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Review

An Approach to Monodisperse Polymeric Particles as Matrices for Immobilization of Biosystems

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Abstract: In this paper, the benefits of using monodisperse polymeric particles as matrices to immobilize biosystems are presented and discussed. The nature of the polymer (natural, synthetic, or semisynthetic) and immobilization techniques were directly related to the performance of this process. In addition, this work reviews the major biological and synthetic entities that have been immobilized on monodisperse polymeric particles and their potential applications available in the literature. The research revealed that enzymes, proteins, cells, and drugs are the main entities immobilized on polymeric matrices. Several physicochemical characterization techniques were discussed to determine the presence of entities after the immobilization process. In addition, some applications of immobilized enzymes in different areas are also presented since this biomolecule was the most frequent entity in terms of immobilization on polymeric matrices. Finally, this review describes the main advances in polymeric materials used as supports for immobilizing biosystems due to their interesting physical and chemical properties.

Keywords: polymeric particles; biomolecules immobilization; support materials; biosensing applications

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1. Introduction

Soft matter science started being investigated by physical chemists and crystallographers around the twentieth century [1,2]. Furthermore, polymers and liquid crystals were the earliest materials explored in this field; perhaps polymers initially received more attention [3].

Conceptually, soft matter involves the majority of organic molecules weakly bonded by intermolecular forces (e.g., van der Waals forces) [4]. In general, all entities under the soft matter denomination include polymers, colloids, gels, liquid crystals, emulsions, foams, surfactants, granular materials, and biological macromolecules [5,6]. On the other hand, soft matter science also considers all materials in which their structural arrangement is found in the mesoscopic domain (1 μm and a few nanometers scale), along with those that can

readily suffer deformation. Taken together, soft matter physics is entirely interconnected with condensed matter physics as well as solid-state physics [7,8].

Currently, the immobilization of biosystems is inserted in this context as a prominent issue in the scientific community in several fields [9,10]. Depending on the area, the research objectives are different; however, in regard to immobilization, three points can be mentioned: (i) support employed in the process; (ii) biosystem of interest to be immobilized; and (iii) application of the immobilized derivative. It is important to emphasize that the term "immobilized derivative" refers to the entity, whatever its nature, immobilized on the support. Additionally, other parameters, such as the immobilization method and biochemical strategies, could be used to obtain potential biocatalysts [11,12].

In the last years, studies involving immobilized biosystems have been conducted due to the alluring benefits of these systems. For instance, biochemists and material scientists are looking for the development of potential biosensing nanoplatforms for the diagnosis and treatment of diseases in the early stages. According to the Scopus database, the number of scientific papers published annually for the descriptors (immobilized biomolecules and polymers) has shown a regular profile, with the number of published documents about 100 per year in the last 10 years (Figure 1a). Indeed, these observations revealed the permanent interest of researchers in this field, applying knowledge specifically to the following subject topics: Biochemistry, Genetics and Molecular Biology, Chemistry, Chemical Engineering, and Materials Science (Figure 1b). In addition, this research field was potentially grouped into two large topics: (i) immobilization and stability of enzymes and (ii) the use of biosensors for the detection of enzyme activity (Figure 1c).

Several polymeric materials have been developed and used to support the immobilization of different kinds of biomolecules, e.g., proteins, enzymes, antibodies, peptides, and nucleic acids. Additionally, synthetic molecules (e.g., antibiotics) and whole cells have also been immobilized on polymeric matrices (Figure 2). The interesting physical and chemical properties of polymers make them good enough as a matrix for immobilizing biosystems, especially enzymes since improved catalytic performance and thermal and pH stabilities have been described [13]. Potential applications for the immobilized derivatives that use this material as a support can be found in several areas, such as the food packaging industry [14], drug release [15], organic synthesis [16], separation and purification [17], water treatment [18], and tissue engineering [19].

The advantages of immobilizing biosystems, particularly enzymes, are well known. However, a valuable biocatalyst needs to preserve the structural stability of the biomolecule and exhibit high catalytic performance. Thus, the success of biocatalyst preparation depends on the properties and support of the biomolecule. The physical nature (synthetic or natural) of a support, in addition to its physical–chemical properties, is crucial. The physical structure (e.g., pore size) of the support, as well as the microenvironment, could also affect the efficiency of immobilization. Additionally, immobilization techniques, including multipoint attachment to support and the presence of one or more spacers, can also play a decisive role in obtaining a bioderived catalyst [20].

Several polymeric particles have been used as supports for immobilization since these materials have functional chemical groups available for binding with the protein or carbohydrate moieties of biomolecules [21]. Chang and Juang reported significant enhancements in the catalytic activity and stability of β-glucosidase after immobilization onto a chitosan–clay composite, a material highly rich in hydroxyl and amino groups alongside remarkable hydrophilicity and high porosity [22].

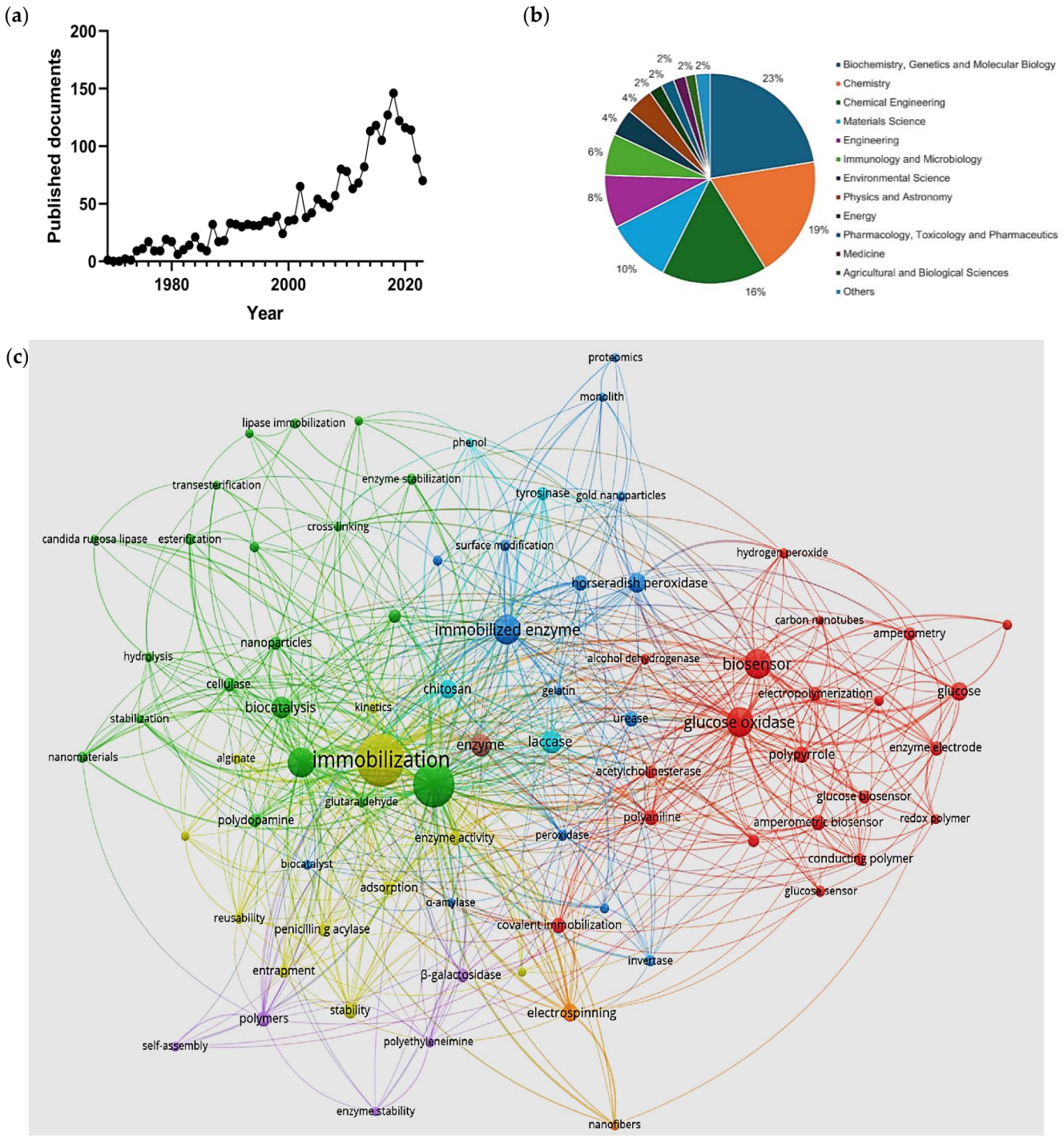

Figure 1. Bibliometric overview of historical research on immobilized biosystems and polymers in terms of (**a**) publication trends, (**b**) subject area, and (**c**) keywords co-occurrence visualization.

In the field of biocatalyst immobilization, another interesting point in the selection of support could be the cost of the polymer matrices. These materials consist of only polymers or other types of materials, e.g., ceramics, employing natural polymers as entity supports. This contributes to the high demand for these materials, mainly by industry, since the operational costs could be reduced by their low cost and wide availability [23]. Several natural biopolymers, such as collagen, chitosan, chitin, alginate, cellulose, and starch, among others, have been widely explored as supports for immobilizing enzymes. The hydrophilic nature and biological safety of polysaccharides also result in their use as interesting matrices [24].

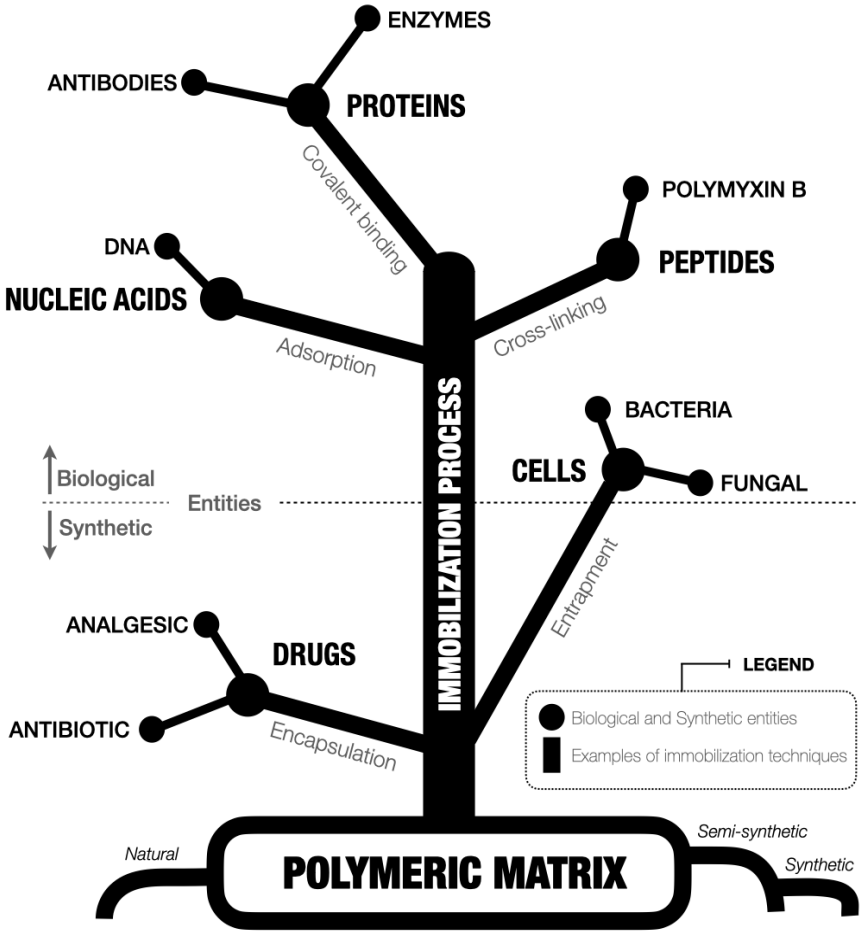

Figure 2. Schematic illustration of the main biological and synthetic entities immobilized by different methods on polymeric matrices of distinct occurrences in nature.

In addition to the benefits already mentioned, several forms (e.g., beads, films, tubes, and fibers) of polymeric material can be prepared and could also contribute to promoting beneficial immobilization conditions for biosystems. Indeed, it is well known that in addition to the surface area of the support, the particle size and the presence of pores, including their size and volume, notoriously influence the ability of the material to load entities [25]. For example, nanofibers can be compared to nanoparticles in terms of their performance as a matrix since they also exhibit porosity and a large surface area for the loading of entities. Additionally, nanofiber biocatalysts preserve catalytic activity considerably, and their separation from the reaction medium is effortless [26].

In this review, we aim to describe the main advances in polymeric materials used to support immobilizing biosystems. We also propose highlighting the interface between the support and the biosystem. Several factors influencing the performance of the biocatalyst are described. Moreover, combinations of immobilization methods and chemical strategies for preparing attractive immobilized derivatives are presented. Furthermore, important physicochemical characterization techniques employed to prove the presence of entities on polymer supports will also be presented. Finally, we recorded the main applications of biocatalysts based on enzymes in different areas. By this means, we intend to highlight the importance of these preparations and draw the attention of the biotechnological and biomedical industries as well as pharmaceutical and food product manufacturing.

2. Polymeric Matrices

Polymeric materials have been studied for several decades for the most diverse applications mentioned above. However, polymer materials for use as matrices for immobilizing biosystems are very common in the literature. In this section, we will briefly mention the polymer classifications. However, we will approach these materials with more emphasis on their classification according to their origin.

From the point of view of their backbone chemical composition, polymers can be classified into two categories: (i) organic and (ii) inorganic. Briefly, organic polymers are composed essentially of carbon and hydrogen atoms together with other atoms (e.g., oxygen, nitrogen, and sulfur). On the other hand, the primary backbone of inorganic polymers commonly consists of silicon, phosphorous, oxygen, or nitrogen atoms. Although a regular quantity of pure inorganic polymers has been investigated, sometimes polymers containing inorganic main chains with organic side groups, called hybrid polymers, have also been explored. The singular chemical properties of these hybrid materials actively depend on the exchange of organic side groups as a result of the hybridization of organic and inorganic components [27].

Regarding the type of polymer material employed to support immobilizing biomolecules, organic polymers have been shown to be the most common materials used for this purpose. Polymers are classified on different bases, that is, based on their origin, backbone, structure, mechanism, monomer, chain topology, and thermal response, among others, as frequently found in the literature [28]. Below, we will describe the polymer classification by the origin of the source and how the highlighted features of these materials could influence their choice of biosystem support.

Simply stated, three categories (natural, synthetic, and semisynthetic) of polymers can be mentioned according to their occurrence in nature since they can be naturally found or synthetically prepared [20]. A concise description with detailed examples is provided below.

This classification by origin provides a systematic framework for understanding the distinct characteristics of each polymer type and their potential as support for biosystem immobilization. Natural polymers, such as polysaccharides and proteins, are valued for their biocompatibility and biodegradability, making them ideal for biological applications. Synthetic polymers, in contrast, are recognized for their enhanced mechanical strength, thermal stability, and chemical resistance, which suit them in more demanding industrial contexts. Semisynthetic polymers combine features of both categories, offering a balance between natural and synthetic properties. This classification highlights the importance of aligning polymer selection with specific application requirements, ensuring optimal performance and functionality.

2.1. Natural Polymers

Natural polymers are materials found in natural sources, such as plants and animals, often exhibiting very complex structures. This category also includes polymers obtained from bacterial synthesis or fermentation (biological processes). Among the advantages of biopolymers is their low cost, which results from their abundance in natural sources and their potential for chemical modifications. Moreover, these polymers exhibit versatile properties, including biocompatibility, multifunctionality, biodegradability, flexibility, and renewability, making them highly suitable for various applications [29]. Additionally, natural polymers are mainly categorized as proteins, polysaccharides, polynucleotides, polyisoprenes, polyesters, or lignin, depending on their source [30]. However, proteins (e.g., collagen and gelatin) and polysaccharides (e.g., chitosan, chitin, dextran, alginates, starch, heparin, cellulose, and pectins) are the primary types of natural polymers employed as supports for biosystem immobilization.

Among polysaccharides, alginate, and chitosan are two biopolymers widely employed in immobilization matrices due to their interesting properties, such as nontoxicity, low cost, and good biocompatibility. A polymer composite based on alginate, chitosan, and citric acid used as a support for the immobilization of acrylamidase to remove acrylamide from roasted coffee was reported by Bedade et al. [31]. The best experimental conditions (5% sodium alginate, 1.5% chitosan, and 0.6 mol/L citric acid) for preparing polymer beads with better mechanical stability were investigated. Citric acid was used as a functionalizer rich in carboxylic groups, and the covalent binding of the acrylamidase enzyme was determined by the carbodiimide method. The immobilized acrylamidase was shown to be effective at degrading acrylamide from coffee [31].

Another polymer composite containing chitosan polysaccharide, collagen protein, and magnetic nanoparticles for lipase immobilization was described by Ziegler-Borowska et al. [32]. Squaric acid, an effective protein cross-linker replacing glutaraldehyde, was proposed to cross-link a mixture of polysaccharides and protein and form a polymer shell on the surface of magnetic nanoparticles. The immobilization of lipase on the polymer magnetic nanoparticles was achieved by covalent binding. The immobilized derivative exhibited better catalytic performance as well as thermal and pH stabilities for the support prepared from squaric acid than for materials cross-linked with glutaraldehyde [32].

Natural polymers derived from plants, animals, or microbes exhibit biocompatibility, biodegradability, and renewability, making them essential in biosystem immobilization. Proteins (e.g., collagen, gelatin) and polysaccharides (e.g., chitosan, alginate) are particularly valued for their nontoxicity and cost-effectiveness. Functionalized composites, such as those incorporating citric acid or squaric acid, further enhance the mechanical stability and catalytic efficiency of immobilized systems, underscoring their role in advanced applications.

2.2. Synthetic Polymers

Synthetic polymers are materials of great interest to several industries for the production of different products commercially. Additionally, synthetic polymers are commonly organic in nature. Many synthetic polymers have been synthesized. However, the polyethylene terephthalate (PET) used in the packaging sector, along with the nylon fibers mainly employed in clothes, are among the main synthetic polymers already prepared. Moreover, the use of these materials as supports for immobilizing several enzymes has been widely reported [33–36].

In addition, polyvinyl alcohol (PVA) is another polymer material used for the immobilization of biomolecules, especially industrial enzymes. Polystyrene, polyisobutylene, polyvinyl chloride, polyamide, polypropylene, poly(acrylic acid), poly(ethylene glycol), polyglycerol, and polyaniline are other synthetic polymers employed as matrices to immobilize biomolecules [37]. A valuable immobilized biocatalyst obtained from lipase immobilized on PVA nanofibers was disclosed by Weiser et al. [38]. The electrospinning approach was applied for PVA nanofiber preparation, as was the case for the immobilization of lipase. The enzyme was entrapped within PVA nanofibers, and its improvement in catalytic activity by molecular imprinting was investigated. For this purpose, polyethylene glycols, nonionic detergents, and organosilanes were used as bioimprinting molecules to modulate the active site of the entrapped lipase. The authors reported an evident enhancement in the biocatalytic properties of lipase by a bioimprinting effect. Furthermore, a low quantity of suitably chosen substrate led to an increase in the activity of the immobilized derivative.

In addition, polyaniline (PANI), obtained from aniline monomers for several polymerization techniques, is a customary example of a synthetic polymer widely used as a support

to immobilize biosystems. A laccase biosensor obtained from magnetic graphene and polyaniline as a composite electrode for the immobilization of the enzyme was reported by Lou et al. [39]. The biocatalyst obtained was employed to investigate hydroquinone in water since this phenolic compound is highly toxic and could be harmful to the environment as well as human health. In this way, a simple, low-cost, and sensitive electrochemical biosensor was prepared by depositing PANI on a magnetic graphene (MG) surface, and the optimal mass ratio (MG:PANI) was 0.1:2. Moreover, electronic microscopy images revealed the three-dimensional structure of the composite material with nanocavities. The developed biosensor has shown promise since it exhibits good electrical properties along with great sensitivity, a good detection limit, and a wide linear range [39].

These advancements highlight the growing importance of synthetic polymers in modern immobilization techniques. Synthetic polymers play a central role in biosystem immobilization due to their structural versatility, durability, and adaptability. Frequently used materials, such as polyvinyl alcohol (PVA) and polyaniline (PANI), exhibit exceptional potential as enzyme supports. Advanced techniques, including electrospinning for PVA nanofibers and composite formation with magnetic graphene for PANI, demonstrate the innovative methods employed to enhance catalytic performance and biosensor sensitivity. The tunable properties of synthetic polymers, such as mechanical strength, chemical resistance, and compatibility with biofunctionalization, provide tailored solutions for diverse industrial and environmental applications. These characteristics underscore the essential role of synthetic polymers in advancing immobilization technologies.

2.3. Semisynthetic Polymers

Semisynthetic polymers are materials prepared from a natural polymer with chemical, enzymatic, or microbiological modifications carried out in a controlled environment [40]. Cellulose derivatives (e.g., cellulose ether and cellulose nitrate) are among the main polymeric materials employed as supports since they present functional groups to bind biosystems. It is still important to mention that cellulose derivatives have been widely used in chemical and biological industries due to their attractive features, such as low cost, nontoxicity, renewability, biodegradability, and biocompatibility [41].

Carboxymethyl cellulose magnetic nanoparticles with core–shell structures were employed to immobilize the prenyltransferase NovQ. The derivative immobilized under the best experimental conditions was tested for vitamin K2 production and used in different protein engineering applications. Interestingly, the hybrid nanocomposite preparation consisted of three steps. First, iron ions are complexed with carboxymethyl cellulose (CMC); after this, nanoparticles are precipitated by the addition of a strong base (NaOH), and finally, a core–shell structure is formed during the oxidation reaction [42].

Silva et al. [43] described the use of cellulose derivatives as a polymeric matrix for phospholipase Lecitase (LU) immobilization. The LU enzyme was immobilized by physical adsorption on cellulose triacetate (CTA). The features of cellulose derivatives, such as biocompatibility and equilibration of hydrophilic/hydrophobic substances, play important roles in enzyme immobilization through adsorption and catalytic activities. Moreover, the hydrophobic nature of the CTA support contributed to a better performance of the immobilization process (above 95%). The immobilized derivative (CTA-LU) exhibited good catalytic activity toward fatty acid methyl ester production (approximately 44%) from soybean oil [43].

Semisynthetic polymers bridge the gap between natural and synthetic materials, offering tailored properties through controlled modifications. Cellulose derivatives, such as cellulose ether and cellulose nitrate, stand out for their functional groups and features like low cost, renewability, and biocompatibility. Examples include carboxymethyl cellulose

nanoparticles for prenyltransferase NovQ immobilization and vitamin K2 production and cellulose triacetate (CTA) for enzyme immobilization with high efficiency and catalytic activity. These cases highlight the adaptability and sustainability of semisynthetic polymers in biosystem immobilization.

3. Hierarchical Structures of Polymeric Matrices

When biosystems are immobilized in a support, chemical transformations can occur and cause modifications in the properties of the entities. These changes are generated by particularities of the material and operational conditions that are submitted; for this reason, a prior understanding of the characteristics of the matrices is essential [44]. Some properties of entities can be altered by their interaction with the support compared to those of free entities; therefore, defining the purpose of using the immobilized derivative and knowing the immobilization technique to be employed are crucial steps.

The matrix is one of the factors responsible for significant interference in the behavior of immobilized biosystems. Several factors must be considered when selecting a support, such as the type of material (ceramic, metallic, polymeric, or composite), the size and shape of the matrix, dispersion, and porosity. The use of these parameters can influence the physical–chemical properties and applicability of an immobilized derivative of interest in industry under desirable conditions when considering the interactions that react with entities.

Another point to be highlighted is the versatility that polymeric matrices can present, especially when combined with other materials, which gives polymeric composites some advantages that can enhance existing properties or offer new additional features, allowing multifunctionality to these materials: (i) a large number of different functional groups; (ii) high processability; (iii) flexibility; (iv) low manufacturing cost; (v) high mechanical and chemical resistance; and (vi) low density. In addition, other parameters, such as low thermal and electrical conductivity and low resistance to temperature, can be observed; however, it is necessary to consider whether these properties are necessary and important when a matrix is produced for the desired application [45–48]. The main criteria for selecting matrices and immobilization techniques are summarized in Figure 3, providing a detailed flowchart of the process.

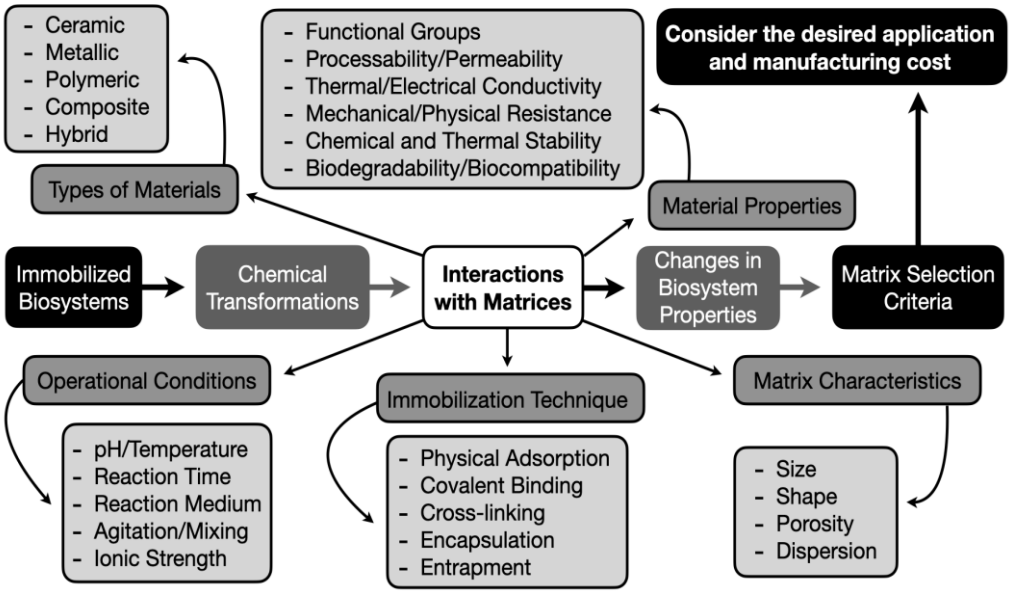

Figure 3. Flow chart detailing the main criteria for selecting matrices and immobilization techniques in biosystems.

Therefore, another issue that must be taken into account is the possible forms of support that can be used for the immobilization of biosystems, which is why, in this subtopic, the highlights are the polymeric matrices and their structural diversity.

Polymeric matrices used as supports for the immobilization of biosystems can be presented in different forms. This variety can be observed using only polymers, whether they are natural [49], synthetic [50] or semisynthetic [51], or, more frequently, polymeric composites, due to the greater possibility of observing superior properties, whether in relation to the improvement in physical–chemical characteristics, such as chemical and mechanical resistance, processability, permeability, biodegradability and biocompatibility [52,53].

These particles are also the main form of support used to prepare immobilized derivatives, mainly on the nanoscale, and these particles are the main form of support for both isolated polymers and polymeric composites. Considering the composites in the form of nano- and microparticles, we can highlight the use of magnetic matrices coated with biopolymers [54,55], semisynthetic [42,56], and synthetic polymers [57,58]; polymeric conjugates with metals [59,60]; mixed polymers [61]; layer-by-layer involving shells, and polymer grafts [44].

Beads are other structures of polymeric matrices that have received increased amounts of attention [62]. These materials are synthesized mainly from chitosan and alginate biopolymers isolated or combined with other materials. These beads can be prepared as carriers for enzyme immobilization using a biopolymer (chitosan) with clay minerals (montmorillonite) to improve several characteristics, such as density [63]. It is possible to obtain beads through the use of two polymers, where both can be natural or through the combination of a synthetic polymer with a natural polymer, for example, alginate-guar gum [64] or poly(vinyl alcohol)-alginate [65], respectively.

Another well-represented way to accomplish this goal is through the use of gels [66] and hydrogels [67] composed of an isolated polymer, which can be found mainly as polymeric composites; these composites can be found only as gels or hydrogels on both the micro [68] and nano [69] scales but also as beads [70] and graft copolymeric micelle/gel systems [71,72].

Polymeric matrices can be used as fibers, mainly as nanocomposites, with more than one polymer; these materials include both synthetic polymers, such as poly(vinyl alcohol)/poly(amidoamine)-montmorillonite (PVA/PAMAM-Mt), used as biosensors [73]; both natural polymers, such as poly(lactic acid)-chitosan/titanium dioxide (PLA-CS/TiO_2), used for cell attachment [74]; and natural and synthetic polymers, such as chitosan/poly(vinyl alcohol), used to optimize the properties of immobilized enzymes [75].

By still focusing on the immobilization of biosystems, the matrices can be used in the form of films with only one polymer, such as silicone polymeric films [50], or with a mixture of polymers, such as poly(pyrrole)/poly(vinyl alcohol) [76]. In addition to these, it is also possible to find in the literature the use of polymeric matrices in the form of a membrane, for example, membranes assembled with hybrid silica nanowires for enzyme immobilization [77]; the biocatalytic membrane of hybrid bioinorganic structure on the surface of poly(dopamine) coated poly(vinylidene fluoride) (PDA@PVDF) and the PDA layer graft of 3-triethoxysilylpropylamine (APTES) modified Fe_2O_3@SiO_2 cubes (FS@cubes), resulting in FS@cubes-PDA@PVDF [78]; bacterial cellulose membrane functionalized through the impregnation of magnetic nanoparticles [79]; and surface of the poly(sulfone) membrane coated with dopamine [80].

Less frequently, we can find microspheres with functionalized polymers, such as cross-linked cellulose (CL-CMs), which have good spherical shapes and monodispersity [81], and microspheres with polymers combined with magnetic particles, such as Fe_3O_4 and glycidyl methacrylate (Fe_3O_4-GMH), which have abundant, large porous structures, surface area

and superparamagnetic properties [82]. Finally, it is also possible to find in the literature the use of multi-walled carbon nanotubes grafted with poly(dopamine) impregnated with magnetic cobalt [83].

4. Monodisperse Polymeric Particles as Matrices for Immobilization

Biosystems immobilized in monodispersed polymeric particles are widely used. However, the scientific community recognizes that it is still a challenge to prepare monodispersed polymeric nanoparticles through a simple approach, especially via emulsion polymerization, due to the complexities of the reaction kinetics and the need for separation of different-sized particles, which lack high dissipation technology, increasing the costs of obtaining them. These challenges, along with the associated methodologies and applications, are summarized in Figure 4, which highlights the key concepts, descriptions, and examples of monodisperse polymeric particles as immobilization matrices.

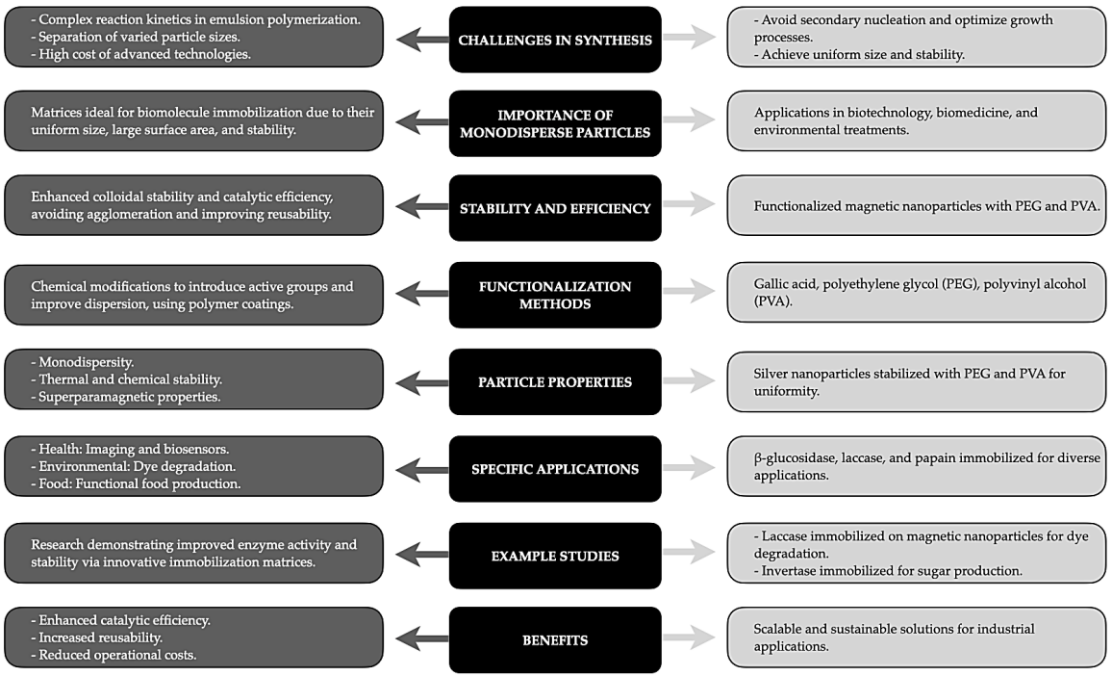

Figure 4. Summary of key concepts related to monodisperse polymeric particles as matrices for biosystem immobilization, with categories displayed at the center, descriptions on the left, and examples on the right.

Faced with the problem of the effects of reaction kinetics on particle size distribution, some research groups have conducted multifarious experimental studies regarding the mechanism and kinetics of different emulsion polymerization systems. To obtain monodispersive polymer nanoparticles, by this system, several principles must be taken into consideration: during the particle growth period, avoid secondary nucleation phenomena and limit the nucleation time to a very short period; during the preparation of monodispersive particles, relatively long competitive growth and self-sharpening processes are necessary, which makes particle coagulation unfavorable [84].

Chemically stable matrices with good dispersion and high enzyme loading are ideal for the immobilization of biosystems [85]. Monodispered magnetic nanoparticles have proven to be excellent candidates for enzymatic immobilization, especially due to the possibility of adjustable porosity, high surface area, and excellent chemical/thermal stability. The enzymatic derivative can yield better results than free enzymes and has diverse applications in the fields of biochemistry, biotechnology, and biomedicine [86].

The colloidal stability of systems with enzymatic particles has received special attention in the context of immobilization since aggregation processes can affect the efficiency of reactions. Therefore, detailed investigations of the charging and aggregation processes under these application conditions must be performed to obtain stable dispersions of composites with significant enzymatic activity [87]. Polymers are used to coat or modify particles of organic or inorganic materials to solve problems with dispersion instability, avoiding the formation of agglomerates [88]. Optimizing the emulsification conditions of aqueous polymeric solutions in a continuous oil-based phase is considered a more affordable method that allows adjustable particle size and morphology [68]. The emulsion polymerization system has been used to obtain monodisperse polymer particles [84].

However, to achieve systems with reproducibility and processability, particles with monodispersity, crystallinity, and superparamagnetic properties have shown great advantages. Therefore, to solve various problems, such as poor stability, scalability, simplicity, and efficiency, and the desire for economic reuse, related to laccase carriers, Iriarte-Mesa et al. proposed the covalent immobilization of laccase on the surface of soluble superparamagnetic iron oxide nanoparticles in water modified with polyacrylic acid and gallic acid. In addition to being an enzymatic derivative with excellent enzymatic activity and higher reactivity during the degradation of industrial azo dyes than the free enzyme, the authors obtained a high colloidal stability. The exchange of binders with a mixture of polyacrylic acid and gallic acid, which made it possible to achieve direct transfer to water, made it possible to guarantee high reproducibility in a very simple way [89].

To demonstrate the ability of the matrices to immobilize biosystems with monodisperse polymeric particles, the authors decided to present some results from studies in which the polymers were used to assist in the stabilization process, as chemical changes are necessary, in addition to strategies for solving the problem of dispersion instability.

An example is the study by Cunha et al. [90] with the synthesis of silver nanoparticles in the presence of polymers (polyethylene glycol—PEG, polyvinyl alcohol—PVA, and chitosan). The polymers were used as stabilizing agents in order to obtain, in addition to matrices with antimicrobial action, adjustable sizes and shapes for the silver nanostructures. It was possible to obtain spherical nanoparticles of uniform shape, small size, and monodispersity using PEG and PVA as protection agents. However, due to the viscosity of chitosan, this phenomenon was impaired [90].

Particles with good spherical shapes and monodispersity were also obtained from cross-linked cellulose with epichlorohydrin as a cross-linker for the immobilization of polymyxin B to use as an endotoxin adsorbent [81]. Chemical modifications for functionalization or physical treatment are often necessary to obtain desired shapes and ensure adequate size and monodispersity [91].

The copolymers of starch and poly(methacrylic acid-co-methyl methacrylate) P(MAA-co-MMA)-starch matrix used for immobilization of l-asparaginase (l-ASNase) were obtained by the starch functionalization process combined with in situ emulsion polymerization, which allowed the uniform distribution of functional groups in the composite, ensuring a homogeneous distribution of l-ASNase [52].

Moradi et al. [92] worked with β-glucosidase immobilized onto amino-tannic acid-modified Fe_3O_4 magnetic nanoparticles (ATA-Fe_3O_4 MNPs) as a biocompatible nanoplatform via the use of modified polyaldehyde pullulan (PAP) as a cross-linker and reported that particle size and uniformity, size and uniformity of particle size, electrostatic colloidal dispersion stability, morphology, and magnetic properties are the most important qualitative indicators of magnetic nanoparticles [92].

In the studies of Yu et al. with magnetic nanoparticles, glycidyl methacrylate (GMA), dimethacrylate ethylene glycol ester (EDGMA), and cross-linking styrene (St), the Fe_3O_4/P

(GMA-EDGMA-St) composite carrier for the immobilization of papain, it was observed that the distribution was relatively uniform, and this was obtained through the dispersion polymerization method. It was also determined that the material does not exhibit any coercive force, the dispersibility is excellent, and the average particle size is on the nanometer scale. The authors argued that increasing the dispersibility of Fe_3O_4-modified oleic acid in polar solvents prevents agglomeration between the particles and claimed that the main reason is that the carboxylic acid group of oleic acid reacts with Fe_3O_4 to change its surface from hydrophilic to lipophilic and forms a protective layer on the outer surface [93].

Waifalkar et al. [54] worked with chitosan-coated sol–gel-derived γ-Fe_2O_3 magnetic nanoparticles (MNPs) for invertase immobilization and reported that biomagnetic materials, in addition to being biocompatible and capable of being functionalized with cells and other biological entities, must retain their magnetic properties for a reasonable period of time in aqueous media at variable pH and temperature and must form stable, nonaggregate dispersions. Furthermore, the ability of particles to withstand aggregation in both a biological medium and a magnetic field to obtain magnetic colloidal ferrofluids can be achieved by coating particles with different surfactants, which can help bind various biological ligands to the nanoparticle surface. Grafting or polymer coatings are also strategies used, and chitosan has allowed better dispersion of NPMs. The superparamagnetic property of MNPs makes them stable against agglomeration in solution and enables them to redisperse rapidly when the external magnetic field is removed [54].

The cellulase immobilized on magnetite carboxymethyl chitosan/calcium alginate, as studied by Jiang et al., was uniformly dispersed, which not only increased the probability of contact between the enzyme and the substrate but also enhanced the response speed and efficiency. It exhibited a small particle size, good dispersion and stability, easy separation, and good cellulase loading through physical adsorption, embedding, and covalent bonding. Its mean hydrodynamic diameter was 56.3 nm, and the loading capacity for cellulase was 3.95 mg/mL [94].

Monodisperse polymeric particles are promising matrices for biosystem immobilization due to their uniform size, high dispersion stability, and large surface area. Polymers are also required as matrices for the immobilization of biosystems due to their chemical inertness in wide ranges of working temperatures, flexibility, lightness, ease of processing, and a better balance between physical and chemical properties than ceramics and metals when used for biomedical applications. Advances in emulsion polymerization and chemical modifications have enabled the synthesis of particles with enhanced colloidal stability and catalytic efficiency. Examples include the use of functionalized magnetic nanoparticles and stabilizing agents such as PEG and PVA to improve uniformity and reusability.

It is important to highlight that the choice of one polymer over another for the immobilization of a biosystem will depend greatly on the particular possibilities of physical and chemical interactions between the polymer matrix and the immobilized biomaterial, aiming to obtain better results for the specific application. The most common interactions involve physical adsorption, covalent binding, cross-linking, and entrapment in gels or membranes. These processes can present some disadvantages, such as syntheses with the use of chemical reagents that are undesirable to the environment (organic solvents, strong acids, etc.) and even low immobilization yield, which will directly affect the possibility of scaling related products to a commercial level.

For example, the entrapment of biosystems in water-soluble polymer matrices that form hydrogels, such as PVA, PVP, and cellulose acetate, can be established at room temperature. The encapsulation film configuration is suitable when the bioelement is fragile at higher temperatures. In addition, this class of polymers can represent a modified release system for the biosystem compared to the isolated bioelement. On the other hand,

these films can swell, causing leakage of the biomaterial to the external environment, and/or have their original conformation undone upon unwanted contact with ambient humidity and become mechanically fragile, causing significant changes in the initial properties designed for the matrix. In this sense, the production of hydrogels from these polymers by incorporating chitosan and proteins has proven to be functional in increasing encapsulation efficiency and providing a more controlled release of the biosystem.

Inorganic polymer matrices can be easily produced by the sol–gel process (gelation reactions at low temperatures), resulting in materials with good biocompatibility for biomedical applications. These matrices can provide stability and maintain the biosystem's activity for its application. On the other hand, these matrices are fragile materials, with the possibility of leakage of the trapped bioelement, low reproducibility, and sensitivity with the action medium.

Acrylic-based polymers and/or polymers with high hydrophobicity are also an alternative for the effective encapsulation of biosystems. For example, these polymers can be synthesized with selective solubility, dependent on the pH of the medium, and can be used as programmed release systems of active substances. On the other hand, without these chemical modifications (which add high cost to these polymers), this class of materials may present limitations such as low sensitivity and high retention of the activity of the confined biosystem.

These developments underscore the potential of monodisperse polymeric particles in innovative applications within biotechnology and biomedicine. In practice, the final choice to use the immobilized biosystem will also depend on the cost–benefit assessment of the technology for the desired application.

5. Biosystem Immobilization Techniques

In this section, the advances in immobilization methods, mainly for enzymes, are mentioned. Going over the potential benefits of immobilization again, the widespread use of immobilized enzymes as a result of better pH and temperature stabilities, high catalytic performance, and reusability is very clear compared to those of their free counterparts. In this way, enzymologists, biotechnologists, and researchers in other fields are employing more than one immobilization technique as a smart strategy to obtain a potential immobilized biocatalyst.

Over the last years, covalent binding, adsorption, and cross-linking have been observed as the most commonly employed immobilization techniques when polymeric materials are used as the matrix. However, these findings were consistent with previous works that used only a single immobilization technique. Below, we will describe some scientific productions where the authors explored more than one immobilization method to obtain a promising immobilized biocatalyst.

The immobilization of papain on hydrogel composites by adsorption and cross-linking was reported by Dai et al. [95]. First, papain was immobilized by adsorption after the hydrogel composite was incubated with the enzyme solution for 24 h at 4 °C. Then, the next stage of immobilization was performed via cross-linking using glutaraldehyde at 4 °C and different contact times to complete cross-linking. Conditions of the immobilization process, such as papain concentration (3 mg/mL), glutaraldehyde concentration (0.75%), glutaraldehyde contact time (1.5 h), and pH immobilization, were also investigated and optimized. The authors performed the papain enzymatic activity tests varying the pH of the medium from 6.0 to 8.0 and obtained the best results at pH = 6.5. Compared to the higher pH range tested (from 7.5 to 8.0), the slightly acidic pH range (from 6.0 to 6.5) proved to be more suitable for the process efficiency.

Additionally, Day and his co-authors revealed an interesting strategy for the preparation of hydrogel composites based on modified cellulose, polyvinyl alcohol (PVA), and SBA-15, which are similar to mesoporous silica materials. The catalytic performance of the immobilized derivative was sensitive to the mass ratio of modified cellulose/PVA and the quantity of SBA-15. That is, a better immobilization performance was achieved for a mass ratio of 1:4 (modified cellulose/PVA) and 0.1 g of SBA-15.

A simple and efficient immobilization protocol for α-amylase on a titania/lignin matrix, including covalent binding and physical interactions such as joint techniques, was described [96]. As highlighted by the authors, the hybrid material prepared from inorganic (titanium dioxide) and organic (lignin) components is novel and interesting in support of immobilizing enzymes because it provides desirable features, such as several reactive functional groups and enhanced mechanical stability. The best operational conditions for enzyme immobilization were 3.0 mg mL^{-1} enzyme concentration, 7.0 immobilization pH, 3 h immobilization time, and 5 °C temperature. The immobilization protocol consisted of mixing the enzyme solution with the hybrid particles under the abovementioned experimental conditions. Strong bonds (covalent binding), as well as weak interactions (hydrogen and ionic), were responsible for fixing the enzyme onto the hybrid surface of the matrix. Moreover, Fourier transform infrared spectroscopy (FTIR) and surface measurements (i.e., surface area, mean pore size, and pore volume) supported the efficiency of the immobilization protocol proposed by the authors.

Zdarta et al. [97] presented an innovative immobilization protocol involving adsorption and encapsulation methods for immobilizing laccase onto poly(l-lactic acid)-co-poly-(ε-caprolactone) (PLCL) nanofibers (Zdarta et al. 2019). The immobilized derivative was used to degrade two anti-inflammatory agents (naproxen and diclofenac) from wastewater. Initially, hydrophilic PLCL nanofibers were prepared by electrospinning with chloroform (10% w/v) and acetate buffer at pH 5 as the oil and water phases, respectively. Like in nanofiber preparation, laccase was encapsulated by emulsion electrospinning. For this purpose, a laccase solution (100 mg/mL) was blended with PLCL nanofibers for 3 h at room temperature under stirring. Adsorption was the second protocol used by the authors to promote the immobilization of laccase on PLCL nanofibers. The enzymes (1 mg/mL) were put in contact with the polymer matrix for 2 h at 4 °C. Scanning electron microscopy (SEM) was used to evaluate the presence of biosystems for both immobilization techniques. SEM images revealed marked changes in the polymer surface and in the diameter of the PLCL nanofibers after immobilization. With respect to the final objective of this work, the immobilized biocatalyst proved to be efficient since it was proven to biodegrade pharmaceuticals under optimal processing conditions. Additionally, electrospinning was also employed to immobilize β-galactosidase on chitosan (CS)/polyvinyl alcohol (PVA) composite nanofibers as a matrix [75].

Click chemistry, like a particular approach for immobilizing biomolecules (e.g., enzymes, antibodies, and nucleic acids), has also been applied recently. The conjugation reaction is simply a cycloaddition reaction between azide and alkyne components catalyzed by copper (I) at room temperature. Additionally, click chemistry is widely employed for assembling architectures and functionalizing the surface of several materials [98]. Polymer-coated gold nanoparticles were used as a matrix to immobilize antibodies by click chemistry, as reported by Finetti et al. [99]. To promote chemical reactions, poly(N,N-dimethylacrylamide) (DMA) was first modified with an alkyne monomer, after which the surface coating process of the gold nanoparticles was conducted. Moreover, an anti-mouse IgG antibody with azido groups was obtained. Clicking was carried out by binding the azido-modified antibody and Au NPs functionalized with the alkyne-modified polymer. The authors described feasible applications for the immobilized antibody; however, an unconventional

biosensing technique denoted the interferometric reflectance imaging system (IRIS), was proposed. Another biomolecule that can be immobilized by click chemistry is heparin, a common anticoagulant.

Wu et al. [100] reported the use of this technique to immobilize heparin with alkynyl groups onto the azide-modified polyisobutylene (PIB)-based thermoplastic elastomer TPE (arb-SIBS-azide). The heparin on the surface of the soft biomaterial (PIB-based TPE) enhanced blood compatibility, which is an important feature of artificial vascular grafts. Additionally, the click chemistry approach was satisfactory due to an improvement in the anticoagulant activity of heparin.

Recently, multienzyme immobilization has become another clever strategy that has been applied to obtain efficient biocatalysts [101]. Random co-immobilization, positional co-immobilization, and compartmentalization are among the major approaches for multienzyme immobilization. Among these methods, positional co-immobilization is promising for obtaining ordered multienzyme immobilization systems. Moreover, polymer-based supports have been widely used as a matrix for complex enzyme systems because of the attractive properties of polymers (e.g., good mechanical strength, flexible morphologies, and stable properties), as well as their ability to control enzyme positions [102]. In this way, a polymer film was used as a matrix to immobilize two enzymes separately and simultaneously via visible light-induced graft polymerization [103]. Trypsin and transglutaminase (TGase) were immobilized on a modified low-density polyethylene (LDPE) film. According to the authors, initially, under UV irradiation, isopropyl thioxanthone (ITX) was put in contact with the LDPE surfaces. Enzymatic solutions prepared in poly(ethylene glycol) diacrylate (PEGDA) were encapsulated by a photografting reaction onto each side of the LDPE film under UV irradiation for 30 min at room temperature. In addition, dual-enzyme-loaded films (DEL films) were shown to be attractive immobilized derivatives since they presented good operational stability (above 87% of the retained activity for both enzymes after four reusability cycles).

Advances in enzyme immobilization techniques have improved stability, catalytic performance, and reusability. Combined methods, such as adsorption with cross-linking or encapsulation, enhance efficiency and adaptability. Polymer-based matrices, like hydrogels and nanofibers, offer functional versatility and mechanical stability, supporting diverse strategies. Innovations like click chemistry and multienzyme systems enable precise biomolecule positioning, reinforcing the importance of polymer-based immobilization for efficient, reusable biocatalysts in industrial and biomedical fields.

Furthermore, a critical analysis of the studies cited in this section revealed that the experimental works focus on the advantages of using techniques for immobilizing biosystems but do not present possible associated disadvantages. For example, the disadvantages of using techniques for trapping biosystems using polymers can involve the possibility of undesirable leakage of the biomaterial from the matrix structure (as in the case of immobilization of biosystems from hydrogel and emulsion preparation techniques) and, in other cases, depending on the type of polymer (those of acrylic and/or hydrophobic nature, for example), even the excessive decrease in the activity of the bioelement with the action medium due to the high resistance to the transfer of active material to the medium. In addition, interactions between carrier and biosystem resulting from adsorption processes (ionic, hydrophobic, hydrogen bonds, and van der Waals forces) are known to represent weak chemical bonds, which can make the final compound unstable compared to those produced by other immobilization techniques. It is important to highlight that the choice of one technique over another will depend mainly on the possible interactions that the biosystem can experience with the polymer, in addition to the economic analysis related to the application of the material resulting from the immobilization process.

6. Biosystems Engaging in Immobilization Process

In the literature, different types of immobilized biological and synthetic entities, such as proteins [104], peptides [81], antibodies [88], enzymes [58], DNA [105], cells [106], and drugs [107], are commonly found. The activity of these entities is very sensitive to microenvironment conditions (e.g., pH, temperature, solvent). However, the immobilization of entities has emerged as a beneficial alternative for improving operational stability, allowing product separation and reusability, among other applications [108].

In fact, enzymes, proteins, cells, and drugs are the major immobilized biological and synthetic entities on polymeric particles [109–114]. Among these biosystems, enzymes were the most commonly used because of their notable and widespread application in several biotechnological processes, for example, as alternatives to chemical catalysts [108].

Waifalkar et al. immobilized invertase on magnetic composites prepared from iron oxide nanoparticles (NPs) and chitosan (MNPs). The magnetic biocatalyst (immobilized invertase on MNPs) performed well in sucrose hydrolysis. Moreover, the operational stability of the immobilized invertase was improved by changing the pH and temperature. In addition, immobilized invertase was successfully reused for 20 cycles, preserving much of its initial activity [54].

Zhang et al. [115] investigated the immobilization of urease by covalently binding to the zwitterionic material poly(carboxybetaine acrylamide) (pCBAA) grafted on magnetite nanoparticles. The immobilized enzyme showed better chemical and thermodynamic stability against the free enzyme, where the residual activity of the immobilized urease was 60% at 70 °C for 2 h, while the free urease activity was only 30% under the same conditions. Additionally, the immobilized derivative exhibited excellent initial activity (up to 80%) after five reuse cycles. From these results, the authors elucidated potential applications for the immobilized enzyme in several areas, such as urea detection in blood serum, urea decomposition in artificial dialysis, the food industry, biosensors, and water treatment [115].

Additionally, Moradi et al. investigated the immobilization of the β-glucosidase enzyme on magnetic nanoparticles modified with amino-tannic acid (ATA-Fe_3O_4). To improve the biocompatibility and enzyme attachment, the magnetic nanocomposite was treated with polyaldehyde pullulan (PAP). The immobilized β-glucosidase showed greater activity at 40 °C and pH 6.0, whereas optimum conditions for the free enzyme were found at 30 °C and pH 5.0. Furthermore, the immobilized invertase was reused ten times, maintaining 83% of the initial activity [92].

Alternatively, other kinds of biomolecules widely immobilized are proteins that have demonstrated powerful effects on various applications, for example, in water treatment processes [116], the food industry [92], and the biomedicine field [110]. Choi et al. [61] immobilized lactoferrin on poly(lactic-co-glycolic acid) nanoparticles coated with heparin-dopamine. The polymeric nanosystem displayed satisfactory results related to its anti-inflammatory effects in vitro and in vivo in an Achilles tendinitis rat model. The immobilized derivative was shown to contribute to decreasing collagen quantity together with decreasing mechanical properties of tendons, avoiding collagen cleavage, and improving tendon-related marker levels [61]. Additionally, exceptional results in the electrochemical detection of hydrogen peroxide in human biological systems were found by covalent immobilization of hemoglobin on polyamidoamine (PAMAM) dendrimers encapsulated with gold nanoparticles [111].

Bacterial or fungal cells have also been immobilized on polymeric matrices, offering advantages such as greater metabolic activity, continuous use, greater cell density, preservation of plasmid-bearing cells, shear stress in the environment, and safety in acidic environments [117]. Biosynthesis [113], environmental pollution [106], and xanthan pro-

duction are widely applied in the food industry [112] and are among the main technological applications of immobilized cells. For instance, Wu et al. [106] described a new biofunctional composite obtained by covalent immobilization of bacteria (Sphingopyxis sp. YF1) on magnetic nanoparticles modified with chitosan (Fe_3O_4@CS). The authors proposed bacterial immobilization for the biodegradation of microcystin-LR (MC-LR) since this is one of the most harmful derivatives present in the water environment. Under optimized experimental conditions, the biofunctional composite exhibited an excellent degradation performance. In addition, the better performance of the immobilized bacteria was also proven through the enhancement of degradation efficiency during six reuse cycles [106].

The immobilization of biosystems, particularly enzymes, offers improved stability, reusability, and functionality. Advances in polymer-based matrices and functionalized nanoparticles have enhanced efficiency and operational stability, enabling diverse applications in biotechnology, biomedicine, and environmental remediation. Enzymes, proteins, and cells have demonstrated significant potential in biocatalysis, pollutant degradation, and therapeutic systems, highlighting the versatility of immobilization strategies.

6.1. Enzyme Immobilization

Owing to their natural features, such as great substrate specificity, high selectivity, high catalytic rate, and soft reaction conditions, enzymes are superior biocatalysts with important functions in the development of biodegradable, biocompatible, and renewable resources. Additionally, the different and attractive uses of enzymes make them highly interesting; for example, in green and sustainable chemical preparation, they could be more cost-effective and environmentally friendly [118]. Thus, enzymes have been widely used in the medical [49] and pharmaceutical [82] fields, in the production of biofuels [79], in the environmental [89] and food industries [94], and in the biological sciences [73], among others. However, for all applications, the immobilized enzyme should be stable and functional in the required process. For this purpose, regulating the stability and catalytic activity of biomolecules is highly important and has received much attention. Additionally, enzyme immobilization has been essential for resolving problems related to enzymatic solubility and reusability in industrial applications. The immobilization technique not only has improved catalytic properties but also has proven to be an effective technology for solving these problems, allowing multiple reuse cycles and the continuous automatic action of enzymes on an industrial scale [119].

According to the relevant enzyme classifications (i.e., hydrolases, ligases, oxidoreductases, transferases, lyases, and isomerases), hydrolases are widely involved in the immobilization of biomolecules on polymeric matrices. In particular, hydrolytic enzymes constitute close to 75% of all global enzymes manufactured, and lipases are the highlighted group of enzymes [120]. Moreover, hydrolytic reactions are most productive when enzymes are immobilized by specific immobilization techniques, such as physical adsorption, covalent bonding, or entrapment [121].

Lipases (EC 3.1.1.3) have been immobilized on several polymeric particles since they are all-purpose enzymes with high catalytic efficiency in many reactions, including hydrolysis, esterification, transesterification, acidolysis, and C–C bond formation [79]. Lipases are ubiquitous in nature and can be extracted from plant, animal, or microbial sources. However, lipases extracted from *Aspergillus niger* are the principal choice due to their economical and widespread use [122]. On the other hand, free lipases should receive special attention due to their low stability, poor reusability, and high production cost, which are sufficient hindrances for their industrial application. One way to increase the stability, improve the catalytic performance, and ensure the reusability of lipase is by immobilizing it on suitable supports, allowing its use on a large scale [79]. Yang et al. [56] reported the immobilization

of lipase on magnetic dialdehyde starch nanoparticles for triglyceride hydrolysis to free fatty acids. The authors revealed that the immobilized derivative had superior acid-base tolerance and thermal stability when compared to its free counterpart. Furthermore, the excellent stability and durability of the immobilized lipases were evidenced by their residual activity being close to 54% after six reuse cycles [56].

Cellulase enzymes are also included in the hydrolase classification. This enzyme has important industrial applications, for instance, in bioethanol production from cellulose hydrolysis to produce fermentable sugars, where cellulose is the key enzyme of the process. Owing to its poor stability and reusability, cellulase is commonly immobilized on appropriate supports for the purpose of enhancing its properties (cellulase efficacy, stability, and reusability) [69]. Jiang et al. [94] prepared a promising bioconjugate by cellulase immobilization on a magnetic carboxymethyl chitosan/calcium alginate composite (MCCCB). Several steps were involved in the preparation of the supports, including the use of a modified hydrothermal method for magnetite synthesis, molecular self-assembly technology for coating with carboxymethyl chitosan, and enzyme immobilization via physical absorption, embedding, and covalent bonding. The catalytic activity of immobilized cellulase was found to be awesome since a superior catalytic performance (>260%) related to free enzymes was observed. In addition, the reusability of MCCCB was also satisfactory because it retained an initial activity near 70% after ten reuse cycles. Finally, immobilized cellulase proved to be a promising biocatalyst able to produce successfully fermentable sugars [94].

Enzyme immobilization is crucial for improving stability, catalytic performance, and reusability, addressing the challenges of free enzymes in industrial applications. Hydrolases, such as lipases and cellulases, stand out for their role in hydrolysis and biofuel production. Polymer-based supports, including magnetic composites, have enhanced enzyme stability and efficiency, enabling their use in scalable and sustainable biotechnological processes.

6.2. Matrix-Biosystem Interface Effects on Bioactivity Performance

The success of a biocatalyst depends on several conditions, and support features are among the most predominant influences on the bioactivity of the immobilized derivative. For instance, physical parameters such as mechanical integrity, surface area, presence of pores, and pore size should be taken into account when selecting the support material [121]. Moreover, obtaining an immobilized derivative with high catalytic performance also involves careful consideration of the microenvironment conditions (e.g., substrate accessibility, enzyme orientation, and balance between associated and dissociated states) where the enzyme is immobilized. Additionally, a more in-depth study should consider the reaction kinetics involving the balance between both enzyme states since this process is strongly related to the catalytic development of the biomolecule [123].

The interfacial interactions (Figure 5) are strongly defined by the chemical and physical properties of the support material and the biosystem. In addition, support features such as topographic structure, size, chemical composition, and active functional groups present on the support surface are potential variables influencing bioactivity. In addition, the pH and temperature in the entity microenvironment, as well as the interaction forces (e.g., hydrophobic and hydrophilic) between the entity and the support, could influence the catalytic efficiency of the immobilized derivative as a consequence of protein conformation changes upon immobilization [124,125].

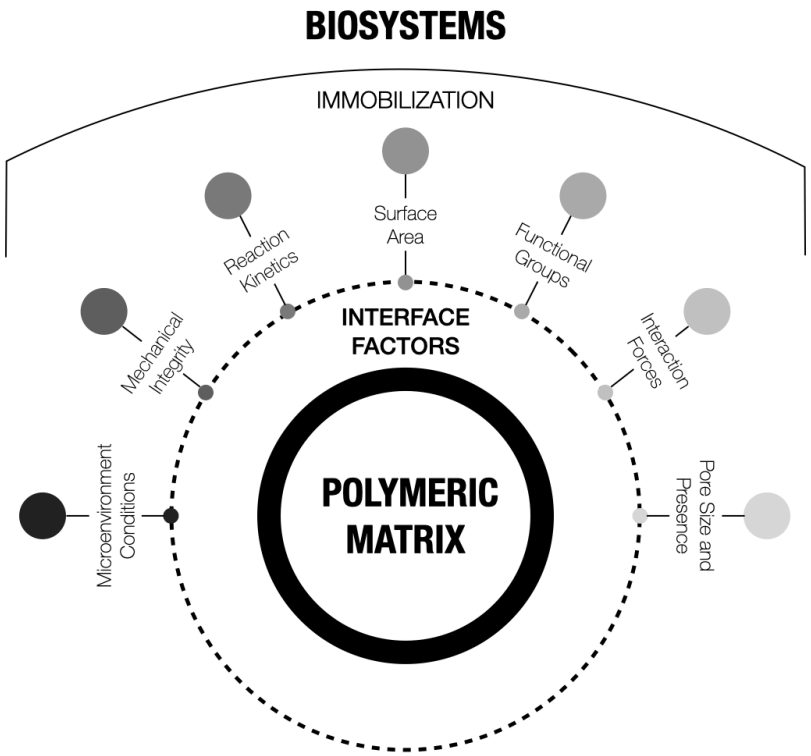

Figure 5. Interface effects in the interaction between biosystems and polymeric matrix.

Porous materials have been shown to be highly useful as supports for immobilizing biosystems since their pore structure allows unrestricted diffusion of substrate and product molecules if their size is lower than the pore size, indicating favorable conditions for effective mass transfer and a positive influence on the catalytic activity of the immobilized derivative [126].

Liu et al. reported a composite preparation of chitosan (CS) and eggshell membrane powder (ESMP) as a support for immobilizing papain. The authors proposed the use of ESMP to improve the porosity of chitosan-based particles once the ESMP is partially dissolved in sodium hydroxide solution and connects with chitosan chains through physical and chemical crosslinking. In addition, highly rich protein residues in ESMP could also provide multisite binding in composites (CSESMs), improving the catchment of biomolecules. Several volume ratios of the components (CS:ESM) were evaluated to determine the best conditions for papain immobilization. Efficient papain immobilization was found for CSESM2 cells at a volume ratio equal to 8:2 (CS:ESM). Under these conditions, an increase of approximately 1.5-fold in the catalytic efficiency of the immobilized derivative was observed. This finding was supported by the presence of amino groups on the ESMP surface, which provided additional reaction sites for the immobilization of the enzyme. Additionally, the authors disclosed that ESMP was responsible for providing greater numbers and larger sizes of pores. It is common knowledge that the presence of pores and adequate size allow beneficial conditions to mass transfer substrate and product molecules. In this way, the mesoporous contribution of ESMP was advantageous for easy diffusion during bioactivity of the biomolecule [127].

Due to its potential features, graphene oxide (GO) has been widely applied in the immobilization of lipases. Zhuang et al. [128] proposed a functionalized GO (FGO) from a chemical reduction process employing disodium guanosine 5′-monophosphate (GMP-2Na) as a safe alternative to conventional reducing agents (e.g., sodium borohydride and hydrazine hydrate), which can cause harmful issues. In addition, chemical reduction of GO was applied to improve the lipase bioactivity after immobilization. For this purpose,

the effect of several concentrations of GMP-2Na (0.1–2.0 mg mL^{-1}) on lipase activity was investigated. The authors observed that a specific quantity of GMP-2Na (0.2 mg mL^{-1}) allowed a suitable degree of hydrophobicity along with valuable bioactivity of the immobilized lipase. A higher GMP-2Na concentration results in excessive hydrophobicity, which lowers the catalytic performance of biomolecules [128].

Another survey reporting GO derivatives as supports for the immobilization of cytochrome c (cyt c) was reported by Patila et al. [129]. In this study, two forms of GO nanoparticles (GONs) (reduced and nonreduced) with chemical modifications (carboxyl and amino groups added) at the material surface were investigated for the immobilization of cyt c. Furthermore, two immobilization methods (i.e., physical adsorption and covalent binding) were also investigated. Summarizing the significant outcomes, the authors outlined that the catalytic performance, immobilization efficiency, and structural changes of immobilized proteins are strongly related to the immobilization approach, GON surface chemistry, and alkyl chain length of the modified material.

In addition, the ability of GO derivatives to immobilize the nuclease P1 via physical adsorption and chemical crosslinking was evaluated by Zhuang et al. [130]. For this purpose, GO nanosheets were modified with amino poly(ethylene glycol) (PEG-NH2) to study the effects of GO functionalization on the catalytic performance of the enzyme. The authors found a greater immobilization capability for GO without modification due to strong electrostatic and hydrophobic interactions between the biomolecule and support. In contrast, the thermal stability, reusability, acid resistance, and degradation effectiveness of the immobilized derivative were greatest for PEG-NH_2-modified GO.

Curiously, Cunha et al. [90] demonstrated the favorable participation of polymer (poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), and chitosan) stabilizers of silver nanoparticles (AgNPs). The composite preparation (polymer-stabilized AgNPs) was evaluated for its ability to support laccase immobilization with the aim of improving the antimicrobial action of AgNPS, which is well known from these metal nanoparticles, as well as drawing attention to the antimicrobial activity of laccase. In other words, the authors aimed to develop hybrid nanosystems capable of exhibiting excellent antibacterial properties and good colloidal stability. The major results revealed the enhancement of AgNP stability by the stabilizing agents due to steric repulsion provided by the polymers. Moreover, immobilized derivatives obtained from PEG- and PVA-AgNPs presented good enzymatic stability. However, problems in mass transfer by biocatalysts involving chitosan have also been reported. Overall, the synergistic effect of the hybrid nanocomposites was satisfactory for antimicrobial activity. The authors attributed these findings to differences in the action times as well as the mechanisms by which the enzyme and AgNPs improved the antimicrobial effect [131].

Interactions between biomolecules and support matrices are critical for the bioactivity and performance of immobilized systems. Surface structure, pore size, and functional groups influence substrate accessibility and enzyme stability. Advances in porous materials and graphene oxide derivatives have improved mass transfer, stability, and reusability. These findings underscore the role of matrix design in optimizing immobilization for industrial and biotechnological applications.

7. Characterization Methods to Confirm the Biosystem Immobilization

Identifying the occurrence of an entity on the immobilized derivative is highly important once a verification affirmative is a strong signal that the immobilization process was successful. Consequently, the researcher will have in their hands a ready biocatalyst to be applied. Several methods of characterization have been employed to investigate the presence of entities after immobilization [132].

It is important to note that knowing the form of interaction of the entity with support is of great interest for initiating the identification process as well as for selecting the appropriate characterization technique. Additionally, more than one technique can be necessary to evaluate the presence of an entity (Table 1), and in general, there is a set of standard techniques used by scientists. For magnetic materials (e.g., association of polymers with magnetic oxides), VSM measurements must be complemented with TGA to correct the value of saturation magnetization (organic phase should not be considered). A comparison of crystal size can be performed using XRD and magnetization measurement techniques. TEM can also provide size values that are close to other techniques already mentioned. FT-IR and spectroscopy Raman are complementary techniques. Therefore, different techniques can verify the reasons for changes in the performance of biosystems after the immobilization process and help to understand and propose alternatives to improve the properties of immobilized derivatives.

Table 1. Characterization methods for confirming the presence of biosystems on monodispersed polymeric particles after the immobilization process.

Characterization Method	Analyzed Properties
FT-IR	Characteristic peaks of functional groups
SEM- EDX and TEM	Size, morphology, chemical composition, and dispersion
XRD	Cristal phase and particle composition, particle size
ZP	Isoelectric point, surface charges, stability of the colloidal system
DLS	Particle size
AFM	Surface of the material
UV–vis	Qualitative and quantitative analysis of compounds
SAXS	Cristal phase and particle size distribution
CD	Detect biomolecule conformation
TGA	Determine the content of functional groups and bond formation
VSM	Magnetization properties measurements (e.g., saturation magnetization, coercivity, and hysteresis curve)

When an entity is immobilized on the matrix, structural changes and physical, chemical, and biological properties may occur, allowing it to present characteristics detectable by measurement and analysis instruments and making it possible to verify the presence of the entity through different characterization techniques when comparing the results obtained before and after the immobilization process. Thus, using appropriate characterization methods, the effectiveness of the immobilization process can be verified, confirming the presence of the entity in the support. Therefore, using methods that analyze the structure, morphology, and composition of materials and/or the entity before and after the immobilization process makes it possible to confirm the success of the biocatalyst preparation.

On the other hand, it is important to mention that the use of the characterization techniques will depend on each situation, that is, the physical phase of the sample (solid or liquid) of the minimum sample quantity necessary to carry out the analysis, which in turn is related to the cost of biocatalyst preparation. The high-cost biosystems are unlikely to be characterized. Therefore, most of the time, the success of biocatalyst preparation is supported by biochemical assays.

Characterization methods confirm biosystem immobilization and reveal changes in structural, physical, and chemical properties. Techniques like spectroscopy, microscopy, and thermal analysis verify entity presence and interactions, guiding improvements in bioactivity and stability for reliable industrial and biotechnological applications.

7.1. Main Characterization Techniques

As mentioned above, a pattern of physicochemical characterization techniques has been used to characterize materials containing immobilized biomolecules. For instance, Fourier transform infrared spectroscopy (FT-IR), scanning electronic microscopy (SEM), transmission electron microscopy (TEM), and X-ray diffraction (XRD) were the major techniques employed to investigate biomolecule occurrence. In addition, several techniques, such as energy-dispersive X-ray analysis (EDX), are used together because they provide complementary information.

7.1.1. Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) is widely used to detect the presence of characteristic vibrational bands of functional groups that are not present before immobilization. Some research works employing this technique for the immobilization of enzymes on polymer-coated magnetic nanoparticles are described below.

Aslani et al. [133] worked with covalently immobilized trypsin on silica-coated Fe_3O_4 magnetic nanoparticles (MNPs-Trypsin) and used FTIR to verify the presence of the enzyme. To confirm this, the authors compared the spectra of the Fe_3O_4, Fe_3O_4@SiO_2, Fe_3O_4@SiO_2-NH_2, immobilized trypsin, and free trypsin samples [133].

Donadelli et al. [134] immobilized soybean peroxidase (SBP) on different silica-coated magnetic nanoparticles (NPA-SBP, NPTAI-SBP and NPTAII-SBP). In this article, for all the samples, the band at 1623 cm^{-1} was attributed to the NH_2 bending mode of the free NH_2 group, confirming the presence of terminal amino groups at the surface of the biocatalyst support. The presence of the enzyme immobilized on the surface of the nanomaterial was consistent with all the signals expected. The authors dedicated the analysis of the signals related to amides I and II present in the range 1750–1550 cm^{-1} because these signals are associated with confirmation of the immobilized SBP. After normalization of the spectra, to increase the intensity ratio of the components, the intensity of the band at approximately 1650 cm^{-1} decreased in the order NPA-SBP > NPTAI-SBP > NPTAII-SBP. This band is mainly the result of C–O stretching vibrations, with minor contributions from out-of-phase C–N stretching vibrations, C–N deformation, and N–H in-plane bending. The decrease in the intensity of the 1650 cm^{-1} signal can be related to the progressive loss of the α-helix conformation of the supported enzyme with decreasing silica amount in the hybrid systems. The structural conformation of the SBP immobilized may have been affected by the thickness of the silica [134].

According to Waifalkar et al. [54], who worked with immobilized invertase on chitosan-coated magnetic nanoparticles (MNPs), it was possible to observe characteristic bands of the chemical groups that successfully revealed the chitosan coating on MNPs and the immobilization of invertase on the support. The presence of the characteristic band of invertase at 1640 cm^{-1} and the peak at 590 cm^{-1} that corresponds to Fe-O are responsible for this difference [54]. Moradi et al. also worked with magnetic nanoparticles modified with amino-tannic acid (ATA-Fe_3O_4 MNPs) and immobilized β-glucosidase (BGL) using poly-aldehyde pullulan (PAP) as a cross-linker, demonstrated the interaction between the enzyme and carrier due to the change in the peak at 520 cm^{-1}, which is associated with the Fe–O units, resulting in displacement caused by the binding of the enzyme to the carrier that occurs through the PAP. Another peak at 800 cm^{-1} that could be observed in the spectrum of the native enzyme was masked by the PAP peak; however, this peak was assigned to C–O–C stretching at α-(1 → 4)-glycosidic linkages, a marker of the "amorphous" region of PAP, which overlaps the amide II bond of the enzyme. The authors also observed that the C=O stretching vibration of amide I in the β-sheet secondary structure of the enzyme masked the characteristic C=N (imine) band belonging to the Schiff base in the BGL-ATA-

Fe_3O_4 spectrum at 1632 cm^{-1}; however, the preservation of the secondary structure, lack of denaturation, and immobilization of the enzyme on the carrier was confirmed by the presence of the amide I band. In addition, it was possible to confirm the presence of the cross-linker (PAP) used for immobilization of the enzyme through two bands at 2858 and 2926 cm^{-1} attributed to C–H stretching vibrations [92].

In the work of Oliveira et al., the enzyme immobilized onto chitosan-coated magnetic nanoparticles was Pectinex Ultra SP-L, which exhibited both hydrolytic and transfructosylating activities. The FTIR spectra of pure chitosan, magnetic nanoparticles without Fe_3O_4-MNPs, and magnetic nanoparticles with chitosan (Fe_3O_4-CS-MNPs) were analyzed, and the pure enzyme and Pectinex immobilized on Fe_3O_4-CS-MNPs were verified. We will compare the results of the last two methods, referring to the presence of the biomolecule. The spectrum of the free enzyme exhibited a peak at approximately 1638 cm^{-1}, which was attributed to C–O and N–H stretching; however, after the immobilization of Pectinex on the Fe_3O_4-CS-MNPs, the peak shifted to 1625 cm^{-1}, indicating that the functional groups of the support and enzyme interacted. According to the authors, these combined spectra demonstrated that CS was bound successfully to MNPs and that Pectinex was bound to Fe_3O_4-CS-MNPs [135].

The chitosan magnetic nanoparticles (CMNPs) with immobilized pectinase, developed by Sojitra et al., presented prominent and characteristic peaks corresponding to the stretching vibration of the C–O–C and Fe–O bonds from CMNPs at 1026.13 and 601.79 cm^{-1}, respectively, in the FT-IR spectrum. In addition, corresponding to the N–H deformation and C–O vibration from chitosan, peaks at 1527.62 and 1334.74 cm^{-1}, respectively, were observed. However, the successful immobilization of pectinase onto CMNPs was confirmed by the presence of the amide-I region of pectinase through the characteristic peak at 1689.64 cm^{-1} [136].

Interventional studies involving animals or humans, as well as other studies that require ethical approval, must list the authority that provided approval and the corresponding ethical approval code.

7.1.2. Circular Dichroism (CD)

Circular dichroism (CD) spectroscopy can be used as a complementary tool to FTIR analysis. CD analysis is suitable for the investigation of proteins and peptides in very dilute solutions that exhibit the alpha-helix as the main structural element. In this sense, Wu et al. used CD to detect protein conformations. For this purpose, we compared the results obtained for lysozyme, CS-NPs, and CS-Lys-NPs. Lysozyme presented a negative band at wavelengths shorter than 240 nm attributed to the α-helical structure and contained two negative minima bands at approximately 208 nm (π–π* transition of the α-helix) and 222 nm (π–π* transition for both the α-helix and random coil). In addition, in the CS-Lys-NP spectrum, a slight decrease in the magnitude of the ellipticity (10^{-3} deg) was observed, which was related to the interactions between the enzyme and the support, indicating an alteration in the lysozyme conformation [104].

7.1.3. Raman Spectroscopy

Raman spectroscopy is a physicochemical characterization technique complementary to FTIR that also provides information on molecular vibrations in polymer-immobilized biosystems.

Biegunski et al. [137] used Raman spectroscopy to study the immobilization of tyrosinase (an enzyme required for melanin production) on poly(indole-5-carboxylic acid) matrix. In the polymer/enzyme composite film, the results showed that the frequency of the characteristic stretching vibration band of the polymer pyrrole ring, at 1335 cm^{-1},

shifted by 15 cm^{-1} compared to the spectrum obtained for the pure polymer film. In addition, the vibration band at 1575 cm^{-1}, initially observed in the enzyme-free film, was not observed in the spectrum of the polymer matrix immobilizing the enzyme. These spectral changes were associated with the presence of covalent bonds of the enzyme with the polymer, involving carboxylic groups, to prove the immobilization.

Liakos et al. [138] successfully immobilized cinnamon (CN), lemongrass (LG), and peppermint (PM) essential oils on electrospun cellulose acetate (CA) polymeric nanofibers for application as antimicrobial wound dressings. The authors investigated the encapsulation of bioactive molecules in the electrospun membranes using Raman spectroscopy. For each of the three blends (CA/CN, CA/GR, and CA/PM) produced, the characteristic vibration bands of the main substances contained in the essential oils, responsible for their respective antimicrobial activities, were detected in the Raman spectra. Then, in vitro bacterial growth tests confirmed the results obtained in the Raman analysis. In summary, the study concluded that wound dressings with immobilized essential oils are effective in inhibiting the growth of *E. coli* bacteria, even when using low concentrations of these biosystems. The work of Cai et al. [139] studied the immobilization efficiency of the model protein bovine serum albumin (BSA) in cysteamine-modified polyaniline (PANI) films for biosensing applications. The changes in the spectrum of the functional composite in relation to the pristine polymer film were associated with the immobilization of BSA mainly by covalent bonds, in addition to physical adsorption. The authors concluded that the BSA immobilized in the modified PANI film is characterized as a potential material for the production of more sensitive biosensors.

7.1.4. Electronic Microscopy (EM) and EDX

Alterations in the size, morphology, chemical composition, and dispersion of the support can be observed when samples are analyzed by scanning electron microscopy (SEM) and/or transmission electron microscopy (TEM). Additionally, these microscopic methods can be performed with energy-dispersive X-ray analysis (EDX) to obtain an approximation of the chemical composition of the sample. When the support contains an immobilized enzyme, for example, it is very common to observe sulfur and/or nitrogen peaks if the bare matrix does not contain these elements.

Ulu et al. [52] worked with immobilized l-asparaginase (l-ASNase) on biodegradable copolymers of starch and poly(methacrylic acid-co-methyl methacrylate) P(MAA-co-MMA). When the sample was analyzed via SEM before the immobilization process, the matrix had a uniform, homogeneous, and crack-free surface, and incorporated starch granules were observed. However, after immobilization, the morphology of the surface of the enzymatic derivative changed from smooth to rough due to the roughness generated by the deposition of the enzyme in the composite, supporting the successful adhesion of l-ASNase to the support. The authors, through the detection of elemental concentrations of carbon (C), oxygen (O), nitrogen (N), and sulfur (S) with EDX, were able to confirm the fixation of the biopolymer on the P(MAA-co-MMA) matrix and the presence of l-ASNase after immobilization. The concentrations of carbon and oxygen present in the P(MAA-co-MMA) support significantly increased after starch functionalization. To prove the presence of the enzyme, a slight increase in the amount of N and S was observed only after the immobilization of the biomolecule. Furthermore, the results showed that the enzymes in the composites functionalized with starch exhibited a homogeneous distribution, which was affected by the distribution of functional groups (NH_2 and SH) of l-ASNase; moreover, the composite had particles of starch distributed evenly [52].

Saxena et al. encapsulated a protein model (urease) on nanoparticles reticulated with Ca^{2+} ions and compared the SEM analyses before and after immobilization; the authors claimed that the particle sizes were not greatly affected by enzyme encapsulation [49].

Magnetic nanoparticles (Fe_3O_4) and Fe_3O_4 coated with silica (Fe_3O_4@SiO_2-NH_2) with the immobilized trypsin enzyme (MNPs-Trypsin) obtained by Aslani et al. [133] were compared to verify the changes in morphology. The results showed that Fe_3O_4 was almost spherical in shape and had an average diameter of 35 nm, while the trypsin-PNMs presented larger particle sizes and seemed to aggregate due to the presence of the enzyme on the surface of Fe_3O_4@SiO_2-NH_2. Using the technique described above, the authors verified the elemental composition via EDX microanalysis and confirmed the presence of Fe, Si, N, and O in the MNP-trypsin sample [133].

Yu et al. [93] immobilized papain in a magnetic Fe_3O_4/P(GMA-EDGMA-St) composite polymer carrier and observed that the magnetically immobilized papain showed good spheroidization and adhesion, indicating that the enzyme was immobilized in the support. Electronic micrography via TEM confirmed the excellent dispensability of papain immobilized in the support [93].

Jiang et al. [94] used TEM to characterize the morphology of the matrix before the immobilization process in a sample of magnetite (Fe_3O_4) carboxymethyl chitosan/calcium alginate (MCCSA) and after cellulase immobilization (MCCCB). The results showed that there was no change in the spherical or ellipsoidal structure of either. Moreover, the intrinsic structure remained unchanged, which was confirmed by the observation of no appreciable aggregation, in addition to a relatively uniform conjugation [94].

TEM images of ionic liquid-modified magnetic carboxymethyl cellulose nanoparticles (IL-MCMC) showed that the size distribution was uniform and dispersed. These results led Suo et al. [124] to suggest that immobilized enzymes can also increase mass transfer due to improved dispersion and increased activity.

7.1.5. X-Ray Diffraction

Changes in the crystalline structure of the material can be observed by X-ray diffraction after the immobilization process since the biomolecule is not crystalline (amorphous); therefore, the diffractogram of the immobilized derivative can present wider peaks, as we can see in the works presented below.

Sojitra et al. [136] immobilized pectinase on magnetic nanoparticles (Fe_3O_4) modified with chitosan (CMNPs). The magnetically immobilized derivative was analyzed, and the diffraction profile showed peaks at 2θ = 30.31°, 35.67°, 41.18°, 57.24°, and 62.84°, corresponding to the peaks obtained for Fe_3O_4, indicating that there was no phase change associated with the immobilization of the enzyme. In addition, the authors calculated the average diameter of the immobilized pectinase on CMNPs to be 38.4 nm using the Debye–Scherrer equation [136].

Like previous results, Moradi et al. [92], when analyzing samples of bare nanoparticle (Fe_3O_4) MNPs, after amino-tannic acid modification (ATA-Fe_3O_4 MNPs), and after immobilization with β-glucosidase (BGL), observed that neither the coating nor the immobilized BGL altered the inverse spinel structure of Fe_3O_4, in addition to demonstrating high purity of the crystal structure [92].

In the same way as previous authors, Oliveira et al. [135] worked with magnetic Fe_3O_4 MNPs coated with chitosan (CS) and immobilized a commercial enzymatic preparation (Pectinex Ultra SP-L). The authors observed that the Fe_3O_4 XRD patterns after interacting with CS (Fe_3O_4-CS-MNPs) and after preparation with the enzyme (Pectinex immobilized in Fe_3O_4-CS-MNPs) showed no detectable changes from those of pure MNPs.

Yu et al. [93] immobilized papain on a magnetic Fe_3O_4/P(GMA-EDGMA-St) composite polymer carrier, analyzed the results of X-ray diffraction and compared the results with those of Fe_3O_4 and Fe_3O_4/P(GMA-EDGMA-St) samples. In all the samples, the position and relative intensity of the diffraction peaks were consistent with those of the standard Fe_3O_4 spectrum, indicating that even after the immobilization process, as well as the work already presented above, the crystal phase remained intact and that the prepared samples had high purity and crystallinity [93].

7.1.6. Zeta Potential (ZP)

Through this characterization method, it is possible to determine the isoelectric point (pI), verify the electrophoretic mobility of the particles, study the surface charges, analyze the stability of the colloidal system, and quantify the electrostatic interactions of the immobilized derivative. Additionally, the isoelectric point (pI) can be used to indicate the degree of repulsion between adjacent particles and similar particles in the dispersion, suggesting the possibility of measuring the existence of aggregation in the suspension.

This technique plays a relevant role in immobilization processes through the correlation between the zeta potential of the biomolecule and the matrix particle and their binding affinities and can be used as a diagnostic tool to predict the coupling of the biological entity to the matrices [132].

For example, the zeta potentials of poly(acrylamide phenylboronic acid)/sodium alginate (PAAPBA/SA) nanoparticles, insulin-loaded NPs, and insulin/GOx-loaded NPs were investigated by Chai et al. [137], and the values were −28.5 mV, −31.2 mV and −35.7 mV, respectively. The difference in PAAPBA/SA was a consequence of the charged phenylboronic acid in PAAPBA and deprotonated carboxylic acid in SA. After insulin encapsulation, the zeta potential of the NPs decreased; however, the most negative surface charge was attributed to the insulin/GOx-loaded NPs due to the negative charges of GOx (pI 4.9) and insulin (pI 5.3) in neutral water [137].

The magnetite (Fe_3O_4) carboxymethyl chitosan/calcium alginate-immobilized cellulase bioconjugate (MCCCB) was obtained by Jiang et al. [94]. The magnetite and nano-Fe_3O_4 samples, after reacting with chitosan carboxymethyl (CCTS) and sodium alginate (SA) (MCCSA), were subjected to zeta potential analysis to confirm the interactions between them. The zeta potential values of Fe_3O_4, MCCSA, and MCCCB were 2.53, −38.04 and −16.52 mV, respectively. The results of MCCSA were attributed to the presence of many –COOH groups on its surface, which have a negative charge under neutral conditions, and the decrease in the zeta potential of MCCCB was due to the significant reduction in –COOH, which was caused by the reaction between cellulase and Ca^{2+}. Furthermore, the successful reaction of magnetite with CCTS or SA and the presence of cellulase in the support were proven by the average increase in the hydrodynamic diameter from 7.62 nm to 168.78 nm through the technique of dynamic light spreading, which will be better discussed below [94].

7.1.7. Dynamic Light Scattering (DLS)

Another way to characterize the support used in the immobilization process is through analysis of the particle size via the DLS technique. This is possible because the size of the biomolecule changes, and thus, it is clear whether the biomolecule was immobilized on the polymeric matrix. Next, we report studies that demonstrate how it is possible to prove that the biomolecule is immobilized on the support using the DLS technique.

In the study, Chai et al. [137] developed an improved glucose-mediated insulin delivery system loaded with glucose oxidase (GOx) immobilized on poly(acrylamido phenylboronic acid)/sodium alginate nanoparticles (NPs). After the immobilization of GOx in

insulin-loaded NPs, the sample was analyzed by DLS, and an increase in size from 190.4 to 201.8 nm was observed, indicating that this increase in particle size was a result of encapsulation of the enzyme. In addition, the insulin/GOx-loaded NPs exhibited good dispersion (polydispersity index—PDI > 0.2) [137].

Like Chai et al., Moradi et al. [92], through sample DLS curves, magnetic nanoparticles (Fe_3O_4 MNPs), after modification with amino-tannic acid (ATA-Fe_3O_4 MNPs), and with the enzyme β-glucosidase (BGL) immobilized (BGL-ATA-Fe_3O_4 MNPs), observed particle diameters of 28.2, 134.1 and 153.7 nm, respectively. These increases in nanoparticle size are attributed to the coating and loading of the BGL [92].

The last study to be cited was carried out by Yu et al., in which the authors analyzed the particle size of Fe_3O_4/P(GMA-EDGMA-St) before and after papain immobilization and found average particle diameters of 161 and 196 nm, respectively. On the basis of these results, the authors were able to confirm the immobilization of the enzyme on the support due to the increase in particle size resulting from the presence of papain [93].

7.1.8. Atomic Force Microscopy (AFM)

With atomic force microscopy, it is possible to analyze the surface of the material used to immobilize the biomolecule and thus identify whether there are differences in the relief at the sample's atomic level. Below are two examples of works in which the authors use AFM and identify changes in the material surface that indicate that the biomolecule was immobilized on the prepared support.

First, we conducted the research by Ulu et al., in which the authors obtained AFM topography images before the starch (MAA-co-MMA) composites were doped, after the P(MAA-co-MMA)-starch was functionalized and after the l-asparaginase (l-ASNase) was immobilized; we observed that the P(MAA-co-MMA) copolymer surface was smooth; however, after starch fixation, it was possible to observe carbohydrate granules. For the sample with the enzyme immobilized, the surface of the support showed an increased roughness, confirming the presence of l-ASNase [52].

Another study by Wu et al. used nanoparticles of chitosan (CS-NPs) with immobilized lysozyme (CS-Lys-NPs). As shown in the AFM images of chitosan (CS), CS-NPs, and CS-Lys-NPs, a circular shape was observed in CS; however, the CS-NPs were apparently smaller in size and corresponded to small dots. However, due to the integration of lysozyme into the CS-NPs, the CS-Lys-NPs were larger at both pH 4 and pH 5. Moreover, the authors concluded that the distribution of nanoparticles per unit of volume was different and that the protonation of the amino groups of CS can be affected by pH [104].

7.1.9. Visible Ultraviolet Spectroscopy (UV–Vis)

In addition to the techniques mentioned above, UV–Vis spectroscopy was used to determine whether the biomolecule was indeed immobilized on the polymeric support, possibly through the presence of characteristic biomolecule peaks that appear when reading the immobilized derivative. For this reason, Wu et al. used UV–Vis spectroscopy on samples of lysozyme, chitosan nanoparticles (CS-NPs), and enzymes (lysozyme) that interact with the CS-Lys-NP support to determine the interaction between the CS-NPs and lysozyme. By analyzing the results, it was only possible to observe peak absorption at 280 nm in the lysozyme, and CS-Lys-NP samples since the absorbance at this wavelength indicates the presence of protein in the samples. The enzyme in the presence of CS-NPs showed a slight redshift of approximately 1 nm and an increase in optical density, demonstrating a higher extinction coefficient than that of free lysozyme. Thus, the authors were able to prove, through UV–Vis spectroscopy, the presence of immobilized lysozyme and the efficiency of the method they developed [104].

7.2. Other Techniques

7.2.1. Small-Angle X-Ray Scattering (SAXS)

With the purpose of exploring the nanostructure of matter through the detection of X-rays scattered throughout the sample at very low angles, Wu et al. studied the potential interactions and conformations between chitosan nanoparticles (CS-NPs) without and with immobilized lysozyme (CS-Lys-NPs) using SAXS. The results showed two typical peaks indicating semicrystalline structures of 45–70 nm and 52–70 nm according to Bragg's law, $d = 2\pi/q$, where d is the real-space distance and q is a vector. This increase in the scattering intensity I(q) reflected the growth of the CS-Lys-NPs compared to that of the CS-NPs, which can influence the compression caused by lamellar "buckling" due to the interaction between lysozyme and the CS-NPs via the N–H and O–H groups [104].

7.2.2. Thermogravimetric Analysis (TGA)

Although TGA is a strategy often used with polymeric composites, especially organic components, it is not a widely used method for the identification of biomolecules, but the application of this procedure could provide information about the presence of organic matter, confirming the immobilization process.

The use of this characterization method to analyze the transformations of the physical and chemical properties of the samples makes it possible to verify the changes in the phase, dehydration, decomposition behavior, and thermal stability of the materials with increasing temperature. When the immobilized derivative is analyzed, it is possible to observe the degradation of the biomolecule when the temperature increases through loss of mass with the evaporation of these components [140].

Although Aslani et al. did not provide detailed methodological information, they worked with the enzyme (trypsin) immobilized on silica-coated Fe_3O_4 magnetic nanoparticles (Fe_3O_4@SiO_2-NH_2) and used this analysis to determine the content of functional groups and bond formation between the trypsin and the matrix [133].

Before presenting the next characterization technique, which is applied to magnetic materials, we would like to register the importance of thermogravimetric analysis, especially when used in combination with other methods, such as vibrating sample magnetometer (VSM). To evaluate the organic (non-magnetic) part of the material, VSM measures only the magnetization of the magnetic part of the matrix.

7.2.3. Vibrating Sample Magnetometer (VSM)

Magnetic nanoparticles (Fe_3O_4) modified with amino-tannic acid (ATA-Fe_3O_4 MNPs) were used to immobilize the enzyme β-glucosidase (BGL) and to study its magnetic properties [92]. By analyzing the hysteresis loops of the Fe_3O_4 MNPs, after coating the nanoparticles (ATA-Fe_3O_4 MNPs) and with the immobilized enzyme (BGL-ATA-Fe_3O_4 MNPs), the authors could conclude that the saturation magnetization was 36.46, 24.49 and 24.03 emu g^{-1}, respectively. This reduction in saturation magnetization was due to the increase in size and weight of the nanoparticles generated by the coating and immobilization of the enzyme. Another piece of information obtained from the magnetic saturation curves was the remanence (the magnetization remaining in zero field after the application of a large magnetic field), which was determined to be 0.057, 0.0076, and 0.123 emu g^{-1}. Furthermore, the coercive force (the intensity of the applied magnetic field required to reduce the magnetization of that material to zero after the magnetization of the sample) was brought to saturation, approximately 50 Oe for all the samples. Considering the results of coercion and remanence, the authors were able to conclude that the samples exhibit a behavior close to superparamagnetism and a capacity for redispersion after removal of the magnetic field because both results were close to zero for all the samples [92].

Another example relates to the magnetic property analysis of a composite Fe_3O_4/P(GMA-EDGMA-St) used to immobilize papain [93]. The samples studied were Fe_3O_4, oleic acid-modified Fe_3O_4, a magnetic Fe_3O_4/P(GMA-EDGMA-St) composite polymer carrier, and magnetically immobilized papain, which presented saturation magnetization values of 68.70, 57.80, 42.10 and 37.60 emu g^{-1}, respectively. The success of the modifications to which each sample was subjected was confirmed by the decrease in the magnetization. In addition, it was suggested that no agglomeration occurred because no hysteresis occurred after the external magnetic field was canceled. These results allow the authors to state that the reuse of the immobilized derivative is advantageous. Finally, it was possible to easily recover the enzymatic derivative using a magnetic field because all the samples showed superparamagnetism and not coercive force [93].

8. Biosystem Immobilization Technology

Monodisperse polymeric particles have been established as excellent matrices for biosystem immobilization due to their biocompatibility, flexibility, biodegradability, stability, low toxicity, and structural versatility. In view of these characteristics, an increasing number of different biomolecules have been immobilized and applied in various fields. Therefore, this Section presents some related research involving the use of these polymeric particles with immobilized biosystems and their possible uses in health, environmental, food, pharmaceutical, and other biotechnology applications (Figure 6).

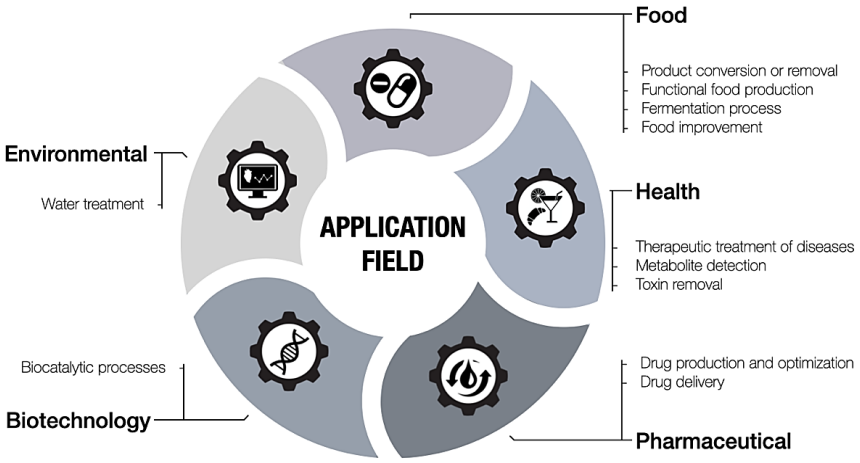

Figure 6. The main application fields of immobilized biosystems on monodisperse polymeric particles and their possible uses in different areas.

Despite the advancements in immobilization technologies and their demonstrated benefits across diverse fields, significant challenges remain. Common limitations include the high cost of production for advanced support materials, scalability issues for industrial applications, and loss of enzyme activity during repeated use. Additionally, ensuring the compatibility of immobilized biosystems with complex systems and addressing regulatory concerns, particularly in pharmaceutical and food industries, are critical barriers. These challenges underscore the need for continued research to optimize support materials, reduce costs, and enhance the operational stability of immobilized biosystems.

8.1. Applications in Health

To present the different possibilities for medical and/or biomedical applications, some examples of biomolecules immobilized on polymeric particles are described.

Ulu et al. investigated the immobilization of the L-asparaginase enzyme (L-ASNase), an efficient antineoplastic agent, on a biocompatible and biodegradable matrix (P(MAA-

co-MMA)-starch). The thermal and pH stability of the immobilized derivative improved, and 60% of the initial enzymatic activity was maintained after 30 days of storage at 25 °C, indicating that the derivative was not toxic. Therefore, the biocomposite material produced becomes a support alternative for immobilizing enzymes that are useful in health applications because of its biocompatibility and biodegradability [52].

Another medical application was reported by Chai et al. [140], who prepared poly(acrylamido phenylboronic acid)/sodium alginate nanoparticles (NPs) via the formation of cycloborates to provide glucose and H_2O_2 responses to NPs. In addition, NPs with dual responsiveness were used to improve glucose-mediated insulin delivery systems loaded with glucose oxidase (GOx). The authors showed a faster release of glucose-responsive insulin with insulin/GOx-loaded NPs than with insulin-loaded NPs. Moreover, through cytotoxicity assays, hemolysis studies, and histopathological examinations, the NPs were shown to exhibit good biocompatibility, associated with the advantages of simple preparation and improved glucose responsiveness, suggesting that these NPs are attractive for subcutaneous insulin delivery. In this way, the immobilized derivative displayed clinical potential and could be applied in the delivery of other therapeutics to treat other health conditions [141].

Li et al. prepared a material with the potential to be used as a support for immobilizing proteins that can be used in magnetic resonance imaging, treatment of hyperthermia by magnetic fluids, and other applications in the biomedical field [110]. The material developed was a fluorescent magnetic nanoparticle (MFNP) with carbon points (CDs), chitosan (CS), and carboxymethylcellulose (CMC). The authors chose bovine serum albumin (BSA) as a protein for immobilization since it has some ability to transport and load fatty acids, porphyrins, bilirubin, and cholesterol. The tests carried out in the present study showed that after the immobilization process, the MFNPs could be reused up to five times while maintaining their properties, which indicated that the material could be tested in industry [110].

The preparation of polymer-magnetic nanoparticles for health applications was described by Zhang et al. [115]. The poly(carboxybetaine acrylamide) (pCBAA) polymer was grafted onto iron oxide (Fe_3O_4) and used as a matrix to immobilize urease. This enzyme was chosen because it has several applications, for instance, in biosensors. The researchers tested the efficiency of the immobilized derivative in detecting urea in blood serum. After immobilization, the enzyme was more resistant (80% of the initial enzyme activity was maintained after five cycles), biocompatible, and more efficient than the free enzyme [115].

Immobilized biomolecules offer significant potential in medical applications, enhancing stability, biocompatibility, and reusability. Examples include L-asparaginase for cancer therapy, glucose oxidase for insulin delivery, and magnetic nanoparticles for imaging and biosensors. These innovations underscore their versatility in addressing health challenges.

8.2. Environmental Applications

In regard to the use of immobilized biosystems in environmental applications, enzymes have received special attention, especially if used for water treatment. Industry-related dyes present in wastewater can negatively affect human health, and their removal is one of the main challenges of nanotechnology. Faced with the relevance of this theme, two examples of enzymes immobilized in magnetic nanoparticles for this purpose are presented.

The first application to be cited is the work of Iriarte-Mesa et al., in which the authors used superparamagnetic iron oxide nanoparticles modified with polyacrylic acid and gallic acid to immobilize the laccase enzyme and applied it to dye degradation (the monoazo dye calmagite (Cg) and the diazo dye Congo Red (CR)). The authors observed that after the immobilization process, excellent enzymatic activity was achieved, and the nanoparticles

exhibited greater reactivity when degrading azo dyes than when degrading free enzymes. The Cg dye had 99% degradation with the immobilized laccase after 18 h of reaction, while the free laccase degraded by only 78% after the same period, which showed the efficiency of the enzyme immobilization process and its potential for application in water treatment [89].

The second work is similar to the previous work, where Donadelli et al. used modified magnetic nanoparticles (MNPs) with tetraethyl orthosilicate (TEOS) and 3-aminopropylthioxysilane (APTES) with immobilized soybean peroxidase (SBP) for the removal of malachite green (MG) from aqueous solution. The researchers tested different amounts of TEOS and the particles that obtained good efficiency in the removal of MG via NPTAI-SBP (2.0 mL of TEOS) and NPTAII-SBP (5.5 mL of TEOS) (88% and 77%, respectively, in 4 h), while NPA-SBP (0.0 mL) achieved only 43% removal under the same conditions. The authors also tested the reuse of NPTAI-SBP and NPTAII-SBP and realized that both can be reused for up to four cycles without a significant loss of initial activity. Among the two materials tested, NPTAII-SBP, which retained 42% of its initial activity after ten cycles, exhibited the best retention, while NPTAI-SBP retained only 3% of its initial activity after 10 cycles. Therefore, magnetic nanoparticles tested by the authors have the potential to be used in effluent treatment [134].

Immobilized enzymes are highly effective in water treatment, improving dye degradation and reusability. Examples include laccase for azo dyes and soybean peroxidase for malachite green, both outperforming free enzymes. These innovations underscore their potential for scalable and sustainable wastewater remediation.

8.3. Applications in the Food Industry

As we have already shown in this review, immobilization favors the use of biomolecules in industrial processes, which is why it is possible to test this method in several situations. In the food industry, enzymes are often used to improve food quality, the synthesis of certain compounds, and other functions. Below, we highlight the use of immobilized enzymes in monodisperse polymeric particles that aim to prolong their useful life and reduce operational costs.

As a first example, β-glucosidase (BGL) is of great interest due to its ability to produce functional food products via food conversion and its ability to improve its use. In the study of Moradi et al., BGL was immobilized in magnetic nanoparticles of Fe_3O_4 (MNPs), which greatly facilitated the process of removing the enzyme from the reaction medium with a magnet. A negative point of Fe_3O_4 MNPs is that they do not contain sufficient reactive hydroxyl groups, making it difficult to bond iron oxide nanoparticles to the enzyme. Therefore, the surface of Fe_3O_4 MNPs has been modified to provide functional groups for enzyme bonding. Researchers have used tannic acid (TA) and incorporated functional amine groups into their structure to allow the creation of covalent bonds. When performing the reuse tests, the researchers noted that after 10 cycles, the immobilized BGL maintained 83% of the initial activity, demonstrating the efficiency of the immobilization process [92].

In another study, we mentioned the use of a bioconjugate of magnetite carboxymethyl chitosan/calcium alginate-cellulase (MCCCB), which was synthesized to improve the activity of cellulase enzymes. Jiang et al. were able to increase the catalytic activity of the enzyme by 267.18% by applying the immobilized enzyme to the hydrolysis of corn stems. The researchers obtained an increase in the yield of fermentable sugars of 698.26% after the immobilization process, thus indicating the efficiency of the material applied. It was still possible to see in the results that the material had good dispersion, stability, easy removal, and a small particle size [94].

In the research conducted by Waifalkar et al., the authors applied magnetic nanoparticles coated with chitosan to immobilize the enzyme invertase and produce inverted sugar.

The immobilized enzymes were able to be reused 20 times without significant loss of enzyme activity, so the enzyme invertase was immobilized as mentioned above and has great potential for use in the industrial production of inverted sugars from sucrose [54].

The enzyme papain can be applied to inactivate rice bran lipase, which causes rancidity in bran and a loss of nutritional value. Yu et al. used immobilized papain in magnetic nanoparticles modified with glycidylmethacrylate polymers (GMA), ethylene glycol dimethacrylate ester (EGDMA) and styrene (St) and observed that it was possible to reuse the immobilized derivative for eight cycles, and the relative activity of the immobilized papain remained above 72%, which is an important point for its application at the industrial scale. Improvements were also noted in relation to pH and temperature stability, and the inactivation of lipase in rice bran was achieved [93].

Oliveira et al. [135] analyzed the reusability of Pectinex immobilized in magnetic nanoparticles of Fe_3O_4-chitosan for the production of fructo-oligosaccharides (FOSs). After six reuse cycles, the immobilized biocatalyst retained 70 and 86% of the residual hydrolytic and transfructosylation activities, respectively, which indicates that the use of this derivative improves the enzymatic activity and thus reduces the operational costs of the synthesis of fructo-oligosaccharides (FOSs). When applied in the synthesis of FOS, the maximum concentration obtained in the laboratory-scale experiments was 101.56 g/L, indicating that the immobilization process provides benefits for the production of FOS by prolonging the life of the enzyme Pectinex [135].

Immobilized enzymes improve efficiency, reusability, and cost-effectiveness in food processing. Notable examples include β-glucosidase for functional foods, cellulase for fermentable sugars, and invertase for inverted sugar production. Additionally, applications such as papain for rice bran stabilization and Pectinex for fructo-oligosaccharides highlight their role in enhancing enzyme stability and reducing operational costs, driving innovation across the food industry.

8.4. Pharmaceutical Applications

The penicillin G acylase (PGA) enzyme is commonly used in industry for the production of antibiotics, but like other enzymes, its enzyme activity is limited when it is used in industrial processes; thus, its performance needs to be improved, with immobilization being an alternative. Below, we will mention two works involving the immobilization of PGA, the results of which are promising for industrial application.

In the first study, Suo et al. immobilized PGA in ionic liquid-modified magnetic carboxymethyl cellulose (IL-MCMC) nanoparticles. After the immobilization process, the enzyme proved to be more stable, and it was possible to reuse it for 10 cycles, with retention of enzymatic activity up to 92.6% [124].

Another study conducted by Yu et al. covalently immobilized PGA in an amino-functionalized magnetic $Ni_{0.5}Zn_{0.5}Fe_2O_4@SiO_2$-$NH_2$ nanocomposite loaded on graphene oxide (GO) also showed good results. The relative activity of immobilized PGA remained > 70% after nine successive cycles [116].

Immobilization enhances the stability and reusability of penicillin G acylase (PGA), which is crucial in antibiotic production. Techniques using magnetic nanoparticles and graphene oxide-based nanocomposites retained over 70% activity after multiple cycles, emphasizing their role in improving pharmaceutical manufacturing efficiency.

8.5. Other Applications

The works reported here have shown that enzymes are the most immobilized biosystems for use in biocatalysis applications. Enzymatic immobilization is a very attractive alternative and has proven promising because it reduces operational costs, is more sustain-

able, allows reuse, and promotes increased catalytic activity and stability. In view of the above, we present two examples of immobilized biocatalysts used in other biotechnological applications.

In the first case, we performed a study by Han et al. [142], in which the magnetic composite nanomaterial GO@Fe_3O_4@4arm PEG NH_2 was successfully synthesized for cellulase immobilization with high loading capacity and operational stability. Compared with free cellulase, immobilized cellulase also demonstrated excellent durability, thermostability, and reusability, and thus, it has attractive potential in industrial applications. Cellulase immobilized with GO@Fe_3O_4@10K 4armPEG NH_2 maintained 45% of its initial activity after seven cycles. The support used in this work proved to be efficient, and the authors suggested tests for the immobilization of other enzymes [142].

Finally, another example of the enzyme L-asparaginase (L-ASNase) immobilized on magnetic particles functionalized with maltose (Fe_3O_4@Au-MS), as well as others mentioned here, can be applied in several areas. In the study by Tarhan et al. [86], the immobilized derivative showed better acid-base tolerance and thermal stability than did the free enzyme. As mentioned above, reusability is essential for the economical use of the enzyme in biotechnological applications, and after 13 cycles, the immobilized L-ASNase retained 50% of its initial activity. Furthermore, the immobilized enzyme showed 64.0% residual activity even after 28 days of storage, which emphasizes its good operational stability, durability, and feasibility for application on an industrial scale [86].

Immobilized enzymes enhance stability, reusability, and efficiency in biotechnology. Examples include cellulase on GO@Fe_3O_4 composites with improved durability and L-asparaginase on magnetic particles with superior stability. These innovations demonstrate their potential to optimize processes and reduce costs in industrial applications.

9. Conclusions

This review described the main advances in polymeric materials used as supports for immobilizing biosystems due to their interesting physical and chemical properties. In addition, polymeric particles were presented in terms of their main features, classifications, hierarchical structures, and interface factors between the matrix and entity. This study also displayed the importance of aligning polymer selection with specific application requirements, ensuring optimal performance and functionality. Semisynthetic polymers that offer tailored properties through controlled modifications become strong candidates as matrices for biosystem immobilization. The topics covered in this review demonstrated the strong repercussions of immobilized biosystems on polymeric matrices in several areas. And considering that enzymes were the major biological entities immobilized on polymeric matrices, this review presented some of their applications in different areas. Biological, biochemical, chemical, and physical concepts were referred to in this material to emphasize the multidisciplinary nature of the immobilized derivative preparation on polymeric matrices. For these reasons, the authors desire that this material will be valuable to scientists and businessmen who work with soft matter and biological sciences.

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