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Colorimetric Measurements of Human Blood Glucose Level in Presence of Nano-Scaled Inorganic Materials

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ABSTRACT

Nowadays, about 415 million people suffer from diabetes throughout the world. So, the diagnosis and control of diabetes through monitoring of blood sugar levels are of most importance. Several nano-scaled inorganic materials, such as iron oxide (Fe_2O_3), cupric sulphide (CuS), CdS and FeS nanoparticles (NPs) have been established as enzyme models i.e., nano-enzyme for measurements of human blood glucose level. These nano-materials behave like horseradish peroxidase enzyme (HRP) which is commonly used at pathological laboratories during blood sample analysis. To test the enzyme-mimic properties, the experiments were carried out through the reaction between per-oxidase substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in the presence of a nano-catalyst, which can be monitored colorimetrically and follow Michaelis–Menten kinetics. Based on this TMB–NPs–H_2O_2 catalysed coloured–reaction, a new analytical method has been explored for identification and quantitative measurement of the concentration of glucose in human blood sample. The probable mechanism of this enzymatic procedure has also been discussed through the detection of hydroxyl radical (OH•). On the basis of the developed methodology, the human blood sugar level can be easily monitored using the attractive nano-enzymes.

Keywords: *Nano-Enzyme; Horseradish Peroxidase; Michaelis–Menten Kinetics; Nonlinear Least Square Fitting; Hydrogen Peroxide; Human Blood Glucose*

Introduction

Diabetes mellitus is a serious disease throughout the world. A higher or lower value of glucose concentration from the normal range (80 to 120 mg/dL or 4.5–6.5 mM) in serum sample is responsible for hyper- and hypo-glycemia disease, correspondingly. So, patients should be cautious to reduce disease-related complications through strict control of blood sugar level, which can be possible through regular diagnosis and monitoring of blood glucose amount (Xu *et al*., 2007 and Badugu; Lakowicz; Geddes, 2004). Again, the persistence of sugar units in human urine samples is similarly more dangerous, because it indicates a serious condition of diabetes. A preliminary screening can easily be done with patients having renal glycosuria which means having high level diabetes (Heller & Feldman, 2008).

Under these circumstances, too many research groups have paid much more attention to developing reliable, cost-effective tools such as titrimetric, spectrophotometric,

electrochemical, etc. (Rivas *et al.*, 2007) for glucose measurement using the horseradish peroxidase (HRP) enzyme (Wang *et al.*, 2000) in the field of medical diagnostics and biotechnology. But one bad luck is that the HRP enzyme holds some drawbacks due to lack of stability, difficult to produce in large quantities and gets easily denatured.

Very recent, Gao *et al.,* (2007) stated that Fe_3O_4 nano-materials show horseradish peroxidase (HRP) enzyme mimetic activity and exposed the entry of different nano-scaled inorganic materials, which have attractive chemical and physical properties, in the bio-medical field.

Keeping this in mind, In the subsequent episode, they have enlarged on details about the enzyme-like activities of our synthesized several nano-scaled inorganic materials and there uses for measurements of human blood glucose level and hydrogen peroxide. How the nano-materials were established as mimic-enzyme by comparing the enzyme kinetic parameters, Michaelis–Menten constant (K_m^{app}) and maximum initial velocity (V_{max}) have been discussed.

Based on these kinetic reactions, this chapter also presented how the calibration curves have been constructed and the blood-sugar level have been measured.

Nano-Scaled Metal Oxides, Metal Sulfides

For colorimetrical measurements of blood-sugar level, the most widely studied nano-scaled inorganic materials include transition-metal oxides, sulfides for instance, iron oxide (Fe_2O_3), cupric sulphide (CuS), CdS and FeS nanoparticles (NPs).

Iron oxide (Fe_2O_3) nanoparticles have been manufactured in laboratories from a trinuclear iron (III) precursor complex through a hydrothermal process using a Teflon PTFE autoclave at 150 °C (Dutta *et al.*, 2012a). The powder X-ray diffraction (XRD) pattern confirmed the formation of the pure maghemite (Fe_2O_3) (JCPDS ID. 39 1346) phase of iron oxide with one major orientation along the (311) plane (Figure 1a). The size and morphology of the nanomaterial has been investigated using transmission electron microscopy (TEM), High-resolution TEM (Figure 1b) which indicate that the sample is composed of well-dispersed hexagonal nanoparticles with an average size about 40 nm.

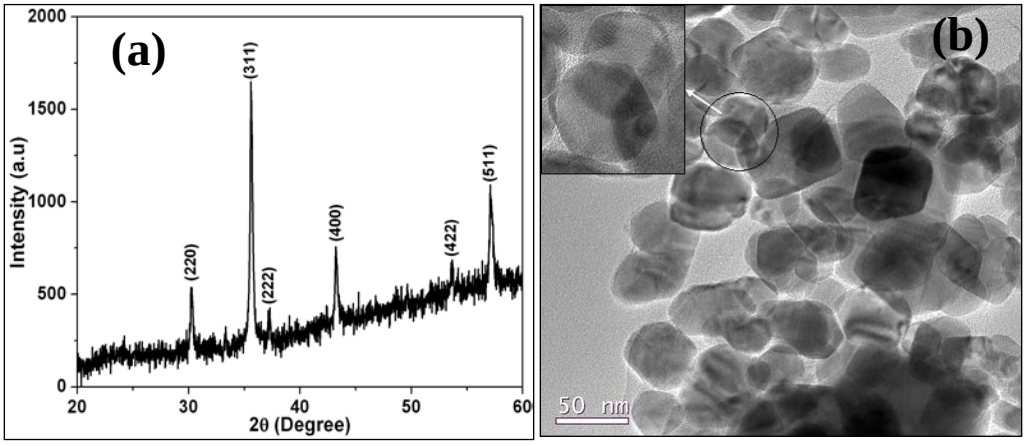


Figure 1: (a) XRD Outline and (b) TEM and HRTEM Pictures (inset) of the Fe_2O_3 NPs (Dutta et al., 2012a)

Another precursor complex $[CuL_2(H_2O)_2]Cl_2$ [HL = pyridine 2-carboxamide] (Dutta *et al.*, 2013) has been used as copper source to prepare cupric sulfide (CuS) nanoparticles via a solvothermal technique using thiourea at pH=8.

CdS NPs (Maji *et al.*, 2012) were synthesized by solvothermal degradation of single-source precursor $[Cd(ACDA)_2]$ using ethylenediamine (EN) or hexadecylamine (HDA) or dimethyl sulfoxide (DMSO) as the solvent at 120 °C.

In another study, FeS NPs (Dutta *et al.*, 2012b) was prepared by dissolution of a trinuclear iron(III) precursor complex in water followed by addition of thiourea, polyvinylpyrrolidone (PVP) and hydrothermal heating in a Teflon PTFE autoclave at 150 °C.

Horseradish Peroxidase–Like Behaviour Measurements

The horseradish peroxidase–like behavior of the proposed nano-scaled inorganic materials was investigated spectro-photometrically through the catalytic reaction of the oxidase substrate 3, 3', 5, 5'–tetramethylbenzidine (TMB) in presence of hydrogen peroxide which generates blue colouration (Figure 2a inset). The reactions were checked by observing the growth of abs (A) value at λ_{max} = 653 nm with time as exposed in Figure 2a. Among the proposed nano-scaled inorganic materials, a comparative performance has been illustrated in Figure 2b and c with iron oxide (Fe_2O_3) and cupric sulfide (CuS) NPs as example and summarized in Table 1.

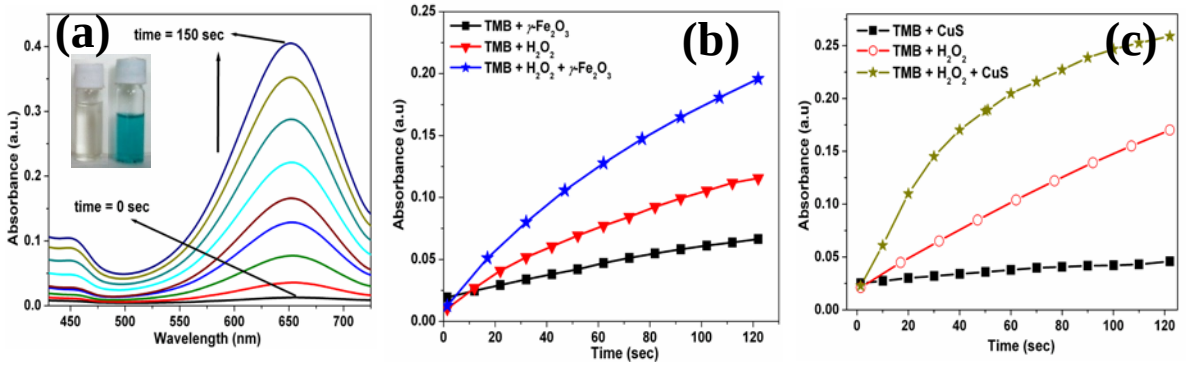


Figure 2: (a) UV–Vis spectral changes of TMB–H_2O_2 system with time, catalyzed by Fe_2O_3 nano-enzyme. Inset: Taking photographs of TMB solutions, before and after the reactions. (b) and (c) absorbance vs. time diagrams of TMB systems using different nano-enzymes under different conditions (Dutta et al., 2013 and Mitra et al., 2014)

To investigate the kinetics of the reaction, the time–stopwatch was started immediately and the absorbance (A) value changing with time was continuously observed at λ_{max} position. The experiments were repeated with fixed amount of H_2O_2 and varying amount of TMB or contrariwise in presence of Fe_2O_3 or CuS nano-enzymes and every-time and the absorbance data were used to calculate kinetic parameters according to Beer–Lambert Law with the help of molar absorption coefficient (ε = 39,100 $M^{-1}cm^{-1}$ at λ_{max} = 653 nm) (Karaseva *et al.*, 2002) of the oxidized product of TMB. It has been found that within the appropriate range of TMB

(Figure 3a, 4a) and H_2O_2 (Figure 3b, 4b) amount, the plot of initial rate vs. TMB or H_2O_2 amount, shows typical Michaelis–Menten like behaviour. i.e.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

E = mimic-enzyme (Fe_2O_3 or CuS NPs), S = substrate (TMB or H_2O_2), ES = enzyme–substrate complex and P = product. The initial rate, $V_0 = k_2[ES]$ can be improved to kinetic equation (2), commonly known as Michaelis–Menten kinetic model.

$$V_0 = \frac{V_{max}[S]}{K_m^{app} + [S]}$$

When the kinetic data were fitted to the above model using nonlinear least squares fitting way, the corresponding parameters (Michaelis–Menten constant) (K_m^{app}) and maximum initial velocity (V_{max}) with TMB as the substrate were acquired. In Table 1, the data have been compared with usually used Horseradish peroxidase (HRP) enzyme and those of previously reported nano-mimetics. All these values can be re-checked from the Lineweaver–Burk (Lineweaver & Burk, 1934) double–reciprocal plot ($1/V_0$ vs. $1/S_0$) (insets of Figure 3 and 4). Actually, Michaelis–Menten constant indicates the enzyme affinity to substrate. Here, a lower value of Michaelis–Menten constant than HRP suggests that the Fe_2O_3 or CuS NPs have greater affinity to TMB. Hence, it has been concluded that the proposed NPs possess inherent peroxidase–like behavior and could be used as artificial peroxidase mimics.

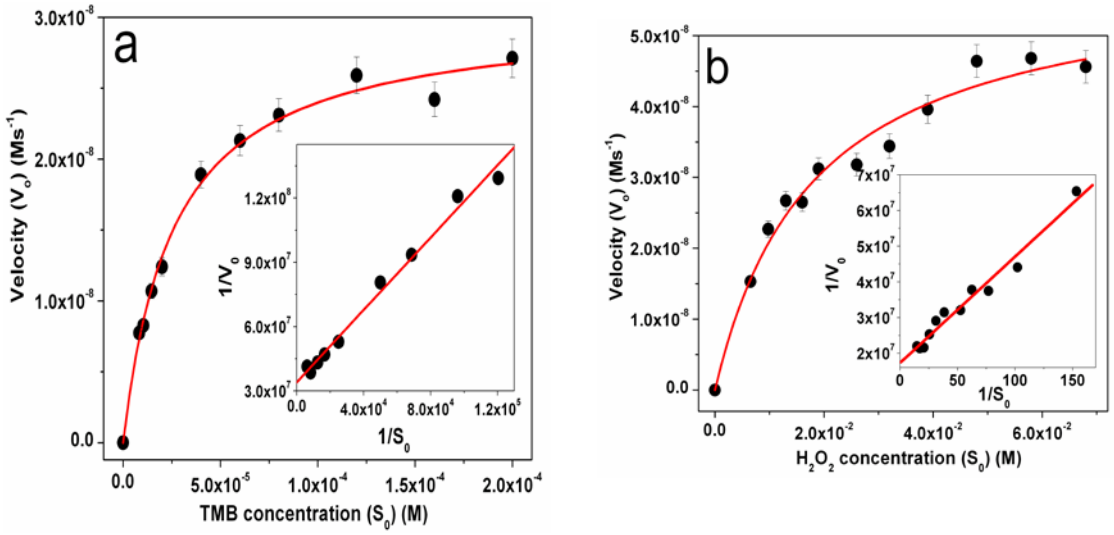


Figure 3: Kinetic analyzed data with the help of the Michaelis-Menten and Lineweaver-Burk model (insets) using γ–Fe_2O_3 NPs (a) changing the amount of TMB with fixed amount of H_2O_2 and (b) changing the amount of H_2O_2 with fixed amount of TMB (Mitra et al., 2014).

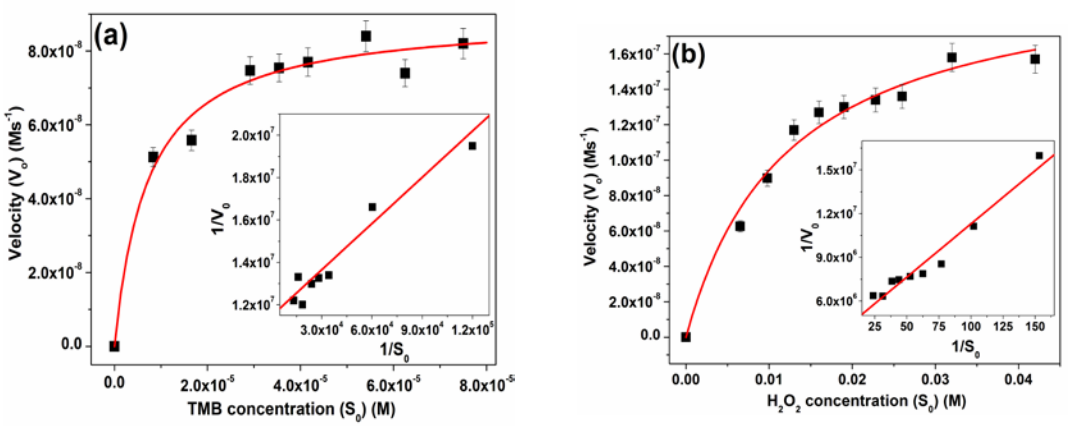


Figure 4: Kinetic analyses plot according to kinetic models via CuS NPs (a) changing the amount of TMB and (b) changing the amount of H_2O_2 (Dutta et al., 2013).

To recognize the mechanism and reason of the enzymatic behavior of the nano-enzymes, Fenton–like operation (He *et al*., 2012) has been proposed and assumed that ferrous ions or cuprous ions, existing at the superficial position of the nano-materials, may react with the substrate with the help of hydrogen peroxide, ensuing in a coloured reaction product.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + OOH^{\bullet} + H^{+}$$

The hydroxyl radical (OH·), produced throughout the reaction sequence, may catalyze the oxidation of the TMB, resulting in a blue color.

Table 1: Assessment of The Kinetic Parameters of The Proposed Nano-Scaled Inorganic Materials Established as Peroxidase-Mimic.

Catalyst	Substrate	K_m^{app} (mM)	V_{max} (M S^{-1})
γ–Fe_2O_3	TMB H_2O_2	0.03 18.0	3.02×10^{-8} 5.9×10^{-8}
CuS	TMB H_2O_2	0.0072 12.0	8.96×10^{-8} 2.09×10^{-7}
HRP	TMB H_2O_2	0.434 3.70	10.00×10^{-8} 8.71×10^{-8}
CdS	TMB H_2O_2	0.0095 3.62	3.57×10^{-8} 5.6×10^{-8}
FeS	TMB H_2O_2	0.0082 9.36	8.70×10^{-8} 1.92×10^{-7}

Colorimetric Glucose Measurements

In this section, the measurement processes of glucose have been demonstrated following the above oxidase–like properties of the CuS or Fe_2O_3 nanoparticles with TMB–H_2O_2 colored-reaction system. Here, a biological enzyme glucose-oxidase (GOx) has been used to oxidize glucose to produce H_2O_2, and that H_2O_2 has been employed in the above TMB–H_2O_2 catalytic reaction in presence of a nano-enzyme instead of external H_2O_2 as follows.

$$\text{Glucose} + O_2 \xrightarrow[\text{pH 7.0}]{\text{GOx}} H_2O_2 + \text{Gluconic acid}$$

$$H_2O_2 + \text{TMB(Colorless)} \xrightarrow[\text{pH 4.0}]{\gamma\text{-}Fe_2O_3\ \text{NPs}} 2H_2O + \text{oxidised TMB(Blue color)}$$

As hydrogen peroxide is the main output of the reaction between glucose oxidase (GOx) and glucose, the amount of H_2O_2 equivalent to amount of oxidized TMB blue color intensity has been employed to measure glucose concentration indirectly. According to Figure 5, a calibration curve has been constructed based on color intensity change i.e., change in abs (A) value at λ_{max} versus glucose concentration in presence of fixed amount of TMB, Gox and nano-enzymes. The response plot is linearly interrelated to glucose concentration from 2-1800 μM using CuS nano-enzyme according to Table 2.

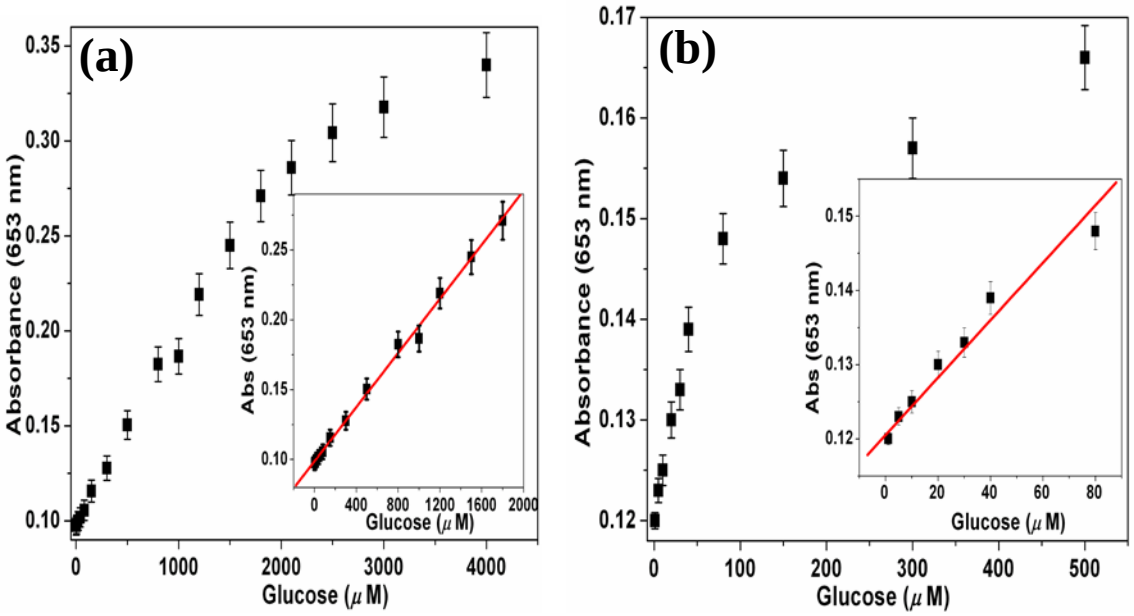


Figure 5: Amount of glucose–response curve using a) GOx/TMB/CuS system and b) GOx/TMB/γ–Fe_2O_3 system. Inset: corresponding linear calibration plot for glucose. (Dutta et al., 2013 and Mitra et al., 2014)

Table 2: Evaluation of Response Parameters towards Glucose Using the Proposed Nano-Scaled Inorganic Materials

Nano-enzyme	Linear range	Detection limit
γ–Fe_2O_3 NPs	1–80 μM	0.21 μM
CuS nanoparticles	2–1800 μM	0.12 μM
Fe_3O_4 nanoparticles	50-1000 μM	30 μM
CdS NPs	18-1100 μM	4 μM
FeS NPs	2-30 μM	0.5 μM

Using the above glucose measurement technique, an attempt has been made to measure glucose concentration in real samples such as blood and excreted urine of healthy human volunteers. In its place of glucose, when the diluted urine and blood solutions have been used with GOx like glucose-GOx system described above, the abs (A) values at λ_{max} = 653 nm were collected. From the calibration curves, the amount of glucose in the above blood and urine are calculated which matched well with pathological lab report (Table 3).

Table 3: Measurements of Sugar Level in Real Samples using γ –Fe2O3 Nano-Enzyme (Dutta et al., 2013)

Sample	Pathological Lab report	Colorimetric measurements
Blood A	5.93 mM (106.0 mg/dl) (GOD-POD process)	6.20 mM (111.5 mg/dl)
Blood B	5.15 mM (92.0 mg/dl) (Hexokinase way)	5.37 mM (96.5 mg/dl)
Urine A	4.90 mM (89.0 mg/dl) (Hexokinase way)	4.73 mM (85.0 mg/dl)
Urine B	5.22 mM (94.0 mg/dl) (Hexokinase way)	5.06 mM (91.0 mg/dl)

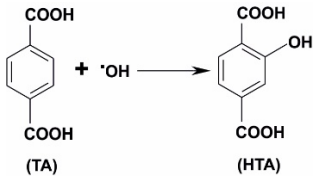
The above glucose reaction may also occur through the production of hydroxyl radicals from the decomposition of hydrogen peroxide similar to the enzyme like activity.

$$O_2 + \text{Glucose} \xrightarrow{\text{GOx}} H_2O_2 + \text{Gluconic acid}$$

$$H_2O_2 \xrightarrow{\text{CuS}} OH\cdot$$

$$\text{TMB (colorless)} + OH\cdot \xrightarrow{\text{CuS}} \text{oxidised TMB (blue)} + H_2O$$

The generation of OH· has been recognized by terephthalic acid (TA) photoluminescence analytical systems (Barreto *et al*., 1994) where TA combined with hydroxyl radicals and formed 2–hydroxy terephthalic acid (HTA) as stated by following equation.



Conclusion

In summary, the proposed nano-scaled inorganic materials are widely explored as an peroxidase–mimic like HRP, which exhibits effective peroxidase–like properties towards conversion of peroxidase substrates TMB with the help of H_2O_2 producing a blue-coloured solution. They show various advantages such as low–cost, easy to synthesis, non–toxic, high catalytic efficiency, excellent steady to biodegradation, and not being liable to denaturation. Based on this TMB–NPs–H_2O_2 catalysed colour–reaction, new analytical platform has been established for glucose identification. Human blood glucose and urine glucose levels have also been measured successfully with the help of glucose oxidase (GOx) and a proposed nano-scaled mimic enzyme. All these studies suggest that the proposed nano-scaled inorganic materials are attractive materials which will help their application in the biomedical and bioengineering fields.

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