Fe-TAML encapsulated MSN as Biomimic Peroxidase for picomole detection of proteins

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By

Abhishek Meena
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Under the guidance of
Dr. Sayam Sen Gupta
Research Scientist, NCL Pune

Department of Chemistry
Indian Institute of Science Education and Research Pune

Certificate

This is to certify that this thesis entitled **"Fe-TAML encapsulated MSN as Biomimic Peroxidase for picomole detection of proteins"** submitted towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research Pune, represents work carried out by **Abhishek Meena** under the supervision of **Dr. Sayam Sen Gupta**

Date: Supervisor

Place: (Dr. Sayam Sen Gupta)

Dedicated to my beloved family…

Declaration

I hereby declare that the matter embodied in the report entitled “Fe-TAML encapsulated MSN as Biomimic Peroxidase for picomole detection of proteins ” are the results of the investigations carried out by me in NCL PUNE, under the supervision of Dr. Sayam Sen Gupta and the same has not been submitted elsewhere for any other degree**.**

Date: **Abhishek Meena**

Place:

Acknowledgments

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Abstract

Detection of biomolecules at very low concentrations is extremely important for early detection of life threatening diseases such as cancer. Standard ELISA assays using the antibody conjugated enzyme horse radish peroxidase (HRP) is routinely used for detection of biomolecules. The role is HRP is to amplify the signal which lowers detection limit. Development of HRP mimics which show more activity that the natural enzyme can bring down the detection limits. We have synthesized mesoporous silica nanoparticles (MSN) containing thousands of small molecule synthetic HRP mimic Fe-TAML that are encapsulated inside their pores. We show that this Fe-TAML encapsulated MSN have k_{cat} values that are 1000-folds higher than the native HRP enzume. Using this nanoparticle, we have developed a highly sensitive and selective linear amplification scheme by using enzyme linked immunosorbent assay (ELISA). Our detection limit of picomolar quantities of proteins is comparable to the best detection systems that have been reported.

KEYWORDS: MSN, detection, ELISA, signal amplification

1. Introduction

Detection of biomolecules at very low concentrations is extremely important for understanding the process of life.[1,2] In this regards, proteins and nucleic acids represents two very important biomolecules which are of great interest because they encode the different information and act as a working unit of life. Among them proteins are of great importance because they function as a working unit for many cellular processes, including storage, signalling, catalysis, and metabolism of energy, which are extremely critical for life processes. Expression of a particular protein works as a biomarker for activity in organisms. Abnormal expression of protein leads to bodily disorders and diseases; hence accurate determination of protein is important for various aspects of life. For treatment of disease, proteins are either supplemented or targeted to maintain their normal expression levels.

In bacterial or viral infections and genetic disorders, proteins levels are changed either through over or under expression of proteins in the body, or with the introduction of exogenous proteins from virus or bacteria. At present we know the normal levels of proteins and what diseases can unbalance these levels. In addition, when a blood sample is taken, it contains several other proteins in high abundance. Hence exclusive identification of a specific protein for a mixture of several thousand of related proteins in blood is an immensely challenging task. If we find out theses specific proteins, a patient can be addressed with proper treatment, long before symptoms of the disease are detected.

Detection and quantification of all proteins found in human fluids would be great for biomedical applications. However, proteins of interest are present only at very low concentration and hence not easily detected. Despite great advancements in proteomic tools, analysing proteins of low abundance still represents a daunting task for the following reasons: (1) Protein effective concentration is constant (protein cannot replicate itself like nucleic acids do, hence use of amplification techniques such as PCR cannot be used to increase their concentrations). (2) Proteins are very sensitive to temperature, pressure, salt concentration and pH.

Interactions between proteins among themselves or with other biomolecules are essential in our body without such interactions body would not function properly. A particular protein selectively interacts with another protein and produces a signal of desired interest. Proteins also have a great ability to discriminate between biomolecules. Certain protein binds only to its designed target, even when other biomolecules are present which has been used in selective detection assays. There are different methods of biomolecule detection and measurement of their concentration is developed by multidisciplinary research. We need sensitive, selective and qualitative detection of targeted protein from the complex mixture of proteins.

Over the last few decade huge amount of research has been done for the sensitive and selective detection of biomolecules. In literature, there are several methods to detect different biomolecules based on their biophysical like size, charge, mass, hydrophobicity and composition. By using these biophysical properties, we can determine the concentration of protein. Several techniques like electrophoresis (size), isoelectric focusing (charge), mass spectrometry (mass), reversed phase chromatography (hydrophobicity) and UV absorbance (composition) has been introduced to detect biomolecules. Also, mass spectrometry combined with 2-D electrophoresis, isoelectric focusing and gel electrophoresis or liquid chromatography has been used for the detection of specific proteins in complex samples. [3]

To trace the biomolecule, there are two basic methods. [4, 5] First is label based method and second is label free method. In label based method, bio molecules are tagged with conventional labelling strategies such as radioisotopes, fluorescent dyes, chemi luminescent molecules and also many novel labels such as inorganic quantum dots, gold nanoparticles, dye-doped silica, and single-walled carbon nano-tubes. On the other side label free methods involves different chemical and physical properties of bio molecule. Label free techniques are surface Plasmon resonance, micro cantilevers, atomic force microscope etc. However, label free methods have not yet been widely adopted due to their uncertainty regarding their sensitivity and specificity whereas label based methods has high sensitivity and specificity for the detection of protein or biomolecule.

A huge amount of research has been done for the development of label based assays and to simplify the assay process with better sensitivity, selectivity and

robustness. One of the approaches is calorimetric assay and has attracted attention because of its low cost, simplicity and short assay duration. Colorimetric assay is particularly based on colour changes in the sample and does not require expensive and sophisticated instruments [6]. The main challenge for colorimetric bio sensing is transforming the non-colored substrate into a colored one. To achieve this aim, various different technique have been developed , such as aggregation-based colorimetric immunoassay[7], lateral-flow colorimetric immunoassay,[8] and enzyme-mediated colorimetric immunoassay.[9] However, aggregation based colorimetric immunoassay and colorimetric lateral-flow immunoassay exhibit low sensitivity because they lack a signal amplification procedure or less intensity of colour. Recently, an enzyme-linked signal amplification strategy has attracted attention and more importance has been paid to advance an enzyme-linked signal amplification strategies.

Catalytic signal amplification is well adopted by many biological assays such as in ELISA, where biomolecule detection is done by a surface bound receptor with an enzyme immobilized (HRP), which will be used for the generation of an easy detectable signal. Different nanomaterial which can be functionalized with many synthetic functional analogues has been used to improve biological assays by providing multivalency [6, 10]. So that for each binding recognition by the analyte and the nanomaterial will provide multitude of reporter signals (turnover number of the catalyst multiplied by the no of catalysts functionalized) as compared to a single catalyst or enzyme. Number of nano-materials that function as enzyme mimics have been developed, the main advantages of these functionalized enzyme mimics are (1) low cost with easy synthesis methods, (2) tuneable catalytic activities, (3) high stability (at different pH, condition, temperature and pressure).Therefore, the robust physical or chemical properties of these nano-materials make them important for enzyme-linked signal amplification strategies.

In last several years different nanomaterials have been developed including gold nanoparticles, magnetic nanoparticles, cerium oxide nanoparticles, carbon nanotubes, graphene oxide, and conjugated polymers[11-18] (Fig.1). Their tuneable high surface area and pore volume make these nanomaterial promising candidates for colorimetric biosensing. Nanomaterial those have peroxidase like activity is of great importance in calorimetric bio sensing assays. Some of the metal nanoparticles, such as iron, gold, cerium can catalyze the reaction of the peroxidase

substrate to produce colour change that has been used in calorimetric assay[19-20] . and recently carbon nano tubes and graphene oxide found to have an intrinsic peroxidise activity that can catalyze the calorimetric reaction[21-22] .

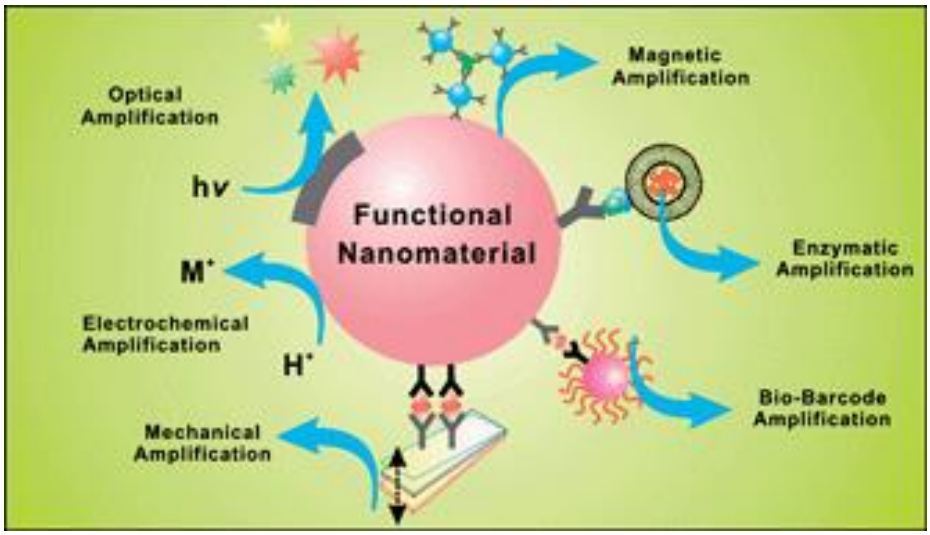


Figure 1: Functional nonmaterial-based amplified bio-detection strategies[23]

Recently, mesoporous silica nanoparticles with immobilised enzyme has come out with high sensitivity in immunoassay [(24)] . Functionalized MSN particles have great importance in recent years due to their tuneable physical and chemical properties such as high surface area, large pore volume and pore size[25-26] . These features make MSN applicable for diverse biomedical applications including protein imaging, protein sensing, catalysis, bone repair, and drug delivery[27-28]. MSN can be efficiently internalized by animal and plant cells. The functionalization of MSNs with organic material or other nanomaterial brings controlled release and molecular recognition to these MSN for drug delivery and bio molecule detection applications, respectively.

1.1 Principal of colorimetric protein detection assay

For the development of any protein detection assay sensitivity and selectivity are key points. For the sensitive detection of protein in a sample an appropriate protein assay method based upon its selectivity with the sample type is required. The main objective of an assay is to develop a method that requires the least pre-treatment and instrumentation. Till date, there are many assay technique have been developed

with its own advantage and disadvantages. Based on application, different methods of protein assay are available in the laboratories; however ELISA assays are mostly use for colorimetric protein detection.

Some important key points or criteria for choosing a protein detection assay include.

1) Assay should be specific so that it specifically binds to target of interest and there should be no interaction with the other proteins present in the assay.

2) The sensitivity of assay should be high so that the low concentration of protein molecules can be detected easily.

3) With different applications in mind, an assay should be stable biologically, chemically (e.g. pH) and physically (temperature).

4) Assay should be fast and simple to carry out.

1.1.1 Enzyme-linked immunosorbent assay (ELISA)

Elisa is technique used to measure the concentration of an analyte (usually antibodies or antigens) in a solution. ELISA assay is quick and simple to carry out and is designed to rapidly carry out a large numbers of samples in parallel. ELISA is a popular choice for the protein detection because its results are selective and sensitive towards detection of biomolecules (Fig 2).

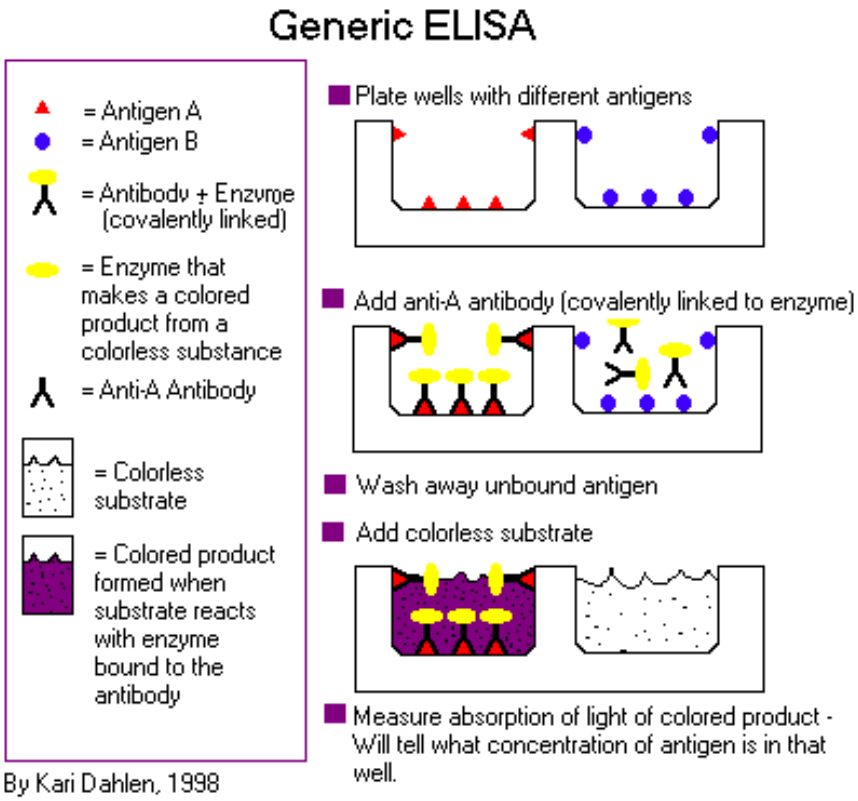


Figure 2: General process of ELISA[29]

1.1.2 Basic ELISA Procedure

ELISA method first begins with a coating step; firstly on the 96 well plates' antigen or an antibody is adsorbed, then enzyme conjugated with antibody binds specifically to the target antigen. After that substrate is added and the signal produced by enzyme-substrate reaction is measured by UV – Vis plate reader. In these types of assay, generally peroxidase enzyme catalyses the substrate to produce signal for detection of analyte. Horseradish peroxidase (HRP), a heme-containing peroxidase is widely used for detection of protein in ELISA method, where the key step of these peroxidase catalysed is to catalysed the conversion of a no luminescent substrate into a luminescent one, thus amplifying the signal by thousands of fold[30] . However, these enzymes are not stable in all conditions (gets denatured upon heating or chemical changes) and relatively its low productivities which hinders the commercial application of HRP. So, there is a need to develop peroxidise HRP mimic enzyme which will be commercial available.

1.1.3 Typical ELISA Formats

There are three main types of ELISA assays direct, indirect and sandwich. It allows certain amount of flexibility which can be adjusted based on the antibodies available, the results required or the complexity of the samples (Fig 3).

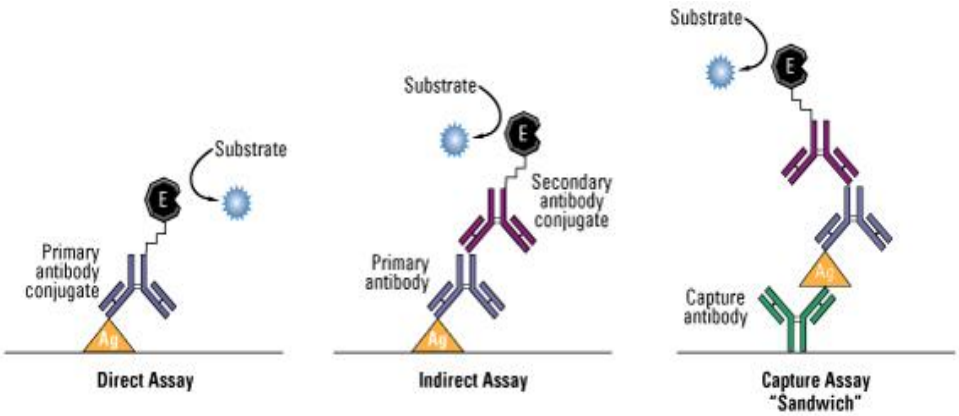


Figure 3: Typical ELISA formats[31]

Direct Assay

An antigen coated to a 96 multiwell plate is detected by an antibody that has been directly conjugated to an enzyme. Main advantages are:

- It is faster with few steps required in the assay (because there is no secondary antibody is use).
- It is less prone to error, since there are few steps.

Indirect assay

Antigen coated to a polystyrene 96 multiwell plate is detected in two stages or layers. First an unlabelled primary antibody which is specific for the particular antigen is applied. Next, an enzyme linked secondary antibody is bound to the first antibody**.** Main advantages are:

- Increased sensitivity as more than one labelled antibody is bound per primary antibody
- Flexibility due to different primary detection antibodies used with a single labelled secondary antibody

Sandwich ELISA

The first antibody, termed as the capture antibody is coated to the polystyrene plate. Next, the protein or sample solution is added to the well. A second antibody layer, the detection antibody, follows this step in order to measure the concentration of the protein. Main advantages are:

- High specificity, since two antibodies are used
- Flexibility and sensitivity, since both direct and indirect detection methods can be used

1.2 Functionalized Mesoporous Silica Nanoparticles (MSN) applications in Catalytic protein detection

Recently, Mesoporous silica nanoparticles (MSN) having unique features has attracted substantial attention for their use as a support for catalyst-immobilization inside their porous matrices. MSN has high surface area with highly uniform pore distribution and high adsorption capacity which helps free diffusion of substrates. Beside those it also has enough chemical, thermal, and mechanical stability and pore size can be easily tuneable. From last two decades, hybrid MSN particles have been considered for a wide range of heterogeneous catalysis reactions. Solid supported catalysts are of great interests due to their recyclability, enhanced catalytic reactivity; and selectivity. Confinement of the catalyst within mesopores

provides a means of introducing size and shape selectivity and thus greater specificity for a reaction. Inorganic, organic and enzymatic catalysis fields have been revolutionized by introduction of mesoporous silica nanoparticles as support due to dramatic increase of contact area and thus contributing to overall reaction yield [32-33]. Furthermore, a mesoporous silica particle offers the opportunity of multifunctionalization and therefore, multiple catalyst immobilizations.

1.3 Encapsulation of Enzymimic Fe(III) complex on Mesoporous Silica Nanoparticles (MSN)

MSNs with different pore size, surface area and structural characteristic are used for their functionalization with enzymes. Functionalization of MSN particles could be attained by physical methods like absorption and chemical methods which involve covalent attachment of enzyme. Enzymes encapsulated inside MSNs particles retain their bio-catalytic activity and are more stable than enzymes in solution and are used in various analyte detections. Different transition metal complexes, noble metal nanoparticles like gold, silver, platinum etc. and oxide metal nanoparticles like Fe_2O_3, CuO, TiO_2 have also been substituted in MSN frame work and are used in catalytic signal amplification for diagnostic analysis.

Enzymes that contain iron as cofactor with peroxidase like activity have great application in protein detection because of their low cost and robust catalytic activity. Peroxidases are found everywhere in nature and are known to activate hydrogen peroxide to perform different oxidations selectively. In various analyte detection, Horseradish peroxidase (HRP), a heme-containing peroxidase, which selectively activates oxidises colourless TMB to blue colour charge transfer complex in presence of H_2O_2 and will amplifying the signal by several folds. However, limited stability of these enzymes and the relatively low productivities features will hinders the commercial application of HRP, because these enzymes gets denatured upon heating or chemical changes and also their preparation, purification and storage are time consuming . So the development of synthetic HRP mimics that posses lower K_m and higher V_{max} values will significantly improves the sensitivity.

We have recently developed $[Fe^{III}(\text{biuret-amide})]^{2-}$ immobilized inside mesoporous silica nanoparticles (Fe-MSNs) that has excellent functional mimics of

the enzyme HRP. Fe-MSNs have several features that make them very attractive as functional mimic for HRP.

(1) Fe-MSNs exhibit excellent reactivity towards 3, 3', 5, 5'-tetramethylbenzidine (TMB) in the presence of H_2O_2 to generate visually detectable green oxidation product at neutral pH.

(2) $[Fe^{III}$ (biuret-amide)$]^{2-}$ catalysts are mostly grafted inside the MSN pores (2–3 nm) which limits their interaction with other enzymes during biological assays.

(3) The amount of immobilized catalyst and the distance between them can be controlled to achieve varying reactivity.

(4) The silanol groups on the outer surface of MSN can be further functionalized to incorporate targeting ligands such as antigens/antibodies for usage in immunoassays like ELISA.

1.3.1 Kinetic and Mechanistic Studies of TMB oxidation Using Fe-MSN Particles as a Catalyst

In enzyme kinetics, Michaelis–Menten kinetics is generally used to derive kinetic parameters. The model serves to explain how an enzyme can cause kinetic rate enhancement of a reaction and explains how reaction rate depend on the concentration of an enzyme and substrate. The basic assumption of Michaelis-Menten kinetics is formation of an enzyme-substrate complex (ES) and the ES complex is present in rapid equilibrium with free enzyme (E) where S is denoting substrate. That reaction is followed by the decomposition of ES to regenerate the free enzyme, E, and the new product, P (Fig 4).

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

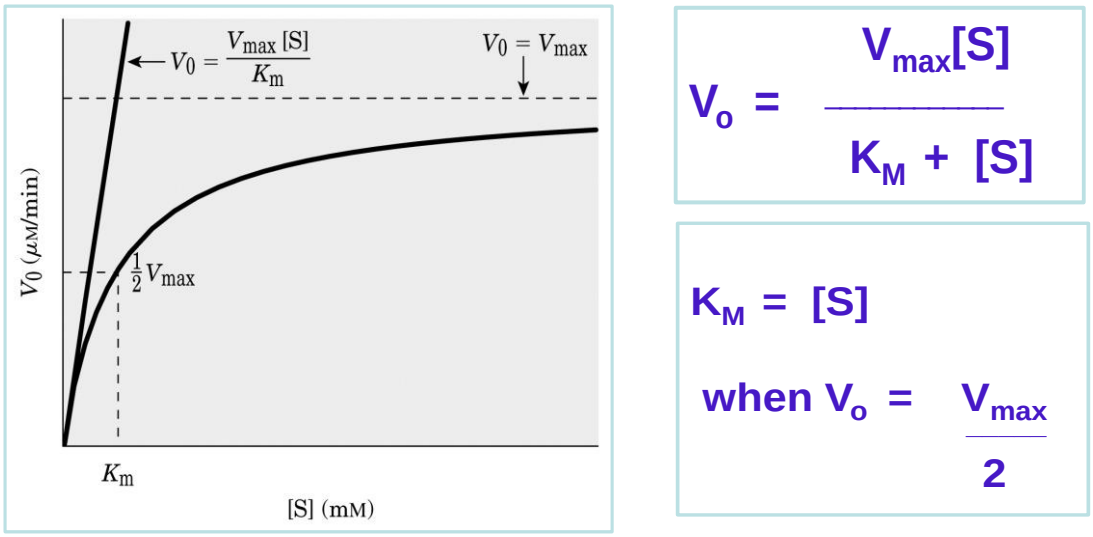
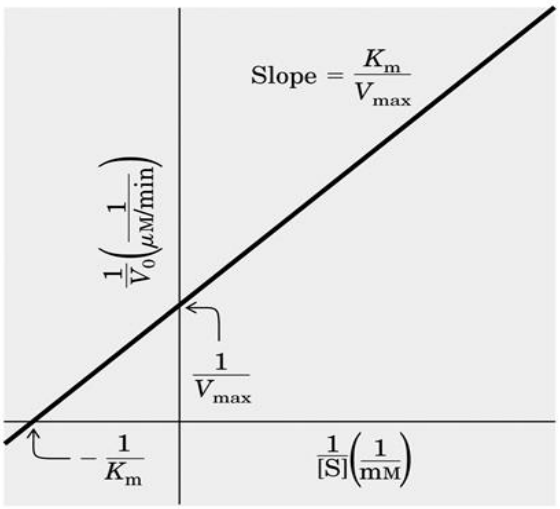


Figure 4: The Michaelis-Menten equation and plot

Here, V_0 is initial velocity; V_{max} represents the maximum velocity achieved by the system, at maximum (saturating) substrate concentrations. K_M (the Michaelis constant) is the substrate concentration at which the reaction velocity is 50% of the $V_{max,}$ [S] Is the concentration of the substrate.

1.3.2 Lineweaver-Burke transformation of the Michaelis-Menten equation

The main regions for common use of Lineweaver-Burke transformation are (1) Velocity vs. Substrate plots and are useful for visually estimating kinetic parameters (2) Hyperbolic curves cannot properly determine the upperlimit of the curve (V_{max}) (3) Transforming the data to a form that can be plotted as a line may improve accuracy and Magnitude of errors can become distorted. Lineweaver-Burke transformation equation is used to determine K_m and V_{max} (Fig. 5).



$$\frac{1}{V_o} = \frac{K_M}{V_{max}[S]} + \frac{1}{V_{max}}$$

Figure 5: Lineweaver-Burke transformation

1.3.3 Important kinetic parameters

K_m (Michaelis constant):

K_m is a constant derived from rate constants, under true Michaelis-Menten conditions; K_m stands for of the dissociation constant of ES. Small K_m means tight binding; high K_m means weak binding.

V_{max} (Maximal velocity):

V_{max} is the maximal rate of the reaction and to reach V_{max}, all enzyme molecules should tightly bind with substrate. V_{max} is asymptotically approached as substrate is increased.

k_{cat} (The turnover number):

k_{cat} is a measure of its maximal catalytic activity and it denotes the number of substrate molecules converted in product per unit time and per molecule of enzyme, on saturating conditions; In this condition $k_2 = k_{cat}$.

$$k_{cat} = V_{max}/E_t$$

k_{cat}/K_m (Catalytic effectiveness of an enzyme):-

The ratio k_{cat}/K_m is often referred to as the 'specificity constant' – is a useful index for comparing the relative rates of enzyme acting on alternative, competing substrates and by using k_{cat}/K_M we compare the catalytic effectiveness of enzymes.

1.4 Antibody antigen in immune response

Antibody recognition of foreign molecules (antigen) is a keystep in the immune response [(ref)]. The immune response is basically how your body recognizes and defends itself against bacteria, viruses, and substances that appear to be foreign and harmful. The immune system protects the body from possibly harmful substances by recognizing and responding to antigens. An antibody is used by the immune system to identify and neutralize foreign objects such as bacteria and viruses. The antibody recognizes a unique part of the foreign target, called an antigen and selectivity of antibody–antigen interactions are among the most finely tuned non covalent bonds exhibited by proteins (Fig. 6). This principle lies for the detection of interested biomolecule from a serum using nanoparticles bound with ligands for antigen/ antibody.

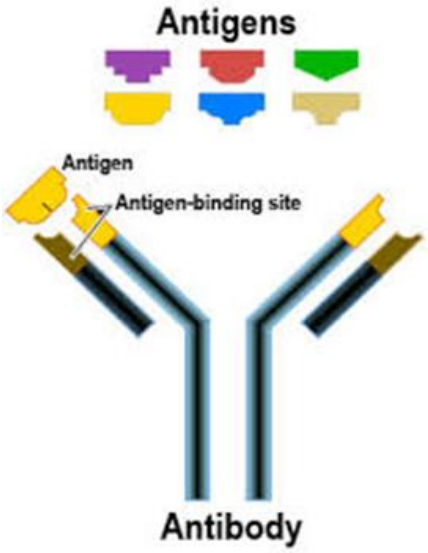


Figure 6: Antigen –Antibody interaction

2. MSN-biuret Fe-TAML as peroxidase mimic for picomole detection of protein

Detection of biomolecules in picomole concentration necessitates development of a particle that possess very high peroxidase activity and is also capable of multivalently displaying receptor ligands (e.g., antibodies for protein detection) on is surface. We therefore designed a mesoporous silica nanoparticle (MSN) that contained several chemically conjugated HRP mimic Fe-TAML inside the pores and antibody molecules on its surface. The presence of large number Fe-TAML complexes inside MSN is expected to have an additive effect to their individual activity and therefore increase the overall peroxidase activity in comparison to HRP enzyme. This high peroxidase activity together with the increased binding of MSN to the target protein due to multivalent display of antibodies on its surface is expected to bring down the detection limits several folds in comparison of commercial antibody-HRP conjugates (Fig. 7).

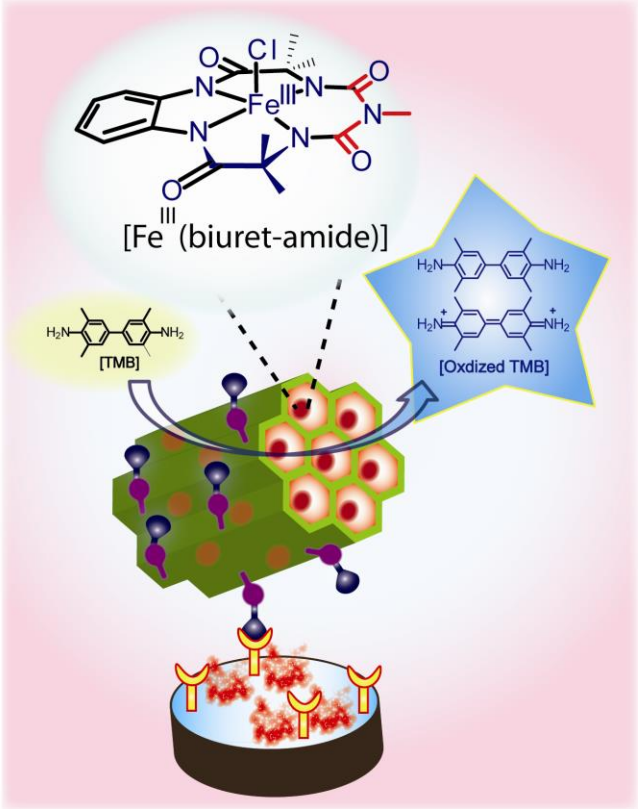


Figure 7: Schematic representation for nanoparticle amplification

Here, we show that TAML encapsulated MSN as biomimic peroxidase for picomole detection of proteins. Firstly, azide functionalized mesoporous silica nanoparticles (MSN) was synthesised by one pot co-condensation method, then amine is grafted outside the surface of MSN. Peroxidase mimic Fe^{III} (biuret-amide)] with alkyne side chain is functionalized inside the 2.3 nm pore channels of MSN using Cu(I) catalyzed azide- alkyne click reaction. Then, antibody (AIgG) was conjugated on the surface of resulting Fe Cat-MSN to bind with the target antigen (IgG) molecule. After the binding, visual colour generation by TMB oxidation was done in presence of H_2O_2, which results signal amplification, colour intensity is measured by UV-Vis plate reader.

2.1 Experimental Section

2.1.1 Materials

Tetraethylorthosilicate (TEOS), Cetyltrimethylammonium bromide (CTAB), Amino guanidine hydrochloride (AG.HCl), 3′, 5, 5′-Tetramethylbenzidine (TBM), 4-Pentynoic acid, streptavidin, and Hydrazine acetate were obtained from Sigma Aldrich. CuSO4, Sodium ascorbate, hydrogen peroxide, Zn dust and $CaCl_2$ were obtained from Merck, India. Human IgG and antigen hIgG were obtained from Jackson Smith and Merck. Alkyne tailed [FeIII-tetraamido-(biuret-amide)] complex, 3-azido-propyltriethoxysilane (AzPTES) and tris(3-hydroxypropyl triazolyl methyl) amine (THPTA) were prepared as reported earlier. In all the experiments, de-ionized water was used.

2.1.2 Synthesis and Characterizations

(a) Synthesis of Azide functionalized MSN x-N_3-MSN)

The azide functionalized MSN (x-N_3-MSN; x stands for % of azide loading) was synthesized by co-condensation of TEOS with AzPTES (3-azidopropyl triethoxysilane) by following procedure reported in literature [33] with slight modifications. The density of azidopropyl groups on the surface of MSN was varied by varying the molar ratio of AzPTES with respect to TEOS during one pot co-condensation synthesis. In a typical batch synthesis for 1% azide loading, CTAB (1

g, 2.744 mmol) was dissolved in 640 mL of water and 2M aqueous NaOH (3.5 mL, 7 mmol). The mixture was stirred thoroughly at 600 rpm for 30 min at 80 °C to dissolve the surfactant completely. To this clear solution, TEOS (4.702 g, 22.56 mmol) was injected rapidly followed by AzPTES (0.0563 g, 0.228 mmol). A white precipitate was observed within 1-2 min after the addition was completed. The resultant reaction mixture was allowed to stir at 600 rpm for 2 h at 80 °C. The hot contents were then filtered and the white residue was washed with copious amounts of water and methanol and dried under vacuum at 100°C over night (yield ~1.7 g). This azide grafted MSN will be simply denoted as 1-N_3-MSN. Similarly, 5-N_3-MSN was synthesized using AzPTES (0.2815 g, 1.142 mmol). TGA was done to determine the azide incorporation in the x-N_3-MSN after removal of template.[33]

(b) Synthesis of Amine functionalized MSN x-N_3-MSN

To put amine groups selectively on the outer surface of the x-N_3-MSN was synthesized by post synthetic grafting method. In short, as-synthesized x-AZP-MSN were subjected to outer surface amine grafting using 3-amino-propyltriethoxysilane (APTES) followed by removal of template [33] to yield amine functionalized x-N_3-MSN (NH_2-x-N_3-MSN, x stands for % of azide loading). 1 g of as synthesized MSN was suspended in 200 mL of dry toluene by sonication for 10 minutes. To this APTES (0.179 g, 1 mmol) was added, and the mixture was stirred for 18 h at 80°C under nitrogen atmosphere. After the completion of reaction, the contents were cooled, filtered and washed with toluene until it became free from APTES. The sample was then dried at 100°C for 8h in a vacuum oven. The template was extracted by stirring the as-synthesized sample (2g) in 400 mL methanol and 4ml concentrated hydrochloric acid at 60 °C for 6 hr. The resulting template removed solid product, was filtered and washed with methanol (100 mL) and 1% triethyl amine in methanol (50 mL). Then it again washed with methanol (50) and dried overnight under vacuum at 100 °C (yield ~0.64 g). This material will be referred as 1-AZP-MSN-NH_2. Similarly, 5-AZP-MSN-NH_2 was synthesized using AzPTES (y = 1.142 mmol). TGA was done to determine the amine incorporation in the NH_2-x-N_3-MSN.

(c) Modification of NH_2-x-N_3-MSN with Fe-TAML by Cu(I) catalyzed Azide-Alkyne Cycloaddition reaction (CuAAC)

For CuAAC, NH_2-x-N_3-MSN was incubated with 3 equivalents of the alkyne tailed [Fe^{III}-tetraamido-(biuret-amide)] complex in 100 mM phosphate buffer containing THPTA (2.5 equivalent), AG.HCl (4 equivalent), $CuSO_4$ (0.5 equivalent) and sodium ascorbate (4 equivalent). In a typical click reaction, NH_2-1-N_3-MSN (10 mg, 1.2 µmol of azide) was incubated with alkyne tailed [Fe^{III}-tetraamido-(biuret-amide)] complex (3 mg, 3.6 µmol) in 1mL, 100 mM phosphate buffer containing THPTA (1.3 mg, 3 µmol), AG.HCl (0.54 mg, 4.8 µmol), CuSO4 (0.16 mg, 0.6 µmol). The reaction mixture was freeze pump thawed thrice and sodium ascorbate (0.96 mg, 4.8 µmol) was added and the mixture was stirred inside microwave for 15min and then at room temperature for 24 h. After completion of reaction, the reaction mixture was centrifuged and the residue was first washed with phosphate buffer twice and then sequentially washed with 10 mM N,N-diethyldithiocarbamate sodium solution in 100 mM phospate buffer and acetone respectively. The last two washings were repeated thrice. Finally, the yellowish white powder obtained was dried at 80°C in vacuum oven for 8 h (Yield: ~9 mg). This [Fe^{III}-tetraamido-(biuret-amide)] functionalized MSN will be simply denoted as x-Fe-MSN x stands for % of azide loading. Similarly, one more click reaction was carried out using NH_2-5-N_3-MSN. The extent of click reaction was estimated by IR spectroscopy (Fig.10).

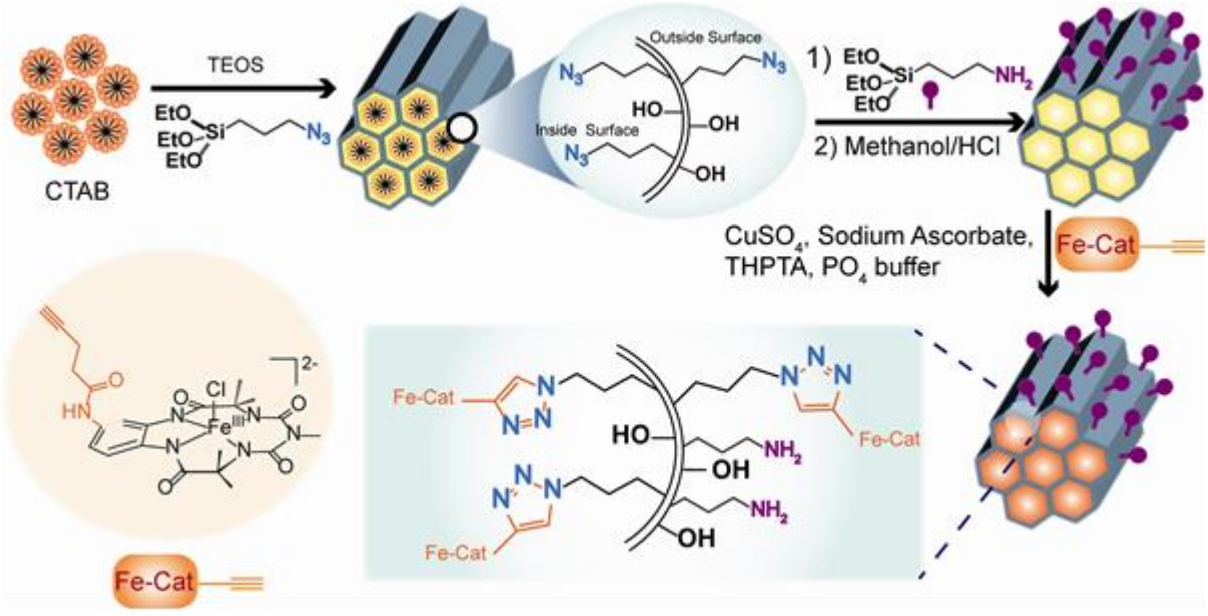


Figure 8: Synthesis of [FeIII(biuret-amide)] functionalized MSN

(d) Synthesis of biotin immobilized Fe-MSN for detection of streptavidin

5-Fe Cat-NH_2-MSN 2mg was mixed with 4mg NHS-Biotin (0.012mmol) in 2ml of 100mM, pH 7.4 PBS and stirred at room temperature for overnight. For the control sample, we used NH_2-5-N_3-MSN instead of 5-FeCat-MSN to prepare simply NH_2-Biotin MSN. After completion of the reaction, both the samples were washed extensively (5times) with the buffer. The samples were then dispersed in buffer and analyzed for colorimetric assay (Fig 9).

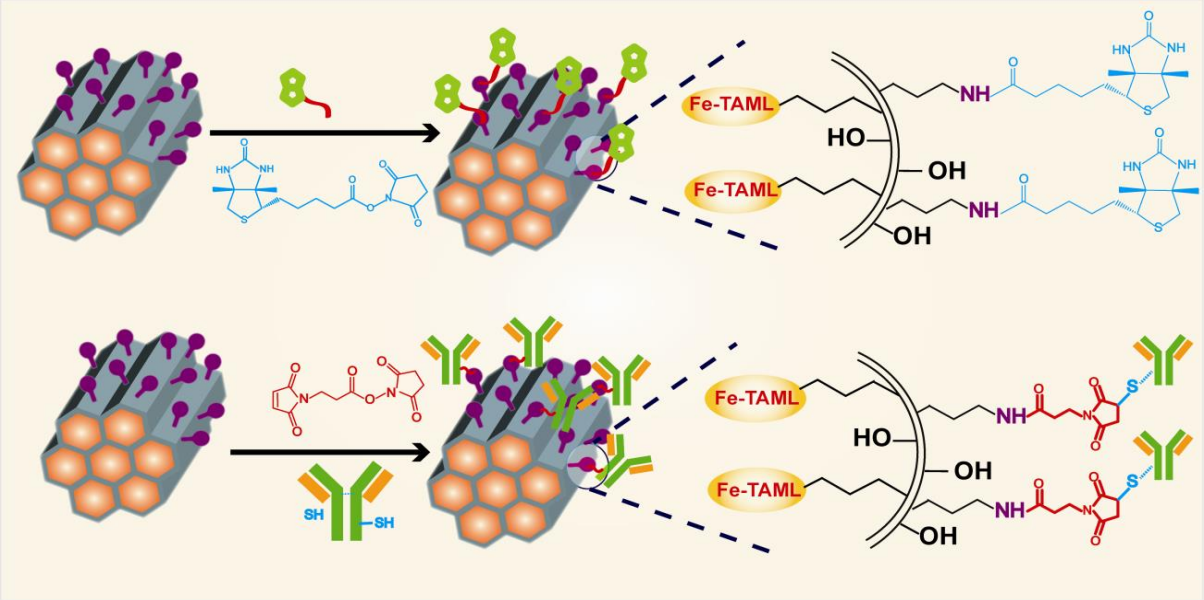


Figure 9: Synthesis of biotin immobilized Fe-MSN for detection of streptavidin

(e) Colorimetric assay of Streptavidin

In ELISA, for the detection of Streptavidin, commonly used 96-well polystyrene (PS) plates were used. In the first step, a 100µl of streptavidin with concentrations ranging from 0.01 to 10^6ng/ml was added to each well and incubated at 4^{o}C overnight. After incubation, the plate was washed three times with 100mM PBS buffer, pH7.4. Each well was filled with 100µl of 1% BSA in PBS as blocking agent to prevent nonspecific interaction between the plate and other protein molecules. After 2hr, the plate was decanted and washed three times with PBS buffer. In the second step, 50µl of Fe-TAML-MSN (0.1mg/ml) was added into wells and incubated at 370C for another 1hr. After washing three times with wash buffer, 100µl substrate solution containing TMB (0.8mM) and H_2O_2 (8mM) was added to each well. After 5min of incubation, green colour observed and ELISA reading was performed in a micro plate reader at 655 nm.

(f) Synthesis of maleimide functionalized Fe-Cat-MSN

To, a dispersed solution of Fe-Cat MSN (2mg) in PBS buffer, maleimide NHS (4mg, 150mmol) dissolved in acetonitrile was added and stirred at room temperature for 12hrs. After completion of the reaction, the particles were centrifuged and washed thoroughly several times with acetonitrile: buffer mixture. At the end of the centrifugation the particles was dispersed in 100mM PBS buffer, pH7.4 and will be used for the conjugation with reduced antibody.

(g) Conjugation of Antibodies to Fe-cat-MSN NPs

The antibodies were reduced with Dithiothreitol (DTT). Reduced antibody fragments with free sulfhydryl groups were conjugated to maleimide activated Fe-MSN through thiol-maleimide reaction. In a typical reaction, 100 µg antibody was mixed with DTT in 200 µL PBS-EDTA (100mM phosphate, 10 mM EDTA and pH 7.2) and incubated at 37 ^{0}C for 2 h. The concentrations of DTT were 30 mM for human antibodies. Reduced antibody fragments were purified by passing through the column The Fe-MSN NPs were mixed with reduced IgG at 1:3 molar ratio (e.g., 72 µg antibody fragments for each 1000 µg maleimide of 50 nm Fe-MSN NPs) in PBS. The antibody concentration in the reaction mixture was fixed at 100 nM. The solution was incubated at room temperature overnight. After conjugation, the solution was centrifuged twice (12000 rpm, 4 ^{0}C and 1 h) and supernatant with unconjugated antibody fragments was discarded. The particles with conjugated antibody were dispersed in PBS buffer and used for antigen detection in immunoassay.

(h)Detection of hIgG using antibody conjugated Fe-CAT –MSN

In ELISA, the plates were prepared by adding 100µl of hIgG with concentrations ranging from 0.01 to 10^6ng/ml to each well and incubated at 4^{o}C for 24hrs. After incubation, the plate was washed three times with PBS. Each well was filled with 100 µL of 1% BSA in PBS and incubated at 37^{0}C for another 2h to prevent nonspecific interactions between the plate surface and other protein molecules. After that plate was decanted and washed three times with PBS buffer and then 50ul of Fe-TAML-MSN (0.1mg/ml) was added into the wells and incubated for another 1hr. The color development was made by addition of 100 µL of mixed substrate solution of 0.4 mM TMB and 8mM of H_2O_2. After 5min of incubation, green colour observed was recorded in a micro plate reader at 655 nm.

(h) Kinetics of TMB Oxidation

The kinetics were monitored in kinetic mode of the spectrophotometer (Agilent 8453) using 1 ml quartz cuvette of 1.00 cm path length at 450 nm (second oxidation product of TMB) in an thermostatted (25.0 ± 0.5 °C) cell housing. We observed that during oxidation three oxidised species (TMB^{2+}, $TMB^{+\cdot}$ And [TMB^{2+}: TMB]) present in equilibrium. Therefore it is very difficult to get exact rate if we consider initial rate for a single oxidised species. To overcome this problem we added acid to get Initial rates of TMB oxidation by shifting the equilibrium towards formation of TMB^{2+}, and by division with the extinction coefficients of 59000 $M^{-1}cm^{-1}$ which stands for oxidation product of TMB at 450 nm. 1 mg of Catalyst was dispersed in 1mL deionized water and was treated as Stock solution. Concentration of the catalyst for 1- Fe-MSN and 5-Fe-MSN is 7.92 x 10^{-13} M and 12.5 x 10^{-14} M .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 peroxidase activity of Fe-CAT MSN , H_2O_2 variations was done from 3×10^{-3} to 40×10^{-3} M while keeping the TMB concentration fixed at 2×10^{-4} M. For TMB variation reveres procedure was followed ([H_2O_2] = 8.0×10^{-3} M; [TMB] = 0.05×10^{-3}– 0.4×10^{-3} M). Phosphate buffer having the appropriate pH of 7.4 was used for all the runs. .The kinetic parameters were calculated according to the equation, $v = V_{max} \times [S]/(K_m + [S])$ 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.

2.2 Results and discussion

(a) Characterization of Fe-TAML immobilized MSN

TEM, and SEM of NH_2-x-N_3-MSN showed formation of well-ordered two-dimensional hexagonal MSN with a spherical morphology having particle size of ~40nm (Fig 10). Nitrogen adsorption–desorption showed the multi-point BET surface area, pore diameter and pore volume to be 844 m^2/g, 2.8 nm and 1.13 cc/g respectively. TGA analysis showed 0.11mmol/g and 0.4 mmol/g of azidopropyl group was incorporated in MSN frame work of 1 and 5% loading respectively. The FT-IR spectra of the azido-functionalized MSN materials (x-N_3-MSN) display an absorbance at ~2100 cm^{-1}, which is the characteristic stretching vibration of an

organic azide (N_3). The presence of this peak in the materials shows that the azidopropyl group was successfully incorporated into the x-N_3-MSN samples (Fig 11).

As-synthesized MSNs were subjected to outer surface amine grafting using 3-amino-propyltriethoxysilane (APTES) followed by removal of template to yield NH_2-MSN. The amount of amine groups grafted on the surface of NH_2-MSN was determined to be 0.78 mmol g^{-1} by elemental analysis. In the second step, NH_2-MSN was coupled with N-(4-Pentynoyloxy)-succinimide to yield alkyne grafted MSNs (ALK-MSN). The amount of catalyst incorporated was estimated by ICP to be 0.048 mmol of [Fe^{III}(biuret-amide)] per gram of Fe-MSN. Efficient removal of copper during the washing has been observed by the absence of copper in ICP analysis.

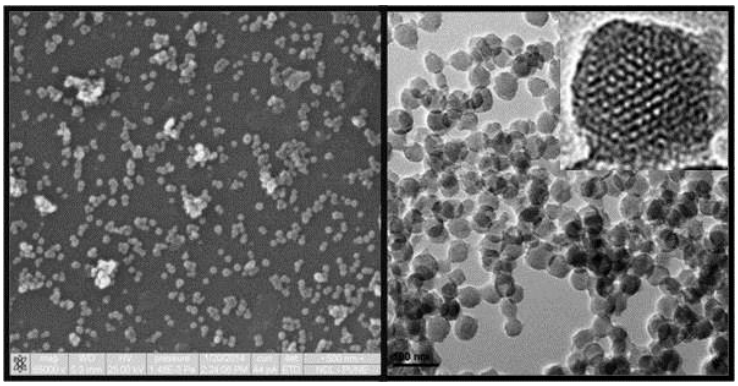


Figure 10: A and B shows SEM and TEM images respectively of N_3-MSN showed well ordered two dimensional hexagonal mesoporous particles ~40nm

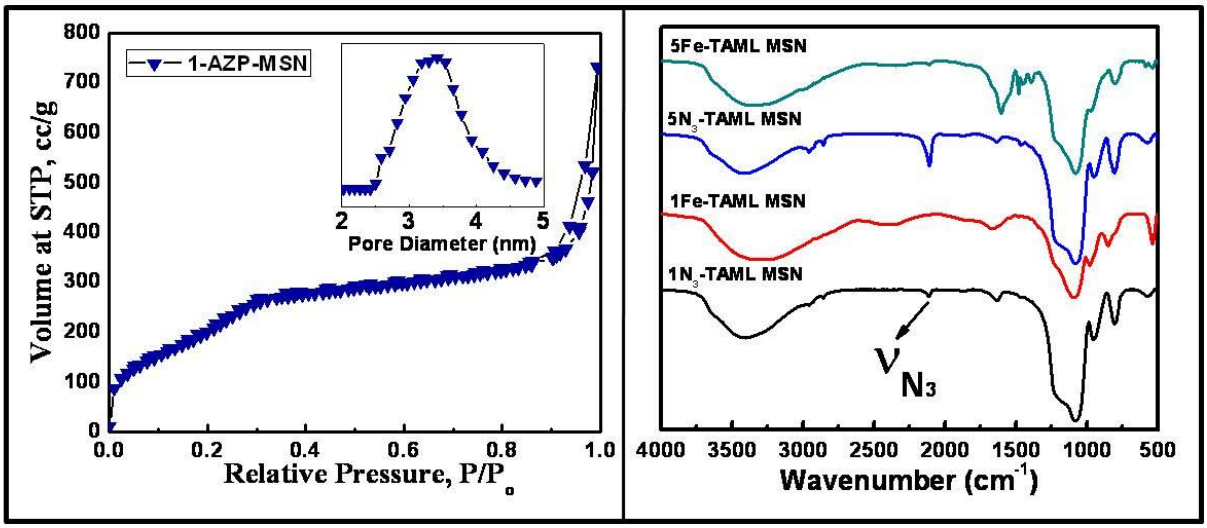


Figure 11: shows N_2 adsorption-desorption isotherm of N_3-MSN exhibit the characteristic type IV isotherm. D showsFT-IR spectra display an absorbance at 2100cm^{-1}, signature peak for organic azide.

(b) Evaluation of kinetic parameters for TMB oxidation by Fe-TAML encapsulated in MSN

The peroxidase activity of 1 -Fe-MSN and 5 -Fe-MSN were evaluated by studying their efficiency for TMB oxidation using H_2O_2 to form the colored oxidized TMB product at 450nm (Fig.12). The plots of initial rate vs substrate concentrations were fitted perfectly to a typical Michaelis–Menten curve (Fig. 13 and 14) and the K_m and V_{max} values are shown in (Table 1). It was observed that the K_m and V_{max} value for our peroxidase mimic 1-Fe-MSN with TMB as a substrate was 0.033 mM and 1.297 X 10^{-7} Ms^{-1} while for 5-Fe-MSN K_m and V_{max} values are 6.67mM and 3.32 X 10^{-7} Ms^{-1} .1-Fe-MSN has a 540 times high catalytic efficiency K_{cat}/K_m and 5-Fe-MSN has a 2300 times high catalytic efficiency K_{cat}/K_m in comparison to commercial HRP. The apparent K_m value of the 5-Fe-MSN with TMB as a substrate is ~4times lower than HRP, suggesting that 5-Fe-MSN have higher affinity for TMB than HRP. In comparison to 1-Fe-MSN the higher K_{cat} and V_{max} values of 5-Fe-MSN using TMB as a substrate indicates that incorporation of increased amounts of biuret modified Fe-TAML led to improved peroxidase activity. Also we studied the peroxidase activity by H_2O_2 variation. The K_m and V_{max} value for our peroxidase mimic 1-Fe-MSN with H_2O_2 as a substrate was 9.62 mM and 1.785 X 10^{-7} Ms^{-1} while for 5-Fe-MSN K_m and V_{max} values are 6.67mM and 3.32 X 10^{-7} Ms^{-1} . The apparent K_m value of the 1-Fe-MSN and 5-Fe-MSN with H_2O_2 as the substrate was higher than HRP, suggesting that the higher concentration of H_2O_2 was required to observe maximal activity. In short, the peroxidase mimic 1-Fe-MSN and 5-Fe-MSN has 65 and 747folds higher K_{cat} than HRP. This increased peroxidase activity of 5-Fe-MSN encouraged us to use this nanoparticle for selective detection of very low protein.

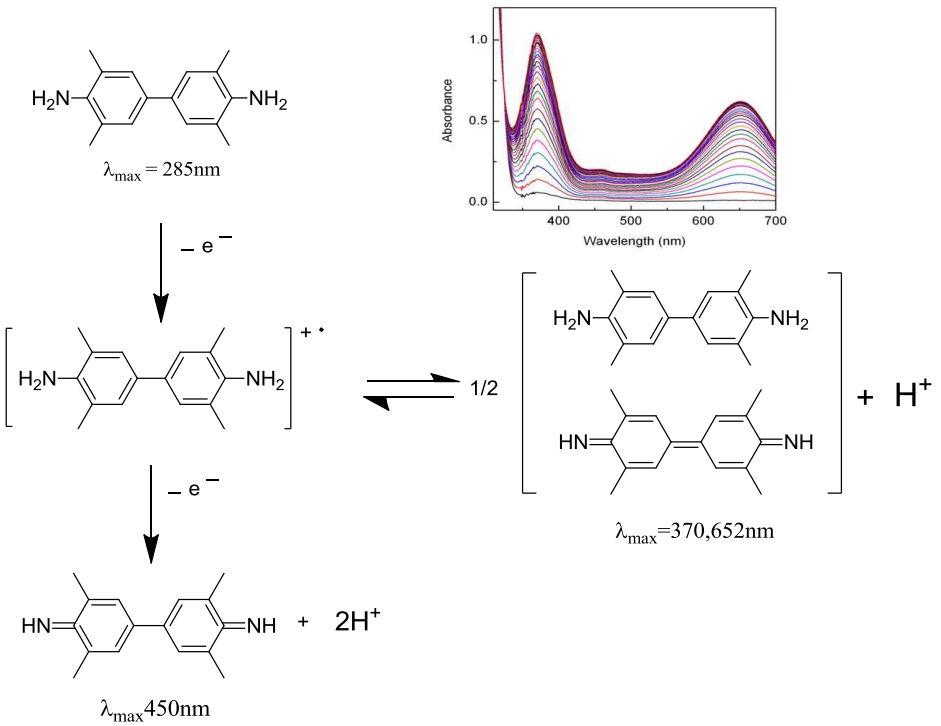


Figure 12: Oxidation Products of TMB [34]

Table 1: Comparison of the kinetic parameters of 1-Fe-MSN ,5-Fe-MSN and HRP. [E] is the enzyme (or MNP) concentration, Km is the Michaelis constant, Vmax is the maximal reaction velocity and Kcat is the catalytic constant, where Kcat Vs max/[E]

	[E](M)	Substrate	K_m(mM)	V_{max} (Ms^{-1})	K_{cat}(s^{-1})	K_{cat}/K_m
HRP	2.5×10^{-13}	TMB	0.434	10.00×10^{-8}	4.00×10^{3}	9.22×10^{6}
HRP	2.5×10^{-13}	H_2O_2	3.70	8.71×10^{-8}	3.48×10^{3}	1.00×10^{6}
5-Fe-MSN	12.5×10^{-14}	TMB	0.122	3.316×10^{-7}	2.65×10^{6}	21.72×10^{9}
5-Fe-MSN	12.5×10^{-14}	H_2O_2	6.67	3.267×10^{-7}	2.60×10^{6}	0.39×10^{8}
1-Fe-MSN	7.92×10^{-13}	TMB	0.033	1.297×10^{-7}	1.64×10^{5}	49.70×10^{8}
1-Fe-MSN	7.92×10^{-13}	H_2O_2	9.617	1.785×10^{-7}	2.25×10^{5}	0.23×10^{8}

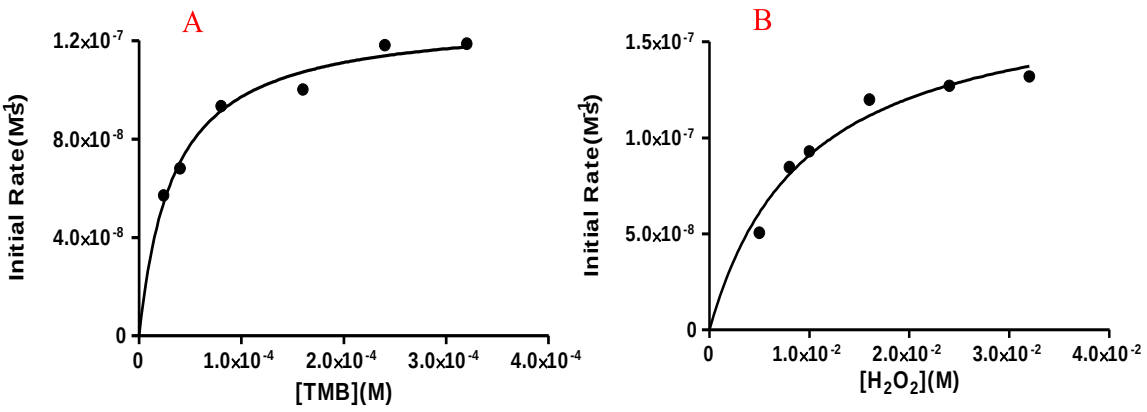


Figure 13: Michaelis Menten fit for TMB (A) and H_2O_2 (B) variation using 1 %-Fe-MSN

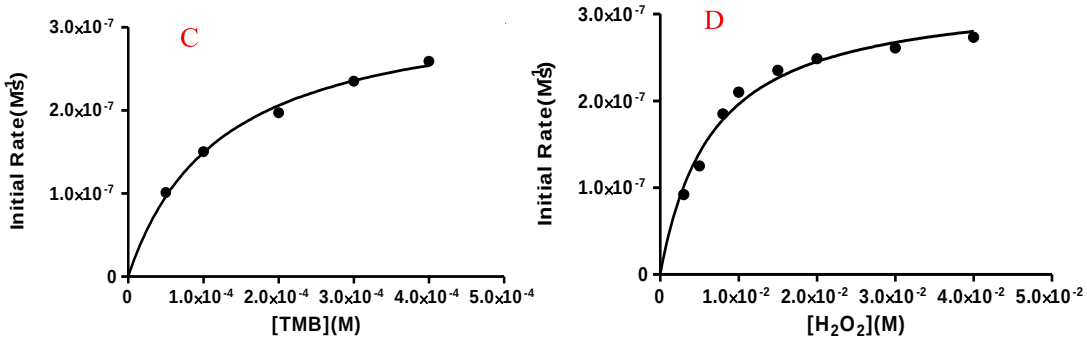
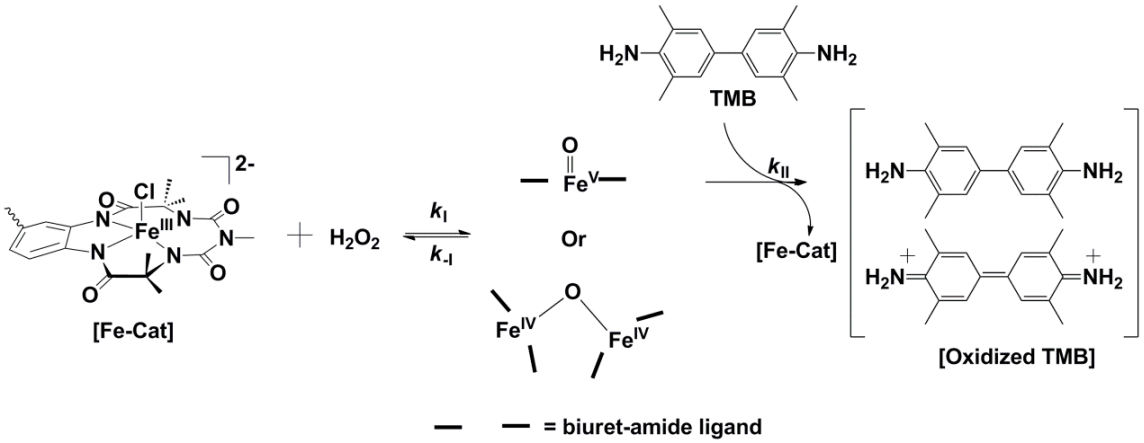


Figure 14: Michaelis Menten fit for TMB (C) and H_2O_2 (D) variation using 5 %-Fe-MSN

Mechanism of TMB oxidation by [Fe^{III}(biuret-amide)]

The proposed mechanism of oxidation of TMB by [Fe^{III} (biuret-amide)] Figure (1) shows that the active intermediate has been proposed to be a high valet iron-Oxo species but the identity of these species has not been confirmed and is proposed the high valent iron-oxo species to be either an iron (V)-Oxo or a μ-Oxo Fe (IV) dimer.



(b) Detection of streptavidin.

It was already known that the [Fe^{III}-tetraamido-(biuret-amide)] complex is a potential peroxidase mimic molecule and in presence of H_2O_2 it rapidly changes colourless TMB to green. It has been already used for glucose and cyanide detection.[ref] To demonstrate the Fe-TAML MSN amplification scheme for the detection of biomolecules, we can perform an assay in which it can be conjugated with the reporter biomolecule, which act as a probe, binds specifically with the target molecule and on the addition of H_2O_2catalyzed the oxidation of TMB which can be used to generate a visual signal that is proportional to the amount of analyte present.

As a proof of concept, we first prepared the Fe-TAML MSN labeled with Biotin for colorimetric assay of Streptavidin (Fig. 15) represents the increase in absorbance of the reporter TMB at 650nm as a function of analyte concentration. A linear dependence of absorbance *vs* analyte concentration was obtained in the range of 0.01 to10^6ng/ml with a limit of detection (LOD) 0.01ng/ml. The proposed sensor exhibited a wide linear range and a low detection limit for the detection of streptavidin biomolecule. The reasons might be as follows: (1) owing to small size Fe-TAML-MSN NPs with high surface area which enhances the loading of Fe TAML catalyst inside the pores (2) porous Fe-TAML-MSN NPs exhibiting superior catalytic performance to the peroxidase substrate TMB in the presence of H_2O_2 realized the signal amplification. This initial result encourages us to go further for the detection of hIgG.

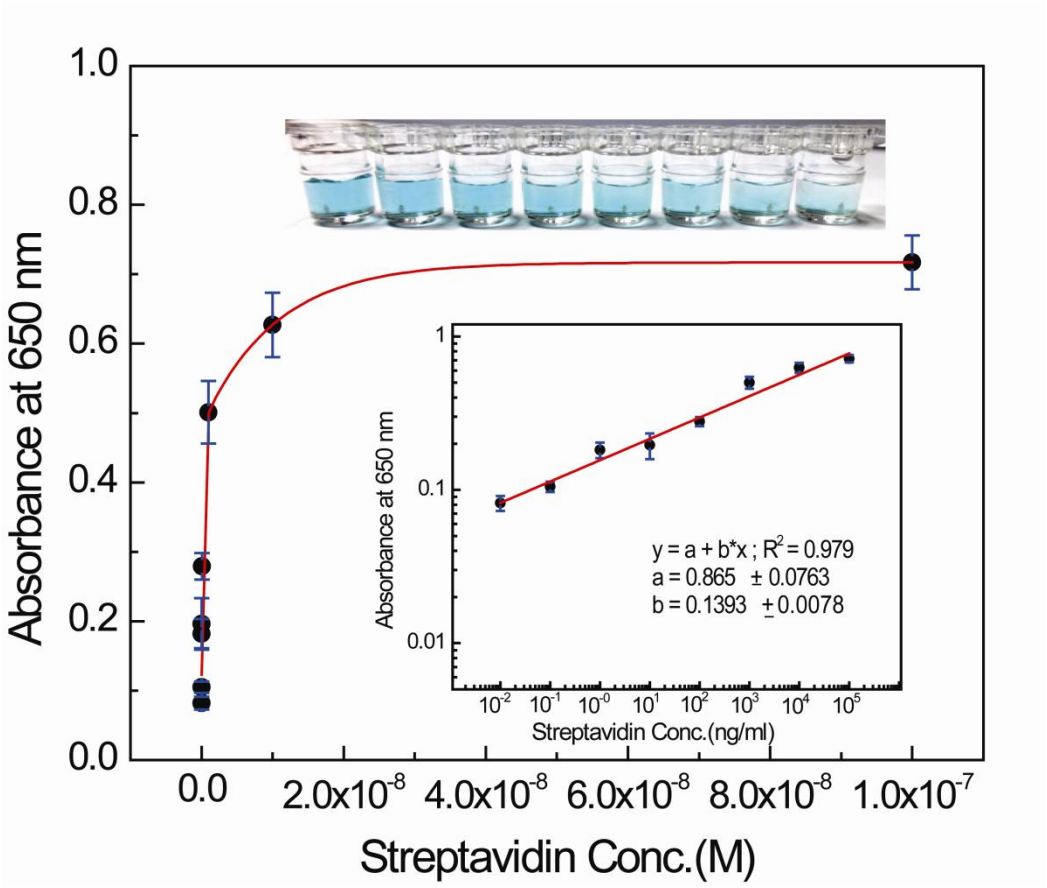


Figure 15: Application of Fe-TAML MSN in direct immunosorbnet assay

(c) Detection of IgG in vitro and in cells

To evaluate the efficiency of this activatablenano-sensor, the as-prepared Fe-TAML-MSN was brought into the ELISA for hIgG detection. For colorimetric immunoassay of hIgG using Fe TAML MSN, various concentrations of hIgG (0.01 to 10^4ng/ml) were captured on antibody-conjugated well plate, and incubated with antibody-conjugated Fe-TAML MSN. As a control experiment, antibody-conjugated MSN was also prepared and used for conventional ELISA. Simultaneously a comparison was done with conventional HRP conjugated hIgG instead of Fe TAML MSN. Fig. 3a shows the correlation between the hIgG concentration and the signals from both Fe TAML MSN and HRP-IgG. The limit of detection (LOD) of Fe TAML MSN was estimated to be ~0.01 ng/mL of hIgG (3.3 pM) while the LOD of conventional ELISA was ~10 ng/mL of hIgG (67 pM) (Fig. 16). The dynamic ranges of conventional HRP-ELISA and Fe TAML MSN were 10-1000 ng/mL and 0.01-1000 ng/mL, respectively (Figs. 3a and S2), representing that the dynamic range of Fe TAML MSN is two-

order wider than that of conventional HRP-ELISA. Interestingly, the upper side of dynamic range was same, but the lower side of dynamic range was extended from 10 ng/mL of ELISA to 0.01 ng/mL of Fe TAML MSN. This suggests that the detection of hIgG in a trace amount became more feasible due to higher ratio of catalyst to antibody in the approach of Fe TAML MSN.

The specificity of the sensor for hIgG was examined under optimal detection conditions (see ESI†). For this experiments CHO-hIgG and the CHO-S cell line was employed. The CHO-S did not express the hIgG while a strong signal was observed for CHO-hIgG cells (Fig. 4A). The results showed that the proposed method has good specificity for the CHO-hIgG cells. Under the optimal conditions, the absorbance intensity was proportional to the logarithmic value of the cell concentration ranging from 1000 cells per mL with a correlation coefficient R of 0.9917 (Fig. 4B). The limit of detection for CHO-hIgG cells was calculated to be 1000 cells per ml and identify the presence and concentration of a target faster and cheaper than using traditional ELISA.

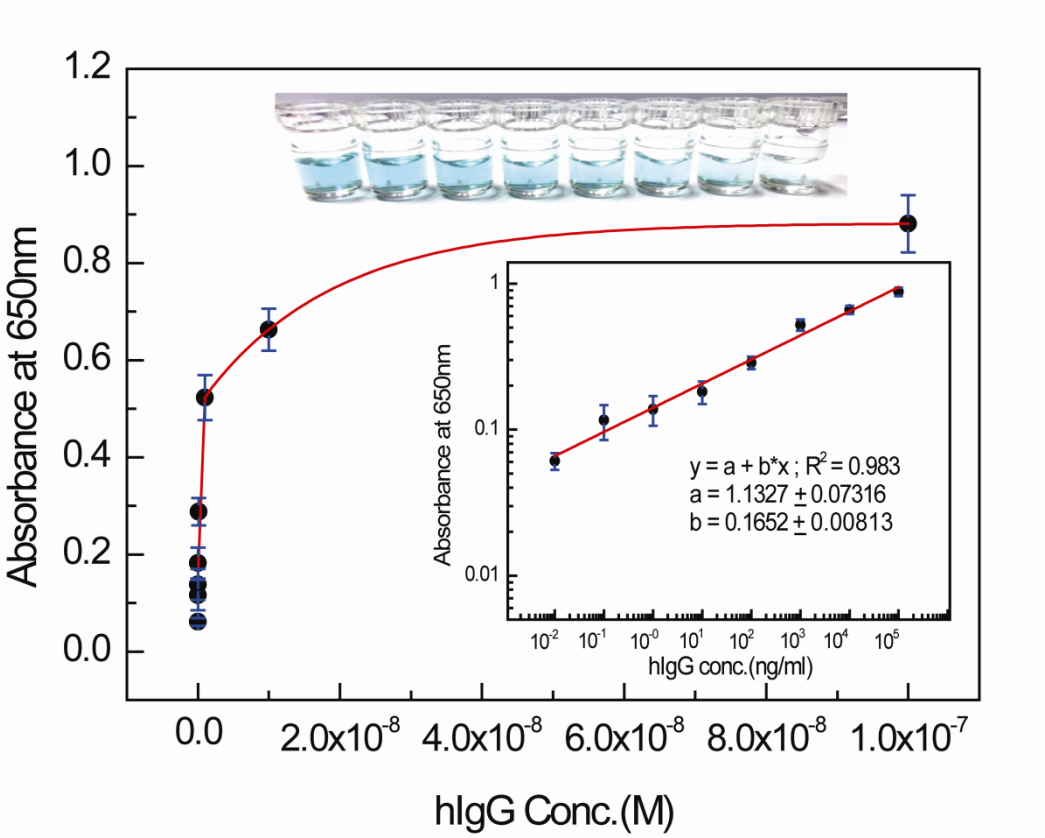


Figure 16: Application of Fe-TAML MSN in direct immunosorbnet assay

3. Conclusion

We have immobilized the [FeIII(biuret-amide)] complex on 40nm mesoporous silica nanoparticles using Cu(I) catalyzed azide alkyne click chemistry. 1-Fe-MSN has a 41 times and 5-Fe-MSN has a 662 times high K_{cat} in comparison to commercial HRP. 5-Fe-MSN nanoparticles are highly effective as a catalyst with K_{cat}/K_m ~ 2300 times higher than commercial HRP. In Further, peroxidase mimic 1-Fe-MSN and 5-Fe-MSN has 65 and 747folds higher K_{cat} than HRP using H_2O_2 as a substrate.This organic inorganic hybrid material functions as an efficient peroxidase mimic and the presence of silanol groups on the outer surface of Fe-MSN was functionalized to install targeting ligands has been used successfully for the colorimetric assay of biotin-strepavidin and h-IgG. The limit of detection (LOD) of 5Fe TAML MSN was estimated to be ~0.01 ng/mL of hIgG (3.3 pM**).**

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