Polymeric-coated Fe-doped ceria/gold hybrid nanocomposite as an aptasensor for the catalytic enhanced colorimetric detection of 2,4-dinitrophenol

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Abstract
The Meisenheimer charge transfer reaction has, for many years, been the predominant mode of interaction to detect explosive compounds via the naked eye. However, this method is majorly sensitive to 2,4,6-trinitrotoluene and insensitive to many other explosive compounds. In this work, we report on the development of a novel aptamer-based amphiphilic polymer-coated Fe-doped ceria/gold (Au) hybrid nanocomposite (NC) as a peroxidase mimetic nanozyme for explosive colorimetric detection. Polymeric-Fe-doped ceria NC was synthesized and incorporated in-situ in the hydrothermal synthetic pot of citrate-Au nanoparticles to form a polymer-Fe-doped ceria/Au NC. A streptavidin (strep)-biotinylated DNA aptamer (B-Apt) binding complex was immobilised on the polymer-Fe-doped ceria/Au NC surface and the modified NC was used as a functional nanozyme in a peroxidase mimic assay to catalyse the oxidation of 3,3',5,5'-tetramethylbenzidine by H₂O₂. Screening of several explosives showed that 2,4-dinitrophenol (DNP) bonded with higher affinity to the aptamer based on the selective greenish colorimetric catalytic reaction observed. The peroxidase mimetic activities of several nanozymes were tested and our analysis showed that the polymer-Fe-doped ceria/Au NC generated enhanced catalytic activity for DNP detection. Several optimisation reactions including the kinetic reaction mechanism was conducted and a limit of detection of 0.45 µg/mL (2.4 µM) was obtained for DNP recognition. The peroxidase mimetic strep-B-Apt-polymer-Fe-doped ceria/Au NC biosensor was successfully applied for the detection of DNP in tap water and river water with satisfactory recoveries in the range of 97 – 117%.

KEYWORDS: Nanocomposite; colorimetric; peroxidase; nanozyme; polymer; detection
1. Introduction
The predominant function of explosive compounds within the industrial setting lies mainly as an energetic material for the mining industry and for military ordnance [1,2]. Within the environmental setting, explosive-contaminated groundwater and soil near military installation sites often constitute health hazards due to the explosive characteristic mutagenicity, toxicity, and biological persistence, while prolonged exposure of trace amounts of explosives can lead to abnormal liver functions and anaemia [3-5]. Global security concerns have also necessitated the need to detect covert explosive devices in (for example) transportation hubs and in conflict zones. Conventional sensor systems used in the detection of explosives can be slow, less sensitive, costly, and sometimes cumbersome to operate especially when analytical systems such as gas chromatography and neutron activation analysis are implemented [6,7]. To meet the growing market needs, it is necessary to develop cost-effective, rapid, selective, and highly sensitive sensor systems for the detection of explosive compounds.

Apart from the conventional methods for explosive detection, sensor detection systems involving the use of nanomaterials is of great interest due to the added degree of attaining enhanced sensitivity, improved selectivity, rapid response, and miniaturisation potentials. A vast majority of reported nanomaterials-based sensor systems for explosives have centred around nitroaromatic explosive compound detection [8-11]. 2,4,6-Trinitrotoluene (TNT) has been the most detected explosive compound due to the ease in which its nitro functional group forms a Meisenheimer complex with an electron-donating amino-functionalized nanomaterial to trigger an orange colorimetric colour reaction [12,13]. Generally, the Meisenheimer complex reaction has been widely exploited for spectroscopic detection of nitroaromatic explosive compounds [14]. However, this method is predominantly selective to TNT and less sensitive to many other explosive compounds. Furthermore, since the Meisenheimer reaction occurs strongly in alkaline solution, the stability of functional nanomaterials could be degraded at such high pH conditions, thus limiting the potential use of such methods in field applications and under harsh conditions. It is therefore of interest to develop novel colorimetric sensor systems that rely on affinity-based biomolecular binding interactions.

Nanozyme, a terminology coined in 2004 by Scrim et al., is an umbrella term describing an emerging class of artificial nanomaterials exhibiting intrinsic enzyme-like catalytic activities [15]. Recent advances in nanotechnology have spawned innovative developments in nanozyme mimetic technology and have empowered researchers with an ever-evolving toolbox of fabricating novel nanomaterials with intrinsic catalytic properties. In 2007, magnetic Fe$_3$O$_4$ nanoparticles (NPs) was first discovered as having intrinsic peroxidase-like catalytic activity [16]. Since then, a plethora of emerging studies have unveiled metal and metal oxide NPs (e.g., gold nanoparticles (AuNPs), CeO$_2$ NPs (nanoceria), nickel oxide NPs and platinum NPs, etc.), carbon-based nanomaterials (e.g., graphene oxide and carbon nanotubes), and various metal-organic frameworks, as having catalytic activities that mimic those of natural enzymes [17,18].
Reported modes of interaction of nanozymes for targeted applications in the fields of biology, chemistry, and medicine have either been of; hydrolase [19], peroxidase [20], superoxide dismutase [21], oxidase [22], or catalase [23]. Amongst the various modes of nanozyme-substrate interactions, peroxidase-mimic assays for sensing applications are the most popular due to the strong affinity between the nanozyme and the substrate in the presence of hydrogen peroxide (H2O2) [24,25]. Most recently, nanozyme peroxidase mimic biosensors based on affinity binding interactions between bio-receptors (such as DNA aptamers (Apt) and antibodies) with target analytes, have emerged as a hot research area. Antibody-based immuno-colorimetric nanozyme peroxidase mimic biosensors have been reported for the detection of influenza virus [26], human epidermal growth factor receptor 2 [27] and prostate specific antigen [28]. Apt-based nanozyme peroxidase mimic biosensors on the other hand, have been reported for the detection of illicit drugs [29], pesticides [30] and proteins [31]. In terms of stability, cost-effectiveness and robustness, Apt receptors are more favourable for nanozyme peroxidase mimic biosensor design and thus represents a new paradigm shift in affinity-based colorimetric biosensor applications.

The sensitivity of a nanozyme biosensor is strongly dependent on the catalytic properties of the nanomaterial used in the construction of the catalytic assay. Although, several single ensemble NPs have been reported as exhibiting catalytic activities as mentioned above, combining two or more nanomaterials to form hybrid nanozymes have shown to trigger enhanced catalytic activities. Examples include reported hybrid nanozyme biosensors for H2O2 and glucose [32], in cancer therapy [33] and in antibacterial application [34].

In this work, we report on the development of a new hybrid nanozyme composed of Fe, ceria and AuNPs as an intrinsic peroxidase mimic for the selective Apt-based catalytic colorimetric detection of 2,4-dinitrophenol (DNP). Firstly, Fe-doped ceria was synthesised via the hot pyrolysis of organic precursors and thereafter coated with amphiphilic polymers. Amphiphilic polymer was chosen to stabilize the nanomaterial and to preserve its catalytic functionality. The as-synthesized polymer-coated Fe-doped ceria nanocomposite (NC) was then incorporated in-situ in the hydrothermal synthetic pot of AuNPs to form a novel polymer-coated Fe-doped ceria/Au NC. We then successfully performed a streptavidin (strep)-biotin (B) anti-explosive biotinylated DNA Apt (B-Apt) immobilisation on the nanozyme surface and used the functionalised nanozyme protein-Apt complex in a peroxidase mimic assay to catalyse the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) by H2O2. Via the catalytic reaction, we have tuned the generated colorimetric reaction for selective DNP detection based on the Apt-DNP affinity binding. To the best of our knowledge, this is the first reported strep-B-Apt-polymer-Fe-doped ceria/Au NC peroxidase mimetic biosensor probe for explosive detection.
2. Experimental

2.1. Materials

Cerium (IV) sulfate hydrate, iron (II) chloride, oleylamine, tannic acid, hexadecylamine, oleic acid, octadecene, poly(isobutylene-alt-maleic anhydride), N,N-diisopropylethylamine, 1,3,5-trinitrobenzene solution (TNB) in acetonitrile (AcCN), 2,4,6-trinitrophenol (TNP) in AcCN, pentaerythritol tetranitrate (PETN) in AcCN, 1,3-dinitroglycerin (DNG) in AcCN, 2,4-dinitrophenol (DNP) in methanol (MeOH), 1,3,5-trinitro-1,3,5-triazinane (RDX) in AcCN, and 1,3,5,7-tetranitro-1,3,5,7-tetraazacyclooctane (HMX) in AcCN were purchased from Sigma Aldrich. n-Octylamine, gold (III) chloride trihydrate (HAuCl₄·3H₂O), tri-sodium citrate dihydrate, terephthalic acid, myristic acid, and sodium borohydride were purchased from Thermo Fisher. TNT in MeOH:AcCN (1:1), 2,4-dinitrotoluene (DNT) in MeOH:AcCN (1:1), triacetone triperoxide (TATP) in AcCN, and hexamethylene triperoxide diamine (HMTD) in AcCN were purchased from AccuStandard. All other chemicals were used as received. B-Apt having the sequence: ATACCAGCTTATTCAATTAGATAGTAAGTGCAATCT [35], was synthesized and purified by Merck.

2.2. Characterization

Ultraviolet/visible (UV/vis) absorption and fluorescence emission measurements were carried out using a Varian Cary Eclipse spectrophotometer. Scanning electron microscopy (SEM) analysis were carried out using a JEOL JSM 7400F field emission scanning electron microscope. Transmission electron microscopy (TEM) analysis were carried using a JEOL JEM-1200EX operated at 80 kV. Fourier transform-infrared (FT-IR) analysis was carried out using an Agilent Cary 630 FT-IR spectrometer. X-ray Diffraction (XRD) analysis was carried out using a Siemens D5000 diffractometer with Cu Kα radiation (λ = 1.54056 nm) and data were obtained in the range of 3-60° using a 0.1° 2θ step size and a 3 sec count time per step with a 0.066° slit width. Dynamic light scattering (DLS) and zeta potential analysis of the Qdots was carried out using Zetasizer Nano ZS series (ZEN3600, Malvern). The colorimetric absorbance signal was recorded using an 800 TS microplate absorbance reader from BioTek.

2.3. Synthesis of organic-phased Fe-doped ceria NPs

The synthesis of Fe-doped ceria was carried out in the organic hot pyrolysis phase by mixing 4 g of iron (II) chloride, 4 g cerium (IV) sulfate hydrate, 5 mL oleylamine, 0.6 g myristic acid, 0.6 g hexadecylamine, 30 mL oleic acid, and 30 mL octadecene in a three-necked flask. The solution was refluxed under vigorous stirring, bubbled with nitrogen gas, and heated for 25 minutes (min) with the temperature reaching ~250 °C. The organic-phased Fe-doped ceria was thereafter harvested and left to cool in a beaker.
2.4. Synthesis of amphiphilic polymer
The amphiphilic polymer was synthesized according to published procedure but with slight modification [36]. Briefly, 20 g poly(isobutylene-alt-maleic anhydride), 6 mL n-octylamine, 10 mL N,N-diisopropylethylamine, and 200 mL chloroform was stirred for 2 hours 45 min under reflux. Thereafter, the polymer synthetic solution was stopped and left to react for ~ 24 hours at room temperature. Purification was carried via ultracentrifugation using acetone, ethanol-acetone, ethanol-acetone-chloroform, ethanol-acetone, and acetone-chloroform. The purified amphiphilic polymer was precipitated out of solution and unreacted products embedded in the supernatant solution was decanted. Finally, the purified polymer was dried in a fume hood and grounded into a fine powder.

2.5. Amphiphilic polymer functionalisation of Fe-doped ceria NPs
Amphiphilic polymer functionalisation of organic-phased Fe-doped ceria was carried out to render the NPs water-soluble. To prepare the polymer reaction, 0.83 g of purified amphiphilic polymer was dissolved in a solution containing 3 g potassium hydroxide and 40 mL MeOH. Thereafter, 0.38 mg of Fe-doped ceria dissolved in 5 mL of chloroform was added into the polymer-methanolic solution and appropriate amount of Milli-Q ultrapure H2O was added to precipitate the hydrophilic polymer-coated Fe-doped ceria from the organic phase solution. The solution was stirred for 10 min and purified via ultracentrifugation using acetone, ethanol-acetone-chloroform, and acetone. The purified polymeric Fe-doped ceria was precipitated out of solution and unreacted products embedded in the supernatant solution was decanted. The final product was left to dry in the fume hood.

2.6. Synthesis of polymer-coated Fe-doped ceria/Au NC
To synthesize the polymer-coated Fe-doped ceria/Au NC, hydrothermal aqueous synthesis of citrate-AuNPs was first carried out and when the AuNPs was formed in solution, the polymer-Fe-doped ceria NC was incorporated in-situ. Briefly, 1 mL 1% HAuCl4·3H2O was first mixed with 79 mL of H2O and stirred for few min. Then, 20 mL aqueous solution containing 4 mL 1% tri-sodium citrate dihydrate, 0.5 mL 1% tannic acid and 15.5 mL of Milli-Q ultrapure H2O was added into the Au salt solution. Citrate-AuNPs were formed within few seconds and 0.2 g of the polymer-Fe-doped ceria NC dissolved in 10 mL of Milli-Q ultrapure H2O, was added in-situ. The solution was heated to ~100 ºC for ~35 min and purified via filtering using a 0.2-micron syringe filter.

2.7. Assay procedure
For DNP peroxidase mimetic colorimetric detection, 50 µL of the polymer-Fe-doped ceria/Au NC (24.5 nM) was mixed with 5 µL strep (0.5 mg/mL), 5 µL B-Apt (10 µM), 150 µL DNP (100 µg/mL), 90 µL TMB (1.2 mM prepared in citrate-phosphate buffer, pH 3) and 60 µL H2O2 in a 96-well plate. After adding the H2O2 solution, the assay solution was allowed to react for 5 min to obtain optimum
catalytic reaction. Photographic pictures of the colorimetric reaction were captured on a smartphone while the absorbance data were recorded using a BioTek absorbance plate reader.

3. Results and discussion

3.1. Fabrication of the polymer-coated Fe-doped ceria/Au NC

Fe-doped ceria NPs was first synthesized in the organic phase and stabilized with organic coordinating ligands as shown in Scheme 1. In our case, oleylamine, hexadecylamine, oleic acid and myristic acid were used as surface capping organic ligands to stabilize the surface of Fe-doped ceria. To solubilize the NPs, we employed amphiphilic polymer coating via the use of a nucleophilic agent with functionalized anhydride polymer backbone. The synthesis of amphiphilic polymer was carried out based on the reaction of poly(isobutylene-alt-maleic anhydride (M_w = 6000 g/mol) backbone with n-octylamine. The desired ratio between the hydrophobic and hydrophilic units was obtained by tuning the relative number of hydrophobic n-octylamine reaction groups. The amphiphilic polymer synthesized in this work consist of relatively 40% repeating units anchoring n-octylamine groups [37] that is index m according to the synthesis description shown in Scheme 1. The advantages of the fabricated protocol are the ability to control the number of functional units needed for robust polymer coating; the lack of utilizing a cross linker in the synthesis step; precise control of the hydrophobic n-octylamine chains and thus over the hydrophobic/hydrophilic assembly of the polymeric coating; and the ability to carry out the polymeric coating strategy without the need for a carbodiimide coupling step.

Our initial investigation showed that the polymer-coated Fe-doped ceria NC exhibited strong oxidase catalytic activity by triggering a bluish colorimetric colour upon reaction with TMB. As this could act as an interference to the peroxidase activity of the sensor, we chose to incorporate citrate-AuNPs into the NC system to eliminate the oxidase activity. This material modification process enabled effective colour discrimination between the control solution and assay solution and also introduced enhanced catalytic activity. The amphiphilic polymer functional groups can form a hydrogen bond with the citrate functional group on the AuNP surface or via hydrophobic/hydrophilic interaction between the n-octylamine groups on the polymer backbone and the citrate groups on the AuNP surface.

3.2. SEM and TEM analysis

Fig. 1 shows the SEM micrograph of the synthesized amphiphilic polymer. It is evident from the surface morphology that the polymer particles appear coarse, compacted with porous particles, and exhibited no definite shape. SEM images of the organic-phased Fe-doped ceria NC and the polymer-Fe-doped ceria NC are shown in Fig. 1B and C, respectively. From the displayed SEM micrographs, the organic-phased Fe-doped ceria NC was characterised by a very dense and coarse particle morphology while the polymer-Fe-doped ceria NC was characterised by cube-like particles. The change in shape morphology can be attributed to the embedded amphiphilic polymers on the NC
surface. The origin of the polymeric nanocube morphology may be attributed to the differences in surface free energies with has direct relation to the crystal faces in the organic-capped and polymer-coated NC [38]. We therefore postulate that the marked difference in surface free energy with respect to the Fe-doped ceria NC facet, played a crucial role in the nanocube shape formation.

TEM micrograph of the polymer-Fe-doped ceria/Au NC shown in Fig. 1D, reveals a well-defined monodispersed particle morphology. The shape of the particles was predominantly spherical, possibly induced by the embedded AuNPs within the NC structure. The estimated particle size processed via Image J software was ~8 nm for the polymer-Fe-doped ceria/Au NC.

3.3. XRD analysis

For comparative purpose, the XRD pattern of the synthesized amphiphilic polymer and the respective nanomaterials was analysed. From the diffraction pattern of the amphiphilic polymer shown in Fig. 2A, no definite diffraction peak was seen, whereas the diffraction pattern of the amphiphilic polymer-coated nanomaterials displayed a strong broad peak at low Bragg angle. The non-appearance of a diffraction peak in the XRD pattern of the amphiphilic polymer may be attributed to the intercalated nature of the polymer chains [39]. The broad diffraction peak observed in the XRD pattern of the polymer-coated nanomaterials can be attributed to the nanocrystallite size of the materials (Fig. 2A). The most notable difference in the diffraction pattern of the polymer-Fe-doped ceria NC and the polymer-Fe-doped ceria/Au NC is the shift to higher Bragg angle for the later. The shift is a common feature associated with surface modification of nanomaterials [40]. In our case, the polymer-Fe-doped ceria NC was surface-modified with AuNPs and we can therefore allude the diffraction shift to this process.

3.4. FT-IR analysis

FT-IR analysis was carried out to probe the functional groups of the amphiphilic polymer and the polymer-coated nanomaterials. As shown in Fig. 2B, the FT-IR spectrum of the amphiphilic polymer was characterised by a peak at 2988 cm⁻¹ which can be attributed to the symmetric telescopic -CH₂ vibration while the peak at 1777 cm⁻¹ can be assigned to the C=O stretching band. For the polymeric nanomaterials, the band at 3354 cm⁻¹ for the polymer-Fe-doped ceria NC and 3357 cm⁻¹ for the polymer-Fe-doped ceria/Au NC can be assigned to the O-H stretching band. For the polymer-Fe-doped ceria NC, the band at 2952 cm⁻¹ can be attributed to the symmetrical -CH₂ vibration while around the same region for the polymer-Fe-doped ceria/Au NC, the peak splits into two bands at 2915 cm⁻¹ and 2847 cm⁻¹ and can be assigned to the symmetric and asymmetric -CH₂ vibration band. The remarkable -CH₂ peak split can be attributed to the presence of the AuNPs in the Fe-doped ceria NC structure. For the polymer-Fe-doped ceria NC, the peak at 1739 cm⁻¹ corresponds to the C=O stretching band while the projected peaks at 1560 cm⁻¹ and 1399 cm⁻¹ corresponds to the functional asymmetric and symmetric -COO group. For the polymer-Fe-doped ceria/Au NC, the C=O and asymmetric -COO stretching bands were shifted to lower wavenumber of 1702 cm⁻¹ and 1524 cm⁻¹.
in comparison to the polymer-Fe-doped ceria NC, while the symmetric -COO band was shifted to higher wavenumber of 1405 cm\(^{-1}\).

3.5. DLS and zeta potential analysis
DLS and zeta potential analysis was carried out to further probe the optical properties of the polymer-Fe-doped ceria/Au NC. DLS was used to determine the hydrodynamic particle size of the polymeric hybrid NC and to use the obtained value to evaluate the degree of monodispersity or polydispersity of the nanomaterial. Generally, hydrodynamic size value ≤100 nm is indicative of a monodispersed colloidal state while values ≥100 nm is indicative of a polydispersed state. Zeta potential on the other hand, was used to determine the colloidal surface charge of the polymeric hybrid NC and to use the obtained value to evaluate the degree of colloidal stability of the nanomaterial. Fig. 2C, shows the DLS histogram plot of the polymer-Fe-doped ceria/Au NC while Fig. 2D show the zeta potential plot. From the DLS analysis, the hydrodynamic particle size of the polymer-Fe-doped ceria/Au NC was ~10 nm while the zeta potential value was ~15 mV. The obtained hydrodynamic value shows that the polymer-Fe-doped ceria/Au NC is colloidally monodispersed while the zeta potential value is indicative of a relatively stable colloidal state [41].

3.6. UV/vis absorption analysis and catalytic activity
The binding effect of the strep-B-Apt on the polymer-Fe-doped ceria/Au NC was studied using UV/vis spectrophotometry. The UV/vis absorption spectra of the polymer-Fe-doped ceria/Au NC, strep-polymer-Fe-doped ceria/Au NC and the strep-B-Apt-polymer-Fe-doped ceria/Au NC are shown in Fig. 3A. From the displayed spectra, it was observed that the UV/vis absorption spectrum of the polymer-Fe-doped ceria/Au NC was characterised by a surface plasmon resonance (SPR) absorption peak around 526 nm which is indicative of the plasmonic AuNP effect in the Fe-doped ceria NC structure. After immobilising the strep protein on the polymer-Fe-doped ceria/Au NC surface, the SPR absorption peak decreased slightly and may be indicative of the protein adsorption on the NC surface. Subsequent binding of the B-Apt on the strep-polymer-Fe-doped ceria/Au NC surface, triggered further slight decrease in the SPR absorption peak and may be indicative of the strep-B-Apt binding on the polymer-Fe-doped ceria/Au NC surface. The apparent lack of peak shift provides strong indication that the strep-B-Apt binding interaction did not alter the dielectric state of the polymer-Fe-doped ceria/Au NC to induce undesired aggregation.

UV/vis absorption was further used to probe the peroxidase mimetic activities towards DNP. We probed different reaction processes so as to assess the efficiency of the catalytic reaction towards DNP detection. As shown in Fig. 3B, no catalytic absorption peak was observed when the following reactions were carried out: (Fig. 3Bii) DNP + TMB + H\(_2\)O\(_2\); (Fig. 3Biii) strep-B-Apt-polymer-Fe-doped ceria/Au NC probe + H\(_2\)O\(_2\) and (Fig. 3Biv) strep-B-Apt-polymer-Fe-doped ceria/Au NC + DNP + TMB. However, a strong catalytic absorption peak around ~658 nm was observed when the strep-B-Apt-polymer-Fe-doped ceria/Au NC was reacted with solutions of DNP, TMB and H\(_2\)O\(_2\) (Fig.3Bv).
From the inset of Fig. 3B, the displayed photographic solution colour showed that the polymer-Fe-doped ceria/Au NC was relatively pale pink while solutions of the non-catalytic reactions were yellowish to varying degree and solution of the catalytic reaction for DNP detection was uniquely green. Thus, the polymer-Fe-doped ceria/Au NC can be used as an efficient peroxidase mimic biosensor platform for DNP recognition based on the strep-B-Apt-DNP affinity binding interaction.

3.7. Assay optimization
The effects of time, TMB, H$_2$O$_2$, Apt and strep concentration were investigated so as to optimize the peroxidase mimic assay for optimum catalytic signal. Fig. 4A shows the effect of time on the catalytic colorimetric detection of DNP using the strep-B-Apt-polymer-Fe-doped ceria/Au NC. From the data, the catalytic absorbance signal increased steadily from 1 min to 5 min for DNP detection. Hence, we chose 5 min as the optimum detection time. The effect of TMB investigated from 0.08 - 1.2 mM, showed that the catalytic signal increased as the concentration of TMB increased (Fig. 4B). Hence, we chose 1.2 mM TMB as the choice concentration for DNP detection. The effect of H$_2$O$_2$ concentration investigated from 0.1 – 3 M, showed that the catalytic signal increased as the concentration of H$_2$O$_2$ increased in the assay system (Fig. 4C). Therefore, we chose 3 M H$_2$O$_2$ as the choice concentration for DNP detection.

Discussion on the effects of strep protein and the B-Apt concentration on the peroxidase mimetic catalytic detection of DNP and the corresponding representative data is provided in the Supplementary Information section (Fig. S1A and B).

3.8. Efficiency of the peroxidase mimetic catalytic reaction
The efficiency of the peroxidase mimetic colorimetric reaction towards DNP detection using the polymer-Fe-doped ceria/Au NC was investigated in comparison to other nanozymes. Each nanozyme was immobilised with the strep-B-Apt binding complex to compare the catalytic efficiency under the same experimental conditions. We used the nanozyme + TMB mixture as the control so as to be able to unravel which nanozyme exhibited oxidase mimetic properties and to then subtract the control signal from the generated analyte colorimetric signal. Fig. 5A shows the photographic colorimetric reaction of each tested nanozyme for DNP detection. From the control solution colour, AuNPs, silver (Ag) NPs and bimetallic AuAgNPs each exhibited slight oxidase mimetic activity based on their mild bluish colour. We also observed oxidase mimetic activity for CeO$_2$NPs and a very strong oxidase mimetic activity for the polymer-Fe-doped ceria NC. However, the control solution of the polymer-Fe-doped ceria/Au NC did not exhibit any oxidase mimetic activity as evident from the clear transparent solution.

We observed respective greenish colour reactions with varying intensity for the strep-B-Apt immobilised CeO$_2$NPs, polymer-Fe-doped ceria NC and the polymer-Fe-doped ceria/Au NC. However, evaluating the corresponding catalytic absorbance signal, with the exception of the polymer-Fe-doped ceria/Au NC, the rest of the tested nanozymes transduced lower catalytic
absorbance signal relative to the absorbance signal of the control solution (Fig. 5B). Even though CeO$_2$NPs and the polymer-Fe-doped ceria NC both transduced green colorimetric reactions, their corresponding catalytic signal was lower than the absorbance signal of the control solution (Fig 5B). Hence, CeO$_2$NPs and the polymer-Fe-doped ceria NC are not suitable nanozymes for the detection of DNP. We have therefore selected the polymer-Fe-doped ceria/Au NC as the choice nanozyme for enhanced DNP detection. Further analysis using UV/vis absorption was carried out to elucidate the blueish reaction exhibited by the control solution of the AuNPs, AgNPs, AuAgNPs, CeO$_2$ NPs and the polymer-Fe-doped ceria NC. The data and corresponding discussion are presented in the Supplementary Information section (Fig. S2).

3.9. Reaction mechanism

Scheme 1 shows the descriptive representation of the detection scheme with respect to the peroxidase mimetic catalytic reaction. After synthesizing the polymer-Fe-doped ceria/Au NC, we immobilised strep protein and an anti-explosive B-Apt on the NC surface. The biotin portion of the B-Apt possesses strong affinity to strep and we used the immobilisation process as a strategy to anchor the Apt on the hybrid NC surface. When DNP was incorporated into the strep-B-Apt-polymer-Fe-doped ceria/Au NC system, the aptamer folds and selectively bind to the target explosive compound. In general reaction terms, the polymer-Fe-doped ceria/Au NC catalysed the oxidation of TMB in the presence of H$_2$O$_2$ to generate a coloured oxidized TMB product. In the case of DNP detection, the explosive affinity binding to the Apt receptor triggered a signal-inducing effect that enabled the catalytic reaction to be selectively tuned for its detection.

A more logical interpretation of the catalytic process between the polymer-Fe-doped ceria/Au NC, TMB and H$_2$O$_2$, involves a generic redox cycle initiation pathway describing both the reduction and oxidation steps. The nanozyme redox cycle can be defined by four sequential reaction equations:

\[
\text{Polymer-Fe-doped ceria/Au NC} + H_2O_2 \rightarrow \text{polymer-Fe-doped ceria/Au NC-I} + H_2O \tag{1}
\]
\[
\text{Polymer-Fe-doped ceria/Au NC-I} + TH_2 \rightarrow \text{polymer-Fe-doped ceria/Au NC-II} + \text{TH}^\bullet \tag{2}
\]
\[
\text{Polymer-Fe-doped ceria/Au NC-II} + BH_2 \rightarrow \text{polymer-Fe-doped ceria/Au NC} + \text{TH}^\bullet \tag{3}
\]
\[
2\text{TH}_2 + H_2O_2 \rightarrow 2H_2O + 2\text{TH}^\bullet \tag{4}
\]

The TH$_2$ in the polymer-Fe-doped ceria/Au NC redox cycle denotes the TMB substrate where TMB acts as the reducing substrate because it is the main donor electron/chromogenic entity in the H$_2$O$_2$ + polymer-Fe-doped ceria/Au NC + TMB redox cycle reaction. From the above equations, activation of the polymer-Fe-doped ceria/Au NC redox cycle occurs via a two-electron oxidation process that is initiated by the oxidizing H$_2$O$_2$ component. The native polymer-Fe-doped ceria/Au NC is converted into a high oxidative intermediate state denoted as polymer-Fe-doped ceria/Au NC-I via the H$_2$O$_2$ oxidizing effect. The oxidizing power of polymer-Fe-doped ceria/Au NC-I then serves as the potent force responsible for converting colourless TMB substrate to a blue coloured product for H$_2$O$_2$.
recognition and a green coloured product for DNP recognition. The Apt-DNP affinity binding enabled the colorimetric catalytic reaction to be tuned towards the explosive target as shown in Scheme 1.

To explain the efficiency of the polymer-Fe-doped ceria/Au NC as an enhanced catalytic nanozyme, we believe the combined metal components of the NC structure, increased free-radical resistance formation attributed to the nanozyme redox activities, and the establishment of an increased structural rigidity to eliminate unwanted environmental conditions, played a crucial role in the enhanced catalytic activity of the polymer-Fe-doped ceria/Au NC over other nanozyme. Furthermore, the ability of the polymer-Fe-doped ceria/Au NC to be unreactive towards oxygen in the presence of TMB to prevent oxidase catalytic reaction, also makes it an efficient functional nanozyme for effective colour discrimination.

To investigate the origin of the green-coloured product for DNP detection, we carried out additional experiments to probe the catalytic reaction of the polymer-Fe-doped ceria/Au NC with TMB and H₂O₂ (without the binding effect of the strep-B-Apt and DNP detection). Fig. S3, shows the reaction of (i) TMB + H₂O₂; (ii) polymer-Fe-doped ceria/Au NC + TMB and (iii) polymer-Fe-doped ceria/Au NC + H₂O₂. From the measured absorption spectra, the tested reactions were non-catalytic and were similar to the absorption spectrum of the polymer-Fe-doped ceria/Au NC (Fig. 3Bi). However, the reaction of the polymer-Fe-doped ceria/Au NC with TMB and H₂O₂, produced a relatively strong and broad catalytic absorption peak around 635 – 666 nm (Fig. S3iv). The corresponding photographic solution colour displayed in the inset of Fig. S3, showed that solutions of the non-catalytic reaction were either colourless or pinkish while solution of the catalytic reaction specific to H₂O₂ was uniquely blue in colour. From the result, it is clearly evident that the binding effect of the strep-B-Apt and DNP presence in the system changed the colour of the oxidized TMB to green.

Further analysis to unravel the generation of hydroxyl radical by the polymer-Fe-doped ceria/Au NC was investigated, and the results are presented in Fig. S4 with corresponding discussion.

### 3.10. Reaction kinetics

The kinetic reaction of the strep-B-Apt-polymer-Fe-doped ceria/Au NC towards DNP detection was studied. Absorbance versus time plots for varying H₂O₂ concentrations (at a fixed TMB concentration) and varying TMB concentrations (at a fixed H₂O₂ concentration) was used to determine the graph slope. The obtained slope values which is a representation of the initial rate of reaction (v) were each divided by the extinction coefficient of TMB, 3.9 × 10⁻⁴ M [42]. Using the Michaelis-Menten equation (Equation 1), respective non-linear plots of v versus varying concentration of TMB and H₂O₂ were plotted.

\[
v = \frac{v_{max} \cdot [S]}{[S] + K_m}
\]

(1)

\[
\frac{1}{v} = \frac{K_m}{v_{max} \cdot [S]} + \frac{1}{v_{max}}
\]

(2)
By plotting $\frac{1}{v}$ versus $\frac{1}{S'}$, the Lineweaver-Burke equation (Equation 2) was used to determine the maximum rate of reaction ($\frac{1}{v_{\text{max}}}$ = intercept) and the Michaelis-Menten constant ($K_m = \text{slope} \times v_{\text{max}}$).

[S] used in the equation denotes the substrate ($H_2O_2$ and TMB in this case) [43]. $K_{\text{cat}}$ denotes the catalytic constant and was determined by multiplying the concentration of the nanozyme by the $v_{\text{max}}$ value. Generally, the lower the value of $K_m$, the stronger is the affinity between the nanozyme and substrate and vice versa. For $v_{\text{max}}$, the higher its value, the higher is the catalytic rate of reaction while for $K_{\text{cat}}$, its value represents the maximum number of oxidised coloured product. Fig. S5A and B shows the steady state non-linear Michaelis-Menten curves while Fig. S5C and D shows the corresponding Lineweaver-Burke plots for $H_2O_2$ and TMB using the strep-B-Apt-polymer-Fe-doped ceria/Au NC. From the Lineweaver-Burke plots, we determined the values of $K_m$, $v_{\text{max}}$, and $K_{\text{cat}}$ respectively. Comparison of our kinetic parameters with other published parameters as shown in Table S1, showed that the $K_m$ value of the strep-B-Apt-polymer-Fe-doped ceria/Au NC towards TMB was lower than some referenced published values. This confirms that the functionalized strep-b-Apt polymeric nanozyme exhibited strong affinity to TMB substrate.

3.11. Selectivity of the catalytic colorimetric assay
For DNP selectivity studies, we probed the catalytic effect of other explosive compounds using the strep-B-Apt-polymer-Fe-doped ceria/Au NC biosensor probe. As shown in Fig. 6A, none of the tested explosives triggered a green colorimetric reaction in similar colour intensity as obtained for DNP under the same experimental conditions. We observed a very mild greenish colour for most of the tested explosives while HMX exhibited a mild bluish-green colour. The measured corresponding catalytic absorbance signal shown in Fig. 6B, revealed that the catalytic signal obtained for DNP was more superior than the signal obtained for the other tested explosive compounds. Although, the other tested explosives generated a measure of catalytic signal as observed from their mild colour reaction, the deep green colorimetric reaction and superior catalytic absorbance signal obtained for DNP indicates that the strep-B-Apt-polymer-Fe-doped ceria/Au NC biosensor probe is more selective to DNP than the other tested explosives.

3.12. Quantitative detection
Quantitative colorimetric detection of trace amount of explosive compound is important [44-48]. Hence, quantitative detection of DNP using the strep-B-Apt-polymer-Fe-doped ceria/Au NC was carried out. Fig. 7A shows that photographic colorimetric reaction of different concentrations of DNP. The greenish colorimetric reaction was visibly apparent across the concentration range of 1 – 100 µg/mL with very slight difference in colour intensity. However, the corresponding catalytic absorbance signal shown in Fig 7B, revealed a more vivid interpretation of the photographic
colorimetric reaction. The catalytic absorbance signal increased progressively as the concentration of DNP increased in the assay system, thus revealing that DNP was quantitatively detected. The limit of detection (LOD) calculated by multiplying the standard deviation of blank measurements \((n = 10)\) and dividing by the slope of the linear plots was as low as 0.45 \(\mu g/mL\) (2.4 \(\mu M\)) for DNP. To the best of our knowledge, there is only one reported colorimetric nanobiosensor for DNP based on the use of an antibody receptor to capture the explosive compound on AuNP surface [49]. However, the LOD for DNP was not reported in the published work, thus making it impossible for us to make a comparison with our obtained LOD. We have however, made a comparison of the analytical parameters for DNP detection using the strep-B-Apt-polymer-Fe-doped ceria/Au NC with other published non-colorimetric methods as shown in Table 1. From the comparison, it is evident that the LOD of DNP obtained in this work is lower than some published values obtained from other analytical detection methods.

3.13. Application for tap water and river water analysis

The efficacy of the strep-B-Apt-polymer-Fe-doped ceria/Au NC biosensor towards DNP detection in real samples was investigated. Tap water obtained in the laboratory and river water obtained locally from the River Tay Estuary in Dundee were used as matrices to test the applicability of the peroxidase mimic biosensor probe towards DNP detection. DNP concentrations of 100, 50, and 10 \(\mu g/mL\) were spiked in tap water and untreated river water and the catalytic signal obtained was compared to the signal obtained for DNP detection in methanol as carried out in this work. As shown in Table 2, DNP was successfully detected at different concentrations in tap water and river water with satisfactory recoveries in the range of 97 – 117%.

4. Conclusions

We have successfully constructed a novel peroxidase mimic apta-biosensor for DNP using amphiphilic polymer-coated Fe-doped ceria/Au NC. Fe-doped ceria was synthesized via the organic hot pyrolysis route and coated with amphiphilic polymers to render the nanomaterials stable and biocompatible. By incorporating the polymer-Fe-doped ceria NC in-situ during the hydrothermal synthesis of AuNPs, we formed the polymer-Fe-doped ceria/Au NC. Electron microscopy and optical-based techniques were used to characterise the NC material. By immobilising a strep-B-Apt binding complex on the polymer-Fe-doped ceria/Au NC, DNP bonded strongly to the aptamer with high affinity. The DNP-aptamer binding affinity was used to selectively tune the green colorimetric catalytic reaction towards DNP recognition quantitatively and selectively when TMB was catalysed by the strep-B-Apt-polymer-Fe-doped ceria/Au NC in the presence of \(H_2O_2\). Application of the peroxidase mimetic strep-B-Apt-polymer-Fe-doped ceria/Au NC biosensor towards DNP detection in tap water and river water was successfully achieved with satisfactory recoveries.
Acknowledgements
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Appendix A. Supporting information
Supplementary data associated with this article can be found in the online version at doi:

References


[50] Z. Sun, Y. Li, Y. Ma, L. Li, Dual-functional recyclable luminescent sensors based on 2D lanthanide-based metal-organic frameworks for highly sensitive detection of Fe³⁺ and 2,4-dinitrophenol, Dyes Pigm. 146 (2017) 263-271.


Table 1. Comparison of the analytical parameters of the strep-B-Apt-polymer-Fe-doped ceria/Au NC peroxidase mimic biosensor probe for DNP detection with other published methods.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Method</th>
<th>Detection time (min)</th>
<th>LOD (µM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D lanthanide-MOF</td>
<td>Fluorescence</td>
<td>-</td>
<td>16.4</td>
<td>50</td>
</tr>
<tr>
<td>Coordination polymers</td>
<td>Fluorescence</td>
<td>-</td>
<td>26.3</td>
<td>51</td>
</tr>
<tr>
<td>Rhodococcus erythropolis HL PM-1</td>
<td>Amperometric</td>
<td>2 min 30 sec</td>
<td>20.0</td>
<td>52</td>
</tr>
<tr>
<td>Polymethine Dyes</td>
<td>Absorption</td>
<td>-</td>
<td>3.7</td>
<td>53</td>
</tr>
<tr>
<td>Strep-B-Apt-polymer-Fe-doped ceria/Au NC</td>
<td>Peroxidase mimic</td>
<td>5 min</td>
<td>2.4</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>Colorimetry</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Analytical performance of the strep-B-Apt-polymer-Fe-doped ceria/Au NC peroxidase mimic biosensor probe for DNP detection in tap water and river water.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>DNP (µg/mL) added</th>
<th>Found (µg/mL)</th>
<th>Recovery (%) ±SD (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap water</td>
<td>100</td>
<td>101.8</td>
<td>~102±2</td>
<td>2.2</td>
</tr>
<tr>
<td>Tap water</td>
<td>50</td>
<td>58.7</td>
<td>117±6</td>
<td>5.7</td>
</tr>
<tr>
<td>Tap water</td>
<td>10</td>
<td>11.7</td>
<td>117±2</td>
<td>2.1</td>
</tr>
<tr>
<td>River water</td>
<td>100</td>
<td>95.9</td>
<td>~96±3</td>
<td>3.0</td>
</tr>
<tr>
<td>River water</td>
<td>50</td>
<td>50.2</td>
<td>~100±2</td>
<td>2.8</td>
</tr>
<tr>
<td>River water</td>
<td>10</td>
<td>9.7</td>
<td>~97±6</td>
<td>6.4</td>
</tr>
</tbody>
</table>
Scheme 1. Schematic representation of the polymer-Fe-doped ceria/Au NC synthesis. Organic-phased Fe-doped ceria is firstly synthesized and thereafter coated with amphiphilic polymers to render the nanomaterial biocompatible. The polymer-coated Fe-doped ceria NC is then incorporated in a Au reduction reaction to form a polymer-coated Fe-doped ceria/Au NC. Further schematic representation of the strep-B-Apt-polymer-Fe-doped ceria/Au NC as a peroxidase mimic.
biosensor for DNP colorimetric detection. In the presence of the strep-B-Apt-polymer-Fe-doped ceria/Au NC, TMB is oxidized by \( \text{H}_2\text{O}_2 \) and this triggered a unique green colorimetric reaction that was selectively tuned for DNP detection based on the Apt affinity to DNP.
Fig. 1. SEM images of the synthesized (A) amphiphilic polymer, (B) organic-phased Fe-doped ceria and (C) polymer-Fe-doped ceria NC. (D) TEM image of the polymer-Fe-doped ceria/Au NC.
A

B

C

Polymer-Fe-doped ceria/Au NC
Polymer-Fe-doped ceria NC
Amphiphilic polymer

%Transmittance

Wavenumber cm$^{-1}$

Intensity

Angle, 2 theta degree

Amphiphilic polymer
Polymer-Fe-doped ceria NC
Polymer-Fe-doped-ceria/Au NC

2988 cm$^{-1}$
2952 cm$^{-1}$
2915 cm$^{-1}$
3357 cm$^{-1}$
2847 cm$^{-1}$
1773 cm$^{-1}$
1702 cm$^{-1}$
1560 cm$^{-1}$
1524 cm$^{-1}$
1405 cm$^{-1}$
1399 cm$^{-1}$
1739 cm$^{-1}$

25
Fig. 2. (A) PXRD pattern and (B) FT-IR spectra of the synthesized amphiphilic polymer, polymer-Fe-doped ceria NC, and the polymer-Fe-doped ceria/Au NC. (C) DLS histogram plot and (D) zeta potential plot of the polymer-Fe-doped ceria/Au NC.
Fig. 3. (A) UV/vis absorption spectra showing the interaction of strep and the B-Apt on the polymer-Fe-doped ceria/Au NC. (B) UV/vis absorption spectra of the polymer-Fe-doped ceria/Au NC (i); DNP (100 µg/mL) + 1.2 mM TMB + 3 M H₂O₂ (ii); strep-B-Apt-polymer-Fe-doped ceria/Au NC + 3 M H₂O₂, (iii) and strep-B-Apt-polymer-Fe-doped ceria/Au NC + DNP (100 µg/mL) + 1.2 mM TMB (iv) and strep-B-Apt-polymer-Fe-doped ceria/Au NC + DNP (100 µg/mL) + 1.2 mM TMB + 3 M H₂O₂ (v).
Fig. 4. Absorbance data showing the effect of (A) time, (B) TMB and (C) $\text{H}_2\text{O}_2$ concentration on the catalytic colorimetric detection of DNP using the strep-B-Apt-polymer-Fe-doped ceria/Au NC.
Fig. 5. (A) Photographic colorimetric response for DNP detection using different nanozyme-based peroxidase mimetic probes. (B) Corresponding catalytic colorimetric absorbance data for DNP detection. Control = nanozyme + TMB (1.2 mM). DNP analysis = nanozyme + strep (0.5 mg/mL) + Apt (10 µM) + TMB (1.2 mM) + DNP (100 µg/mL) + H₂O₂ (3 M).
Fig. 6. (A) Photographic colorimetric response of the strep-B-Apt-polymer-Fe-doped ceria/Au NC for selective DNP detection in comparison to other analytes and the (B) corresponding catalytic absorbance signal. [DNP and other explosives] = 100 µg/mL.
Fig. 7. (A) Photographic colorimetric response of the strep-B-Apt-polymer-Fe-doped ceria/Au NC biosensor probe for quantitative DNP detection and the (B) corresponding calibration absorbance signal plot. Inset of Fig. 6B: Linear calibration plot.