Cytocompatible peroxidase mimic CuO:graphene nanosphere composite as colorimetric dual sensor for hydrogen peroxide and cholesterol with its logic gate implementation

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Faster and easy detection of cholesterol still remains a challenge. Thus, by introducing colorimetric sensor for detection of cholesterol may lead to the fabrication of a ready to use sensing strip. Our present work demonstrates the use of a cytocompatible CuO:Graphene nanosphere (CuO:GNS) composite as a peroxidase mimic for detection of H2O2 and free cholesterol. The synthesized CuO:GNS composite was investigated systematically for structural, morphological and functional aspects. The proposed methodology involves detection of H2O2 produced during oxidation of free cholesterol in the presence of cholesterol oxidase. The nanocomposite sensor has shown excellent detection sensitivity for cholesterol and has demonstrated a linear response in the range of 0.1 mM–0.8 mM with LOD as low as 78 μM. This nanocomposite sensor also detected a very low concentration of H2O2 (0.01–0.1 mM) with LOD of 6.88 μM. An AND logic gate system based on CuO:GNS and Cholesterol input was also proposed. The CuO:GNS was found to have better cytocompatibility than standalone CuO.

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1. Introduction

Cholesterol plays an important role in a range of physiological functions in the body such as maintaining cell membranes, production of bile acids, a precursor in several biochemical pathways and as a construction unit in the hormonal system [1]. The abnormality in cholesterol concentration can lead to various diseases such as coronary heart disease, arteriosclerosis, myocardial infarction, hypertension etc. [2,3]. The routinely practised quantification of cholesterol concentration has been a vital need for clinical diagnosis [4]. Hence, there is a considerable interest in making new cholesterol biosensor [5–7].

Nanomaterials are getting vital attention as enzyme mimics owing to their unique properties, high stability under abrasive conditions, low cost, ease of synthesis and their utilization in a wide range of applications [8]. Although, natural enzymes have very high specific activity but their extraction, purification, expenses, storage and inability to sustain harsh conditions, limits their applications [9].

Metal and metal oxide nanoparticles are shown to have enzyme mimic activity [10,11] and has gained much attention for their applications towards biological systems [12,13] and sensors [14]. The CuO nanoparticles are being studied extensively for enzyme mimic [15], sensing [16] or catalytic properties [17–19]. Recently, various enzyme mimic inorganic nano-hybrid combinations and nanocomposites have been explored due to their enhanced properties compared to their solo counterparts [9,20–22].

Graphene quantum dots (GQD) are the graphene sheets with planner size less than 100 nm and due to edge effect and quantum confinement, the GQD’s are reported to have excellent luminescence, remarkable mechanical properties, good chemical resistance and excellent biocompatibility [23–25] but enzymatic activity of graphene is relatively less explored [26]. Graphene has gained significant attention in the area of chemical sensing due to its carbon base and 2D structure [27]. Graphene is expected to be an ideal substrate for anchoring of metallic and metal oxide nanoparticles for enhanced performance [28]. The large surface area and perfect two-dimensional carbon structure of graphene results in a higher catalytic response of its composites [29]. The catalytic activity of graphene can be tuned by tuning its shape, size and thickness [30].
The sensing response of nanoparticles is also shown to have a shape dependency [31].

The majority of work reported in biosensor area is based on amperometric detection of analytes [32], but these methods suffer from manufacturing issues, narrow range of diagnosis and sometimes the detected concentrations fall considerably below the physiological range which requires quite high sample dilution [33]. On the other hand, the colorimetric sensors have the advantage of efficiency, convenience and no requirement of sophisticated instruments [34].

The sensing behaviour of CuO is extensively reported towards glucose, lactate, hydrogen peroxide, volatile organic compounds and gases [17,18,35–39], but its composite with graphene nanosphere is not yet explored. The toxicity associated with metal oxide nanoparticles limits their applicability and cytocompatible alternatives are being sought. CuO nanoparticles are reported to have genotoxicity and can cause DNA damage. In view of this, efforts are being made to reduce the toxicity of CuO [40]. There are various reports available for toxic impacts of CuO [41] but its graphene composite is rarely studied [42].

The area of molecular logic gates is growing rapidly. The development of molecular logic gates having chemical signals as input and optical signals as measurable output are gaining significant interest recently. The conventional silicon based electronics is reaching its limits due to physical constraints and hence the development of new molecular logic gates is need of the hour [43].

Herein, we report a simple, cytocompatible and highly efficient dual sensor for colorimetric detection of H₂O₂ and cholesterol. The synthesized CuO:GNS nanocomposite not only mimic peroxidase activity but also give enhanced sensing response as compared to standalone CuO nanoparticles with reduced toxicity. To the best of our knowledge, this is the first report on the use of CuO:GNS nanocomposite as a dual sensor for cholesterol and H₂O₂ detection. The proposed methodology has shown excellent selectivity towards cholesterol. The methodology is also utilised as molecular logic gate to implement AND logic function.

2. Experimental

2.1. Materials and instrumentation

Copper acetate monohydrate, Sodium hydroxide, Hydrogen peroxide and Phenol were purchased from Merck. 4-aminoantipyrine (4-AAP), 3,3′,5,5′-tetramethylbenzidine (TMB), cholesterol and cholesterol oxidase were purchased from sigma-aldrich. Rhodamine-123 and 2,7-dichlorofluorescin diacetate (DCFH-DA) was purchased from sigma-aldrich. All chemicals were used as received without further purification. The water used was purified by a Sartorius Milli-Q system. Synergy H1 microplate reader from BioTek was used for absorption measurements. IR spectra [4000–400 cm⁻¹] were recorded with a Bio-Rad FTS 3000MX instrument on KBr pellets. Spectrophotometric measurement was performed on a Varian UV–vis spectrophotometer (model: Carry 100) using a quartz cuvette with a path length of 1 cm. Thermogravimetric analyses were performed on a Metler Toledo thermal analysis system. The measurements were done at a heating rate of 5 °C/min from 25 °C to 1000 °C under flowing nitrogen environment. Powder X-ray diffraction studies were carried out on Rigaku Smart Lab X-ray diffractometer using CuKα radiation (1.54 Å). TEM was done using Philips CM 200 Transmission Electron Microscope.

2.2. Synthesis of CuO nanoparticles

The synthesis of Copper oxide nanoparticles was based on a simple chemical co-precipitation method reported earlier [44]. Briefly, copper acetate monohydrate (0.02 M) was dissolved in water and 500 μL glacial acetic acid was added to the solution. The mixture was stirred, heated and refluxed for two hours. NaOH aqueous solution was added to above reaction. The final concentration of NaOH was 0.05 M. The addition of NaOH resulted in quick precipitation and color of the solution turned black. The mixture was centrifuged at 7000 rpm for 30 min and washed thrice with absolute ethanol and dried in the air. The synthesized CuO nanoparticles were fairly soluble in water.

2.3. Synthesis of graphene nanosphere (GNS)

GNS was synthesized using ultrasonication assisted method in which graphite powder was mixed with 20 mL of H₂SO₄·HNO₃ (3:1) solution and sonicated for 3 h, during sonication the temperature was raised from RT to 60 °C. Further, the solution was heated at 90 °C and stirred vigorously. After 90 min the reaction was stopped and the solution was left to cool down. The pH of the solution was adjusted using NaOH. The supernatant was dialysed using 3.5 kDa dialysis tubes for 4 days. Finally, a yellow coloured solution was obtained. The effect of H₂SO₄·HNO₃ on the interlayer spacing of graphite was reported earlier [45]. The catalytic response of graphene is size and shape dependent [30]. The accurate control of the size of graphene is still a challenge. The size of graphene nanosphere can possibly be altered by different sonication/mechanical condition, varying etching time, heating rate and time [46–49].

2.4. Preparation of CuO:GNS nanocomposite

A dark brown coloured solution of water soluble CuO nanoparticle (5 mL) was mixed with the yellow coloured solution of GNS (5 mL) and stirred vigorously and sonicated for 15 min. The mixture was further heated for 30 min left to cool down. Within 30 min CuO:GNS nanocomposite settles down. The supernatant was discarded and the composite was air dried.

2.5. Determination of peroxidase activity

A micro-assay was used to investigate the catalytic behaviour. A stock solution of TMB (5 mM) was made in absolute ethanol. Stock solutions of CuO and CuO:GNS (0.2 mg/mL) were made in water. In a typical experiment 76 μL of stock TMB (final concentration 750 μM) was added to 320 μL PBS (pH = 7.4) and incubated for 2 min. To this 84 μL of H₂O₂ was added. The above solution was divided into two wells of 96 well plate (240 μL each). 10 μL of CuO and CuO:GNS solution was added to well 1 and 2 respectively. Corresponding control was kept without adding H₂O₂. The plate was read after 5 min using a microplate reader and respective control was reduced.

2.6. H₂O₂ detection using CuO:GNS nanocomposite

The stock solutions of Phenol (6 mM) and 4-AAP (12 mM) was prepared in 20 mM PBS. CuO:GNS stock solution of 0.2 mg/mL and H₂O₂ solution of various concentrations were prepared. In a typical experiment, a multiwell plate was used where each well was supplied with 70 μL of Phenol and 30 μL of 4-AAP along with 100 μL of 20 mM PBS and 5 μL of CuO:GNS. H₂O₂ ranging from 0.01 mM to 0.1 mM in steps of 0.01 mM was added to each well in such a way that the final volume was maintained at 250 μL. The multiwell plate was incubated at 37 °C for 40 min and was read using a microplate reader.
2.7. Cholesterol sensing

The stock solutions of Cholesterol (in absolute ethanol) and Cholesterol Oxidase (ChOx) (0.5 mg/mL in 20 mM PBS) were prepared. The stocks of Phenol, 4-AAP and CuO:GNS prepared for H₂O₂ detection were used as it is. The experimental assay was designed to be of 250 µL hence multiwell plate was used. In a typical experiment different concentration of cholesterol ranging from 0.1 mM to 1.0 mM was incubated with 5 µL of ChOx at 37 °C. The dilution of cholesterol was performed in 20 mM PBS. 70 µL Phenol, 30 µL 4-AAP and 5 µL of CuO:GNS was added to above incubation mixture. The solution was further incubated at RT and transferred to a multiwell plate which was read using a microplate reader.

2.8. Cytotoxicity studies

The toxicity of CuO and CuO:GNS was analysed using MTT assay [50], ROS generation and mitochondrial membrane potential studies [51]. For MTT assay the dose of CuO and CuO:GNS (0.1 µg/mL to 100 µg/mL) was given for a period of 24 h. The ROS generation and mitochondrial membrane potential were studied at a concentration of 50 µg/mL of CuO and CuO:GNS.

3. Results and discussion

A freshly prepared Copper oxide (CuO) and Graphene nanosphere (GNS) was taken as a precursor for the synthesis of (CuO:GNS) composite by using ultrasonic assisted method (Scheme 1). The CuO:GNS composite was employed as a colorimetric sensor for detection of H₂O₂ and Cholesterol. Further, the cytotoxicity and ‘AND’ logic implementation was studied.

3.1. Characterization of CuO:GNS nanocomposite

The structural, optical and thermal properties of CuO, GNS and CuO:GNS was investigated using Powder X-ray diffraction (PXRD), Transmission Electron Microscopy (TEM), Fourier Transform Infrared Spectroscopy (FT-IR), UV–vis spectroscopy and Thermogravimetric analysis.

The structural characterisation was performed using PXRD patterns of nanoparticles and nanocomposite as shown in Fig. 1A. The XRD pattern of CuO revealed high phase purity and crystalline nature with characteristic peak broadening of nanomaterials. The peaks were well indexed as a monoclinic crystal structure (JCPDS Card no. 80–1916) and the average crystallite size determined using the Debye–Scherer equation for the most intense peak (111) was found to be 8.5 nm. The PXRD pattern of GNS shows a characteristic peak at 2θ = 24.37° (d spacing = 3.65 Å) corresponding to 002 plane of graphene. The PXRD pattern of CuO:GNS composite shows, peaks corresponding to CuO at 2θ = 38.58°, 35.57° and 48.56° along with a broad peak at 2θ = 24.9° which corresponds to GNS. The rest of the CuO peaks, in the composite, are of low intensity which prevails that the interaction resulted in a decreased crystallinity. It indicates a good interfacial interaction between the composite components.

Fig. 1B depicts the FT-IR spectra of CuO, GNS and CuO:GNS. The frequency modes obtained at 532 and 595 cm⁻¹ are due Cu(II)-O bond vibrations. The peak around 2922 cm⁻¹ indicates the presence of –COO group of acetic acid. The peak around 1630 in GNS and CuO:GNS composite can be assigned to C=O stretching mode of
graphene. The strong broad peak near 3440 cm\(^{-1}\) is due to O–H bond stretching of the adsorbed water molecule.

The morphology of synthesized CuO, GNS and CuO:GNS composite were examined by TEM (Fig. 1C). The CuO particles were nearly spherical in shape with an average diameter of approx. 8 nm which is in accordance with the crystallite size estimated by PXRD. The GNS were also found to be spherical with an average diameter of approx. 60 nm. Interestingly, the GNS were attached to each other resulting in a random network like structure (Fig. 1C). The GNS was seemed to be converted in a sheet-like morphology in the composite as evident by TEM micrograph, whereas the CuO particles retained their size and shape in composite and appeared to be reinforced in the graphene sheet.

The UV–vis spectra of CuO, GNS and CuO:GNS are depicted in Fig. 1D. The CuO dispersed in water shows absorption peak at 275 nm. The band gap of CuO was determined to be 2.91 eV (Fig. S1), that is larger than the reported band gap of bulk CuO (1.2 eV). The increase in band gap can be attributed to the quantum confinement effect. The GNS has given a long absorption edge which was also present in CuO:GNS.

The TGA of CuO, GNS and CuO:GNS are depicted in Fig. 1E. The total weight loss up to 1000 °C was 17.5% and 25.8% in case of CuO and GNS respectively, whereas 11.2% weight loss was observed in the case of CuO:GNS indicating increased thermal stability of the composite due to positive synergistic effect.

3.2. Peroxidase mimic activity

The peroxidase mimic activity of CuO nanoparticles and CuO:GNS composite was determined using the catalytic oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) in the presence of hydrogen peroxide based on the following reaction [52] (1)
It is to be noted that both CuO and CuO:GNS composite can act as a catalyst for the oxidation of peroxidase substrate TMB which was confirmed by a sudden color change. The solution containing TMB and H$_2$O$_2$ undergoes a color change from transparent to blue by the addition of CuO or CuO:GNS which shows an absorption peak at 652 nm. The catalytic activity of CuO:GNS composite is found to be 60% higher than that of CuO nanoparticles as estimated by absorbance at 652 nm (Fig. 2).

3.3. H$_2$O$_2$ and cholesterol detection

The detection of Hydrogen peroxide is of significant importance owing to its presence as an intermediate in several processes. A colorimetric process in which phenol is coupled with 4-aminoantipyrine (4-AAP) in the presence of hydrogen peroxide and a peroxidase enzyme, yields a red coloured product 1, which was reported by Trinder (reaction 2) [53]. A similar colorimetric process was explored for H$_2$O$_2$ and Cholesterol detection using peroxidase mimic CuO:GNS composite.

Fig. 2. Absorption spectra of TMB catalytically oxidized by CuO and CuO:GNS.

Fig. 3. A) Absorbance change with increasing concentration of H$_2$O$_2$ B) Linear correlation between concentration of H$_2$O$_2$ and absorbance.
It can be seen from Fig. 3A that the increasing concentration of H$_2$O$_2$ in the presence of CuO:GNS results in increasing absorbance intensity due to higher production of a red coloured product confirming the role of CuO:GNS as a peroxidase. A linear relation in absorbance and concentration of H$_2$O$_2$ is found to be in the range 0.01 mM to 0.1 mM (Fig. 3B). The detection limit of the method is calculated to be 6.88 µM. The ESI–MS (m/z) of production of 1 is given in Fig. S2.

**Scheme 2.** Schematic illustration of colorimetric Cholesterol detection process.

**Fig. 4.** A) Absorbance change with increasing concentration of Cholesterol B) Linear correlation between concentration of Cholesterol and absorbance.
Cholesterol oxidase is an enzyme that catalyses the chemical reaction

\[ \text{Cholesterol} + \text{O}_2 \rightarrow \text{Choles} \rightarrow 4 - \text{en} - 3 - \text{one} + \text{H}_2\text{O}_2 \]

Hydrogen peroxide is the main product of oxidation of cholesterol by cholesterol oxidase, hence the \text{H}_2\text{O}_2 detection using \text{CuO:GNS} composite can be coupled with this cholesterol oxidation process to determine the presence of cholesterol (Scheme 2). The \text{H}_2\text{O}_2 released during cholesterol oxidation was detected using a reaction between 4-AAP and phenol as depicted in the Scheme 2. It was observed that the increasing concentration of cholesterol resulted in increasing absorption intensity at 490 nm and the same red coloured product formation (Fig. 4A). The proposed methodology gave a linear relation for cholesterol detection in the range of 0.1 mM-0.8 mM as depicted in Fig. 4B. The limit of detection (LOD) of the process for cholesterol was found to be 78 \:\text{\mu M}. The LOD is calculated using the following equation \[ \text{LOD} = 3.3(\sigma/S) \] \quad (1)

where \( \sigma \) and \( S \) are standard error and slope of calibration curve respectively.

Fig. 5 represents the time-dependent absorption changes in different reaction mixtures which show a continuous increase in absorbance at 490 nm as the reaction proceeds in the presence of \text{CuO:GNS} composite. However, no obvious change is observed without \text{CuO:GNS} composite, which reveals that the sensing behaviour is solely due to \text{CuO:GNS}.

3.4. Specificity and repeatability of the sensor

The specificity of the proposed methodology towards cholesterol was tested using its relative activity with other interfering species such as urea, uric acid, ascorbic acid, glucose and sucrose. The absorbance intensity for 1 mM of all the interfering species is plotted against absorbance intensity for 0.1 mM cholesterol. As evident by Fig. 6(a), the sensor is highly selective towards cholesterol detection without being affected by other species. To determine the consistency of the results obtained by the sensor, the experiment was repeated 6 times using the same set of samples made in same conditions. The measurements were performed in triplicates and the mean was calculated (Fig. 6(b)).

3.5. Stability of the sensor

The sensor has been tested for its activity at different temperatures. The relative activity was tested in the temperature range \( -20^\circ \text{C} - 90^\circ \text{C} \). The sensor is found to be stable at a wide range of temperature with maximum activity at around 40 \^\circ \text{C} (Fig. S3). The robustness of the \text{CuO:GNS} composite makes it a good alternative for other peroxidase where the environmental factor such as high temperature is a bottleneck for their application under harsh conditions.

On comparison with the recently reported probes, our \text{CuO:GNS} based dual sensor has demonstrated better performance in terms of the limit of detection (Table 1). Moreover, the sensor also has the advantage of facile synthetic approach.

3.6. Implementation of the logic system

An aqueous solution of 4-AAP, phenol and cholesterol oxidase with input cholesterol and \text{CuO:GNS} can be used to construct an absorbance based logic system. For AND logic implementation, a solution of working concentrations of Phenol, 4-AAP, and ChOx in PBS was used [Input (0,0)]. The addition of \text{CuO:GNS} to this solution was used as [Input (1,0)]. The addition of cholesterol to [Input (0,0)] was treated as [Input (0,1)]. The addition of both \text{CuO:GNS} and cholesterol to (0,0) were treated as [Input (1,1)]. The inputs were allowed to react at RT and output was measured on a microplate reader.

Fig. 7(a) represents the truth table followed by the logic system. Fig. 7(b) represents the absorbance intensity at a different input
Table 1

Performance comparison of different sensors for detection of Cholesterol.

<table>
<thead>
<tr>
<th>Material</th>
<th>Linear Range</th>
<th>LOD</th>
<th>Method</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au Nanocomposite</td>
<td>0.5–450 mg dL⁻¹</td>
<td>0.235 mg dL⁻¹</td>
<td>Electrochemical</td>
<td>[55]</td>
</tr>
<tr>
<td>ChOx/CS–GR/GCE</td>
<td>5–1000 mmol L⁻¹</td>
<td>0.715 mM</td>
<td>Electrochemical</td>
<td>[36]</td>
</tr>
<tr>
<td>AgNPs/GCE</td>
<td>3.9–773.4 mg dL⁻¹</td>
<td>0.99 mg dL⁻¹</td>
<td>Electrochemical</td>
<td>[56]</td>
</tr>
<tr>
<td>Chitosan capped CdS</td>
<td>0.64–12.9 mM</td>
<td>0.47 mM</td>
<td>Electrochemical</td>
<td>[57]</td>
</tr>
<tr>
<td>CNT/Gold</td>
<td>0.18–11 mM</td>
<td>0.02 mM</td>
<td>Electrochemical</td>
<td>[58]</td>
</tr>
<tr>
<td>ZnO@ZnS</td>
<td>0.4–3.0 mM</td>
<td>0.02 mM</td>
<td>Amperometric</td>
<td>[06]</td>
</tr>
<tr>
<td>CuO:GNS composite</td>
<td>0.1–0.8 mM</td>
<td>78 μM</td>
<td>Colorimetric</td>
<td>This Work</td>
</tr>
</tbody>
</table>


signal. The corresponding logic symbol is shown in Fig. 7(c).

3.7. Cytocompatibility of the CuO:GNS sensor

In order to investigate the biocompatibility of synthesized CuO:GNS, its cytotoxicity was compared to standalone CuO using MTT assay and ROS generation.

As exhibited in Fig. 8, CuO:GNS shows significantly better cell viability than standalone CuO. A very low concentration of CuO (~40 μg/mL) cause cell death to more than 50% of cells while more than 90% cells are live at this concentration of CuO:GNS. More than 50% cells are found live at a high concentration 100 μg/mL of CuO:GNS.

The reason of reduced toxicity of CuO:GNS was studied using the level of ROS generation and mitochondrial membrane potential. The breast cancer cell line MCF-7 treated with CuO and CuO:GNS (50 μg/mL) was incubated with 20 μM DCFH-DA. DCFH-DA reacts with ROS inside the cell and forms a green fluorescent compound dichlorofluorescein (DCF) [59], which is imaged under a fluorescence microscope. As illustrated in Fig. 9, the relative proportion of cells having ROS induction is considerably less in the case of CuO:GNS than CuO. In the case of CuO treatment, almost all cells are showing green fluorescence indicating high ROS levels, while the relative proportion of fluorescent cells as compare to non-fluorescent is less in the case of CuO:GNS treatment indicating fewer levels of ROS (Fig. 10).

Further, the mitochondrial membrane potential, which is known to reduce during apoptosis was studied using the intensity of Rhodamine-123 in CuO and CuO:GNS treated DU145 and MCF–7 cells. The intensity of red fluorescence was reduced in CuO exposed cells as compared to CuO:GNS exposed cells indicating a reduction in mitochondrial membrane potential. High fluorescence intensity in CuO:GNS treated cells indicates non-apoptosis while CuO is causing apoptotic cell death.

4. Conclusions

In summary, a simple and cost-effective methodology for the fabrication of a cytocompatible CuO:GNS nanocomposite is reported. The composite was systematically characterized and
investigated for its peroxidase mimic activity. Further, the composite was employed as a colorimetric sensor for $\text{H}_2\text{O}_2$ and cholesterol. The sensor has shown excellent sensitivity and selectivity towards cholesterol with LOD values of 78 $\mu$M and towards $\text{H}_2\text{O}_2$ with LOD of 6.88 $\mu$M. The sensor has found to be robust and stable at varied temperature. An AND logic gate was proposed using cholesterol and CuO:GNS as inputs. The composite CuO:GNS was found more biocompatible than CuO.
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2016.08.169.

References


Fig. 10. Mitochondrial membrane potential assessment following exposure to CuO and CuO:GNS.

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