# Enzyme Immobilization and Applications of Magnetic Nanoparticles in Smart Enzyme Immobilization

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## ABSTRACT

Immobilized enzymes are more vigorous and more resistant to changes in the environment as compared to enzymes free in solution. For efficient enzyme consumption their properties are enhanced by immobilization in the industrial processes. Magnetic nanoparticles are highly appreciated due to their large surface to volume ratio, ease of separation and high surface area. Immobilization of enzymes onto the magnetic nano-carriers progressively established new catalytic system. Recent advances in the enzyme immobilization improvement and maintenance have been considered in this review. Modern applications based on immobilization of enzymes onto magnetic nanoparticle are also discussed in this review.

**Keywords:** Enzyme Immobilization, Smart Enzyme Immobilization, Magnetic Nanoparticles.

## I. INTRODUCTION

In living systems, chemical species' conversion is encouraged with the help of enzymes that are pervasive catalytic agents. These catalytic agents are eco-friendly, biocompatible and are renewable resources imitative (Sheldon & Van Pelt, 2013). In biological cells crowd of diverse reactions are catalyzed by them. By-product formation can be reduced by carrying out processes involving enzymes with high selectivity and rates under trivial conditions in water like functional pH, atmospheric pressure and ambient temperature. Enzyme which has high catalytic efficiency is formed from vibrant cells (Pires-Cabral et al., 2010). In biological processes and numerous industries i.e. chemical, food and pharmaceutical, the applied use of enzymes is renowned more advantageous as compared to chemical catalyst because of its properties like adaptable activity, high specificity and catalytic efficacy (Zhao et al., 2011). Though their use in industries is restricted because of short lifespan, high separation cost and reduced stability. Consequently, applied enzyme applications can be enhanced by improving their stability (Malmiri et al., 2012).

To enhance the stability of enzyme there have been various strategies named as enzyme immobilization, protein engineering, medium engineering and enzyme modification (Huang et al., 2003). Since last four eras to make enzyme effectively applicable in industrial processes enzyme immobilization has been exploited and demonstrated, chiefly valued in improving enzyme stability, activity and specificity of substrate. Molecules of enzymes are attached or integrated into or onto large configurations by enzyme immobilization. This attachment or integration can be done by support binding, cross-linking and encapsulation (Ansari & Husain, 2012). Unique method of immobilization has been considered the one in which enzymes smart immobilization occurred through covalent binding to smart polymers or stimulus responsive and magnetic particles. Environmental fluctuations e.g. pH, ionic strength and temperature influences smart polymers and causes major changes in their organization (Galaev & Mattiasson, 2001). Magnetic decantation used to isolate the magnetic particles from reaction mixture. For evolving new system of catalysis important advancement has been done in which enzymes are immobilized onto the magnetic nano-carriers (Govan & Gunko, 2014). Magnetic particles are favorable applicants for the carrier bound immobilization of

enzyme because of their biocompatibility, deficiency of toxicity and significantly their magnetic properties (Huang *et al*., 2003; Kouassi *et al*., 2005). Magnetic particles at nano-scale enlightened the capacity of loading significantly and released diffusion restriction because they have extensive super Para-magnetism, huge surface to volume ratio and high surface area as compared to magnetite (Zhou *et al*., 2013).

Magnetic nanoparticles are advantageous more in a sense that on the supports they can control the enzyme alignment ideally. Non-permeable magnetic nanoparticles in comparison with polymeric supports have additional properties like no difficulties of exterior diffusion. These additional properties making their consumption at large industrial scale more economical particularly in solid-liquid systems (Xu *et al*., 2014). Magnetic nanoparticles and their bio-conjugate materials have been raising concern from last decade in biomedical applications like hyperthermia treatment, drug delivery, biosensors, enzymatic assays, and cell separation, environmental remediation applications and biocatalyst applications (Horak *et al*., 2007). The advances made throughout the last few years in the stabilization of enzyme based on magnetic nanoparticles are considered in this review.

## Enzyme immobilization

In industrial applications few applied problems are accompanying with the use of enzymes regardless of their many benefits as compared to ordinary catalysts. Cost of enzyme separation and purification as compared to traditional catalysts is much higher so generally they are considered as expensive. When isolated enzymes from their natural environs they have to face many denaturing situations as they are protein in nature (Pierre, 2004). They show inhibitory action in response to process circumstances e.g. pH, trace level materials and temperature because of their sensitivity which make them expensive. Most enzymes also cause contamination of product and their recovery ruled out for further use because in homogenous system of catalysis they function only when they are dissolved in water (Cherry

& Fidantsef, 2003). To overwhelm these drawbacks immobilization of enzyme was used successfully. A

heterogeneous enzyme immobilized system is created when fixation of enzymes occur onto or within the solid supports in the practical process of enzyme immobilization (Massolini & Calleri, 2005). In the living cells most of enzymes in their immobilized form are attached to membrane, organelle structures and cellular cytoskeleton, and impersonating their natural mode. Enzyme structure stabilizes and as a result maintaining their activities by solid support system. Therefore, immobilized enzymes are more vigorous and more resistant to the changes in the environment as compared to the free enzymes (Cao *et al*., 2003). Additionally, heterogeneous system of immobilized enzymes permits the enzymatic processes to operate continuously, easy recovery of enzymes and products, greater diversity of bioreactor designs, numerous recycles of enzymes and rapid termination of reactions.

Alternatively, because of substrate retrieving difficulty immobilized enzymes usually have higher Michaelis constant and lower activity as compared to their free forms (Kress *et al*., 2002). Generally, immobilized enzymes are more unwavering and can handle easily as compared to free enzymes in solution. Additionally, enzymes do not contaminate the products of reaction and it is particularly beneficial in pharmaceutical and food industries. On immobilization intensely reduction occurs in the autolysis process rate in the matter of proteases. These modifications resulted on applying the procedure of enzyme immobilization, where the molecule of enzyme undergoes the changes in their structure and microenvironment formed in which enzyme works (Koeller & Wong, 2001). Furthermore, varieties of biotechnological products formed on the basis of immobilization methods and have applications in the bio-affinity chromatography, therapeutics, biosensors and diagnostics (Petkar *et al*., 2006; Wiemann *et al*., 2009). Immobilized enzymes are used by only 20% of bio-catalytic processes despite of their clear advantages and long history (Straathof *et al*., 2002). Immobilization methods have two important properties with respect to industries and these are easiness and cost-effectiveness. Alternatively, industries required very stable derivatives preparation because of extensive reuse of immobilized enzymes and ensured that these derivatives should also have functional properties such as activity and selectivity for the given reaction (Bickerstaff, 1997). The advantages associated with enzyme immobilization

are high stability, continuous use, continuous product separation, improved process control and minimum reaction time. There are some disadvantages that are also associated with enzyme immobilization such as mass transfer limitations, lowered activity, lowered efficacy against insoluble substrates enzyme conformational changes (Singh *et al*., 2013).

## Methods of enzyme immobilization

Conventionally, methods used for immobilization of enzymes summarized in Fig. 1 (Sheldon

Van Pelt, 2013) are adsorption, covalent binding, entrapment (encapsulation), cross linking and affinity immobilization.
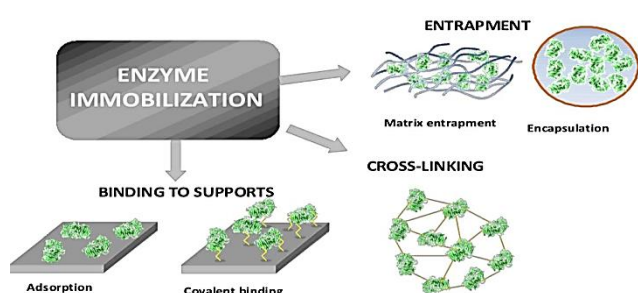


Fig. 1: Methods of enzyme immobilization

## Adsorption

Salt linkages and hydrophobic interactions resulted in enzyme adsorption. For physical adsorption dried the enzyme on the surface of electrode or immersed the enzyme on support. Enzymes proteolysis, contact with hydrophobic interfaces and accumulation are protected by adsorption (Spahn & Minteer, 2008). Eco-friendly supports like micro-crystalline cellulose, kaolin with high stability of enzyme, thiol functionalized micro/mesoporous materials and coconut fibers having property of high cation exchange and good capacity of holding water have been used by researchers (Brigida *et al*., 2010). On the pore walls silanols existence enabled immobilization of enzyme by hydrogen bonding hence making successful use of silanized molecular sieves as support for adsorption of enzyme (Mitchell & Ramirez, 2011). Most extensively familiar maybe is to use slides coated with polylysine or nitrocellulose membranes for binding electrostatically. Neither the target protein amendment nor the reagents additional coupling is

required for this kind of enzyme immobilization. For better immobilization of enzyme numerous chemical modifications can do with presently used supports (Persson *et al*., 2000). Reversible and comparatively weak interactions are involved in non-covalent immobilization. Severe conditions such as high ionic strength and high concentration of reactant and product in industries usually made very fragile physical binding with carrier to keep enzyme fixed. Consequently, adjacent media contaminated and activity lost with the passage of time because proteins leached out from the support (Karagulyan *et al*., 2008). Predominantly when this non-covalent immobilization method used in sensor devices and analytical assays it resulted in recyclability and toughness of whole systems. Activity of protein immensely lost because of proteins denaturation and changes in structure when adsorption of protein occurred on the support surfaces. Moreover, extreme flocking additionally minimized the activity because density of immobilized proteins packing is not in control (Huang *et al*., 2011).

## Covalent binding

Enzymes because of their degree of reactivity on the basis of diverse functional groups and their amino acids side chains form more stable covalent association with supports. Stability and high specific activity with precise alignment of protein have been resulted when linkages of enzyme used surfaces modified with peptide (Singh *et al*., 2013). Enzyme leakage is prohibited from the surface of support matrix by the covalent binding (Sheldon & van Pelt, 2013). Enzymes that are bound covalently received thermal stability from spacer arm glutar-aldehyde and carbohydrate moiety of cyanogen bromide activated with Sepharose and cyanogen bromide agarose (Hsieh *et al*., 2000; Cunha *et al*., 2008). Enzymes covalent binding to amended carriers of silica gel stated biocatalysts with high activity and stability (Szymanska *et al*., 2009). Covalent binding of enzymes with diverse supports such as chitosan and meso-porous silica have improved thermal stability and half-life. Due to adjustable nano sizes and thermal strength alcohol dehydrogenase binded covalently on the nanofibers of attapulgite like hydrated magnesium silicate (Zhao *et al*., 2011). Silicon coated enzymes' covalent interactions have been unscrambled with the help of bio-catalytical membranes. Covalent binding helped magnetic nano-

clusters to achieve the diverse locations of immobilized enzymes on them and due to their reusability, stability and durability they have some uses in the pharmaceutical industries (Yusdy *et al*., 2009).

## Entrapment

Enzymes incarceration within the gels or fibers with the help of covalent or non-covalent bonds is called entrapment (Singh *et al*., 2013). Mechanical stability improved and leakage of enzymes prevented by using Alginate-gelatin-calcium hybrid carriers, this making encapsulation process more efficient (Shen *et al*., 2011). The world of enzyme immobilization has been revolutionized with widespread applications of encapsulation by nanostructured supports such as pristine materials and electro-spun nano fibers in the field of biofuels, fine chemistry, biosensors and biomedicine (Wang *et al*., 2009; Wen *et al*., 2011). In chitosan the entrapment of *Candida rugosa* has been stated with the ability to improve enzyme activity and efficiency of entrapment with augmentation, leaching and friability inhibition. This support because of its hydrophilic nature has high affinity for protein, nontoxic, acquiescent to chemical amendment and biocompatibility (Erdemir & Yilmaz, 2011). Mesoporous silica because of high capacity of adsorption, uniform distribution of pore, high surface area and pore size tunability is characterized to carry out entrapment (Ispas *et al*., 2009). Wavering silane additives activity improved when magnetite nanoparticles and lipase do instantaneous entrapment with biomimetic silica. *Candida rugosa* lipase entrapped with matrices of sol-gel having polymers of supra-molecular calixarene because of their carrying and selective binding abilities (Chen *et al*., 2011).

## Cross linking

Cross linking agent played important roles during the immobilization of enzymes to conserve the functional and structural properties. Stable covalent bond subunits and their solubility in aqueous solvents making gultar-aldehyde as bifunctional cross linking agent (Cirillo *et al*., 2014). Because of enhanced porosity and surface area more residual activity is displayed by enzymes crosslinking to electro-spun nano-fibers. Biocatalyst immobilization got a revolving point owing to the use of

such supports of nano-diameters (Huang *et al*., 2008; Sakai *et al*., 2010). It has been stated that enzyme precipitation of aggregates of cross-linked enzymes has been occurred by the addition of ionic polymers or organic solvents from the aqueous solution (Sheldon, 2011). Formation of cross-linked enzyme aggregates is shown in Fig. 2(Sheldon & Van Pelt, 2013).
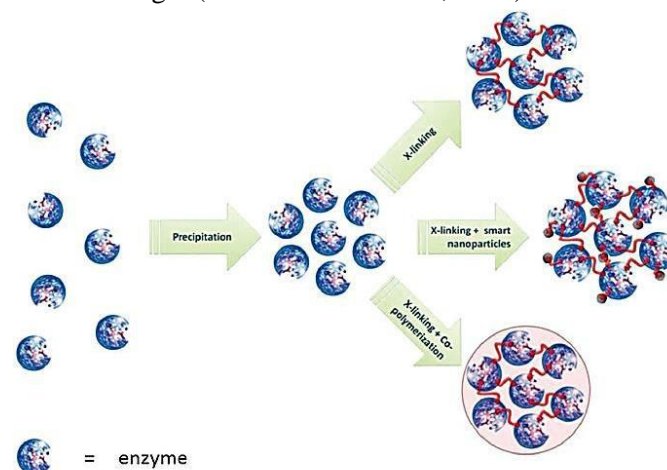


Fig. 2: Formation of cross-linked enzyme aggregates

## Affinity immobilization

Underneath the diverse functional circumstances the specificity of enzyme to its support is exploited by the affinity immobilization. The affinity immobilization is carried out in two ways: either the affinity is developed by the conjugation of enzyme toward the matrix or for the protein of interest the affinity ligand is pre-coupled to the matrix (Sardar *et al*., 2000). For the immediate enzyme purification the affinity absorbents can also be used (Ho *et al*., 2004).

Enzymes higher quantities which are responsible for improved efficacy and stability are anchoraged by complex support affinity such as multilayered concanavalin A linked with agarose and porous beads coated with chitosan that is alkali stable (Shi *et al*., 2003; Sardar & Gupta, 2005). Non-covalent forces like Vander Waals forces, hydrogen bonding and coulombic forces have improved exponentially the enzyme reusability and capacity of binding that lead to the creativeness of bio-affinity layering (Haider & Husain, 2008).

## Smart enzyme immobilization

Through covalent binding immobilization of enzymes can be done to magnetic particles and smart polymers (stimulus-responsive). Fluidized bed reactors that are stabilized magnetically and magnetic decantation can be used to detach the particles from the reaction mixture (Yiu & Keane, 2012).

## Smart polymers

Smart polymers are water soluble, synthetic and functional polymers that can also be called as stimulus responsive (Galaev & Mattiasson, 2001). The suitable stimulus i.e. ionic strength, temperature, magnetic or electric field, addition of a chemical species or pH cause intense changes in the solubility of these polymers those are reversible. All the hydrogel systems and smart polymers according to their sensitivity can be categorized into five sets such as twofold stimuli responsiveness polymers, phase sensitive, light sensitive, heat sensitive and pH sensitive smart polymers (Ghaz-Jahanian *et al*., 2015). Enzyme immobilization, bio-separation, tissue engineering and drug delivery are some important uses of smart polymers (Mahajan & Aggarwal, 2011). Smart polymeric behavior does not change by immobilizing enzyme on them and these bio-conjugates are thus known as smart biocatalysts. Chitosan, j-carrageenan and alginate are such naturally occurring polymers but it is also possible to synthesized smart polymers chemically. A biocatalyst is obtained by covalent or non-covalent binding of enzymes with these polymers as shown in the Fig. 3(Bickerstaff, 1997).
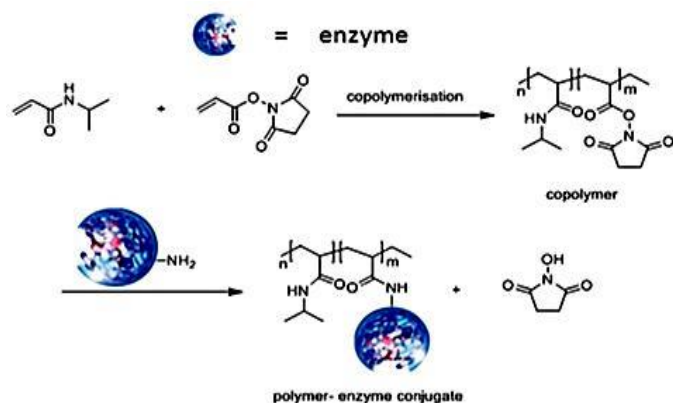


Fig. 3: Polymer-enzyme conjugates as thermo-responsive biocatalysts

## Magnetic particles

For binding of enzymes, drugs, proteins and antibodies magnetic particles have been used progressively as carriers. Procedures of magnetic immobilization have great influence in different fields of biotechnology and biomedicine for biologically active compounds. Target cells and molecules can be captured or modified for affinity ligands by immobilized biomolecules (Bickerstaff, 1997). Enzymes and proteins immobilization on magnetic particles is of great concern. Worthy results have been obtained by using magnetic polymer microspheres and copolymers with magnetic particles. The advancement of new catalytic system has noteworthy improvement from last few years in which magnetic nano-carriers are immobilized (Dyal *et al*., 2003).

## Magnetic nanoparticles

For the development of enzyme stabilization usually huge surface area is provided by the variety of nanostructures for the enzyme immobilization. Magnetic nanoparticles (MNPs) are predominantly important and useful group of nanocarriers (Govan & Gunko, 2014). Nano-scale magnetic particles have distinct qualities i.e. large surface to volume ratio, super-para-magnetism, reducing the diffusion limitation, high surface area and ease of separation (Xu *et al*., 2014). Beneficial properties and high activity has been shown by enzyme immobilization on magnetic nanoparticles. Biocompatibility and low toxicity of maghemite ($\gamma$-$Fe_2O_3$) and magnetite ($Fe_3O_4$) made them most frequently used magnetic particles (Zhou *et al*., 2013). Several materials for the immobilization of enzyme have been made as more reasonable hosts by the innovations in nano- and hybrid-technology (Singh *et al*., 2013). Functional parts of magnetic nanoparticle carriers are magnetic core, functional outer coating and surface coating to defend magnetic core (Vatta *et al*., 2006).

Catalytic magnetic reclamation and immobilization of enzyme is empowered by magnetic nanoparticles. The preservation of these materials is allowed by the catalyst binding to magnetic nanoparticles for reuse after the completion of reaction (Govan & Gunko, 2014). As compared to aforementioned techniques in order to

eradicate the catalyst this will not require an extra purification procedure and making itself a green catalyst (Baig & Varma, 2013). The enzyme immobilization onto the magnetic nanoparticles has numerous technical benefits. Especially, the cost production is reduced by the magnetic repossession of dynamic material. In contrast to unsupported proteins the immobilized enzymes show improved pH stability, higher activity and temperature stability (Hola *et al*., 2015). As compared to free enzyme, greater activity showed by immobilized enzymes when tested against varied pH and temperature ranges as shown in Fig. 4(Chen *et al*., 2014).
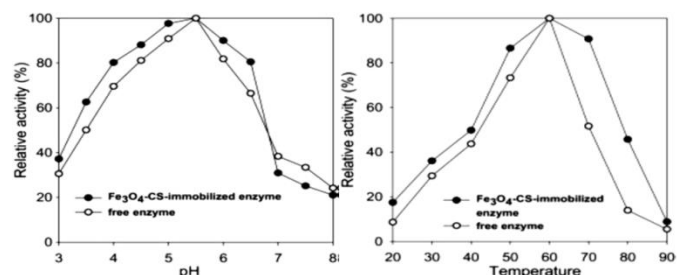


Fig.4: Change in enzymatic activity with changing pH and temperature to compare enzymes immobilized on MNPs with the free enzyme

## Magnetic properties

Magnetic vulnerability is defined as the ratio of induced magnetism to the applied magnetic field which is the basis of organization of magnetic material's properties. Magnetism is divided into five categories named as diamagnetism, para-magnetism, ferromagnetism, ferrimagnetism and anti-ferromagnetism (Indira & Lakshmi, 2010). Domains partitioned the ferromagnetic materials into zones. These domains' moments are orientated arbitrarily in an un-magnetized sample but they tried to arrange them in the direction of applied external magnetic field. Domain walls development become unfavorable energetically when then size of particle reached to critically minimum size even at nano scale range (Willard *et al*., 2004). In magnetization rotation of spins caused fluctuations and particles with these characteristics are called as single domain. Super-paramagnetic particles are formed when thermal variations affected the spins of further reduced size particle. Exposure of external magnetic field causes magnetization of individual particles in the super-paramagnetic materials and large surface area is provided by particle size for functionalization (Vatta *et al*. 2006).

## Applications of magnetic nanoparticles

Magnetic nanoparticles have variety of applications among several nanostructures. Low toxicity, biocompatibility and super-paramagnetism are unique characteristics of magnetic nanoparticles (Ashtari *et al*., 2012). They have potential applications as shown in Fig. 5 like drug delivery, immobilization of cells and enzymes, bio-separation systems, biosensors and immunoassays (Govan & Gun'ko, 2014). Cell sorting/targeting, cell sensing and magnetic resonance imaging have been new applications of magnetic nanoparticles in the field of biomedicines (Chomoucka *et al.,* 2010). They also have applications in the extraction of targeted cells from the biological samples and cultures, empowering magnetic catalyst recovery and support immobilization. For enzymes continuous operation they can stabilize easily in the fluidized bed reactor (Baig & Varma, 2013).
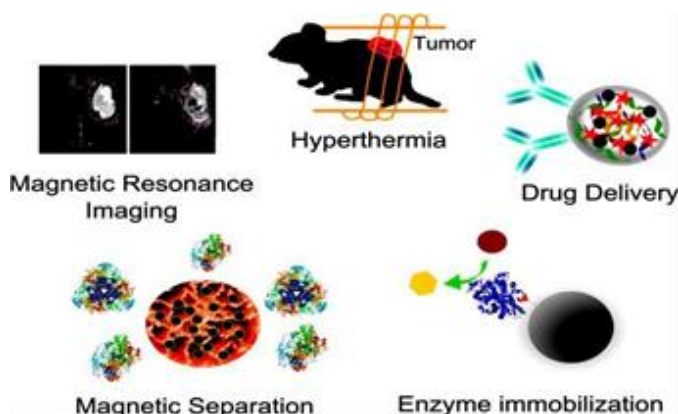


Fig. 5: Applications of magnetic nanoparticles

## Applications of magnetic nanoparticles in smart enzyme immobilization

Highly developing areas of biotechnology research encompassing enzymes in magnetic nano systems. Designs of various types of magnetic nanoparticles with diverse arrangement of core-shell are outlined graphically in Fig. 6 (Hola *et al*., 2015). In few abridged nanoparticle assemblies the size of magnetic nanoparticles varied from few nanometers to several hundred nanometers. These nano-composites have potential application in the food industry as food processing units. They also have application in the

generation of biofuels as renewable "bio-catalysts" (Ansari & Husain, 2012).



Fig. 6: General types of MNPs arrangements with polymers, molecules and inorganic NPs

Immobilization of enzymes on magnetic nanoparticles has several applications that are summarized in Table 1.

**Merits of immobilized enzymes on magnetic nanoparticles**

For commercial applications immobilized enzymes are beneficial due to cost-effectiveness, ease in handling, reusability and ease of enzyme separation from the reaction mixture (Ansari Husain, 2012). Ideal regulation of enzyme configuration on the support material is an important advantage of magnetic nanoparticles. Non-porous nanoparticles are more competitive in the solid-liquid systems for large scale industries because they have no problems of external diffusion (Xu *et al.*, 2014). Moreover, magnetic nanoparticles display low or no allergenicity. Under operational and storage conditions they show higher stability towards autolysis and denaturation by heat (Sheldon & van Pelt, 2013).
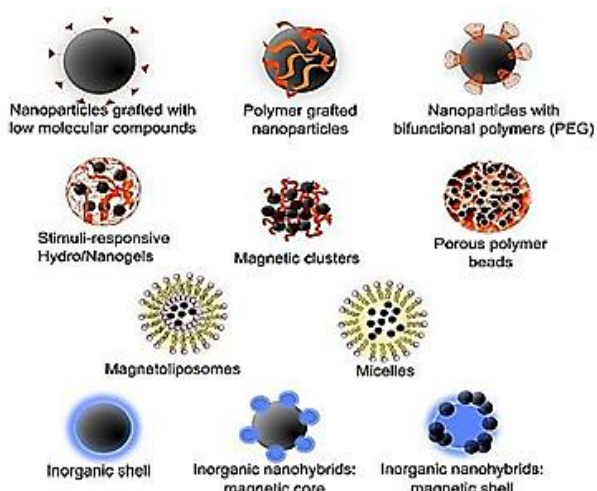
Table 1: Examples of enzymes immobilized on MNPs and their application

| Enzyme | Magnetic carrier | NP precipitation method | Characteristics of NP | Immobilization Method | Kinetic parameters | Applications | References |
|---|---|---|---|---|---|---|---|
| Alkaline Phosphatase | $Fe_3O_4$ | Co-precipitation | ___ | Cross-linking | Relative activity (%): 38, 44 | Plasmid dephosphorylation | Saiyed *et al.* (2007) |
| α-Amylase | $Fe_3O_4$ @ cellulose | Co-precipitation | Nanoparticle: 2.5–22.5 nm | Covalent Attachment | ___ | Degradation of starch | Namdeo & Bajpai (2009) |
| Cellulase | Carboxylic acid functionalized $Fe_3O_4$ | Co-precipitation | Nanoparticle: polydisperse 13.28 ± 3.9 nm Conjugated enzyme: 13.31 ± 3.2 nm | Cross-linking | Relative activity (%): 30.2 | Green synthesis of cellulosic ethanol | Jordan *et al.* (2011) |
| Cholesterol Oxidase | Gamma-$Fe_2O_3$ @ $SiO_2$@ APES | Co-precipitation | ___ | Cross-linking | Free enzyme Ea = 13.6 kj/mol Immobilized enzyme Ea = 9.3 kj/mol | Analysis of total Cholesterol In serum | Kouassi *et al.* (2005) |
| D-Amino acid oxidase | $Fe_3O_4$ @ APES | Co-precipitation | Nanoparticles: spherical, 12 ± 2 nm Conjugated enzyme: spherical, 13 ± 3 nm | Cross-linking | Recovered activity (%) 63 | ___ | Hsieh *et al.* (2009) |
| Esterase | $Fe_3O_4$ @ APES | Co-precipitating | Nanoparticles: 11.9 nm | Cross-linking | Free enzyme Km: 34 mM Vm: 1.4 | ___ | Ashtari *et al.* (2012) |
| Lipase from *Thermomyces lanuginose* 6 | Amino functionalized $Fe_3O_4$ | Co-precipitation | Nanoparticles: spherical or Ellipsoidal 11.2 nm Conjugated enzyme: 12.7 nm | Covalent binding | Recovered activity (%): 71 | Enzymatic transesterification of soybean oil | Xie & Ma (2010) |

**Demerits of immobilized enzymes on magnetic nanoparticles**

Magnetic nanoparticles instead of their associated benefits also have some disadvantages such as conformational changes of enzyme, mass transfer limitations, lowered activity, changes in properties, lowered efficacy and enzyme denaturation. Additionally, stable derivative preparation is required for the use of

immobilized enzymes at industrial scale for long duration (Singh *et al*., 2013).

## II. CONCLUSION

Recent advances made in the stabilization of enzyme based on magnetic nanoparticles are considered in this review. Enzymes that are immobilized magnetically due to their properties such as ease of reusability and separation have benefits for commercial applications. Non-porous nanoparticles are more competitive for large scale industries because they have no problems of external diffusion. Regardless of associated benefits enzyme immobilization also suffering from some difficulties in low efficacy, denaturation and mass transfer. For long term enzymes applications in industries these issues have to be addressed in the further research.

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