Biocompatible bimetallic Au-Ni doped graphitic carbon nitride sheets: A novel peroxidase-mimicking artificial enzyme for rapid and highly sensitive colorimetric detection of glucose

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\textbf{Abstract}

We report herein, a novel two dimensional (2D) nanocomposite sensor towards detection of glucose via a simple colorimetric detection technique. The graphitic carbon nitride (g-C\textsubscript{3}N\textsubscript{4}) sheets are decorated with Au-Ni bimetallic nanoparticles with size $\sim$11 nm by a simple chemical reduction technique. The Au-Ni/g-C\textsubscript{3}N\textsubscript{4} nanocomposites is found to possess intrinsic peroxidase like activity capable of catalysing the oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) in presence of H\textsubscript{2}O\textsubscript{2} and exhibits substrate affinity much higher than biological horseradish peroxidase as well as other reported nanozymes. Also, there is a scanty of literature reports on bimetallic g-C\textsubscript{3}N\textsubscript{4} based nanocomposites towards detection of any biomolecules related to human life. The peroxidase like activity combined with glucose oxidase (GO\textsubscript{x}) helped in developing a simple, sensitive and a specific colorimetric method for glucose detection with a detection limit of 1.7 $\mu$M and a linear detection range between 0.5–30 $\mu$M. The colorimetric method for detection of glucose was further successfully extended towards detection of glucose in real serum samples with comparable results to standard hospital method. Additionally, the nanocomposites are non-cytotoxic towards human umbilical vein endothelial cell line (HUVEC) which demonstrates its high biocompatibility and suitability for biosensor applications. Our results thus demonstrate that our method based on Au-Ni/g-C\textsubscript{3}N\textsubscript{4} is reliable, economical, feasible and highly efficient.

1. Introduction

Metallic nanoparticles exhibits fascinating properties owing to their unique physical and chemical properties like high surface to volume ratio, surface plasmon resonance (SPR), super-paramagnetism and quantum confinement which makes them applicable in a host of interesting applications like catalysis [1], surface plasmonics [2,3], biosensor [4], diagnostics [5], optoelectronics etc. [6,7]. Another promising area of intense research is the use of nanomaterials as enzyme mimics [8–11]. The pioneering work led by Yan et al. in 2007 reported Fe\textsubscript{3}O\textsubscript{4} nanoparticles to possess intrinsic enzyme like activity similar to natural peroxidase and was capable of catalysing a chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB) in presence of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) to produce a characteristic blue colour compound 3,3′,5,5′-tetramethylbenzidine diimine (TMBDI) [12]. Hence, nanomaterials could best serve as artificial enzyme mimetics which are stable, less costly, exhibits excellent efficiency, selectivity [13] and can surpass the disadvantages associated with natural enzymes like denaturation and digestion by environmental changes [14]. This less cost effective and simple colorimetric assay can be utilized as a platform for biosensor application towards detection of important biomolecules like glucose [9,15], ascorbic acid [16], H\textsubscript{2}O\textsubscript{2} [8,15,17], amino acids [18], Hg\textsuperscript{2+} [19] etc. Detecting glucose concentration in real time has gained remarkable attention as alteration in its concentration is associated with a number of diseases of eyes, kidneys, blood vessels, heart, etc. [20]. Hence, monitoring glucose level in our body is vital. Following the study by Yan et al., numerous peroxidase mimicking nanomaterials were designed such as metal nanoparticles like Au [21], Ag [22], Pt [23], Cu [24], MnO\textsubscript{2} nanosheets [15], Ce(OH)CO\textsubscript{3} [17] etc. which produces the blue colour product that can be easily detected by bare eyes.

Bimetallic nanoparticles, a new class of materials have received considerable importance because of their improved catalytic performance than their monometallic counterparts. Their advantage lies in...
their capability to systematically tune their composition, coordination environment and the electronic states [25]. Consequently, bimetallic nanomaterials like Au-Pt nanostructures [26], Au-Pd nanostructure [27], Cu-Ag/rGO nanocomposites [28], Cu-Pt nanorods [29], Bi-Au nanoparticles [30] etc. are reported to possess excellent peroxidase like activity.

Amongst bimetallic systems, Au-Ni has proved to be an important catalytic system for several reactions like carbon reforming [31], CO oxidation [32], water-gas shift reactions [33], hydrodechlorination [34] etc. Au nanoparticles because of their distinct physical and chemical properties have been investigated as scaffolds for constructing novel chemical and biological sensors and are also found to be effective in stimulating the catalytic activity of artificial enzymes [35,36]. Au nanoparticles are also used towards colorimetric assays owing to their unique plasmonic properties [37,38]. Moreover their high extinction coefficients helps in improving the sensitivity and detection limits of the targeted analytes [39,40]. Again, addition of a second metal to Au greatly improves its catalytic activity. Individual Ni nanoparticles find applications in several areas like in organic catalysis [41], electro-chemical hydrogen evolution reactions [42] etc. It is thus promising to combine a second metal like Ni possessing individual interesting properties for enhancing the catalytic activity of Au.

Recently, two dimensional (2D) graphitic carbon nitride (g-C3N4), an analogue of graphene has attracted the attention of researchers because of significant properties such as appreciable thermal and chemical stability, tunable electronic structure [43], high biocompatibility and high fluorescence quantum yield [44,45]. Its constituents like carbon and nitrogen are amongst the abundant elements on earth and thus g-C3N4 is environment friendly, non-toxic and inexpensive [46]. The high degree condensation of the tri-s-triazine unit of g-C3N4 endows it with strong photoluminescence properties which coupled with its large surface area makes it a suitable podium for developing g-C3N4 based nanomaterials like Au-Pt nanostructures [26], Au-Pd nanostructure [27], Cu-Ag/rGO nanocomposites [28], Cu-Pt nanorods [29], Bi-Au nanoparticles [30] etc. are reported to possess excellent peroxidase like activity.

Precursor urea (15 g) was calcinated in a silica crucible at 550 °C at a ramping heat of 5 °C/min for 3 h. The obtained yellow solid product was ultrasonicated in 15 mL of ethanol along with 15 mL of hexane to result in instant colour change from yellow to wine red corresponding to the targeted analytes [39,40]. Again, addition of a second metal to Au greatly improves its catalytic activity. Individual Ni nanoparticles find applications in several areas like in organic catalysis [41], electro-chemical hydrogen evolution reactions [42] etc. It is thus promising to combine a second metal like Ni possessing individual interesting properties for enhancing the catalytic activity of Au.

2. Materials and methods

2.1. Materials

Urea (Sigma Aldrich, Germany), gold (III) chloride trihydrate (HAuCl4, Sigma Aldrich, Germany), nickel (II) acetylacetonate (Ni(acac)2, 95% Sigma Aldrich, USA), oleylamine (> 70%, Sigma Aldrich, USA), 1-octadecene (90%, Sigma Aldrich, USA), sodium borohydride (NaBH4 > 95%, TCI, Japan), triphenyl-phosphine (TPP, 99%, Sigma Aldrich, USA), 3,3’,5,5’-tetramethylbenzidine (TMB, Sigma Aldrich, USA), 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, TCI Chemicals, Japan), glucose oxidase (GOx, Sigma Aldrich, USA), o- (+) glucose (> 99.5%, Sigma Aldrich, USA), fructose (Sigma Aldrich, USA), maltose (Sigma Aldrich, USA), lactose (Sigma Aldrich, USA), L-ascorbic acid (Sigma Aldrich, USA), l-cysteine (≥ 99%, Sigma Aldrich, USA), dopamine hydrochloride (Sigma Aldrich, USA) were used as received.

2.2. Synthesis of g-C3N4 nanosheets

Precursor urea (15 g) was calcinated in a silica crucible at 550 °C at a ramping heat of 5 °C/min for 3 h. The obtained yellow solid product was ultrasonicated in 15 mL of ethanol along with 15 mL of hexane to obtain dispersed g-C3N4 nanosheets.

2.3. Synthesis of Au-Ni/g-C3N4 nanocomposite

Au-Ni bimetallic nanoparticles was synthesized on g-C3N4 sheets by a solvothermal reduction technique using metal precursors Ni(acac)2 and HAuCl4.3H2O. At first, 0.3 mmol of Ni(acac)2 was added to 6 mL of oleylamine solution preheated to 45 °C. Separately, 0.3 mmol of HAuCl4.3H2O in 2 mL of oleylamine and 1 mL of 1-octadecene solution was added to the Ni solution under vigorous stirring for 20 min. To the resulting solution, 0.0189 g of NaBH4 (0.01 M) was added which resulted in instant colour change from yellow to wine red confirming the reduction of Au precursors to Au nanoparticles. At this point, the temperature of the reaction was increased to 210 °C and maintained at this temperature for another 30 min. At 210 °C, solid triphenyl phosphine (TPP, 0.2 mmol) was added which helped in the reduction of Ni on preformed Au and the solution transformed to a black colloidal solution which was kept at that temperature for another 30 min for the complete reduction of Ni ions to obtain Au-Ni nanoparticles. The reaction mixture was cooled down and ethanol was added to it followed by centrifuging and then finally the Au-Ni nanoparticles were dispersed in hexane. The g-C3N4 solution obtained previously was mixed with the Au-Ni nanoparticles dispersion followed by further ultrasonication for 2 h to obtain Au-Ni/g-C3N4 nanocomposite. The entire reaction mixture passed through a 0.2 μm membrane filter and kept in a refrigerator at 4 °C for further use.

Table 1

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was filtered and then washed with toluene: ethanol in the ratio of (1:3/v:v) to remove excess solvent and subsequently dried in an air oven to obtain the desired Au-Ni/g-C3N4 nanocomposite. To confirm that Au-Ni does not undergo leaching during the synthesis process, the filtrate was subjected to atomic absorption spectroscopy (AAS) analysis to verify leaching of Au-Ni. The results of the AAS analysis confirmed the absence of Au-Ni in the filtrate thus approving no loss of Au-Ni during the synthesis process.

2.4. Instrumental techniques

Details information on different instrumental techniques is discussed in the Supporting Information (SI).

2.5. In-vitro cytotoxicity effect of Au-Ni/g-C3N4 nanocomposite on HUVEC cell line

The cytotoxicity assay was performed to study the biocompatibility of the nanocomposites which is a major concern in biosensor application as the nanocomposites might possibly come in contact with cells or blood during the biosensor application. HUVEC cells were purchased from Hi-Media (India) and were cultured to confluence using HiEndoXL™ endothelial cell expansion media with 5% CO2 at 37 °C. The cells were incubated for 24 h after reaching confluence and then mixed with nanocomposites of Au/g-C3N4, Ni/g-C3N4, Au-Ni/g-C3N4 and g-C3N4 with different concentrations ranging from 5 to 200 μg mL⁻¹ and again incubated for another 24 h. The old medium was aspirated and 200 μL of Alamar Blue solution was added to each well and incubated further for 4 h at a temperature of 37 °C. The optical density of each well was calculated using a microplate reader in the absorbance range of 570-600 nm. Similar conditions were repeated three times and the well without any treatment was taken as a control.

2.6. Flow cytometry analysis and assessment of cell death

The flow cytometric analysis was performed in a flow cytometer (CytoFLEX S, Beckman Coulter, USA). The effect of nanocomposites on the distribution of apoptotic and necrotic cells was investigated by using annexin V-FITC and propidium iodide (PI) binding bioassay. In order to distinguish the living cells (annexin V−/PI−), early apoptotic/primary apoptotic cells (annexin V+/PI−), late apoptotic/secondary apoptotic cells (annexin V+/PI+), and necrotic cells (annexin V−/PI+), quadrants positioning on annexin V/PI dot plots was carried out. Therefore, in the total apoptotic proportion, the percentage of cells with annexin V+/PI− and annexin V+/PI+ fluorescence were included. Different concentrations of nanocomposites (Au/g-C3N4, Ni/g-C3N4, Au-Ni/g-C3N4 and g-C3N4) with concentrations ranging from 5 to 200 μg mL⁻¹ were used to treat the cells which were then incubated for 24 h followed by suspending the treated cells in 1 mL binding buffer (1 ×). 5 μL annexin V-FITC and 10 μL PI was incubated for 15 min at room temperature in a dark and 400 μL binding buffer (1 ×) was added to each sample. The FITC and PI fluorescence was measured through an ow cytometer (10,000 events were acquired).

2.7. Peroxidase like catalytic activity of Au-Ni/g-C3N4 nanocomposite

For investigating the peroxidase like catalytic activity of Au-Ni/g-C3N4 nanocomposite, a series of experiments was carried out using Au-Ni/g-C3N4 which could catalyse the oxidation of chromogenic peroxidase substrate TMB or ABTS possessing opposite charges in presence of H2O2. Typically, to a sodium acetate buffer (pH 4 for TMB and pH 7 for ABTS), TMB/ABTS (0.5 mM), 50 μL H2O2 (30%) and Au-Ni/g-C3N4 nanocomposites (5 mg/L) was added respectively, and incubated for 20 min followed by examining the oxidation product of TMB using a UV−vis spectrophotometer (Shimadzu, Japan) at 652 nm and that for ABTS at 414 nm. Different parameters such as effect of catalyst concentration, pH of the reaction medium and temperature on peroxidase like catalytic activity were analysed.

Steady state kinetic analysis was assessed by changing the concentration of either TMB/ABTS or H2O2 at a time while keeping the other constant. The Michaelis-Menten curves for Au-Ni/g-C3N4 nanocomposite were obtained over a certain concentration range of TMB/ABTS or H2O2. The Michaelis-Menten constant (Km) is obtained using the Lineweaver-Burk double reciprocal plot:

\[
\frac{1}{v} = \frac{K_m}{V_{\max}} \left(\frac{1}{[S]}\right) + \frac{1}{V_{\max}}
\]

Where, \( v \) = initial velocity, \( V_{\max} \) = maximal reaction velocity and \([S]\) = substrate concentration provides the binding affinity of enzyme to the substrate. The Michaelis-Menten constant (Km) is the concentration of substrate at which reaction rate is half of \( V_{\max} \).

2.8. Catalytic procedure for the colorimetric detection of glucose

Glucose detection was realized as follows: glucose oxidase (GOx) (1 mg mL⁻¹, 0.1 mL) was added to glucose solutions (0.1 mL) of different concentrations in the range 0.001−3 mM in phosphate buffer (10 mM, pH 7, 0.5 mL) and incubated at 37 °C for 30 min followed by the addition of 0.3 mL TMB (20 mM) 2.5 mL of sodium acetate buffer (pH 4, 0.2 M) and Au-Ni/g-C3N4 nanocomposite dispersion to the above glucose solutions. The entire resulting solution was allowed to incubate further at 35 °C for 30 min and the absorbance at 652 nm was recorded. The selectivity of the Au-Ni/g-C3N4 nanocomposite towards glucose was examined considering six analogues glucose molecules namely maltose, fructose, lactose, dopamine, l-cysteine and ascorbic acid. For studying the same, 10 fold higher concentrated solutions of these molecules were used instead of glucose and the experiments were performed in a similar manner as for glucose detection. The absorbance change at 652 nm was monitored followed by comparing with the absorbance of glucose.

For further effective study, glucose was detected in real clinical blood serum samples using our colorimetric method and the obtained results were matched with that determined using standard hospital method. For the experiment, the blood samples were initially centrifuged at 4000 rpm for 5 min and then the supernatant was diluted ten times with 10 mM phosphate buffer (pH 7) to carry out further experiments similar to that used for glucose detection in standard samples and finally the absorbance at 652 nm was noted.

3. Results and discussions

3.1. Formation and characterization of Au/g-C3N4, Ni/g-C3N4 and Au-Ni/g-C3N4 nanocomposites

The size, morphology and the crystallinity of the nanoparticles was studied with the help of the TEM analysis. The TEM and the HRTEM images of the Au-Ni bimetallic nanoparticles decorated g-C3N4 sheets are shown in Fig. 1A (a−c) which demonstrates the polydisperse distribution of the nanoparticles on the g-C3N4 sheets with an average size of 11.08 ± 4 nm as determined using Image J software (Fig. 1A (d)). The particles have a variation of size from diameter 3.9 nm to large particles with diameter 30 nm. The HRTEM image confirms the presence of clear lattice fringes corresponding to both Au (111) with interplanar spacing 0.230 nm and Ni (111) with interplanar spacing 0.203 nm. From this it is evident that the Au-Ni/g-C3N4 nanocomposite consists of only bimetallic Au-Ni nanoparticles. The polycrystalline nature of the Au-Ni/g-C3N4 nanocomposite was analysed through the selected area electron diffraction (SAED) pattern which shows different planes of Au and Ni that can be assigned to fcc (111), (200), (220) and (222). The elemental distribution of the Au-Ni bimetallic nanoparticles
on g-C3N4 sheets was studied with the help of HAADF-STEM images (Fig. 1B). The as obtained elemental mapping further confirms the presence of both Au and Ni in a single nanoparticle and distributed over the g-C3N4 sheets. Thus, both HRTEM and HAADF-STEM analysis confirms that Au-Ni/g-C3N4 nanocomposite consists of only bimetallic Au-Ni nanoparticles. The TEM images of the monometallic Au/g-C3N4 and Ni/g-C3N4 are shown in the Fig. S1 of the Supporting Information (SI). The Au/g-C3N4 has an average particle size of 8.6 ± 0.2 nm with particle size ranging from 3.37 nm to 11.24 nm (Fig. S1(c)). The particles are spherical in shape containing clear lattice fringe of 0.230 nm corresponding to the (111) plane of Au. The TEM images of Ni/g-C3N4, however shows the presence of larger particles that are agglomerated and the average particle size of the particles are found to be 90 ± 4.6 nm with particle sizes ranging from 62.7 nm to 140.45 nm. The HRTEM image (Fig. S1(e)) shows clear lattice fringe of 0.203 nm for (111) plane of fcc Ni.

Fig. 1. (A) TEM images of Au-Ni bimetallic nanoparticles decorated on g-C3N4 (a–c); size distribution histogram (d); HRTEM image (e), SAED pattern (f) of Au-Ni/g-C3N4 nanocomposite (B) HAADF-STEM elemental mapping of Au-Ni/g-C3N4 nanocomposite.
The FESEM images provide information on the surface morphology of the nanocomposites. The FESEM images of Au-Ni/g-C3N4 nanocomposites are shown in Fig. 2A. The images show smooth surface with irregularly curved layers of g-C3N4. The curved surfaces of g-C3N4 sheets are decorated with spherical shaped nanoparticles of Au-Ni. The corresponding EDX data confirms the existence of both Au and Ni finely distributed on the g-C3N4 sheets (Fig. 2B). The FESEM images of monometallic Au/g-C3N4 and Ni/g-C3N4 nanocomposites are present in
The XRD patterns of Au-Ni/g-C3N4, Au/g-C3N4 and Ni/g-C3N4 nanocomposites are presented in Fig. 3. The diffractions patterns for monometallic Au/g-C3N4 appear at 2θ value of 38.2, 44.4, 64.7, 77.6 and 81.7 corresponds to the fcc Au(111), Au(200), Au(220), Au(311) and Au(222) planes, respectively. For monometallic Ni/g-C3N4, 2θ value appears at 44.3 and 51.8 corresponds to the fcc Ni(111) and Ni (200), respectively. In comparison to monometallic Au/g-C3N4 and Ni/ g-C3N4 nanocomposites, the diffraction patterns for bimetallic Au-Ni/g-C3N4 nanocomposites are found to be broader than those of the individual Au, an observation reported by Chen et al. for alloyed Ni-Au nanoparticles [55]. The diffractions for bimetallic Au-Ni/g-C3N4 nanocomposite appear at 2θ value lying between that of the monometallic counterparts at 38.22, 44.40, 50.84, 64.68 and 77.74 corresponding to the Au(111), Au(200) and Ni(111), Ni(200), Au(200), Au(311) and Ni (220), respectively. The diffractions peak for Ni(111) and Ni(220) at 2θ value of 44.40 and 77.74 overlapped with those of (200) and (311) planes of fcc Au. In all the two nanocomposites, another two diffractions appear at 20.74 and 27.68 corresponding to the (100) and (002) planes of g-C3N4 which thus confirms the presence of g-C3N4 in their structure. Thus, the XRD pattern clearly confirms the formation of Au-Ni bimetallic nanoparticles decorated on the 2D g-C3N4 sheets.

The presence of different functional moieties on the Au-Ni/g-C3N4 nanocomposite was confirmed using DRIFT spectroscopy as shown in Fig. S3 of (SI). The g-C3N4 shows strong absorption band at 981 cm⁻¹ which is attributed to the vibration from the triazine ring, one typical peak in g-C3N4. The bands at 1605 cm⁻¹ corresponds to the stretching modes of C=N while the bands at 1226 cm⁻¹ and 1373 cm⁻¹ are consistent with the aromatic C-N stretching. The broad bands above 2850 cm⁻¹ arise due to the primary and the secondary amino groups of g-C3N4. For Au-Ni/g-C3N4 nanocomposite, the absorption positions for all the absorption appears similar to those of g-C3N4, which indicates that the g-C3N4 retains its structure after the formation of the Au-Ni/g-C3N4 nanocomposite. Moreover the intensity of the peak at 810 cm⁻¹ decreases in Au-Ni/g-C3N4 as compared to that in pure g-C3N4 indicating decrease in the g-C3N4 content in the Au-Ni/g-C3N4 nanocomposite.

Study on the chemical composition and the oxidation states of the bimetallic Au-Ni/g-C3N4 and monometallic Au/g-C3N4 and Ni/g-C3N4 nanocomposites were carried out with the help of the XPS analysis. The low resolution survey spectrum for Au-Ni/g-C3N4 nanocomposite is shown in Fig. S4a. The survey spectrum of Au-Ni/g-C3N4 nanocomposite displays prominent peaks corresponding to C1s (285.15 eV), N1s (398.74 eV), O1s (531.03 eV), Au4f (83.19) and Ni2p (855.80 eV). Fig. 4 presents the high resolution XPS spectra of C1s, N1s, Au4f and Ni2p of the Au-Ni/g-C3N4 nanocomposite. The high resolution spectrum for the C1s (Fig. 4a) exhibits two strong peaks at 284.75 eV and 287.80 eV corresponding to the C–NH₂ and C–N–C functional groups, respectively [54]. The high resolution spectrum of N1s can be deconvoluted into three peaks corresponding to C–N=C at 399.45 eV, C–NH at 400.88 eV (Fig. 4b) [55]. For the high resolution spectrum of Au, two distinct peaks occur at 83.90 eV and 87.67 eV corresponding to Au4f7/2 and Au4f5/2, respectively [Fig. 4c] [56]. The high resolution XPS spectrum for Ni2p indicates two major peaks at 855.89 eV and 873.40 eV corresponding to the Ni2p3/2 and Ni2p1/2, respectively for Ni(OH)₂ phase [Fig. 4d]. These findings are in well agreement with the reported literature [57,58]. Two extra peaks centred at 861.71 eV and 879.83 eV corresponds to the satellite peaks of Ni2p3/2 and Ni2p1/2, respectively. The XPS spectra indicate the formation of Ni in the hydride form in Au-Ni/g-C3N4 nanocomposite as confirmed from the presence of O1s in the survey spectrum. The XPS analysis thus clearly confirms the formation of Au-Ni bimetallic nanoparticles on g-C3N4 sheets. Table 2 indicates the composition for the bimetallic Au-Ni/g-C3N4 nanocomposite as well as the monometallic Au/g-C3N4 and Ni/g-C3N4 nanocomposites. The high resolution XPS spectra for monometallic Au in Au/g-C3N4 and Ni in Ni/g-C3N4 are shown in Fig. S4(b) and (c), respectively.

3.2. Investigation of cytotoxic effect of Au-Ni/g-C3N4 nanocomposites on HUVEC cell line

The cytotoxic effect of g-C3N4 based nanocomposites was examined using HUVEC cell culture model. Cells were treated with or without different concentrations of all the nanocomposites (Au-Ni/g-C3N4, Au/ g-C3N4, Ni/g-C3N4 and g-C3N4) at a dose of 5–200 µg mL⁻¹ for 24 h followed by examining the cell viability using Alamar blue reduction bioassay. Results (Fig. 5) demonstrates that none of the treatments cause any significant cytotoxicity effect compared to control (untreated), which suggest the potential biocompatibility of all these g- C3N4 based nanocomposites.

Using flow cytometry techniques and Annexin V-FITC/ PI staining, investigation was carried out to study whether the treatment of g-C3N4 based nanocomposites caused any cell death (apoptotic or necrotic) on HUVECs. Fig. 6 showed that treatment with different nanocomposites (Au-Ni/g-C3N4, Au/g-C3N4, Ni/g-C3N4 and g-C3N4) at a dose of 5 and 200 µg mL⁻¹ for 24 h did not cause any Annexin V-FITC/ PI staining and thus no cell death was observed compared to those seen in the control cells. This study again supported the excellent biocompatibility of all these g-C3N4 based nanocomposites.

3.3. Intrinsinc peroxidase like activity of Au-Ni/g-C3N4 nanocomposites

The peroxidase like activity of the Au-Ni/g-C3N4 nanocomposite was studied through the oxidation reaction of the TMB or ABTS in presence of H₂O₂ to produce the coloured oxidized products of TMB and ABTS showing absorption maxima at 652 nm and 414 nm, respectively.

The oxidation of TMB or ABTS was studied in different systems to demonstrate the peroxidase like catalytic activity of the Au-Ni/g-C3N4 nanocomposite. Fig. 7(a,b) display the oxidation of TMB and ABTS using Au-Ni/g-C3N4 nanocomposite. From both the figures it is clearly observed that presence of both H₂O₂ and Au-Ni/g-C3N4 nanocomposite is required for the oxidation of TMB as well as ABTS.

The optimal conditions for peroxidase-like activity of the Au-Ni/g-C3N4 nanocomposite were determined considering different parameters such as pH, temperature and catalyst loading. The effect of catalyst loading on the oxidation of TMB was studied in the range 3–7 mg L⁻¹ and that for ABTS was studied in the range 3–9 mg L⁻¹ as shown in Fig. S5a and S5b, respectively. Fig. S5a indicates that the absorbance at 652 nm increases gradually as the amount of Au-Ni/g-C3N4 is increased from 3 to 5 mg L⁻¹. Maximum absorbance was observed at Au-Ni/g-C3N4 concentration of 5 mg L⁻¹. However, as the amount of Au-Ni/g-C3N4 increases, the extent of oxidation decreases as indicated by the
decrease in the absorbance. Same observation was observed for the oxidation of ABTS and maximum absorbance at 414 nm was obtained using 7 mg L$^{-1}$ of the Au-Ni/g-C$_3$N$_4$ nanocomposite (Fig. S5b). The pH of the buffer was varied between 2 to 9 and shown in Fig. S5c and S5d for TMB and ABTS, respectively. Maximum TMB oxidation is observed at pH 4 and that for ABTS is observed at pH 2. The effect of temperature was studied at temperatures 25 °C, 35 °C, 45 °C, 55 °C and shown in (Fig. S5(e,f)). The TMB oxidation was found to increase from 25 °C to 35 °C and beyond this the catalytic oxidation was found to be decreased. Maximum ABTS oxidation was found at 35 °C and that of ABTS was found at 25 °C. Thus, the optimum conditions for TMB oxidation using Au-Ni/g-C$_3$N$_4$ nanocomposite was found at pH 4, 5 mg L$^{-1}$ of catalyst loading at 35 °C and that for ABTS was found at pH 2, 7 mg L$^{-1}$ catalyst loading and at 25 °C.

Table 2
Chemical composition (at.%) of Au-Ni/g-C$_3$N$_4$, Au/g-C$_3$N$_4$, Ni/g-C$_3$N$_4$ and g-C$_3$N$_4$ as determined by XPS analysis.

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<td>68.84</td>
<td>24.25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g-C$_3$N$_4$</td>
<td>75.47</td>
<td>24.53</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.4. Kinetics of the peroxidase mimetic catalytic activity of Au-Ni/g-C$_3$N$_4$ nanocomposites

Enzyme kinetics is determined using the steady state method in presence of H$_2$O$_2$ and TMB or ABTS as the substrates. Typical Michaelis Menten curves using Au-Ni/g-C$_3$N$_4$, Au/g-C$_3$N$_4$ and Ni/g-C$_3$N$_4$ nanocomposites were obtained for both TMB or ABTS and H$_2$O$_2$ by changing the concentration of one substrate while keeping the other constant. A series of experiments were carried out by varying the concentration of either TMB/ABTS or H$_2$O$_2$ at a time while keeping the other constant. The obtained absorbance values were converted to the concentration using the Beer Lambert’s law:

$$A = e_{\text{MBD}} \times c \times L$$

Where, $e_{\text{TMBD}} = 39,000$ M$^{-1}$ cm$^{-1}$ for TMB and 36,000 M$^{-1}$ cm$^{-1}$ for ABTS [59]. The corresponding Lineweaver-Burk plots were obtained for both TMB/ABTS and H$_2$O$_2$ from the Michaelis-Menten curves and this gives the value of $V_{\text{max}}$ and $K_m$. The $K_m$ and the $V_{\text{max}}$ values were determined from the Lineweaver- Burk plot where the intercept of the plot gives the $V_{\text{max}}$ value and the slope gives the value of $K_m$. The Michaelis Menten constant $K_m$ is a very important parameter as it gives the binding affinity of a substrate with the enzyme.

Fig. 8 represents the steady state assay and the catalytic mechanism of Au-Ni/g-C$_3$N$_4$ nanocomposites examined using TMB and ABTS as substrates. In Fig. 8a, the H$_2$O$_2$ concentration was fixed and the TMB concentration was varied and that in Fig. 8b, the TMB concentration was fixed and H$_2$O$_2$ concentration was varied. The corresponding Lineweaver-Burk plots were fitted as shown in the insets and the values
of the obtained $V_{\text{max}}$ and $K_m$ are shown in Table 3. The low value of the $K_m$ indicated high affinity of Au-Ni/g-C$_3$N$_4$ towards TMB and high $K_m$ value for H$_2$O$_2$ indicated weak affinity towards H$_2$O$_2$. We further proceeded towards performing the kinetic assay of Au-Ni/g-C$_3$N$_4$ using ABTS as the chromogenic substrate (Fig. 8c,d). Similar to TMB, the catalytic behaviour of Au-Ni/g-C$_3$N$_4$ was found to follow Michaelis-Menten equation towards ABTS. Fig. 8c is obtained by fixing the H$_2$O$_2$ concentration at varying ABTS concentration and that in Fig. 8d, the ABTS concentration was fixed and H$_2$O$_2$ concentration was varied. The corresponding Lineweaver-Burk plots and the $V_{\text{max}}$ and the $K_m$ values prove that the affinity of Au-Ni/g-C$_3$N$_4$ towards TMB was higher than that towards ABTS molecule. The variation in the affinity of Au-Ni/g-C$_3$N$_4$ towards the substrates is due to the difference in charge on TMB, ABTS and Au-Ni/g-C$_3$N$_4$ nanocomposite. The TMB molecule is positively charged whereas the ABTS is negatively charged. The zeta potential analysis of Au-Ni/g-C$_3$N$_4$ in a wide pH range (pH 2–12) as shown in Fig. S6 gives us an idea of the negative charge prevailing on its surface. At pH 2 i.e., the optimum pH for maximum ABTS oxidation, the surface of Au-Ni/g-C$_3$N$_4$ is negatively charged and since ABTS molecule itself is negatively charged so due to the electrostatic repulsions the affinity of Au-Ni/g-C$_3$N$_4$ to bind with the ABTS molecule is less. The interaction between TMB and Au-Ni/g-C$_3$N$_4$ nanocomposite is found to be higher than that of ABTS because at pH 4 (the optimum pH for TMB oxidation), the surface charge on Au-Ni/g-C$_3$N$_4$ is negative and since TMB molecule is positively charged there is a strong possibility of electrostatic attraction between the TMB molecule and the Au-Ni/g-C$_3$N$_4$ nanocomposite resulting in better substrate binding affinity of the nanocomposite with TMB. The Michaelis-Menten curves and the Lineweaver-Burk plot were also obtained for the corresponding monometallic Au/g-C$_3$N$_4$ (Fig. S7) and Ni/g-C$_3$N$_4$ (Fig. S8) nanocomposites with both TMB and ABTS to establish the efficiency of the bimetallic Au-Ni/g-C$_3$N$_4$ nanocomposite. For the bimetallic nanocomposite, the $K_m$ values are found to be lower than those of their monometallic counterparts indicating high catalytic activity of the Au-Ni/g-C$_3$N$_4$ nanocomposite. For comparison, the $K_m$ and $V_{\text{max}}$ values for TMB, ABTS and H$_2$O$_2$ determined for Au-Ni/g-C$_3$N$_4$, Au/g-C$_3$N$_4$ and Ni/g-C$_3$N$_4$ nanocomposites are given in Table 3. The $K_m$ value for Au-Ni/g-C$_3$N$_4$ with TMB is found to be 0.16 mM which is much lower than that of Au/g-C$_3$N$_4$ (0.27 mM) and Ni/g-C$_3$N$_4$ (0.49 mM). The low affinity of Au-Ni/g-C$_3$N$_4$ with TMB indicates strong affinity of the enzyme to TMB. Also, the $K_m$ value of Au-Ni/g-C$_3$N$_4$ for H$_2$O$_2$ as substrate in presence of TMB was found to be 4.47 mM, whereas that using Au/g-C$_3$N$_4$ and Ni/g-C$_3$N$_4$ are found to be 11.37 mM and 19.91 mM, respectively. The $K_m$ value for Au-Ni/g-C$_3$N$_4$ with ABTS is found to be 0.51 mM, which is much lower than that of Au/g-C$_3$N$_4$ (0.73 mM) and Ni/g-C$_3$N$_4$ (0.96 mM). Also, the $K_m$ value of Au-Ni/g-C$_3$N$_4$ for H$_2$O$_2$ as substrate in presence of ABTS was found to be 7.74 mM, whereas that using Au/g-C$_3$N$_4$ and Ni/g-C$_3$N$_4$ are found to be 15.16 mM and 22.59 mM, respectively. The enhanced catalytic activity of the bimetallic Au-Ni/g-C$_3$N$_4$ as compared to Au/g-C$_3$N$_4$ and Ni/g-C$_3$N$_4$ nanocomposites towards peroxidase like catalytic activity can be attributed to the synergistic effects between Au, Ni and g-C$_3$N$_4$. Such type of effects has been previously reported for high catalytic activity in several cases using bimetallic nanoparticles [60,61]. The synergistic effect accounts from the electronic charge...
Fig. 6. Flow cytometric analysis of Annexin V/PI-stained HUVEC cells using (A) 5 μg mL$^{-1}$ and (B) 200 μg mL$^{-1}$ concentration of g-C$_3$N$_4$, Au-Ni/g-C$_3$N$_4$, Au/g-C$_3$N$_4$ and Ni/g-C$_3$N$_4$ nanocomposites.
transfer between Au and Ni. The ionization potentials for Au and Ni are 9.22 eV and 7.63 eV, respectively and as such there is a possibility of electron transfer from Ni atoms to Au atoms that leads to increase in the electron charge density on Au and thus both Au and Ni can act as active catalytic sites for the peroxidase like activity [62]. Also the strong interaction between Au and Ni with the support g-C3N4 accounts for its high activity. g-C3N4 has many delocalized electrons which could also donate its lone pairs to the metal thus providing a good network for electron mobilization and easy absorption by TMB. The amino groups present on the TMB offers many lone electron pairs that could pass the electrons to the nanocomposites and thus provide easy access of electrons to reduce H2O2. H2O2 gets converted to H2O and consequently rate of TMB oxidation increases. These factors contribute towards the high performance of our synthesised Au-Ni/g-C3N4 nanocomposite to peroxidase mimicking activity.

3.5. Analysing the mechanism for peroxidase like catalytic activity of Au-Ni/g-C3N4 nanocomposite

The mechanism of peroxidase like catalytic activity of the Au-Ni/g-C3N4 nanocomposite was studied considering TMB as the substrate. The peroxidase like catalytic reaction of Au-Ni/g-C3N4 nanocomposites owing to their small size and large
surface area facilitates the adsorption of TMB on its surface which donates lone pair electrons from its amino group to the Au-Ni/g-C3N4 resulting in enhanced electron density as well as movement of electrons in the Au-Ni/g-C3N4 nanocomposites. Simultaneously the electron transfer from Au-Ni/g-C3N4 to H2O2 molecules increases and consequently the rate of TMB oxidation by H2O2 increases [63,64]. Thus, the peroxidase like catalytic activity of the nanocomposites arises from their ability to transfer electrons between TMB and H2O2. Acidic media facilitates the decomposition of H2O2 into OH radical which in turn oxidizes TMB to blue coloured TMDBI. In order to clarify the catalytic reaction mechanism, it is essential to confirm the reactive oxygen species produced during the reaction. The evidence for the formation of OH radical was studied by photoluminescence probing techniques, which is highly sensitive and selective method, widely used in the detection of OH radical. In this method fluorescent probe molecule tertephthalic acid (TA) in presence of H2O2-Au-Ni/g-C3N4 system produces OH radical which reacts with TA to form highly fluorescent 2-hydroxyterephthalic acid exhibiting maximum emission at 426 nm when excited at 315 nm. Thus the photoluminescence study provides ample evidence for the generation of OH radical that helps in the oxidation of TMB to form the blue coloured product. The results thus clearly indicate that Au-Ni/g-C3N4 nanocomposites could decompose H2O2 to generate the OH radical.

The formation of the OH radical in the course of the reaction can be further illustrated by performing the scavenging experiment by introducing isopropanol as the scavenger of OH radicals. Isopropanol was added at a molar concentration of 0.1 mmol L\(^{-1}\) to the reaction mixture containing TMB, H2O2 and Au-Ni/g-C3N4. The experimental conditions were similar to that discussed in Section 2.7. The addition of isopropanol which acts as a scavenger of the OH radical modifies the reaction course resulting in lowering of the rate of the peroxidase activity of the Au-Ni/g-C3N4 nanocomposite. Fig. 9(b) shows the effect of scavenger on the peroxidase like activity of the Au-Ni/g-C3N4 and it is evident that in absence of scavenger i.e., for the blank sample, the peroxidase like catalytic activity of Au-Ni/g-C3N4 in presence of H2O2 and TMB shows absorbance for TMDBI at 652 nm with an absorbance intensity of 1.72 which however decreases when isopropanol is present in the reaction to 0.361. This proves that isopropanol scavenges the formation of OH radical formed in course of the reaction and thus the oxidation of TMB also decreases. From the above experimental result it is clearly observed that hydroxyl radicals act as the main reactive species in the peroxidase like enzymatic reaction.

### 3.6. Detection of glucose, its selectivity and its analysis in real blood samples

Considering the peroxidase like catalytic activity of our synthesized Au-Ni/g-C3N4 nanocomposite and its higher affinity to bind with TMB than with ABTS, a colorimetric method for glucose detection was developed. As H2O2 is the foremost product in the GOx catalysed reaction, so the detection of glucose by this method can be realized in two sequential steps: (1) first step involves oxidation of glucose to gluconic acid by GOx in pH 7 and simultaneous production of H2O2, (2) second step is the reduction of H2O2 by Au-Ni/g-C3N4 in presence of TMB at pH 4 and the formation of the oxidized product TMDBI that can be determined using UV-Vis absorption at 652 nm. The calibration curve for the glucose detection is shown in Fig. S9. The limit of detection for glucose is determined from the standard glucose calibration curve calculated by the formulation of LOD = 3S/K at the signal-to-noise ratio of 3, where “S” is the standard deviation of experimental data with n = 9 and “K” is the slope of the calibration curve. The regression equation for glucose detection using Au-Ni/g-C3N4 is given a linear fitting with R\(^2\) value 0.995. The linear range for glucose detection was found between 0.5–30 \(\mu\)M and the limit of detection for glucose based on the above formulation is found to be 1.7 \(\mu\)M. The efficiency of glucose detection using Au-Ni/g-C3N4 catalyst was also compared with

---

**Table 3**

Kinetic parameters (Km and Vmax) of different enzyme mimics obtained from Lineweaver Burk double reciprocal plot.

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Substrate</th>
<th>Km (mM)</th>
<th>Vmax ([10^{-8} \text{ M s}^{-1}])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au-Ni/g-C3N4</td>
<td>TMB</td>
<td>0.16</td>
<td>2.34</td>
</tr>
<tr>
<td>Au-Ni/g-C3N4</td>
<td>H2O2</td>
<td>4.47</td>
<td>6.16</td>
</tr>
<tr>
<td>Au/Ni/g-C3N4</td>
<td>TMB</td>
<td>0.27</td>
<td>1.27</td>
</tr>
<tr>
<td>Au/g-C3N4</td>
<td>H2O2</td>
<td>11.13</td>
<td>3.44</td>
</tr>
<tr>
<td>Ni/g-C3N4</td>
<td>TMB</td>
<td>0.49</td>
<td>0.75</td>
</tr>
<tr>
<td>Ni/g-C3N4</td>
<td>H2O2</td>
<td>19.91</td>
<td>1.38</td>
</tr>
<tr>
<td>Au-Ni/g-C3N4</td>
<td>ABTS</td>
<td>0.51</td>
<td>4.79</td>
</tr>
<tr>
<td>Au-Ni/g-C3N4</td>
<td>H2O2</td>
<td>7.74</td>
<td>4.94</td>
</tr>
<tr>
<td>Au/g-C3N4</td>
<td>ABTS</td>
<td>0.73</td>
<td>3.43</td>
</tr>
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<td>Au/g-C3N4</td>
<td>H2O2</td>
<td>15.16</td>
<td>3.54</td>
</tr>
<tr>
<td>Ni/g-C3N4</td>
<td>ABTS</td>
<td>0.96</td>
<td>2.40</td>
</tr>
<tr>
<td>Ni/g-C3N4</td>
<td>H2O2</td>
<td>22.59</td>
<td>2.37</td>
</tr>
</tbody>
</table>

---

**Fig. 9.** (a) Photo luminescent excitation and emission spectra of TA, TA + Au-Ni/g-C3N4, TA + H2O2 and TA + Au-Ni/g-C3N4 + H2O2 (b) Effect of isopropanol on the peroxidase like catalytic activity of Au-Ni/g-C3N4 nanocomposites.
monometallic Au/g-C₃N₄ and Ni/g-C₃N₄. The regression equation for glucose detection using Au/g-C₃N₄ and Ni/g-C₃N₄ the R² values are found to be 0.994 and 0.986, respectively. For Au/g-C₃N₄, a detection limit of 3.55 μM with a linear range from 2 – 50 μM was recorded (Fig. S10a), while a detection limit of 6.28 μM with a linear range from 5 – 100 μM was obtained for Ni/g-C₃N₄ (Fig. S10b). Comparative table for glucose detection in presence of other reported enzyme mimics is presented in Table S1.

The selectivity experiment of the Au-Ni/g-C₃N₄ nanocomposite towards glucose and other analogues of glucose like maltose, fructose, lactose, dopamine, L-cysteine and ascorbic acid were carried out as discussed in Section 2.8. The UV–vis curves shown in Fig. 10(a) illustrates that the absorbance of the glucose analogues were negligible in comparison to glucose even at higher concentrations. The results demonstrate the better selectivity of Au-Ni/g-C₃N₄ nanocomposite towards glucose detection although all the analogues have similar electron lone pairs and molecular size as glucose. The reason for the selectivity of the reaction actually lies in the use of GOx in the reaction which has a high degree of specificity to oxidize glucose [24]. As such the absorbance barely increases for maltose, fructose, lactose, dopamine, L-cysteine and ascorbic acid, as shown in the bar diagram of Fig. 10(b). On this basis we thus developed a highly selective colorimetric method for the detection of glucose.

Using the same colorimetric method, we extended our investigation towards colorimetric detection of glucose in real clinical serum samples of human volunteers. The absorbance obtained from the UV–vis curve for the real samples is shown in Fig. 11. The concentration of glucose in the two blood serum samples as determined from the glucose calibration curve was found to be 6.1 and 4.62 mM. The results obtained were compared to that determined using standard hospital method and are shown in Table S2. The concentration of glucose as determined by our colorimetric method is in well agreement to that determined using standard hospital method and therefore our method can be well used for detecting glucose in real serum samples.

Thus, we developed a novel and biocompatible nanzyme based on Au-Ni/g-C₃N₄ nanocomposites that can be successfully utilized for the detection of glucose with very low detection limit and can also be further implemented for detecting glucose precisely in real samples like blood serum. As a novel peroxidase mimic, the Au-Ni/g-C₃N₄ nanocomposites show several advantages over HRP and other peroxidase nanomimetics such as high substrate affinity, selectivity and catalytic efficiency. The accurate quantitative assay of glucose in human serum samples also indicates the potential for their practical application. Again paper based microfluidic devices have attracted considerable attention due to their capillary based self-pumping ability, low cost, ease of availability and is widely used in point-of-care (POC) diagnostics [66,67]. Using Au-Ni/g-C₃N₄ nanocomposites, an easy to fabricate, portable and user friendly paper based analytical device can be constructed for reliable and sensitive detection of glucose [68]. Further, by tuning the synthesis process the optical properties of the plasmonic Au based Au-Ni/g-C₃N₄ nanocomposite can be modified to obtain efficient plasmon resonance properties that can provide new prospects to develop more competent colorimetric sensors for the ultrasensitive detection of target molecules [69].

4. Conclusion

In conclusion, 2D g-C₃N₄ sheets were designed with bimetallic Au-Ni nanoparticles with an average size ~ 11 nm by through a simple chemical reduction technique and characterized using different sophisticated analytical tools. The Au-Ni/g-C₃N₄ nanocomposites possessed intrinsic peroxidase like activity capable of catalysing the oxidation of peroxidase substrates 3,3′,5,5′-tetramethylbenzidine (TMB) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in presence of H₂O₂. Combining the peroxidase like catalytic activity of the Au-Ni/g-C₃N₄ nanocomposites with glucose oxidase (GOₓ), a simple, sensitive and a selective colorimetric analytical method was designed which helped in detecting glucose upto a limit of 1.7 μM with linear detection range between 0.5 to 30 μM. The monometallic Au/g-C₃N₄ and Ni/g-C₃N₄ nanocomposites exhibited a detection limit of 3.55 and 6.23 μM, respectively. Owing to the synergistic effects of Au and Ni, the bimetallic Au-Ni/g-C₃N₄ nanocomposites were found to be catalytically more efficient than their monometallic counterparts. Also, the colorimetric glucose detection method using Au-Ni/g-C₃N₄ can be successfully utilized for the detection of glucose in real serum samples.
with comparable outcomes to that obtained from standard hospital method. Moreover, all these nanocomposites did not cause any cytotoxic effect towards HUVEC cell line thus making the materials highly biocompatible and suitable for biosensor application. Thus, such type of bimetallic nanoparticles-graphitic carbon nitride nanocomposites can be successfully utilized for other futuristic applications in the area of biochemistry and biotechnology.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.snb.2019.01.048.

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