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PAPER

Novel porous silica encapsulated Au nanoreactors as peroxidase mimic for one-pot glucose detection†

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The peroxidase family of natural enzymes facilitate H_2O_2 reduction by one electron transfer through aromatic substrates, if chromogenic, they can be used in the colorimetric detection of H_2O_2 and subsequently glucose in tandem with glucose oxidase. Au nanoparticles encapsulated within porous silica exhibited peroxidase mimetic activity and kinetic parameter evaluation indicates an excellent affinity for H_2O_2. It is also shown to have a detection capability for glucose in very low concentrations and could be used for glucose detection in a one-pot assay. The material is synthesised by using cation-ended thiol-stabilised ultra small gold clusters as precursors. Removal of the thiols creates micropores within the silica, making this material unique in that the active centres are protected inside the silica, yet are accessible to reactant molecules. This characteristic makes the material ideal as a detection tool where attrition resistance will be advantageous.

Introduction

Enzymes form an important class of compounds, catalyzing many biologically significant reactions. Peroxidases, like glutathione peroxidase, horseradish peroxidase, *etc*., constitute one such family of enzymes utilising hydrogen peroxide to oxidise a spectrum of organic compounds.[1] This property has meant that these enzymes are very useful in waste water treatment, since toxic organic compounds can be oxidised easily to reduce their adverse effect on the environment.[2] Another important property is the production of chromogenic species during redox reactions. In the presence of H_2O_2, peroxidases facilitate one and two electron transfer processes, which turn amine substrates like benzidine to blue and yellow in colour.[3] This phenomenon is exploited in detection tools for H_2O_2 and can be extended to glucose detection in tandem with glucose oxidase, employing colorimetry. However, using natural enzymes for these catalytic processes is limited due to their inherent instability, and their complex separation and synthesis steps. Hence inorganic materials which mimic natural enzymes have recently been gaining importance due to their stability and ease of synthesis. Oxide nanoparticles, like Fe_3O_4, CuO, CeO_2 and $BiFeO_3$ nanoparticles,[4] as well as carbon based materials[5] are found to be peroxidase mimics and very recently, positively charged Au nanoparticle colloids are reported to possess intrinsic peroxidase activity.[6] Interestingly, the surface charge and nature of the grafted ligand were found to play an important role in the peroxidase mimicking and a two-step assay was employed for glucose detection.

In utilising noble metal nanoparticle colloids (NPs) for catalysis, the most adverse effect occurs *via* loss of activity due to agglomeration and subsequent variations in surface properties.[7] Moreover, the presence of surface functionalisation restricts a widespread use of such materials in catalysis under harsh conditions.[8] To exploit the excellent properties of the nanoparticles for a wide range of reaction conditions, it is imperative to protect them from agglomeration, while, at the same time, keeping the surface properties intact and surface sites accessible to reactant molecules. This is, to an extent, achieved in supported catalysts but the propensity of noble metals for sintering as well as leaching of the active metal sites also plague these systems.[9] However, recently nanoparticles encapsulated in porous oxides have attracted attention for their potential to address these issues.[10] Even though the coating of nanoparticles with oxides, especially silica is a well advanced field of research,[11] is frequently used to stabilise the nanoparticles, such a material will not be conducive for catalytic processes. The main drawback is the complete encapsulation of the NPs by dense oxide layers, which deny the reactant molecules access to the active surface sites. This has been overcome by post-synthetic etching of the silica layers using strong base, creating mesopores in the silica shell.[12] However, the particle size of the Au NPs achieved in this work are much bigger, whereas the Au particles

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of sizes 1–5 nm are suggested to be electronically and structurally advantageous for catalytic processes.[13]

In this scenario, we have been developing an alternate simple method for controlling the particle size of Au NPs, as well as encapsulating them in porous silica to enable reactant molecule access to their surfaces. For this, thiol protected Au clusters were encapsulated in silica, whereby porosity can be generated *in situ* by removing the organics by calcination. Such a synergism of the silica matrix porosity and the Au NP stability makes this material ideal for catalysis. Moreover, this material has the added advantage of active sites being protected within the inert oxide layer, leading to attrition resistant catalysts. Due to this unique characteristic, such materials will be ideal for catalytic detection tools for repeated use. In addition, the resultant active particles are protected against further agglomeration and leaching by the encapsulating silica. In this paper we report a detailed study of H_2O_2 reduction kinetics by Au NPs encapsulated in porous silica as well as their utilization in glucose detection. The catalyst was found to be stable and active in a range of pH and temperatures.

Experimental

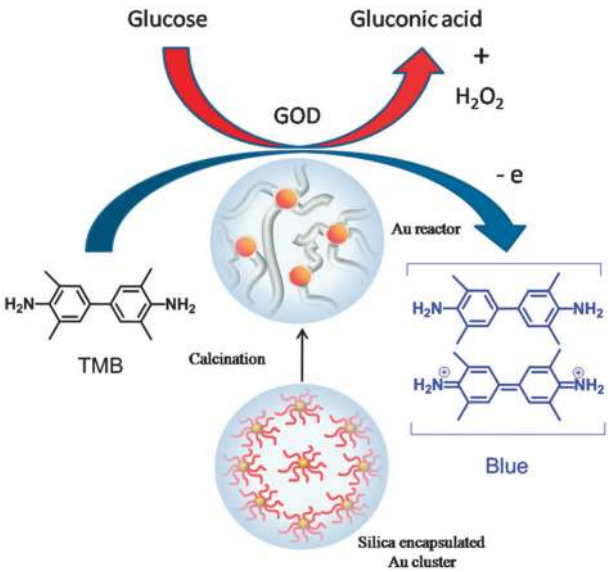
The synthesis of the Au@p-SiO_2 is reported elsewhere,[14] details given in ESI.† The amount of Au present in the sample was estimated by inductively coupled plasma spectroscopy, ICP-AES on Spectro Akos, FHS-12. Thermogravimetric analysis was carried out on a Mettler Toledo thermogravimetric analyzer. Accurately weighed 5–6 mg of the sample was heated from room temperature to 400 °C under air (50 mL min^{-1}) with a ramping temperature rate of 5 °C min^{-1}. The kinetics of TMB oxidation were monitored in kinetic mode of the spectrophotometer (Perkin–Elmer −λ35) using 1 mL quartz cuvette of 1 cm path length at 650 nm (one of the absorption peaks of oxidized product of TMB) in a thermostated (25.0 ± 0.5 °C) cell housing. 1 mg of the catalyst was dissolved in 1 mL double distilled water and was treated as stock solution. The concentration of H_2O_2 was calculated by dividing the UV absorbance at 240 nm by the characteristic molar extinction coefficient (ε = 43.6 dm^3 $M^{-1}cm^{-1}$). To investigate the mechanism assays, H_2O_2 variation was done from 5.0×10^{-5} to 2.0×10^{-3} M, while keeping the TMB concentration fixed at 5.75×10^{-3} M. For TMB variation the reverse procedure was used ([H_2O_2] = 5.0×10^{-5} M; [TMB] = $5.75 \times 10^{-3} - 1.4 \times 10^{-3}$ M). In all kinetic runs, the catalyst concentration was kept constant. 30 μL stock catalyst solution was added in each set of H_2O_2 variation and TMB variation. Phosphate buffer having the appropriate pH of 1.9, 2.3, 2.5, 3.5, 4.4, 4.8 and 6 was used for different runs. For each set, after the reaction, the solution pH was measured with a pH meter (LABINDIA, PICO+) with a calibrated electrode.

For glucose calibration, 60 μL of 5.5 mg mL^{-1} glucose oxidase [Sigma Aldrich (G7141-10KU); from *Aspergillus niger*] and 600 μL glucose with different concentrations, 270 μL TMB of 20 mM TMB in 0.1 M HCl, 30 μL of stock catalyst solution (1 mg mL^{-1}) and 40 μL 500 mM phosphate buffer at pH 4.0 were incubated at 55 °C in a water bath for 15 min. The resulting solution was used for absorption spectroscopy measurements at 650 nm using a Perkin–Elmer −λ35 spectrophotometer.

Results and discussion

We have recently reported the synthesis of water dispersible ultra small Au clusters of size < 2 nm using a novel cationic ammonium ended thiol ligand.[14] Arrays of these clusters could be encapsulated within reasonably monodisperse silica spheres of size 25–30 nm. The clusters were deployed in a sufficiently space separated manner and individually encapsulated by silica. In this way, the formation of a core shell material with a thick shell of silica and multiple clusters agglomerating in the core could be avoided. These clusters were utilized as precursors for the synthesis of nanoreactors encapsulated within the spheres on calcination. TGA analysis showed a substantial decrease in weight loss in the calcined sample when compared to the as-synthesised sample, indicating the removal of organics (ESI†). The abundance of organic ligands within the silica spheres ensured the formation of pores in the silica matrix. The architecture, where clusters are encapsulated individually in silica, enabled better control of the particle sizes by minimising sintering during calcination. The material was found to have micropores of a size of ~1 nm and an average Au particle size could be controlled to be ~3 nm.[14] Elemental analysis by ICP-AES indicated that the composition of the sample is 5 wt% Au/SiO_2.

The encapsulated Au in porous silica, henceforth mentioned as Au@p-SiO_2, was tested for its catalytic activity in H_2O_2 reduction and subsequent glucose sensing. Au NPs act as peroxidase mimics and provide H_2O_2 adsorption sites. In the presence of a chromogenic electron donor, like 3,3,5,5-tetramethylbenzidene (TMB), H_2O_2 reduction is proposed to take place, aided by partial electron transfers to the Au surface.[15] The consequent change in colour of the oxidation product of TMB by one electron transfer is followed easily by UV-Vis spectroscopy. This catalytic ability of Au NPs, to act as a peroxidase mimic, can be put to use for a range of applications in environmental and biological detection tools. One such use is in glucose detection in tandem with enzyme glucose oxidase (Scheme 1). Glucose oxidase (GOD) is the enzyme responsible for oxidizing glucose to gluconic acid and H_2O_2. Even very small concentrations of glucose can be detected by following the H_2O_2 produced during its oxidation. These consecutive reactions are exploited in designing a detection tool for glucose. We have already reported H_2O_2 reduction catalysed by the Au@p-SiO_2 system, proving the presence of the porosity in the silica, enabling reactant molecules access to the Au NPs encapsulated within.[14] We have proceeded with kinetic studies to understand the enzyme mimetic behaviour of the system by following the peak at 650 nm in the UV-Vis spectrum, which indicates one of the oxidation products of TMB. Initial rates of TMB oxidation were calculated from the linear absorbance *versus* time plots using the extinction coefficients of 39 000 $M^{-1}cm^{-1}$ which stands for the oxidation product of TMB[16] at 650 nm when the conversion of the TMB did not exceed 10–20%. Concentrations of H_2O_2 and TMB were varied and the effect followed as above (Fig. 1A, and B). The range of concentration of TMB was fixed between 1–8 mM to obtain better calibration data. To solubilize TMB in these concentrations, highly acidic solutions were used and the kinetic studies were carried out at a pH of 2.5 and at room temperature using 0.25 mM of



Scheme 1 Formation of Au@p-SiO_2 nanoreactors from ultra small cluster arrays encapsulated within silica spheres and their utilization in glucose detection. GOD oxidises glucose to gluconic acid and H_2O_2 and the H_2O_2 produced oxidises TMB to the coloured product. The reactant molecules can access the Au particle surface through micropores in the silica and facilitate electron transfer, enabling the colorimetric detection of the oxidised product.

Au (1 mg mL^{-1} of 5 wt% Au@p-SiO_2). The experimental points in the above studies were fitted perfectly to the Michaelis–Menten equation defining enzyme kinetics:

$$V = V_{max} \times [S]/(K_m + [S])$$

where V stands for initial rate or initial velocity, V_{max} is the maximal velocity, [S] is the concentration of the substrate and K_m is the Michaelis constant.

For both [H_2O_2] and [TMB] variations, the rates were found to follow the above equation with very good fit and the kinetic parameters calculated from the curve are given in Table 1. The Michaelis–Menten constant K_m indicates enzyme affinity towards the substrate,[4] while V_{max} stands for the maximal initial velocity. The K_m values, with respect to H_2O_2 as substrate, was much lower than those reported for HRP as well as other nanoparticles systems.[4–6]

A comparison of this kinetic value is given in ESI,† and this indicates a higher affinity of the catalyst to H_2O_2, which suggests that the detection capability is higher for much lower concentrations of H_2O_2 when compared to other reported systems.[4–6] However, with respect to TMB, the trend was reversed. The results also prove that the catalytic activity of Au NPs in H_2O_2 reduction is enzyme mimicking with respect to both the substrates, H_2O_2 and TMB. So, a mechanism of two electron transfer similar to peroxidise enzymes can be envisaged for the Au@p-SiO_2 catalysts.[17]

Natural enzymes are known to be stable only in a narrow range of pH and temperature, which restricts their utilization in applications involving harsh conditions. The rate of thermal denaturation of HRP is very high above 70 °C,[18] and its protein structure is reported to be unstable below pH 4.5.[19] Hence, any alternative developed should be stable at a wide

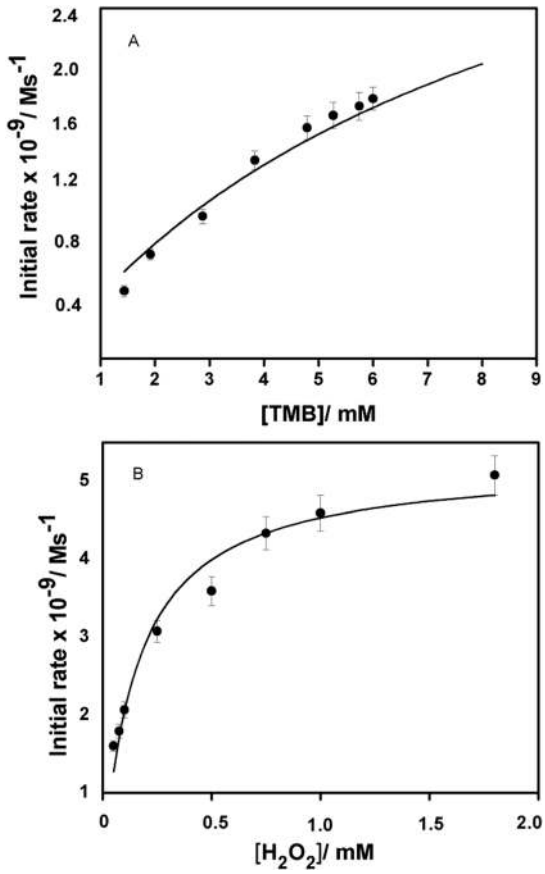


Fig. 1 Steady-state kinetic assays of Au@p-SiO_2. (A, B) The initial rate of the reaction was measured using 30 μg mL^{-1} Au@p-SiO_2 in a reaction volume of 1 mL (pH 2.5) at 25 °C. Error bars represent the standard error derived from three repeated measurements. (A) The concentration of H_2O_2 was 5.0×10^{-5} M and TMB concentration was varied. (B) The concentration of TMB was 5.75×10^{-3} M and H_2O_2 concentration was varied.

Table 1 Kinetic parameters for TMB Oxidation by peroxidise mimic Au nanoreactors

Substrate	V_{max}/Ms^{-1}	K_m/mM
TMB	4.21×10^{-9}	8.70
H_2O_2	5.24×10^{-9}	0.156

range of temperatures and pH. Inorganic nanomaterials are proposed to be good alternatives and such nanomaterials, like Fe_3O_4, CuO and positively charged Au NPs, were found to be stable over a reasonable range of these conditions. This was tested for Au@p-SiO_2 by incubating the catalyst at pH values from 2.5 to 10 as well as temperatures to 90 °C and further, following the H_2O_2 reduction using TMB at 30 °C. Interestingly, this material was found to be stable and active after treatments at 90 °C and pH 10 (ESI†).

Au NPs reported hitherto showed optimal activity in the acidic range and we also tested the activity of our system in the pH range 2–6. Further pH increase was not amenable due to the high concentrations of TMB used. The material showed highest activity at a pH of 4.5 and the activity was found to increase exponentially with temperature (Fig. 2). The silica

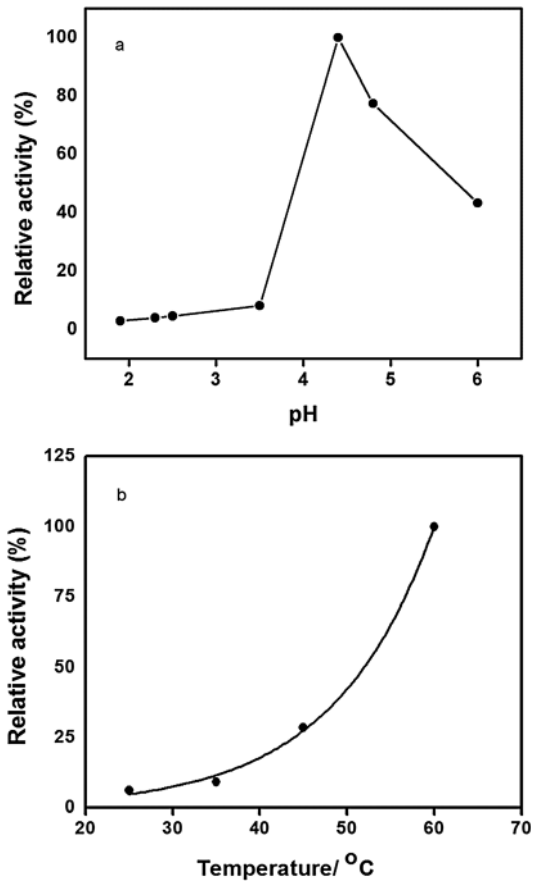


Fig. 2 (a) Dependence of the peroxidase like activity of Au@p-SiO_2 on pH and (b) on temperature. The maximum point was set as 100% in terms of initial rate.

surface will be negatively charged at pH values above the isoelectric range of 3.5 and hence an electrostatic interaction is possible with TMB molecules, which would be protonated in acidic conditions. This may lead to an enhanced interaction between the catalyst and substrate. However, pure silica without Au nanoreactors did not show any activity, ruling out any electron transfer between the silanols and TMB molecules (ESI).† Further testing of this hypothesis was carried out using 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as the chromogenic substrate. The catalyst was found to be active in the presence of ABTS as substrate at a pH of 4.5 (Fig. 3). This may indicate that surface interactions, although may be present, may not have a substantial effect on activity. This observation is further corroborated by the literature report of positively charged Au cysteamine nanoparticles showing high activity at pH 4.5.[6]

As mentioned earlier, H_2O_2 detection can be extended to the design glucose detection tools. Au@p-SiO_2 could be used in a simple one-pot reaction to detect glucose. The colorimetric response of the assays was observed after incubation of the catalyst, TMB, GOD and different concentrations of glucose at 55 °C and pH 2.5 for 15 min. GOD is reported to be stable under these conditions.[20] The response with variations in glucose concentrations, in comparison to the control solutions without catalyst, was even visible by the naked eye. Also, the assay was found to be very specific to glucose, the control

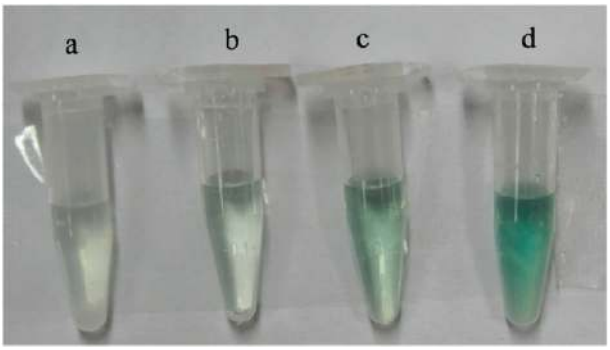


Fig. 3 Images for the oxidation of ABTS using Au nanoreactor and silica. In a typical experiment 10 μL 18 mM ABTS, 170 μL 160 mM phosphate buffer of pH 4.5, 100 μL H_2O_2 of 2 M (for a, b, c) and 8 M (for d), 20 μL of Au catalyst (stock solution 1 mg mL^{-1}) (for c, d), 1 mg silica (for a), no Au catalyst (for b) were added and incubated in a 30 °C water bath for 10 min.

experiments with another sugar (mannose) did not produce any blue colour (Fig. 4).

The calibration was carried out by following the 650 nm absorbance peak in the UV-Vis spectra as in the case of H_2O_2 calibration. The result shows a linear increase in the absorbance in the range 20 μM to 0.5 mM and reaches a saturation point after 1 mM (Fig. 5). The linear response region shows that the lower detection limit for glucose for this material is 20 μM, which is comparable to other nanomaterial based catalysts.[4–6] It is noteworthy here that Au@p-SiO_2 has high activity in a one-pot reaction, as compared to a complicated two-step method reported for other Au NP based materials. This can be attributed to the overall enhanced stability and surface properties of the silica encapsulated Au NPs. For this material to be employed in any detection tool, attrition resistance is an advantage. We propose that encapsulation of the active Au particles inside silica helps in preventing attrition and this would be reflected in the reusability of the catalyst. Recyclability of this catalyst was tested for three colorimetric detection cycles with repeated centrifugation and washing. The activity was found to be intact, which indicated a robust catalyst structure (ESI).† The material showed saturation in activity at physiological conditions of 2.5 mM of glucose under the concentration levels of TMB used in the current study. However, these studies show the potential of using this catalyst

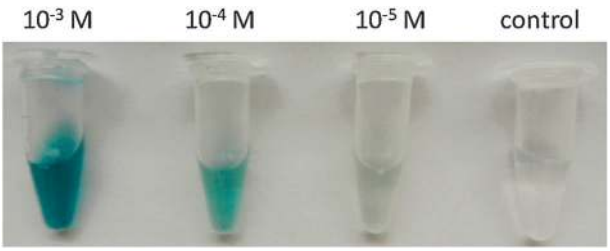


Fig. 4 Typical images of detection of glucose at different concentrations: (a) 10^{-3} M, (b) 10^{-4} M, (c) 10^{-5} M and (d) 10^{-3} M mannose. Experimental conditions: 60 μL of 5.5 mg mL^{-1} glucose oxidase and 600 μL glucose with different concentrations, 270 μL TMB of 20 mM TMB in 0.1 M HCl, 30 μL of stock catalyst solution (1 mg mL^{-1}) and 40 μL 500 mM phosphate buffer 4.0 were incubated at 55 °C water bath for 15 minutes.

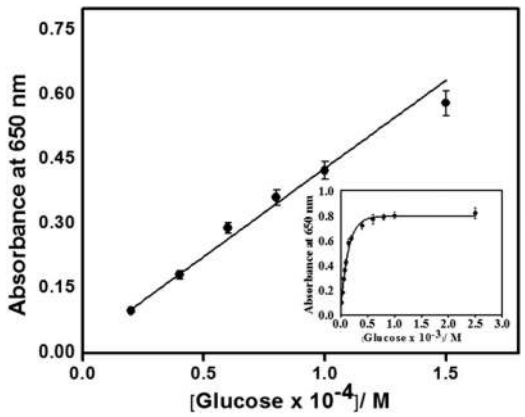


Fig. 5 Linear calibration plot between the absorbance at 650 nm and concentration of glucose. The inset shows the dependence of the absorbance at 650 nm on the concentration of glucose in the range 20 μM to 2.5 mM.

in glucose detection in biological systems, like blood, with appropriate changes in concentration levels of TMB.

Conclusions

In summary, a stable and novel catalyst based on Au nanoparticles is developed for H_2O_2 and glucose detection. Ultra small clusters of Au thiolate are used as precursors for porous silica encapsulated Au nanoparticle catalysts. The catalyst was found to be a peroxidase mimetic nanomaterial following Michaelis–Menten kinetics for enzyme catalysed reactions. The activity was found to be maximum at a pH of 4.5 and increased exponentially with temperature. Incubation stability was also found to be reasonably high in the range of pH 2.5–10 and up to 90 °C. Kinetic parameters revealed that the affinity of the catalyst to H_2O_2 is very high compared to other reported systems, leading to a minimal concentration for maximum activity. The combined effect of the porosity of the silica coating and the control of the particle size of the active Au nanoparticles has made this material ideal for catalysis. Also, the silica layer protecting the active Au particles is advantageous for detection tools due to the attrition resistance.

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