the emission spectra of carbon dots overlap with the absorption of bilirubin as evidenced by Fig. S5 (Supplementary Material). Moreover, the net charges present on the surface of carbon dots were analysed by Zeta potential analysis (Table S3 Supplementary Material) and the results revealed higher positive values for NiCD and CuCD which may be due to the doping of nickel and copper ions on the surface of carbon dots⁵³. Due to the higher affinity of copper to bilirubin and the relative positive surface charge of CuCD, the sensitivity of CuCD for the detection of bilirubin is better than NiCD and CD^{15,32}.

Selectivity of carbon dots to fluorescent detection of bilirubin

To investigate the performance of the synthesized metal-doped carbon dots towards bilirubin detection over other potential biomarkers present in human serum, a selectivity analysis was conducted. Testing biomolecules and metal ions is critical to ensure the selectivity of the bilirubin detection system based on carbon dots due to the complex composition of biological samples such as blood and serum. Biomolecules like glucose, uric acid, creatinine, ascorbic acid etc. present in these matrices exhibit high chances of interaction with carbon dots or bilirubin, potentially causing non-specific binding or cross-reactivity to cause fluorescence quenching, leading to inaccurate results. Similarly, metal ions such as Fe³⁺, Mg²⁺, Cu²⁺ etc. can further affect the optical properties of carbon dots through ionic interactions or redox reactions, influencing the performance of the developed sensing system. By systematically testing the effect of these potential interfering substances, the specificity and selectivity of the bilirubin sensor can be ensured, while also verifying its robustness, practicality and reliability in real-world biological systems. Triplicates ($n=3$) of the experiments were conducted to study the effects of different biomolecules and metal ions on the selectivity of carbon dots to bilirubin. The response of potential interference substances including biomolecules and metal ions at elevated concentrations were tested. In the bar diagrams, blank indicates the PL intensity of the corresponding carbon dots, while the responses of each of the biomolecules and metal ions are represented sequentially. Biomolecules such as uric acid (UA) – 560 μM, ascorbic acid (AA) – 200 μM, creatinine (Crt) – 240 μM, dopamine (DA) – 500 pM, glucose (Glu) – 8 mM, urea (Urea) – 10 mM, glycine (Gly) – 500 μM, L-alanine (L-Ala) – 500 μM, L-aspartic acid (L-Asp) – 20 μM, L-cystine (L-Cys) – 100 μM, and vitamin B12 (Vit. B12) – 500 pM were tested and compared with 20 μM bilirubin (BR). Figure 5

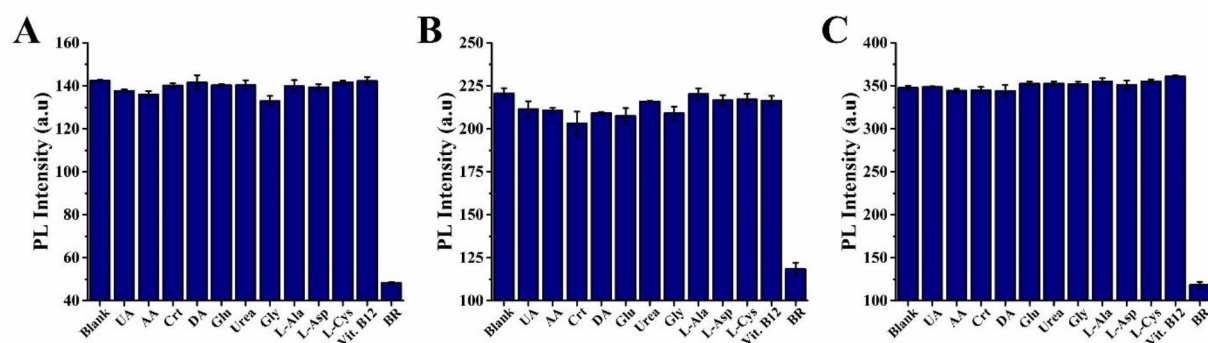


Fig. 5. Selectivity study of the carbon dots (A) CD (B) NiCD and (C) CuCD towards potential interference biomolecules in serum uric acid (UA), ascorbic acid (AA), creatinine (Crt), dopamine (DA), glucose (Glu), urea (Urea), glycine (Gly), L-alanine (L-Ala), L-aspartic acid (L-Asp), L-cystine (L-Cys), and vitamin B12 (Vit. B12) in comparison with bilirubin.

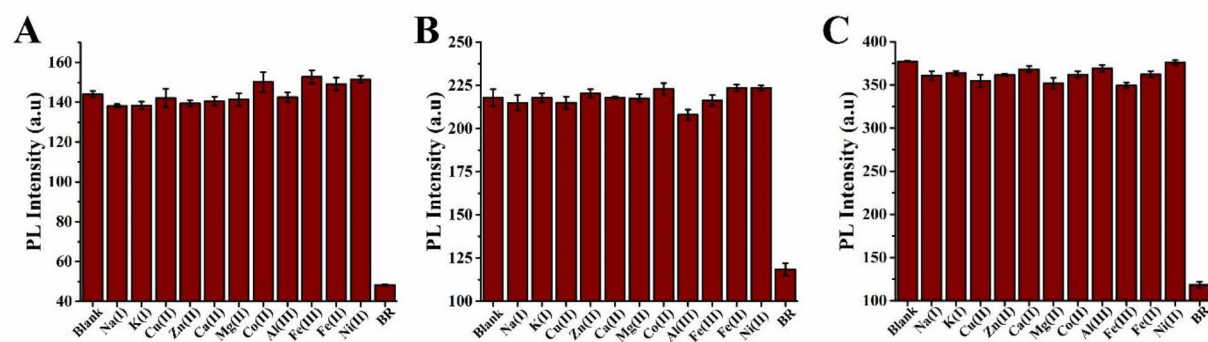


Fig. 6. Selectivity study of the carbon dots (A) CD (B) NiCD and (C) CuCD towards potential interference metal ions in serum Na(I), K(I), Cu(II), Zn(II), Ca(II), Mg(II), Co(II), Al(III), Fe(III), Fe(II), and Ni(II) in comparison with bilirubin.

demonstrates the effect of various tested biomolecules on the fluorescence intensity of the carbon dots. The PL intensity of blank (carbon dots) remains nearly unchanged in the presence of the biomolecules, except for bilirubin, implying that the other tested biomolecules do not cause fluorescence quenching in carbon dots and therefore, do not interfere with the detection of bilirubin. Hence, the selectivity of bilirubin to carbon dots, even in the presence of other potential biomolecules commonly present in biological samples can be confirmed.

Metal ions present in serum such as Na(I) – 160 mM, K(I) – 6.5 mM, Cu(II) – 30 μ M, Zn(II) – 25 μ M, Ca(II) – 3.5 μ M, Mg(II) – 2 mM, Co(II) – 0.05 μ M, Al(III) – 0.5 V, Fe(III) – 35 μ M, Fe(II) – 10 μ M, and Ni(II) – 0.03 μ M were also tested and compared with 20 μ M bilirubin (BR). Figure 6 illustrates the effect of various tested metal ions on the PL intensity of the carbon dots. Similar to the response of the series of tested biomolecules, the PL intensity of carbon dots in the presence of the metal ions also closely resembles that of blank in contrast to the significant quenching effect observed with bilirubin. This indicates that the tested metal ions do not cause any fluorescence quenching in the carbon dots, and, hence do not interfere with bilirubin detection. Statistical parameters such as mean, standard deviation (SD) and standard deviation of mean (SDoM) have been calculated for the triplicates of results obtained on testing the effects of different biomolecules and metal ions on the C-dots. The error bars represented by SDoM demonstrated values below 5% for metal ions and below 6% for biomolecules, indicating high reproducibility and precision of the measurements. These minimal variations validate the reliability and robustness of the bilirubin sensor, making it well-suited for accurate and dependable performance in real-world scenarios. The distinct fluorescence quenching of carbon dots caused by bilirubin, compared to the minimal effects from other tested interference species, confirms the exceptional selectivity and specificity of the metal-doped carbon dots to bilirubin highlighting their potential for use in real biological sample analysis.

Detection of bilirubin in spiked serum samples

For real-time application, the method has been utilized for the sensing of bilirubin in complex biological samples such as the human serum. Bilirubin-spiked human serum samples were added to the carbon dots in a 1:1 ratio and the fluorescence spectra were recorded for different concentrations of bilirubin-spiked serum samples.

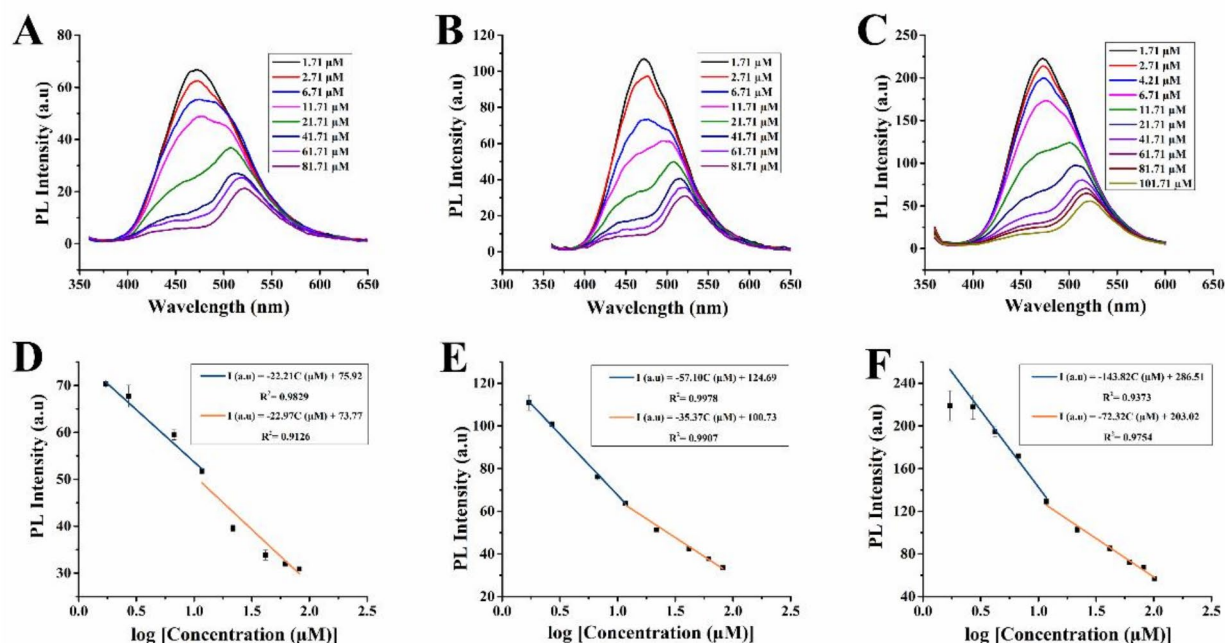


Fig. 7. PL emission spectra of the carbon dots (A) CD (B) NiCD and (C) CuCD with increasing concentration of bilirubin spiked serum ($\lambda_{\text{ex}} = 350 \text{ nm}$). Dual linear plots of log of concentration of bilirubin spiked serum (μM) with PL intensity of carbon dots (D) CD (E) NiCD and (F) CuCD.

Figure 7A–C represent the quenching of fluorescence of carbon dots CD, NiCD and CuCD with the addition of human serum spiked with bilirubin similar to the trend observed in laboratory bilirubin samples. The tested bilirubin concentration in the serum was $1.71 \mu\text{M}$, to which known concentrations of bilirubin were added and tested. The calibration plots obtained from the quenching of fluorescence of C-dots have been depicted in Fig. 7D–F. CuCD exhibited a sensitivity of $143.82 \text{ a.u./log } \mu\text{M}$ and $72.32 \text{ a.u./log } \mu\text{M}$ in the dual linear ranges $1.71\text{--}11.71 \mu\text{M}$ and $11.71\text{--}101.71 \mu\text{M}$. From the calibration plots and values of sensitivity, it is clear that CuCD exhibits the maximum linear range and sensitivity compared to CD and NiCD and hence can be used as a reliable system for the detection of bilirubin in serum.

Conclusion

In summary, a simple one-pot hydrothermal method has been developed for the successful detection of bilirubin in human serum samples. The detection of bilirubin was through static and IFE-based fluorescence quenching mechanisms. The method involved the development of highly selective carbon dots doped with metals, nickel and copper and the maximum sensitivity, LOD and LOQ were exhibited by copper-doped carbon dots (CuCD). The carbon dots were also successfully tested in bilirubin spiked serum samples in order to prove their applicability to detect bilirubin in spiked serum samples. Hence, a highly sensitive and selective system has been developed for the fluorimetric detection of bilirubin in human serum.

Data availability

The data that support the findings of this study are available on request from the corresponding author, T G Satheesh Babu, upon reasonable request.

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Author contributions

LDA: Methodology, Investigation, Formal Analysis, Data Curation, Writing – Original Draft; SM: Investigation, Methodology; PVS: Validation, Writing – Review & Editing, Supervision; TGS: Conceptualization, Supervision, Writing – Review & Editing, Resources, Funding Acquisition.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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