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Generating Luminescent Graphene Quantum Dots from Tryptophan: Fluorosensors for Hydrogen Peroxide in Cancer Cells

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Abstract: Herein, we report a single step synthesis of highly fluorescent Graphene Quantum Dots (GQDs) using tryptophan and glycerol as precursors via pyrolysis. The morphological and functional characterization of the prepared GQDs was performed using PXRD, FTIR, TEM, XPS and zeta potential measurements. The prepared GQDs found their practical application in ultrasensitive detection of an emerging potential cancer biomarker, H₂O₂, by exploiting the fluorescence quenching behaviour of H₂O₂. To evaluate the detection sensitivity, a series of various concentrations of H₂O₂ was spiked to biomatrices like, serum and MCF-7 (human breast cancer cell line) cell lysate medium. A remarkably low limit of detection (LOD) was found in serum medium (139.5 pM) which further improved in MCF-7 cell lysate medium (LOD 61.43 pM). Moreover, the sensing capacity of the GQDs was further validated in presence of various physiological variables such as glucose, cholesterol, insulin and nitrite. Sensing assay was also carried out in HaCaT (human keratinocyte cell line) cell lysate medium to compare the performance of our prepared sensor but the non-linearity of the F₀/F versus H₂O₂ concentration plot pointed towards the conduciveness of the MCF-7 cell lysate medium for sensitive detection of H₂O₂. The mechanism behind the sensing was also explored using spectroscopic methods.

Keywords: Graphene Quantum Dots; Cancer biomarker; H₂O₂; Fluorescence; MCF-7; HaCaT

1. Introduction

Cancer is the second most abundant cause of human mortality taking the place only after cardiovascular diseases. Cancer still accounts for one in four fatalities in wealthy nations, despite major global spending in research. Over 14 million cancer diagnoses were reported worldwide in 2012; this number is projected to rise to nearly 22 million annually over the next 20 years [1]. Cancer emerges from our own tissues, complicating both detection and treatment methods due to the similarities between the diseased tissue and healthy tissue. Despite this fact, the mortality rate due to cancer is greatly reduced by early detection of the disease. In order to enhance the prognosis for cancer patients, early diagnosis is crucial [2].

At present, clinical detection of cancer primarily relies on imaging techniques or morphological analyses of cells (cytology) or tissues (histopathology) that are suspected to be diseased. Imaging techniques applied to cancer detection, including X-ray, computed tomography (CT), magnetic resonance imaging (MRI), endoscopy, and ultrasound, have low sensitivity and are limited in their ability to differentiate between benign and malignant lesions [3]. These methods, which mainly rely on imaging to assess tumour status, offer scant information on the development of cancer and scant surveillance of malignant cells in the early stages [4]. Therefore, a major challenge is encountered for the development of sensitive assays before the disease becomes symptomatic.

One promising approach in the early detection of cancer is to identify and detect prominent biomarkers in the blood or other body fluids that correlates with the presence of cancer. Measuring the levels of particular cancer biomarkers from a patient's blood, urine, faeces, or saliva could enable the detection of cancer at the early stages of the disease, identification of tumour recurrence and prediction of a patient's risk to a new or existing cancer. So far protein biomarkers are recognized as gold standards for cancer diagnosis. The objective of biomarker analysis is early cancer detection so that the patient can receive apt therapy. Several conventional techniques for quantitative analysis of biomarkers have been recognized using different methods like electrochemical immunoassay, differential pulse voltammetry (DPV), mass spectrometry [5], chromatography [6], enzyme-linked immunosorbent assay (ELISA) [7], chemiluminescence, photoelectrochemical method, etc. However, most of these techniques are inconvenient for reasons like time intensive labour cost, need of special handling, use of large and expensive instruments and complex operations and often lack selectivity [8]. The World Health Organisation (WHO) advises that diagnostic techniques

should be accessible, not reliant on expensive technology, user-friendly, sensitive, specific, quick, and reliable [9].

Recent research within the field of nanotechnology addresses the limitations of the currently available methods for cancer diagnosis through several analytical techniques. Graphene Quantum Dots (GQDs), a class of carbon-based nanomaterials distinguished by the presence of the graphene structure, offer a number of advantages due to their exceptional chemical, physical, and optical properties, enabling the development of ultrasensitive and selective biosensor devices [10]. Due to quantum confinement and edge effects, GQDs, as a new kind of quantum dots (QDs), have attracted tremendous research interest due to their high aqueous solubility, stable photoluminescence (PL), low cytotoxicity, good biocompatibility as well as superior resistance to photobleaching as compared to traditional semiconductor QDs and organic dyes [10-14]. In addition, their 2D surface shows strong noncovalent interactions with adsorbed biomolecules through p-p interactions, electrostatic forces, or hydrogen bonding, which provide a chemically tunable platform for bioconjugation [15-17]. Therefore, GQDs have found extensive applications as fluorescent sensors in recent years.

The preparation techniques for GQDs that have emerged in recent years can be broadly divided into two groups: top-down and bottom-up, in accordance with the source materials. Using a top-down method, GQDs are produced by cleaving of bulk carbon materials such as graphene, graphene oxide (GO), coal, soot, carbon nanotubes (CNTs), carbon fibers, etc [18]. This method uses a variety of techniques to carry out the synthesis, such as the acidic-oxidation process, solvothermal, hydrothermal, and microwave assisted methods [19]. Less control over the stability and shape of the QDs as well as the use of strong acid which might result in the creation of hazardous gases like dinitrogen tetroxide (N_2O_4) and nitrogen dioxide (NO_2) — are the key disadvantages of top-down approach [18]. In contrast to a top-down technique, a bottom-up approach produces GQDs by gradually converting tiny organic molecules into GQDs through condensation reactions followed by pyrolysis [19]. This method allows for greater control over the structural integrity and stability of the QDs [20]. The initial synthesis method by Dong and group that used citric acid carbonization provided a quick and economic way to produce GQDs [20]. This procedure was modified by Li et al. to manufacture N-functionalized GQDs utilizing glycine as precursor in ethylene glycol wherein glycine served as a source of both nitrogen and carbon [21]. A recent paper describes a green synthesis of glycine based N-GQDs by Trpkov et al., wherein ethylene-glycol was substituted with an even more cost-effective medium, glycerol [22]. The addition of heteroatoms, such as

nitrogen, to GQDs offers an alluring means of raising luminescence efficiency. Zhang et al. synthesized tryptophan-functionalized GQDs with citric acid as the carbon source and tryptophan as the modifier and nitrogen source [23].

As a possible biomarker for cancer, H_2O_2 is the main focus of the current investigation. H_2O_2 is found in the highest concentrations in breast cancer samples, followed by colon and cervical malignancies. When extracellular stimuli activate nonphagocytic cells along the course of disease development, H_2O_2 is created as a putative biomarker. Under normal physiological conditions, a low concentration of H_2O_2 within allowed limits can kill bacteria in order to maintain cell function stability while posing no risk to the human body. Pathological conditions, on the contrary, induce cells to produce more H_2O_2 (10-50 μM), which promotes the proliferation of cancer cells, epithelial cells, and angiogenesis [24].

The present work focuses on paving a facile and fast route to develop low-cost and highly sensitive GQDs as a novel sensing platform for the detection of H_2O_2 by fluorescence spectroscopy. Fluorescence spectroscopy is a useful technique for the detection of biomarkers. It is widely used in biological and biomedical applications due to its high spatial and temporal resolution. Here we report a simplistic and effective strategy to prepare amino acid-functionalized GQDs by thermolysis treatment on tryptophan and glycerol at 200°C for 20 min wherein the tryptophan molecule served both as carbon and nitrogen source. Furthermore, the GQDs were successfully applied to detect H_2O_2 in presence of various physiological parameters.

2. Materials and methods

2.1. Materials

Tryptophan was procured from Sisco Research Laboratories Pvt. Ltd. Glycerol anhydrous, H_2O_2 and dextrose anhydrous each were purchased from Merck, India. Sodium nitrite used was of A.R. grade. Cholesterol was obtained from LOBA Chemie, India. Ringer-Lactate solution (RL) (aqueous solution of sodium lactate, sodium chloride, potassium chloride, calcium chloride) and semisynthetic human insulin actrapid and huminsulin were obtained from local pharmacy. Lyophilized human serum used as commercial reference material was obtained from Lyphochek Assayed Chemistry Control (Bio-Rad). Dialysis tubing cellulose membrane (molecular weight cut-off ~14000 kDa) was purchased from Sigma-Aldrich, USA. Triple distilled water was used throughout the experiment.

2.2. Instrumentations

A Horiba fluoromax plus spectrofluorometer was used to perform fluorescence spectroscopic measurements. Fourier Transform Infrared (FTIR) spectroscopic data was recorded using Perkin-Elmer instrument in the range of 3500 cm^{-1} to 400 cm^{-1} . A JEOL JEM-2100F transmission electron microscope (TEM) with 200 kV electron source was used to get the TEM images and selected area diffraction (SAED) pattern of the synthesised GQDs. A Rigaku SmartLab automatic high resolution multipurpose PC Controlled X-Ray diffractometer system with Cu anode as the source with K- α wavelength of $1.54060\text{ \AA}$ (current 40 mA, voltage 40 kV) was used to perform powder X-ray diffraction (PXRD) analysis. A Malvern Zetasizer Nano ZS instrument was used for zeta potential (ζ) measurement. A REMI Elektrotechnik Ltd. R-4C centrifuge was used for centrifugation purposes. Fluorescence lifetime experiments were done using a time-correlated single-photon counting (TCSPC) Horiba Jobin Yvon spectrophotometer with a nanosecond diode LED of 300 nm (IBH, nanoLED-03) as light source. The data stored in a multichannel analyzer was routinely transferred to IBH DAS-6 decay software. A multi-exponential fitting program supplied by IBH was used to analyze the fluorescence decay curves while taking reduced chi-square values into account.

A Kratos Axis Ultra DLD system was used to collect XPS spectra using monochromatic Al K α X-ray source operating at 120 W (10 mA x 12 kV). Data was collected with pass energies of 160 eV for survey spectra, and 40 eV for the high-resolution scans with step sizes of 1 eV and 0.1 eV respectively. Samples were mounted by pressing in to a recess made by a copper washer mounted using double sided adhesive tape (Scotch 665) mounted on to a clean glass microscope slide which was taped to a standard Kratos sample bar. The system was operated in the Hybrid mode, using a combination of magnetic immersion and electrostatic lenses and acquired over an area approximately $300 \times 700\text{ }\mu\text{m}^2$. A magnetically confined charge compensation system was used to minimize charging of the sample surface, and all spectra were taken with a 90° take off angle. A base pressure of ca. 5×10^{-9} Torr was maintained during collection of the spectra. Data was analysed using CasaXPS (v2.3.26) [25] after subtraction of a Tougaard polymer background and using modified Wagner sensitivity factors as supplied by the manufacturer. For charge calibration, the lowest C(1s) signal was set to an energy of 284.5 eV, typical of graphitic like carbon.

2.3. Synthesis of GQDs

Tryptophan (0.5 g) and glycerol (10 mL) were taken in a 100 mL beaker and heated at 200 °C for 20 minutes using a heating mantle with constant stirring on a magnetic stirrer (250 rpm) and then allowed to cool to room temperature. The brownish-yellow product thus obtained was subjected to dialysis for 24 hours using a dialysis membrane. Then the GQDs processed by dialysis were stored at room temperature as stock solution for further characterization and use. After purification and drying, the total yield of the as-prepared GQDs was 8% of the weight of tryptophan (main precursor).

2.4. Characterization of GQDs

The inherent features of the synthesised GQDs were identified by characterising them utilising various analytical methods. The GQD stock solution was diluted 1000 times with triple distilled water in a micro centrifuge tube and a drop from the aforementioned diluted solution was then drop-casted on a carbon-coated copper grid. The grids were dried under IR lamp and were used to record the TEM micrographs, selected area electron diffraction (SAED) pattern and lattice fringes to observe their structural morphologies like size, shape and crystallinity. Zeta potential (ζ) measurement was carried out using the same diluted solution to determine the effective charge developed on the surface of the GQDs. The measurement gives the idea of the stability of the GQDs in fluid suspension. The surface functionalities were tapped by FTIR spectroscopy and for that purpose a drop from the GQD stock solution was drop-casted on a quartz plate and the FTIR spectra were recorded. To determine the crystal orientation of the GQDs, a solid powder sample of the GQDs was placed in a grooved rectangular glass holder and PXRD analysis was performed. XPS analysis was performed to probe the elemental composition and bonding at the surface of the synthesised GQDs.

2.5. Quantum yield (QY) calculation

Using Rhodamine B in water as a reference dye, the fluorescence QY of the GQDs in water was determined at an excitation wavelength of 254 nm using the following equation [26]:

$$QY_{GQDs} = QY_R (A_R/A_{GQDs}) (F_{GQDs}/F_R) (n_{GQDs}/n_R)^2 \quad (1)$$

where QY is fluorescence quantum yield, F is the observed integrated fluorescence intensity, A is the absorbance of the solution at the excitation wavelength, n is the refractive index of

the solvent, while subscripts ‘R’ and ‘GQDs’ refer to the reference used (Rhodamine B, QY= 0.31 in water) and the sample respectively.

Table 1: Specific values of parameters for calculating QY of the GQDs

Sample	Integrated fluorescence intensity (F) (arbitrary units)	Absorbance (A)	Refractive index of solvent (n)	Quantum Yield (QY)
Rhodamine B	252387	0.258	1.33	0.31
GQDs	71282	0.236	1.33	0.0957

2.6. Photostability of GQDs

The photostability of the GQDs was investigated by monitoring the change in fluorescence intensity with irradiation time under UV light. The photostability of GQDs was tested by continuously exposing the sample to UV light (254 nm) for 6 hours.

2.7. Sensing of H₂O₂ by GQDs

At room temperature, fluorescence spectroscopy was used in order to explore the biomarker sensing ability of the GQDs. As working medium, reconstituted serum, 1000-fold diluted in RL medium, was used for each set of experiments in order to keep the reaction conditions at par with biological significance. 0.2 mL of GQD stock was added to 1.8 mL working serum medium in a quartz cuvette. At an excitation wavelength of 254 nm, different known aliquots of H₂O₂ (20 nM) were successively added to this mixture with proper mixing, and their subsequent fluorescence spectra were instantly measured. Fluorescence spectroscopy was also used to validate further the sensing capacity of the GQDs in presence of various physiological variables such as glucose, cholesterol, insulin and nitrite. Simulations of various pathophysiological conditions involving elevated levels of glucose, cholesterol, and nitrite were carried out; their concentrations were maintained higher than normal physiological limits to impersonate conditions like diabetes, hypercholesterolemia, nitrosative stress respectively. Again, as pancreatic β -cells release insulin when glucose level increases in bloodstream, further simulations were carried out in presence of two forms of insulin, actrapid and huminsulin to monitor its contribution towards H₂O₂ sensing by the GQDs. For the aforementioned purpose, 0.02 mL each of 1 mM dextrose and 1 mM cholesterol were

mixed with the working serum medium. Aliquots of H₂O₂ were sequentially added to it and fluorescence spectroscopic analysis was carried out. Similarly, 0.002 mL each of actrapid (40 mIU) and huminsulin (40 mIU) were added to the working serum medium and sensing assay of H₂O₂ was carried out. In an identical manner, 0.02 mL of a 220 µM sodium nitrite solution was added to the working serum medium, and thereafter sensing assay of H₂O₂ was conducted. Similarly, sensing assays were also carried out in cell lysate medium. The preparation of cell lysate is described in the following section.

The Stern-Volmer equation (eqn. 2) was used to examine the quenching of the intrinsic fluorescence of GQDs by H₂O₂ both in absence and presence of high concentrations of biomolecules such as glucose, insulin, cholesterol, and nitrite.

$$F_0/F = 1 + K_{SV} [Q] \quad (2)$$

where, F₀ and F are the relative fluorescence intensities of GQDs in absence and presence of the quencher (H₂O₂) respectively and [Q] is the concentration of the quencher (H₂O₂). The slope of the plot of F₀/F vs. [Q] gives the value of Stern-Volmer quenching constant K_{SV}. Limit of detection (LOD) and limit of quantification (LOQ) of H₂O₂ were also calculated using the following equations (3 and 4) with the help of slope (S) and standard deviation (σ) of the calibration curve of the responses.

$$LOD = 3.3 (\sigma/S) \quad (3)$$

$$LOQ = 10 (\sigma/S) \quad (4)$$

2.8. Cell culture

Human breast cancer cell line, MCF-7 were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Fetal bovine serum), 1% penicillin-streptomycin and 1% L-glutamine at 37 °C in a humidified incubator with 5% CO₂.

The human keratinocyte cell line, HaCaT were obtained from National Centre for Cell Science (NCCS), Pune, India, and were cultured in DMEM medium supplemented with 10% FBS, 1% penicillin-streptomycin in a humidified incubator at 37 °C with CO₂ (5%).

2.9. Cell lysate preparation

MCF-7 cultured cells were removed from the growth medium and then washed with ice-cold PBS buffer (pH 7.4), and scraped. The cells were pelletized and was suspended in 150 μ L of ice-cold Radio Immunoprecipitation Assay (RIPA) (1% IGEPAL CA-630, 50 mM of Tris HCl at pH 7.4, 150 mM NaCl, 0.5% deoxycholate, 5 mM EDTA, 0.1% sodium dodecyl sulfate [SDS] supplemented with a protease inhibitor cocktail) lysis buffer for cell lysis. The cell lysate was kept on ice for incubation for about 30 minutes. The cell lysate was then transferred to a micro centrifuge tube and centrifuged at 12000 rpm for 30 minutes at 4 °C. The clear supernatant obtained after centrifugation was collected and stored at -20 °C for further experiments. The same protocol was also followed for the lysis of cultured HaCaT cells and subsequent storage of the supernatant to carry out further experiments.

3. Results and discussion

3.1. Design of highly fluorescent GQDs

The goal of this work is to create a straightforward bottom-up procedure for the synthesis of highly fluorescent GQDs. The use of a green and natural material is the primary aspect of our design. GQDs, an intriguing class of carbon nanomaterials, have shown promise as a new platform for fluorescent (FL) biosensing. However, various labor-intensive, time-consuming, and expensive techniques for making GQDs restrict their potential for wide applications. The precursor needs to meet our criteria for selection, which include being non-toxic, common or natural, having nitrogen groups for simple surface modification of the GQDs during their applications, and not requiring any additional capping operations. We carried on with tryptophan after considering these factors. Additionally, the glycerol used in the synthesis serves as the reaction medium at the same time serves as one of the reactants (Fig. 1). It can also facilitate thermal conduction throughout the reaction, aiding in the ultimate formation of uniform products.

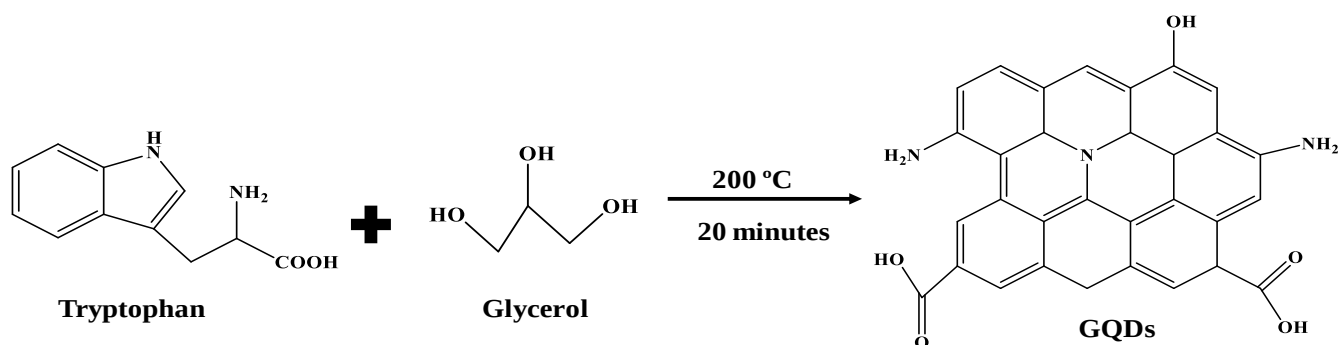


Fig. 1. Schematic representation of synthesis of GQDs

3.2. Photostability of GQDs

From Fig. S1 it can be seen that the GQDs retained fluorescence activity up to 94% when subjected to UV exposure up to 4 hours. After that the fluorescence intensity of the GQDs decreased drastically implying photo degradation of the GQDs. Thus, the GQDs remained photo stable for 4 hours.

3.3. Characterisation of GQDs

3.3.1 TEM analysis

The morphologies of the as-prepared GQDs were characterized by TEM. As shown in Fig. 2a, uniform quantum dots are formed, and the as-prepared GQDs exhibit a size ranging from 5-6 nm. Fig. 2b shows the HRTEM image of the GQDs with a clear lattice fine structure, indicating its high crystallinity. The inter-layer separation (d-spacing) calculated from ImageJ software comes out to be 0.37 nm, which is greater than graphite ($d = 0.34$ nm) and is due to the introduction of oxygen containing functionalities like epoxy (C-O-C) and hydroxyl (-OH) groups [27,28]. Bright concentric rings that appear in the SAED pattern of the GQDs are an indication that these nanoparticles are polycrystalline in nature. The SAED pattern (Fig. 2c) was examined using ImageJ software, and their corresponding hkl planes were determined. Narrow particle size distribution with size 5-6 nm could be seen from particle size histogram (Fig. 2d).

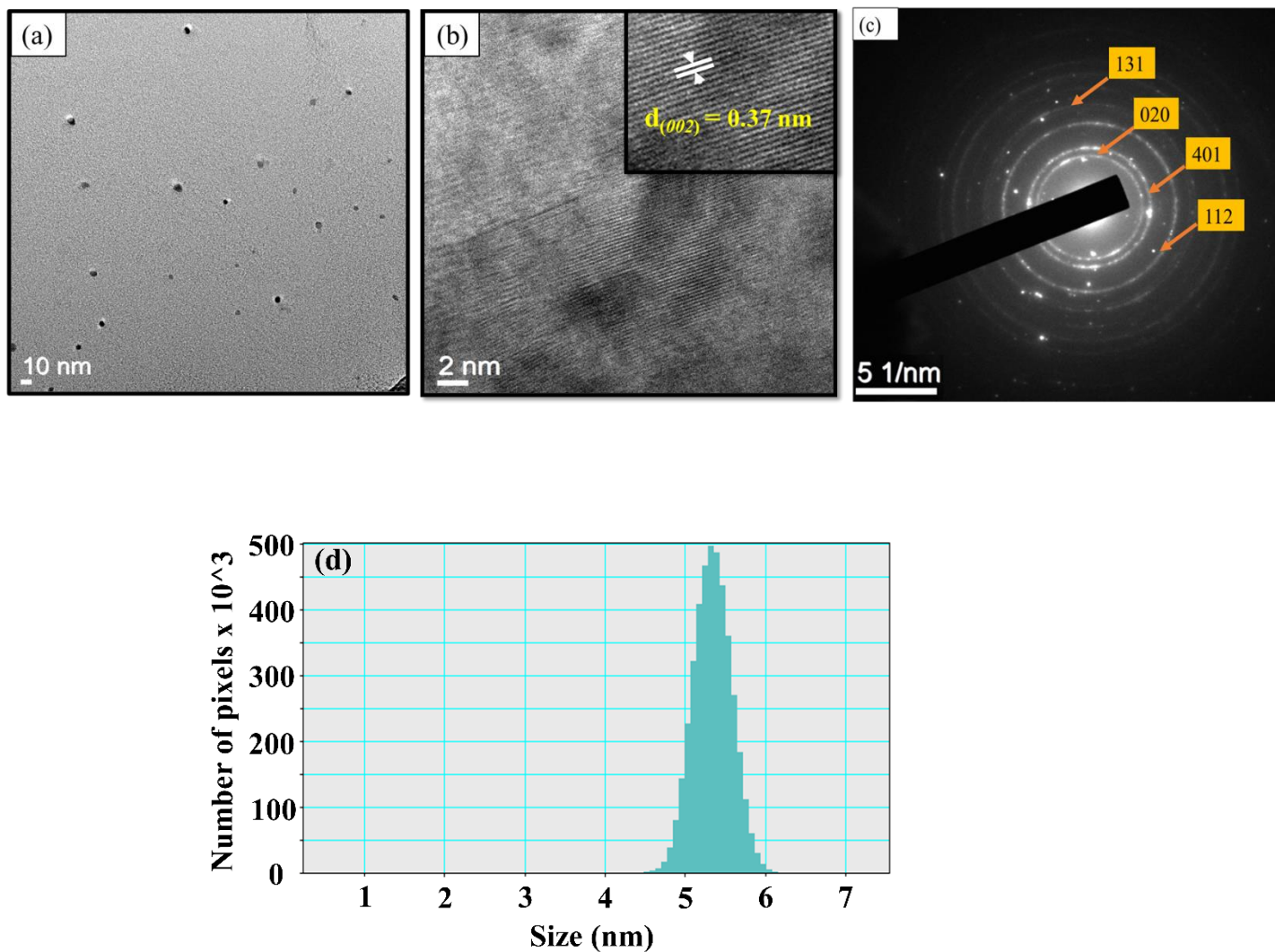


Fig. 2. (a) TEM image of GQDs, (b) Lattice fringes of GQDs, (c) SAED pattern of GQDs, (d) Histogram of GQDs

3.3.2. Zeta potential measurements

Zeta potential is a crucial tool for determining a nanoparticle's surface condition and for foretelling its long-term durability. The magnitude of zeta potential gives an indication of the potential stability of the colloidal system. When all the particles in suspension have a large negative or positive zeta potential then they tend to repel each other thereby preventing aggregation. The zeta potential value of the synthesized GQDs was found to be -16.4 mV which can be ascribed to the surface oxygen-containing carboxyl and hydroxyl functional

groups which made the surface negatively charged. According to the guidelines categorizing the nanoparticle dispersions, such value of zeta potential indicates that the GQDs are relatively stable in the solution [29,30].

3.3.3. PXRD analysis

The X-ray diffraction pattern of the GQDs is shown in Fig. 3. The presence of crystalline lattice in GQDs is indicated by the appearance of pronounced peaks in the spectra. In order to designate potential hkl planes, the peak positions from the PXRD data were compared with the database from the Joint Committee on Powder Diffraction Standards (JCPDS). PXRD data with possible (hkl) planes and corresponding JCPDS numbers are summarized in Table 2. The diffraction pattern of the GQDs have a strong and sharp (002) peak centred at $2\theta = 30.912^\circ$ suggesting that carbonizing tryptophan would produce graphite structure [31]. Fig.3 shows that more intense peaks come from 2θ values of 30.259° and 53.269° corresponding to (111) and (113) planes. From this data it could be said that lattice structure of GQDs is mostly end- centered having orthorhombic lattice system. When Bragg's equation is applied to PXRD data, interlayer crystallographic lattice planes (d-spacing values) are found. These values correspond closely to those found in the SAED pattern of the GQDs using ImageJ software.

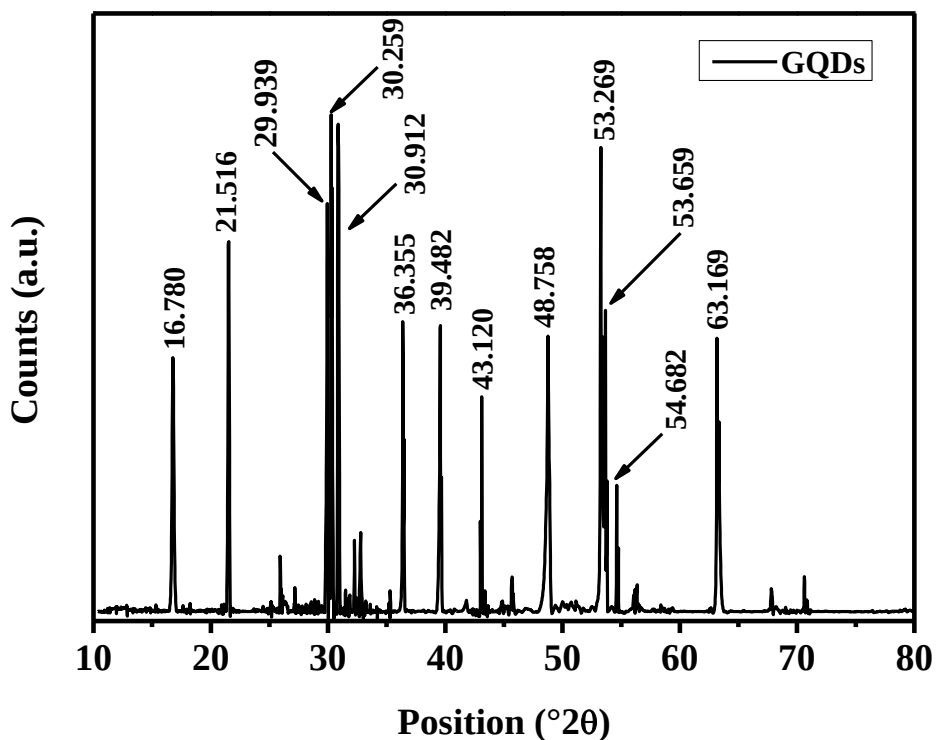


Fig. 3. PXRD pattern of GQDs

Table 2: PXRD data of the prepared GQDs with possible assignment of JCPDS no.

Actual 2θ	Obtained 2θ	Possible (hkl) plane	Possible System	Possible Lattice	JCPDS No.
29.869	29.939	(020)	Orthorhombic	Primitive	89-8490
30.260	30.259	(111)	Orthorhombic	End-centered	89-8488
30.142	30.912	(002)	Orthorhombic	End-centered	89-8489
48.649	48.758	(112)	Orthorhombic	End-centered	89-8492
53.410	53.269	(113)	Orthorhombic	End-centered	89-8489
54.881	54.682	(022)	Orthorhombic	End-centered	89-8492
53.680	53.659	(221)	Orthorhombic	Primitive	89-8490
63.136	63.169	(131)	Orthorhombic	Primitive	89-8491

3.3.4. FTIR analysis

The chemical structure of the as-synthesised GQDs was investigated using FTIR spectroscopy. Fig. 4 displays the FTIR spectrum of the GQDs and Table 3 shows the list of

possible functionalities that are present in GQDs. The broad bands at around 3000–3400 cm^{-1} correspond to N–H and O–H stretching vibrations [32]. The spectrum demonstrates that the GQDs contain abundant surface functional groups like carbonyl (C=O) and epoxy (C–O–C) at 1647 cm^{-1} and 1046 cm^{-1} respectively. The peaks at 1456 cm^{-1} and 1209 cm^{-1} can be attributed to C=C stretching and C–O stretching respectively. In addition, the bands at 2944 cm^{-1} and 2878 cm^{-1} arise due to stretching vibration of C–H bond. The vibrations under 1000 cm^{-1} represent out-of-plane C–H bending of the aromatic system [33]. The presence of the oxygen containing groups (hydroxyl, carbonyl, epoxy) endows the GQDs with good hydrophilicity. It should be emphasised that these hydrophilic functional groups will act as a self-passivation layer around the GQDs to facilitate their superior water-solubility and boost photoluminescence.

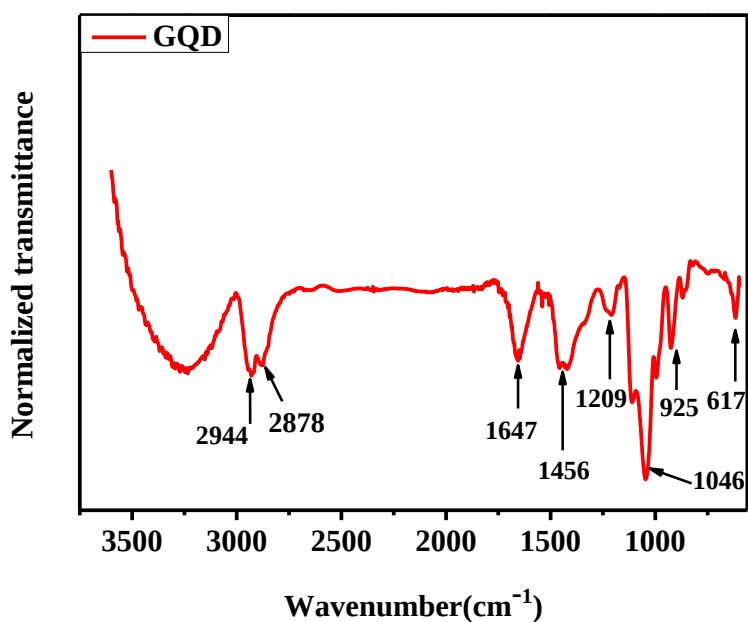


Fig. 4. FTIR spectrum of GQDs

Table 3: Peaks of FTIR spectrum showing different functionalities

Wave number (cm ⁻¹)	Possible functionality	Reference
1046	C–O–C	[34]
1209	C–O stretching	[35]
1456	C=C	[36]
1647	C=O	[37]
2878	C–H stretch	[38]
2944	C–H stretch	[39]
3000–3400	N–H and O–H stretching	[32]
under 1000	out-of-plane C–H bending	[33]

3.3.5. XPS analysis

To further investigate the chemical structure of the GQDs, XPS analysis was performed. Table 4 shows the elemental composition of the GQDs in terms of atomic percentage.

Table 4: Survey spectrum derived elemental analysis (at%) result of the synthesised GQDs

Element	C 1s	O 1s	N 1s
Atomic percentage (%)	81.88	9.98	5.6

The C 1s XPS spectrum (Fig. 5A) of GQDs shows three major peaks at 284.5 eV, 286.2 eV and 288.3 eV, which are attributed to C–C [40], C–N/C–O [40], and C=O [41] bonding respectively. Additional structure above 290 eV can be attributed to the shake up π - π^* satellite peaks or the aromatic ring structure [42], [43]. The N 1s spectrum is comprised of two peaks, centred at 399.8 eV and 401.6 eV [44] (Fig. 5B) and are assigned to the presence of C–N/NH_x and protonated NH₃ or C–N containing functions neighbouring an oxygenated function respectively. The O 1s spectra (Fig. 5C) reveals three peaks with binding energies of 531.3, 532.1 and 533.5 eV. The lower energy peak can be ascribed to quinone and carbonyl type functions, and consistent with the C 1s chemistry. The peak at 533.5 eV [41], [45] may be attributed to carboxylic functions. However, as some S and Si are present, these signal may also comprise of Si–O and S–O bonds (present as impurities) nevertheless, there is significant modification of the carbon network to conclude that the surface of GQDs contains

abundant oxygen containing functional groups like hydroxyl and carbonyl thus imparting the GQDs its high aqueous solubility.

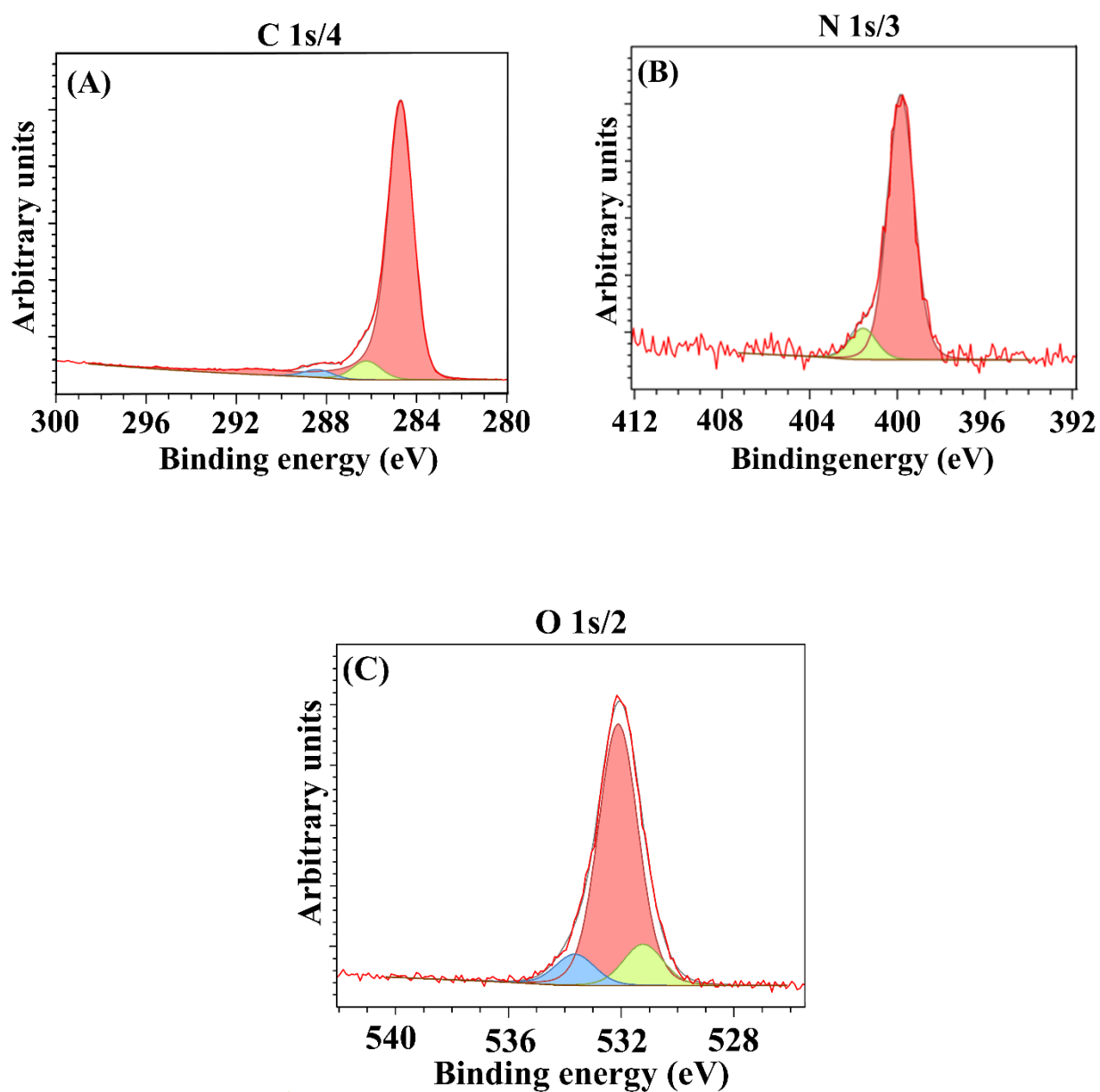


Fig. 5. (A) C 1 s (B) N 1 s and (C) O 1 s XPS spectra of the synthesised GQDs

3.4. Fluorescence Sensing Study of H₂O₂ by GQDs

3.4.1 In serum medium

To prove the feasibility of our GQDs for use as a fluorescence detection platform, the variation of fluorescence intensities of the GQDs with increasing H₂O₂ concentrations were measured (Fig. 6a). The fluorescence intensity of the GQDs gradually decreases with increasing concentration of H₂O₂. The plot in Fig.6b also shows that there is a good linear relationship between F_0/F (where F_0 and F are the relative fluorescence intensities of GQDs in absence and presence of H₂O₂ respectively) vs. H₂O₂ concentration over the range 39.92-582.5 pM with a limit of detection 139.5 pM. The linear regression equation was $F_0/F = 0.0003[\text{H}_2\text{O}_2] + 1.046$, with a coefficient of determination $R^2 = 0.962$. The limit of detection was based on the equation $\text{LOD} = 3.3 (\sigma/S)$, where σ is the standard deviation and S is the slope of the calibration curve of the responses. This result indicates that the GQDs can be used as a sensor platform for H₂O₂ at ultra-trace scale by tracking the changes of fluorescence intensity.

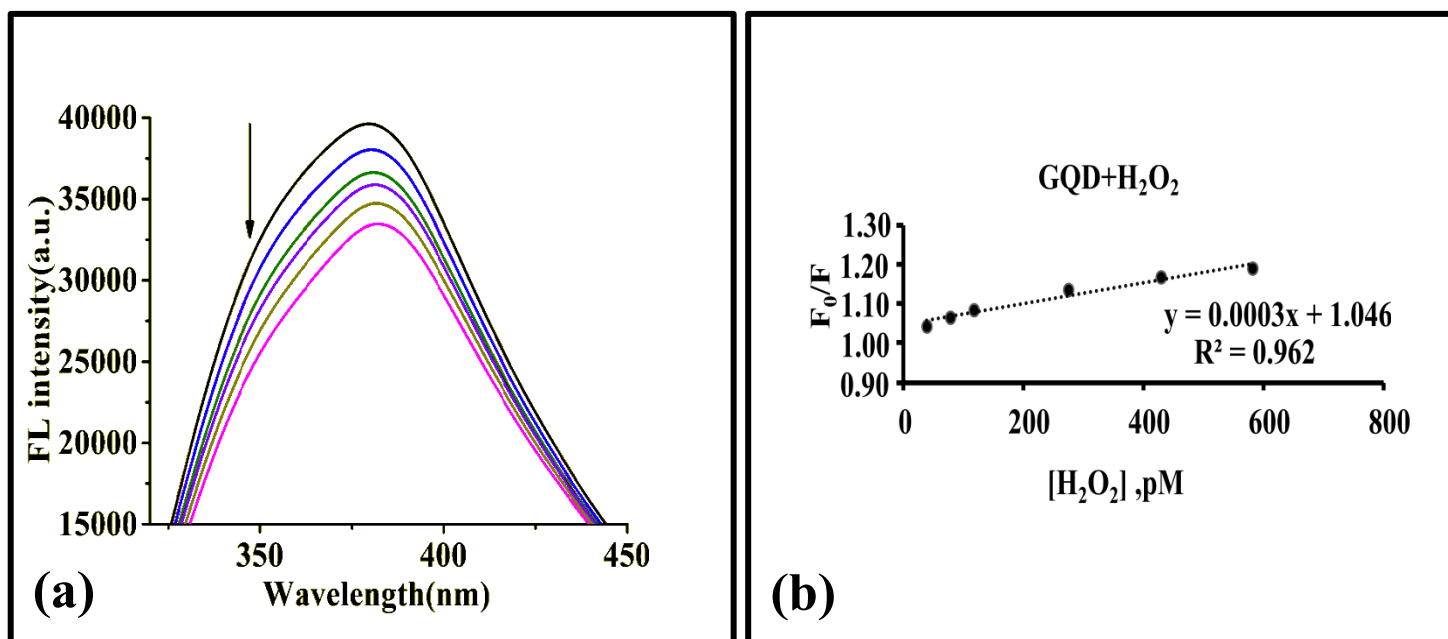


Fig 6. (a) Fluorescence spectra of GQDs in serum medium with increasing concentrations of H₂O₂ in the range of 39.92-582.5 pM ($\lambda_{\text{ex}} = 254 \text{ nm}$, $\lambda_{\text{em}} = 380 \text{ nm}$); (b) Stern-Volmer plot of F_0/F versus H₂O₂ concentration for GQDs

3.4.2. In MCF-7 and HaCaT cell lysate medium

To evaluate and compare the sensing in practical situations, the MCF-7 cell lysate was spiked with a series of various concentrations of H_2O_2 and the plot of F_0/F versus H_2O_2 concentration was drawn. It is clearly visible that the fluorescence intensity of the GQDs monotonically decreases with increasing concentration of H_2O_2 over the concentration range 39.92-582.5 pM (Fig.7a). The plot in Fig.7b shows that there is a good linear relationship between F_0/F and H_2O_2 concentration with a limit of detection 61.43 pM. The linear regression equation was $F_0/F = 0.0003[\text{H}_2\text{O}_2] + 1.096$, with a coefficient of determination $R^2 = 0.993$.

To compare the performance of our GQDs in normal cells, we also have carried out the sensing assay in HaCaT cell lysate medium. Here, with increasing concentration of H_2O_2 , the fluorescence intensity of the GQDs did not show a prominent change (Fig. 8a). Also, the F_0/F versus concentration plot shown in Fig. 8b portrayed non-linearity. This is a much anticipated event as the HaCaT cell medium does not provide the required environment for H_2O_2 sensing as is possible in MCF-7 cell lysate medium which is conducive for sensitive interaction of H_2O_2 with GQDs. The observations can be explained by the fact that in response to oxidative stress, normal activity of certain enzymes, like heme oxygenase-1 (HO-1) [46], superoxide dismutase (SOD) [47], glutathione peroxidase (GPX) [47], catalase [47], etc., in HaCaT cells show their cytoprotective properties thereby suppressing generation of reactive oxygen species (ROS).

On the other hand, high concentration of estrogen is found to be associated with increased breast cancer risk [48] and estrogen induces rapid formation of mitochondrial ROS in MCF-7 cells [49]. Therefore, it may be deduced that the estrogen induced ROS-mediated signalling in MCF-7 cells is what drives the interactions of H_2O_2 with GQD in MCF-7 cell lysate medium. GQDs therefore cement its potential as an H_2O_2 -responsive cancer sensing unit. This also proves that our proposed sensor can be utilized as an effective probe for the identification of cancer cells from normal cells. A detailed mechanism is described in a later section.

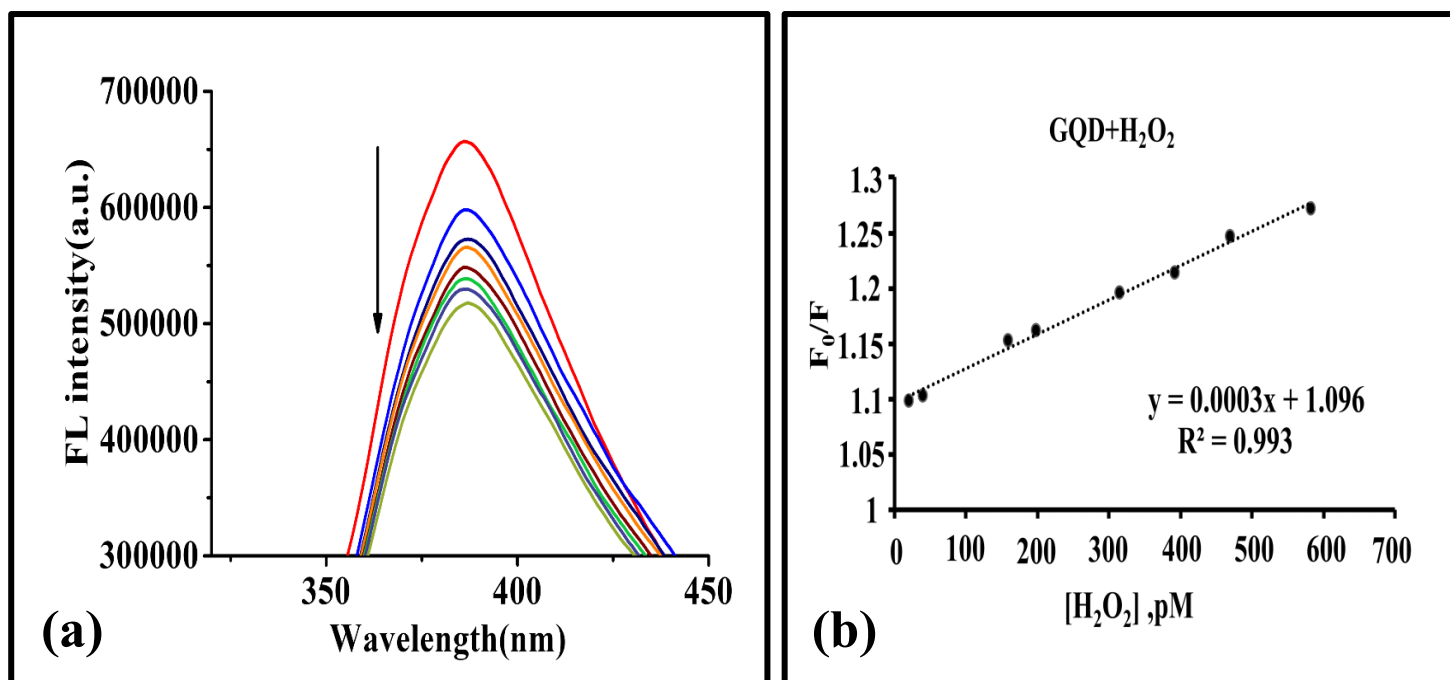


Fig 7. (a) Fluorescence spectra of GQDs in MCF-7 cell lysate medium with increasing concentrations of H_2O_2 in the range of 39.92-582.5 pM ($\lambda_{\text{ex}} = 254 \text{ nm}$, $\lambda_{\text{em}} = 386 \text{ nm}$); (b) Stern-Volmer plot of F_0/F versus H_2O_2 concentration for GQDs

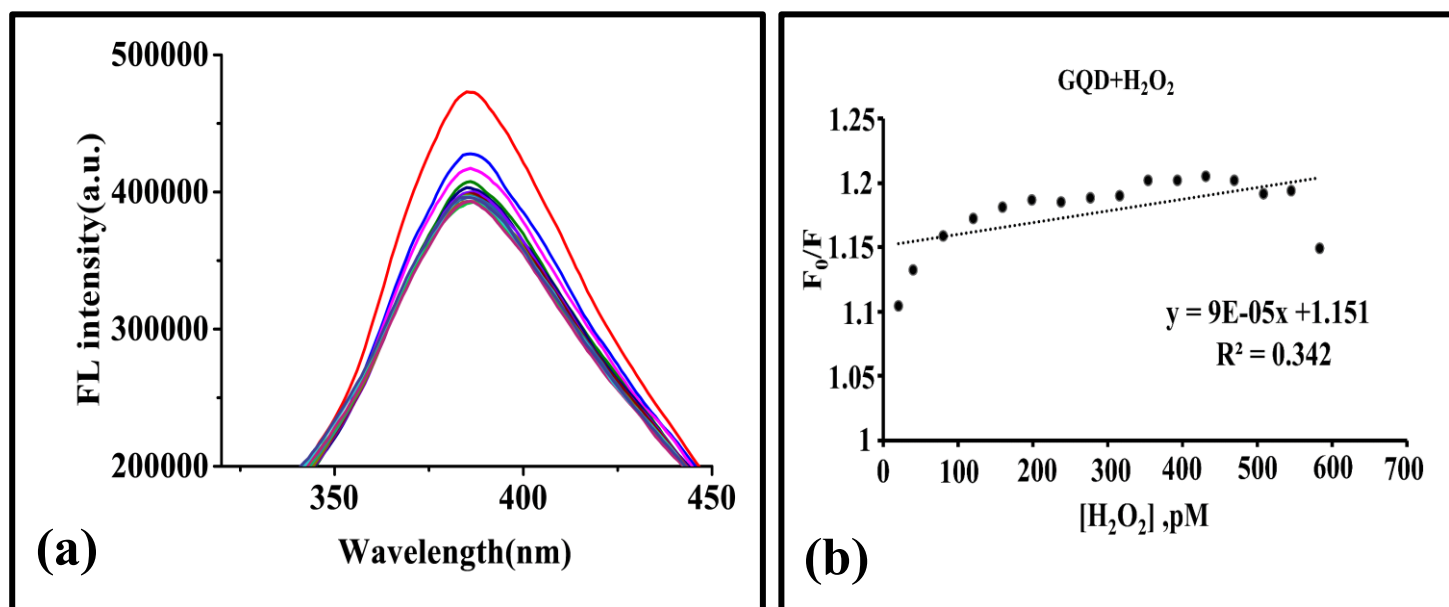


Fig 8. (a) Fluorescence spectra of GQDs in HaCaT cell lysate medium with increasing concentrations of H_2O_2 in the range of 39.92-582.5 pM ($\lambda_{\text{ex}} = 254 \text{ nm}$, $\lambda_{\text{em}} = 386 \text{ nm}$); (b) Stern-Volmer plot of F_0/F versus H_2O_2 concentration for GQDs

3.5. Limit of detection (LOD) determination

Interaction between the GQDs and H₂O₂ was monitored fluorometrically at an excitation wavelength of 254 nm in both serum and MCF-7 cell lysate medium. The fluorescence intensity of the GQDs gradually decreased with increase in concentration of H₂O₂ in both the media. Also, in presence of comorbidity causing biomolecules such as dextrose, cholesterol, insulin and nitrite ions, decrease in fluorescence intensity is observed with increase in H₂O₂ concentration which has been illustrated in Fig. S2 and S3. Calculated LOD value of each condition set in both serum and MCF-7 cell lysate medium is shown in the following Table 4. LOD obtained in presence of interfering biomolecules is much less as compared to an otherwise healthy condition. Thus, this detection method could also be applied efficiently to patients having these comorbidities. The lowest LOD value in serum medium was observed in presence of nitrite (125.0 pM). Thus, serum samples from patients having high nitrite in their serum will show even more sensitive detection of H₂O₂. However, even if the patient serum is free from this biomolecule, H₂O₂ sensing could still be enhanced efficiently by the GQDs upon its *in vitro* addition to the serum samples. It is very clear from the data presented in Table 4 that sensing assay carried out in cell lysate medium offers highly sensitive detection of H₂O₂. Table 5 and Table 6 show different analytical parameters associated with H₂O₂ sensing by the GQDs along with their R² values in serum and MCF-7 cell lysate medium respectively.

Table 4: Limit of detection (LOD) values for H₂O₂ in different conditions with GQDs

Medium	LOD (pM)					
	Conditions*					
	N	D	C	A	H	N _t
Serum	139.5±6.978	215.1±10.75	161.6±8.084	224.6±11.23	179.2±8.962	125.0±6.252
MCF-7 cell lysate	61.43±3.072	188.7±9.438	86.39±4.320	79.13±3.959	105.9±5.296	166.1±8.308

Conditions*: N = with increasing concentration of H₂O₂, D = with increasing concentration of H₂O₂ in presence of 1 mM dextrose, C = with increasing concentration of H₂O₂ in presence of 1 mM cholesterol, A = with increasing concentration of H₂O₂ in presence of 40 mIU actrapid, H = with increasing concentration of H₂O₂ in presence of 40 mIU huminsulin, N_t = with increasing concentration of H₂O₂ in presence of 220 µM nitrite

Table 5: Various analytical parameters for the fluorometric detection of H₂O₂ in serum medium by QDs

Conditions	Slope (S)	Intercept (C)	R²	Standard deviation (σ)	LOD (pM)	LOQ (pM)
H₂O₂	3×10⁻⁴	1.046	0.962	0.013	139.5	422.9
H₂O₂ + Dextrose	6×10⁻⁵	1.045	0.933	0.007	215.0	651.7
H₂O₂ + Cholesterol	9×10⁻⁵	1.021	0.949	0.004	161.6	489.9
H₂O₂ + Actrapid	1×10⁻⁴	1.036	0.918	0.010	224.6	680.8
H₂O₂ + Huminsulin	8×10⁻⁵	1.051	0.947	0.004	179.2	543.1
H₂O₂ + Nitrite	9×10⁻⁵	1.022	0.972	0.003	125.0	378.8

Table 6: Various analytical parameters for the fluorometric detection of H₂O₂ in MCF-7 cell lysate medium by GQDs

Conditions	Slope (S)	Intercept (C)	R ²	Standard deviation (σ)	LOD (pM)	LOQ (pM)
H ₂ O ₂	3×10 ⁻⁴	1.096	0.993	0.005	61.43	186.1
H ₂ O ₂ + Dextrose	3×10 ⁻⁴	1.213	0.933	0.017	188.7	571.9
H ₂ O ₂ + Cholesterol	3×10 ⁻⁴	1.231	0.981	0.005	86.39	261.7
H ₂ O ₂ + Actrapid	2×10 ⁻⁴	1.263	0.984	0.005	79.13	239.8
H ₂ O ₂ + Huminsulin	7×10 ⁻⁴	1.226	0.967	0.022	105.9	321.0
H ₂ O ₂ + Nitrite	2×10 ⁻⁴	1.049	0.964	0.008	166.1	503.5

3.6. Mechanism of H₂O₂ sensing by GQDs

The possible detection mechanism of H₂O₂-induced fluorescence quenching of the GQDs can be explained on the basis of interactions between highly oxidizing hydrogen peroxide and electron rich GQD centres. Upon UV excitation, H₂O₂ decomposes to generate ROS in the medium which interact with the GQDs and instantly get reduced to O₂ by the electrons present in GQDs that have crossed the band gap. As a consequence, the radiative recombination of the photoinduced electrons and holes is not possible any further leading to quenching of fluorescence intensity of the GQDs.

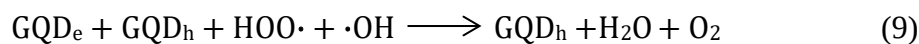
In MCF-7 lysate, ROS production is upregulated due to higher production of estrogen. Hence, H₂O₂ undergoes higher decomposition and thus the fluorescence quenching of the GQDs by H₂O₂ is also high in MCF-7 lysate. However, in normal cells, the presence of

several ROS scavenging enzymes like, HO-1, SOD, GPX, etc., diminish the possibility of H₂O₂ decomposition [46,47] thereby nullifying the interactions of the decomposition products with GQDs to show spectral sensing. On the other hand, in lyophilized human serum medium, the said interactions are observed, but to a lesser extent as compared to the MCF-7 lysate medium. This can be attributed to the absence of both ROS scavenger enzymes as well as estrogen in the medium. Production of free radicals solely occurs here due to the decomposition of H₂O₂ in presence of UV light. The possible reactions occurring in the medium are shown below.

Decomposition of H₂O₂ in presence of light



Decomposition of H₂O₂ in presence of GQDs and light



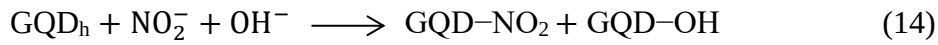
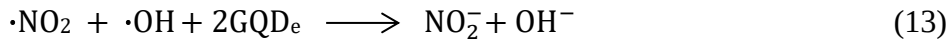
In presence of NO₂⁻, decomposition of H₂O₂



Plausible mechanism of quenching in presence of nitrite

In presence of nitrite as the nitrite ion reacts with H₂O₂ to form peroxynitrite (ONOO⁻) which in turn decomposes to produce nitrogen dioxide radical (·NO₂) which gets reduced to nitrite ions upon interacting with the free electrons of GQD. The holes of GQD (GQD_h) available in the system get neutralized by the electron rich NO₂⁻ and OH⁻ species. In the resulting GQD–NO₂ and GQD–OH species, higher fluorescence quenching occurs owing to its association with higher electronegative moieties. The presence of nitrite group embedded in GQDs has been confirmed from the FTIR spectrum of H₂O₂ and NO₂⁻ treated GQDs which has been presented in Fig. S4 along with a table (Table S1) showing the list of possible functionalities present.





Fluorescence quenching usually originates from dynamic or static quenching. One can distinguish between static and dynamic quenching using fluorescence lifetime measurements. The measurement of fluorescence lifetimes is the most definitive method to distinguish static and dynamic quenching [50].

The time-resolved fluorescence spectroscopy was carried out to explore the H_2O_2 induced fluorescence quenching mechanism of the GQDs. The average fluorescence lifetime is calculated from the equation (15)

$$\tau_{avg.} = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i \quad (15)$$

where α_i represents amplitude, τ_i represents decay time and $\tau_{avg.}$ represents average fluorescence lifetime. Fig 9. shows the fluorescence decay of the GQDs in the absence and presence of H_2O_2 . The fluorescence lifetime of the GQDs in absence of H_2O_2 is found to be 4.76 ns. Upon addition of H_2O_2 , the lifetime of the GQDs decreases to 4.293 ns, suggesting that dynamic fluorescent quenching occurs through a fast electron transfer between GQDs and H_2O_2 . Since dynamic quenching is a rate process acting on the entire excited-state population, the mean decay time of the entire excited-state population decreases. As evident from the decay parameters (Table 7), GQDs displayed a bi-exponential fit in the absence and presence of H_2O_2 with favourable χ^2 values.

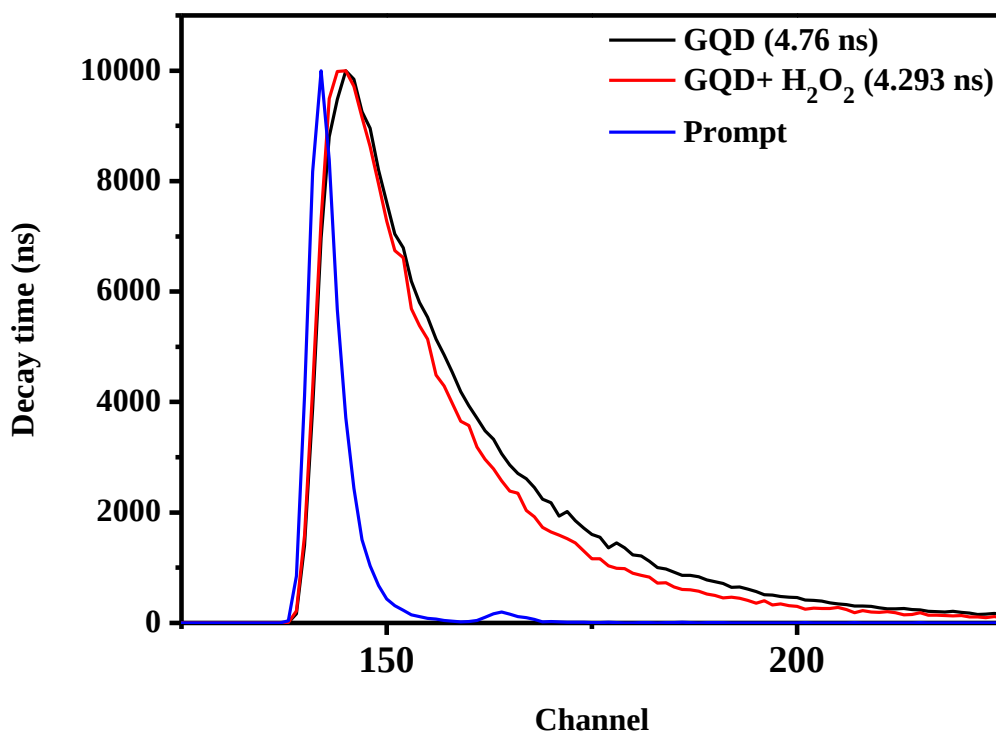


Fig 9. Fluorescence decay of GQDs as the function of time in absence and presence of H_2O_2

Table 7: Time-resolved fluorescence decay parameters of GQDs

Sample	τ_1 (ns)	α_1	τ_2 (ns)	α_2	$\tau_{avg.}$ (ns)	χ^2
GQDs	2.351	62.64	6.296	37.36	4.76	0.958
GQDs+ H_2O_2	2.072	70.39	6.095	29.61	4.293	0.943

3.6.1. TEM analysis of H_2O_2 treated GQDs

The instability in GQDs is caused by an increasing number of holes being produced in the lattice as a result of the increased interaction of conduction band electrons with H_2O_2 and subsequent production of free radicals. Consequently, the surface of GQDs that have interacted with H_2O_2 are now electron-deficient and reorganize to compensate for the electron deficit from other GQD regions in order to establish stability. Thus, it can be clearly observed in the TEM images (Figs. 10 a & 10 b), that the discrete GQD particles agglomerate when they are treated with H_2O_2 .

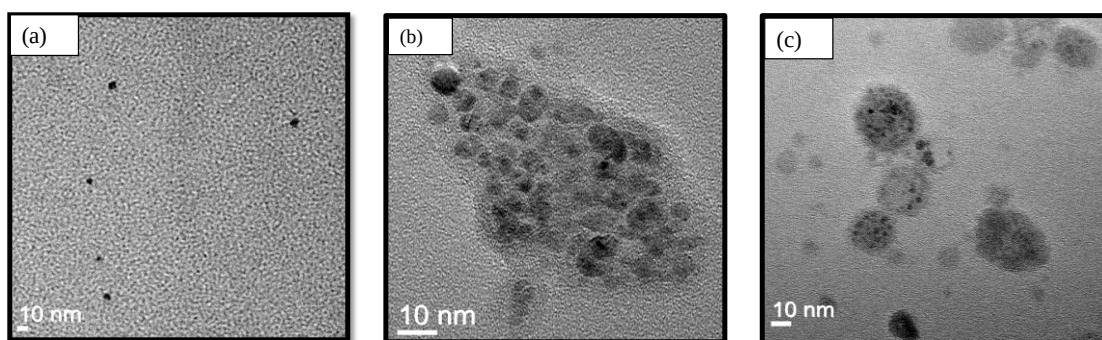


Fig 10. TEM image of GQDs (a) without H_2O_2 (b) with H_2O_2 (c) with H_2O_2

3.6.2. FTIR analysis of H_2O_2 treated GQDs

It is proposed that H_2O_2 can be annihilated at the graphitic domains of GQDs. GQDs by virtue of their peroxidase-like catalytic activity, reduces H_2O_2 to H_2O and O_2 . Thus, the broad band appearing in the $3000\text{--}3400\text{ cm}^{-1}$ region due to N–H and O–H stretching vibrations in the FTIR spectrum of GQDs intensifies due to increased intermolecular hydrogen bonding with the produced H_2O from H_2O_2 . Oxidative cleavage of 1,2 cis-diols by H_2O_2 results in the formation of carbonyl centres which might be the reason of the carbonyl peak that appears at 1647 cm^{-1} in GQDs to increase in intensity. The C=C peak at 1456 cm^{-1} in GQDs gets shifted to 1400 cm^{-1} with a slight decrease in intensity suggesting interaction of the sp^2 carbon nanodomains with H_2O_2 . The band appearing due to presence of epoxy group at 1046 cm^{-1} in GQDs diminishes on interaction with H_2O_2 due to cleavage of the epoxy bonds. The peak at 1057 cm^{-1} appearing in the spectrum of H_2O_2 treated GQDs can be attributed to the C–O stretching vibration [51]. A prominent peak due to the C–H bending vibration of the aromatic system appears at 824 cm^{-1} which may have appeared due to disruption of graphitic core of the GQDs by H_2O_2 . The spectral data are presented in table 3.

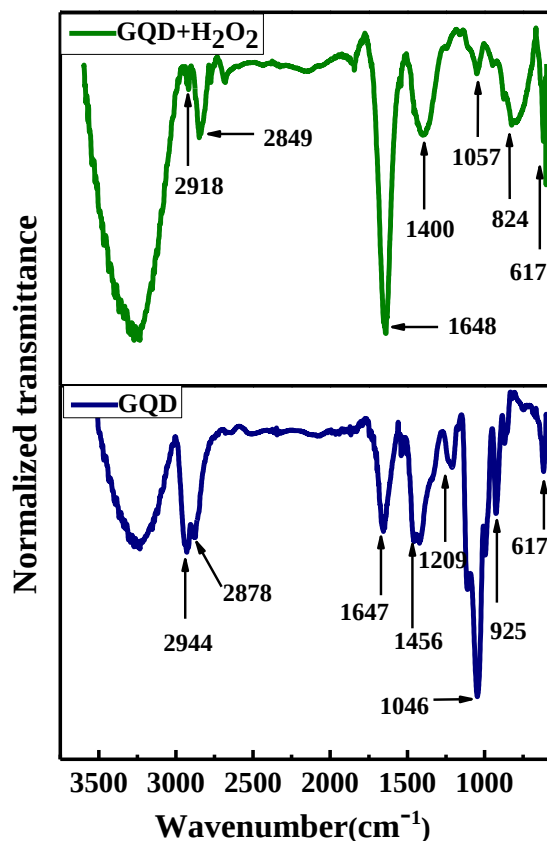


Fig 11. FTIR spectra of GQDs (navy blue), GQDs treated with H₂O₂ (olive green)

4. Comparison of the analytical performance of the present H₂O₂ sensing with some of the reported procedures

Different techniques have been used to produce the H₂O₂ sensor up until now. In this work, our major goal is to create a sensor that can detect ultra-trace amounts of H₂O₂ in the presence of comorbidity-causing biomolecules at elevated concentrations. Table 8 compares the suggested sensor's LOD to that of the previously reported ones. In terms of extremely low detection limits and robust analytical properties, the performance of the presently recommended sensing tool is significantly better than that of the earlier constructed sensors indicated in the table. The present method has an additional advantage of its applicability in actual cancer cell lysate medium, which is again different from that in healthy cell lysate.

Table 8: A comparative study of H₂O₂ sensing by different reported methods with the proposed method

S.No.	Sensor Material	Method	Medium	Limit of Detection (LOD)	Linear range	Reference
1.	*PVA/GQD nanofibrous membrane	Optical	PBS****	1.0 μ M	0.05 to 35 mM	[52]
2.	GQDs@AgNPs**	Optical	Aqueous	-	0-100 μ M	[31]
3.	Nitrogen doped GQDs (N-GQDs)	Optical	PBS	0.212 M	0.1-1 M	[17]
4.	Pd NPs decorated N-GQDs@N-doped Carbon Hollow Nanospheres	Electrochemical	PBS	20 nM	upto 1.4 mM	[53]
5.	GQDs/Gold electrode	Electrochemical	PBS	0.7 μ M	0.002-8 mM	[54]
6.	N-GQDs	Optical	H ₃ PO ₄ -NaH ₂ PO ₄ buffer	5.3 μ M	20-1170 μ M	[55]
7.	N-GQDs/Rhodamine B	Optical	Human serum	0.46 μ mol/L	4.0-40 μ mol/L	[56]
8.	N-GQDs	Electrochemical	PBS	63 nM	0.3–100.0 μ M	[57]
9.	GQD-PNF***-GO****	Electrochemical	PBS	1.056×10^{-6} M	0.2×10^{-3} to 12.2×10^{-3} M	[58]
10.	GQDs	Optical	MCF-7 cell lysate	61.431 pM	39.920-582.524 pM	Our work

*PVA: Polyvinyl alcohol, **NP: Nanoparticle, ***PNF: Peptide Nanofibers,

****GO: Graphene oxide, *****PBS: Phosphate-buffered saline

5. Conclusion

In summary, we have successfully utilized tryptophan and glycerol as precursors to synthesise highly fluorescent GQDs with a high quantum yield of 9.57% ushering in a new era of easy and environmentally friendly N-functionalized GQD synthesis. The GQDs demonstrated a highly efficient and sensitive detection ability to recognize H₂O₂ with an ultra-trace detection limit of 139.5 in serum medium. The limit of detection was even lower

when sensing assay was carried out in MCF-7 cell lysate medium (LOD 61.43 pM). Also, in presence of various physiological variables significantly low LOD values were obtained. Again, the sensing assay carried out in HaCaT cell lysate medium did yield fruitful results in terms of its unfavourable environment to sense H₂O₂ thereby cementing the potential of our proposed sensor as an H₂O₂-responsive cancer sensing unit.

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