Amalgamated gold-nanoalloys with enhanced catalytic activity for the detection of mercury ions (Hg^{2+}) in seawater samples

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ABSTRACT

Mercury (Hg) is extremely toxic, and continues to cause major threats to aquatic life, human health and the environment. Hg^{2+} mainly derives from seawater as a product of atmospheric deposition, therefore there is great demand for sensing approaches that can detect Hg^{2+} in seawater samples. Herein, we demonstrate that the peroxidase-mimicking activity of gold nanoparticles (AuNPs) or so-called nanozymes, can be exploited for the detection of Hg^{2+} ions in various water samples. In a high electrolyte environment, the catalytic activity for the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) was significantly diminished due to poor stability of the bare-AuNPs. This activity was reduced by ~ 73.7% when the NaCl concentration was higher than 1.168%, which is much lower than that of seawater (~ 3.5%), thus presenting its unsuitability for detecting Hg^{2+} in harsh water matrices. To overcome this limitation, AuNPs were first functionalized with oligo-ethylene glycol (OEG), of which their colloidal form presented high stability in NaCl concentrations up to 20% and across a wide range of pHs from 1–14. Interestingly, the catalytic activity of OEG-AuNPs for the oxidation of TMB was strongly suppressed by the coating, but enhanced upon formation of Au-Hg amalgamation. This novel finding underlies a straightforward, sensitive, and highly selective detection platform for Hg^{2+} in water samples. The approach could detect the exposure limit level for Hg^{2+} in drinking water (i.e., 2 ppb for tap and bottled water) as set by the United States Environmental Protection Agency (EPA) and the World Health Organization (WHO). When Hg^{2+} was spiked into a 3.5% saline solution and a coastal seawater certified reference material (CRM), the detection limits were found to be 10 and 13 ppb, respectively, which exceed the Hg^{2+} concentrations commonly found within seawater (~ 60–80 ppb). The whole procedure takes less than 45 min to conduct, providing a highly innovative, rapid and low-cost approach for detecting Hg^{2+} in complex water matrices.

KEYWORDS

gold nanoparticles, nanozyme, peroxidase-like, mercury detection, water samples, seawater

1 Introduction

Mercury (Hg) is a naturally occurring contaminant found in air, soil and water. It is a toxic heavy metal released into the environment via natural (e.g., forest fires, volcanic eruptions) and anthropogenic (e.g., burning fossil fuels, gold mining) sources. The Arctic ice-caps store twice as much Hg as the atmosphere, ocean and soil combined. Thus, climate change could contribute to the release of up to 15 million tonnes of Hg into the oceans [1]. Inorganic mercury (Hg^{2+}) can transform into neurotoxin methylmercury (MeHg) by bacterial conversion within water bodies. These extremely toxic compounds are highly stable and accumulate within marine systems, causing serious damage to major organs including the brain, nervous system, heart, kidneys and endocrine system when ingested, even at low concentrations [2]. Therefore, it is critical to monitor oceanic Hg^{2+} levels rapidly, to help prevent the consumption of contaminated seafood.

The United States Environmental Protection Agency (EPA) [3] and the World Health Organization (WHO) [4] have set maximum contaminant levels (MCLs) at 0.002 mg/L (2 parts per billion, ppb) in drinking water. However, the average level

of oceanic mercury is reported to be between 60–80 ppb in shallow waters, where many consumable aquatic animals exist [5]. Methods exploited for the detection of Hg^{2+} in drinking water and seawater, include inductively coupled plasma mass spectrometry (ICP-MS) [6], energy-dispersive X-ray (EDX) spectroscopy [7] and high-performance liquid chromatography (HPLC) [8], which are limited by complex sample preparation and expensive, bulky instrumentation. Gold nanoparticles (AuNPs) have come to the forefront of sensing technologies due to their inherent plasmonic and catalytic properties. These properties allow for the development of highly robust, sensitive and rapid colorimetric bioanalytical platforms for Hg^{2+} detection, with the advantages of low cost, simple procedures and reduced need for complex readout instrumentation [9].

Previously, plasmonic AuNPs have been widely exploited for the colorimetric detection of Hg^{2+} through T-Hg²⁺-T co-ordination chemistry [10–12] and interactions with molecules such as cysteamine [13], tween-20 [14], papain [15] and thioctic acid [16]. Additionally, AuNPs can mimic the activities of natural enzymes (termed nanozymes), for example horseradish peroxidase (HRP) [17], glucose oxidase [18–20], superoxide dismutase [21] and catalase [22], which have been well documented for

detecting metal ions, including Pb, Co, K and Hg [23–25]. However, some of these mimicking behaviours can exhibit relatively low catalytic activity [26]. In the presence of Hg^{2+} ions, AuNPs demonstrate strong peroxidase-mimicking behaviour for the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) substrate, due to Au-Hg amalgamation [27–30]. These important findings have resulted in the development of fluorescence [31] colorimetric [32] and paper-based sensors [33] and the exploitation of catalytically-active nanomaterials including DNA-Ag/Pt nanoclusters [34] and manganese dioxide (MnO_2) nanorods [35] for the detection of Hg^{2+} ions.

The size, shape and surface charge of AuNPs can strongly influence their catalytic activities, and these parameters are affected by electrolytic environments [36–39]. In fact, most applications for Hg^{2+} ion detection focus on low-electrolyte matrices including drinking water [40] and river water [41]. Limited applications to high salinity matrices (i.e., seawater) may be attributed to the nanomaterials suffering from stability issues in complex biological systems and reduced sensitivity, as a result of AuNP aggregation [42]. To improve stability, AuNPs are commonly modified with synthetic polymers including, polyethylene glycol (PEG) [43, 44] and oligoethylene glycol (OEG) [45, 46].

Herein, we demonstrate the reduced peroxidase-mimicking activity of aggregated bare-AuNPs, regardless of the formation of Au-Hg nanoalloys, which have previously demonstrated strong peroxidase-mimicking activity [30]. To overcome the detrimental effects to catalytic activity and improve applications to high electrolyte mediums, the AuNPs were functionalised with oligo-ethylene glycol (OEG). The obtained OEG-functionalised AuNPs (OEG-AuNPs) not only show excellent stability in high salt concentrations and in different pHs, but also suppressed catalytic activity. Interestingly, in the presence of Hg^{2+} ions the catalytic activity of the OEG-AuNPs is recovered, resulting in a highly selective and sensitive assay for the detection of Hg^{2+} in complex water samples. The approach takes less than 45 min to conduct, requires common reagents to perform and can be conducted at room temperature, thus highlighting its low-cost and practical procedures. In addition, the colorimetric response can be analysed without the need for complex equipment, therefore can improve the on-site analysis of complex seawater samples, where Hg^{2+} contamination is most abundant. These features of the developed approach are advantageous as compared to traditional methods.

2 Experimental section

2.1 Chemicals and reagents

Sodium citrate tribasic dehydrate ($\text{HOC}(\text{COONa})_2$ aq), gold (III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 99.9%), mercury (II) perchlorate hydrate ($\text{Hg}(\text{ClO}_4)_2 \cdot x\text{H}_2\text{O}$, 99.998%), O-(2-Mercaptoethyl)-O'-methyl-hexa(ethylene glycol) ($\text{C}_{15}\text{H}_{32}\text{O}_7\text{S}$, 95%), sodium chloride (NaCl), hydrochloric acid (HCl), sodium hydroxide (NaOH), 3,3',5,5'-tetramethylbenzidine (TMB), hydrogen peroxide (H_2O_2), nitric acid (HNO_3), sodium borohydride (NaBH_4), phosphate buffered saline (PBS), acetic acid (CH_3COOH) and sodium acetate (CH_3COONa) were all purchased from Sigma Aldrich (UK). TEM carbon coated, copper grids were purchased from Agar Scientific (UK). Syringe filters (0.22 μm) were purchased from Merk Millipore (Germany). Natural mineral water (Acqua Panna) was purchased from a local food establishment (Spar Store, Belfast, UK). Coastal seawater Hg^{2+} certified reference material (CRM) was purchased from LGC Standards (UK).

2.2 Analysis instrumentation

Ultraviolet-visible spectroscopy (UV-vis) measurements were performed using a Cary 60 spectrophotometer (Agilent Technologies, USA). Absorbance measurements were carried out using a Tecan Safire² plate reader (Tecan, Switzerland). AuNP size and zeta-potential measurements were carried out using a Zetasizer NanoZS (Malvern, UK). Transmission electron microscopy (TEM) measurements were conducted on a Joel JEM 1400 Plus Transmission Electron Microscope, operated at 100 kV. Fourier-transform infrared (FTIR) spectroscopy measurements were conducted on a Nicolet iS5 FTIR Spectrometer (ThermoFisher Scientific, UK). Elemental mapping, high-angle annular dark-field-scanning transmission electron microscopy (HAADF-STEM) and energy-dispersive X-ray spectroscopy (EDS) were carried out using a TALOS FEI High Resolution Transmission Electron Microscope (HRTEM), operated at 200 kV (ThermoFisher, UK).

2.3 Synthesis and functionalisation of gold nanoparticles (AuNPs) with oligo-ethylene glycol (OEG)

AuNPs are synthesised using the Turkevich method, with minor adjustments [47]. Briefly, 1 mM HAuCl_4 was dissolved in 100 mL of deionised water (dH_2O) and heated until rapidly boiling. Upon reflux 10 mL of 1% sodium citrate was quickly added to the boiling solution, under vigorous stirring. The solution was removed from the heat upon a colour change from yellow to wine-red, which indicates the citrate reduction of gold ions. For functionalisation, typically 0.5 mM OEG was incubated with 2 mL of as-prepared AuNPs overnight at room temperature. The particles were centrifuged at 16,100 rcf twice for 20 min, to remove unbound OEG. The pellet containing OEG-particles was re-suspended in dH_2O and stored at 4 °C for further use.

2.4 Analysis of AuNP catalytic activity in the presence of different NaCl concentrations

In a typical experiment, AuNPs were pre-treated with various concentrations of NaCl (0–0.5 M), and 1.25 mM TMB/2% H_2O_2 solutions were added to the mixture. All samples were incubated at room temperature for 10 min, followed by absorption measurements, kinetic analysis and dynamic light scattering (DLS) analysis.

2.5 Catalytic activity of bare-AuNPs and OEG-functionalised AuNPs in the presence of Hg^{2+} ions

Prior to analysis, a stock solution of Hg^{2+} was prepared at 20,000 ppm in 0.02 M HNO_3 (stored at 4 °C). In a typical experiment, 85 μL of Hg^{2+} ions (0–1 ppm) were transferred to a 96-well microtitre plate. Bare-AuNPs (15 μL , 1.7×10^{-9} M) were subsequently added and incubated for 2 min at room temperature to allow amalgamation. Finally, 1.25 mM TMB/6% H_2O_2 was added and incubated for a further 10 min at room temperature. Similarly, the Hg^{2+} concentrations (0–1 ppm) were spiked into a saline solution (containing 3.5% NaCl, pre-filtered using a 0.22 μm syringe filter to remove impurities) and the previous procedure repeated to analyse the performance of Au-Hg amalgam in high electrolyte conditions. For OEG-AuNP, various concentrations of Hg^{2+} were prepared in dH_2O and transferred to a microtitre plate (150 μL). The OEG-AuNP conjugate was subsequently added (0.5 μL , as-prepared), followed immediately by 0.2 M NaBH_4 and incubated at room temperature for 5 min. Finally, 1.25 mM TMB/6% H_2O_2 was added and incubated for a further 10 min at room temperature. Prior to analysis, the plate was sealed and sonicated for 3 min

to remove any bubbles formed as a result of hydrogen released from NaBH_4 .

2.6 Detection of Hg^{2+} ions in different water samples using OEG-AuNPs

Bottled and tap water were collected from a local establishment and the laboratory, respectively and filtered using a $0.22\ \mu\text{m}$ syringe filter to remove any particulate impurities. A 3.5% saline solution to simulate the salinity of seawater was prepared as mentioned previously. After filtration the 3.5% saline solution was used to prepare spiked water samples with Hg^{2+} concentrations in the range of 0–1 ppm and the assay was repeated. Additionally, the same Hg^{2+} concentrations were also spiked into a certified coastal seawater reference material (CRM) to validate the applicability of the approach.

3 Results and discussion

3.1 Gold nanoparticles (AuNPs) exhibit a strong peroxidase-like activity for the oxidation of TMB substrate

AuNPs (average diameter ~ 13 – $20\ \text{nm}$) were synthesised using the sodium citrate reduction of HAuCl_4 , with minor adjustments [47]. The particles synthesised by this method exhibit a net negative charge ($\zeta = -48.1\ \text{mV}$), and a deep ruby-red colour (Fig. 1(a), cuvette far left) showing a UV–vis absorbance band at $519\ \text{nm}$ (Fig. 1(b), red line). The concentration of the synthesised AuNPs ($\text{OD}_{520\ \text{nm}} = 1.0$) was $5 \times 10^{-9}\ \text{M}$, as calculated by their mean diameter and corresponding extinction coefficient value, as described previously [48]. Figure 1 indicates that after 10 min in the presence of 2% H_2O_2 , TMB was not oxidized unless AuNPs were present. With the addition of AuNPs to TMB/2% H_2O_2 , the rate of the oxidation reaction is greatly enhanced, which can be observed at wavelengths 370 and $650\ \text{nm}$ (Fig. 1(b), blue line). Additionally, kinetic analysis can also confirm the increased reaction rate over a 10 min incubation in the presence of AuNPs, at a fixed wavelength of $370\ \text{nm}$ (Fig. 1(c), blue triangles). Due to the overlapping of plasmonic peak from AuNP aggregation ($\sim 650\ \text{nm}$) with the

second peak from oxidised TMB (oxTMB), the most prominent peak at $370\ \text{nm}$ was selected for analysis [17]. From these results, it is evident that the presence of AuNPs notably enhances the rate of TMB oxidation therefore, confirming the strong peroxidase-mimicking activity of bare-AuNPs.

The catalytic mechanism of the negatively charged AuNPs can be explained by the decomposition of H_2O_2 . During the oxidation of TMB, TMB acts as a hydrogen donor whilst H_2O_2 acts as the recipient of the electron, to split H_2O_2 into H_2O and O_2 . Citrate-capped AuNPs speed up this process as they possess an extra electron from gold, which can be readily transferred to the adsorbed O_2 on the surface of the AuNPs, weakening the O–O bond. This leads to H_2O_2 being split into double hydroxyl radicals which can be stabilised by the AuNP, via a partial electron exchange interaction. The newly formed hydroxyl radicals oxidise TMB, thus contributing to the nanozymes peroxidase-like activity [49], producing a blue coloured product with two very distinct absorption peaks at 370 and $650\ \text{nm}$. Furthermore, TMB substrate is positively charged and has strong affinity to the negatively charged AuNP, thus electrostatic interactions facilitate the reaction further [50].

3.2 The peroxidase-like activity of AuNPs is inversely proportional to the particle aggregation state

As reported in the scientific literature, the catalytic activity is inversely proportional to the nanoparticle size. Therefore, it is rational to hypothesise that nanoparticle aggregation would also strongly influence this property. To investigate the hypothesis, AuNPs were treated with different NaCl concentrations ranging from zero to $0.5\ \text{M}$ (~ 0 – 2.92%) to induce particle aggregation. To confirm NaCl-induced aggregation, TEM was conducted on AuNPs in the absence and presence of $0.5\ \text{M}$ NaCl (Figs. S1(a) and S1(b) in the Electronic Supplementary Material (ESM), respectively). Figure 2(a) (top) illustrates the colour change of the AuNP solution from red to blue. The UV–vis analysis data (Fig. S2 in the ESM) indicates that the LSPR peak of the AuNPs shifts to a longer wavelength, with partial and complete aggregation occurring after the addition of $0.06\ \text{M}$ NaCl ($\sim 0.35\%$) and $0.1\ \text{M}$ NaCl ($\sim 0.58\%$), respectively. To examine the effect of AuNP aggregation on the peroxidase-like activity,

Figure 1 Evidence of bare-AuNPs exhibiting a strong peroxidase-like activity. (a) A colour photograph of colloidal AuNP solution ($\text{OD}_{520\ \text{nm}} = 1.0$), 2% H_2O_2 alone, TMB (1.25 mM) alone, 1.25 mM TMB and 2% H_2O_2 mixture, 1.25 mM TMB/2% H_2O_2 in the presence of AuNPs. All solutions were kept at room temperature, and the image was taken after 10 min. (b) Corresponding UV–vis spectra. (c) Kinetic analysis of the solutions after 10 min at a fixed wavelength of $370\ \text{nm}$.

Figure 2 The peroxidase-like activity of colloidal AuNPs ($OD_{520\text{ nm}} = 1.0$) in the presence of NaCl. (a) (top) Colour photograph of AuNP aggregation with different concentrations of NaCl. (below) colour photograph of peroxidase-like activity of the corresponding AuNPs after aggregation and addition of 1.25 mM TMB/2% H_2O_2 . (b) UV-vis analysis demonstrating peaks at 370 and 650 nm indicative of oxTMB product. (c) Absorbance at 370 nm as a function of NaCl concentration. (d) Kinetic analysis over a 10 min incubation illustrating the peroxidase-like activity of AuNPs after NaCl-induced aggregation. (e) Correlation between the average size of AuNP clusters Vs. the catalytic activity (absorbance at 370 nm produced by oxTMB), affected by varying the NaCl concentration and measured using DLS. Standard deviation (σ) was calculated from triplicate samples ($n = 3$).

1.25 mM TMB and 2% H_2O_2 was introduced to the AuNPs pre-treated with different NaCl concentrations. Figures 2(a) (below) and 2(b) demonstrate as the concentration of NaCl increases there is a decline in the peroxidase-like activity, witnessed by a reduction in peak absorbance at both 370 and 650 nm. Figure 2(c) also highlights the correlation between NaCl concentration and reduced peroxidase-like activity at a fixed wavelength of 370 nm. The AuNPs witness $\sim 73.7\%$ decrease in catalytic efficiency when faced with NaCl concentrations above 1.168% (~ 0.2 M). Therefore, these results can confirm that the peroxidase-like activity is strongly dependent on the particle aggregation state. Additionally, it was important to confirm that this reduction in catalytic activity was attributed to particle aggregation and not by the addition of NaCl. The same samples were centrifuged to remove NaCl in the surrounding medium, and the catalytic activity of the pellet (Fig. S3(a) in the ESM) and supernatant (Fig. S3(b) in the ESM) was analysed. The results confirm that in the absence of NaCl the catalytic activity of the aggregated nanoparticles remained the same and the supernatant containing NaCl did not exhibit any peroxidase-like activity. To further investigate the reduction in reaction rate over time, kinetic analysis was conducted. Figure 2(d) further illustrates the depletion in reaction rate over a time period of 10 min, with increasing NaCl concentrations. Figure 2(e) highlights the average hydrodynamic size of the

aggregated clusters gathered from DLS, compared with the absorbance values at 370 nm in the presence of NaCl. These results confirm that when aggregation is induced with NaCl the size of the particles or particle clusters dramatically increases (blue line), thus having a negative impact on the catalytic activity of the nanozyme (black line).

3.3 When the AuNPs are aggregated, their catalytic activity is suppressed regardless of the presence of Hg^{2+} ions

Figure 3(a) indicates that the catalytic activity of the bare-AuNP was significantly enhanced by the presence of Hg^{2+} ions at 1 ppm (cyan line) after 10 min incubation with 1.25 mM TMB and 6% H_2O_2 (working concentration). Similarly, the kinetic analysis confirms the enhancement of AuNP catalytic activity in the presence of Hg^{2+} ions over a 10 min incubation period (Fig. 3(b), cyan triangles). To investigate further steady-state kinetics were carried out to investigate the peroxidase-like activity of Au-Hg amalgam in comparison to the bare-nanozyme (Fig. S4(a) in the ESM). The results suggest that the maximum reaction velocity of AuNPs is greatly reduced in the absence of Hg^{2+} ($V_{\max} = 2.34 \times 10^{-6} \text{ M}\cdot\text{min}^{-1}$). However, AuNPs continue to catalyse the oxidation reaction in the presence of Hg^{2+} (1 ppm) at a much faster rate, due to the formation of Au-Hg amalgam ($V_{\max} = 7.70 \times 10^{-6} \text{ M}\cdot\text{min}^{-1}$) and the catalytic efficiency of the

Figure 3 The catalytic activity of bare nanozyme (0.85×10^{-9} M) in the presence of Hg^{2+} ions. (a) Full wavelength spectra of bare-AuNPs in the absence (blue line) and presence of Hg^{2+} ions (cyan line) after incubation with 1.25 mM TMB and 6% H_2O_2 , 1.25 mM TMB/6% H_2O_2 mixture alone (red line) and 1 ppm Hg^{2+} ions only (black line). (b) Kinetic analysis of Hg^{2+} ions (black squares), 1.25 mM TMB/6% H_2O_2 (red circles), AuNPs and 1.25 mM TMB/6% H_2O_2 (blue triangles) and AuNPs with 1.25 mM TMB/6% H_2O_2 in the presence of 1 ppm Hg^{2+} ions (cyan triangles). All experiments were conducted over a 10 min incubation period at room temperature. (c) Linear relationship between Hg^{2+} concentration and the enhanced catalytic activity of the bare-nanozyme ($R^2 = 0.996$) in dH_2O . (d) Catalytic performance of the bare nanozyme for the detection of Hg^{2+} ions in a dH_2O matrix (red bars) and in seawater conditions (3.5% NaCl, black bars).

AuNPs could be enhanced by 62.2% in the presence of Hg^{2+} ions (Fig. S4(b) in the ESM). These results confirm that the formation of Au-Hg amalgam can significantly improve the peroxidase-mimicking potential of the bare-nanozyme, which agrees with the previous report [30]. This mechanism could therefore be employed for the detection of Hg^{2+} ions in a dH_2O matrix (Figs. 3(c) and 3(d), red bars). However, to investigate the capabilities of the bare-nanozyme for the detection of Hg^{2+} ions in harsh water matrices, Hg^{2+} concentrations were spiked into saline solution (3.5% NaCl) prior to interaction with the nanozyme. The results in Fig. 3(d) (black bars) indicate that the strong electrolyte environment induces the aggregation of AuNPs, resulting in suppressed catalytic activity of the nanozyme by up to 88.4%, even in the presence of Hg^{2+} . This can be attributed to the high electrolyte environment increasing the particle size or size of aggregated clusters, thus reducing their surface area-to-volume ratio and subsequently their enzyme-mimicking properties, as reported previously [51]. Therefore, these results suggest that the aggregation state of the AuNP has more influence on their catalytic activity than the formation of Au-Hg amalgam.

3.4 OEG functionalisation improves the colloidal stability of the nanozyme, thereby enabling the detection of Hg^{2+} ions in harsh water matrices

As discussed, AuNP aggregation has a detrimental effect on the catalytic activity regardless of the presence of Hg^{2+} , thus in order to detect Hg^{2+} in a high electrolyte water sample it

was crucial to stabilise the bare-nanozyme with OEG. The functionalisation of AuNPs with OEG was conducted according to a previous method with minor adjustments [45]. The OEG molecule (O-(2-mercaptoethyl)-O'-methyl-hexa(ethylene glycol)) was functionalised onto the AuNP surface via chemisorption of thiol groups. A shift in LSPR peak from 519 (bare-AuNP) to 522 nm (OEG-AuNP) was observed (Fig. S5 in the ESM), indicating an increased refractive index at the particle surface due to adsorbed OEG molecules. FTIR was conducted to characterise the functionalisation of AuNPs with OEG (Fig. S6 in the ESM). The result indicates the presence of ethylene glycol molecules on the surface of AuNPs, thus confirming the successful functionalisation.

The OEG-AuNPs show excellent stability in a range of NaCl concentrations (0–20%, ~ 0 –3.4 M) (Fig. S7(a) in the ESM) and pHs (1–14) (Fig. S7(b) in the ESM), and can remain stable for several months in dH_2O when stored at 4 °C. The stability of the particles can be attributed to the hydrophilic nature of ethylene glycol forming a protective layer around the AuNP surface, which shields from high electrolyte environments. The OEG-AuNP when tested in acidic conditions (pH 1–3) a broadening in LSPR peak was observed (~ 50 nm). This slight shift in wavelength can be attributed to a reduction in interparticle distance, however not particle aggregation, as the UV-vis spectra (Fig. S7(b) in the ESM) can confirm particle stability in acidic conditions. Nonetheless, functionalising the AuNPs with OEG can provide stability which exceeds the limits required for a seawater matrix, as the pH of seawater is ~ 7 –8 and the NaCl concentration is $\sim 3.5\%$ (~ 0.6 M). Also,

the bare-nanozyme lacks stability over 0.3% (0.05 M) NaCl, which is much lower than the concentration found in seawater and highlights the improvements in particle stability after functionalisation with OEG. Additionally, the zeta-potential of the bare-AuNP was -48.1 mV, whilst the zeta-potential of OEG-AuNP in the absence and presence of 10 ppm Hg^{2+} was -40.8 and -38.7 mV, respectively. The change in zeta potential can be attributed to the desorption of capping agent, citrate ions which have a strong negative charge due to the formation of Au-S bonds, after the addition of the OEG molecules. The results also indicate that in the presence of Hg^{2+} the charge of the OEG-AuNP becomes less negative, due to the desorption of capping agent and the two positive charges held by Hg^{2+} ions [52]. Overall, this demonstrates the excellent stability of OEG-AuNP and their potential for detecting Hg^{2+} in a wide range of environments and matrices.

Figure 4 presents the catalytic activity of OEG-AuNP in the absence and presence of Hg^{2+} . After 30 min incubation with 1.25 mM TMB/6% H_2O_2 , the OEG-AuNP demonstrates suppressed catalytic activity (Fig. 4(a), red line) in comparison to the bare-AuNP, confirmed by the two distinct absorption peaks at 370 and 650 nm (Fig. 4(a), black line). In the absence of Hg^{2+} the OEG-AuNP cannot catalyse the oxidation reaction of TMB, as the hydrophilic ethylene glycol layer shields the surface, therefore blocking the transferring of oxygen atoms required for the reaction. However, in the presence of Hg^{2+} ions (10 ppm), the catalytic activity of OEG-AuNP is recovered by $\sim 75\%$ after 30 min incubation (Fig. 4(b), blue triangles). This suggests that the enhanced peroxidase-like activity of the stabilised OEG-AuNP could also be attributed to Au-Hg amalgam formation, due to the strong aurophilic interactions which exist between Au and Hg.

Figure 4 Experiment to confirm the suppression of OEG-AuNPs ($\text{OD}_{520\text{nm}} = 0.5$, 0.85×10^{-9} M) nanozyme activity, and recovery after incubation with Hg^{2+} ions (10 ppm). (a) Full wavelength spectra of bare-AuNP (black line) and OEG-functionalised AuNP (red line) after incubation with 1.25 mM TMB/6% H_2O_2 for 30 min. (b) Scanning kinetic analysis of bare-AuNP (black squares), OEG-functionalised AuNP (red circles) and OEG-AuNP incubated with Hg^{2+} ions (10 ppm) (blue triangles) all with 1.25 mM TMB/6% H_2O_2 at a fixed wavelength of 370 nm over 30 min.

TEM was carried out to characterise the size of bare-AuNPs (Fig. S1(a) in the ESM), and OEG-functionalised AuNP (Fig. S1(c) in the ESM). As we have discussed previously, aggregation of AuNPs has a strong effect on their catalytic activity. Therefore, it was important to ensure that the preparation of OEG-AuNP, or addition of Hg^{2+} ions was not affecting the aggregation state of the AuNPs, as this would be detrimental to the catalytic efficiency, and overall sensitivity of the assay. The TEM images (Fig. S1 in the ESM) and corresponding UV-vis absorption spectra (Fig. S8 in the ESM) can confirm that OEG and Hg^{2+} ions, do not dramatically influence the AuNP aggregation state. Interestingly, in the presence of Hg^{2+} the particles appear to coalesce (Fig. 5(a), red arrows) transforming the shape of the spherical AuNPs, however this does not cause particle aggregation. Further characterisation techniques were conducted to confirm the formation of Au-Hg amalgam, including HRTEM (Fig. 5(b)), HAADF-STEM (Fig. 5(b), inset 1), elemental mapping (Fig. 5(b), inset 2–3) and EDS (Fig. 5(c)). It is observed from the HRTEM image (Fig. 5(b)) the formation of an Au-Hg nanostructure, and the elemental mapping (Fig. 5(b), inset 1–3) performed using HAADF-STEM imaging indicates the presence of both Au and Hg within one nanostructure. The EDS spectrum (Fig. 5(c)) could further confirm the composition, highlighting the presence of both Au and Hg within the same amalgamated Au-Hg nanostructures.

Thus far, the results can support that our sensing mechanism is based on the ability of Hg^{2+} (ions) to become reduced to

Figure 5 Characterisation of OEG-AuNP in the presence of Hg^{2+} (100 ppm). (a) TEM analysis indicates particle coalescence upon amalgamation (red arrows). (b) HRTEM. Inset: (1) HAADF-STEM, elemental mapping of (2) Au and (3) Hg^{2+} . (c) EDS of the area in inset 1.

Hg⁰ (metal) to allow the formation of Au-Hg amalgamation, through strong aurophilic interactions. The metallic alloy can facilitate the oxidation of TMB substrate further in the presence of H₂O₂, through an electron transfer process which occurs at the surface of the Au-Hg nanostructure, resulting in enhanced reaction rates and an intense (vivid)-blue coloured product. However, Hg²⁺ ions are most abundant and problematic within seawater causing serious concerns for seafood consumers. Findings in the research indicate that the Au-Hg alloy is not able to catalyse for the oxidation of TMB substrate in seawater conditions (i.e., 3.5% NaCl), due to particle aggregation, thus making this method unsuitable for detecting Hg²⁺ ions in harsh water matrices. When the AuNPs are functionalised with OEG, the particles are not only stable at high salt concentrations and various pHs, but interestingly the nanozyme activity is inhibited and only recovered by the presence of Hg²⁺ ions (Scheme 1). The amalgamation-promoting nanozyme activity of OEG-AuNPs can provide a sensitive and specific detection platform for detecting Hg²⁺ in different conditions, including high salt concentrations, varied pH and improve the approaches applicability to harsh water matrices. Extensive kinetic analysis of the catalytic activity of OEG-AuNPs in the presence of various concentrations of Hg²⁺ ions is presented in Fig. S9 in the ESM. The results indicate that OEG-AuNP do not exhibit strong peroxidase-like activity unless Hg²⁺ ions are present.

3.5 Sensitivity and selectivity of the Au-Hg amalgam in water samples

Figure 6(a) demonstrates the calibration curve ($R^2 = 0.999$) and linear fitting of catalytic activity increase as a function of Hg²⁺ concentration ($R^2 = 0.996$) in a dH₂O matrix. Under optimal assay conditions the calibration curve provided a working range from 10 ppb Hg²⁺ to 200 ppb Hg²⁺, and the relationship between the absorbance at 370 nm against Hg²⁺ concentration was linear in a range between 10 ppb and

60 ppb Hg²⁺. The limit of detection (LOD) was calculated at a signal to noise ratio of 3 times the standard deviation (σ) of the blank, with a minimum detection limit of 0.9 ppb Hg²⁺ in dH₂O conditions (Table 1). The selectivity of the colorimetric assay was tested against thirteen metal ions (K⁺, Zn²⁺, Na⁺, Mn²⁺, Ag³⁺, Al³⁺, Co²⁺, Ca²⁺, Fe²⁺, Mg²⁺, Bi³⁺, Li⁺, Sn⁴⁺) to ensure the assay was specific to Hg²⁺ detection (Fig. 6(b)), and to confirm that the enhancement of OEG-AuNP nanozyme activity was only attributed to the presence of Hg²⁺ ions. The concentrations of all metal ions including Hg²⁺ were fixed at 10 ppm. When the concentration of metal ions was over 10,000 times the assay LOD, the nanozyme activity of the OEG-AuNP was not recovered or enhanced in the presence of any other metal ions, except Hg²⁺ ions. This excellent selectivity can be attributed to the high affinity Hg has for Au, and their ability to amalgamate. Thus, in a real water matrix the assay should not be hindered by the presence of other metal ions, which confirms its applicability to complex water samples.

To determine the applicability of the assay in real water matrices, drinking water (tap and bottled water) samples were analysed, and a saline solution (3.5% NaCl) to represent seawater. Hg²⁺ concentrations ranging from 0–1 ppm were spiked into all water matrices and the calibration range and linear fittings were determined (Fig. S10 in the ESM). The results showed good linearity between Hg²⁺ concentrations and the absorbance at 370 nm, with LODs identified as 2 ppb ($R^2 = 0.997$), 2 ppb ($R^2 = 0.995$) and 13 ppb ($R^2 = 0.994$) for tap water, bottled water and saline solution, respectively (Table 1). For validation purposes a coastal seawater CRM was also spiked with Hg²⁺ concentrations ranging from 0–1 ppm. The results also showed good linearity ($R^2 = 0.988$) and a LOD of 10 ppb was detectable (Fig. S11 in the ESM), which is slightly lower than that of the saline solution. The coefficient of variation (CV) (%) was calculated to confirm assay repeatability and was based on triplicate samples over consecutive days ($n = 3$), using the

Scheme 1 Working principle of the sensing mechanism for the colorimetric detection of Hg²⁺. Above row: bare-AuNPs can strongly catalyse for the oxidation of TMB substrate in the presence of H₂O₂. However, in the presence of 3.5% NaCl (to simulate the salt concentration of seawater) the catalytic activity of the AuNP is significantly reduced due to particle aggregation, even with the formation of Au-Hg amalgam. Below row: functionalising the AuNPs with OEG provides improved stability in high electrolyte environments. However, the catalytic activity of the OEG-AuNP is suppressed, and only recovered in the presence of Hg²⁺ ions. The mechanism can be attributed to the formation of Au-Hg amalgam, which can provide a colorimetric sensor applicable to seawater conditions.

Figure 6 Illustrating assay sensitivity and specificity in dH₂O. (a) Analysis of OEG-AuNP nanozyme activity with increasing concentrations of Hg²⁺ ions. Inset: the linear relationship between Hg²⁺ concentration and enhanced nanozyme activity ($R^2 = 0.996$). Data was normalised relative to the blank sample (zero). (b) Analysis of OEG-AuNP nanozyme activity in the presence of additional metal ions (all at 10 ppm).

Table 1 Nanozyme activity of OEG-AuNP as a function of Hg²⁺ concentration, analysed in water matrices after 10 min incubation with 1.25 mM TMB/6% H₂O₂. The table demonstrates the sensitivity of the assay in dH₂O, tap water, bottled water, saline solution and certified coastal seawater reference material (CRM). Validation was also conducted to assess the coefficient of variation (%) and % recovery (both calculated from the average of triplicate samples ($n = 3$) over three consecutive days)

Matrix	LOD (ppb)	Hg ²⁺ concentration (ppb)	CV (%)	Recovery (%)
dH ₂ O	0.9	20	2.3	89.6
		40	5.5	98.6
		80	3.0	97.1
		10	14.7	87.9
Tap water	2	20	7.1	82.4
		40	17.3	75.8
		10	11.5	103.9
Bottled water	2	20	6.4	105.3
		40	13.6	111.8
		20	10.1	103.6
Hg ²⁺ seawater CRM	10	60	5.6	97.8
		100	5.7	95.8
		40	7.4	91.0
Saline solution	13	80	9.8	93.0
		160	12.9	123.7

average (x) and σ ($n = 3$). The CV% for all water samples fell below 17.3%. The % recovery was also calculated to confirm the reliability of the assay and was based on an average of triplicate spiked samples over consecutive days ($n = 3$) against the developed calibrations (known concentrations). The % recovery results all fell into an acceptable range of 76% to 124%. Therefore, the results from the spiked sample analysis and validation can confirm that the assay is repeatable and reliable.

The LOD had increased ~ 2 times in tap and bottled water in comparison to the dH₂O conditions, however these limits can nonetheless meet the MCLs for Hg²⁺ in drinking water (2 ppb), as set by the EPA and WHO. Additionally, the LOD in seawater CRM and saline solution had increased to 10 ppb and 13 ppb, respectively however this can be attributed to matrix effects within the complex water samples, more specifically the decomposition of hydrogen peroxide in the presence of chloride interfering with the oxidation reaction [53]. However, due to the excellent stability of OEG-AuNPs and the recovery of nanozyme activity in the presence of Au-Hg nanoalloys, it was still possible to quantify LODs for oxTMB in complicated water matrices, using spectroscopy. Furthermore, the concentration of Hg²⁺ in seawater is commonly between 60–80 ppb in shallow waters, therefore the assay should be capable of detecting within this range and highlights its potential to analyse real seawater samples. Finally, dangerous levels of Hg²⁺ within seafood are most abundant within larger species (e.g., tuna, shark, swordfish), with levels ranging from 100 ppb (min) to 4.5 ppm (max) [54]. Therefore, the assay shows not only good applicability to drinking water and seawater, but also has the potential for seafood analysis in the future.

4 Conclusion

Overall, a sensitive and highly specific colorimetric assay for the detection of Hg²⁺ ions, which is based on recovering the nanozyme activity of OEG-functionalised AuNPs was successfully developed. We discovered that bare-AuNPs witness $\sim 73.7\%$ decline in catalytic efficiency when faced with NaCl concentrations above 1.168%, which is much lower than the concentration of NaCl in seawater (3.5%). In addition, steady-state kinetic parameters reveal that the peroxidase-like activity of the bare-nanozyme could be enhanced by 62.2% in the presence of Hg²⁺. However, when faced with high concentrations of electrolytes the catalytic activity of the Au-Hg amalgam was significantly reduced (by up to 88.4%). OEG-AuNPs demonstrated high stability in a range of NaCl concentrations (0–20%) and pHs (1–14), thus can improve the applications of Hg²⁺ detection to complex water matrices. Surprisingly, the catalytic activity of the OEG-AuNP was suppressed due to the ethylene glycol layer surrounding the nanoparticle surface, however it was found that this activity could be recovered in the presence of Hg²⁺ ions.

Characterisation techniques confirmed that the sensing mechanism was due to Au-Hg amalgamation, which allowed the development of a highly specific colorimetric assay with sensitivity down to parts per trillion level (0.9 ppb in dH₂O). Real sample analysis could determine detection limits for Hg²⁺ as 2 ppb in drinking water (tap and bottled water) and 10–13 ppb in high salinity samples (seawater CRM and saline solution, respectively). Due to the excellent stability of OEG-AuNPs the sensitivity was not influenced dramatically by the matrix, demonstrating the assays effectiveness for water analysis. In addition, the total assay time takes 45 min, requires only a few reagents and can be conducted at room temperature, highlighting its low-cost and practicality. Overall, the assay

has the ability to improve the on-site analysis of drinking water, seawater samples and potentially seafood samples. Thus, it contributes to the protection of human health, aquaculture and the environment from toxic Hg^{2+} contamination.

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Electronic Supplementary Material: Supplementary material (TEM images of (a) stable bare-AuNP and (b) aggregated AuNPs, in the presence of dH_2O and 0.5 M NaCl, respectively (c) OEG-functionalised AuNPs (Fig. S1); UV–vis spectra of AuNP treated with different NaCl concentrations (0–0.5 M) (Fig. S2); the effect of NaCl on AuNP peroxidase-like activity (Fig. S3); Michaelis-Menten kinetic analysis (Fig. S4); UV–vis analysis of bare-AuNP and OEG-AuNP (Fig. S5); FTIR spectra for OEG-AuNP characterisation (Fig. S6); full wavelength spectra to examine the stability of AuNP functionalised with OEG in the presence of 0–20% NaCl and PBS buffer pH 1–14 (Fig. S7); UV–vis analysis to confirm the stability of bare-AuNP and OEG-AuNP in the presence of Hg^{2+} ions (10 ppm) (Fig. S8); scanning kinetic analysis of OEG-AuNP nanozyme activity in the presence of Hg^{2+} concentrations (Fig. S9); nanozyme activity of OEG-AuNP as a function of Hg^{2+} concentration, analysed in water matrices demonstrating the sensitivity and applicability of the assay (Fig. S10); Linear relationship between spiked Hg^{2+} concentration and recovered nanozyme activity for OEG-AuNP analysed in a coastal seawater Hg^{2+} certified reference material (CRM) (Fig. S11)) is available in the online version of this article at <https://doi.org/10.1007/s12274-020-2731-y>.

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