Synthesis of blue fluorescent carbon dots for sensitive and selective detection of glucose in biological samples

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Abstract. This Article describes a technique for the preparation of blue fluorescent carbon dots (CDs) showing fluorescence emission at 450nm. CDs were prepared using ultra-sonication technique using citric acid and glycerol as precursors. Since glucose quenches the fluorescence of CDs, depending upon this fact, a fluorescent sensor for glucose determination has been established. Under optimized conditions, linear response in the range of 0.1-100µM was obtained for glucose concentration. Limit of detection is found to be 0.08µM. This method was promisingly used for the glucose determination in serum albumin samples and urine samples from different human beings.

Keywords
Fluorescence, Glucose, Carbon dots, UV-Visible, FTIR, HR-TEM.

1. Introduction
Glucose is an essential biomolecule which plays a vital role as a source of energy in cell function and regulation of metabolism. Glucose is an important metabolic intermediate of other complex biomolecules. Glucose level in urine and blood is a measure of human fitness states [1-3]. Investigation of urine and blood glucose level is very crucial because abnormal level of glucose can related to hyperglycaemia, hypertension, and vision problem; increase the risk of heart disease and stroke, kidney disease, and diabetes (type-II) [4]. Diabetes mellitus, now a day a frequent disease occurs due to malfunction of pancreas and is spreaded worldwide. Reports of W.H.O. states that 347 million diabetic people were found in 2013 year and 3.8 million people leads to death every year, this number will be doubled in 2030 [5]. Blood sugar (glucose) levels both in diabetic human being and healthy people lies in the millimolar range, biological contains potential interfering ions and analogues, thus very sensitive method is required for detection of glucose in the low concentration range [6]. Several techniques for determination glucose have been existed like mass spectrometry [7], fluorescence [8], electrochemistry [9], Surface Enhanced Raman Scattering (SERS) [10], chemiluminiscence [11], and colorimetry [12]. Out of these methods fluorescence based detection have attracted great attention due their low cost, good stability, easeness of handling, and excellent sensitivity. Alotts of fluorescence sensors have been applied for determination glucose like organic dyes [13], nanoclusters [14], and Quantum dots [15]. However, most of the existing methods require complex modification, more cost of
experiment, unappreciable limit of detection, less sensitivity and not applicable for biological samples. So, to develop the fluorescent sensor with high sensitivity, lower limit of detection, having high selectivity, cost-effective method is of great significance. Quantum dots have been also used as fluorescent sensor but toxicity, high cost, and complex method of preparation, limits their applicability. Carbon quantum dots (CDs) having various advantages over QDs, like biocompatibility, water solubility, chemical inertness, extremely tunable photoluminescence and electrochemiluminescence [16], That’s why CDs are used now a days in the place of toxic QDs and organic dye mediated sensors.

In this study, fluorescent probes (CDs) have been synthesized without surface modification for glucose determination in human serum and urine samples. The Carbon dots were prepared using citric acid and glycerol as precursors using ultra-sonication. Synthesized fluorescent probe was successfully used for detection of glucose in the real samples.

2. Experimental section

2.1. Chemicals and reagents

Citric acid and glycerol were procured from Sigma Aldrich Pvt. Ltd. Mumbai. Glucose, ribose, maltose, fructose, xylose, galactose, lactose, arabinose were purchased from Himedia Chemical Pvt. Ltd. Standard solution of different metal ion were purchased from Merck Chemical Pvt. Ltd. During all the experimental procedures ultrapure water was used from Millipore Merck water purification system. All chemical employed are of AR grade, without any refinement.

2.2. Apparatus

HR-TEM images (High Resolution Transmission electron microscopy) were recorded from JEOL JEM-2100 transmission electron microscope (JEOL, Japan). UV-Visible spectra were measured using Thermo-Fischer spectrophotometer in the 200-600 nm of wavelength range. Fourier transform infrared spectra were recorded on Thermo-Fischer DRS-FTIR instrument, in the range of 1000-4000 cm⁻¹. Fluorescence spectra were recorded using Carry-Eclipse (Agilent technologies) from 350-550 nm wavelength range. pH measurement was made by using Hanna digital pH meter. Eszet ultrasonicator was applied for ultrasonication process.

2.3. Synthesis of Blue fluorescent carbon dots (CDs)

The CDs were synthesized from glycerol and citric acid using ultra-sonication technique. In the experiment 1g citric acid and 10 ml glycerol are mixed with 10 ml distilled water resulting solution was stirred for 10 minutes, then transferred in a ultra-sonication chamber for 6 hours, then the reaction mixture was cooled at room temperature. The obtained raw CDs after reaction was dialyzed in a tubing membrane of 1000 Da, mol. Wt. cut-off for 48 hours at room temperature for removing impurities. Obtained purified CDs were kept at 4°C for experiment.

2.4. Characterization of CDs

A UV-Visible band between 200nm to 360nm is the characteristic of carbon nanomaterial. A UV-Visible band at around 240nm is because of the π-π* transition of sp² aromatic carbon atom. CDs shows fluorescence band at 450 nm as shown in fig. 1(a). Dilute solution of CDs is light yellow in colour and shows blue fluorescence when exposed to long wavelength ultraviolet light (365nm). CDs show excitation wavelength dependent fluorescent behaviour.
Fluorescence excitation was started from 305nm and ends at 360nm of excitation wavelength, fluorescence intensity increases on increasing wavelength then reaches to maximum and decreases further, fluorescence intensity found maximum at 350nm excitation wavelength as shown in fig. 1(b). These shifts in fluorescence band and intensity are due to the different surfaces of CDs. The steadiness of nanoparticle was also analysed at wide range of pH (2-10.0), CDs show tremendous stability at all pH. Functional groups can be identified using FTIR spectrophotometer. Broad bands at around 3450 cm\(^{-1}\) and 3100 cm\(^{-1}\) are due to O-H and C-H stretching. Bands at 1450 and 1680 cm\(^{-1}\) are due to C-H bending and C=O functional groups. These observations shows that surface of CDs consists of –COOH, –OH and amide groups\[17\] as shown in fig. 1(c). Morphology of synthesized CDs was analysed by HR-TEM (High Resolution Transmission electron microscope). Particles of CDs are of spherical shape having sizes between 1nm to 5nm with average diameter of 3.2nm respectively as shown in fig. 1(d).

Figure 1 (a) UV-Visible absorption spectra of synthesized CDs, (inset: photographs of CDs under ordinary light and ultra-violet light respectively); (b) Excitation spectra of Fluorescent CDs under various wavelengths; (c) FTIR spectra of CDs; (d) image of the CDs under HR-TEM (inset: histogram showing size distribution of CDs).
2.5. Calculation of Quantum yield of CDs

The quantum yield calculation of CDs is carried out using equation given below-[18]

\[ Q = Q_R \frac{I_0 D_R}{I_R D n^2} \]

Where \( Q \) is quantum yield of CD, \( Q_R \) represent the quantum yield of reference, \( I \) stand for optical density of CDs and \( I_R \) is optical density of reference which is obtained by UV-Vis spectrophotometer. The quantum yield of CDs was found to be 4.8 upon 350 nm excitation wavelength in reference quinine sulphate which has quantum yield 54% in 0.1M H\(_2\)SO\(_4\)(n=1.33).

2.6. Detection of glucose using CDs fluorescent probe

In the experiment different concentrations of glucose solutions were added to the solution of CDs and incubated at room temperature for 30 minutes, and then fluorescence spectrum was measured employing excitation wavelength350nm. Concentrations of glucose vary in each sample from 0 µM to 1000 µM.

2.7. Glucose detection in biological samples

The urine samples of diabetic human beings were collected from Pt. Jawaharlal Nehru Medical College Raipur(C.G.) and urines of healthy human beings were obtained from healthy individuals after and before oral glucose tolerance test. Before the analysis obtained samples were stored at -20°C in deep freezer. Samples were centrifuged at 15000 rpm for 15 minutes, and then supernatant was diluted 10 times. 100 µL of following samples was employed for detection of glucose as described above.

3. Results and discussions

3.1. Limit of detection of glucose (LOD)

When various solutions of glucose were added to CDs solution then fluorescence of CDs was quenched linearly. It was found that with increasing concentrations of glucose fluorescence of CDs decreased. Concentration of glucose ranges from 0-1000 µM, indicating that quenching of fluorescence is related to concentration of glucose added. The calibration graph was plotted by using fluorescence quenching efficiency \((F_0-F)/F_0\) versus glucose concentration (fig. 2 a, b, c) where \( F_0 \) is the fluorescence intensity in absence of glucose \( F \) is the fluorescence intensity in the presence of glucose. A good linear correlation obtained from 0.1 to 100 µM concentrations of glucose. By using regression equation LOD was found to be 0.08 µM of glucose, with a correlation coefficient \( R^2 \) of 0.996. These results prove that the fluorescent probe CDs is a suitable sensor for highly sensitive detection of glucose.

For the improved detection of glucose by proposed method, reactions conditions are optimized such as temperature, pH, incubation time, and concentration of CDs. Quenching efficiency was checked at different pH values it is observed that between 7-8 pH quenching efficiency was maximum i.e. more interaction between fluorescent sensor and analyte molecule(glucose).
3.2. Mechanism of quenching for fluorescent probe

Possible mechanism of fluorescence quenching may be dynamic or static quenching, in dynamic quenching there is FRET (Froster Resonance Energy Transfer) between excited state of donor and acceptor molecules. In static quenching there is complex formation between fluorofore and analyte molecule. Here the possible mechanism of quenching is static quenching because –COOH groups which are present on the surface of CDs gives esterification reaction with –OH groups of glucose molecule.

3.3. Selectivity investigation of the fluorescent probe (CDs) for detection of glucose

For assessment of selectivity of fluorescence probe(CDs) for glucose, the effects of certain biomolecules( ribose, arabinose, maltose, lactose, xylose, fructose, mannose, galactose) and some other interfering materials which exists in human urine (urea,Mg^{2+},uric acid,Na^+ascorbic acid , K^+ ,Zn^{2+},Cl^-,Ca^{2+}) were tested. On addition of these biomolecules and interfering ions to CDs solution, fluorescence quenching is negligible. Fluorescence quenching efficiency \( (F_0-F)/F_0 \) was compared with glucose, it is found that 100-times more concentration of such ions does not cause appreciable quenching the fluorescence of CDs.The
high selectivity imputed to the specificity of CDs for glucose. These results reveal that CDs is very selective for differentiating glucose from other ions and molecules which are present in the biological samples.

Figure 3 Selectivity of glucose (100 µM); (a) in the presence of various analogues of glucose (1000 µM); (b) in the presence of various potential interfering analogues.

3.4. Real sample analysis
To evaluate the applicability of fluorescent probe (CDs) for the detection of glucose in biological samples, glucose concentration in human urine and serum samples from healthy and diabetic patients were analysed. Concentrations of glucose in human urine were detected by glucometer and compared with present method; results show that there is good agreement between results obtained by CDs and glucometer. To further investigate the accuracy of present method following experiments were performed. Recovery experiment suggests that recoveries of glucose concentrations 98.9 - 102.6% were obtained and relative standard deviation (RSD) was found to be below 5%, these results are acceptable for quantitative analysis of glucose in biological samples.

Table 1. Glucose determination in human serum samples and recovery experiment

| Spiked(mM) | Detected(mM) (mean±SD) | Glucometer(mM) (mean±SD) | Average recovery(%) |
|-----------|------------------------|--------------------------|---------------------|
| 0         | 5.45±0.07              | 5.51±0.06                |                     |
| 2.0       | 7.34±0.06              | 7.49±0.11                | 99.6                |
| 4.0       | 10.38±0.05             | 10.46±0.05               | 99.3                |
| 8.0       | 13.42±0.09             | 13.52±0.08               | 98.9                |
| 10.0      | 15.43±0.12             | 15.30±0.12               | 102.6               |

*aMean of three values of standard deviation bStandard deviation*

Determination of glucose in also carried out using fluorescent probe (CDs) and compared there results with convenient glucometer which is used for glucose estimation. Samples were
collected three diabetic human beings then samples were also collected from healthy human beings before and after 2hr for taking oral doses of 50g tolerance test. Results obtained shows that fluorescent probe (CDs) is very sensitive and here incubation time was also minimized to 30 min.

Table 2. Glucose determination in human urine from diabetic and healthy human beings

| Samples \(^{x,y}\) | Glucometer (mM) | Present Method (mM) | RSD (n=3, %) |
|------------------|------------------|---------------------|-------------|
| DH 1             | 6.82             | 6.78                | 1.66        |
| DH 2             | 8.12             | 8.02                | 2.21        |
| DH 3             | 18.36            | 18.40               | 3.86        |
| HH 1             | <LOD undetected  | 0.48                | 2.64        |
| HH 2             | <LOD undetected  | 0.70                | 1.89        |
| HH 3             | 3.46             | 3.48                | 3.62        |
| HH 4             | 4.32             | 4.28                | 2.04        |

\(^{x}\)Samples from DH1 to DH3 are urine from diabetic human beings

\(^{y}\)Samples from HH1 to HH4 are urine from healthy human beings, before (HH1, HH2) taking oral doses of 50g and after 2hr (HH3, HH4) for taking oral doses of 50g of glucose.

We compared our results with previously existing nanoparticle based sensing method for glucose detection including C-dots/AgNPs[19], SiNPs[20], dsDNA-CuNPs-SG1[21], AuNPs-GOQD[22], MnO2-nanosheet[23], CeO2 NPs[24], ZnO nanoparticles [25], and CDs[1], all these method includes complex preparation and low sensitivity than present method expect MnO2-CD nanocomposite[26], whose LOD is lower than our method.

4. Conclusions

In the present study fluorescent nano-biosensor was synthesized using green ultrasonication method. Synthesized CDs is dispersible in water, show strong blue fluorescence, it is photostable, CDs is very sensitive and selective for glucose than other analogues of glucose and potential interfering ions which are present in biological sample (serum and urine), this method is accurate enough for glucose detection, and this method shows various advantages than most of existed methods. CDs were used without any further modifications, so it reduces cost and time both. This method provides an alternate way to determine glucose in biological samples.

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