Chapter

# Magnetic Bio-Derivatives: Preparation and Their Uses in Biotechnology

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## Abstract

In this chapter, the authors will show different proposals of magnetic bio-derivatives and its applicability in biotechnology. The historical context of immobilized enzymes, as well as highlighting the main advantages and disadvantages of each, will be mentioned. Besides, iron oxides and composite materials will be presented as support for biomolecules immobilization. Composites are effortlessly prepared including many materials capable of providing advantages to the magnetic derivatives. Enzymes covalently linked to these magnetic particles combine their catalytic properties with reaction specificity, reusability, and possible reactor construction. In addition, proteins can also be purified by these magnetic composites containing specific ligands allowing reactors and reuses too. Some characterization techniques used to study the magnetic material and derivative immobilized will be described as well. Altogether, an engaging presentation about the interesting features of magnetic bio-derivatives will highlight their uses in biotechnology field as well as in others.

**Keywords:** iron oxides, composite, enzyme, protein, immobilization, purification

## 1. Introduction

Even today, the widespread use of magnetic particles in areas such as biotechnology, engineering, material sciences, biomedicine, and microbiology, among others, still is disclosed in the literature. So, a large part of the scientific community including biomedical, biologists, and pharmacists, in addition to chemists and physicists, is looking for novel applications for magnetic bio-derivatives obtained from biomolecules immobilized on iron oxide particles. The use of magnetic particles is further than matrices for biomolecules immobilization such particles can also be used as a potential contrast agent in magnetic resonance imaging (MRI), drug delivery, magnetic hyperthermia, photothermal therapy, and food treatment in order to better the organoleptic properties [1].

The great importance of developing attractive and promising magnetic bio-derivatives as well as bringing to light new applications is evident. Our research group has proposed a considerable number of magnetic materials from inorganic (e.g., diatomaceous earth) or organic (e.g., azocasein) compounds and iron oxides as support to enzyme immobilization or protein purification. Recently, magnetic

diatomaceous earth coated with polyaniline (mDE@PANI) exhibited good features as a matrix for covalent immobilization of three industrial enzymes (invertase, trypsin, and β-galactosidase) [2]. This magnetic composite was also promising for the treatment of boldo tea after immobilization of the enzyme tannase [3]. Alves et al. [4] reported a novel magnetic composite from magnetite and azocasein to trypsin purification of fish Nile tilapia (crude extract). The obtainment of antioxidant peptides for use in food products was possible by immobilization of protease on magnetic support [5]. These are some of our research involving magnetic materials and their applications that we will address in more detail later.

### 1.1 Historical background: enzyme immobilization on magnetic particles

In 1916, the first scientific report on immobilization of enzymes was announced. This finding involved the invertase, a hydrolytic enzyme, which preserved their catalytic behavior after being absorbed on charcoal or aluminum hydroxide surface [6]. Robinson et al. [7] reported the first work of the use of magnetic particles as support to enzyme immobilization in 1973. The authors purposed two magnetic materials from iron oxide (magnetite) and another compound as a matrix to immobilize the enzymes: α-chymotrypsin and β-galactosidase for applications in bioreactors. A year later, Van Leemputten and Horisberger [8] immobilized trypsin and invertase on functionalized magnetite. Since then, magnetic separation has become an increasingly popular tool for the process of separating biological molecules and cells.

According to IUPAC gold book [9], an immobilized enzyme is defined as "a soluble enzyme bound to an insoluble organic or inorganic matrix, or encapsulated within a membrane in order to increase its stability and make possible its repeated or continued use." An efficient and robust immobilized derivative must preserve good retention of the catalytic activity, possess greater thermal and operational stability, be reused without considerable loss of activity, allow the easy separation of products and enzyme, and be resistant to microbial attack. In addition, a derivative immobilized on magnetic particles has advantages such as (i) easy and fast separation of the reaction medium by application of an external magnetic field, (ii) enzyme which is not stressed since conventional methods of separation (e.g., centrifugation and filtration) can be avoided, and (iii) large loading of biomolecules onto small particles (nanoparticles (NPs)) as a consequence of their high surface area.

Since the last decade, several scientific works about immobilization of enzymes have been published. In the first month of 2019, over 100 articles with the keyword "enzyme immobilization" have been reported in the PubMed database.

### 1.2 Immobilization strategies: Choosing the better approach

The choice of the immobilization method is as important as the nature of biomolecule (e.g., biochemical properties) and the experimental conditions chosen to obtain an immobilized derivative with desired features. So special attention should be given to immobilization approaches since the applicability of the immobilized derivative depends on this. The characteristics of the support are also relevant; however, it will be discussed later.

Overall, the methods of immobilization are categorized as irreversible and reversible since those interactions between enzyme and support are from weak physical adsorption to strong covalent bonds. Irreversible immobilization is understood as the attachment of the biocatalyst to the support with retention of the biological activity. However, the detachment of the biocatalyst will lead to the loss of its activity. Covalent bond, entrapment or microencapsulation, and cross-linking belong to this category. Already the adsorption and the affinity methodologies are

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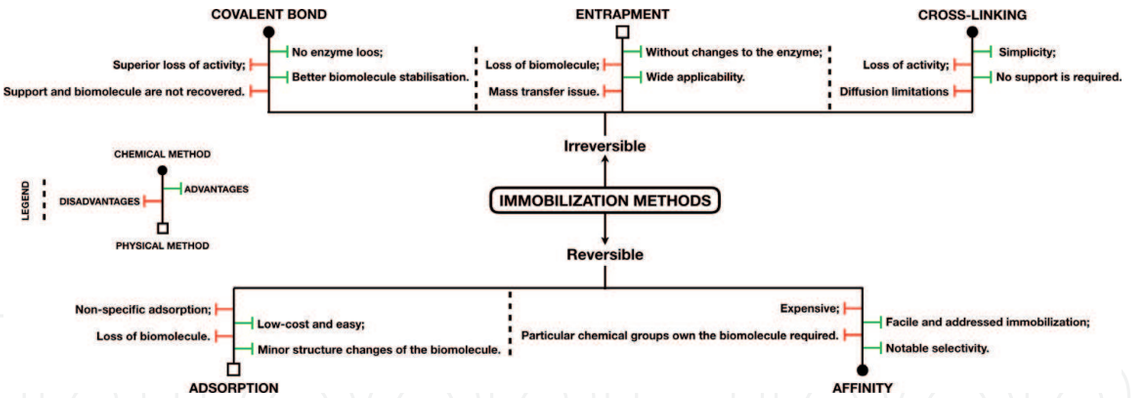


**Figure 1.**
*Scheme with major strategies used for enzyme immobilization. Advantages and disadvantages are also highlighted.*

associated with the reversible category. Additionally, the immobilization methods can be classified into chemical and physical according to support binding. **Figure 1** displays the most used immobilization methods together with some advantages and disadvantages.

Currently combinations of two or more immobilization methods can be employed to obtain derivative immobilized with features desired. Thus, the limitations from one type of immobilization could be avoided. Briefly, the main features of the immobilization methods will be mentioned as follows.

- **Covalent bond**. This method is based on the formation of covalent bonds between the biocatalyst and the support. For this, the presence of active chemical groups on surface of both components (i.e., biomolecule and support) is necessary. It is important to mention that these functional groups in the biocatalyst are not responsible for their catalytic activity. Moreover, the use of an activating agent, e.g., glutaraldehyde, which will lead the covalent attachment is required. Covalent bond is one of the most immobilization technique used.

- **Entrapment**. In this approach, the biocatalyst is restricted within a polymeric network. The substrate and products can reach the biocatalyst since their molecular weight is low. So, this method is suitable when mass transfer limitations through polymeric network are not a problem. Porous gels, fibers, and microencapsulation are some strategies to entrapping the biocatalyst. The immobilization by entrapment could be by essentially physical forces or include covalent bonding.

- **Cross-linking**. An arrangement of insoluble aggregates with high molecular weight is formed by a simple process involving the biocatalyst and bi- or multifunctional reagents or ligands. Due to this method which did not use a support and involve covalent bonds, conformation changes of the biocatalyst are possible leading to the loss of their activity. Glutaraldehyde, a bifunctional agent, is the most generally used for this immobilization technique. Cross-linked enzyme aggregates (CLEAs) have emerged as attractive alternative to produce physical aggregates with preservation of biocatalyst structure and hence their catalytic activity.

- **Adsorption**. This simple, fast, and inexpensive method leads to the formation of noncovalent interactions. Electrostatic adsorption is the linkage approach more used. Unfortunately, the interaction between the biocatalyst and the support may be modulated by some operational parameters such as ionic strength, pH, and temperature. Therefore, this approach is more appropriate

when the physical adsorption of the biocatalyst is carried out in hydrophobic environments.

- **Affinity**. The immobilization by affinity interaction requires that the biocatalyst as well as the support present specific chemical groups on their surfaces, that is, the presence of complementary species, e.g., streptavidin-biotin interaction. As a consequence of the specific interaction, notable selectivity is a major benefit of this method. However, this procedure is expensive since antibodies or lectins can be used.

### 1.3 Why covalent immobilization?

There is not a universal method of immobilization and support for the enzymes and their uses in biotechnology. Several factors could drive the choice of a particular method, e.g., physicochemical properties of support or different features of substrates and products. As shown in **Figure 1**, all methods present advantages and disadvantages. However, why should the covalent bond be chosen? Covalent bonding is the most used strategy of the irreversible category. The removal of the covalent immobilized enzyme from the support without affecting its catalytic activity as an attempt to recover either the biomolecule or the support is meaningless due to hard process usually involved to disrupt the covalent bond. Moreover, better thermal and operational stability, as well as major resistance to pH, temperature, and solvent variations, are some of the well-known benefits of the immobilization approach. A good reason to choose this method is when a system with high stable protein coverage is recommended. Obtainment of the product of high purity, i.e., no contaminants including the enzyme, is another excuse to employ the covalent bond for immobilization.

## 2. About the particles used as support

In order to prepare an immobilized derivative, at least the biomolecule, the support, and the method of immobilization are required. Choosing the support is an essential step in the immobilization process since the characteristics of the material can influence in the performance of the biocatalyst. Even today there is no general rule for selecting the ideal support to attach the biomolecule. However, materials with characteristics such as chemical inertia, hydrophilic character, low cost, mechanical resistance, and resistance to microbial attack are widely used. Often in the literature, terms as matrix and carrier are found as synonyms of support.

Magnetic particles as a carrier to biomolecule immobilization are desirable materials due to easy separation of the biocatalyst from the reaction medium by application of an external magnetic field. Among the particles with magnetic property, the iron oxides, in particular, magnetite ($Fe_3O_4$), are the materials with notable uses in the immobilization process. Maghemite ($\gamma$-$Fe_2O_3$) and hematite ($\alpha$-$Fe_2O_3$) are iron oxides derived from magnetite and can be found together with it.

### 2.1 Iron oxides: Why use it as a support of biomolecules?

Until now several magnetic particles obtained from pure iron oxide or a mixture of phases and composites materials have been presented as attractive support to enzyme immobilization or protein purification. To understand why these particles are so attractive, it might be useful to review the characteristics of the magnetite. The iron oxide is inorganic in nature, which presents a ratio of ferric/ferrous ions equal to 2:1, in stoichiometric magnetite. The ions positively charged (ferric,

denoted as $Fe^{3+}$, and ferrous, expressed as $Fe^{2+}$) are distributed in two different sites with a crystal inverse spinel structure, formed by a cubic network face centered (fcc) of oxygen anions. Tetrahedral sites are filled by $Fe^{3+}$ ions, while the octahedral sites are occupied by $Fe^{3+}$ and $Fe^{2+}$ ions [10].

According to the magnetic response, iron oxides can be classified as ferromagnetic, antiferromagnetic, paramagnetic, and ferrimagnetic. At room temperature, bulk magnetite is a ferrimagnetic material due to the combination of ferromagnetic and antiferromagnetic comportments that happens below the Curie temperature (850 K) [11]. This iron oxide presents a saturation magnetization ($M_s$) value between 92 and 100 emu $g^{-1}$. Maghemite is also a ferrimagnetic material ($M_s$ = 60–80 emu $g^{-1}$), while the hematite is weakly ferromagnetic ($M_s$ minor than 1 emu $g^{-1}$). It is important to mention that there is a correlation between the magnetic properties of the material with their size. So, when the magnetite presents nanometer size (below 15 nm), the superparamagnetic behavior is observed [12].

In addition to physicochemical and magnetic properties of the magnetite, using these particles as a carrier of the biomolecules could avoid laborious procedures such as decantation, centrifugation, or filtration.

### 2.2 Synthesis of iron oxides

At present, many synthesis methods to produce iron oxide particles have been developed. Evaluating the preparation method together with the features desires of the material (i.e., size, shape, size distribution, and surface chemistry) is very important once the magnetic property, as well as applications of material synthesized, depends on it. Moreover, the degree of structural defects (i.e., the presence of impurities) and distribution of the defects are also related to the synthesis methodology [13]. In general, the main preparation methods of the iron oxide particles can be classified as chemical, physical, and biological. Among these methodologies, the chemical route is the most used due to high yield along with low-cost production. Coprecipitation, hydrothermal, microemulsion, sonochemical, thermal decomposition, sol-gel synthesis, and electrochemical decomposition are some of the chemical methods [14]. A complete description about the most common preparation methods of the iron oxide particles together with their benefits and drawbacks can be found in the literature [1, 12, 14].

The "coprecipitation method" presents interesting characteristics that make it the chosen one among the chemical route category. For instance, this technique is simple and inexpensive and can be used for large-scale production. Briefly, iron oxide particles are produced by addition of a precipitating agent (e.g., ammonium hydroxide) to the solution containing a mixture of ferrous and ferric salts (e.g., $FeCl_2.4H_2O$ and $FeCl_3.6H_2O$) at room temperature or high temperature. A molar ratio equal to 2:1 for $Fe^{3+}/Fe^{2+}$ ions is generally used. The obtainment of a black precipitate ($Fe_3O_4$) is a good indication that the synthesis was successful. The size, shape, composition and magnetic property of the particles can be modified depending on the iron precursors, iron concentrations, ferric/ferrous ratio, precipitating agent, pH, temperature, ionic strength, stirring velocity, surfactants addition, and working under inert atmosphere [10, 15].

Processes of the nucleation and the growth of crystals are involved in this technique. Both processes should take place separately, i.e., first the nucleation and then the growth. According to Wu et al. [10], pH is an important parameter to address toward the nucleation (solution pH minor than 11) and the growth (solution pH major than 11) of crystals. Even though the coprecipitation method is widely used to produce iron oxide particles with high saturation magnetization, some limitations like broad and large size distribution, poor crystallinity,

aggregation, and tendency to oxidize can be cited. For instance, the formation of impurities (e.g., maghemite) depends on the initial and final solution pH as well as the reaction temperature [16]. Moreover, impurities are also observed in very small particles (minor than 20 nm) because of the high surface area/volume ratio, which allows a great number of surface atoms. This happening could lead to the formation of maghemite, for example, as a consequence of the oxidation of $Fe^{2+}$ to $Fe^{3+}$ ions. The maghemite can also be present when the magnetic sample is stored for an extensive period (6 months) as well as exposure to high temperatures (superior to 180°C) [16]. Since maghemite is also a ferrimagnetic material but with a slightly minor saturation magnetization than magnetite, the presence of maghemite in the magnetic sample may not be considered a disadvantage. However, the presence of hematite is an unwanted impurity since this oxide is weakly ferromagnetic and exhibits low saturation magnetization.

Our research group has used the coprecipitation method for the obtainment of magnetic particles to be used as a matrix for biomolecules immobilization [17, 18]. Among these materials, some magnetic composites were also synthesized. For this, materials (without magnetic property) can be added to the $FeCl_3.6H_2O/FeCl_2.4H_2O$ mixture before the magnetization aiming different purposes: magnetic particle composite synthesis.

Maciel et al. [19] synthesized a magnetic levan; that is, under a polymer of fructose, the iron oxide particles were formed. The magnetic levan particles were treated with sodium periodate ($NaIO_4$) and employed as a support for trypsin covalent immobilization.

Rêgo et al. [20] have reported a gum magnetic from *Parkia pendula* seeds as a matrix for concanavalin A (Con A) covalent immobilization. For this to be possible, seed gum was included in the solution containing the $Fe^{3+}$ and $Fe^{2+}$ ions. Afterward, it was functionalized with $NaIO_4$ allowing the covalent immobilization of the lectin Con A.

Mercês et al. [21] described the process to convert Dacron to magnetic Dacron-hydrazide (mDAC). Heparin (HEP) was activated by carbodiimide and N-hydroxysuccinimide and covalently linked to mDAC (mDAC-HEP). Human antithrombin was then purified by affinity chromatography using the mDAC-HEP.

Alves et al. [4] prepared a magnetic composite from azocasein and iron oxide particles (mAzo). The presence of azocasein, azo-dye-insoluble casein derivative, on the surface of the magnetic particles allowed trypsin to be purified by affinity binding. The enzyme forms complex with the modified substrate but does not hydrolyze it. Washing the enzyme-azocasein-magnetic particles removes unspecific proteins of the mixture. Afterward, the complex is disrupted by increasing the ionic strength, and the enzyme is collected in the supernatant.

Due to the inorganic nature of the iron oxides, the magnetic particles do not have chemical groups to enable biomolecules to bind covalently. Therefore, additional procedures should be performed for this purpose (functionalization). Two approaches can be carried out: (1) coating them with polymers containing these chemical groups and (2) adding materials encompassing these chemical arms during or after the magnetic particle synthesis (composites). In this context, Cabrera et al. [22] used the 3-aminopropyltriethoxysilane (APTES) as a silane agent to available amine groups on the surface of the magnetic diatomaceous earth (mDE). The treatment with APTES was carried out after the synthesis of the mDE particles. The composite material (mDE-APTES) showed efficiency as a matrix to immobilize invertase. Furthermore, Cabrera et al. [2] have also reported a simple, effective, and inexpensive synthesis methodology to obtain a magnetic composite made from mDE particles coated with polyaniline (mDE@PANI). The coating with PANI was carried out after preparation of the mDE particles. Three industrial enzymes (invertase, β-galactosidase, and trypsin) were successfully immobilized using glutaraldehyde as

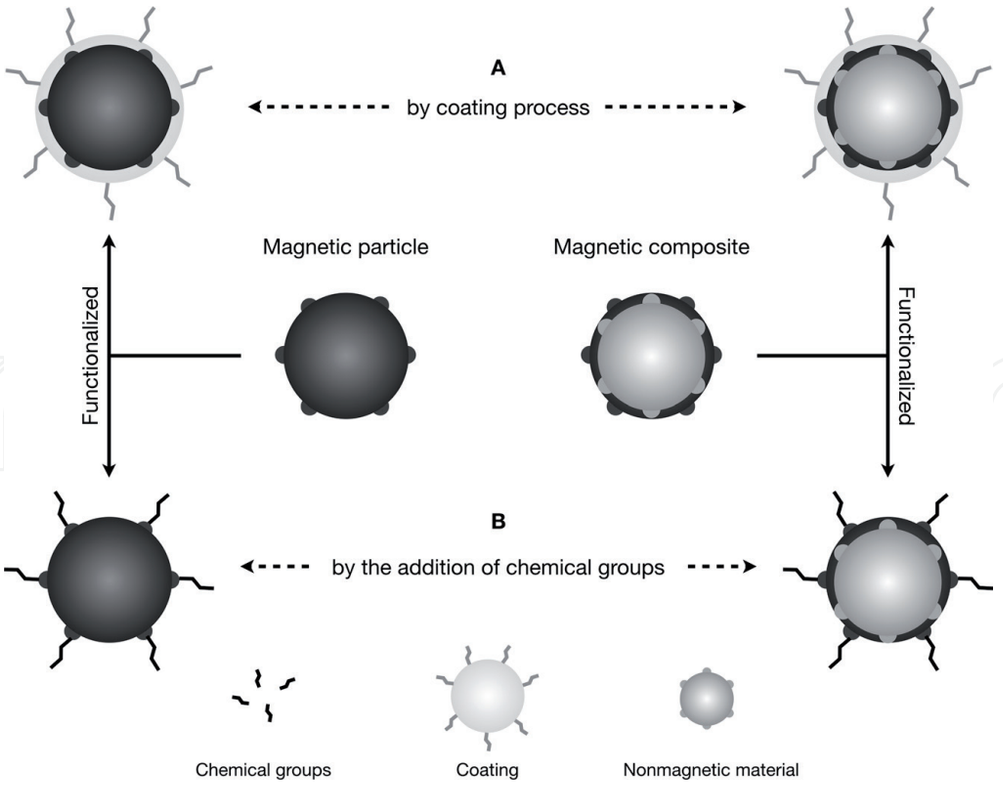


**Figure 2.**
*Approaches to prepare functionalized magnetic particles. (A) Functionalized by coating process and (B) functionalized by the addition of chemical groups.*

a chemical arm on the mDE@PANI particles. All magnetic bio-derivatives displayed superior performance (related to catalytic activity and stability) compared to the free enzyme. Thus, the mDE@PANI particles showed potential as a matrix to immobilize other biomolecules. Tannase was also covalently immobilized onto the mDE@PANI particles [3]. This enzyme catalyzes the hydrolysis of tannins present in several beverages. **Figure 2** displays the preparation of functionalized magnetic particles for biomolecule immobilization or protein purification.

## 2.3 Micro or nano iron oxide particles?

Before proceeding with the immobilization process, essential aspects such as material and surface science, biomolecule chemistry, and reaction engineering should be evaluated. A satisfactory immobilization will be achieved when all these aspects are properly integrated. However, this is not very easy due to the multidisciplinary nature of problems along with the main effects occurring in different length scales from nanometer to millimeter [23].

It is well known that an immobilized derivative presents, in the majority of times, minor retention of their catalytic activity when compared to its free counterpart. This happens for two reasons: (i) conformational changes of the biomolecule due to covalent bond with the support and (ii) problems arising of the catalytic reaction occur in a heterogeneous environment [24]. So, analyzing the behavior of biomolecule immobilized could help in choosing the most suitable particle size for the material used as support.

In the last times, the "nanoimmobilization" is widely used for several researchers to indicate nanostructures as support for the immobilization of biomolecules. The employment of NPs presents various benefits due to unique physical properties resulting from their nanometer size (below 100 nm). Higher surface area, significant biomolecule loading, superior mass transfer resistance, and minor diffusion

problems are some of the advantages of the use of nanoparticles as a matrix. Also better stability and performance of biocatalyst immobilized along with a low protein unfolding were also reported [25, 26]. Some disadvantages for the NPs (e.g., large-scale application and price of material preparation) have been presented [26]. On the other hand, magnetic nanoparticles (MNPs) have been used as a support of several biological molecules as well as present superparamagnetism (i.e., magnetic response is only observed after application of magnetic field) and can be recovered by the use of a magnet [27]. So, the magnetic property as a plus feature to the nanoparticles would make them more attractive not only for potential uses in biotechnology [14] but also for a lot of biomedical applications including magnetic hyperthermia [28], drug delivery [29], contrast agent in magnetic resonance imaging (MRI) [30], and cellular therapy [31].

As above mentioned (Section 2.2), some factors influence the size (micro or nano) of iron oxide particles. For instance, Maciel et al. [17] assessed the effect of the temperature and the nature of the precipitating agent (strong base) to produce iron oxide nanoparticles. The authors employed sodium hydroxide (NaOH) as the precipitating agent and carried out the synthesis at low temperature (50°C). Small magnetic nanoparticles with a diameter near to 15 nm were obtained and used as a matrix to immobilize trypsin. The magnetic bio-derivative displayed about 90% retention of specific activity after five reuses. Despite Cabrera et al. [2] have reported mDE@PANI nanoparticles (~12 nm) as a promising matrix to immobilize trypsin, the immobilized derivative (mDE@PANI-TRYP) retained 75 and 60% of its initial activity after five and nine cycles of reusability, respectively. The decrease of catalytic activity could be attributed to the loss of the magnetic bio-derivative (mDE@PANI-TRYP) during the washing process after each reusability cycle. After coating with polyaniline, the mDE@PANI showed better stability in suspension. Thus, working with very small particles and good colloidal stability can lead to loss of the immobilized derivative.

Therefore, the choice of micro or nanoparticles as support will depend on several factors including colloidal stability of the particles, operating conditions, and application of the immobilized derivative, among others.

## 3. The chemistry of materials

In this section, a brief presentation of differential physicochemical techniques used to characterize the support and/or to demonstrate the efficiency of the immobilization methodology will be described. The description of theoretical basis, equipment, and conditions of analysis of the techniques are not the purpose of this material. The main information obtained from each analysis method as well as some examples will be presented below.

### 3.1 Magnetization measurements

In general, a material with magnetic property is analyzed by the magnetization measurement technique in order to quantify this property. The saturation magnetization ($M_s$), remanent magnetization ($M_r$), and coercivity ($H_c$) are among the main data obtained by this technique. Additionally, the presence of a hysteresis loop, as well as the $M_r$ and $H_c$ parameters, could help to assess the magnetic behavior of a material. For instance, a superparamagnetic material presents $M_r$ and $H_c$ values near to zero.

The inclusion of materials into the magnetic particles decreases their magnetization power although they still can be attracted by an external magnet. The

saturation magnetization for the magnetic composite prepared from levan polymer and iron oxide particles was reduced tenfold. The happening can be attributed to the difficult alignment of magnetic dominions in the composite material due to the coating process. Furthermore, the addition of the levan increased the particles sizes varying from 20 to 60 μm for magnetite only and 100–200 μm for magnetic levan composite. The authors used this magnetic composite to immobilize trypsin by covalent binding [19].

Gregorio-Jauregui et al. [32] also showed a decrease in the saturation magnetization values as the amount of polymer (chitosan) in the particles was increased. For instance, bare magnetic nanoparticles (without chitosan—0 w/v%) and magnetic nanoparticles coated with chitosan (0.5 w/v%) presented a $M_s$ near to 70 and 45 emu $g^{-1}$, respectively. Furthermore, the authors suggested that these findings could be associated with the direct relation between crystallinity and magnetization in magnetic particles. That is, magnetic materials with a good degree of crystallinity will present a large saturation magnetization. However, the addition of chitosan polymer (poor crystallinity) leads to a decrease of magnetic response.

Surface modification processes, including immobilization of enzyme, were evaluated by Defaei et al. [33]. The authors synthesized magnetic nanoparticles coated with silica and functionalized with naringin (MNP@$SiO_2$/NA). This material was employed as support to immobilize α-amylase (MNP@$SiO_2$/NA/AA). After each modification process a decrease on saturation magnetization values was observed due to the increase of thickness of the shell layer on the magnetic nanoparticles. So, the saturation magnetization values were 38, 27, and 22 emu $g^{-1}$ for MNP@$SiO_2$, MNP@$SiO_2$/NA, and MNP@$SiO_2$/NA/AA, respectively.

### 3.2 X-ray diffraction (XRD)

X-ray diffraction (XRD) is an important method used for analyzing the intermolecular structure of ordered materials. However, this technique is not appropriate for quantifying the degree of order. Magnetic materials such as bare iron oxide particles as well as magnetic composites can be characterized by XRD analysis in order to evaluate the presence of different components in the sample. XRD can also be used to estimate the particle size by using the Scherrer equation, for example.

By using XRD technique, to differentiate between magnetite and maghemite is not possible since the iron oxides present similar standard XRD patterns. According to the International Center of Diffraction Data (reference code: ICDD 019-0629), the crystal planes at (111), (220), (311), (400), (422), (511), (440), (620), and (533) corresponding to the 2θ peaks at 18.44, 30.30, 35.67, 43.37, 53.80, 57.35, 62.97, 71.43, and 74.48° are attributed to both magnetite and maghemite [17]. For instance, Gregorio-Jauregui et al. [32] could not differentiate by XRD technique the iron oxides present in magnetic nanoparticles coated with chitosan. The authors attributed the presence of magnetite due to the black color of the magnetic composite. Furthermore, the coating with chitosan did not affect the crystalline structure of the magnetic nanoparticles.

Cabrera et al. [2] assessed by XRD the chemical composition (qualitative data) as well as the crystalline structure of magnetic diatomaceous earth coated with polyaniline (mDE@PANI) nanoparticles. The XRD pattern of the mDE@PANI sample displayed characteristic peaks for crystalline and amorphous silica along with albite, polyaniline, and magnetite. The iron oxide was the predominant crystalline phase. Additionally, the authors reported that the coating process with PANI did not affect the crystallinity degree of the magnetic sample since the narrow peaks were preserved.

Díaz-Hernández et al. [34] reported the use of magnetite nanoparticles coated with chitosan ($Fe_3O_4$@chitosan) as support for immobilization of enzymes. In spite of XRD pattern which displayed a low peak at 18° probably related to maghemite, the authors concluded that magnetite was present in the $Fe_3O_4$@chitosan nanoparticles. Therefore, the XRD technique revealed that the addition of chitosan polymer did not affect the crystal structure of the magnetic sample. Moreover, the authors carried out the XRD spectrum for enzyme immobilized by cross-linking. The XRD pattern for the immobilized derivative displayed broad peaks and with low intensity, but all peaks were in agreement with magnetite. This finding could be attributed to the amorphous nature of the biomolecule immobilized.

### 3.3 Mössbauer spectroscopy (MS)

Mössbauer spectroscopy (MS) is a sensitive technique to the iron ionic state and environments. MS can distinguish the ferric ($Fe^{3+}$) and ferrous ($Fe^{2+}$) ions because of their different isomer shifts. Thus, magnetite, maghemite, and hematite, for example, can be detected in a magnetic sample. Since magnetite mainly presents potential biotechnological and biomedical applications, it is very important to know the main iron oxide phases present in the sample. Therefore, the MS technique is very useful for monitoring the processes of preparation and modification of the iron oxides at different sizes. However, small magnetic nanoparticles (below than 10 nm) must be analyzed at low temperatures in order to block the superparamagnetic relaxation of them [35].

Cabrera et al. [36] proposed two magnetic composites as a matrix to immobilize invertase via covalent bonding. For this, clay minerals such as montmorillonite (MMT) and diatomaceous earth (DE) were used as a nonmagnetic component. Using MS technique, it was possible to evaluate the main iron oxide phases present in the magnetic composites (mMMT and mDE). Mössbauer results revealed a mixture of magnetite and maghemite in equal proportion for the mMMT particles, while a pure magnetite phase was observed in the mDE particles.

Storage conditions can also lead to changes in magnetic property due to phase transformation of iron oxides. Rümenapp et al. [37] described strong oxidation of the bare magnetic nanoparticles on the fourth day of preparation. The complete oxidation to maghemite was observed in the fourth week. In order to avoid the oxidation process, the authors suggested a coating process on the magnetic surface. Iron oxide nanoparticles without and with polyaniline (PANI) coating were assessed by Maciel et al. [17] using MS analysis. The authors described the presence of maghemite in the two samples. In addition, the coating with PANI did not change the chemical nature of the magnetic sample. Similarly, Cabrera et al. [2] functionalized with PANI a magnetic composite from diatomaceous earth and proposed it as promising support to enzyme immobilization. Due to the great catalytic performance of the magnetic bio-derivatives, the authors evaluated the magnetic behavior by MS technique. It is not common to analyze the magnetic sample containing the biomolecule may be due to the small amount of biomolecule immobilized in most of the time. MS results showed the magnetite as the major iron oxide phase in the magnetic composites (mDE and mDE@PANI). Moreover, the hematite was not detected in the samples.

### 3.4 Scanning electronic microscopy (SEM) and transmission electronic microscopy (TEM)

The structural and morphology characterization can be performed by scanning electronic microscopy (SEM) and transmission electronic microscopy (TEM). SEM can be employed to investigate the morphological surface of the support as well as the biomolecule. However, information related to the internal structure of the

sample cannot be obtained by this technique. Additionally, SEM also provides the particle size. TEM is mainly used to determine the particle size, size distribution, and particle shape and to evaluate the effectiveness of the functionalization process (e.g., coating with a polymer) along with the thickness of the coating. In general, the particle size values found by TEM are in agreement with those obtained by XRD.

Cabrera et al. [2] used SEM technique to display physical changes in the size and surface of diatomaceous earth (DE) after the magnetization and functionalization processes. Strong operating conditions such as high temperature could lead to the destruction of DE frustules along with a rougher surface. A surface modification as the coating process with PANI on a magnetic core along with the morphology, shape, and size of material can be evaluated by using TEM. Maciel et al. [17] described a synthesis methodology to obtain magnetic nanoparticles coated with PANI as a matrix to immobilize trypsin. TEM results displayed a magnetic system with a spherical shape and particle size near to 15 nm.

Defaei et al. [33] demonstrated by SEM and TEM techniques the immobilization of α-amylase on magnetic nanoparticles. A slight increase in the particle size after the immobilization process suggested the effective enzyme immobilization. Moreover, the presence of oxygen, sulfur, and nitrogen atoms in the sample containing the enzyme by energy-dispersive X-ray spectroscopy (EDX) confirmed the presence of the biomolecule.

Gregorio-Jauregui et al. [32] reported the use of scanning transmission electron microscopy (STEM) technique to determine the size and morphology of magnetic nanoparticles coated with chitosan. The magnetic system presented a small size (9.9–11 nm) and spherical morphology. The STEM analysis combines the principles used by both SEM and TEM techniques, and it can also be used to locate biomolecules as well as active groups inside the matrix. For this, a high-angle annular dark field (HAADF) detector is coupled to STEM since the contrast is associated with the atomic number [38]. Mayoral et al. [39] demonstrated the presence of lipase immobilized inside the pores of silica by STEM-HAADF.

### 3.5 Thermal gravimetric analysis (TGA)

Thermal gravimetric analysis (TGA) is a thermal analysis technique which provides quantitative data (i.e., thermal stability, reaction rates, oxidation process, components quantification, and kinetic of decomposition). In general, TGA is used to study changes in physical and chemical properties, e.g., oxidation, dehydration, and decomposition, as a function of temperature [40]. Thermal stability of magnetic particles used as a matrix for immobilization of biomolecules is evaluated by weight loss (decomposition process) of the sample. An additional process (functionalization) carried out on the surface of magnetic particles can also be observed by this technique. That is, the amount of organic material in a sample can be detected and quantified. For instance, Aydemir and Güler [41] have reported the use of magnetic bio-derivative (laccase immobilized on magnetic chitosan-clay composite) for phenol removal. Structural characterization of the magnetic composite by TGA revealed superior thermal stability of chitosan after addition of clay and magnetite nanoparticles. Moreover, at 230°C the composite (magnetic chitosan-clay beads) presented a weight loss of 16% corresponding to the decomposition and elimination of the polymeric component. Neri et al. [42] used magnetic polysiloxane-polyaniline (mPOS-PANI) particles as support to immobilize β-galactosidase. TGA technique was employed to evaluate the coating process with PANI and thermal resistance of the mPOS (without PANI) and mPOS-PANI particles. Thermal degradation of PANI was observed near to 325°C. The magnetic samples (mPOS and mPOS-PANI) displayed a remaining weight loss probably due to the removal of the solvent.

| Technique | Information type | Parameter[a] | Easy access to technique? |
|---|---|---|---|
| Magnetization measurement | Quantitative | Saturation magnetization, remanent magnetization, and coercivity | Yes |
| X-ray diffraction (XRD) | Qualitative and quantitative | Chemical composition, crystallinity degree, crystalline phase, and crystalline size | Yes |
| Mössbauer spectroscopy (MS) | Qualitative | Specific to differentiate between iron oxide phases (e.g., magnetite, maghemite, and hematite) | No |
| Electronic microscopy (SEM and TEM) | Qualitative and quantitative | Particle size, morphology, size distribution, and shape | Yes |
| Thermal gravimetric analysis (TGA) | Quantitative | Mass change in processes such as oxidation, decomposition, and dehydration | Yes |

[a] *Some parameters provided by the technique.*

**Table 1.**
*Main features of some physicochemical techniques to characterize a magnetic material or magnetic bio-derivative.*

TGA can also be employed to determine the degree of functionalization of the support, the effectiveness of the immobilization method, and structural information of biomolecule after the immobilization [40]. An attractive nanobiocatalyst consisting of α-amylase (AA) immobilized on magnetic nanoparticles (MNP@$SiO_2$) functionalized with naringin (NA) was proposed [33]. The functionalization and immobilization processes were assessed by TGA analysis. The results for the MNP@$SiO_2$/NA (without enzyme) and MNP@$SiO_2$/NA/AA (with enzyme) showed that the major weight loss was associated with the removal of the organic moieties. Moreover, the difference in weight loss between these samples was used to evidence the α-amylase immobilization.

In order to conclude this section, **Table 1** exhibits a summary with the main information about the physicochemical techniques above described. The reader can use the information contained therein to evaluate the desired parameters. It is important to mention that these techniques can be applied in both magnetic materials with and without the biomolecule. Moreover, other techniques can also be performed to characterize a magnetic bio-derivative.

## 4. Biotechnological applications

The immobilization of different biomolecules in magnetic material has been used for several biotechnological applications in biomedicine [5], environment [43], and food industry [22] (**Figure 3**). At present, magnetic bio-derivatives have found potential uses in biodiesel production. Knowing it as a renewable, biodegradable, noninflammable, and nontoxic product, the biodiesel has been studied as an attractive substitute to the diesel fuel based on petroleum. Lipase immobilized on magnetic particles has been used to produce biodiesel by enzymatic transesterification [44, 45]. Moreover, magnetic microreactors employing immobilized enzymes are widely investigated to study and manipulate bioprocesses. For instance, the prediction of pharmaceutical response in animal models could be avoided by using enzymatic microreactors in culture systems. The authors advice reading the review article to find more information about magnetic enzyme microreactors [46].

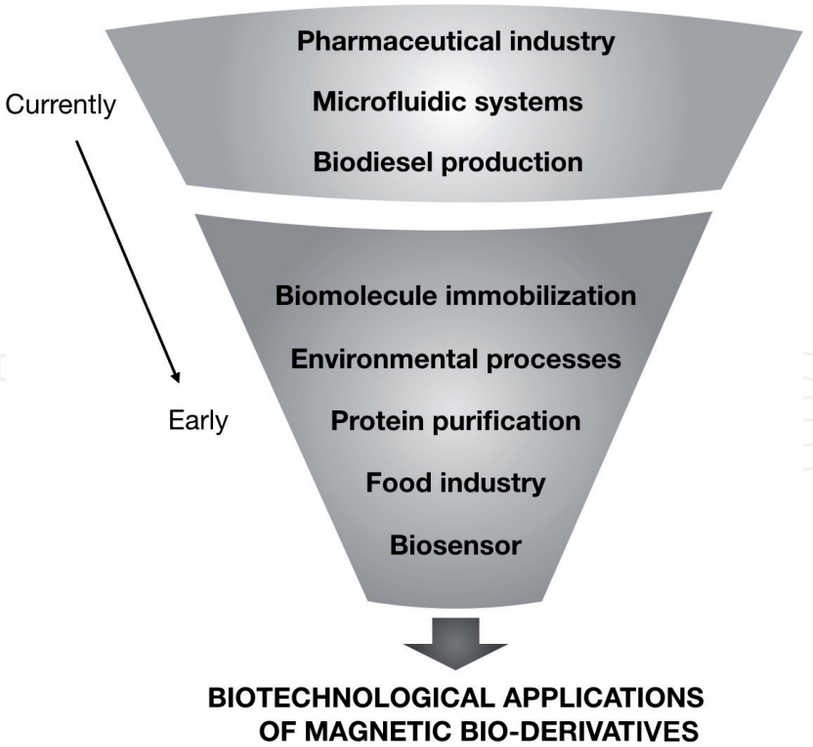


**Figure 3.**
*Major biotechnological uses of the magnetic bio-derivatives.*

Biocompatibility, specificity, ability to recognize other molecules, and operational stability presented by biomolecules such as enzymes [2], carbohydrates [21], antibodies [47], antigens [48], peptides [49], DNA [50], and glycoprotein [20], among others, are properties that make biomolecules attractive to biotechnological applications. The possibility of reuse, the easy removal of the magnetic bio-derivative from the reaction medium, the greater stability of the biomolecule, the high productivity, and the low-cost of production are some of the advantages of the immobilization.

Considering the above, the topics below exhibit the most recent developments in our research group. Applications such as trypsin purification, obtaining of antioxidant peptides, affinity purification of antithrombin, high sucrolytic activity by invertase, and purification of glycoproteins are discussed.

### 4.1 Trypsin purification using magnetic particles of azocasein-iron composite

The purpose of this work was to develop a trypsin purification strategy based on affinity binding with ferromagnetic particles of azocasein composite (mAzo) [4]. Trypsin was purified when the fish crude extract (2 mL) obtained from intestines of fish Nile tilapia (*Oreochromis niloticus*) was exposed to the magnetic bio-derivate (mAzo: 100 mg) for 2 h, removed from the reaction medium with the aid of a magnetic field, and washed with buffer (0.1 M Tris-HCl) seven times to remove unbound protein and three times under high ionic strength (3 M NaCl) to leach off the protein. The specific activity of the free enzyme present in the crude extract was 60-fold lower than that of the preparation. The optimum performance suggests that the mAzo composite, besides being reused, can be applied to purify trypsin from other sources.

### 4.2 Optimization of *Penicillium aurantiogriseum* protease immobilization on magnetic nanoparticles for antioxidant peptides' obtainment

In this work, magnetic nanoparticles coated with polyaniline were used to immobilize protease from *Penicillium aurantiogriseum* and applied to the production

of antioxidant peptides derived from bovine casein [5]. The casein was hydrolyzed using the magnetic bio-derivative, uncovering its peptides that were sequenced and had antioxidant properties tested through 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging and hydrogen peroxide scavenging assays. After being reused for five times, the magnetic bio-derivative was able to maintain more than 74% of the initial activity. Hydrolyzed casein presented similar peptide when used; the free and immobilized enzyme and prevalent peptides could be sequenced. When comparing the reactive oxygen species (ROS) scavenging activity, the hydrolysates presented 2.5 times more than nonhydrolyzed casein, which allows the use of an immobilized protease to obtain natural ingredients derived from casein attractive, indicating potential use in the production of functional foods.

### 4.3 Synthesis and characterization of magnetized Dacron-heparin composite employed for antithrombin affinity purification

The focus of this work was to produce a Dacron magnetic composite with heparin (mDAC-HEP) for the use in the purification of antithrombin from human plasma [21]. To obtain mDAC-HEP, heparin was activated by carbodiimide and N-hydroxysuccinimide, allowing covalent attachment. Purified antithrombin was released from the magnetic bio-derivative using solutions with increasing ionic strength (NaCl). The affinity properties of mDAC-HEP after 2 years of storage were preserved, and the magnetic bio-derivative was able to be reused for at least tenfold. The presence of the expected antithrombin size (58 kDa) was revealed in the bands by electrophoresis of the eluates. The composite synthesis was considered easy, low-cost, magnet-based affinity purification steps, and reusable.

### 4.4 High sucrolytic activity by invertase immobilized onto magnetic diatomaceous earth nanoparticles

The purpose of this work was to produce magnetic diatomaceous earth nanoparticles for invertase immobilization (mDE-APTES-invertase) using an easy and low-cost method that could offer high sucrolytic activity [22]. To obtain the results of high residual specific activity (92.5%), an experimental design was made with the objective of achieving the best immobilization conditions. This activity was able to be 2.42 times higher than other derivatives reported in the literature. The thermal and storage stability of the immobilized invertase was verified, and after 120 days of storage, the enzymatic derivative retained 80% of the activity, whereas the free enzyme lost practically all the activity. After ten reuses mDE-APTES-invertase retained 60% of residual activity. Considering the ease of obtaining the matrix and its efficiency, this nanocomposite proves promising to immobilize invertase from different origins and other biomolecules.

### 4.5 Magnetic *Parkia pendula* seed gum as a matrix for concanavalin A lectin immobilization and its application in affinity purification

The main objective of this work was to obtain a magnetic matrix with *Parkia pendula* seed gum to covalently immobilize concanavalin A [20]. The application of this magnetic composite was to obtain glycoconjugates through affinity purification. The obtained gum in the process was magnetized and activated with $NaIO_4$. Concanavalin A immobilized on the magnetic composite was used for the recognition of bovine serum fetuin glycoprotein. A glucose solution (300 mM) was used to carry out the election of the fetuin, and confirmation was made via SDS-PAGE.

A lectin immobilization efficiency of 63% was achieved and 14% fetuin purification. The magnetic composite is promising, given the results obtained, to be used with magnetic polysaccharide matrix to immobilize other lectins. Because it is a magnetic system, it can be used for affinity purification, and its recovery is performed easily and quickly with the help of a magnetic field.

## 5. Conclusions

This chapter has gathered primary and interesting information about immobilization of biomolecules, how to choose the best immobilization methodology, the support for the immobilization, and, among the magnetic materials, why iron oxide particles are highly used as a matrix to immobilization. Several physicochemical techniques were mentioned and described highlighting their features and the main information provided. Lastly, we presented some magnetic bio-derivatives with potential biotechnological applications which could be applied in other areas. So the authors hope that this material has been useful not only for the enzymologists but also to scientists working with applied surface science.

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## Conflict of interest

The authors confirm that there are no conflicts of interest in this work.

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