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Review

Natural polymers: suitable carriers for enzyme immobilization

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Summary. Enzyme immobilization onto support carriers has the potential to overcome some of the limitations of soluble enzymes in practical applications. Various materials have been used as carriers, such as inorganic matrices, as well as natural and synthetic polymers. Production of carriers from natural biopolymers and their derivatives has been the focus of research worldwide, and a summary of their applications for enzyme immobilization is presented in this paper. Enzymes, or cells as an enzyme source, are entrapped inside a three-dimensional polymeric network, called a hydrogel, that is able to retain large amounts of water. This network can be formed by chemical cross-linking, ionotropic gelling in the presence of cation, or in thermo reverse polymerization, depending on the polymer in use and its physico-chemical characteristics. The most frequently used biopolymers as carriers for immobilization include alginate, cellulose, chitosan, collagen, xylan, pectin, and others.

Keywords: alginate, biopolymer, carrier, enzyme immobilization, hydrogel.

INTRODUCTION

Enzymes are macromolecular biocatalysts that conduct and accelerate chemical reactions in biological systems (Liu and Chen 2016). Due to their characteristics, ease of production, high activity, unique selectivity, substrate specificity, mild reaction conditions and low energy consumption, enzymes can be catagorised as green chemistry with low environmental impact, and are biocatalysts that are widely used in diverse fields (Ahmad and Sardar 2015; Zdarta et al. 2018). The ability to catalyze complex chemical processes under mild conditions is exploited both for fundamental research and industrial applications (e.g. the food industry, pharmaceutical industry and clinical analysis) (Guisan 2006). Furthermore, enzymes are water-soluble, biodegradable and are becoming increasingly more affordable with the development of biotechnology and production of recombinant proteins (Liu and Chen 2016).

Regardless of these benefits, broader industrial applications have been constrained by specific limitations of soluble enzymes, such as instability, difficult and tedious recovery procedures and the inability to reuse enzymes, all of which results in increased costs. Since reusability in industrial applications is required, separation of products from reaction mixtures and re-purification of enzymes is expensive and may lead to contamination and reduction of catalytic activity (Liu and Chen 2016). In order to overcome these limitations, enzymes can be immobilized on different carriers (Homaei et al. 2013). According to Tosa et al. (1966), immobilized enzymes are defined as "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeat-

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edly and continuously". Enzyme immobilization provides increased stability and resistance to non-physiological conditions, such as increased temperature, extreme pH or the presence of organic solvents, as well as easy recovery from reaction media and reusability (Tumturk et al. 2008). Separation of enzymes from reaction products allows process control and reusability, which is required for industrial treatments and also prevents contamination of the products with other proteins (Mohamad et al. 2015). Cost reduction of both the enzyme and obtained reaction products is significant, and is a crucial factor for catalytic processes and applications (Mohamad et al. 2015).

The performance of an immobilized enzyme system depends on the characteristics of the support material and its interaction with the enzyme. An ideal carrier for enzyme immobilization should be stable, resistant to mechanical forces, inert, affordable and biocompatible (Liu and Chen 2016). All carriers used for enzyme immobilization can be generally divided into three groups (Table 1): inorganic matrices, synthetic and natural polymers (Liu and Chen 2016).

The present review will focus on natural and chemically modified polymers. Natural biopolymers that have been used as carriers for enzyme immobilization include alginate, cellulose, chitosan, collagen, xylan, pectin, starch, carrageenan and agarose (Ahmad and Sardar 2015). The gelling mechanism and properties of the resulting gel vary depending on the characteristics of the polymer used and available functional groups. Ionotropic gelation in the presence of specific divalent cations is the preferred immobilization mechanism for natural polymers like alginate and carrageenan. On the other hand, for polymers such as agar, agarose and gelatine, enzyme entrapment is achieved through thermo-reverse polymerization (Datta et al. 2012; Ahmad and Sardar 2015). Some disadvantages of enzyme immobilization include enzyme leakage through large pores in the polymeric network, diffusion limitations of substrates and products due to small pore sizes, or enzyme inactivation during polymerization (Bezerra et al. 2015).

BIOPOLYMERS

A major advantage of natural polymers and their derivatives in biomedical applications, as well as enzyme or cell immobilization, is their non-toxicity, biocompatibility, and biodegradability: which results in minimal environmental contamination and chemical stabilization (Ogushi et al. 2007; Liu and Chen 2016). In aqueous solution, these biopolymers are capable of forming inert and stable gels of various shapes and sizes, even at low concentrations (Homaei et al. 2013). Biopolymers are easily obtained from natural sources and are considered renewable biomaterials. The most commonly used biopolymers for enzyme immobilization are polysaccharides and their derivatives (e.g. oxidized, aminated, esterified, acylated, hydroxylated, carboxylated, alkylated or acetylated).

HYDROGELS

Hydrogels are insoluble, highly hydrated, three-dimensional networks composed of natural or synthetic hydrophilic polymer chains (Drury and Mooney 2003; Caló and Khutoryanskiy 2015). This polymeric network is capable of absorbing and retaining water from 10% to thousands of times its dry weight (Hoffman 2012). Due to their characteristics, hydrogels have been widely used in a variety of biomedical and biotechnological fields, including: drug delivery, tissue engineering and both enzyme or cell immobilization. Although diverse natural polymers have been used to prepare hydrogels, more favourable methods for immobilization of enzymes are based on physical restriction, encapsulation or entrapment in polymeric networks (Tumturk et al. 2008; Rother and Nidetzky 2014). Cross-linking of polymers and the formation of a three-dimensional network can be achieved through covalent chemical cross-linking, hydrogen bonding, van der Waals interactions or physical entanglements (Qiu and Park 2012).

Table 1. Main characteristics of the three types of support materials and selected examples.

Inorganic materials	temperature, pH and mechanical stability, operational stability, inertness, good absorption properties, uncomplicated synthesis, inexpensive, large specific surface area with possible functionalization
	silica, inorganic oxides, minerals, carbon-based materials
Synthetic polymers	different functional groups available, high purity, synthesized from selected monomers, designed structure
	polyvinyl alcohol (PVA), ion exchange resins, polyacrylamide, polyamide, polystyrene
Natural polymers	biocompatibility, abundantly available in nature, presence of numerous reactive functional groups, biodegradability and minimal environmental contamination, nontoxicity, high affinity for proteins
	alginate, cellulose, chitosan, collagen, xylan, pectin, starch, carrageenan, agarose, dextrans

ALGINATE

Alginates are naturally obtained polysaccharides and the most frequently used biopolymers for enzyme immobilization via hydrogel entrapment, due to their mild gelling properties and nontoxicity (Rowley et al. 1999; Tumturk et al. 2008). Alginate is an unbranched polysaccharide that is isolated from the cell wall of brown algae, and contains a backbone composed of (1-4)-linked β-D-mannuronic acid and α-L-guluronic acid monomers that produce M units and G units, respectively (Rowley et al. 1999; Datta et al. 2012). These monomers are arranged in regions composed of consecutively alined M or G units forming homopolymeric M-blocks and G-blocks, or are randomly organized in heteropolymeric structures called MG blocks (Drury and Mooney 2003). Depending on the origin of alginate, the M and G content and sequential distribution varies along the chain (Rowley et al. 1999).

Ionotropic alginate gelation in aqueous solution occurs in the presence of divalent cations (eg. Ca^{2+}, Ba^{2+} or Sr^{2+}). Contiguous alginate chains cooperatively bind Ca^{2+} ions between their G-blocks. Interactions of polyanions with cations through ionic bonds results in the gelling of alginate solutions and creation of ionic interchain bridges (Rowley et al. 1999). The so-called "egg-box" model is used to describe complex formation between alginate and divalent ions, where each ion is surrounded and interacts with two G-residues from one chain and two G-residues from the opposing polymer chain (Fig. 1) (Kristiansen et al. 2009).

Resulting hydrogels and their properties depend on the gelling conditions used and can be optimized by altering parameters such as pH, divalent ion concentration, as well as the molecular weight and chemical composition of the alginate (Kristiansen et al. 2009).

Horseradish peroxidase (HRP) was successfully encapsulated in a semi-permeable calcium alginate membrane and used for removal of phenol from synthetic wastewater (Alemzadeh et al. 2009). Peroxidase from rice seedling shoots was immobilized using the entrapment method in calcium alginate beads (Nahakpam et al. 2008). Glucose isomerase was entrapped inside nonmodified calcium alginate beads and modified beads with glutaraldehyde (Tumturk et al. 2008). To prevent enzyme leakage from the carrier and improve efficiency, HRP was covalently immobilized onto activated alginate beads (oxidized and aminated) (Spasojević et al. 2014). Alginate was modified by oxidation with sodium periodate and subsequently, tyramine was bound to created aldehyde groups in a reductive amination reaction. The resulting modified alginate with amino and phenol groups was successfully used for HRP immobilization in tyramine-alginate hydrogel microbeads (Prodanovic et al. 2015).

CELLULOSE

Cellulose is the most abundant biopolymer and a major component of plant cell walls (Bezerra et al. 2015; Liu and Chen 2016). Cellulose has a linear structure composed of repeating β(1→4) linked D-glucose, dual hydrophilic/hydrophobic polysaccharides with a specific number of hydroxyl groups on the surface. In order to be used as a carrier, new functional groups can be introduced by chemical modification, enzymatic or microbiological method. The most frequently used cellulose derivatives include carboxymethylcellulose (CMC), cellulose acetate (CA), and cellulose nitrate (Liu and Chen 2016). Introduced functional groups (amino, aldehyde, carboxyl or epoxy) are ready to covalently attach enzymes. Due to difficulties dissolving cellulose, entrapment of enzymes is limited only to soluble derivatives (CA).

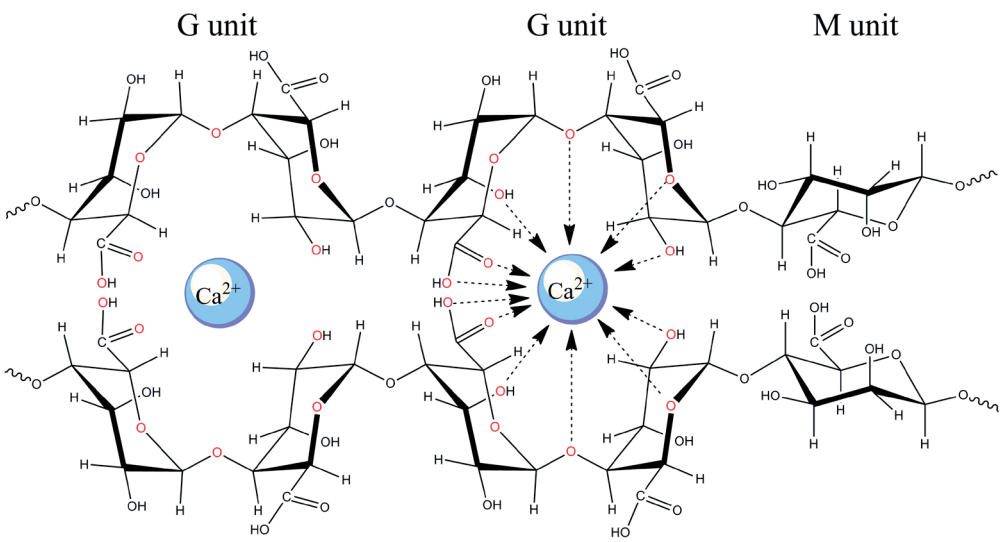

Fig. 1. Schematic view of alginate complexation with divalent cations and formation of the so-called "egg-box" model.

Catalase was immobilized by entrapment within cellulose acetate beads and used for decomposition of H_2O_2 in milk (Yildiz et al. 2004). Cellulose has been used as a support for the immobilization of different enzymes: lipase (Girelli et al. 2012), glucose oxidase (Yabuki et al. 2012), trypsin (Nikolic et al. 2010), polyphenol oxidase (Arica 2000), and horseradish peroxidase (Isobe et al. 2011).

CHITOSAN AND CHITIN

Chitin, the second most abundant polymer in nature and primary component of arthropod exoskeletons and fungal cell walls; is a linear polysaccharide composed of β-1,4 linked 2-acetamido-2-deoxy-2-amino β-D-glucose (or N-acetyl-D-glucosamine) (Krajewska 2004; Bezerra et al. 2015). Chitin can be N-deacetylated by alkaline treatment with sodium hydroxide, resulting in chitosan: a cationic polymer containing positively charged primary amino groups that are rarely found in natural polymers (Bezerra et al. 2015). During the immobilization process, positively charged groups form electrostatic interactions with negatively charged groups present on the surfaces of proteins. Although chitin has been tested as a carrier for enzyme immobilization, deacetylated chitosan is more popular due to these primary amino groups. Bi-functional cross-linking reagents such as glutaraldehyde have been covalently attached and used for preactivation of amino groups (Bezerra et al. 2015).

Beta-galactosidase was immobilized on a chitosan-based carrier and used for hydrolysis of lactose from milk (Vieira et al. 2013). Chymotrypsin (Adriano et al. 2008), β-galactosidase (Lima et al. 2013), catalase (Kaushal et al. 2018), and penicillin G acylase (Adriano et al. 2005) have been covalently immobilized on an activated chitosan support via glutaraldehyde cross-linking. Three different activation reagents (glycidol, glutaraldehyde and epichlorohydrin) were used for covalent immobilization of microbial lipase onto a chitosan support (Rodrigues et al. 2008). Invertase has been covalently immobilized via glutaraldehyde and glutaraldehyde/ethylenediamine activated chitosan to obtain a biocatalyst with improved activity and thermal stability (Hsieh et al. 2000).

XYLAN

Xylan is a branched hemicellulose composed of β-(1→4) linked D-xylose monomers with side chains comprised of L-arabinofuranose or D-glucuronic acid (Spasojevic et al. 2019). Amyloglucosidase has been successfully encapsulated in tyramine-carboxymethylxylan hydrogel microbeads created in an enzymatic emulsion polymerization reaction. Modified xylan was introduced with new functional groups (carboxyl, amino and phenol) for better interaction with the immobilized enzyme (Spasojevic et al. 2019). Xylan from spruce has been modified using carbodiimide activation and subsequently conjugated with tyramine. After enzymatic crosslinking of the introduced phenolic groups with HRP, the spruce xylan-based gel has been used for cell encapsulation (Kuzmenkoa et al. 2014).

COLLAGEN AND GELATIN

Collagen is the most abundant animal protein, and is important for fiber formation in the extracellular matrix of connective tissues (Bezerra et al. 2015; Hanachi et al. 2015). Although it is suitable as a biomaterial for biomedical applications, enzyme immobilization has rarely been reported. Amino acid (carboxyl and amino) functional groups are available for interaction with enzymes or participation in activation reactions with a cross-linking molecule. Gelatin, obtained after partial hydrolysis of collagen, has also been used as a support through covalent interaction with enzymes or for hydrogel entrapment (Bezerra et al. 2015). Alkaline phosphatase was immobilized on collagen by cross-linking with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, affording a biocatalyst with increased thermal stability (Hanachi et al. 2015). Lipase has been immobilized onto magnetic collagen nanoparticles prepared by coprecipitation with Fe_3O_4, yielding a biocatalyst with magnetic separation abilities and increased pH and temperature tolerance (He et al. 2017).

PECTIN

Pectin is a natural branched heteropolysaccharide, traditionally used as a gelling agent (Jana et al. 2011). Pectin is derived from the middle lamella and plant cell wall, and is commercially extracted from citrus peels or apple pomace (Sriamornsak 2003). The polymer structure is composed of three distinct domains: linear homogalacturonan (HGA) and branched rhamnogalacturonan-I (RG-II) and rhamnogalacturonan-II (RG-II) (Munarin et al. 2012). HGA is an unbranched α-1,4-linked-D-galacturonic acid (GalpA) with different degrees of methylesterification and acetylation that makes up more than 60% of the total pectin in the cell wall (Caffall and Mohnen 2009). Similar to the "egg-box" model of alginate, unmethylated C-6 residues of GalpA are negatively charged and in the presence of divalent ions form stable hydrogels over a wide range of pH values (Caffall and Mohnen 2009; Munarin et al. 2012).

Glucoamylase and α-amylase were covalently co-immobilized on a pectin-based support carrier resulting in enhanced thermal and pH stability: the resulting immoblized enzymes were used for starch hydrolysis (Jadhav and Singhal 2013). Lipase has been immobilized onto a pectin support that was

activated with sodium metaperiodate (Batista et al. 2014). Soybean peroxidase has been immobilized by entrapment inside tyramine-pectin hydrogel microbeads created in an emulsion polymerization reaction (Prokopijevic et al. 2017).

STARCH

Starch is the most abundant storage reserve polysaccharide in plants, and is composed of α-D-glucose units which form two distinct components: linear amylase (connected with α-1-4 and α-1-6 glycoside bonds) and branched amylopectin (α-1-4 linked chains with α-1-6 side chains) (Hoffmann et al. 2011; Bezerra et al. 2015). Few reports have mentioned using starch alone as an enzyme immobilization support (Bezerra et al. 2015). On the other hand, in combination with other polymers, starch has been added as a thickener to improve microencapsulation efficiency and prevent uncontrolled enzyme release from the carrier (Hoffmann et al. 2011).

Lipases have been immobilized onto the surface of starch films (Hoffmann et al. 2011). It has been reported that immobilization of peroxidase from bitter gourd by entrapment within calcium alginate–starch hybrid gel beads had increased pH and temepature tolerance (Matto and Husain 2009).

CONCLUDING REMARKS AND PERSPECTIVES

Interest in the application of biopolymers as carriers for enzyme immobilization as well as for other biomedical fields, such as drug delivery or tissue engineering, continues to grow (Ogushi et al. 2007; Jana et al. 2011). Immobilized enzyme systems operate under mild conditions and their wide-range of potential applications is constantly increasing. The development of new materials that are capable of producing hydrogels via chemical modifications of natural biopolymers is an expanding and challenging area of biomedical engineering. Advantages of developing biocatalysts created by enzyme entrapment inside hydrogels composed of natural polymers and their derivatives include all the benefits of an unbound or carrier-free enzyme with preserved conformation and catalytic activity of the soluble enzyme form, and at the same time, retaining all of the benefits of immobilized enzymes, such as reusability and easy separation from the reaction medium. Additionally, multi-enzyme immobilization for multi-step cascade biotransformations is carried out simultaneously within the polymeric network. Furthermore, biopolymers are non-toxic, abundantly available from natural sources, and are biodegradable and environmentally friendly. The only disadvantage of natural polymers is that sometimes they display poor mechanical stability (Bilal and Iqbal 2019). Both natural polymers and their modified derivatives are promising candidates for applications as carriers for enzyme or cell immobilization, and offer new possibilities for the development of novel biocatalytic systems.

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