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Chapter 2

Micro- and Nanocarriers for Immobilization of Enzymes

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Abstract

Two types of micro- and nanocarriers for immobilization of enzymes for biotechnological and biomedical applications are described: magnetic nanoparticles and cross-linked enzyme aggregates (CLEAs). Nanosized structures with their large surface and smaller size volume ratio, which is dependent on their strong magnetic dipole, give key features that make magnetic nanoparticles useful in many biotechnological and biomedical applications. They are therefore used as carriers to which different active substances can bind. The preparation of the magnetic nanoparticles, possible surface coating methods, and functionalization with different materials are described. Enzyme immobilization methods, such as adsorption, affinity binding, chelation, or metal binding or covalent binding, enable the preparation of efficient and stable enzyme bound to magnetic nanoparticles. Such a product may be used among bioreactor applications for targeted drug delivery in biosensors or bioimaging and magnetic resonance imaging. Preparation of CLEAs, the microsized enzyme structures without a carrier, is described as well. Their main advantage is very simple preparation, where two steps, precipitation of the enzyme and cross-linking, are joined. A broad spectrum of enzymes for CLEA preparation has been used and many biotechnological reactions are catalyzed. The improvement in CLEA preparation to enhance their stability and operability is also shown.

Keywords: Biotechnology, Biomedicine, Enzymes, immobilization, carriers, nanotechnology.

1. Introduction

Thirty years ago, the first chemical immobilization of proteins and enzymes was performed. Immobilized proteins are currently used for processing of several products in different areas: from food industry to environment and medicine. Their use is also in many bioanalytical and...
biomedical applications, which include the use of immobilized antibodies or antigens in bioaffinity chromatography, of immobilized receptors or ligands, and of immobilized cells in biosensors.

Because free enzymes show poor stability towards heat and pH, they offer limited possibilities of its reusability and recovery. To improve those main characteristics, new approaches are constantly producing new ways of improvement in enzyme stability and reusability with growing tools, such as immobilization techniques. New immobilization techniques offer new improved materials, which provide high-performance biocatalysts and favorable environments in which immobilized enzymes can work. These environments include much wider pH and temperature range and give more activity and stability than free enzymes [1–5]. Numerous applications of magnetic micro- and nanoparticles in biotechnology and biomedicine have been expanding and growing over the last decade due to their tremendous potential in targeting and delivery systems and due to their advantages in biosubstance binding. The development and synthesis of such materials have contributed immensely to several research disciplines, such as pharmaceutical, biomedical, and biotechnological to physics and electronics, to advanced materials, and to chemical science in general in a form of imaging agents, sensors, drug delivery targets/vehicles, and diagnostic tools. A great contribution was made to improve engineering skills in surgical diagnosis, therapy, and treatment [6–9]. The advantages and applicability of nanoparticles has opened up a large scope of studies. The main advantages of nanoparticles, compared to larger-sized particles, are their high surface-to-volume ratio and hence higher surface energy and excellent magnetic properties and, of course, their small size (approximately 1–100 nm). Their inorganic magnetic core is usually surrounded by layers of functional coatings. Their large surface and smaller size volume ratio, which is dependent on their strong magnetic dipole, gives key features that make them useful in many biotechnological and biomedical applications. Magnetic nanoparticles are therefore used as carriers to which different active substances can bind. They also have high coercivity and high magnetic susceptibility and are often bound together in aggregates, which give them many new possibilities in properties and functionalization. High surface area of magnetic nanoparticles allows the improvement of its bioapplicability and bioavailability, which allows larger dosage of bulk drugs and its pharmacokinetic properties and increase of vascular circulation. In nanoparticle applications, their properties are mainly used to develop drugs for targeted delivery systems and for diagnostic purposes, as they are a good contrast device for magnetic resonance imaging (MRI). Magnetic nanoparticles are able to concentrate in a specific tissue or anatomical location. The results of clinical studies may help us determine the key substances for immobilization on magnetic nanoparticles with the aim to gain better control of four healing processes: migration and proliferation of fibroblasts, deposition of extracellular matrix, angiogenesis, and transformation (scarring). At the same time, the optical properties of magnetic nanoparticles are used as alternatives to organic dyes for imaging purposes and also provide enhanced target specificity, which makes them an attractive drug delivery vehicle with an advantage and possibility of monitored drug release. Magnetic nanoparticles coated with different materials give more advantages in biological applications and provides an improvement in properties. Those properties can provide less cytotoxicity, improve biocompatibility, and provide better conjugation with other biologically active substances. Because
magnetic nanoparticles are generally toxic to a living system, when they penetrate through membranes and interfere with basal metabolic reactions within the cell, a form of a coating on top of the core particle can make the magnetic nanoparticle less toxic and more biocompatible. The conjugation of biologically active substances and other suitable proteins onto its surface can improve many bioapplications. Magnetic nanoparticles modified with different surface substances are able to improve the surface chemistry of the nanoparticles to increase its affinity to bind different biologically active substances, proteins, DNA complexes, drugs, receptors, etc. All these modifications have led to improvements in the synthesis and preparations of magnetic nanoparticles that have to be in sync with a certain biological system. Therefore, biocompatibility and cytocompatibility are the most important aspects to increase the therapeutic value in using magnetic nanoparticles as drug delivery vehicles and furthermore in controlling drug release [10–14].

Another interesting area are micro- and nanosized enzyme cross-linked aggregates without additional carrier. Due to their extremely high activity, they are welcome in biotechnology and in biomedicine applications as biosensors.

1.1. Immobilized enzymes in biotechnology

Over the past decades, immobilized enzymes have affected numerous technologies in industrial processes. The ability of the enzymes to catalyze the reaction has made them indispensable to science for many years. Because one of the most important roles of enzymes as natural biocatalysts is their ability to enhance the rate of all chemical reactions, they increase the rates of chemical reactions without their altering or being consumed by the reaction and without changing the equilibrium between reactants and products. The most valuable properties of immobilized enzymes are their ability of easy reusability, lower degradation, higher control over the reaction rate, and ability to prevent contamination of the substrate with enzyme or protein. Also, the immobilization of the enzymes has been shown to improve the stability of the enzymes and to lengthen their half-life. It offers the enzymes to work in a larger range of environments, which improves enzyme stability against temperature, solvents, pH, contaminants, and impurities. The immobilization of the enzymes increases the productivity of biocatalysts and enhances their features, which makes them more attractive and highly applicable in all kinds of evolving biotechnologies.

The enzyme, the matrix, and the mode of attachment are the main components of an enzyme immobilization, where the main advantages are the improvement of enzyme stability, increase of volume-specific enzyme loading, and simplification of biocatalyst recycling [1,3].

Immobilized enzymes have been employed in reactions in either a batch or a continuous flow reactor. Batch reactors are applicable for smaller productions. They are suitable for expensive enzymes, as they can be used in small amounts and are not wasteful. Continuous reactors are suitable for increasing the time of reactions as well as the efficiency of a reaction using biocatalysts.

With recent advances and improvements in biotechnology, immobilized enzymes have been used in a wide range of applications. The advances include the improvements of immobiliza-
tion techniques, new methods for the production or breakdown of compounds using biocata-
lytic reactions, methods of targeted drug delivery and tumor identification, and advances in
using immobilized enzymes in biosensors. The most frequent enzyme immobilization is used
for the synthesis of chemicals, where the industrial applications are straightforward. Through
biocatalyzed reactions, they provide a reusable method for the production of chemicals and
can break down harmful or undesired compounds. To develop stable and recoverable enzymes
for multipurpose biotechnological applications and to increase their utilization as industrial
biocatalysts, biocatalysts must be obtained from enzymes isolated from microbes, which
naturally exist in extreme environments. Another way of obtaining stable enzymes is genetic
engineering, which involves isolation. The most common and best way of obtaining stable
enzymes is achieved by immobilization, protein engineering, and chemical modifications.
Protein engineering has been able to produce enzymes that differ from their native forms in
only several or one amino acids. This approach is offering many possibilities of stabilizing
enzymes by changes in amino acid sequences [15–25].

1.2. Immobilized enzymes in biomedicine

Currently, the routine use of immobilized proteins or enzymes in medical field is in the
diagnosis and treatment of various diseases. Immobilized antibodies, receptors, and enzymes
are used as biosensors for the detection of different bioactive compounds in diagnosis.
Encapsulated enzymes are used in different types of bioreactors for the separation of metab‐
olites from body fluids or for the improvement of metabolic deficiency in the human body.
Artificial cells and the development of controlled drug delivery systems for dosing of proteins
or enzymes is also an application of immobilized enzymes in biomedicine. One of the appli‐
cations of immobilized enzymes in medicine are also enzyme-based electrodes. High specif‐
icity and reactivity of an enzyme towards its substrate is used in biosensors, which have high
reliability, sensitivity, accuracy, and ease of handling and are relatively low cost compared to
conventional analytical methods. These biosensors are currently used in the clinical diagnosis
of glucose [26–30], exalate [31,32], urea [33,34], glutamate [35,36], theophylline [37], creatine
and creatinine [38,39], cholesterol [40,41], and bilirubin [42,43].
The application of immobilized enzymes in bioreactions is in the convection of inborn errors
of metabolism, cancer treatment, and separation of waste products from body fluids. Enzyme
or enzymes could be immobilized on solid support, encapsulated in sol-gel support, or
alternatively cross-linked without a support to form the bioreactor.

Some applications of bioreactors used in human medicine are in areas of

a. Degradation of organophosphate in organophosphate poisoning by the use of phospho-
triesterases [44,45],
b. Conversion of alcohol to acetate in alcohol poisoning by the use of alcohol dehydrogenase
(ADH) and acetaldehyde dehydrogenase [46,47],
c. DNA damage repair in skin aging and cancer by DNA repair enzyme (liposome) [48,49],
d. Hydrolysis of phospholipids in hypercholesterolemia by the use of phospholipase A\(_2\) (liposome) [50], and
e. Conversion of ifosfamide or oxazaphosphorine to tumoricidal metabolites in tumors by the use of cytochrome P\(_{450}\) (cells encapsulated in cellulose sulfate formation) [9,51,52].

2. Magnetic nanoparticles as nanocarriers

Recent developments in life sciences in the last decade have produced numerous applications of magnetic nanoparticles in different fields of science and technology (biomedicine, biotechnology, biochemistry, etc.) and offer exciting new possibilities for applying new methods in an ever-growing and developing field of nanomedicine.

Superparamagnetic iron oxide (magnetite and maghemite) nanoparticles are the most used particles in several research disciplines due to their advantages in biosubstance binding and their tremendous potential in targeting and delivery systems. The main advantages of nanoparticles, compared to larger-sized particles, are their high surface-to-volume ratio and hence higher surface energy and excellent magnetic properties. Magnetic nanoparticles have been widely used in enzyme immobilization because of their unique properties, which among others include superparamagnetism and easy separation [29–31]. Together with the bound enzymes, they are interesting for the use in bioreactors. Among other advantages of magnetic nanoparticles, such as biocompatibility, small size, low toxicity, and, especially, superparamagnetism, a significant reduction of the biocatalyst amount used in a reaction was achieved at nicotinamide adenine dinucleotide hydrate (NADH) oxidation in a microreactor using ADH-loaded magnetic nanoparticles. In a reactor system with an oscillating magnetic field and an actively moving ADH-loaded magnetic nanoparticles, 100% NADH conversion was achieved for residence time of just 2 min [53].

![Image of magnetic nanoparticle surrounded by layers of functional coatings](http://dx.doi.org/10.5772/63129)

Figure 1. Magnetic nanoparticle surrounded by layers of functional coatings.
Magnetic nanoparticles are typically crystals of inorganic elements for which the largest characteristic dimension is approximately 1 to 100 nm. Their inorganic magnetic core is usually surrounded by layers of functional coatings (Figure 1).

Frequently used magnetic nanoparticles are also cobalt and nickel magnetic nanoparticles. Cobalt is a ferromagnetic element commonly used as an alloying element in permanent magnets. Nickel magnetic nanoparticles are of great interest due to their superparamagnetic and optical properties. There have been many protocols for the synthesis of cobalt or nickel magnetic nanoparticles, which include thermal decomposition, reverse micelles, chemical reduction, and polyol reduction, and for cobalt magnetic nanoparticles also sonochemical synthesis [54–62]. There are also many alloy-bimetallic magnetic nanoparticles that include various systems, such as iron-cobalt, nickel-cobalt, iron-platinum, and cobalt-platinum.

However, because of their low toxicity and biocompatibility, the most used magnetic nanoparticles are iron oxide nanoparticles [10,13,63,64]. Iron, being a ferromagnetic element, has the highest saturation magnetization at room temperature; therefore, iron magnetic nanoparticles exhibit superparamagnetic behavior in the size range below 20 nm. In general, naked iron oxide magnetic nanoparticles are often highly reactive and can easily undergo degradation and oxidation, which leads to poor stability in strong acidic solutions and results in an iron oxide shell. Also, leaching can occur, which limits the reusability and lifetime of such materials [12,65–68]. The aggregation of the particles is also one problem, which is a result of large surface-to-volume ratio that presents another limitation. This limitation results in low surface energy that can occur due to strong magnetic attractions between particles and would result in the loss of activity or certain decrease of it.

To prevent and overcome such limitations, many various approaches can be used to modify the surface of magnetic nanoparticles. These approaches and techniques can improve and optimize their biocompatibility and biodegradability and, more importantly, can transfer a hydrophobic system into a hydrophilic one. To prevent oxidation and avoid instability in ambient conditions, iron magnetic nanoparticles must be protected by applying suitable coatings with materials that are chemically stable and biocompatible, such as gold (Au). Au shell makes a versatile surface for biofunctionalization due to its strong affinity towards thiols that are often present in biological molecules. Many versatile materials have been used in the application of these improvements. Surfactants, such as oleic acid, citric acid, lauric acid, alkylsulfonic acids, and alkylphosphonic acids, are commonly used. Also used as coatings are different polymers, such as polyethylene glycol (PEG), polyvinylpyrrolone (PVP), and polyvinyl alcohol (PVA). More importantly, also natural materials, such as chitin, dextran, alginate, albumin, gelatin, starch, liposomes, and ethyl cellulose, are widely used and applied as coatings onto iron oxide magnetic nanoparticles, which are bound chemically or by multiple hydrogen bonds and therefore develop hydrophilic system [69–75]. Coatings on iron magnetic nanoparticles do not only influence the colloidal stability but also the biological functionality of nanoparticles. Good colloidal stability and the prevention of particle aggregation in a pH area of approximately 7.4 are the most important requirements for biomedical applications. Molecules on the surface of nanoparticles can interact in different ways.
“Encapsulation” of nanoparticles in polymers can apply through polymerization in a suspension of nanoparticles or magnetic nanoparticle synthesis occurs in a polymeric media or polymer molecules are grafted at the surface of nanoparticles [76].

“Coating through hydrophobic interactions” is a strategy also named bilayer functionalization. Magnetic nanoparticles that are synthesized by thermal decomposition are capped by hydrophobic surfactants that are coated with amphiphilic molecules [77].

“Interactions using coupling agents or covalently binding” present an alternative to physisorption, as physisorbed molecules are easily desorbed when nanoparticles are introduced to physiological media and nanoparticles coated with adsorbed molecules tend to aggregate in phosphate-buffered saline (PBS), which is caused by the displacement of carboxylic acid groups from the nanoparticle surface by phosphate salts [78–80].

To improve coating stability, other high-affinity surface-capping agents are being explored (silane group, carboxylate, carboxylate and OH groups, catechol, hydroxamic acid, phosphonate groups, and inorganic coatings, which include silica, Au, and other metals and metal oxides). A silica shell is mostly used for the modification and protection of magnetic core, where it can prevent unwanted interactions, such as acidic corrosion. Silica coating has many advantages and can provide better stability under aqueous conditions and better biocompatibility [72,81,82].

Surface silanization is a commonly used method to introduce functional groups onto magnetic nanoparticles. The silanization process can widely improve and help forward to low cytotoxicity and high stability under acidic conditions and can ease the surface chemical modification. Reactions of silanization can be carried out in aqueous media or organic solvents at moderate temperatures. In the reaction mechanism, silane-based molecules such as 3-aminopropyl trimethoxysilane (APTMS) are covalently bound to the surface of nanoparticles to where other bioactive molecules can be further bound. The main advantage of a silane agent is its biocompatibility and a high density of surface functional end groups (alcohol, amine, thiol) to which other biocompatible components can bind and be functionalized [76,80,83].

Another agent used for the modification of nanoparticle surface is carboxylic acid group, which can be employed with oleic acid used as a surfactant. Carboxylic groups are reportedly more active than hydroxyl groups. However, when the hydroxyl group is ortho-positioned regarding the carboxyl group, both groups will coordinate to the iron atom, forming a chelating structure. Hydroxamic acid derivates are mainly used in ligand exchange process to coat nanoparticles in situ with fatty acids during synthesis. Using bifunctional phosphonic acid groups with polar end groups (−COOH, −NH₂, −OH) provides functionality on the surface and a hydrophilic and stable system.

The size of the magnetic carriers can be engineered by their preparation technique. Chitosan-coated magnetic nanoparticles were prepared using three different methods: microemulsion process, suspension cross-linking technique, and covalent binding method [84]. The size of the particles varied with the preparation method and was increased by the increase in the concentration of chitosan and decreased by the increase of the cross-linker concentration. Particles coated with chitosan had a spherical shape and a size range of 40 to 350 μm using the
microemulsion process, 10 to 50 μm using the suspension cross-linking technique, and 50 to 100 nm using the covalent binding method. Magnetic nanoparticles coated with chitosan with the covalent binding method were the most suitable for practical applications due to their sufficiently high values of amino groups (2.48 mmol/g), which is important for protein immobilization [85].

Immobilization methods developed for enzyme/protein binding onto iron magnetic nanoparticles are mainly divided into physical immobilization, covalent conjugation, and biologically mediated specific interactions.

2.1. Adsorption

2.1.1. Physical adsorption

Physical immobilization, including physical adsorption, is considered as the simplest method for protein or enzyme immobilization. This method is based on the adsorption of enzyme or protein on the surface of water-insoluble carriers, which include porous carbon, ion-exchange matrices, or polymeric aromatic resins (Figure 2). Furthermore, the method can easily be carried out without any additional coupling agents, surface treatment, or enzyme/protein modification. Also, it makes no conformation changes to the enzyme and is simple and cheap. It can simply be performed by dipping the material into a certain solution containing our target bioactive substances [86–88]. In recent years, many physically immobilized enzymes based on iron magnetic nanoparticles were developed. For example, hybrid magnetic Fe₃O₄-SiO₂-poly(ethylene oxide)-maltose nanoparticles were used as affinity adsorption carriers for the direct separation of maltose binding protein-fused heparinase I. The results demonstrated that the mentioned nanoparticles were suitable carriers for affinity purification and immobilization in a single step, showing high adsorption capacity [89]. Also, glucose oxidase was immobilized by employing tannic acid-modified CoFe₂O₄ magnetic nanoparticles. Using strong protein and tannic acid binding, glucose oxidase immobilization was carried out via physical adsorption, where it showed excellent reusability and where immobilized glucose oxidase maintained at 60% of its initial activity [90]. Feruloyl esterases (FAE) were also successfully immobilized on magnetic iron nanoparticles via physical adsorption. Optimal immobilization conditions were significantly influenced by pH 6, where the maximum activity of immobilized FAEs were observed [91]. Because physical adsorption is a simple and mild method, it involves weak interactions, where enzymes are attached to the matrix through hydrogen bonds [92], van der Waals forces [93,94], and hydrophobic interactions [95]. Thermomyces lanuginosa lipase (TLL) was immobilized via physical adsorption onto the polyethyleneimine-coated iron magnetic nanoparticles that were prepared for the chelation of three metal ions. The immobilized lipase obtained more than 60% of initial activity after 10 cycles and retained about 80% of initial activity after 14 days of storage [96].
Forces involved in noncovalent immobilization result in a process that can be reversed by changing the environmental conditions, such as pH, temperature, ionic strength, polarity of the solvent, and concentration of bioactive molecules. Immobilization by adsorption usually preserves the catalytic activity of the enzyme, which makes them economically attractive. However, because these interactions in physical adsorption are relatively weak, they often result in breaking away from the support or in enzyme leakage from matrix, which can lead to the loss of activity and denaturation of the protein or enzyme.

2.1.2. Affinity binding

Affinity binding has well been applied to enzyme immobilization, where the most important advantage is the selectivity of the interactions. The method often requires covalent binding of an expensive ligand to the matrix. For example, an oriented immobilization of chloroperoxidase on iron magnetic nanoparticles was achieved by layer-by-layer controlled assembly through specific interactions of avidin-biotin affinity binding [97].

2.1.3. Chelation or metal binding

Another method of reversible immobilization is also presented in chelation or metal binding, where metal salts or hydroxides on the surface of organic carriers are bound by coordination with nucleophilic groups on the matrix. The metal salt or hydroxide is precipitated on the support (chitin, alginate, silica-based carriers) by heating or neutralization. Some of the positions on the metal remain free, as it is impossible for the matrix to occupy all positions on the metal. This occurs because of the steric factors. The method is simple and can obtain relatively high remaining specific activities of the enzyme (30–80%). For example, β-D-glucosidase was immobilized on iron magnetic nanoparticles coupled with agarose via metal ion affinity between alkaline amino groups located on the surface of the β-D-glucosidase and the Co²⁺ chelated on the carrier [98]. Another example shows that metal chelate ions (Co²⁺, Cu²⁺, Pd²⁺) increase the capacity of enzyme immobilization, where polyethyleneimine-coated iron magnetic nanoparticles were prepared via chelation [96].
2.2. Covalent binding

Covalent immobilization is the most widely used immobilization method and an attractive one, as it can be regulated with specific functional groups to bind enzymes or proteins. The main advantage of covalent immobilization is that the bonds formed between the enzyme and the matrix are of stable nature, so the enzyme cannot be released into the solution and the enzyme leakage is absent or limited. There are several immobilization protocols that are being researched using covalent binding and applying it in enzyme immobilization [1,99–101]. For improving enzyme activity, a coupling reaction can be applied using coupling agents, such as glutaraldehyde (GA) [72,99,102–105] or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) [106,107]. GA and EDC are because of their functional group (aldehyde group) mostly covalently cross-linked to magnetic nanoparticles and enzymes/proteins due to their interaction with both functional groups (Figure 3). However, in some cases, the presence of a coupling agent can also cause conformational changes in proteins, which can result in a decrease of enzyme activity.

![Covalent binding](image)

Figure 3. Covalent immobilization of the enzyme.

There are many researches performed, where enzymes were covalently immobilized onto iron magnetic nanoparticles. For example, recombinant trehalose synthase was immobilized onto glutaraldehyde-activated silanized iron magnetic nanoparticles by covalent binding, where it retained 82% of its initial activity after successive 12 repeated cycles of reaction [108]. Another research reports the covalent coupling of lipase using EDC onto chitosan magnetic nanoparticles, where the immobilization of lipase was performed by the formation of an amide bond between the carboxy group of lipase and the primary amino group of the nanoparticle. Lipase immobilized onto the magnetic support demonstrated high catalytic activity and retained 78% of its initial activity [109]. Also, superparamagnetic nanoparticles were surface modified with a mixture of two polymers for lipase immobilization, where the lipase was covalently bound to the surface of nanoparticles via EDC/N-hydroxsulfosuccinimide sodium salt (sulfo-NHS) activation and retained 82% of its initial activity [73]. Another study reports recombinant acetyl xylan esterase (rAXE) being covalently immobilized with chitosan magnetic nanoparticles using glutaraldehyde and demonstrating better stability at thermal and pH ranges than soluble
free rAXE. The immobilized rAXE was stable for about 90% of activity in aqueous phase and retained only 60% of its initial activity after 10 cycles of reuse [105]. Furthermore, serratiopeptidase enzyme was immobilized by covalent binding through glutaraldehyde after aminofunctionalization of magnetic nanoparticles. The immobilized enzyme showed 67% of residual activity and good storage stability [110]. α-Acetolactate decarboxylase (α-ALDC) was covalently immobilized to paramagnetic Fe₃O₄-Si-NH₂ nanoparticles using 1-carboxyldimiazole (CDI). Such immobilized α-ALDC retained almost 65.35% of its initial activity and 31.05% of its initial activity after five times of reusing [111]. Another study reports of cholesterol oxidase (COX) immobilization to magnetic fluorescent core-shell structured nanoparticles, where immobilization consisted of silanization, linker molecule deposition, and enzyme coupling of GA and 3-aminopropyltriethoxysilane (APTES). Therefore, immobilized COX maintained 80% of its initial activity and 71% of its initial activity after seven consecutive operations [103]. AiiA, which is a 28-kDa lactonase, was covalently immobilized onto magnetic nanoparticles, where immobilized recombinant AiiA (r-AiiA) was evaluated for its ability to hydrolyze and inhibit bacterial quorum sensing [112]. An interesting research was presented where pullulanase was immobilized onto magnetic chitosan activated nanoparticles prepared by in situ mineralization, showing that, although the immobilization by electrostatic adsorption can preserve higher activity of the enzyme (87%), the poor stability of the immobilized pullulanase cannot be avoided. Covalent immobilization by cross-linking maintained 70% of initial activity but gave smaller activity losses and more improvement between enzyme and substrate affinity and gave more stability, as the covalent attachment formed between amino groups of the enzyme and support showed more catalytic activity than between the carboxy groups of the enzyme and support [113]. Another study describes COX covalently immobilized onto magnetic nanoparticles of maghemite further functionalized with silica and amino-silane molecules. Therefore, prepared nanoparticles immobilized with COX maintained up to 60% of initial activity [114], where another study shows how Aspergillus niger xylanase A (XyLA) was covalently immobilized onto chitosan-coated magnetic nanoparticles by forming covalent bonds between the aldehyde group of glutaraldehyde and the amino group of the enzyme. The retained activity of immobilized XyLA was 56.5% of its initial activity [115]. The immobilization of yeast ADH (YADH) from Saccharomyces cerevisiae onto chitosan-coated magnetic nanoparticles was performed by covalent binding, where different preparation conditions were taken into account, such as immobilization time, pH, and enzyme concentration. Finally, the optimum reaction temperature was 30°C with pH of 7.4 and the retained activity of immobilized YADH was 65% of its original initial activity [71]. Another work presents α-amylase being covalently immobilized using glutaraldehyde onto magnetic nanoparticles using gum acacia as the steric stabilizer. GA magnetic nanoparticles have been demonstrated to be capable of being reused for at least six cycles while retaining about 70% of the initial activity [116]. Another lipase immobilization was performed by both physical adsorption and covalent binding onto magnetic nanoparticles, of which the surface was grafted by block copolymer of 2-dimethylaminoethyl methacrylate (DMAEMA) and glycidyl methacrylate (GMA). The activity recovery of the immobilization lipase reaches 43.1% at the protein loading of 0.5 mg (per mg of support) and retains 73% of its initial activity at 65°C for 8 h and its residual activity is reportedly more than 55% after six consecutive cycles [117].
On the contrary, a biosorbent was successfully prepared by the immobilization of *Phanerochaete chrysosporium* with iron oxide magnetic nanoparticles and calcium alginate, which is capable of removing Pb(II) ions from solutions, as *P. chrysosporium* was reported for the biosorption of heavy metals, taking advantages of favorable heavy metal affinity. In this case, the immobilization assists native microbes in improving their biosorption capacity. The biosorption efficiency was reportedly highly time and pH dependent and affected by initial Pb(II) concentration [118].

2.3. Targeted drug delivery using magnetic nanoparticles

There are many advantages when talking about targeted drug delivery systems based on magnetic nanoparticles. Compared to other conventional drug carriers, magnetic nanoparticles have a better retention ability and are therefore promising simulative carriers that can transport a drug to the desired place in the body. Nanotechnology in biomedicine is mostly used for intravenous delivery, especially to cancer tissues. Another advantage of magnetic nanoparticles for targeted drug delivery is that nanoparticles as carriers can also be coated with different biodegradable organic polymers to enable interaction by binding with active molecules and to achieve a sufficient drug load. Thus, such system allows biodegradability, biocompatibility, nontoxicity, and prolonged circulation of a specific therapeutic or drug agent. Once the drug carrier is concentrated at our desired tissue location, the drug can be released either by enzymatic activity or by changes in physiological conditions, such as temperature, pH or osmolality, as can be seen in Figure 4.

An extensive research has been made regarding targeted drug delivery systems using magnetic nanoparticles. For example, magnetic molecularly imprinted polymer was synthesized using polydopamine, which was used for controlled 5-fluorouracil delivery in a spontaneous model of breast adenocarcinoma in BALB/c mice in the presence of an external magnetic field [119]. Another study investigates magnetic nanoparticles functionalized with thiol-terminated polyethylene glycol, loaded with anticancer drug doxorubicin (DOX) used as a biodegradable system for targeted delivery [120], whereas another research explores the same drug DOX delivered with β-cyclodextrin assembled magnetic nanoparticles, which were
fabricated with layer-by-layer method to develop a versatile biocompatible platform for selective loading, targeted delivery, and pH-responsive release of mentioned drug [8].

2.4. Magnetic nanoparticles in biosensors

Iron magnetic nanoparticles can also be coated with fluorescent materials or with silica, metal, or a polymer. Magnetic nanoparticles coated with such materials are used as bioanalytical sensors. In developing new biosensors, Au nanoparticles show good mechanical resistance and electrical properties as well as good biocompatibility. As all magnetic nanoparticles, they have large surface area for enzyme or protein immobilization. Biosensors contain layers that are bioselective, which can react with a target biomolecule that transforms the biologic interaction into physical signal (optical, chemical, electrical, thermal, etc.). The principle of biosensors is represented in Figure 5.

![Figure 5. Principle of biosensors: transforming biological interaction into electrical signal.](http://dx.doi.org/10.5772/63129)

A study proposes a biosensor using glassy carbon electrode modified with Au nanoparticles and tyrosinase, which is covalently immobilized in a dihexadecylphosphate film. Therefore, modified electrode is used to determine catechol concentrations by amperometry in natural water samples [121].

Biosensors are also applied to the determination of hepatitis B virus (HBV) DNA in urine and blood plasma samples. As described in a recent study, a magnetite and Au nanoparticle-modified paste electrode was prepared for the immobilization of thiol-modified HBV probe DNA. Therefore, developed biosensor could measure target HBV DNA virus concentration with low concentrations [122]. Later on, an ultrasensitive electrochemical DNA biosensor based on in situ labeling Au nanoparticles on the free terminal of hairpin DNA was developed, with good regeneration, stability, and hybridization selectivity [123]. Another paper describes Au nanoparticles being used to enhance electrochemical DNA biosensor, where the goal was to improve the sensitivity of biosensor by detecting small sequences in large amounts of double-stranded DNA [124]. Magnetic fluorescent core-shell nanoparticles immobilized with COX were prepared to be used as a sensing material with application in multiparameter fiber-optic biosensor based on enzyme catalysis and oxygen consumption [125].
2.5. Bioimaging and MRI using magnetic nanoparticles

MRI is a commonly used medical imaging technique that is a powerful and noninvasive tool used for the visualization of structures and functions in tissue. MRI is based on the detection of proton relaxation and an external magnetic field. Different enzymes and proteins immobilized on magnetic nanoparticles are largely applied in MRI imaging mostly because of their biocompatibility, superparamagnetism, low toxicity, colloidal stability at physiological conditions (37°C, pH 7.4, PBS buffer), and well-controlled surface charges. For example, magnetite nanoparticles were functionalized with mebrofenin, as it is known as a liver targeting function. Therefore, functionalized nanoparticles coated with silica were prepared as liver targeting contrast agents in MRI [126]. In another study, magnetic nanoparticles were used as drug carriers, aiming to form a magnetically controlled nanoplatform [127].

3. Cross-linked enzyme aggregates (CLEAs) as microcarriers

Many biotechnological approaches for production cover the synthesis of drugs. A lot of these enantiopure compounds, such as anticancer, antiviral, anti-infective, antipsychotic, antiarhythmic, cholesterol-lowering agents, calcium channel blockers, ACE inhibitors, and many others, may be synthesized enzymatically.

Via immobilization of enzymes, stabilization occurs. Intermolecular interactions are prevented [100] and the enzyme structure becomes more rigid due to multipoint covalent attachment [1]. In multimeric enzymes, the dissociation of the subunits is prevented [128] and inhibition is reduced [100]. However, structural properties such as size, specific area, and porosity that dictate the resistance to mechanical stress are also important.

The most used cross-linking agent for enzyme immobilization is glutaraldehyde, as it is available in commercial quantity and is relatively cheap. Although its chemistry has been not fully understood, it is generally accepted that the inter- and intramolecular cross-linking reaction occurs between the free amino groups of lysine (Lys) residues on the enzyme or protein molecule and the carbonyl group of glutaraldehyde resulting in a Schiff base formation [129].

3.1. Carrier-free immobilization

The CLEA method is used for the dual purpose of combining both the purification and immobilization of enzyme in one step [130]. The first step is enzyme precipitation with the most suitable precipitating agent and the second step is enzyme cross-linking with a cross-linking reagent. An example of CLEA preparation is presented in Figure 1, where the scheme illustrates the preparation of CLEAs from horseradish peroxidase (HRP) [131]. Beside the precipitating agent and cross-linker, some other compounds are added to form the enzyme clusters. The reason for their use is described below (Figure 6).
As mentioned before, the first step of CLEA preparation is the precipitation of the enzyme from the solution with the most suitable precipitating agent. Which precipitating agent is the most suitable one for a certain enzyme? This question may be answered after a simple experiment where the enzyme is precipitating with different precipitating agents and then resuspended again. The precipitating agent that gives the highest residual activity of the resuspended enzyme is the most suitable one. The most commonly used precipitating agents are acetone, methanol, ethanol, propanol, 2-propanol, and ammonium sulfate.

CLEAs are easily prepared from crude enzyme extracts, and the costs of (often expensive) carriers are circumvented. Many studies employed purified or partially purified enzyme samples for CLEA preparation [132–139]. On the contrary, it was successfully demonstrated that the lipase from *Penicillium expansum* (PEL)-CLEAs can be prepared directly from the fresh fermentation broth or its lyophilizate [140]. They generally exhibit improved storage and operational stability towards denaturation by heat, organic solvents, and autoproteolysis and are stable towards leaching in aqueous media. Furthermore, they have high catalyst productivities (kilogram product per kilogram biocatalyst) and are easy to recover and recycle. In CLEAs, immobilized enzymes are much more resistant to shear and extreme pH values than the soluble enzymes as well.

The particle size of CLEAs is usually between 5 and 50 μm, but it can be varied by changing the preparation parameters, such as precipitating agent or cross-linker concentration. Although with a smaller size of the clusters higher reaction rates are obtained, sometimes applications dictate the formation of bigger clusters. It is obvious that bigger particle sizes result in better filterability and smaller pressure drop in packed bed reactors. Table 1 represents particle size variations of CLEAs from cellulase (from 26 to 161 μm) in the dependence from the used precipitating agents.
Table 1. Particle size deviation of CLEAs from cellulase.

<table>
<thead>
<tr>
<th>Precipitating agent</th>
<th>Particle size of CLEA clusters (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propanol</td>
<td>26</td>
</tr>
<tr>
<td>Ethanol</td>
<td>43</td>
</tr>
<tr>
<td>Acetone</td>
<td>46</td>
</tr>
<tr>
<td>Tetrahydrofurane</td>
<td>67</td>
</tr>
<tr>
<td>Methanol</td>
<td>87</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>161</td>
</tr>
</tbody>
</table>

However, the concentration of the cross-linking agent must be carefully optimized to prevent the enzyme activity loss. Namely, too big particle size of the enzyme preparation, which is formed with high glutaraldehyde concentration, may affect the mass transfer of the substrates to contact with the enzymes in the inner side. In this case, inner enzymes of the enzyme preparation lose the opportunity to form complexes with the substrates, which results in the apparent activity loss of the enzyme.

Scanning electron microscopy showed a uniform structure of the cross-linked α-amylase aggregates. The diameter of CLEAs was about 250 nm with a small deviation (Figure 7). CLEAs
can form larger clusters that do have mass transfer limitations, especially in fast UV-based assays. One CLEA particle can contain up to 8 million enzyme molecules. The size of these clusters can be up to 100 μm, making them visible with the bare eyes. The number of CLEAs in a cluster is much less uniform than enzymes in an aggregate; it can vary from a few to many hundred thousands [141].

A broad spectrum of enzymes for CLEA preparation has been used. The majority of CLEAs that have been described involve enzymes, which belong to the class of hydrolases (proteases, amidases, lipases and esterases, glycosidases), oxidoreductases (oxidase, peroxidase), or lyases (nitril hydratase, hydroxinitril lyase), have been successfully immobilized as CLEAs [142]. The reason is probably because they are the enzymes that have the most industrial applications and are the simplest enzymes to work with.

With enzymes containing few or no accessible Lys residues, cross-linking may be insufficient and lead to CLEAs that are unstable towards leaching in aqueous media. One way to overcome this problem is to add polyamines, such as polyethyleneimine, which are then coimmobilized with the enzyme [143]. Problems can also be encountered in CLEA formation when the protein concentration in the enzyme preparation is low. In such cases, CLEA formation can be promoted by the addition of a second protein, such as bovine serum alumin (BSA), as a so-called proteic feeder [144]. A typical enzyme with low content of amine residues is aminoacylase [145]. Therefore, the immobilization of aminoacylase was improved by adding BSA to yield coaggregates [146]. Using BSA as a proteic feeder is also a solution in cases when the enzyme activity is vulnerable to high concentrations of glutaraldehyde required to obtain aggregates [139,144]. Namely, a high concentration of glutaraldehyde may reduce the activity of the obtained CLEAs, as free amino groups present in the proteic structure may react with this cross-linker [147] or it can bind amino acids associated with the active site.

These drawbacks can be prevented by the use of molecules with a large amount of Lys residues in the surface, such as BSA. With such a solution, the possibility of cluster formation, as in the case of excess of glutaraldehyde, with mass transfer limitations, may be prevented. Shah et al. prepared CLEAs of *Pseudomonas cepacia* lipase (PCL) with BSA addition. The recovered activity relative to that of free enzyme was 100% in contrast with the same preparation without BSA, which retained only 0.4% of activity [16]. More recently, the synthesis of CLEAs of lipase B from *Candida antarctica* (CALB) showed difficulties in the cross-linking step due to the low content of surface Lys on CALB [18]. The addition of BSA as a feeder allowed an effective cross-linking step and permitted to greatly stabilize CLEAs.

To enhance the activity of CLEA used enzymes, it is possible to use a modification of the CLEA synthesis protocol using proteic feeder. The enzyme could be added after cofeeder aggregates are formed and cross-linked. A slightly cross-linked layer of the enzyme is formed over the cofeeder cores, which might lead to enhanced enzyme-substrate contact (Figure 8). This approach was successfully demonstrated by Guauque Torres et al. [147] using TLL. The layer methodology led to high increase in retained activity compared to non-layered TLL CLEAs.
3.2. CLEA formation using carriers

There may appear some difficulties using CLEAs in a reaction system, as it is difficult to isolate and recover CLEAs from the reaction system only by centrifugation or filtration, especially when the substrate particles are in the same size range as CLEAs [148]. Therefore, some new reactor styles were designed [149–152]. Another way is to improve the operability and mechanical stability of CLEAs. For that reason, CLEAs of papain were prepared in commercial macroporous silica gel from simple adsorption, precipitation, and one-step cross-linking [148].

Another example was when CALB was adsorbed and cross-linked on a polypropylene carrier [153]. Such enzyme preparation maintained its activity when dispersed in ionic liquids, such as [BMIm][NO$_3$], which denatured the free enzyme. This is an important fact, as ionic liquids are, to an increasing degree, being accepted as reaction solvents, including biocatalytic transformations [154,155]. However, ionic liquids that contain strongly coordinating anions, such as [BMIm] nitrate or acetate, interact sufficiently strong with CALB to dissolve the enzyme but also cause its deactivation [156,157].

Laccase-based CLEAs were stabilized through the formation of a surrounding polymeric network made of chitosan and 3-aminopropyltriethoxysilane (EPES-lac). The average size of EPES-lac particles was about 100 μm and with a spherical shape. The thermostability of the resulting enzyme polymer engineered structures of laccase CLEAs (EPES-CLEA) were more than 30 times higher than that of free laccase and CLEAs at pH 3 and 40°C. The formation of a polymer network around laccase-based CLEAs had a desirable effect on the biocatalysts formed in regard to chemical and physical characteristics, stability under harsh conditions, and kinetic parameters [158].

3.3. A broad spectrum of biotechnological reactions may be catalyzed with CLEAs

With CLEA of purified nitrilase from Escherichia coli harbouring gene of Pseudomonas putida, the hydrolysis of (RS)-mandelonitrile to (R)-mandelic acid, a versatile chiral building block,
was performed by Kumar et al. [130]. (R)-mandelic acid is namely used as a chiral synthon for the production of antitumor and antiobesity agents [159].

The industrial production of enantiopure L-amino acids from N-acyl DL-amino acid can be performed using L-aminoacylase (N-acyl amino acid amidohydrolase or acylase I; E.C. 3.5.1.14), which has excellent chiral specificity and is one of the top 10 enzymes used in biotechnology [160]. This enzyme from *Aspergillus* sp. is useful for a broad spectrum of reactions, such as enantioselective hydrolysis of amino acid esters and amides [161], regioselective alcoholysis of carboxylic acid esters [162–164], acylation of primary and secondary alcohols [165–168], and acylation of amines [165–169]. Immobilization of L-aminoacylase from *Aspergillus niger* using coaggregation with polyethyleneimine and subsequent cross-linking with glutaraldehyde resulted in stable aminoacylase-polyethyleneimine CLEAs (AP-CLEA) with significantly higher temperature and storage stability of the enzyme without affecting its enantioselectivity [170].

The enzymatic synthesis of β-lactam antibiotics, such as ampicillin, is generally performed under conditions, such as lower pH and temperature, which are far from the optimum of the enzyme [171]. With the aim of achieving a high synthesis/hydrolysis ratio at high conversion and a high productivity and space-time yield, penicillin G acylase was immobilized in the form of CLEAs using ammonium sulfate or tert-butanol as precipitation agents [132].

For obtaining advanced intermediates of β-lactam antibiotics such as desacetyl-7-ACA and desacetyl cephalosporanic acid, a highly active CLEA of the purified rAXE from *Bacillus pumilus* CECT5072 with strong operational stability was produced. The operational stability of this CLEAs was with a half-life of 45 cycles. Therefore, this new methodology should decrease the industrial cost of CLEAs both in terms of biocatalyst production and reusability [133].

Penicillin G acylase (penicillin amidohydrolase; E.C. 3.5.1.11) is widely used in the industrial synthesis of 6-aminopenicillanic acid (6-APA) by the enzymatic deacylation of penicillin G [172,173]. The free enzyme is known to have a limited thermal stability and a low tolerance toward organic solvents [174] and this drawback could be circumvented by the immobilization of the enzyme. In the synthesis of ampicillin, CLEAs of penicillin G acylase were more efficient catalysts than cross-linked crystals (CLECs) of the same enzyme and maintained their activity in organic solvents [175].

It is well known that lipases are the most commonly used enzymes in industrial scale. Esterification reactions to produce industrially important products such as emulsifiers, surfactants, wax esters, biopolymers, modified fats and oils, structured lipids, and flavorers [146], esters of short-chain alcohols and short-chain fatty acids, which are important aroma compounds [170], esters of short-chain alcohols and long-chain fatty acids, which are valuable oleochemicals that may be used as lubricants, diesel fuel, and antistatic reagents [138], and esters of long-chain fatty acids and polyhydric alcohols such as glycerol, sorbitol, and other carbohydrates, can be catalyzed by lipases. Therefore, there exist much interest in their immobilization methods. CLEAs from *Candida rugosa* lipase (CrL) using glutaraldehyde as the cross-linker had some advantages, such as thermal stability, good reusability and enhanced
stability, and better dispersibility in organic solvents, which were reflected in improved operational performance compared to the free enzyme in the synthesis of esters from a variety of alcohols and fatty acids [138].

The third generation of biodiesel from microalgae is interesting due to the advantages of easy cultivation and fast reproduction, high oil content, no competition for water and land resources, and being more ecofriendly [176]. PEL-CLEAs catalyzed biodiesel production from microalgal oil in the IL [BMIm][PF6] with a conversion of 85.7%, demonstrating that they can be taken as a promising catalyst for this application [140].

CLEAs may be used in the environmental care as well. Mushroom tyrosinase was used for the enzymatic treatment of phenolic wastewater. Catalyzed by the enzyme immobilized in the form of CLEAs, phenolic compounds such as phenol, p-cresol, p-chlorophenol, and bisphenol A can be efficiently eliminated, with a complete conversion, superior to other processes catalyzed by the same enzyme [177].

CLEAs of laccase are promising biocatalysts for the decolorization of synthetic dyes in aqueous solution [178]. Namely, industrial applications of laccase for dye decolorization require large amounts of readily available crude or purified laccase, enzyme stability under operational conditions, and recyclability [1,128,179], which can be achieved by enzyme immobilization in CLEA formation. The use of free laccases in such applications has disadvantages, such as their relatively short lifetime and their instability under harsh environment (e.g., temperature, organic solvents, and salts) [4].

In the case of removal of hydrogen peroxide residue from milk, production of gluconic acid, phenyl pyruvic acid, and dihydroxyacetone phosphate catalase has been used in combination with oxidases [180–184]. However, the single use and the instability of free catalase in harsh reaction conditions are not economically viable. The reusability of catalase plays an important role in the industrial applications and can be achieved by immobilization. Therefore, bovine liver catalase was immobilized as cross-linked catalase aggregates (CLEA-CAT) via precipitation with ammonium sulfate and then cross-linking with glutaraldehyde. Finally, BSA as the proteic feeder was used. Although the catalase had the low Vmax value after immobilization process, the thermal and storage stabilities of CLEA-CAT-BSA derivative were comparably higher than those of free CAT. CLEA-CAT-BSA derivative was reused for 400 cycles in the batch-type reactor and the total amount of H2O2 decomposed was 1.2×10^4 mol H2O2 throughout 400 consecutive uses [185].

There are also some unusual examples of CLEA use, such as biocatalytic synthesis of silver nanoparticles from silver nitrate. For this biocatalytic synthesis, immobilized NADH-dependent nitrate reductase as CLEAs was used based on the bioreduction ability of nitrate reductase [186]. At the same time, immobilization improved the thermal stability of the enzyme.

Another advantage of this type of immobilized enzymes is the preparation of combined CLEAs, so called combi-CLEAs, which is the possibility to coimmobilize two or more different enzymes in a single CLEA. In this case, CLEAs are capable to catalyze two or more biotransformations, independently or in sequence as catalytic cascade processes.

A good example is the bioconversion of sucrose to trehalose (Figure 9).
Figure 9. Biotransformation of sucrose to trehalose using combi-CLEAs in comparison to the use of individual enzymes for each reaction step.

Combi-CLEAs of amylosucrase (AS), maltooligosyltrehalose synthase (MTS), and maltooligosyltrehalose trehalohydrolase (MTH) were successfully established with acetone and glutaraldehyde (GA) as a precipitant and a cross-linker to achieve the one-step biocconversion of sucrose to trehalose [187]. The stability of this enzyme preparation was quite good, as reusability of about five cycles without any activity loss was obtained. This reaction is important in the food, cosmetic, and pharmaceutical industries, because trehalose is known to have high water-holding activity and this characteristic makes it applicable to the development of additives, stabilizers, and sweeteners [188].

4. Conclusion

The stability of enzymes is important for biotechnological applications and can be achieved via immobilization methods. Large amounts of research have been devoted into exploring the synthesis and modification of magnetic nanoparticles. They have been proven to be a successful tool for the immobilization of enzymes, proteins, drugs, and other biologically active molecules because of their biocompatibility, biodegradability, colloidal stability, low toxicity, and nanometer scale. Magnetic nanoparticles that are properly surface functionalized can be applied into various fields of science, mostly benefiting in biomedicine and biotechnology, improving targeted drug deliveries of active therapeutic agents, and exploring and developing new strategic applications to improve and increase our development in the future. Another interesting possibility for enzyme immobilization is CLEA formation. With their micrometer size and unique advantages, where there is no need for carriers and pure enzymes, they are applicable in different kinds of bioreactors and biosensors.

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