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Gold nanoparticles decorated single walled carbon nanotubes nanocomposite with synergistic peroxidase like activity for D-alanine detection

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Abstract

In this report, gold nanoparticles decorated single walled carbon nanotubes (SWCNTs) nanocomposite was shown to possess synergistic intrinsic peroxidase like activity and enhanced affinity towards H_2O_2 oxidation. The gold nanoparticles decorated SWCNTs nanocomposite were characterized by high catalytic activity, enhanced stability of gold nanoparticles and improved dispersion of SWCNTs. Subsequently, this nanocomposite was proved to be a novel peroxidase mimetic with great potential to catalyze oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of H_2O_2 to yield a blue color product. As a proof of concept, gold nanoparticles decorated SWCNTs composite was used as a robust nanoprobe for the detection of D-alanine with improved analytical characteristics. Taking into account the valuable intrinsic peroxidase activity of nanohybrid, the present work may find widespread applications in the field of sensors and biosensors for diverse applications.

Keywords: SWCNTs/gold particle nanocomposite; peroxidase like activity; synergic effect; D-alanine detection; colorimetric assays

1. Introduction

Natural enzymes have remained a topic of great interest for researchers owing to their catalytic properties, and as well as substrate specificity. However, the catalytic activity of natural enzymes is directly influenced by different parameters such as temperature, acidity and inhibitors[1]. Their significance is further limited due to their high cost and time consuming preparation, purification and storage steps[1-4]. Thus, more attention is paid to the discovery and development of new enzyme mimics during the last few years. The peroxide enzymes mimics such as cyclodextrin[5], porphyrin[6], hemin[7, 8], DNAzyme[9], and hematin[10] were largely used as catalysts for the determination of H_2O_2.

During recent years, the growing field of nanotechnology has resulted in the development of a variety of nanomaterial with improved catalytic properties due to their large surface-to-volume ratio[11]. The enzyme mimics of transition metal oxides and sulfides such as graphene oxide [12], cupric oxide [13], V_2O_5 nanowires [14], Fe_3O_4 [3], $BiFeO_3$ [15], polymer-coated CeO [16] and FeS nanostructures [17] have been successfully integrated to impart intrinsic peroxidase activity for sensing applications. Moreover, hybrid nanocomposite materials with well-defined structure have been investigated to realize the synergic effect by combining the properties of two materials or to achieve cooperatively enhanced performance for various applications. In this context, a variety of inorganic nanomaterials have been incorporated with different supports to achieve nanohybrids of desired functionalities. Typically, some of these nanocomposites have been

explored to possess synergistic peroxidase like activity to replace the natural enzyme [18]. There is a great interest to design and fabricate new nanomaterials with enzyme like activities, and to subsequently use them for sensing applications.

Recent studies have demonstrated the catalytic activity of carbon nanotubes even in the absence of catalytic factors[19]. The intrinsic peroxidase like activity of SWCNTs has received much attention to design biofuel cells and biosensors of novel characteristics. Similarly, noble metal nanomaterials such as gold and silver having several to tons of metal atoms have become emerging area of scientific research due to their optical properties, biocompatibility and low toxicity. Interestingly, recent work by Wang et al has explored the peroxidase like activity of gold nanoparticles for xanthine detection[20]. Therefore, in the light of superiority of SWCNTs and gold nanoparticles, the decoration of gold nanoparticles on SWCNTs was expected to possess new and enhanced catalytic properties that cannot be achieved by either component alone. To the best of our knowledge, the peroxidase like activity of SWCNTs/gold nanoparticles nanocomposite has not been explored in the literature so far. To demonstrate the feasibility of nanocomposite, the synergistic peroxidase like properties of the gold nanoparticles decorated SWCNTs nanocomposite were further employed for the determination of D-alanine detection. D-alanine belongs to D-amino acids family. Each amino acid exists in two isomeric forms based on the possibility of forming two different enantiomers around the central carbon atom. The two isomeric forms are known as D- and L-forms analogous to right handed and left handed configurations. L-amino acids are produced in the cell and subsequently incorporated into the proteins. L-amino acid oxidase is used to catalyze the reaction of L-aminoacids, while D-amino acids are converted by the D-amino acid oxidase. D-amino acids (DAAs) are known to have important physiological roles in central nervous system[21] and insulin regulation [22]. Besides this,

71 their concentration is monitored due to the correlation of DAAs with several diseases. Therefore,
72 it is of vital importance to detect concentration of DAAs in biological samples with great
73 precision and accuracy. Various analytical methodologies have been employed to monitor level
74 of DAAs which includes High Performance Liquid Chromatography, Gas Chromatography and
75 electrochemical detection methods. [23] Alternatively, colorimetric methods based on the use of D-
76 amino acids oxidase can be employed for monitoring of DAAs. D-amino acids oxidase oxidizes
77 amino acids into imino acid and H_2O_2 in the presence of oxygen. The peroxidase catalytic
78 oxidation of generated H_2O_2 in the presence of TMB results in the formation of a blue colored
79 product that can be monitored for colorimetric detection of DAAs. Herein, we have proposed a
80 new, simple and sensitive method for the colorimetric determination of DAAs in which the
81 combined catalytic effect of gold NPs and CNTs was used for the quantification of H_2O_2 instead
82 of commonly used natural enzyme. (see eq. 1 & 2).

83 $$\text{D-amino acids} + O_2 \xrightarrow{\text{DAAO}} H_2O_2 + \text{Imino acids} \quad (1)$$

84 $$\text{TMB} + H_2O_2 \xrightarrow{\text{Au NPs / CNTs}} \text{Oxidized TMB} + H_2O \quad (2)$$

85 D-alanine was selected as a model DAA to demonstrate the applicability of proposed
86 nanocomposite as peroxidase mimetic. The proposed method can be very easily extended for the
87 detection of other D-amino acids, as D-amino acids oxidase is a generic enzyme for D-amino
88 acids oxidation. The same chemistry could also be integrated to other H_2O_2 colorimetric
89 detection based sensing methodologies.

90 **2. Experimental**

91 2.1 Chemical and apparatus

D-amino acids oxidase (DAAO), D alanine, 3,3′,5,5′-Tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) solution were obtained from Sigma Aldrich. Chitosan, Single Walled Carbon Nanotubes (SWNC) and acetic acid were also purchased from Sigma Aldrich. Chloroauric acid ($HAuCl_4.3H_2O$ and all other chemicals were purchased from Fisher scientific. Interfering compounds including uric acid, absorbic acid, glycine and glucose were purchased from Sigma. All chemical were of analytical grade and used as received. Working solutions were achieved by serial dilution of the stock solution. All solutions were made using deoinized water. 96 Well Microplates were obtained from Greiner bio-one. Colorimetric measurements were performed with a lab systems Multiskan EX micro titer plate reader. UV/Vis Spectrophotometer (Perkin-Elmer Lambda) was used to characterize the proposed reaction.

2.2 Synthesis of SWCNTs-gold nanocomposite

SWCNTs-gold nanocomposite synthesis was performed with the dissolution of chitosan powder in acetic acid solution with stirring for 1h at room temperature to achieve a completely dispersed solution. Afterwards, 10 mg of SWCNTs were added in 20 mL of chitosan solution, and resulting mixture was sonicated for 2h prior to 10 min of centrifugation to obtain the well dispersed SWCN. Further, 1 mL of 25 mM $HAuCl_4$ was added to the above obtained dispersion under intense stirring for 10 min. The mixture was heated up to 80°C, until the color of the solution was stabilized and did not change[24]. The synthesized SWCNTs-gold nanocompoiste was subsequently employed in the construction of H_2O_2 and D-alanine biosensors to replace the commonly used Horseredish peroxidase (HRP) enzyme.

2.3 Measurement of SWCNTs-gold nanocomposite activity towards H_2O_2

TMB solution was used to determine the reactivity of SWCNTs-gold nanocomposite. Experiments were carried out using 10 μL of nanocomposite in a reaction medium containing H_2O_2 and TMB. The oxidation reaction by nanocompiste was characterized by a blue color product (diimine, one electron oxidation product) with an absorption wavelength of 652 nm. In order to achieve the concentration dependence response, and to determine the nanocomposite sensitivity, H_2O_2 in the range of 0.5 to 25 μM was incubated in the reaction mixture and absorption values were used to draw a calibration curve. Kinetic measurements were carried out by measuring the absorbance at various times, and were subsequently used to obtain the kinetic parameters.

2.4 Bioassay for D-alanine measurement

D-alanine detection was carried out as follows: firstly, 85 μL of DAAO solution and 85 μL of D-alanine solution with varying concentration strength were mixed in the wells of 96 microplates and incubated for a time period of 30 min at room temperature. Then 20 μL of TMB and 10 μL of SWCNTs-gold nanocomposites were successively added to the D-alanine reaction solution. Finally the mixed solution was incubated for a time period of 20 min at room temperature for standard curve measurements. D-alanine contents were determined in fruit Juice samples to demonstrate the applicability of the proposed method for real sample analysis.

3. Results and Discussion

To obtain an insight on the peroxidase like activity of SWCNTs and gold nanocomposite, catalytic oxidation of H_2O_2 in the absence or presence of chromogenic substrate TMB was investigated. SWCNTs and gold particle nanocomposite resulted in excellent catalytic properties for the oxidation of H_2O_2 in the presence of TMB. As can be seen from Fig. 1, the reaction for

the oxidation of TMB did not proceed in the absence of catalysts, demonstrating the suitability of composite for H_2O_2 detection. In the contrary, the presence of SWCNTs and gold nanoparticles composite significantly increased the rates of reaction and a deep blue colored solution was observed with an absorption wavelength of 652 nm (Fig 1). However, the SWCNTs and gold nanocomposite system resulted in negligible color change under same experimental conditions in the presence of TMB. These above findings suggest that SWCNTs and gold particles composite possess peroxidase like activity that can be explored to construct H_2O_2 based biosensors to replace the natural enzyme.

3.1 Optimization of analytical parameters

Like natural enzymes, the catalytic activity of artificial enzymes was also dependent on the amount of nanocomposite, concentration of TMB, H_2O_2 and pH of the reaction mixture. The maximum catalytic activity of the nanocomposite was achieved under following optimal experimental conditions: pH 7.0, room temperature, 10 μL of nanocomposite, 400 μM TMB and 30 mM H_2O_2 (supporting information, Fig S1). These results are in close proximity to the previously described values for other NP-based peroxidase mimetics and HRP. After optimization of these initial parameters, optimal conditions were employed to perform the subsequent assays.

For assessing the catalytic mechanism and acquiring kinetic parameters, the catalytic activity of SWCNTs and gold particles nanocomposites was carried out by enzyme kinetics methodology in the presence of TMB and H_2O_2. Experiments were performed under varying concentration of one substrate and constant concentration of other substrate. Michaelis-Menton curves were obtained for varying concentrations of two substrates (supporting information, Fig 2a and 2b for TMB and H_2O_2 respectively). The kinetic parameters such as maximum initial

158 velocity (Vm) and Michaelis-Menton (Km) were calculated from the Lineweaver-Burk plots and
159 are listed in the table 1. The comparison of kinetic parameters revealed that the Km value of
160 SWCNTs and gold nanocomposite towards H_2O_2 was 64 folds lower than that of SWCNTs and
161 39 times lower as compared to gold nanoparticles. These results provide evidence that a lower
162 concentration of H_2O_2 is needed for nanocomposite as compared to SWCNTs and gold
163 nanoparticles to achieve the maximum catalytic activity. Km value is a representative of the
164 enzyme affinity towards substrate conversion. The decreased Km value is directly related to
165 better catalytic efficiency towards H_2O_2 oxidation, suggesting that SWCNTs and gold particles
166 nanocomposite has more affinity for H_2O_2 as compared to SWCNTs and gold nanoparticles. The
167 enhanced affinity can be related to the improved peroxidase like activity of nanocomposite, and
168 subsequently, this novel material may find wide spread applications in various fields. The
169 enhanced enzyme like activity of gold nanoparticles decorated single walled carbon nanotubes
170 may be attributed to the improved stabilization and dispersion of the nanocomposite in the
171 detection medium. It can be predicted that the electronic structure of SWCNTs is preserved upon
172 gold nanoparticles coating, leading to a synergistic effect. The other phenomena such as Au NPs
173 co-tunneling effects and the Au NPs-induced energy-band modulation of the SWCNTs may also
174 contribute to improve the biomimetric properties of nanocomposite against hydrogen peroxide
175 oxidation[25].
176
177
178
179

Table 1 A comparison of the K_m and V_m values

	K_m [mM]		V_m [10^{-8} MS^{-1}]	
Catalyst	**TMB**	**H_2O_2**	**TMB**	**H_2O_2**
SWCNTs /Gold particle Nanocomposite	0.48	0.65	14.2	5.8
HRP[26]	0.434	3.7	10	8.71
Carbon nanotubes[27]	0.02	41.42	-	-
Gold nanocluster[20]	0.00253	25.3	6.23	7.21

To further evaluate the process of SWCNTs and gold nanocomposite catalysis, experiments were performed over a wide range of TMB and H_2O_2 concentrations. The double reciprocal of velocity against one of the component concentrations were achieved while the concentrations for other substrates were fixed. The catalytic activity of SWCNTs/gold particles nanocomposite was investigated for different concentrations of H_2O_2 under optimal experimental conditions. The absorbance of reaction mixture increased with the increasing concentration of H_2O_2. Similarly, the reaction rate of TMB with H_2O_2 was observed at varying concentration of TMB. As provided in the supporting information (Fig 2c and 2d) , the slopes of the lines are parallel , revealing a ping pong mechanism and indicating the proposed nanocomposite binds and reacts with the first substrate and then releases the first product prior to its reaction with other substrate.

3.2 Assays for detection of hydrogen peroxide and D-Amino acids

Based on the intrinsic and synergic peroxidase like properties of SWCNTs/gold particles nanocomposite, a simple colorimetric method to detect H_2O_2 and D-alanine employing the catalyzed color reaction was designed. As the absorbance of TMB is proportional to the

197 concentration of H_2O_2, it can be a facile approach to quantitatively measure H_2O_2 at 652 nm.
198 Figure 3a represents the calibration curve for varying concentrations of H_2O_2 ranging from 0.5
199 μM to 25 μM. The color variation can be seen as inset of Fig 3a, indicating that this approach
200 can offer a convenient way to monitor H_2O_2 by naked eye with a visual limit of detection of 1.5
201 μM. The analytical parameters including linearity, limits of detection and precision were carried
202 out under optimal experimental conditions. Figures of merit are included in the Supplementary
203 table 1.

204 DAAs detection is of vital importance in the clinical analysis, and generally
205 DAAO is used to catalyze the oxidation of DAAs to produce imino acids and hydrogen peroxide
206 in the presence of oxygen. In the proposed work, SWCNTs/gold particles nanocomposite was
207 used to catalyze H2O2 in the presence of TMB to obtain a blue color product. The color
208 variation/intensity from the converted TMB can be monitored for the indirect measurement of
209 DAA. The obtained results for DAA detection with our nanocomposite are presented in
210 supplementary table 1, while figure 3b presents the calibration curve along with visual inset. The
211 response was linearly proportional to DAA concentration from 0.1 μM to 25 μM, with a
212 detection limit of 0.05 μM. The obtained limit of detection was lower than the LOD of
213 previously reported method for DAA detection [28]. The naked color changes were also obvious to
214 monitor the level of DAA. Furthermore, the specificity of the proposed method was
215 demonstrated against common interfering compounds including uric acid, ascorbic acid, glycine
216 and glucose. As can be seen from the Fig 3, the absorbance of these interfering compounds was
217 not obvious even when they were used at much elevated concentration as compared to DAA.
218 These results show that the proposed nanocomposite based colorimetric method has very good

selectivity for DAA detection, which is attributed to the specificity of DAAO towards DAA catalysis.

In order to demonstrate the applicability of the SWCNTs/gold particles nanocomposite as a peroxidase mimetic, the developed approach was used to detect DAA in the fruit juice samples. The obtained results with recovery values are included in the Table 2. The average recovery values for three DAA spiked concentrations were from 95 % to 98%. Similarly the precision of the method was also presented in the table 2. The relative standard deviation values were obtained for each concentration level. Good recovery values and good precision values for DAA detection based on proposed nanocomposite reveals that the peroxidase like activity based colorimetric approach was useful to reduce the matrix effect of fruit sample. It is obvious that the proposed method may find spread applications in various fields particularly in sensor and biosensor field. In comparison with previously reported nanomaterials based oxidase mimics[18, 29-36], SWCNTs/gold particle nanocomposite has the best analytical characteristics in terms of sensitivity and linear range. The analytical performance of our purposed methods is comparable to the assay based on BSA-stabilized gold nanoparticles oxidase like activity[20]. However, the gold nanoparticles assays suffer from aggregation phenomena, and require specific experimental conditions. Table 3 provides a comparison between the analytical performance of our purposed method and previously reported assays with peroxidase like activity **(Table3).**

241 **Table 2.** Recovery percentages obtained with designed colorimetric assay

DAA added (µmol/L)	DAA found (µmol/L)	R.S.D %	R.E %	R%
0.15	0.143	5	4.66	95.33
5	4.89	3.2	2.2	97.8
12.5	12.1	3.4	3.2	96.8

242 R.S.D % = relative standard deviation percentage; R.E % = relative error percentage; R% = recovery

243 percentage.

244 **Table 3.** A comparison between the analytical performance of our purposed method and

245 previously reported oxidase like mimics towards H_2O_2 detection

Sr No	Nanomaterial	Limit of detection (µmol/L)	Linear range (µmol/L)	Ref
1	Graphene oxide-Fe2O3 magnetic nanocomposite	0.32	1-50	[18]
2	MWCNT-PBin	0.1	1-1500	[29]
3	PtPd nano dendrites supported on graphene nanosheets	0.1	0.5-150	[31]
4	BSA-stabilized Au nanocluster	0.02	0.5 – 20	[20]
5	Au@Pt core/shel nanorods	44	44-1000	[30]
6	Co3O4/rGO nanocomposite	1	1-100	[32]
7	Fe3O4 magnetic nanoparticles	3	5 -100	[33]
8	Chitosan stabilized silver nanoparticles	0.1	5-200	[34]
9	Positively charged gold nanoparticles	0.5	2 -200	[35]
10	Porphyrin-Fe2O3 nanocomposite	1.07	5-80	[36]
11	Gold nanoparticles decorated SWCNTs	0.08	0.5-25	Present work

246

247 **4. Conclusion**

We have reported a new combination of artificial enzyme for colorimetric determination of D amino acids through the catalytic oxidation of H_2O_2. In the proposed method, the synergistic effect of gold nanoparticles and SWCNTs has shown excellent intrinsic peroxidase activity which is much higher than the sum of individual catalytic effect of both nanomaterials. The rate of oxidation of TMB was dependent on time, pH, the concentrations of H_2O_2 and TMB and the catalyst. The method showed good sensitivity, selectivity and linearity for the determination of D-amino acid in the range of 0.1 – 25 μM. The enzyme-like catalysis is proved to be a good competitor of natural enzymes due to robustness and good stability under rigorous experimental reaction conditions. Moreover the assay is simple and cheap, making it suitable and applicable for various applications in different domains.

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338

339 **Figure Captions**

340 **Figure 1.** UV/Visible spectra and color evolution of different reaction systems; (a) H_2O_2 +
341 nanocomposite; (b) TMB + nanocomposite; (c) H_2O_2 + TMB+ nanocomposite

342 **Figure 2.** Steady state kinetic assay of the proposed nanocomposite ; a) TMB concentration was
343 varied under fixed concentration of H_2O_2 and nanocomposite; b) H_2O_2 concentration was varied
344 under same concentration of TMB and H_2O_2; c and d) double reciprocal plot of nanocomposite
345 activity with the concentration of one substrate fixed and the other varied

346 **Figure 3.** The calibration plots for ; a) H_2O_2 and b) D-alanine detection: Inset; images of end
347 colored product under varying concentration of two analytes

348 **Figure 4.** Selectivity analysis for D-alanine detection with following analyte concentration; 5
349 μM D-alanine and 1 mM for the rest of interfering compounds

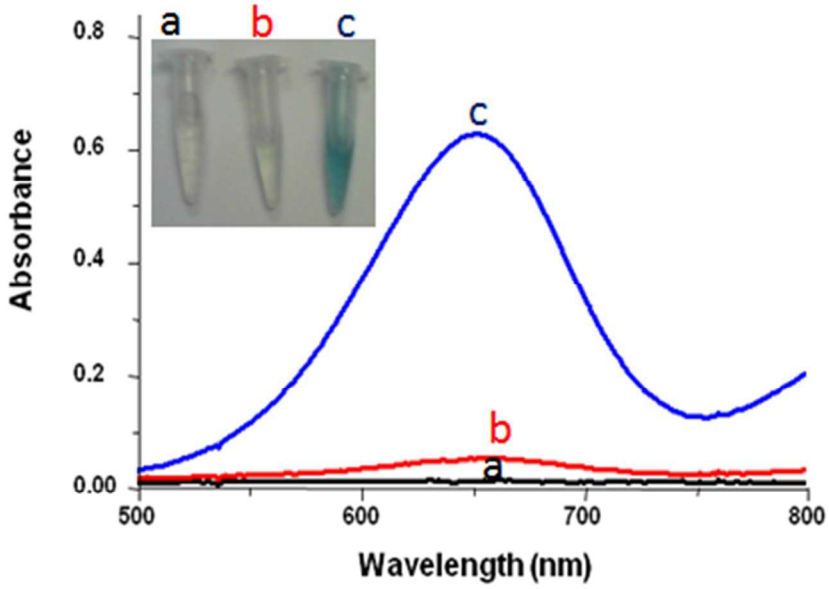

Figure 1

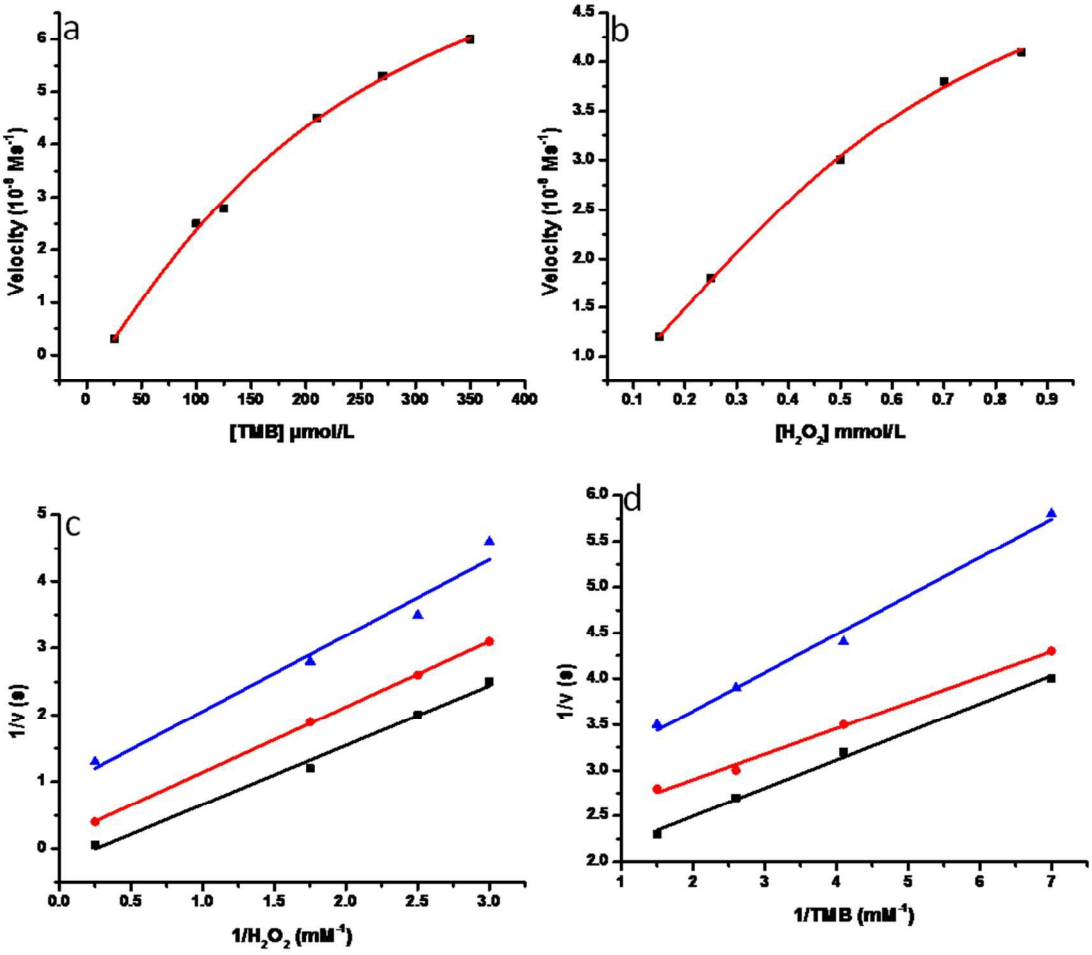

Figure 2

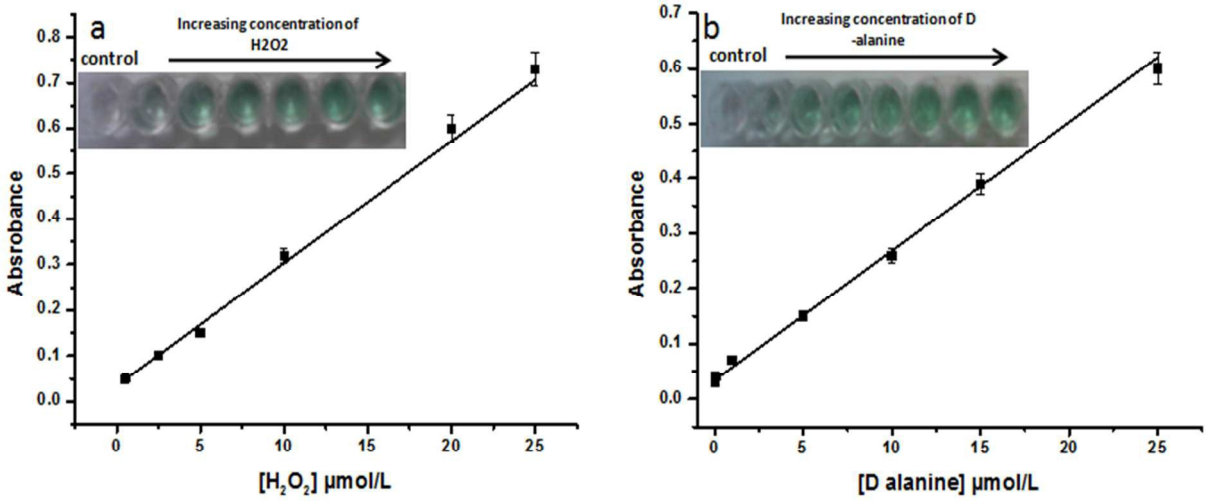

Figure 3

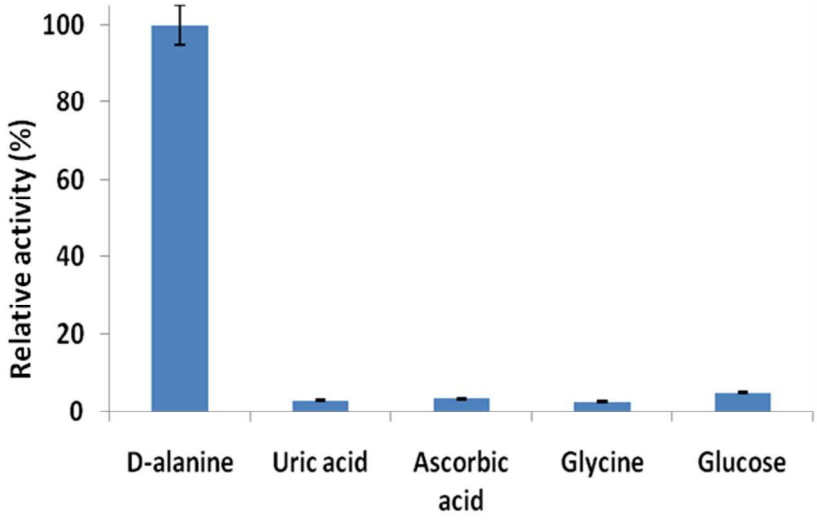

Figure 4