Volume 6, Issue 6, 2016, 1585 - 1606 ISSN 2069-5837
Biointerface Research in Applied Chemistry
www.BiointerfaceResearch.com
Review Article Open Access Journal
Received:22.08.2016 / Revised: 22.09.2016 / Accepted: 15.10.2016 / Published on-line: 05.11.2016

Magnetic nanoparticles as a tool for the immobilization/stabilization of hydrolases and their applications: An overview

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ABSTRACT
Magnetic nanoparticles due to their unique properties have emerged as an excellent support for the immobilization/stabilization of enzymes. Various kinds of required modifications in magnetic nanoparticles have been made in order to use them for immobilization of enzymes, hydrolases. Most of the magnetic nanoparticles bound hydrolases exhibited significantly very high stability towards heat, pH and other kinds of denaturants. Hydrolases immobilized on magnetic nanoparticles were found more resistant to denaturation mediated by organic solvents and less inhibitory to their specific inhibitors compared to free enzymes. Enzymes immobilized on magnetic supports have obtained remarkably high operational stability and maintained good activity even after several repeated uses in batch processes. Enzymes from varying subclasses of hydrolases have successfully been immobilized on the surface of modified and unmodified magnetic nanoparticles or entrapped within the network of polymeric support containing magnetic nanoparticles. In this manuscript an effort has been done to review latest literature in the area of research. The magnetic nanoparticles bound enzymes have independently been used in various fields such as biomedical, biotechnological, environmental, bioanalysis, therapeutic and several other applications.
Keywords: *Enzymes; hydrolases; immobilization; magnetic nanoparticles; reusability; stabilization; thermostability.*

1. INTRODUCTION
Enzymes are naturally occurring biocatalysts present in all living organisms and they catalyze plenty of biochemical reactions. These reactions may be within the cell, nucleus or outside cell. Enzymes require specific environment for their catalytic action. The enzymes have shown their wide spectrum applications in varying disciplines such as medical, biochemical, analytical, environmental, fuel and food industry and in other biotechnological fields [1-3]. The use of soluble enzymes at industrial level is restricted due to their high cost, limited stability, poor reusability and difficulty to use in continuous reactors. Enzyme immobilization technology offered an effective way to solve such problems, which not only improves enzymes catalytic efficiency and operational stability but also enhanced their reusability, separation and continuous applications at industrial scale [4, 5]. To date, a large number of carriers and methods have been considered for the purpose of enzyme immobilization in order to use them in various fields [6, 7]. The performance of immobilized enzymes depend on different factors such as method and support material used, enzyme and support pretreatment procedures and reaction conditions required during immobilization. However, the application of enzyme technology at large scale is rarely noticed during industrial processing. The main reasons are the high cost of immobilization and poor performance of immobilized enzymes [5, 8-10].

The industrial importance of hydrolases exceeds that of other classes of enzymes. The main area of application of hydrolases is the dissolution of biopolymers such as starch, pectin, cellulose, chitin and protein; in many cases it has been desired to obtain complete solubilization of macromolecules. The immobilization of enzymes acting on macromolecules is not suitable due to steric hindrances between enzyme active site and macromolecular structure of their substrates. The other problem of the enzymes catalyzing the hydrolysis of insoluble substrates is the difficulty in the separation of immobilized enzymes from the reaction mixture [11]. Nanobiocatalysis is relatively a new field that is a combination of nanotechnology and biocatalysis which is fastly growing as the latest discipline of nanobiotechnology [12-14]. Nanoparticles (NPs) are quite fascinating molecules due to their novel properties such as biocompatibility, large surface area, very high surface-to-volume ratio and modifiable surfaces [15, 16]. Magnetic nanoparticles (MNPs) along with other properties of NPs have additional advantages such as easy recovery and help in separation of enzyme from reaction mixture simply by applying an external magnetic field [17, 18].

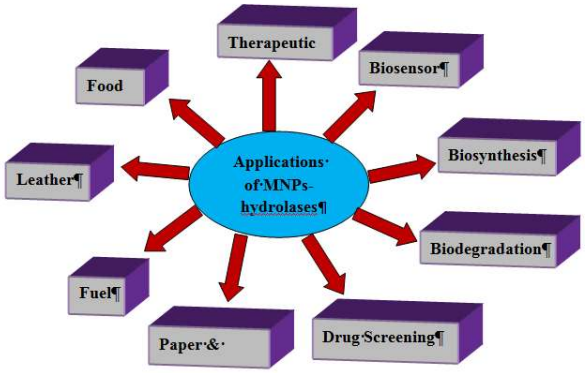

Figure 1. Demonstrates various applications of MNPs bound hydrolases.

Magnetic carriers provide an easy recycling of enzymes and make them suitable to work in aqueous, water miscible and water immiscible organic solvents. Moreover, by applying an external magnetic field the movement behavior and direction of the immobilized enzyme can be easily controlled in order to improve

their catalytic efficiency as compared to traditional mechanical stirring procedures [19-21]. Figure 1 summarizes various applications of MNPs bound hydrolases.

In this manuscript an effort has been made to survey the literature based on immobilization of hydrolases on various types of simple and modified MNP supports. The effect of nanomaterials binding on the activity and stability of enzymes belong to various sub-classes of hydrolases such as carbohydrases, lipases, proteinases or proteases, ureases and other types of hydrolases after immobilization has been summarized in detail. The applications of immobilized enzyme preparations in varying fields have also been illustrated. The merits and demerits of the MNPs immobilized enzymes and their future developing prospects are also discussed.

2. HYDROLASES

Hydrolase (EC 3) is a specific class of enzymes that works to control the process of hydrolysis in living organisms. There are many types of hydrolases, each catalyzing different reactions. These enzymes are divided into various subclasses, depending on the particular bonds they act upon during chemical processes. Hydrolases catalyze breakdown of different types of substrates into smaller molecules in the presence of water. These substrates include phosphate esters, fats, proteins, cellulose, starch and nucleic acids [22]. Some more specific hydrolases also catalyze reactions that break ether (C-O) bonds, carbon-nitrogen (C-N) bonds other than peptide bonds, acid anhydride bonds, carbon-carbon (C-C) bonds and phosphorus-nitrogen (P-N) bonds.

3. CARBOHYDRASES

Table 1 summarizes MNPs support immobilized carbohydrases, their mode of immobilization and improved properties.

Table 1. MNPs immobilized carbohydrases, their mode of immobilization and improved properties.

Name of enzyme	Name of support	Mode of immobilization	Property/properties Enhanced	Reference
Diastase	Ferric impregnated silicon oxide particle	Adsorption	Excellent reusability	[26]
Glucoamylase	Amino-functionalized MNPs, functionalized MCNTs	Adsorption, covalent & metal ion affinity binding	Superior stability & high reusability	[32, 33]
Thermophilic Bacillus sp. strain TS-23 α-amylase	Adipic acid-modified MNPs	Bioaffinity binding	Excellent thermostability & reusability	[23]
Amylase	MMIPs		Very high reusability	[24]
AMG	Magnetic single-walled carbon nanotubes, MNPs	Adsorption, covalent binding	Enhanced substrate affinity, high thermal & storage stability, & reusability	[30,31]
β-glucosidase	Magnetic Fe_3O_4 NPs	Covalent binding	Wider pH & temperature ranges of activation, better thermal & storage stability	[37]
Cellulase from *Trichoderma reesei*	Activated magnetic support	Covalent binding	K_m decreased, High temperature-optima, good reusability	[47]
Cellulase	Molecular imprinted supermagnetic Fe_3O_4@SiO_2 NPs	Adsorption	High catalytic efficiency & temperature optima, better thermal stability	[48]
Kluyveromyces fragilis β-galactosidase	The magnetic PGMA, EGDA & HEMA nanobeads	Covalent binding	High synthetic activity, excellent reusability	[52]
Aspergillus oryzae lactase	Darboxylic acid functionalized MNPs	Covalent binding	Decreasing size of MNPs increased lactase bound activity	[53]
Invertase	Polyvinylimidazole -grafted MNPs	Adsorption	Enhanced thermal, operational & storage stability	[57]
β-mannosidase	MNPs	Covalent binding	V_{max} increases, K_m decreases	[58]
Pectinase	Amino functionalized silica-coated MNPs	Covalent binding	Enhanced stability	[60]

3.1. α-Amylase. The α-amylase (EC 3.2.1.1) catalyzes hydrolysis of α-D-1,4 glycosidic bonds present in starch, glycogen and other related polysaccharides. It breaks down long-chain carbohydrates into dextrins and different gluco-oligosaccharides of variable

lengths. The systematic name for this enzyme is 1,4-α-D-glucan glucanohydrolase and the other name is glycogenase. The adipic acid-modified MNPs support was used for efficient immobilization of C-terminally lysine-tagged α-amylase from thermophilic Bacillus sp. strain TS-23. Soluble amylase was active in the range of 45-70^{o}C and had an optimum-temperature at 60^{o}C, while the thermal stability of bound enzyme was remarkably enhanced upon immobilization. The immobilized enzyme exhibited better storage stability as compared to free enzyme and was reused 20-times without any loss in its initial activity [23]. Lee et al. [24] investigated the immobilization of amylase on the magnetic molecularly imprinted polymers (MMIPs). The activities of both bound template and rebound enzyme were confirmed by measuring glucose production via starch hydrolysis, at different temperatures, for MMIPs with different compositions. The highest hydrolytic activity of MMIPs was 1545.2 U g^{-1}. MMIPs have advantages of high surface area, suspension, easy removal from reaction and rapid reload of enzyme as compared to conventional catalytic process. Khan and coworkers [25] immobilized porcine pancreatic α-amylase on Fe_2O_3-MNPs. The MNPs bound enzyme maintained 94% of the original activity. Immobilized enzyme showed lower pH optimum at pH 6.0 while the free α-amylase had its pH-optima at pH 7.0. Optimum temperature for the immobilized enzyme shifted towards higher temperatures, 50^{o}C compared to native enzyme, 40^{o}C. The immobilized enzyme obtained greater resistant to inactivation mediated by different metal ions and chemical denaturants. Immobilized α-amylase hydrolyzed 92% starch in a batch process in 8 h at 40°C whereas the free enzyme hydrolyzed only 73% starch under similar experimental conditions. MNPs bound α-amylase retained 83% activity after its 8-repeated uses. Diastase was immobilized onto ferric impregnated silicon oxide particle. The MNPs immobilized enzyme was characterized for its catalytic activity and kinetic behavior. MNPs-bound enzyme was successfully reused more than 50-times without any significant loss in its original activity [26].

3.2. Amyloglucosidase/glucoamylase.

Glucoamylase (1,4- α-D-glucan glucohydrolase, EC 3.2.1.3) catalyzes hydrolysis of α-1,4 and α-1,6 glucosidic bonds to release β-D-glucose from non-reducing ends of starch and related poly- and oligosaccharides [27]. Fungal glucoamylases are commonly exploited for the production of glucose and fructose syrups. α-Glucosidase was covalently immobilized onto magnetic nanospheres using glutaraldehyde. Immobilized α-glucosidase exhibited very high activity and stability and was reused for 10-times without any significant decrease in its activity [28]. *Aspergillus niger* amyloglucosidase (AMG) was coupled to the silver-iron oxide NPs via sonication. The immobilization brought a multi-fold increment in the enzyme specific activity, 807 U mg^{-1} compared 69 U mg^{-1} for soluble enzyme. The immobilized enzyme retained remarkably very high activity when stored at room temperature for long time [29]. Goh et al. [30] immobilized AMG on magnetic single-walled carbon nanotubes. Enzyme was attached to the support by physical adsorption and covalent attachment. Immobilized AMG retained its full activity when stored at 4°C for at least one month. AMG from *Aspergillus niger* was directly immobilized by novel method of crosslinked enzyme aggregate onto MNPs. AMG was covalently linked to the MNPs to form a monolayer of AMG (MNPs-AMG), followed by crosslinked aggregates with free AMG, which was not immobilized to yield MNPs with high enzyme loading (MNPs-AMGn). Very high recovery, 92.8% of enzyme activity was achieved under standard experimental conditions, in MNPs-AMGn using 14-times less support as compared to the amount of support needed by traditional procedure. MNPs-AMGn exhibited greater affinity for substrate, thermal and storage stability and reusability [31].

Zhao and colleagues [32] evaluated covalent attachment of glucoamylase on the amino-functionalized MNPs via glutaraldehyde coupling. Two novel regenerated strategies of supports were successfully developed to regenerate them at the end of life of immobilized glucoamylase. The results showed that the strategies for the regeneration of supports are viable and the regenerated support can be further reused for the immobilization of enzymes other than glucoamylase. The regenerated strategies also offer an attractive and flexible alternative to regenerate other traditional supports at the end of life of immobilized enzyme. In a further study these workers immobilized glucoamylase on the functionalized magnetic carbon nanotubes (MCNTs) by adsorption, covalent binding and metal-ion affinity interactions. The MCNTs immobilized glucoamylase exhibited superior stability and reusability than free enzyme. Moreover, the finding of the work showed that the metal-chelate dendrimer provided an efficient route to immobilize enzymes via metal-ion affinity interactions. The applicability of regenerated supports in this study is also significant for the immobilization of other enzymes [33].

3.3. Cellobiases/β-glucosidase.

β-1,4 glucosidase or cellobiase (EC 3.2.1.21) hydrolyzes β-1,4 bonds present in cellobiose. This enzyme is one of the components of cellulases [34]. Wang et al. [35] evaluated the effects of silica-derived nano-supports; core shell magnetite NPs and SBA-15 on the activity and stability of cellobiase. The cellobiase immobilized on both nano-supports demonstrated an improvement in its stability under low pH and high temperature conditions. SBA-15 had higher amount of bound protein than the core shell magnetite NPs, but exhibited lower specific activity than core shell magnetite NPs, it might be due to change in silica surface properties promoted during support synthesis. Zhou et al. [36] developed a novel and efficient β-glucosidase immobilization method using magnetic Fe_3O_4 NPs as a carrier. The immobilized β-glucosidase showed wider pH and temperature ranges of activation, higher accessibility of substrate, better thermal and storage stability as compared to soluble enzyme. The enzyme-MNPs composite was easily separated magnetically during its repeated uses. MNPs bound β-glucosidase exhibited its potential for industrial applications. Verma et al. [37] have covalently immobilized a thermostable β-glucosidase from *Aspergillus niger* on the functionalized MNPs and the obtained yield of enzyme immobilization was 93%. This preparation was used for biofuel production. Immobilized enzyme exhibited different pH-optima but the same temperature optima compared to free enzyme. Thermal stability of immobilized enzyme was enhanced at 70°C. The MNPs immobilized enzyme retained more than 50% enzyme activity after 16-repeated uses, and the bound enzyme produced maximum glucose from cellobiose in 16 h. A novel magnetic Fe_3O_4 NPs coupled with agarose was synthesized using co-

precipitation via alkaline condition and span-80 surfactants in organic solvent. Imino diacetic acid (IDA) was first attached to the MNPs via epichlorohydrin (ECH) and then chelated with metal ions. Among them, the Co^{2+}-chelated MNPs (MNPs-ECH-IDA-Co^{2+}) showed the second highest enzyme adsorption capacity of 1.81 mg g^{-1} particles and achieved largest activity recovery of 117% g^{-1} protein in immobilization of β-glucosidase. K_m and V_{max} of the immobilized β-glucosidase were 0.904 mM and 0.057 μmol min^{-1}, respectively and its activation energy was much lower than the soluble enzyme. Moreover, the immobilized enzyme exhibited enhanced thermal and operational stability. MNPs bound enzyme maintained nearly 90% activity even after 15 repeated uses in batch processes. This study demonstrates that the immobilized β-glucosidase has an excellent future in industrial applications [38].

The magnet directed enzyme/prodrug therapy was developed by coupling β-glucosidase to aminated starch-coated iron oxide MNPs using glutaraldehyde. MNPs conjugated enzyme maintained 85.54%±6.9% activity and exhibited remarkably high thermostability. The animal investigation findings demonstrated that β-glucosidase-MNPs showed preferable pharmacokinetics properties in relation to MNPs. A significant quantity of β-glucosidase-MNPs was specifically delivered into a subcutaneous tumor of a glioma-bearing mouse. The enzyme activity of the applied β-glucosidase in tumor lesions showed as high as 20.123±5.022 mU g^{-1} tissue with 2.14 of tumor/non-tumor β-glucosidase activity [39]. β-Glucosidase was linked to aminated magnetic iron oxide NPs via glutaraldehyde and further PEGylated via N-hydroxysuccinimide (NHS). β-Glucosidase-MNPs and polyethleneglycol (PEG)-β-glucosidase-MNPs retained 73.0% and 65.4% of its activity, respectively. Both magnetophoretic mobility and pharmacokinetics studies demonstrated that PEG-glucosidase-MNPs *in vitro/in vivo* showed greater stability than the β-glucosidase-MNPs. *In vivo* magnetic targeting of PEG-β-glucosidase-MNPs has been evaluated by magnetic resonance imaging and electron spin resonance analysis in a mouse model of subcutaneous 9L-glioma. The accumulation of PEG-β-glucosidase-MNPs in tumor tissue was successfully achieved, with an iron content of 627±45 nmol Fe g^{-1} tissue and β-glucosidase activity of 32.2±8.0 mU g^{-1} tissue [40].

3.4. Cellulase.

Cellulase (EC 3.2.1.4) catalyzes hydrolysis of cellulose and other related polysaccharides. It hydrolyzes 1,4-β-D-glycosidic bonds in cellulose, hemicellulose, lichenin, and cereal β-D-glucans. This enzyme is produced by various bacteria, fungi and protozoans. Cellulases hydrolyze cellulose into monosaccharides, simple sugars such as β-glucose, or shorter polysaccharides and oligosaccharides. There are various synonyms, derivatives and specific enzymes associated to the name "cellulase" for example endo-1,4-β-D-glucanase, β-1,4-glucanase, β-1,4-endoglucan hydrolase and cellulase A etc. Cellulase was immobilized on the carbodiimide activated Fe_3O_4 MNPs. The immobilized enzyme showed optimum temperature at 60°C and extensive pH-optimum from 3.94 to 5.50. The result demonstrated that immobilized enzyme exhibited better thermal and storage stability [41]. Liao et al. [42] immobilized cellulase on polyvinyl alcohol (PVA)/Fe_2O_3 MNPs which retained very high activity. Immobilized cellulase along with wet ball milling was used to degrade microcrystalline cellulose and it produced 1.89 mg mL^{-1} glucose which was at least three-times as compared to the sum of individual yield. The immobilized cellulase maintained 40% activity after 4 repeated uses. The findings of the work have shown that the immobilization of cellulase along with wet ball milling is a novel method to improve remarkably cellulose hydrolysis efficiency. Cellulase was immobilized on MNPs via glutaraldehyde. Fourier transform infrared (FTIR) analysis had confirmed covalent binding between residual amine groups of Fe_3O_4 MNPs and cellulase. The immobilized enzyme retained significantly greater fraction of activity in broad pH and temperature ranges and improved storage stability. The obtained low K_m value for immobilized cellulase exhibited higher affinity for cellulosic substrate. The immobilized cellulase better hydrolyzed steam-exploded corn stalks than the bleached sulfite bagasse pulp [43]. Jordan et al. [44] evaluated covalent immobilization of cellulase on carbodiimide activated Fe_3O_4 MNPs. The maximum binding was 90% at low enzyme loadings (1-2 mg) and the enzyme-to-support saturation point took place at a weight ratio of 0.02. Immobilized enzyme showed high stability over a wide range of temperatures and the optimum temperature was at 50°C. The ionic forces between the enzyme and support caused a change in the optimum pH from 4.0 to 5.0. The cellulase was immobilized on superparamagnetic NPs via ionic bonds with binding efficiency of 95% and adsorption capacity of 31 mg g^{-1} NPs. The results demonstrated that the stability and activity of the cellulase were enhanced via physical adsorption to MNPs. It is evident from the findings of the work that immobilized enzyme can be used in variety of application at wide ranges of temperatures and pH [45].

Three cysteine-tagged cellulases were co-immobilized on AuNPs and Au-doped magnetic silica NPs for the hydrolytic degradation of cellulose. The biochemical properties, stabilities, activities and reusability of these co-immobilized systems were enhanced as compared to those of mixtures of free cellulases [46]. *Trichoderma reesei* cellulase was immobilized on the activated magnetic support by covalent binding. MNPs retained about 94% protein of cellulase. Immobilized enzyme showed same pH-optima, pH 4.0 and higher temperature-optima at 60°C as compared to soluble enzyme. The K_m of the immobilized enzyme decreased. The immobilized enzyme retained 50% of the initial activity after its 5th repeated use. Immobilized cellulase hydrolyzed greater percentage of pretreated hemp hurd biomass as compared to free enzyme. The immobilized enzyme had merit over the free enzyme in terms of reusability and longer storage stability [47]. The molecular imprinted supermagnetic Fe_3O_4@SiO_2 NPs were used as support for the immobilization of cellulase. The adsorption of cellulase on MNPs was quite fast and specific. The immobilization yield was 95% and half-life of immobilized cellulase was 3.3-fold of the free-enzyme at 70°C. The immobilized cellulase has same pH-optima, higher temperature-optima, better thermal stability and higher catalytic efficiency as compared to native enzyme. The results strongly suggest that the immobilized cellulase on molecular imprinted Fe_3O_4@SiO_2 was successfully used for the production of bioethanol, paper & pulp and in pharmaceutical industry [48]. Cellulase was covalently attached to chitosan coated magnetic Fe_3O_4 MNPs support by using glutaraldehyde. The amount of cellulase immobilized on MNPs was 112.3 mg g^{-1}. The immobilized cellulase had higher operational stability than free

enzyme over broad spectrum of temperature and pH and retained good activity on its repeated uses [49].

The immobilization of cellulase cocktail in the form of cross-linked enzyme aggregates was done onto amine functionalized core-shell Fe_3O_4@silica MNPs. There was a slight change in the optimum-pH of the cellulase cocktail towards acidic side while the temperature-optima remained unaltered at 65ºC. Carboxymethyl cellulase (CMCase) activity of immobilized enzyme at higher side of pH and temperature-optima was remarkably different from those of soluble cellulase. Immobilized cellulase retained about 65% of its initial activity at higher temperature up to 80ºC. Crosslinked cellulase aggregates-MNP exhibited improved thermal and operational stability than native enzyme. MNPs bound crosslinked cellulase maintained 30% CMCase activity after 6-repeated uses [50]. *Aspergillus niger* cellulase was immobilized on β-cyclodextrin-coupled MNPs by silanization and reductive amidation. The immobilized cellulase gained supermagnetism due to the MNPs. Activated MNPs retained 90% of the added enzyme activity. Ionic liquid, 1-butyl-3-methylimidazolium chloride was introduced into hydrolytic process in order to improve performance of immobilized cellulase. The rate of immobilized cellulase catalyzed hydrolysis of rice straw was enhanced from 1.629 to 2.739 $g\,h^{-1}\,L^{-1}$ in presence of ionic liquid. Immobilized cellulase had very high reusability and it was reused 16-times without a significant loss in its activity. Magnetized cellulase can be recycled by magnetic field more easily than free enzyme and immobilized enzyme retained 85% of its initial activity in the presence of high concentration of glucose, 15 $g\,L^{-1}$. The results showed that the immobilized cellulase hydrolyzed more rice straw continuously compared to soluble enzyme. The amount of harvested glucose was 20-times higher than the hydrolysis obtained by free enzyme [51].

3.5. β-Galactosidase/lactase.

β-Galactosidase (EC 3.2.1.23) catalyzes hydrolysis of β-galactosides into monosaccharides. Substrates of different β-galactosidases include ganglioside GM1, lactosylceramides, lactose and various glycoproteins. It is also called β-gal. Lactase is often confused as an alternative name for β-galactosidase, but it is a sub-class of β-galactosidase. The magnetic poly(glycidylmethacrylate) [PGMA], ethylene glycol dimethacrylate (EGDA) and hydroxyethyl methacrylate (HEMA) nanobeads were used as support for covalent attachment of *Kluyveromyces fragilis* β-galactosidase. The enzyme was immobilized 145.6 mg g^{-1} of support with recovery of 72.6% activity. The loading capacity of this novel support for *K. fragilis* β-galactosidase was improved 2.6-folds as compared to Eupergit® C, commercial epoxy support. The immobilized *K. fragilis* β-galactosidase showed high catalytic activity during synthesis of galacto-oligosaccharide and a total of 2,240 g galacto-oligosaccharide were generated g^{-1} of immobilized enzyme during consecutive batch process of 10-times. The immobilized biocatalyst maintained 81.5% of its initial activity after its10-repeated uses [52]. Talbert and Goddard [53] studied influence of particle size on *Aspergillus oryzae* lactase activity when it was covalently bind to MNPs of varying sizes. Lactase was attached to carboxylic acid functionalized MNPs (18 nm, 50 nm and 200 nm in diameter) via carbodiimide. After attachment the retention of initial activity was 73%, 39% and 14% for 18 nm, 50 nm and 200 nm MNPs, respectively. The apparent K_m was not significantly altered as a function of particle size while the apparent k_{cat} decreased with increasing particle size. By decreasing particle size of MNPs resulted in increasing the bound activity of lactase. This work has emphasized that the particle size of NPs has played a significant role in the binding of enzymes on their surfaces.

3.6. β-Glucuronidase.

β-Glucuronidase (EC 3.2.1.31) is a member of glycosidase family that catalyzes hydrolysis of complex carbohydrates. It is commonly present in microorganisms, plants and animals. The physiological importance of this enzyme in the metabolism of sulfated glycosaminoglycans is well known and its genetic deficiency in humans leads to a disease called mucopolysaccharidosis type VII [54]. Bifunctional graphene/γ-Fe_2O_3 hybrid aerogels was employed to immobilize β-glucuronidase for biocatalytic conversion of glycyrrhizin into glycyrrhetinic acid monoglucuronide or glycyrrhetinic acid. This bound enzyme showed high catalytic activity and excellent reusability [55].

3.7. Invertase.

Invertase (EC 3.2.1.26) catalyzes hydrolysis of sucrose into fructose and glucose which is also known as inverted sugar syrup. The systematic name for the enzyme is β-fructofuranosidase. The other commonly used names are saccharase, glucosucrase, β-fructosidase, invertin, sucrase and acid invertase. Invertase was immobilized on polyamidoamine dendrimer functionalized on the surface of superparamagnetite NPs. The amount of immobilized invertase on the surface-hyperbranched MNP was up to 2.5 times (i.e., 250%) as much as that of MNPs modified with only amino silane. Immobilized invertase exhibited improved thermal, operational and storage stabilities [56]. Different metal ions; Cu^{2+}, Zn^{2+}, Cr^{2+}, Ni^{2+} ions were chelated on polyvinylimidazole-grafted iron oxide MNPs and then the metal-chelated MNPs were used for the adsorption of invertase. The maximum invertase immobilization capacity of the modified MNPs beads was 142.856 mg g^{-1} at pH 5.0. Immobilized enzyme was more resistant to denaturation mediated by pH and heat. The immobilized enzyme retained significantly high stability on long time storage than the native enzyme [57].

3.8. β-Mannosidase.

β-Mannosidase (EC 3.2.1.25) catalyzes hydrolysis of terminal non-reducing β-D-mannose residues in β-D-mannosides. The systematic name for the enzyme is β-D-mannoside mannohydrolase. This enzyme also has other names such as mannanase, mannase, β-D-mannosidase and lysosomal β-A mannosidase. Recombinant human β-mannosidase (rhMANB) is an important glycosidase that degrades mannose-linked glycoproteins and mannan polysaccharides. rhMANB was purified and covalently attached to MNPs. MNPs bound enzyme retained nearly 65% of the original activity. The V_{max} and K_m of immobilized rhMANB was observed 3.0-fold higher and 2.024-fold lower, respectively compared to free enzyme. Metal chelators; oxalic acid, citric acid and ascorbic acid had no inhibitory effect on the activity of immobilized enzyme, while ethylenediamine tetraacetic acid decreased the activity of the enzyme. The results obtained by thin-layer chromatography demonstrated that immobilized rhMANB was more efficient than the soluble enzyme in hydrolyzing mannobiose, mannotriose,

mannotetrose, mannopentose, galactoglucomannan and locust bean gum. MNPs suspended gel-permeation chromatography exhibited that 29% locust bean gum hydrolyzed efficiently during flow in the column. The immobilized rhMANB was significantly more useful in gelling and saccharification of mannan polymers at industrial level [58].

3.9. Pectinase.

Polygalacturonases (EC 3.2.1.15) hydrolyze α-1,4-glycosidic bonds in polygalacturonic acid chains. The interest on specific inhibitors of pectinase and the versatility of magnetic carrier for enzyme immobilization supported the construction of an immobilized enzyme reactor. $CoFe_2O_4$ amino-derivatives were used for the immobilization of *Leucoagaricus gongylophorus* pectinases [59]. Seenuvasan et al. [60] have evaluated covalent binding of pectinase onto amino functionalized silica-coated MNPs via glutaraldehyde. The morphological and phase change of MNPs after immobilization were characterized by X-ray diffraction (XRD) analysis. The various surface modifications and pectinase binding onto NPs were confirmed by FTIR spectroscopy. The maximum activity of immobilized pectinase was obtained at its weight ratio of 19.0×10^{-3} mg bound pectinase mg^{-1} NPs. Immobilized enzyme exhibited markedly very high stability as compared to free enzyme. The fabrication of Fe_3O_4-chitosan-pectinase nanobiocatalyst was performed by covalently attaching pectinase to the carboxyl group activated chitosan-coated MNPs. At the weight ratio of about 19.8×10^{-3} mg bound pectinase mg^{-1} activated chitosan-coated MNPs, the activity of fabricated nanobiocatalyst was found to be maximum [61].

4. LIPASE

Lipases catalyze hydrolysis of lipids. These are subclasses of the esterases. Lipases perform significant roles in the digestion, transport and processing of dietary lipids in most of the living organisms [62]. Genes encoding lipases are even present in certain viruses. Most lipases act at a specific position on the glycerol backbone of lipid substrate (A1, A2 or A3) (small intestine). Human pancreatic lipase is one of the main enzyme that breaks dietary fats in digestive system, converts triglyceride substrates found in ingested oils to monoglycerides and two fatty acids. Lipases are engaged in various types of biological functions ranging from routine metabolism of dietary triglycerides to cell signaling and inflammation. Table 2 depicts MNPs support bound lipases, their mode of immobilization and improved properties.

Dyal and coworkers [63] have reported the stability and enzymatic activity of *Candida rugosa* lipase (CRL) immobilized on γ-Fe_2O_3 MNPs. The immobilization strategies were either reacting enzyme amine groups to the NP surface acetyl or amine groups. In the former, the enzyme was attached via C=N bond, while in the latter it was linked using glutaraldehyde. The activity of immobilized CRL was determined by following ester cleavage of *p*-nitrophenol butyrate. The covalently immobilized enzyme was stable and reactive over 30 d. Magnetic siliceous mesocellular foam functionalized with octyl groups was prepared and used for adsorption of lipase. Most of the active lipases from crude solution were adsorbed on the magnetically separable, hydrophobic siliceous mesocellular foam. The resolution of 1-phenylethanol acylated with vinyl acetate was achieved in 1.5 h using resultant magnetic biocatalyst, whereas only 30% conversion was obtained by free lipase under similar experimental conditions. Enzyme entrapped into nanopores of this foam can be recycled magnetically for at least 7-times without any loss in its activity and enantioselectivity [64]. Jiang et al. [65] immobilized CRL on MNPs supported ionic liquids having different cation chain length (C_1, C_4 and C_8) and anions (Cl^-, BF_4^- and PF_6^-). MNPs supported ionic liquids were obtained by covalent binding of ionic liquids-silane on magnetic silica NPs. The particles are superparamagnetic with diameter of about 55 nm. Large amount of lipase (63.89 mg 100^{-1} mg carrier)) was loaded on the support via ionic adsorption. Activity of the immobilized CRL was evaluated by catalysis of esterification between oleic acid and butanol. The activity of bound CRL was 118.3% compared to soluble enzyme. Immobilized lipase retained 60% of its initial activity even when the temperature was up to 80°C. Moreover, immobilized enzyme retained 60% of its original activity after 8-repeated uses, while no activity was detected after 6-cycles for the free enzyme. Magnetic Fe_3O_4-chitosan was used to immobilize lipase. The adsorption capacity of lipase was 129 mg g^{-1} NPs and the maximal activity was 20.02 μmol min^{-1} mg^{-1} proteins and activity retention was as high as 55.6% at a certain loading amount [66]. Magnetic particles have been coated with silica and charged with Cu^{2+} ions via a multidentate ligand, IDA, for the immobilization of His-tagged *Bacillus stearothermopilus* L1 lipase. The specific activity of the immobilized enzyme was found to conform to the following order: Cu^{2+}-charged silica-magnetic NP>silica magnetic NP>Cu^{2+}-charged silica gel>silica gel. Cu^{2+}-charged silica-magnetic NP immobilized lipase retained nearly 70% of the initial activity after 5-repeated uses while the silica gel immobilized enzyme lost its full activity just after 3-reuses [67].

Table 2. MNPs immobilized lipases, their mode of immobilization and improved properties.

Name of enzyme	Name of support	Mode of immobilization	Property/properties Enhanced	Reference
CRL	γ-Fe_2O_3 MNPs	Covalent Binding	Enhanced thermal & storage stability	[63]
Lipase	Magnetic siliceous mesocellular foam functionalized with octyl groups	Adsorption	Enhanced operational stability/reusability	[64]
CRL	MNPs supported ionic liquids	Adsorption	Enhanced thermo-stability/reusability	[65]
Lipase	Magnetic Fe_3O_4-chitosan NPs	Adsorption		[66]
B. stearo-thermopilus L1 lipase	Cu^{2+}-charged silica-MNPs	Bioaffinity binding	Enhanced reusability	[67]

Name of enzyme	Name of support	Mode of immobilization	Property/properties Enhanced	Reference
Lipase	Nanosized magnetic support	Covalent binding	Good thermal stability & reusability	[68]
Burkholderia cepacia Lipase	Superparamagnetic NPs	Adsorption & chemi-sorptions	Good reusability	[69]
Lipase	PDA coated MNPs	--	Enhanced pH & thermal stability, high reusability	[70]
Lipase	Magnetic supports	-	High stability in organic solvents	[71]
Lipase	Super paramagnetic MNPs-modified by gluconic acid	Covalent binding	Enhanced pH and heat stability, reusability	[72]
Lipase	Magnetic microspheres	Adsorption, covalent binding	Enhanced resistance to heat & pH inactivation, superior reusability in nonaqueous medium	[74]
Lipase	Superparamagnetic S1 and S2 silica particles	Covalent binding	Enhanced thermostability & high reusability	[78]
*Thermus thermophilus*WL recombinant lipase	3-APTES-modified Fe_3O_4@SiO_2 supermagnetic NPs		High resistance to heat, pH, metal ions, inhibitors & detergents; good reusability, low K_m	[80]
CRL	NH_2/COOH groups activated Fe_3O_4 clusters MNPs-loaded alginate composite beads Silica-based β-cyclodextrin on MNPs	Covalent binding Entrapment Entrapment	Very high thermal stability and reusability Enhanced pH stability/reusability Very high conversion & enantioselectivity behavior	[81] [87] [94]
Lipase	Fe_3O_4 NPs via EDC & Sulfo-NHS	Covalent binding	Very high thermal stability & reusability	[85]
Lipase	Fe_3O_4@SiO_2 NPs	Covalent binding	Higher relative activity, better stability, broader pH range & high reusability	[88]
Lipase	Carbon coated cobalt NPs	Covalent binding	Excellent stability & reusability	[89]
PPL	COOH functionalized silica-coated MNPs	Covalent binding	Enhanced thermal stability	[91]
Lipase	Superparamagnetic polymer emulsion particles	Covalent binding	Enhanced thermal stability/good reusability	[91]
Lipase	Core-shell super-paramagnetic NPs	------	Good reusabilty	[92]
Thermomyces lanuginose lipase	Succinated PEI grafted on silica coated Fe_3O_4 MNPs	Adsorption/covalent binding	Good reusability and high stability in organic solvents	[93]
Lipase	MNPs@hydroxyapatite /PEI/β-cyclodextrin nanocomposites	Covalent binding	High stability, reusability, uses in synthesis of ethyl valerate	[95]

Cui and colleagues [68] synthesized a facile nano-sized magnetic carrier and this support was used for covalent immobilization of lipase via glutaraldehyde. The immobilized lipase exhibited good thermal stability and reusability. The lipase loading amount and activity recovery were 43.6 mg g^{-1} support and 58.2%, respectively. Rebelo et al. [69] investigated the immobilizatiom of lipase from *Burkholderia cepacia* on superparamagnetic NPs using adsorption and chemisorption procedures. This immobilized lipase was efficiently applied as recyclable biocatalyst in the enzymatic kinetic resolution of (RS)-1-(phenyl)ethanols via transesterification reactions. (R)-esters and the remaining (S)-alcohols were obtained with excellent enantiomeric excess (>99%), which corresponds to a perfect process of enzymatic kinetic resolution (conversion 50%, E> 200). The transesterification reactions catalyzed by *B. cepacia* lipase immobilized by the glutaraldehyde method showed best results in terms of reusability, preserving the enzyme activity (conversion 50%, E> 200) for at least 8-repeated uses. Polydopamine (PDA) coated MNPs was used for the immobilization of lipase and immobilized enzyme showed 73.9% yield with loading capacity as high as 429 mg g^{-1} support. Immobilized lipase exhibited enhanced pH and thermal stability compared to free enzyme. Moreover, immobilized enzyme was easily isolated from the reaction mixture by magnetic separation and retained more than 70% of initial activity even after 21-repeated uses [70]. Magnetic support immobilized lipase was successfully used in organic

solvents for longer duration. The potential of enzyme-MNPs hybrids and their applications in the synthesis of fine chemicals, i.e., precursors for drugs have been discussed [71]. Superparamagnetic NPs surface-modified with gluconic acid was used for covalent immobilization of lipase. The immobilized lipase had better resistance to pH and heat inactivation in comparison to free enzyme. Immobilized enzyme showed high activity in broad spectrum ranges of pH and temperature. Thermal and storage stability of the enzyme improved upon immobilization. Immobilized lipase exhibited higher activity after repeated uses and bound enzyme was easily recovered by magnetic separation [72]. Magnetic microspheres with carboxyl groups were prepared by copolymerization of vinyl acetate, acrylamide and acrylic acid in presence of oleic acid-coated Fe_3O_4 MNPs. The effect of microsphere surface properties on lipase immobilization was investigated by preparing a series of microspheres with different hydrophobic/hydrophilic surface properties and by accommodating molar percentages of different monomers. The results showed that microspheres with different hydrophobicities/hydrophilicities had different immobilized ratios and activity recovery. The microspheres having hydrophilic characteristics had a much higher lipase binding efficiency than the microsphere containing hydrophobic characteristics. However, this study further demonstrated that moderate hydrophobicity/hydrophilicity of microsphere surface was highly important for increasing activity recovery [73]. CRL was successfully immobilized on superparamagnetic microsphere. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed to understand the mode of immobilization, whether it is covalent immobilization or physical adsorption. The results exhibited that the polymerization was successfully done and CRL was immobilized on magnetic microspheres via ionic adsorption and covalent binding under mild conditions. The immobilized lipase exhibited high activity recovery (69.7%), better resistance to heat and pH inactivation in aqueous phase as well as superior reusability in nonaqueous medium [74].

Burkholderia sp. C20 lipase was immobilized on Fe_3O_4 core with silica shell. The NPs treated by dimethyl octadecyl [3-(trimethoxysilyl) propyl] ammonium chloride were used as a support for lipase immobilization. The protein binding efficiency on alkyl-functionalized Fe_3O_4-SiO_2 was estimated as 97%, while the efficiency was only 76% on non-modified Fe_3O_4-SiO_2. Maximum adsorption capacity of lipase on alkyl-functionalized Fe_3O_4-SiO_2 was estimated as 29.45 mg g^{-1} based on Langmuir isotherm. The hydrolytic kinetics, using olive oil as substrate, of the lipase immobilized on alkyl-grafted Fe_3O_4-SiO_2 followed Michaelis-Menten model with a maximum reaction rate and K_m of 6251 U g^{-1} and 3.65 mM, respectively. Moreover, the immobilized lipase was used to catalyze transesterification of olive oil with methanol to produce fatty acid methyl esters, which obtained a substrate conversion of over 90% within 30 h in a batch process when 11 wt% immobilized lipase was employed. The immobilized lipase was reused for 10-repeated cycles without any significant loss in its transesterification activity [75]. Xun et al. [76] immobilized *Pseudomonas fluorescens* lipase onto Fe_3O_4 MNPs via hydrophobic bonding. Enzyme loading and immobilization yield were determined as 21.4±0.5 mg g^{-1} and 49.2±1.8%, respectively. Immobilized lipase was successfully employed for the resolution of 2-octanol with vinyl acetate used as acyl donor. The preferred isomer for the enzyme was (R)-2-octanol under optimum experimental conditions and the highest enantioselectivity (E=71.5±2.2) was achieved with a higher enzyme activity (0.197±0.01 μmol $mg^{-1}min^{-1}$). The findings indicated that immobilized lipase was simply separated from reaction media by magnetic steel and retained 89% of its original activity and there was no change in enantioselectivity after 5-repeated uses.

CRL was then directly immobilized on the magnetite NPs coated by alkyl silanes of different alkyl chain lengths via hydrophobic interaction. Furthermore, the catalytic activities of lipases immobilized on trimethoxyl octadecyl silane (C_{18}) modified Fe_3O_4 were a factor of 2 or more than the values reported from other surface immobilized systems. The activities of lipases immobilized on C_{18} modified NPs retained 65% after 7-repeated uses; it showed a significant enhancement in stability. Lipase immobilized MNPs facilitated easy separation and recycling with high activity retaining. The activity of immobilized lipases increased with increasing alkyl chain length of the alkyl trimethoxy silanes used in surface modification of magnetite NPs. Lipase stability was also improved via hydrophobic interaction. Alkyl silane modified magnetite NPs are thus novel carriers for enzyme immobilization enabling efficient enzyme recovery and recycling [77]. Lipase was immobilized on uniformly sized superparamagnetic single-shell nonporous (S_1) and double-shell mesoporous silica nanocomposite particles with ~130 nm magnetite cluster cores. Mesoporous particles were synthesized with two pore sizes; 2.44 and 3.76 nm, designated as S2 and S3 particles, respectively. The finding exhibited that the enzyme loading capacities of mesoporous structures, i.e. S_2 and S_3 were higher than that for nonporous particles (S_1). Immobilized lipases maintained nearly 90% of their intial activities. The thermal stability of bound enzymes was remarkably superior with each matrix. It was noticed that the rate of enzyme catalyzed reaction was not influenced by silica matrix used for enzyme immobilization. However, the rate of immobilized enzyme catalyzed reaction was lower as compared to free enzyme. The bound lipases exhibited more than 76% of their original activity after 5-repeated uses [78].

CRL was immobilized onto magnetic alginate/chitosan nanospheres by electrostatic adsorption and covalent bonding. A layer-by-layer assembly process was made to coat the immobilized lipase with covering layers comprised of alginate and chitosan. Immobilized enzyme preparations obtained by these two methods were far superior as compared to preparation obtained only by simple electrostatic adsorption. Additional layering of immobilized CRL with covering layers exhibited an enhancement in enzyme stability [79]. Song and coworkers [80] studied expression of a recombinant thermostable lipase from *Thermus thermophilus* WL in *Escherichia coli* and this enzyme was immobilized onto 3-APTES-modified Fe_3O_4@SiO_2 supermagnetic NPs. The immobilized enzyme showed high resistance to temperature, pH, metal ions, enzyme inhibitors and detergents compared to soluble enzyme. The K_m for the immobilized lipase found to be lower than the free enzyme. It demonstrated that the immobilization improved affinity of the enzyme for its substrate

and immobilized lipase exhibited good reusability. It retained more than 79.5% of its original activity after its 10-repeated uses. In a further study, CRL was immobilized on Fe_3O_4 nanocrystals clusters activated with amino or carboxyl groups. The immobilized CRL exhibited significantly very high thermal stability and reusability as compared to soluble enzyme [81]. Ghasemi and coworkers [82] prepared MNPs by co-precipitation of Fe+3/Fe+2 (2:1; molar ratio) with a mean size of 40 ± 5 nm. The MNPs and also hydroxylated nonporous glass beads were similarly activated by glutaraldehyde to crosslink lipase from *Thermomyces lanuginosus*. The yield and efficiency for immobilized lipase on MNPs were calculated as 72 ± 2.4 and $63 \pm 3.5\%$, whereas a yield and efficiency of 60 ± 2.1 and $55 \pm 4.1\%$, respectively, were measured for the corresponding parameters of the immobilized enzyme on the glass beads. Immobilized lipase showed an insignificant change in K_m and a remarkable decrease in V_{max} as compared to soluble enzyme. This immobilized lipase was compared with six other commercially available lipases for regioselective acetylation of prednisolone. The highest and lowest yields of the product were observed for Novozym 435 and immobilized *Thermomyces lanuginosus* lipase on glass beads, respectively. Immobilized lipase retained its bioacetylation activity of prednisolone in 5-repeated uses. Mukherjee et al. [83] demonstrated a simple method for the immobilization of CRL on superparamagnetic Fe_3O_4 NPs. The PEI coated Fe_3O_4 NPs were used for the adsorption of CRL via electrostatic interactions. The procedure involved simultaneous purification and immobilization of enzyme. Immobilized enzyme exhibited 110× higher transesterification activities in low-water media. It was also efficient in kinetic resolution of (±)-1-phenylethanol with eep of 99% and E = 412 within 24 h. The immobilized lipase was reused 4-times without any loss in its activity. An environmentally benign process, immobilized lipase mediated transesterification has attracted considerable attention for biodiesel production [84].

Lipases were covalently attached to the Fe_3O_4 MNPs via 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) activated coupling to synthesize magnetically responsive lipases. The binding of Fe_3O_4 MNPs with lipases was demonstrated by electrophoresis and zeta potential analyses. The optimum binding was obtained when 200 µL lipase and 10 µL EDC solution were taken for 1 mL of Fe_3O_4. The molar ratio of EDC and Sulfo-NHS was 1:2.5 and the reaction time was 2 h. The immobilized lipase showed greater thermal stability and reusability [85]. *Candida antarctica* Lipase B was immobilized directly and as a crosslinked aggregates of enzyme on MNPs. The hydrolytic and biosynthetic activities of the resulting magnetic nanobiocatalysts were evaluated in aqueous and organic media. The hydrolysis of triglycerides and the transesterification reactions to synthesize biodiesel and biosurfactants were carried out using crosslinked aggregates of lipase immobilized on magnetic support [86]. CRL was entrapped in magnetic iron oxide NPs-loaded alginate composite beads. The magnetic alginate composite beads entrapped lipase exhibited lower enzyme activity and specific activity as compared to enzyme entrapped in alginate beads. The results revealed that the magnetic alginate composite beads immersed in acidic solution, pH 3.0 were found more stable than alginate beads. Lipase entrapped in magnetic alginate composite beads was easily collected by an external magnet and was reused at least six-times [87].

Liu et al. [88] described immobilization of lipase on Fe_3O_4@SiO_2 NPs and the immobilized enzyme was characterized and compared with soluble enzyme. There was no remarkable change in optimal pH, temperature and K_m after the immobilization of enzyme. However, the immobilized lipase showed greater fraction of activity in the range of pH 7.0-9.5. The immobilized enzyme exhibited significantly very high stability at higher temperatures and the relative activity of immobilized enzyme was 5.8-fold as compared to soluble lipase at 70°C after 10 h incubation. The bound enzyme has advantages like higher relative activity, better stability, broader pH range and easy recovery. It revealed that Fe_3O_4@SiO_2 NPs immobilized lipase can be exploited at industrial level. Carbon coated cobalt NPs were chemically functionalized (diazonium chemistry), activated for bioconjugation (N,N-disuccinimidyl carbonate) and was used for the successful covalent immobilization of lipase B on the magnetic nanosupport. Conjugated enzyme demonstrated good activitiy and stability and was effectively recycled in short times from milliliter to liter scales [89]. Zhu et al. [90] used carboxyl functionalized silica-coated MNPs for the immobilization of porcine pancreatic lipase (PPL) via EDC/NHS coupling reaction. The immobilization of enzyme on the nanocomposite was probed by atomic force microscopic analysis. Immobilized enzyme activity, reusability, thermo- and storage stability were remarkably superior as compared to free enzyme. The K_m and V_{max} values (0.02 mM, 6.40 $U \cdot mg^{-1}$ enzyme) showed enhanced activity of the immobilized PPL compared to free enzyme (0.29 mM, 3.16 $U \cdot mg^{-1}$ enzyme). Moreover, immobilized PPL retained 60% of its initial activity at 70°C. PPL-MNPs nanocomposite was applied in the enzyme inhibition assays using orlistat and two natural products isolated from oolong tea as the test compounds. The superparamagnetic polymer emulsion particles were prepared by copolymerization of butyl methacrylate, vinyl acetate and EGDA in presence of seeded emulsion; a copolymer of styrene, GMA and 2-HEMA and superparamagnetic Fe_3O_4/SiOx NPs or Fe_3O_4-APTES NPs via a two-step process, without using any emulsifier. The obtained magnetic emulsion particles were used for the immobilization of lipase by two strategies on the available magnetic composites directly (S-1) or using glutaraldehyde as a coupling agent (S-2). The thermal stability and reusability of immobilized lipase based on S-2 were significantly very high than that of S-1 [91].

Lipase was immobilized on the high surface area core-shell superparamagnetic NPs. It has been fabricated and used as an efficient reusable catalysts for selective production of pharmaceutically important chiral isomers from meso-cyclopent-2-en-1,4-diacetate [92]. Further similar group described synthesis of β-cyclodextrin grafted PEI nanocomposite with magnetic cores and the prepared nanocomposite was used for lipase immobilization. MNPs@hydroxyapatite/PEI/β-cyclodextrin bound lipase exhibited high thermal, pH and storage stability. Lipase immobilized on this magnetic nanocomposite was successfully used for synthesis of ethyl valerate in organic medium [93]. Ozyilmaz et al. [94] prepared macrocyclic compound with magnetic property by immobilizing silica-based β-cyclodextrin on

MNPs. β-Cyclodextrin-grafted MNPs was encapsulated along with *Candida rugosa* lipase in sol-gel matrices using alkoxysilane precursors. The cyclodextrin-based, encapsulated lipase had very high conversion and enantioselectivity behavior as compared to sol-gel free lipase. It was also noticed that very good enantioselectivity (E=399) was achieved for encapsulated lipase with magnetic β-cyclodextrin that has an ee value of S-naproxen acid of nearly 98%. Magnetically separable nanospheres consisting of PEI and succinated PEI grafted on silica coated magnetite MNPs were used for physical adsorption or covalent attachment of *Thermomyces lanuginose* lipase via glutaraldehyde or hexamethylene diisocyanate. The results showed that MNPs@PEI-GLU immobilized lipase was best biocatalyst and it retained 80% activity after 12-repeated uses. The MNPs@PEI-GLU immobilized lipase was applied for the synthesis of ethyl valerate and the immobilized enzyme catalyzed esterification within 24 h in *n*-hexane and solvent free media as 72.9% and 28.9%, respectively [95].

5. PHOSPHOLIPASE A_1

Phospholipase A_1 (EC 3.1.1.32) is a type of phospholipase which removes 1-acyl group. It hydrolyzes phospholipids into fatty acids. There are 4 classes, which are sub-divided on the basis of type of reaction they catalyze. In particular, phospholipase A_1 (PLA1) specifically catalyzes the cleavage at SN-1 position of phospholipids, forming a fatty acid and a lysophospholipid [96]. PLA1 immobilized onto MNPs was used for the optimization of soybean oil degumming process. The optimum enzymatic degumming was obtained by 0.10 g kg^{-1} doses of enzyme, 2.13 mL 100^{-1} g of added water, at 56°C for 6.3 h. Magnetically immobilized PLA1 catalyzed reduction of phosphorus and free fatty acids contents of degummed soy oil to 10.38 mg kg^{-1} and 1.09 100^{-1} g under optimal experimental conditions, respectively [97]. In a further study same group has investigated the immobilization of PLA1 onto magnetic Fe_3O_4/SiOx-g-PGMA-NPs and the obtained immobilization efficiency was 64.7%. The immobilized PLA1 had a broader pH-activity profile, pH 4.5-6.5 and was markedly more stable at 45-55°C for 7 h. The optimum-temperature for the immobilized enzyme was shifted from 50°C to 60°C. The immobilized enzyme retained above 80% soybean oil degumming activity after its 10-repeated uses at 55°C and pH 6.0. The remaining phosphorus concentration was 9.6 mg kg^{-1}, which fulfills oil safety standard and suitable for physical refining of soybean oil [98].

6. PROTEOLYTIC ENZYMES

Table 3 demonstrates MNPs supports bound proteases, their mode of immobilization and improved characteristics.

Table 3. MNPs immobilized proteases, their mode of immobilization and improved properties.

Name of enzyme	Name of support	Mode of immobilization	Property/properties enhanced	Reference/s
Alkaline protease	3-APTES-functionized iron oxide MNPs	Covalent binding	Enhanced reusability	[99]
α-chymotrypsin	NH_2-functionalized &COOH-functionalized superparamagnetic nanogels	Covalent binding	Good thermal & storage stability, higher $K_{m\ \&}$ reusability, low V_{max}	[100, 101]
α-chymotrypsin Thermolysin, Papain	Various γ-Fe_2O_3/ Fe_3O_4 MNPs	Covalent binding	Good reusability, γ-Fe_2O_3 MNPs better support than Fe_3O_4 MNPs	[102]
Sulfolobus solfataricus carboxypeptidase	Silica-coated iron oxide NPs	Bioaffinty Binding	Very high stability to heat & organic solvents denturation	[104]
Bacillus subtilis Keratinase	PEG-supported Fe_3O_4 superparamagnetic NPs	Covalent binding	4-fold enhanced activity, very high thermal & storage stability & reusability	[108]
Papain	Functionalized MNPs decorated by carboxymethylated chitosan	Covalent binding	Enhanced activity, high stability against pH & heat, improved storage stability & reusability	[110]
	Fe_3O_4NPs & Au NPs embedded on cellulose nanocrystals	Covalent binding	Good reusability	[111]
	Magnetic gold nanocomposites	Chemi-sorbed Covalent binding	Increased K_m & V_{max}	[112]
	3-CPTMS modified silica-coated MNPs		Enhanced activity, pH, heat & storage stability & reusability	[113]
rtPA	PAA-coated magnetite	Covalent binding	High stability	[120]
Raffinose-modified trypsin	Monodispersed superparamagnetic maghemite NPs	Covalent binding	Enhanced thermostability & decreased autolysis.	[127]
Trypsin	MNPs modified GO nanocomposites	Covalent binding	High storage stability	[130]
	PGMA, grafted from MNPs	Covalent binding	Excellent activity, repeatability & stability	[134]
	Superparamagnetic carboxymethyl chitosan	Covalent binding	Excellent reusability	[133]

Name of enzyme	Name of support	Mode of immobilization	Property/properties enhanced	Reference/s
	Magnetosomes	Covalent binding	Enhanced thermal & storage stability, reusability,	[132]
Serratio-peptidase	NH_2-functionalized MNPs, chitosan NH_2-functionalized MNPs	Covalent binding	Good storage stability, enhanced drug delivery via membrane, increased anti-inflammatory effect	[117,118]

6.1. Alkaline protease.

Alkaline protease was coupled to the surface of 3-APTES functionalized MNPs via glutaraldehyde. The results showed that the surface of modified MNPs had a good capability of magnetic separation so that alkaline protease was properly immobilized on its surface. The maximum immobilization yield was obtained when 7000 U g^{-1} alkaline protease was incubated with support at 40^{o}C for 1.5 h. The enzymatic activity after immobilization was 3352 U g^{-1} and the activity recovery was 48%. Moreover, immobilized enzyme exhibited 34.2% activity after five-repeated uses [99].

6.2. Alpha-chymotrypsin.

Chymotrypsin (EC 3.4.21.1) is a digestive enzyme component of pancreatic juice acting in the duodenum where it performs protein hydrolysis. It preferentially cleaves peptide amide bonds where the carboxyl side of the amide bond, P_1 position, is an aromatic amino acid; tyrosine, tryptophan and phenylalanine. These amino acids contain an aromatic ring in their side chain that fits into a 'hydrophobic pocket', S_1 position, of the enzyme. It is activated in the presence of trypsin. The hydrophobic and shape complementarity between the peptide substrate P_1 side chain and the enzyme S_1 binding cavity accounts for the substrate specificity of this enzyme. Chymotrypsin also hydrolyzes other amide bonds in peptides at slower rates, particularly those containing leucine and methionine at the P_1 position.

Hong et al. [100] obtained stabilization of α-chymotrypsin by covalent immobilization on the amine-functionalized magnetic nanogel. The amino groups containing superparamagnetic nanogel was achieved by Hoffman degradation of the polyacrylamide gel-coated Fe_3O_4 NPs prepared by facile photochemical *in situ* polymerization. Chymotrypsin was then covalently bound to the magnetic nanogel with reactive amino groups by using EDC as coupling reagent. The binding capacity was 61 mg enzyme g^{-1} nanogel as determined by BCA protein assay. The measured specific activity of the immobilized enzyme was 0.93U mg^{-1} min^{-1}, 59.3% as that of free chymotrypsin. The immobilized enzyme had better tolerance to heat and pH denaturation. The immobilized enzyme exhibited good reusability, thermal and storage stability. The immobilized enzyme had greater K_m as compared to free enzyme, whereas the V_{max} was low for the immobilized enzyme than the soluble enzyme. In a further study, these workers conjugated α-chymotrypsin onto the novel carboxyl-functionalized superparamagnetic nanogels, prepared via facile photochemical *in situ* polymerization, by EDC as coupling reagent. The binding capacity of enzyme was 30 mg g^{-1} support and 37.5 mg enzyme g^{-1} nanogel as determined by TG analysis and bicinchroninic acid (BCA) protein assay, respectively. Specific activity of immobilized enzyme was 0.77 U mg^{-1} min^{-1}, 82.7% [101]. Xin et al. [102] synthesized various γ-Fe_2O_3/Fe_3O_4 MNPs based immobilized proteases by three different immobilization procedures including A) direct binding, B) with thiophene as a linker, and C) with triazole as a linker. The oligopeptides syntheses catalyzed by MNPs immobilized proteases were systematically evaluated. These findings exhibited that MNPs immobilized α-chymotrypsin by both immobilization strategies A and B provided good reusability for Z-Tyr-Gly-Gly-OEt synthesis. MNPs bound α-chymotrypsin was easily reused 5-times without a remarkable loss in its catalytic activity. For the synthesis of Z-Asp-Phe-OMe, MNPs bound thermolysin via thiophene linker provided best recyclability. For the immobilized papain, however both strategies A or B exhibited an immobilized enzyme for the first cycle of Z-Ala-Leu-NHNHPh synthesis in good yield but their subsequent activity was rapidly decreased. It was demonstrated that γ-Fe_2O_3 MNPs was a better matrix for the immobilization of proteases compared to Fe_3O_4 MNPs. It might be due to their smaller particle size and higher surface area. α-Chymotrypsin was covalently immobilized on Fe_3O_4@Au-core/shell NPs via thiol and amine groups present on the surface of protein. However, the enzyme activity was decreased to 30%, thus it suggested some conformational changes near to active site of the enzyme [103].

6.3. Carboxypeptidase.

Carboxypeptidase (EC 3.4.16-3.4.18) cleaves a peptide bond at the carboxy-terminal end of a protein or peptide. Humans, animals, and plants contain several types of carboxypeptidases that have diverse functions from catabolism to protein maturation. Carboxypeptidases also function in blood clotting, growth factor production, wound healing, reproduction, and many other processes. Sommaruga et al. [104] studied the effect of superparamagnetic iron oxide NPs bioconjugation on the catalytic properties of a thermostable carboxypeptidase from the hyper thermophilic archaeon *Sulfolobus solfataricus* which showed catalytic properties that are useful in synthetic processes. The enzyme was immobilized onto silica-coated iron oxide NPs via NiNTA-His tag site-directed conjugation. The immobilized enzyme exhibited significantly high stabilization against denaturation mediated by heat and organic solvents. N-blocked amino acids were produced remarkably in high yields by immobilized enzyme compared to free enzyme. The synthesized nanobioconjugate exhibited enhanced stability in aqueous media at room temperature as well as in different organic solvents. The improved stability in ethanol opens new avenues for using immobilized enzyme during synthesis of N-blocked amino acids.

6.4. Enterokinase.

Enteropeptidase (EC 3.4.21.9) is an enzyme produced by cells of duodenum and involved in human and animal digestion. It

is also known as enterokinase and it is secreted by intestinal glands following the entry of ingested food passing through the stomach. Enteropeptidase converts inactive trypsinogen into active trypsin, resulting in the subsequent activation of pancreatic digestive enzymes [105]. Absence of enteropeptidase results in intestinal digestion impairment. Enteropeptidase is a serine protease consisting of a disulfide-linked heavy-chain of 82-140 kDa that anchors enterokinase in the intestinal brush border membrane and a light-chain of 35-62 kDa that contains the catalytic subunit. Enteropeptidase is a part of the chymotrypsin-clan of serine proteases and is structurally similar to these proteins. Enterokinase was immobilized on the iron oxide MNPs coated with biopolymers. Two different strategies have been adopted for covalent coupling of enzyme, namely carbodiimide and maleimide activation. Immobilization of enterokinase via carbodiimide coupling retained higher activity. The immobilized enzyme proved to cleave a control fusion protein and remarkably simplify separation of enzyme from the reaction mixture [106].

6.5. Keratinase.

Keratinases (EC 3.4.99) are proteolytic enzymes in nature. This enzyme is classified as proteinase of unknown mechanism. Recently, some of the worker defined keratinase as serine protease due to its 97% sequence homology with alkaline protease and it is also inhibited by same inhibitor that inhibits serine protease [107]. Keratinases are produced only in the presence of keratin containing substrate. It mainly attacks on the disulfide (-S-S-) bond of the keratin substrate. The keratinase production by various microorganisms was reported by a number of earlier workers. It showed that keratinases were obtained from fungi, Streptomyces and bacteria at alkaline pH and almost thermophilic temperatures. These enzymes have wide range of substrate specificity. These enzymes degrade other fibrous protein fibrin, elastin, collagen and other non fibrous protein like casein, bovine serum albumin gelatin etc. *Bacillus subtilis* keratinase was immobilized onto PEG-supported Fe_3O_4 superparamagnetic NPs. The highest enzyme activity was observed when enzyme was immobilized onto cyanamide-activated PEG-assisted MNPs. The activity of enzyme increased 4-times upon immobilization as compared to free enzyme. Moreover, the immobilized enzyme showed remarkably very high thermal and storage stability and recyclability. The leather-industry-oriented application of immobilized enzyme was investigated for removal of hair from goat-skin [108].

6.6. Papain.

Papain (EC 3.4.22.2) is a cysteine protease present in papaya (*Carica papaya*) and mountain papaya (*Vasconcellea cundinamarcensis*). Papain belongs to a family of related proteins with broad spectrum activities, including endopeptidases, aminopeptidases, dipeptidyl peptidases and enzymes with both exo- and endo-peptidase activity. Members of the papain family are widespread found in baculovirus, eubacteria, yeast and practically all protozoa, plants and mammals [109]. Functionalized Fe_3O_4 NPs layered with carboxymethylated chitosan and this modified support was used for covalent immobilization of papain. Magnetic measurement revealed that the resultant papain-bound NPs were superparamagnetic with a saturation magnetization of 59.3 emu g^{-1}. The immobilized papain showed enhanced activity, better tolerance to variations in pH and temperature and improved storage stability as well as good reusability as compared to free enzyme [110]. A nanocomposite consisting of magnetite NPs and Au NPs embedded on cellulose nanocrystals was used as a magnetic support for the covalent immobilization of papain and facilitated recovery of immobilized enzyme. The nanocomposite was successfully used for the immobilization and separation of papain from the reaction mixture. The optimal enzyme loading was 186 mg protein g^{-1} CNC/Fe_3O_4NPs/AuNPs, significantly higher than the previously reported results. The immobilized enzyme retained 95% activity after 35 d storage at 4°C, compared to 41% activity maintained by soluble counterpart under identical storage conditions [111].

MNPs were modified with 3-(mercaptopropyl) trimethoxy silane. Moreover, the citrate stabilized gold NPs were chemisorbed on the thiol coated MNPs to synthesize magnetic gold nanocomposites. Positive charge containing papain was immobilized on the surface of negatively charged magnetic gold nanocomposites via electrostatic interaction. K_m and V_{max} of the immobilized papain were increased as compared to free enzyme. The loading amount of papain on this magnetic nanocomposite was 54 mg g^{-1} support and the activity recovery of the immobilized papain reached to 47±5% compared to soluble enzyme [112]. Mosafa et al. [113] investigated covalent immobilization of papain on the (3-chloropropyl) trimethoxysilane (3-CPTMS)-modified silica-coated MNPs. The maximum immobilization was obtained at 27.3°C, pH 7.1, concentration of papain 3.3 mg mL^{-1} and immobilization time 10 h. The immobilized papain exhibited enhanced enzyme activity, better tolerance to varying pH and temperature, improved storage stability and good reusability. Both soluble and immobilized forms of the enzyme were effectively used in the clarification of pomegranate juice.

6.7. Proteinase K.

Proteinase K (EC 3.4.21.64) is a broad-spectrum serine protease [114]. The enzyme was discovered in 1974 in extracts of fungus *Engyodontium album* (formerly *Tritirachium album*). Proteinase K is able to digest native keratin (hair), hence, the name "Proteinase K". The predominant site of cleavage is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino groups. It is commonly used for its broad specificity. This enzyme belongs to peptidase family S8. An assay with proteinase K immobilized to magnetic-COOH micro- and NPs can provide a simple as well as cheaper method. The stability of the newly developed proteinase K magnetic reactor, noticed during kinetics measurements, was highly satisfactory. The apparent K_m values for soluble and immobilized enzyme from Lineweaver-Burk plots were 4.25 mM and 1.28 mM, respectively. Human growth hormone was digested using newly prepared magnetic proteinase K reactor and matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)-mass spectrometry (MS) analysis of the digests showed satisfactory efficiency. Controlled digestion of PrP(c) from Mov mouse cell line was demonstrated by western blotting [115].

6.8. Serratiopeptidase.

Serratiopeptidase is a proteolytic enzyme and produced by enterobacterium *Serratia* sp. E-15. It has also other names such as serralysin, serratiapeptase, serratia peptidase, serratio peptidase, or serrapeptidase [116]. This microorganism was originally isolated

in late 1960s from silkworm *Bombyx mori* L. (intestine). Serratiopeptidase is present in the silkworm intestine and allows the emerging moth to dissolve its cocoon. Kumar et al. [117] immobilized serratiopeptidase on Fe_3O_4 MNPs and used this immobilized enzyme for its targeted delivery. The enzyme was immobilized by covalent binding via glutaraldehyde after amino functionalization of MNPs. Magnetic targeting of enzyme bound to MNPs enhanced delivery (permeation) of drug via membrane *in vitro* and increased anti-inflammatory effect on carrageenan induced paw oedema in rats *in vivo* at much lower doses of enzyme required for treatment compared to the doses of free enzyme. Serratiopeptidase was immobilized on chitosan amino-functionalized MNPs by covalent binding via glutaraldehyde. Immobilized enzyme was targeted *in vitro* in modified diffusion cell and *in vivo* in rats. Maximum protein and enzyme loading on enzyme MNPs were 264 mg g^{-1} and 325 U g^{-1}, respectively. The maximum 52 enzyme molecules were attached to each particle and it retained nearly 68% enzyme activity. There was no change in K_m and V_{max} but it retained good storage stability. Magnetic targeting of immobilized enzyme enhanced delivery of drug via membrane *in vitro* and the increased anti-inflammatory effect on carrageenan-induced paw oedema in rats *in vivo* at much lower doses of enzyme than the doses of the free enzyme required for treatment. The enzymatic preparation of MNPs exhibited stimulated effects at lower concentrations with magnetic targeting [118].

6.9. Tissue plasminogen activator (tPA).

tPA (EC 3.4.21.68) is a serine protease and found in endothelial cells, the cells that line the blood vessels. This enzyme catalyzes conversion of plasminogen to plasmin, the major enzyme responsible for clot breakdown. Because it works on the clotting system, tPA is used in clinical medicine to treat embolic or thrombotic stroke [119]. The antidote for tPA in case of toxicity is aminocaproic acid. tPA may be manufactured using recombinant DNA technology, therefore tPA created in this way may be referred as recombinant tissue plasminogen activator (rtPA). Ma et al. [120] evaluated the feasibility and efficacy of target thrombolysis by using rtPA covalently bound to MNPs and retained to the target site *in vivo* by an external magnet. rtPA was immobilized to polyacrylic acid-coated magnetite (PAA-MNP) via carbodiimide-mediated amide bond formation. The thrombolytic activity of PAA-MNP-rtPA with rtPA equivalent to 0.2 mg kg^{-1} was examined by flowmetry in a rat embolic model. Intra-arterial administration of PAA-MNP-rtPA maintained iliac blood flow within 75 min to 82% of that before clot lodging. However, the equivalent amount of PAA-MNP or free rtPA showed no improvement on hemodynamics. Immobilized rtPA exhibited no change in the levels of hemoglobin, hematocrit, or blood cell count after 2 h. The covalent binding of rtPA to PAA-MNP provided stable rtPA and predictable amount of rtPA around target site under direction of magnet; this procedure obtained reproducible and effective target thrombolysis with <20% of a regular dose of rtPA. Yang et al. [121] employed low-toxicity magnetic nanocarriers composed of a shell of poly [aniline-co-N-(1-one-butyric acid) aniline] over a Fe_3O_4 MNPs core to transfer rtPA for targeted thrombolysis. Nearly 276 µg of active rtPA was bound mg^{-1} of magnetic support. The storage stability of immobilized rtPA was remarkably improved at 4°C and 25°C. *In vitro* thrombolysis testing by a tubing system demonstrated that magnet-guided carrier-rtPA exhibited significantly improved thrombolysis compared to free rtPA and decreased time of clot lysis from 39.2±3.2-10.8±4.2 min. Moreover, magnet-guided support-rtPA at 20% of its regular dose maintained blood flow during 15-25 min of treatment in a rat embolism model without creating hematological toxicity. This magnet targeting accelerated thrombolysis can be employed for therapeutic applications in thromboembolic diseases.

6.10. Trypsin.

Trypsin (EC 3.4.21.4) is a serine protease from PA clan superfamily, found in the digestive system of many vertebrates, where it hydrolyzes proteins. Trypsin is secreted by pancreas as an inactive precursor, trypsinogen. Trypsin cleaves peptide chains mainly at carboxyl side of amino acids lysine or arginine, except when either is followed by proline. It is used for numerous biotechnological processes. The process is commonly referred to as trypsin proteolysis or trypsinization and proteins that have been treated with trypsin are said to be trypsinized. Bilkova et al. [122] evaluated preparation of an easily replaceable protease micro-reactor for micro-chip application. Magnetic particles coated with poly(N-isopropylacrylamide), polystyrene, poly(2-HEMA-co-EDMA), PGMA, [(2-amino-ethyl)hydroxymethylen]biphosphonic acid, or alginic acid with immobilized trypsin were employed for heterogeneous digestion. The properties were optimized, with the constraint of allowing immobilization in a micro-channel by a magnetic field gradient. To obtain the highest digestion efficiency, sub-micrometer spheres were organized by an in homogeneous external magnetic field perpendicularly to the direction of channel. The performance of the proteolytic reactor was monitored by taking five-model (glyco)-proteins ranges in molecular mass from 4.3-150 kDa. Digestion efficiency of proteins in different conformations was evaluated using SDS-PAGE, high performance capillary electrophoresis, reverse phase-HPLC and MS. The compatibility of the micro-chip IMER system with total and limited proteolysis of high-molecular-weight (glyco)-proteins was confirmed. These findings have opened new avenues for automated, high-throughput proteomic micro-chip devices.

A novel and facile procedure was developed for the immobilization of enzyme on MNPs and its application to fast protein digestion via a direct MALDI-TOF-MS analysis was demonstrated. Different aldehyde groups functionalized MNPs were obtained by treating amine-functionalized MNPs via glutaraldehyde. Finally, immobilization of trypsin onto aldehyde-functionalized MNPs was done by reaction of aldehyde groups with amine groups of trypsin. MNPs coupled trypsin was conveniently used for protein digestion. The digestion efficiency was demonstrated by peptide mapping analysis of three model proteins. The process of digestion is very facile due to easy manipulation of MNPs. Full protein digestion was noticed in a short time, 5 min, without any complicated reduction and alkylation procedures. Since MNPs immobilized trypsin is very cheap and highly reproducible, it may find much potential in proteome research [123]. Jeng et al. [124] constructed a high concentration trypsin-modified MNPs and the preparation was used for rapid and efficient digestion of proteins at high temperature. The time needed for digestion was minimized to less than 10s. Trypsin-MNPs composite was collected magnetically

from reaction mixture after digestion. Digested peptides were characterized by MALDI-TOF-MS. Protein digestion was standardized by using trypsin-MNPs (5 μg μL^{-1}) at 57°C; significantly high peptide coverage was evaluated for protein identification e.g., 98% for lysozyme. However, a high concentration of trypsin-MNPs was used for digestion, the short digestion time led to much lower amounts of trypsin peptides being produced by self-digestion. Consequently, interference in MS analysis of peptide ions was remarkably decreased.

The immobilization of trypsin onto aldehyde-functionalized MNPs was achieved via reaction of the aldehyde groups with amine groups of trypsin. The prepared MNPs were then locally packed onto glass microchip by the use of a strong magnetic field applying a magnet to form an on-chip MNPs packing bed. Capability of proteolytic micro-reactor was demonstrated by cytochrome c, bovine serum albumin (BSA) and myoglobin as model proteins. The digestion products were characterized by MALDI-TOF-MS with sequence coverage of 83%, 43% and 79% observed, respectively. Complete protein digestion was achieved in a short time, 10 s under the flow rate of 5 μL min^{-1}. These findings are expected to open new avenues for the analysis of proteolysis as well as a new application of MNPs. It is easy to replace the NPs and make the new micro-reactor. It takes less than 1 min under the condition of extra magnetic to form a new packing bed. The packing bed can be used for at least 5-times without any treatments. Additionally, since the preparation and surface functionality of MNPs is of low-cost and reproducible, the preparation method and application approach of the MNPs may find much potential in proteome research. This micro-reactor was also successfully used for analysis of a reversed-phase liquid chromatography fraction of rat liver extract. Six proteins were identified after a database search [125]. A novel microwave-mediated protein digestion method was explored using MNPs bound trypsin. The MNPs worked as not only support for the immobilization of trypsin but also excellent microwave irradiation absorber and thus significantly improved microwave-assisted digestion efficiency. BSA, myoglobin and cytochrome c were used to standardize this digestion conditions and by this method peptide fragments were produced within 15 s which were identified by MALDI-TOF-MS. Moreover, the enzyme was easily removed by an external magnetic field and reused. It was reported that the magnetic support bound trypsin still maintained high activity even after 4 reuses. This procedure was further used to digest proteins from the extract of rat liver in order to confirm the efficiency of the method. The complete proteome was digested by immobilized trypsin in 15 s which was analyzed by liquid chromatography (LC)-electrospray ionization-multi stage (MS)/MS direct analysis. The entire shotgun proteomic experiment was fined in only 1 h with the identification of 313 proteins ($p<0.01$). This new method of protein digestion by immobilized trypsin assisted by microwave is remarkably useful for large-scale proteomic analysis [126].

Monodispersed superparamagnetic maghemite NPs was used for the immobilization of thermostable raffinose-modified trypsin using glutaraldehyde. The covalent binding of trypsin resulted into an enhancement in its thermostability and elimination of autolysis. Raffinose-modified trypsin catalyzed fast digestion of proteins in solution and their identification by MALDI-TOF-MS [127]. Miao et al. [128] demonstrated a new method by combining trypsin-immobilized MNPs and microwave-assisted protein digestion of human lens tissue. The digested proteins were identified by LC and MS. The lens proteins were digested under optimized conditions determined using BSA as the standard protein, before LC and MS analysis. This new method helped in the identification of 26 proteins whereas only 11 proteins were identified by earlier traditional digestion method in solution in 12 h. γ-Crystallin, β-crystallin and SOD 1 proteins are related to cataract development, identified by microwave-assisted method but not by traditional method. The MNPs bound trypsin was easily separated from the reaction mixture. This new digestion method has shown its potential at large-scale proteomic analyses. Trypsin-coated MNPs (EC-TR/NPs) were prepared via a simple multilayer random crosslinking of trypsin molecules onto MNPs. This enzyme preparation was highly stable and was simply collected by a magnet from reaction mixture after the digestion of protein. EC-TR/NPs exhibited a very little loss of trypsin activity after multiple uses and continuous shaking, whereas the conventional immobilization of covalently attached trypsin on NPs resulted in a rapid inactivation under identical experimental conditions due to denaturation and autolysis of trypsin. A single model protein, a five-protein mixture and a whole mouse brain proteome were digested at atmospheric pressure and 37°C for 12 h or in combination with pressure cycling technology at room temperature for 1 min. EC-TR/NPs showed equally to or better than soluble enzyme in terms of both identified peptide/protein number and the digestion reproducibility in all these experiments [129].

Jiang and colleagues [130] prepared magnetic Fe_3O_4 NPs modified GO nanocomposites (GO-CO-NH-Fe_3O_4) by making covalent bonds between amine groups of functionalized Fe_3O_4 and carboxylic groups of GO. This modified magnetic GO support was used to immobilize trypsin via π-π stacking and hydrogen bonding. The binding capacity of trypsin was as high as 0.275 mg mg^{-1}. GO-CO-NH-Fe_3O_4 is also working as an excellent microwave irradiation absorber together with an immobilizing support. The protein digestion efficiency was further improved by microwave assistance. Reactor containing immobilized enzyme digested standard proteins efficiently within 15 s, with sequence coverage better than those obtained by conventional in solution digestion in 12 h. Since trypsin was immobilized under mild conditions, the enzyme activity of immobilized enzyme reactor preserved at least for a month. Moreover, due to good hydrophilicity of GO, no peptide residue was observed in the sequent digestion of BSA and myoglobin. Qin et al. [131] obtained a novel immobilized trypsin by using hairy noncross-linked polymer chains hybrid MNPs. This immobilized trypsin was ultra fast, highly efficient proteomic digestion and facile ^{18}O labeling for absolution protein quantification. The flexible noncross-linked polymer chains not only provide large amount of binding sites but also work as scaffolds to support 3-dimensional trypsin immobilization which leads to increased loading amount and improved accessibility of immobilized trypsin. For complex proteomic samples, obviously increased digestion efficiency and completeness was demonstrated by 27.2% and 40.8% increase in the number of identified proteins and peptides as well as remarkably reduced undigested proteins residues compared to that

obtained by conventional free trypsin digestion. The successful application in absolute protein quantification of enolase from *Thermoanaerobacter tengcongensis* protein extracts using ^{18}O labeling and MRM strategy further exhibited the potential of this hybrid NP immobilized trypsin for high throughput proteome quantification.

Magnetosomes from microorganisms were selected as a magnetic support for covalent immobilization of thermostable trypsin conjugates. Prior to covalent immobilization, bovine trypsin was modified by binding to α-, β- and γ-cyclodextrin. Modified trypsin was bound to the magnetic support via amine groups using EDC and sulpho-NHS as coupling reagents. Immobilized trypsin exhibited enhanced resistance to high temperatures, eliminated autolysis, unchanged pH-optimum and significant storage stability and reusability. The proteolytic performance was demonstrated in solution digestion of model proteins such as horseradish peroxidase, BSA and hen egg white lysozyme followed by MS. It has been demonstrated that both magnetic immobilization and chemical modification enhanced the properties of trypsin making it a potential tool for protein digestion [132]. The immobilization of trypsin onto the superparamagnetic carboxymethyl chitosan, Fe_3O_4 (PEG+CM-CTS) NPs was performed. The immobilized trypsin exhibited less affinity to the Nα-benzoyl-L-arginine ethyl ester as compared to soluble enzyme. However, the MALDI-TOF MS analysis demonstrated that immobilized trypsin has digested BSA more efficiently under mild conditions. It reduced significantly digestion time from 12 h to 15 min. Immobilized enzyme maintained 76.3% of its activity after its 6-repeated uses [133]. Shen and coworkers [134] developed a biocompatible reactive polymer, PGMA, grafted from MNPs by atom transfer radical polymerization method and used this support for the immobilization of trypsin for microwave-assisted digestion. The MNPs immobilized enzyme exhibited excellent activity, repeatability and stability. It was noticed that the digestion efficiency of immobilized trypsin was enhanced by the lengthened polymer brushes due to increased immobilization amount. The digestion efficiency of the immobilized biocatalyst has been demonstrated by taking cytochrome c as a model protein and the microwave-assisted digestion was completed within 15s. The enzymatic digestion of protein corona was the primary step to achieve identification of components of protein corona for the bottom-up proteomic approaches. MNPs immobilized trypsin was used for tryptic digestion of protein corona. It showed that the number of identified bovine serum proteins on the commercial Fe_3O_4 MNPs was increased to 13% for the immobilized trypsin after 1 h digestion as compared to overnight digestion obtained by soluble trypsin. The immobilized trypsin was used for the identification of human plasma protein corona. The results demonstrated an effective digestion of human plasma proteins and the identification of 149 human plasma proteins related to putative critical pathways and biological processes [135].

7. OTHER HYDROLASES

Table 4 demonstrates MNPs support bound other hydrolases, their mode of immobilization and improved properties.

Table 4. MNPs immobilized other hydrolases, their mode of immobilization and improved properties.

Name of enzyme	Name of support	Mode of immobili-zation	Property/properties	Reference/s
ALP	MNPs Fe_3O_4 via carbodiimide	Covalent binding	Enhanced storage stability, high efficiency of plasmid dephosphorylation	[136]
ALP	Superparamagnetic NPs	Covalent binding	Useful for inhibitor screening	[137]
L-asparaginase	Superparamagnetic dextran MNPs	Covalent binding	Improved thermostability & resistance to proteolysis	[139]
L-asparaginase	Synthetic hydrogel-MNPs	Entrapment	Enhanced cells permeability of enzyme	[139]
Beta-lactamase	Silica-encapsulated MNPs	Covalent binding	Excellent recovery & reusability	[141]
His-tagged epoxide hydrolase	Magnetic silica assembled with NiO NPs	Bioaffinity binding	Very high reusability	[143]
Recombinant esterase	MNPs via glutaraldehyde	Covalent binding	High reusabilty	[145]
PGA	Polymer brush-grafted magnetic microspheres	Entrapment	High thermal stability & enhanced tolerance to pH	[147]
PGA	Magnetic cellulose porous microspheres	Entrapment	High catalytic efficiency, thermal & pH stability, good reusability	[148]
Formylglutathione hydrolase	Superpara-magnetic core-shell NPs, Fe_3O_4@Au.	Covalent binding	Activity enhanced two-times	[152]
Phytases	Fe_3O_4 MNPs	Covalent binding	Excellent thermal & operational stability and reusability	[150]
Urease	Phosphonomethyl IDA coated MNPs via carbodiimide	Covalent binding	High reusability	[154]

Name of enzyme	Name of support	Mode of immobili-zation	Property/properties	Reference/s
Urease	Cobalt oxide MNPs Co_3O_4	Adsorption	High selectivity, reusa-bility & stability biosensor	[155]
Urease	MNPs functionalized by siloxane via active thiol or thiol-and-alkyl moieties	Covalent binding, adsorption, entrapment	Alkyl functions permitted increased adsorbed enzyme	[157]

7.1. Alkaline phosphatases.

Alkaline phosphatase (EC 3.1.3.1) hydrolyze phosphate groups from many types of molecules, including nucleotides, proteins and alkaloids. The process of removing phosphate group is called dephosphorylation. This enzyme is abbreviated as ALP, ALKP and ALPase. As the name suggests, it is only effective in alkaline medium and sometimes it is also referred as basic phosphatase. Saiyed et al. [136] investigated direct binding of ALP on Fe_3O_4 MNPs in presence of carbodiimide using two different methods. ALP binding efficiency was in the range of 80-100% by both immobilization techniques. The retained activities were in the range of 20-38% by shaking method and 30-43% with sonication method, respectively. The immobilized enzyme preparations were more stable than free enzyme for at least 16-week storage. Moreover, enzyme immobilized on MNPs was successfully employed for dephosphorylation of plasmid DNA before it was used for ligation reaction. The use of immobilized ALP for plasmid dephosphorylation allows easy manipulation, reduces procedural time and also avoids exposure of reaction mixture to high temperatures. MNPs immobilized ALP was considered for enzyme inhibition study by taking theophylline, L-tryptophan and D-tryptophan as model inhibitors. The enzyme kinetics indicated that DL-tryptophan has chiral discrimination inhibition while L-tryptophan worked as an uncompetitive inhibitor for ALP. The result also exhibited that theophylline behaved noncompetitively and showed remarkably high inhibitory activity than L-tryptophan. The protocol described, allows easy manipulation, reduces procedural time and can be adapted to high-throughput screening of enzyme reactions and inhibitors [137].

7.2. L-asparaginase.

Asparaginase (EC 3.5.1.1) catalyzes the hydrolysis of asparagine to aspartic acid. These enzymes are expressed and produced by many microorganisms. Asparaginases can be used in various industrial and pharmaceutical applications. These enzymes are recorded as most important medication needed in a basic health system. Asparaginase is sold as a drug under the brand name Elspar for the treatment of acute lymphoblastic leukemia and is also used in some mast cell tumor protocols. This enzyme can be administered as an intramuscular, subcutaneous or intravenous injection without fear of tissue irritation. Asparaginase was immobilized on the superparamagnetic dextran MNPs. The effects of temperature and chymotrypsin on immobilized L-asparaginase were compared with those of soluble enzyme. Immobilized L-asparaginase showed improved thermal stability and high resistance to proteolysis by chymotrypsin [138]. Teodor and coworkers [139] constructed a novel system based on a biocompatible polymer with organic-inorganic structure capable of vectoring support for biologic active agents, L-asparaginase. The synthesized hydrogel-MNPs with entrapped L-asparaginase were characterized by using several techniques. The cytotoxicity of NPs was monitored and also the interactions with microorganisms. The size of hydrogel-MNPs entrapped L-asparaginase was below 30 nm in dried stage. These particles were capable of penetrating into cells and tissues.

7.3. β-Lactamase.

β-Lactamases (EC 3.5.2.6) break that ring open, deactivating the molecule's antibacterial properties. These enzymes are produced by some bacteria and are resistance to β-lactam antibiotics like penicillins,cephamycins and carbapenems (ertapenem). Some antibiotics have a common element in their molecular structure: a four-atom ring known as a β-lactam. β-Lactam antibiotics are normally employed to treat a broad spectrum of Gram-positive and Gram-negative bacteria [140]. β-Lactamases from Gram-negative organism was immobilized onto silica-encapsulated iron oxide MNPs of controlled dimension. It has been demonstrated that the relatively smaller sized silica-coated MNPs obtained by the microemulsion technique can hold big enzyme like β-lactamase, via chemical linkages on the silica overlayer without hindering the active site of enzyme. MNPs bound β-lactamase showed excellent recovery and reusability [141].

7.4. Epoxide hydrolase.

Epoxide hydrolase (EC 3.3.2.9.) catalyzes conversion of epoxides to trans-dihydrodiols, which can be conjugated and excreted from the body. It is also known as epoxide hydratase and it functions in detoxification during drug metabolism. Epoxides produced during degradation of aromatic compounds. Deficiency of this enzyme in patients receiving aromatic-type anti-epileptic drugs such as phenytoin is reported to lead to DRESS syndrome [142]. Choi et al. [143] immobilized a recombinant triple-point mutated fish microsomal epoxide hydrolase containing a His-tag on the magnetic silica assembled with NiO NPs. The immobilized epoxide hydrolase retained nearly 90% of its original activity after 10-repeated uses. (S)-styrene oxide with 98% enantiopurity was repeatedly prepared with more than 50% theoretical yield by magnetically separable high-performance mutated enzyme.

7.5. Esterase.

An esterase is a hydrolase that splits an ester bond in between an acid and an alcohol in the presence of water. Several kinds of esterases are available but differ in their substrate specificity, protein structure and biological functions. Gold-coated MNPs was manufactured and used as a support for immobilization

of hexa-arginine-tagged esterase. Immobilized esterase activity was evaluated by monitoring hydrolysis of *p*-nitrophenol butyrate. However, the immobilized enzyme exhibited a lower activity (60%) compared to free enzyme, the original activity of the enzyme was retained even after its 7-repeated uses [144]. A recombinant esterase from *Pseudomonas putida* IFO12996 was covalently coupled to MNPs via glutaraldehyde. The immobilized esterase maintained 63% of its activity and it showed same temperature and pH stability as free esterase. The immobilized esterase hydrolyzed methyl dl-β-acetylthioisobutyrate to give d-β-acetylthioisobutyric acid with enatiometric excess value of 97.2% and enantioselectivity value of 245. It maintained 84% of activity and 40% of conversion after 10-repeated uses [145].

7.6. Penicillin G acylase (PGA).

Penicillin G acylase (EC 3.5.1.11) is a hydrolytic enzyme that acts on the side chains of penicillin G, cephalosporin G and related antibiotics in order to produce β-lactam antibiotic intermediates, 6-amino penicillanic acid and 7-amino des-acetoxy cephalosporanic acid with phenyl acetic acid as a common by-product. These antibiotic intermediates are most important building blocks for semi-synthetic antibiotics, such as ampicillin, amoxicillin, cloxacillin, cephalexin, and cefatoxime [146]. PGA catalyzes following reaction.

Penicillin G+ H_2O ⇌ Phenyl acetic acid + 6-Aminopenicillanic acid

PGMA-co-(glycerol monomethacrylate)]-grafted magnetic microspheres prepared by graft random copolymerization via ATRP from polymer microspheres with dispersed Fe_3O_4 NPs. PGA was immobilized on polymer brush-grafted magnetic microspheres. The immobilized PGA prepared with initial GMA/glycerol monomethacrylate ratios of 40/60 to 60/40 had greater catalytic activity than that prepared with higher proportions of GMA in initial monomer mixture. The immobilized PGA showed high thermal stability and enhanced tolerance to pH changes [147]. PGA was successfully immobilized in magnetic cellulose porous microspheres. The immobilized enzyme showed highly effective catalytic activity, thermal stability and enhanced tolerance to pH changes. The cellulose microspheres loaded enzyme was easily collected by applying magnetic field. Thus, it makes the preparation more easy reusable [148].

7.7. Phytases.

A phytase is a type of phosphatase that catalyzes hydrolysis of phytic acid, myo-inositol hexakisphosphate. It is indigestible, organic form of phosphorus that is found in grains and oil seeds and releases a usable form of inorganic phosphorus. Phytases are present in animals, plants, fungi and bacteria but majority of the phytases have been characterized from fungi [149]. Greiner et al. [150] carried out covalent immobilization of three phytases from different origin onto Fe_3O_4 MNPs. Binding efficiencies in all three phytases were more than 70% relative to the number of aldehyde groups available on the surface of MNPs. Temperature stability of all immobilized phytases was enhanced while the pH dependence of enzyme activity remained unchanged. Maximum catalytic activity of the immobilized phytases was found at 60°C (rye), 65°C (*Aspergillus niger*) and 70°C (*Escherichia albertii*). The immobilized enzymes exhibited same substrate specificities and unique myo-inositol phosphate phosphatase activities as their soluble counterparts. However, the catalytic turnover number dropped drastically for the immobilized phytases. The amount of the desired partially phosphorylated myo-inositol phosphate isomer has been simply controlled by contact time of substrate and immobilized enzymes. The immobilized phytases showed significantly very high operational stability by retaining almost full activity even after their 50-repeated uses.

7.8. S-formylglutathione hydrolase.

S-formylglutathione hydrolase (EC 3.1.2.12) belongs to the family of hydrolases, specifically those acting on thiolester bonds. The systematic and trivial names are same for this enzyme as S-formylglutathione hydrolase. This enzyme participates in methane metabolism. The methylotrophic yeast *Candida boidinii* exhibits *S*-formylglutathione hydrolase activity, which participates in glutathione-dependent formaldehyde oxidation during growth on methanol as the main carbon source [151]. It catalyzes the following reaction.

S-Formylglutathione + H_2O ⇌ Glutathione + Formate

Immobilization of S-formylglutathione hydrolase from the psychrophilic *P. haloplanktis* TAC125 was done on the gold coated surface of modified superparamagnetic core-shell NPs, Fe_3O_4@Au. This enzyme was originally annotated as a putative feruloyl esterase, an enzyme that releases ferulic acid (an antioxidant reactive towards free radicals such as reactive oxygen species) from polysaccharides esters. The immobilized enzyme was found twice more active than its soluble counterpart in solution [152].

7.9. Urease.

Ureases (EC 3.5.1.5) are found in several bacteria, fungi, algae, plants and some invertebrates, as well as in soils. Ureases are nickel-containing metalloenzymes of high molecular weight. They hydrolyze urea into CO_2 and NH_3 [153]. The reaction occurs as follows:

$$(NH_2)_2CO + H_2O \rightarrow CO_2 + 2NH_3$$

Urease was immobilized on the phosphonomethyl IDA coated MNPs via carbodiimide crosslinking. The catalytic activity of the immobilized urease was comparable to the free enzyme. The immobilized urease showed remarkably very high activity after 6-repeated uses [154]. Wazir [155] has developed an electrochemical biosensor based on urease immobilized on cobalt oxide MNPs Co_3O_4 for the biosensing of urea. Urease was physically adsorbed onto MNPs Co_3O_4. The electrochemical response of the urease/Co_3O_4/Cu biosensor against a standard reference electrode (Ag/AgC1) was evaluated for logarithmic concentration of urea from 0.1-80 mM. The expected biosensor showed maximum output response at neutral pH and the stable output signal was obtained nearly in 12 s. Moreover, the manufactured biosensor exhibited high selectivity, reusability and stability. A potentiometric biosensor for the measurement of atrazine pesticide was developed by using urease immobilized onto the insulator-semiconductor electrode and different additional materials such as glutaraldehyde crosslinking agent, BSA, coated

Fe_3O_4 MNPs, cationic poly(allylamine hydrochloride) and anionic poly(sodium 4-styrenesulfonate) polyelectrolytes. The concentration of atrazine was measured by its inhibitory effect on urease biosensor. An incubation time of 30 min was selected to examine inhibitory effect of different concentrations of atrazine on urease biosensor. The obtained results showed that the detection limit of atrazine via inhibition of urease was nearly 0.13 μM with a dynamic concentration range from 10^{-2}-10^{-7} M [156]. Urease was immobilized on the surface of MNPs functionalized by siloxane layers via active thiol or thiol- and-alkyl moieties. Immobilization of urease was performed by three different methods such as covalent binding, adsorption and entrapment during hydrolytic polycondensation reaction. For entrapment the enzyme was taken into solution prior to functionalization of the magnetite. The amount of enzyme entrapped was more than 700 mg g^{-1} MNPs, but its activity was decreased compared to soluble enzyme in between 10 and 18%. Covalent binding of urease was almost as efficient as entrapment but its remaining activity was 75%. Urease immobilized by adsorption on the surface of thiol-functionalized particles retained nearly 97% of the original activity. Introduction of alkyl functions permitted increase of the amounts of adsorbed enzyme, but its activity was somewhat decreased [157].

8. CONCLUSION AND FUTURE PROSPECTS

Numerous kinds of hydrolases have been successfully immobilized on the surface of modified and unmodified MNPs and entrapped into polymeric matrices along with MNPs. Covalent binding and physical adsorption both methods have been involved in surface immobilization of hydrolases. Various kinds of chemical compounds have been used for the functionalization of supports and for making covalent bonds between MNPs support and hydrolases. It has been concluded from this review work that in majority of the immobilized hydrolases, the stability of the enzymes was enhanced against different types of chemical and physical denaturants. It has also been noticed that the hydrolases immobilized to MNPs carrier were recalcitrant to inhibition caused by their specific inhibitors. The reusability and operational stability of the bound enzymes were also significantly enhanced. Hydrolases immobilized to the MNPs support have been employed in several bioanalytical, biomedical, therapeutic, industrial, pharmaceutical, biotechnological and biosynthetic fields. In few cases, it has been noticed that the immobilization yield of the bound enzymes was quite low. Such types of problem must be solved by selecting proper procedure of activation of MNPs supports or by using an appropriate crosslinking agent.

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10. ACKNOWLEDGEMENT

All kinds of help during writing of this manuscript from the Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, India is gratefully acknowledged.