

## Manipulation of Fe/Au Peroxidase-Like Activity for Development of a Nanocatalytic-Based Assay

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**Abstract:** *Nanoparticles have been discovered to have intrinsic peroxidase-like catalytic activity that shows beneficial applications in a biosensor. The aim of this study is to investigate the synthesised Fe/Au nanoparticles' peroxidase-like activity and further evaluate them for development of a nanocatalytic-based assay specifically designed to detect 17 $\beta$ -estradiol in water. The peroxidase-like activity of the synthesised Fe/Au nanoparticles was optimised using the H<sub>2</sub>O<sub>2</sub>-ABTS system and was characterised using Michaelis-Menten kinetics. Then, the nanoparticles surface was functionalised with aptamers for specific conjugation with the target analyte, 17 $\beta$ -estradiol. The feasibility of this assay was tested at different concentration of aptamer-tagged Fe/Au nanoparticles and 17 $\beta$ -estradiol. Also, assessment of this assay was conducted with potentially interfering materials and spiked real tap water samples. Results obtained from absorbance data reveal that the Fe/Au-17 $\beta$ -estradiol complex significantly hampered the peroxidase-like catalytic activity of the nanoparticles. The absorbance intensity declined drastically after aptamer-tagged nanoparticles (Fe/Au-fl-apt) "captured" the targets and formed nanoparticles-analytes complexes. This assay showed good accuracy and reproducibility for detection of 17 $\beta$ -estradiol concentration ranging from 3 to 272 ng/L. Furthermore, the aptamers used in this study were very selective towards the target analyte and related compounds showed little to no interference. Thus, a simple, rapid and sensitive detection assay, specific for 17 $\beta$ -estradiol was developed using a new detection strategy by manipulation of nanoparticles' peroxidase-like activity.*

**Keywords:** Fe/Au nanoparticles, peroxidase-like activity, nanocatalytic-based assay, detection of 17 $\beta$ -estradiol

## 1. INTRODUCTION

Recently, many nanoparticles have been reported to show enzyme mimetic activity such as magnetite,<sup>1</sup> Au nanoparticles,<sup>2</sup> Fe<sub>3</sub>O<sub>4</sub>-Au<sup>3</sup> and Ag<sub>3</sub>PO<sub>4</sub>.<sup>4</sup> These enzyme-like nanoparticles display a beneficial property as they could potentially replace peroxidase in various applications, including hydrogen peroxide dependent systems.

Besides the growing interest in nanoparticles' catalytic property, nanoparticles have long been known for their excellent ability to conjugate with biomolecules such as enzymes, DNAzymes, antibodies and aptamers. These biomolecules can be immobilised on the modified nanoparticle surfaces by direct conjugation to the surface of some nanoparticles and to surface-bound stabilising ligands or coatings, either directly or using small cross-linking molecules and other intermediaries.<sup>5</sup> Aptamers are short synthetic oligonucleotides that could be chemically synthesised for any targets and show many advantages, for examples easy to modify, high stability and cost effective.<sup>6,7</sup>

Analytical assays that utilise a hydrogen peroxide dependent system by catalysing the oxidation of certain substrates have been extensively used for numerous applications. This analytical assay has become a powerful detection tool especially in immunoassay studies. Recently, replacement of horseradish peroxidase with nanoparticles as a peroxidase mimetic has demonstrated improvement in biosensors. Zhang's group presented work with  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanoparticles modified by Prussian blue (PBMNPs) that were further conjugated with staphylococcal protein A (SPA) and showed a potential application in bio-detection. Here, the PBMNPs served as an inexpensive horseradish peroxidase, HRP.<sup>8</sup> A recent study by Liu et al.<sup>9</sup> reported that the incorporation of casein on magnetic nanoparticles helped to improve the affinity towards both H<sub>2</sub>O<sub>2</sub> and 3,3',5,5'- tetramethylbenzidine (TMB) thus resulting in a simple, inexpensive, highly sensitive and selective method for glucose detection.

While prior work has demonstrated the capability of nanoparticles to conjugate with the target analytes and has utilised nanoparticles' peroxidase-like activity for an analytical assay, we observed that a similar strategy could be adapted for development of a nanocatalytic-based assay. It has been reported that peroxidase-like activity of iron oxide nanoparticles is dependent on the surface attributes of the nanoparticles.<sup>10</sup> Therefore, in this study we report on a detection strategy formulated by the nanoparticles capability to conjugate with analytes and then the nanoparticles-analytes catalytic activity was evaluated using a hydrogen peroxide dependent system. We envisaged that the conjugation of target analytes to the Fe/Au nanoparticles would hinder the peroxidase activity resulting in an assay response that was inversely proportional to the analyte concentration. This

is based on the fact that the formation of this complex would create a gap with substrate molecules and lower the nanoparticles catalytic activity by reducing the affinity of nanoparticles for substrate molecules.

To evaluate the feasibility of this assay, the synthesised nanoalloy, Fe/Au nanoparticles were initially assessed for their peroxidase-like activity using the hydrogen peroxide dependent system, H<sub>2</sub>O<sub>2</sub>-ABTS. A specifically designed aptamer was attached to the Fe/Au nanoparticles to confer specific conjugation abilities for the target analyte, 17 $\beta$ -estradiol. It is known as an endocrine disrupting chemical (EDCs) that has the greatest estrogenic activity.<sup>11</sup> When 17 $\beta$ -estradiol was successfully "captured" by aptamer-tagged nanoparticles and formed Fe/Au-17 $\beta$ -estradiol complex, the catalytic activity of this complex was measured using the hydrogen peroxide dependent system.

This is the first work reported, to our knowledge, to use this approach for development of such a detection assay. Hence, it is hoped that this study would initiate a comprehensive research exploration of nanoparticles' peroxidase-like activity in nanosensor systems and further develop a simple, reliable and sensitive detection assay.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals

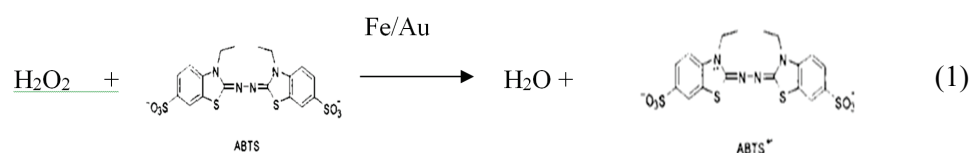
Chemical reagents used in this experiment were ferrous sulfate heptahydrate (FeSO<sub>4</sub>.7H<sub>2</sub>, ReagentPlus®, > 99%), hydrogen tetrachloro-aurate (III) (HAuCl<sub>4</sub>.3H<sub>2</sub>O,  $\geq$  99.9%), sodium hydroxide (NaOH, ACS Reagent,  $\geq$  97%), trisodium citrate (Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>, anhydrous,  $\geq$  98%, GC), nitric acid (HNO<sub>3</sub>, ACS Reagent 70%) diluted to 65%, phosphate buffered saline (PBS) buffer (tablet), Tween 20 (viscous liquid), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS, 10 mg/tablet), 30% H<sub>2</sub>O<sub>2</sub>, PBS buffer (tablet), Tween 20 (viscous liquid), sodium acetate anhydrous (NaAc), MES buffer ( $\geq$  99.5%), 17 $\beta$ -estradiol (98%), estriol ( $\geq$  97%), nonylphenol (99.8%) and carbaryl (99.8%). These chemical reagents were purchased from Sigma Aldrich. Thiolated aptamer (SH-apt) was adapted from Wang, Wang and Dong<sup>12</sup> with slight modification and was synthesised at Alpha DNA (Montreal, Quebec) and the probe aptamer (fl-apt) was adapted from Kim et al.<sup>13</sup> with slight modification and was synthesised from Eurofins MWG Operon LLC (Huntsville, AL). All reagents were analytical grade and were used as received without further purification.

## 2.2 Preparation of Fe/Au Nanoparticles

The formation of Fe/Au nanoparticles is based on electrostatic interaction between positively charge  $\gamma\text{-Fe}_2\text{O}_3$  as in Nurdin et al.<sup>14</sup> and negatively charge citrate-capped AuNPs. Initially, they were synthesised separately.  $\gamma\text{-Fe}_2\text{O}_3$  were synthesised using a reverse co-precipitation method adapted from Mahmed et al.<sup>15</sup> with slight modification and then were treated with  $\text{HNO}_3$  (6 mol). AuNPs were synthesised using the Turkevich method by mixing 500  $\mu\text{L}$   $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$  (10 mM) in a solution containing 300  $\mu\text{L}$   $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$  (100 mM) and 10 mL deionised water. Then, 12 mg/mL of  $\gamma\text{-Fe}_2\text{O}_3$  were mixed with 2.5 mL citrate-capped AuNPs for 1 h. Then, the nanoparticles were separated by a permanent magnet overnight. The separated Fe/Au nanoparticles were stabilised with 3.5 mL of PBS-T (10 mM, pH 4) by vigorous mixing for 1 h. Subsequently, the solution was centrifuged for 10 min at  $6.5 \times 1000$  rpm and the Fe/Au nanoparticles were separated using a permanent magnet and washed several times with PBS-T and stored at  $4^\circ\text{C}$  until use.

## 2.3 Measurement of Fe/Au Peroxidase-Like Activity

The prepared Fe/Au nanoparticles weighed to approximately 50 mg and were added into 1 mL PBS buffer (10 mM, pH 4). The extent of reaction was indicated by a green colour development and was measured by absorbance at 414 nm based on the presence of the oxidised ABTS ( $\text{ABTS}_{\text{ox}}$ ) in the solution, using microplate reader (Multiskan Ascent, Labsystems). To calculate the amount of absorption that is contributed by the Fe/Au catalytic reaction, the obtained absorbance needed to be subtracted from the control absorbance. The catalytic reaction by Fe/Au is as follows:



## 2.4 Steady-State Kinetic Analysis

The kinetic analysis of Fe/Au and  $\gamma\text{-Fe}_2\text{O}_3$  nanoparticles with ABTS as the substrate was performed by adding 10  $\mu\text{L}$  of nanoparticles aliquot, 10  $\mu\text{L}$  of  $\text{H}_2\text{O}_2$  (100 mM) and different concentrations (0.9, 1.8, 3.6 and 4.5 mM) of ABTS reagent. For kinetic analysis with  $\text{H}_2\text{O}_2$  as the substrate, 10  $\mu\text{L}$  of nanoparticles aliquot, 30  $\mu\text{L}$  of ABTS (18.2 mM) and different concentrations (0.5, 1.5, 2.5 and 5.0 mM) of  $\text{H}_2\text{O}_2$  were used. Both kinetic analyses were performed in 165  $\mu\text{L}$

reaction buffer (10 mM NaAc, pH 5). The green colour developed as the reactions proceeded was monitored kinetically in a 96-well microplate at room temperature for 300 sec with 60 sec of time interval. The Michaelis-Menten constant was estimated based on a Hanes-Woolf plot:

$$\frac{[S]}{v} = \frac{[S]}{V_{max}} + \frac{K_m}{V_{max}} \quad (2)$$

where  $v$  is the initial velocity,  $V_{max}$  is the maximal reaction velocity,  $[S]$  is the concentration of substrate and  $K_m$  is Michaelis constant for the particular enzyme being investigated.

## 2.5 Development of Nanocatalytic-Based Assay

### 2.5.1 Surface functionalisation with aptamer

Fe/Au nanoparticles were weighed to approximately 50 mg and were added to 1 mL MES buffer (10 mM, pH 4). Subsequently, 5  $\mu$ mol of the thiolated aptamer (SH-apt) was added to the solution and this was incubated by mixing at room temperature for 1 h. To remove the unbound SH-apt, the solution was centrifuged for 5 min and was washed twice with PBS-T, pH 4 and was further separated using a permanent magnet. 1 mL MES buffer, pH 7 was added to the Fe/Au-SH-apt nanoparticles. The next step of this strategy was to attach the probe aptamer (fl-apt) to the Fe/Au-SH-apt nanoparticles, where 5  $\mu$ mol of fl-apt was added to the solution. The interaction was allowed at room temperature for 1 h. To remove the unbound fl-apt, the solution was centrifuged for 10 min and was washed twice with PBS-T, pH 4 and was separated using permanent magnet. Finally, 1 mL PBS buffer, pH 4 was added to the aptamer-tagged nanoparticles (Fe/Au-fl apt) and stored at 4°C until use. The presence of bound aptamers on the nanoparticles surface was evaluated using a UV-VIS spectrophotometer (Diode Array spectrophotometer, HP) based on the presence of fluorescent molecules, cyanine dyes (Cy5.5). Two types of aptamers used in this study are SH-apt (SH-C6-TCTCTTGGACCC) and fl-apt (AGAGAACCTGGG-GCT-TCC-AGC-TTA-TTG-AAT-TAC-ACG-CAG-AGG-GTA-GCG-GCT-CTG-CGCATT-CAA-TTG-CTG-CGC-GCT-GAA-GCG-CGG-AAG-C-(Cy5.5)).

### 2.5.2 Assessment of nanocatalytic-based assay

10  $\mu$ L of the prepared Fe/Au-fl-apt nanoparticles (approximately 50 mg) was incubated with 100  $\mu$ L of 17 $\beta$ -estradiol (100 nmol) and 890  $\mu$ L PBS buffer (10 mM, pH 4) at room temperature for 1 h to allow Fe/Au-17 $\beta$ -estradiol complex formation. To remove the unbound 17 $\beta$ -estradiol, the solution was

centrifuged for 10 min and was washed twice with PBS-T, pH 4, and was further separated using a permanent magnet. Then, the nanoparticles-analytes catalytic activity was measured by adding 50  $\mu\text{L}$  of Fe/Au-17 $\beta$ -estradiol aliquot, 10  $\mu\text{L}$  of  $\text{H}_2\text{O}_2$  (100 mM), 30 $\mu\text{L}$  of ABTS reagent (18.2 mM), and 110  $\mu\text{L}$  reaction buffer (10 mM NaAc, pH 5) in 96-wells microplate. The absorbance signal at 414 nm was measured using the microplate reader after 5 min of interaction. High absorbance intensity indicated that a strong catalytic activity was present.

Further assessment was done by testing a serial dilution of 17 $\beta$ -estradiol (0.01, 0.1, 1.0 and 10 nmol) at different Fe/Au-fl-apt nanoparticles concentrations (33, 17 and 2.5 mg/mL) in PBS buffer (10 mM, pH 4) with total volume of 1 mL. To remove the unbound 17 $\beta$ -estradiol, the solution was centrifuged for 10 min and was washed twice with PBS-T, pH 4, and was further separated using a permanent magnet. Then, the nanoparticles-analytes catalytic activity was measured as discuss previously.

In this study, the experimental assay was measured in triplicate ( $n = 3$ ) for inter- assay and two assays run ( $n = 2$ ) for determination of intra-assay variability. To assess the selectivity of this assay, a cross-reactivity study was conducted with potentially interfering materials such as estriol, nonylphenol (4NNP) and carbaryl and was evaluated at 100 nmol concentrations for each EDCs. To further test this assay in real water sample, different concentration of 17 $\beta$ -estradiol (0.1, 1 and 10 nmol) was added into filtered tap water (F) and non-filtered tap water (NF). Then, 10  $\mu\text{L}$  prepared Fe/Au-fl-apt nanoparticles (approximately 50 mg) was added into the contaminant solution with total volume of 1 mL. The detection was conducted at room temperature for 1 h. To remove the unbound 17 $\beta$ -estradiol, the solution was centrifuged for 10 min and was washed twice with PBS-T, pH 4, and was further separated using a permanent magnet. Then, the nanoparticles-analytes catalytic activity was measured and detection efficiency was calculated as follows:

$$(A_0 - A)/A_0 \quad (3)$$

whereas  $A_0$  and  $A$  are absorbance intensity at 414 nm before and after various concentration of 17 $\beta$ -estradiol are added in tap water samples.

### 2.5.3 Characterisation

For morphology and heterostructural analysis, the nanoparticle samples were sent to the Canadian Centre for Electron Microscopy (CCEM), McMaster University, where high-resolution transmission electron microscopy (HRTEM, JEOL) was used at an accelerating voltage of 200 kV, and the Energy Dispersive X-Ray Analysis (EDX) technique was used to identify the elemental composition

of the materials. Zeta potential was employed using a Malvern Zetasizer to obtain information on the nanoparticles surface charge.

### 3. RESULTS AND DISCUSSION

#### 3.1 Analysis of Fe/Au Peroxidase-Like Activity

Fe/Au nanoparticles are prepared by a strong electrostatic interaction between  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> and AuNPs that subsequently formed a metastable heterogeneous structure as characterised using TEM and EDX (S1). Due to the fact that both nanoparticles ( $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> and AuNPs) exhibit a good peroxidase-like activity, it is of interest to investigate the catalytic activity of the synthesised Fe/Au nanoparticles. The catalysis performance of the synthesised nanoparticles was examined using ABTS, a non-carcinogenic chromogenic substrate and is oxidised in the presence of peroxidase and H<sub>2</sub>O<sub>2</sub> to a green reaction product with maximum absorbance at approximately 414 nm.

The optimum pH value for nanoparticles' catalytic activity was found at pH 5 and a linear absorbance of Fe/Au concentration (0.995) was obtained (S2). We also observed that increase of nanoparticles concentration also contribute to nanoparticles aggregation and we can speculate that due to the increase of nanoparticles-substrate interaction that lead to surface modification, the nanoparticle tends to aggregate since Fe/Au shows metastable structure.

#### 3.2 Steady-State Kinetic Analysis

The steady-state reaction rates at different concentrations of substrate and co-substrate (ABTS and H<sub>2</sub>O<sub>2</sub>) were obtained by calculating the slopes of initial absorbance changes with time. The reaction was determined in 10 mM NaAc buffer, pH 5 at room temperature for 300 sec. Absorbance data were back-calculated to concentration by the Beer–Lambert Law using a molar absorption coefficient of 36,000 mol<sup>-1</sup> cm<sup>-1</sup> for ABTS-derived oxidation products at 414 nm.<sup>16</sup> As a comparison, the steady state kinetics for  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanoparticles were also examined to investigate the surface modification impact on catalytic activity.

The reaction catalysed by Fe/Au and  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanoparticles displayed Michaelis–Menten kinetics (S3). The Michaelis constant ( $K_m$ ) and maximal reaction velocity ( $V_{max}$ ) were obtained from the slope and intercept of the extrapolated straight line with the horizontal axis in the Hanes-Woolf plot (S4). A high  $K_m$  value represents a weak affinity whereas a low  $K_m$  value suggests high affinity.<sup>17</sup>  $K_m$  value of Fe/Au with H<sub>2</sub>O<sub>2</sub> as the substrate was significantly lower than  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> (Table 1) shows that Fe/Au nanoparticles have a strong affinity

towards  $\text{H}_2\text{O}_2$ . In addition, the corresponding  $K_m$  values of the Fe/Au nanoparticles also indicate that the catalytic reaction can reach the maximum rate at a lower concentration of  $\text{H}_2\text{O}_2$ . According to Voinov et al.,<sup>17</sup>  $\text{Fe}_3\text{O}_4$  nanoparticles were significantly more effective in producing hydroxyl radicals ( $\cdot\text{OH}$ ) than the  $\gamma\text{-Fe}_2\text{O}_3$  nanoparticles at the same ratio of the nanoparticle total surface and reaction volume. The peroxidase-like activity mostly originates from ferric ions which have a low rate constant and is thus a rate-limited reaction process.<sup>8</sup>

Study conducted by Sun et al.<sup>3</sup> demonstrated that  $\text{Fe}_3\text{O}_4$ -Au nanocomposites exhibited better catalytic activity than pure  $\text{Fe}_3\text{O}_4$  resulting from the specific electronic structure at the nanoparticles interfaces. They proposed that the AuNPs changed the electron structure at the interface, which may accelerate the electron transfer. Moreover, the partial electron transfer from  $\text{Fe}_3\text{O}_4$  to Au facilitates  $\text{H}_2\text{O}_2$  adsorption and activation. Hence, it is most likely the synthesised  $\gamma\text{-Fe}_2\text{O}_3$  catalytic activity in this study was enhanced upon surface modification with AuNPs as proven by the strong affinity of Fe/Au nanoparticles towards  $\text{H}_2\text{O}_2$ .

Conversely, the  $K_m$  value for Fe/Au nanoparticles with ABTS as the substrate was slightly higher than  $\gamma\text{-Fe}_2\text{O}_3$ , suggesting that the Fe/Au nanoparticles had a slightly lower affinity for ABTS. The plausible reason for this phenomenon is due to the difference in charges present on the Fe/Au and  $\gamma\text{-Fe}_2\text{O}_3$  nanoparticles' surface. Results obtained from zeta potential measurements showed that the  $\gamma\text{-Fe}_2\text{O}_3$  nanoparticles have a positive surface charge (+17.80 mV) while Fe/Au nanoparticles have a negative surface charge (-24.70 mV) (S5). It is known that ABTS contains two negatively charged sulfonic groups, thus, exhibiting higher affinity toward a positively charged nanoparticles surface.<sup>10</sup> However, the effect of charge difference was relatively small because of the slight difference in  $K_m$  values, and suggested that the surface modification by Au deposition help to enhance the interaction between nanoparticles and ABTS.

Therefore, the intrinsic peroxidase-like activity of Fe/Au nanoparticles is largely influenced by surface modification. From this information, we could utilised the nanoparticles surface modification flexibility and manipulate the catalytic property to develop an analytical assay. Development of the detection assay is further discussed in the next subsection.



Table 1: Comparison of the kinetic parameters of  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> and Fe/Au nanoparticles.

Nanoparticles	Substrate	$K_m$ (mM)	$V_{max}$ (mols <sup>-1</sup> )
$\gamma$ -Fe <sub>2</sub> O <sub>3</sub>	H <sub>2</sub> O <sub>2</sub>	0.137	$2.892 \times 10^{-7}$
	ABTS	0.674	$9.400 \times 10^{-8}$
Fe/Au	H <sub>2</sub> O <sub>2</sub>	0.021	$5.935 \times 10^{-8}$
	ABTS	1.019	$9.226 \times 10^{-8}$

### 3.3 Development of Nanocatalytic-Based Assay

#### 3.3.1 Analysis of aptamer-mediated conjugation

The probe aptamer that contained a fluorescent dye-labeled 17 $\beta$ -estradiol (fl-apt) was easily attached at the SH-apt complementary site by interaction between the nucleobases. After the nanoparticles were successfully functionalised with the probe aptamer, Fe/Au-fl-apt would be able to "capture" the 17 $\beta$ -estradiol molecules in solution by conjugation at site-specific sequence, and subsequently form Fe/Au-17 $\beta$ -estradiol complexes (S6).

The SH-apt would covalently attach to the Fe/Au nanoparticles based on the Au-SH chemisorption interaction where the sulfur atom of a thiol contributes a lone pair of electrons to the empty orbitals of Au atoms at an interface.<sup>5</sup> Another advantage of introducing a thiolated-aptamer is to avoid aptamer desorption from the nanoparticle's surface and loss the nanoparticle functionality. On the other hand, the attachment of the fl-apt would facilitate the conjugation process between nanoparticles and 17 $\beta$ -estradiol molecules due to the presence of 17 $\beta$ -estradiol site-specific sequence. The attachment of the fl-apt to thiolated-tagged nanoparticles can be determined using cyanine dye, Cy5.5. This dye excites at 675 nm and emits at 695 nm.<sup>18</sup> The fluorescence emission intensity at 695 nm was measured from the visible light absorption spectrum. We could observe a broad band ranging from 600 to 700 nm suggesting that the fl-apt was successfully attached to the thiolated-tagged nanoparticles and no obvious peak was observed for Fe/Au nanoparticles (S7). The possible reason for its low absorbance intensity might be due to the quenching effect of Fe/Au nanoparticles, as both Fe and Au nanoparticles can be efficient fluorescence quenchers as demonstrated by many analytical analysis studies.<sup>19,20</sup>

#### 3.3.2 Detection of 17 $\beta$ -estradiol

A simple nanocatalytic-based assay to detect 17 $\beta$ -estradiol was devised as illustrated in Figure 1. In general, this assay requires two simple steps, immobilisation and catalytic reaction process. The immobilisation process is based on the conjugation of aptamer-tagged nanoparticles with 17 $\beta$ -estradiol. The

bound  $17\beta$ -estradiol formed a complex at the nanoparticle surface and this was easily separated from the solution using a permanent magnet. Then, the catalytic property of Fe/Au- $17\beta$ -estradiol was measured using the  $H_2O_2$ -ABTS system in a 96-well microplate. The absorbance signal was measured at 414 nm as an indicator that oxidised ABTS was present in the solution.

From result in Figure 2, we can observe that a slight difference could be found after Fe/Au nanoparticles were functionalised with probe aptamer (Fe/Au-fl-apt). The effect of surface functionalisation with probe aptamer is negligible due to its low impact on the nanoparticles catalytic activity. Inversely, the absorbance intensity was significantly decreased after nanoparticles-analytes complex was formed. Low absorbance intensity indicates that weak catalytic activity is involved. Thus, it clearly shows that the peroxidase activity of Fe/Au nanoparticles is disrupted or hindered by Fe/Au- $17\beta$ -estradiol complex formation. This forms the basis of the assay, where a reduced signal should be proportional to an increase in  $17\beta$ -estradiol concentration, and vice-versa.

To further evaluate the feasibility of this assay, a serial dilution of  $17\beta$ -estradiol (0.01, 0.1, 1.0 and 10 nmol) was tested at different concentrations of aptamer-tagged nanoparticles (33, 17 and 2.5 mg/mL). As expected, the absorbance intensity was inversely proportional to the  $17\beta$ -estradiol concentrations (Figure 3). Therefore, we could suggest that steric hindrance effects are most likely contributing to the disruption of nanoparticles catalytic activity. According to the literature, for small-molecules targets like  $17\beta$ -estradiol, aptamers often form a cage surrounding the ligand.<sup>21,22</sup> Thus, it is speculated that when aptamer-tagged nanoparticles "captured"  $17\beta$ -estradiol, the aptamer became folded and formed a cage surrounding the target. Assuming many cages were formed at the Fe/Au nanoparticles surface resulting in molecular crowding, its effect on the nanoparticles' catalytic activity is substantial. A study by Pitulice et al.<sup>23</sup> revealed that the volume occupied by the crowding agent has a significant impact on the rate of ABTS reaction by  $H_2O_2$  and catalysed by HRP. The  $V_{max}$  and  $K_m$  of the Michaelis-Menten plot decay along with the growth of obstacle concentration.

It is known that interaction between the nanoparticles and substrate is important to ensure the catalytic process can occur, similar to enzyme-substrate interaction principals. The intermolecular steric hindrance considerably affects the catalytic activity of nanoparticles because the active centre (catalytic site) is less accessible to the substrate molecules. Since the substrates are unable to move into close proximity, interaction between nanoparticles and substrate molecules is interrupted causing weak apparent catalytic activity.

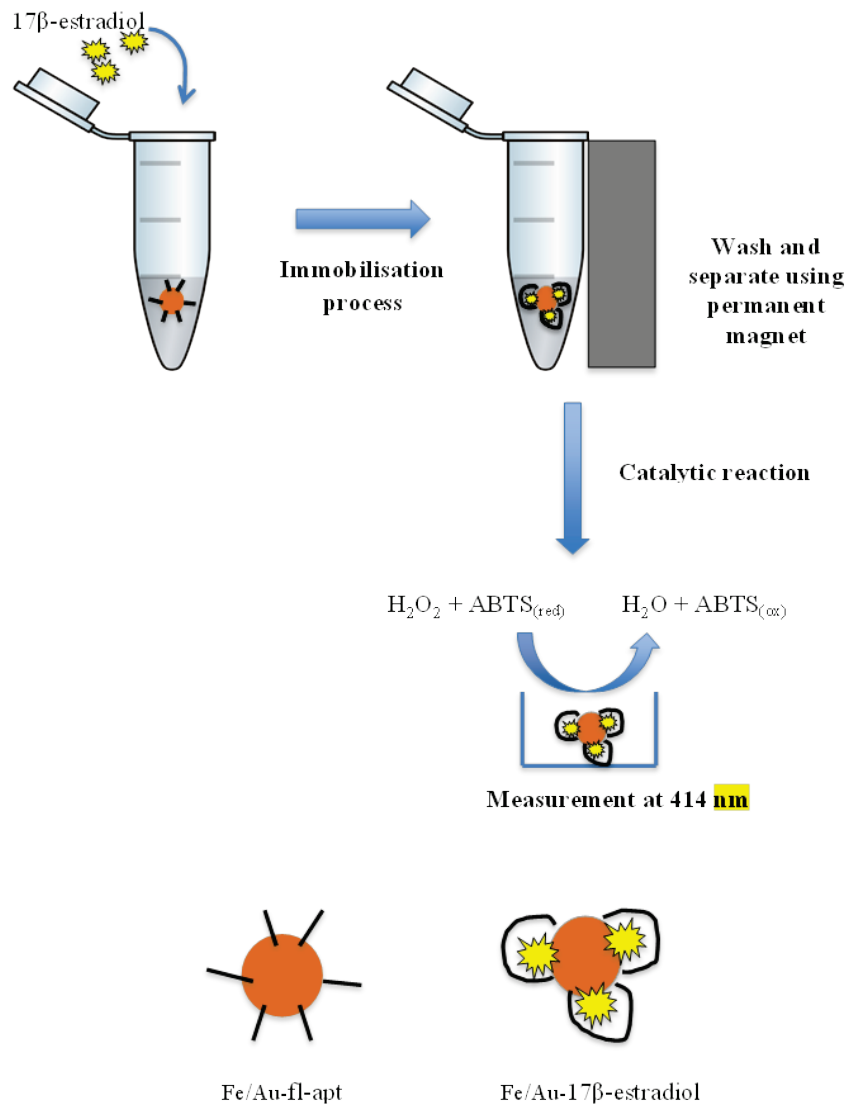


Figure 1: Illustration of the detection strategy for a nanocatalytic-based assay that consists of two main steps i.e. an immobilisation process and a catalytic reaction process, measured at 414 nm wavelength for 5 min of reaction.

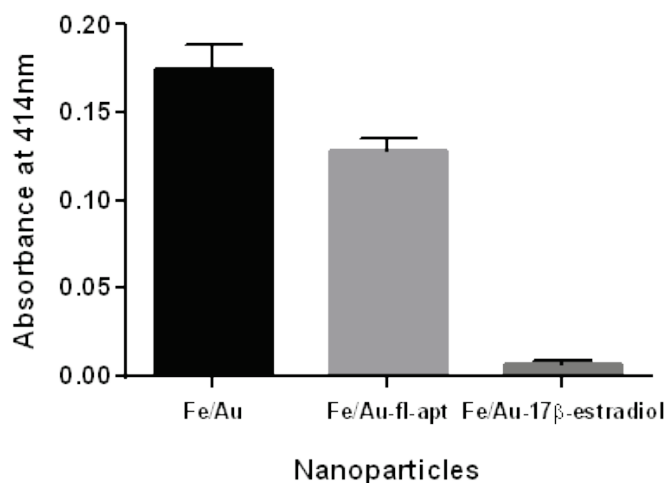


Figure 2: Absorbance intensity at 414 nm for unmodified (Fe/Au) and modified nanoparticles (Fe/Au-fl-apt; Fe/Au-17 $\beta$ -estradiol) with each nanoparticles concentration approximately 12.5mg/mL (error bars represent standard deviation [S.D.] of the absorbance mean).

At low aptamer-tagged nanoparticles concentrations (2.5 mg/mL), a linear correlation coefficient was obtained, suggesting a better sensitivity for 17 $\beta$ -estradiol detection. Further evaluation for batch-to-batch reproducibility was conducted (S8). A low CV (%) can be observed for 17 $\beta$ -estradiol concentration (0.01 to 1 nmol) indicating that the developed assay could be used repeatedly, and further revealed the possibility of batch preparation. A study by Atkinson<sup>24</sup> reported that 17 $\beta$ -estradiol was found at maximum concentrations in raw sewage (Ottawa and Cornwall, Ontario, Canada) at 66.9 ng/L. For this assay, the detection limit is ranging from 3 to 272 ng/L based on the calculation of 17 $\beta$ -estradiol molecular weight (272.4 g/mol). Therefore, this assay shows a practical use for detection of 17 $\beta$ -estradiol in environment particularly in wastewater.

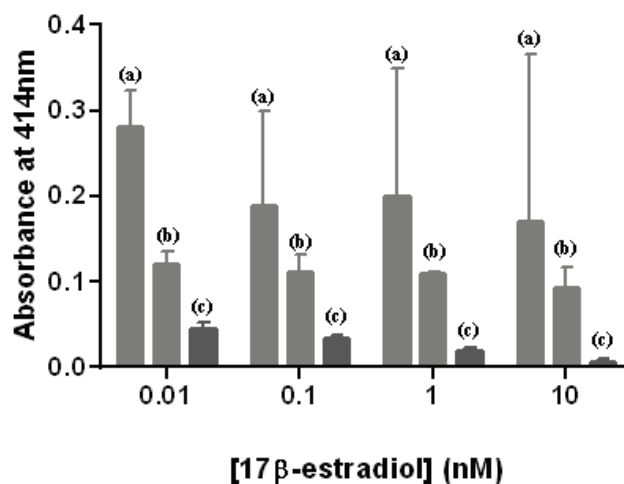


Figure 3: Serial dilution of 17β-estradiol at different aptamer-tagged nanoparticles concentration, (a) 33, (b) 17 and (c) 2.5 mg/mL (error bars represent S.D. of the absorbance mean).

### 3.3.3 Specificity test

To determine the specificity of the probe aptamer used in this study, several related endocrine disrupting chemicals (EDCs) such as carbaryl, 4NNP and estriol were studied by incubation with the Fe/Au nanoparticles. Then, the nanocatalytic-based assay was measured for each EDCs sample. As shown in Figure 4, the results from this assay show a slight absorbance difference between the blank control (Fe/Au-fl-apt) and ones containing other EDCs. In contrast, the absorbance declined substantially when 17β-estradiol was incubated with the nanoparticles, indicating a good selectivity of this assay. Therefore, the nanocatalytic-based assay using the probe aptamer attached on Fe/Au nanoparticles surface could specifically detect 17β-estradiol.

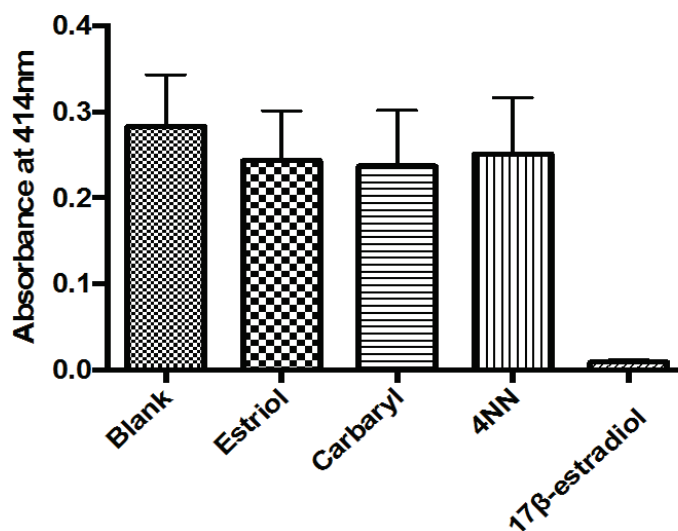


Figure 4: Cross-reactivity study with estriol, carbaryl and nonylphenol (4NNP). All the samples were evaluated at 100 nmol of each EDCs with approximately 12.5 mg/mL of Fe/Au-fl-apt nanoparticles concentration (error bars represent S.D. of the absorbance mean).

### 3.3.4 Tap water test

Based on this preliminary study to evaluate the detection efficiency in real water samples, the detection efficiency of Fe/Au-fl-apt nanoparticles show considerably good responses towards 17β-estradiol in filtered tap water (F) compared to non-filtered tap water (NF). The plausible reason is because the presence of other particles in NF that possibly interfere significantly the Fe/Au nanoparticles catalytic activity. Surprisingly, high detection efficiency of 17β-estradiol at concentration 10 nmol was obtained for NF (Figure 5). Thus, this preliminary testing in tap water provides a good starting point for detection of 17β-estradiol in real water samples using Fe/Au-fl-apt nanoparticles.

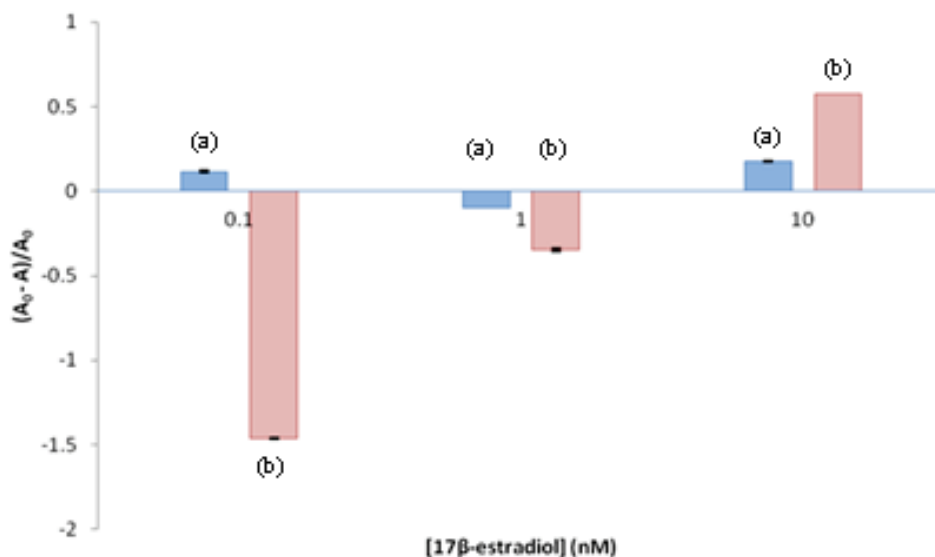


Figure 5: Detection efficiency of Fe/Au-fl-apt nanoparticles with various concentration of 17β-estradiol in tap water samples. Two types of tap water samples were tested, (a) NF and (b) F. All the samples were evaluated with approximately 12.5 mg/mL of Fe/Au-fl-apt nanoparticles concentration (error bars represent S.D. of the absorbance mean).

#### 4. CONCLUSION

In summary, we investigated the synthesised Fe/Au nanoparticles' intrinsic peroxidase-like activity and the potential manipulation of this interesting property for development of a nanocatalytic-based assay. A catalytic reaction by the Fe/Au nanoparticles for the oxidation of ABTS by H<sub>2</sub>O<sub>2</sub> showed a typical Michaelis-Menten kinetic form and exhibited a good catalytic efficiency. We also functionalised the nanoparticles by attachment of a specific aptamer to "capture" a target analyte, 17β-estradiol that would form a nanoparticles-analytes complex. The formation of this complex significantly influenced and decreased the nanoparticles' catalytic activity as shown by drastic declines in the absorbance intensity. We suggest that steric effects might be a plausible reason for this phenomenon since the active centre, which is the catalytic site, is less accessible to the substrate molecules once the target analyte has complexed with the nanoparticle. We also suggest that 17β-estradiol concentrations ranging between 3 to 272 ng/L are detected with this assay with good accuracy and reproducibility. However, a study of optimised sensing conditions needs to be done, in order to improve the assay performance, as this is our first attempt to investigate the practicality of this assay.

Our study provides a new way of utilisation of the nanoparticles' peroxidase-like activity for development of a simple, rapid and sensitive detection assay that appears to specifically detect 17 $\beta$ -estradiol in aqueous solution.

## 5. ACKNOWLEDGEMENTS

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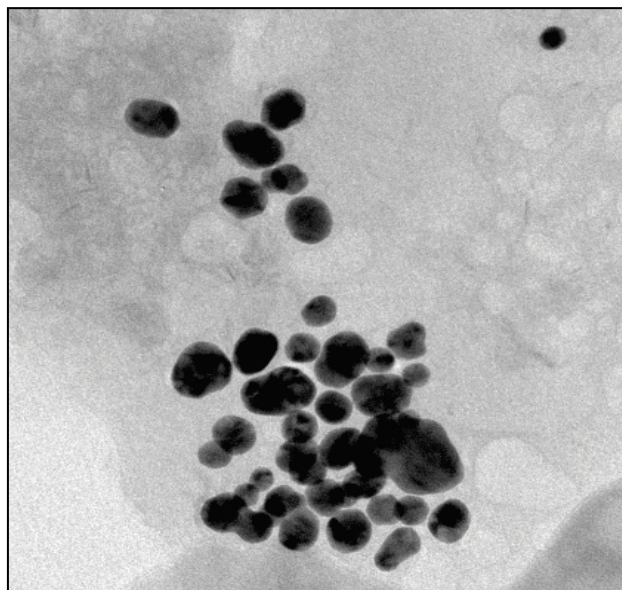


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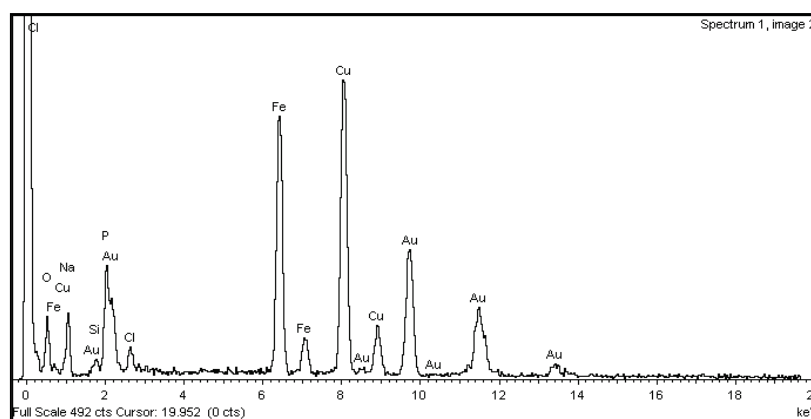
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## APPENDIX

### Supplementary Materials 1 (S1)

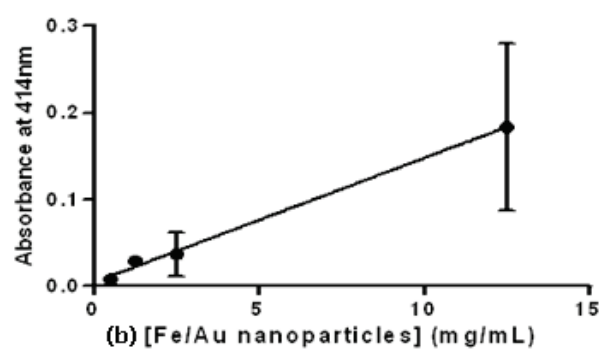
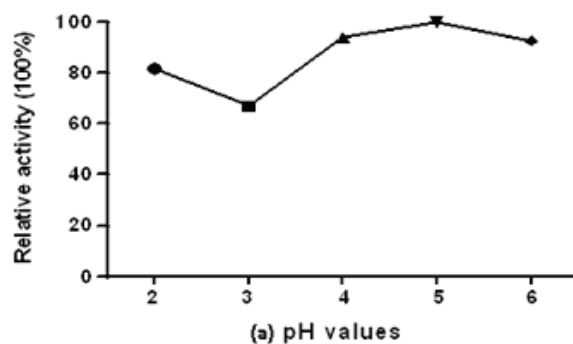


TEM image of metastable Fe/Au nanoparticles.



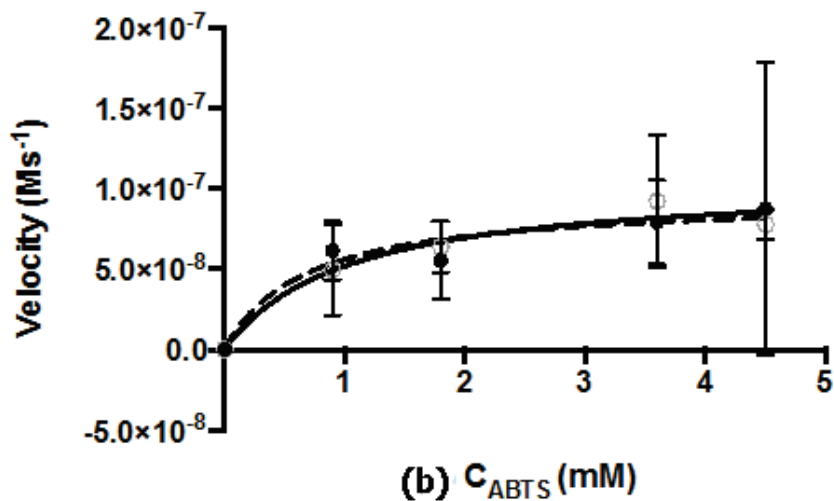
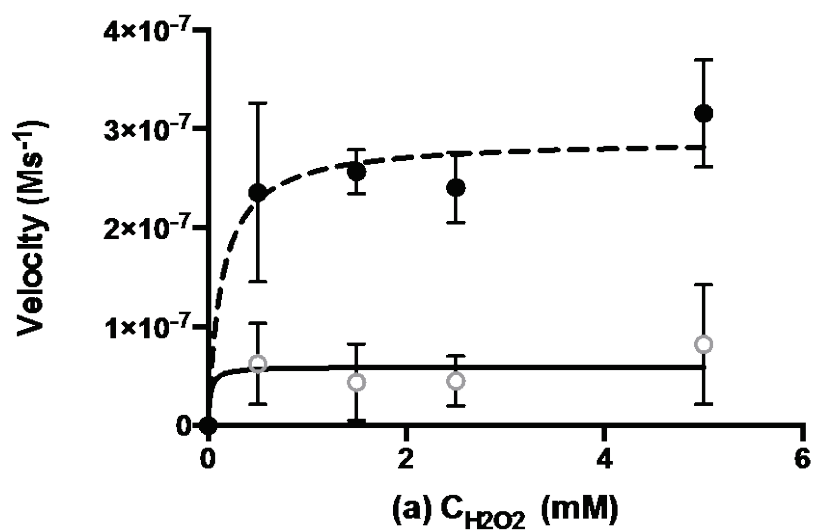
EDX analysis of metastable Fe/Au nanoparticles.

## Supplementary Materials 2 (S2)



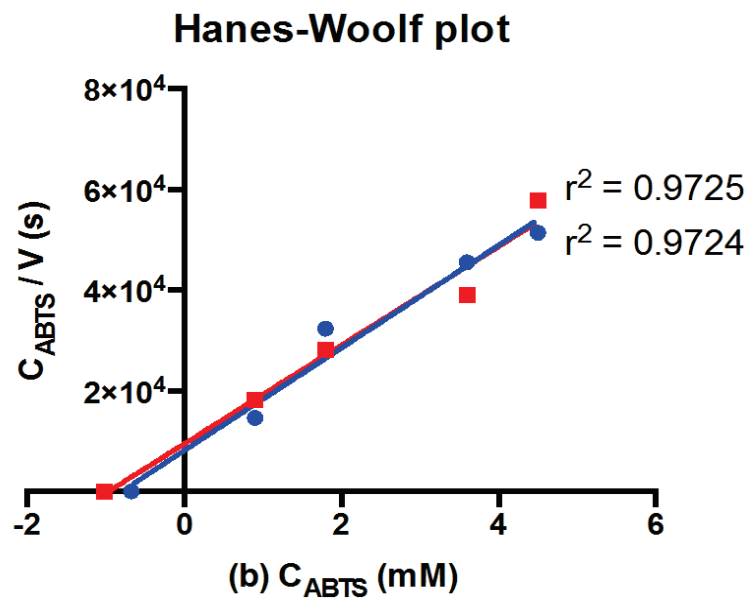
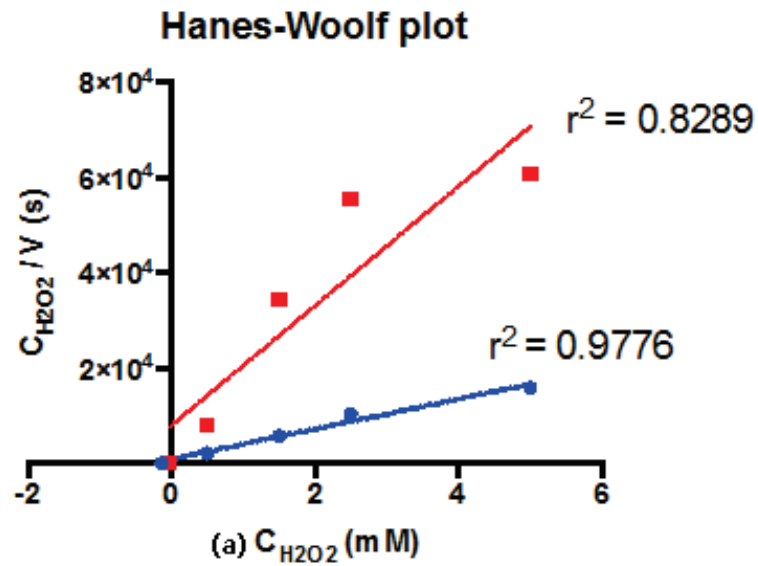
- Effect of different pH value on Fe/Au peroxidase-like activity.
- Effect of different concentration of Fe/Au on peroxidase-like activity (error bars represent S.D. of the absorbance mean).

Supplementary Materials 3 (S3)



Steady-state kinetics of  $\gamma-Fe_2O_3$  (dashed line) and Fe-Au nanoparticles (black line) with (a) ABTS concentration at 1.8 mM and varied  $H_2O_2$  concentration and (b)  $H_2O_2$  concentration at 5mM and varied ABTS concentration (error bars represent S.D. of the velocity mean).

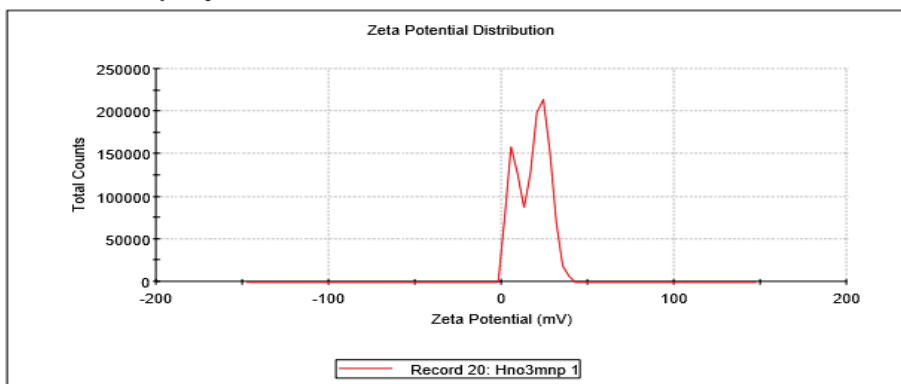
## Supplementary Materials 4 (S4)



Hanes-Woolf plot of  $\gamma\text{-Fe}_2\text{O}_3$  (blue) and Fe/Au nanoparticles (red) (a) with ABTS concentration at 1.8 mM and varied  $\text{H}_2\text{O}_2$  concentration and (b) with  $\text{H}_2\text{O}_2$  concentration at 5mM and varied ABTS concentration.

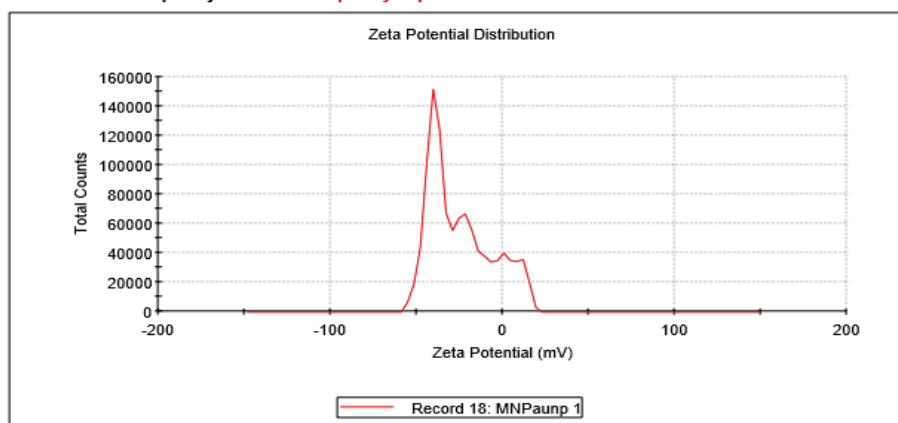
**Supplementary Materials 5 (S5)**

	Mean (mV)	Area (%)	Width (mV)
<b>Zeta Potential (mV): 17.8</b>	<b>Peak 1: 22.6</b>	<b>66.2</b>	<b>5.75</b>
<b>Zeta Deviation (mV): 9.12</b>	<b>Peak 2: 7.28</b>	<b>33.8</b>	<b>3.69</b>
<b>Conductivity (mS/cm): 0.165</b>	<b>Peak 3: 0.00</b>	<b>0.0</b>	<b>0.00</b>
<b>Result quality : Good</b>			

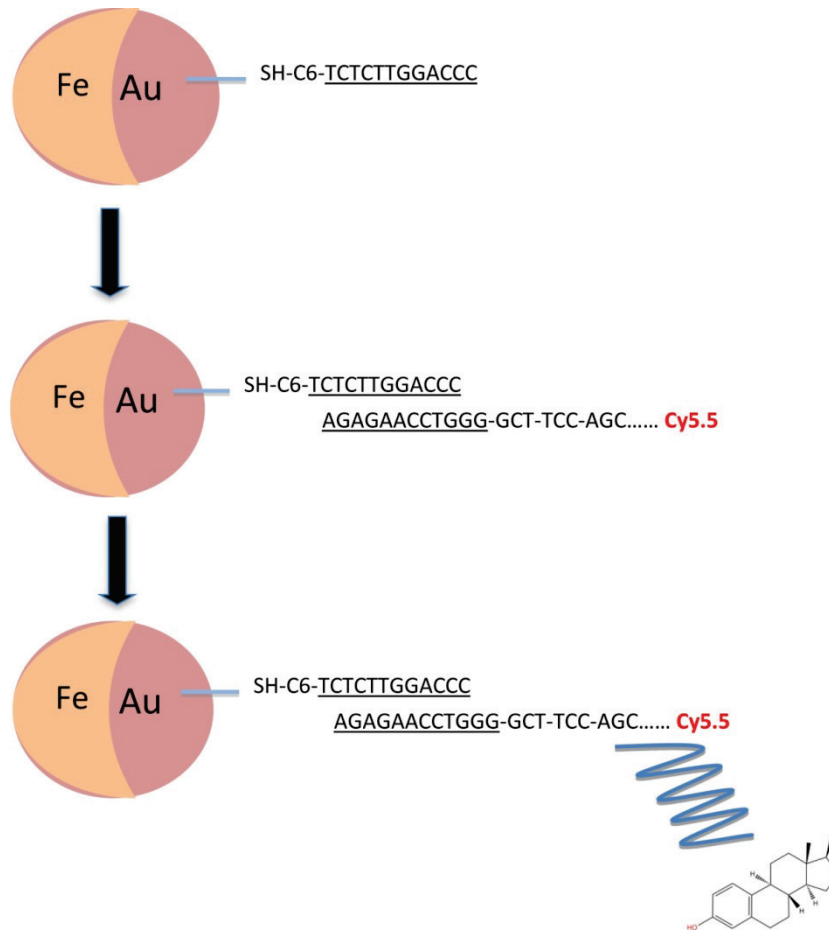


DLS measurement of  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> surface charge.

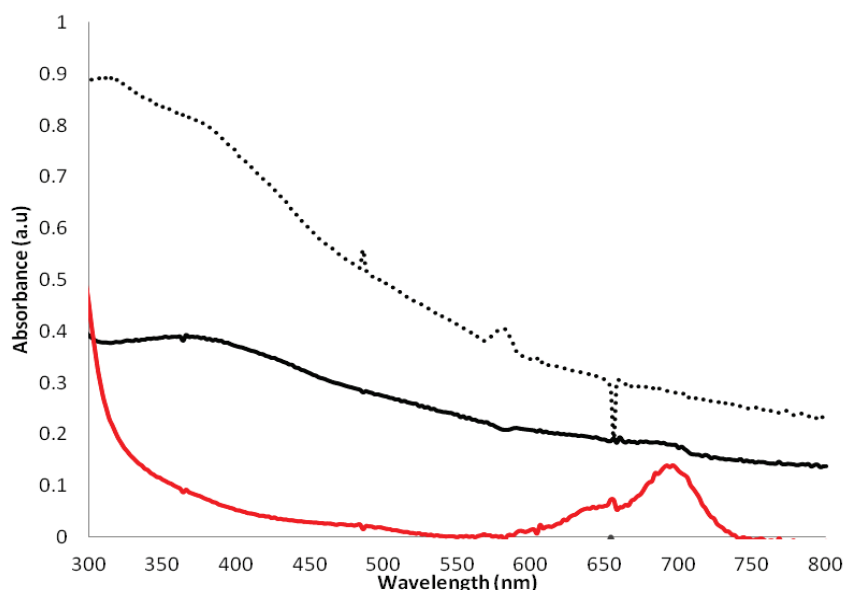
	Mean (mV)	Area (%)	Width (mV)
<b>Zeta Potential (mV): -24.7</b>	<b>Peak 1: -39.3</b>	<b>47.6</b>	<b>5.85</b>
<b>Zeta Deviation (mV): 18.4</b>	<b>Peak 2: -19.4</b>	<b>29.7</b>	<b>7.03</b>
<b>Conductivity (mS/cm): 0.299</b>	<b>Peak 3: 0.747</b>	<b>14.9</b>	<b>5.17</b>
<b>Result quality : See result quality report</b>			



DLS measurement of Fe/Au nanoparticles surface charge.

**Supplementary Materials 6 (S6)**

A sequential attachment of thiolated aptamer (SH-apt) and probe aptamer (fl-apt) to functionalise Fe/Au nanoparticle surfaces followed by conjugation of  $17\beta$ -estradiol at the site-specific sequence to generate Fe/Au- $17\beta$ -estradiol complex.

**Supplementary Materials 7 (S7)**

UV-Vis spectrophotometer measurement of Fe/Au nanoparticles (black dots), Fe/Au-fl-apt (solid black) and probe aptamer, fl-apt as a sample control (solid red).

**Supplementary Materials 8 (S8)**

Intra-assay coefficients of variation (CV) for serial dilutions of  $17\beta$ -estradiol at 2.5 mg/mL of aptamer-tagged nanoparticles for batch-to-batch reproducibility determination.

[ $17\beta$ -estradiol] (nM)	Mean (n = 2)	S.D.	CV (%)
0.01	0.047	0.003	7
0.10	0.029	0.008	26
1	0.018	0.001	4
10	0.012	0.009	77