Chapter

Alginate-Based Applications in Biotechnology with a Special Mention to Biosensors

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Abstract

The exploitation of alginate and its composites as immobilisation support matrices in multiple applications remains a promising field that has the potential to create advanced functional materials from sustainable natural sources. They are non-toxic, allow sol-gel transformation, are biocompatible, have remarkable ion exchange properties, are biodegradable, and are amenable to chemical functionalisation. Alginate and its derived composites have numerous biotechnological and biomedical applications, including biomolecule or cell immobilisation, tissue engineering, drug delivery, wound dressing, and biosensors. Alginate can rapidly crosslink into a stable 3D water-insoluble network called hydrogel with polyvalent cations. Blending alginate with other materials to produce composite materials with improved or novel physico-chemical properties remains an ongoing research endeavour. For instance, natural and synthetic polymers or nanoparticles have been incorporated into alginate-yielding composite material with enhanced physical strength, controlled porosity, improved interaction between the alginate support and the biomolecules, and the impartation of other features such as electrical and magnetic responsiveness, among others. Immobilisation strategies are discussed herein, including their innovations and future research perspectives.

Keywords: alginate, encapsulation, hydrogels, immobilisation, alginate composite, biosensors

1. Introduction

1.1 Alginate: Structure and chemical properties

Alginate belongs to a class of linear polysaccharides consisting of a repeated alternating 1–4 glycosidic bond linked to hexuronic acids: α-L-guluronic acid (G-block) and β-D-mannuronic acid (M-block) subunits are randomly distributed along the chain. They can be arranged into homopolymeric sequences of MM or GG blocks and heterogenous or alternating MG blocks, as shown in **Figure 1** [1]. The physiological and rheological properties of alginate are strongly influenced by the uronic acid

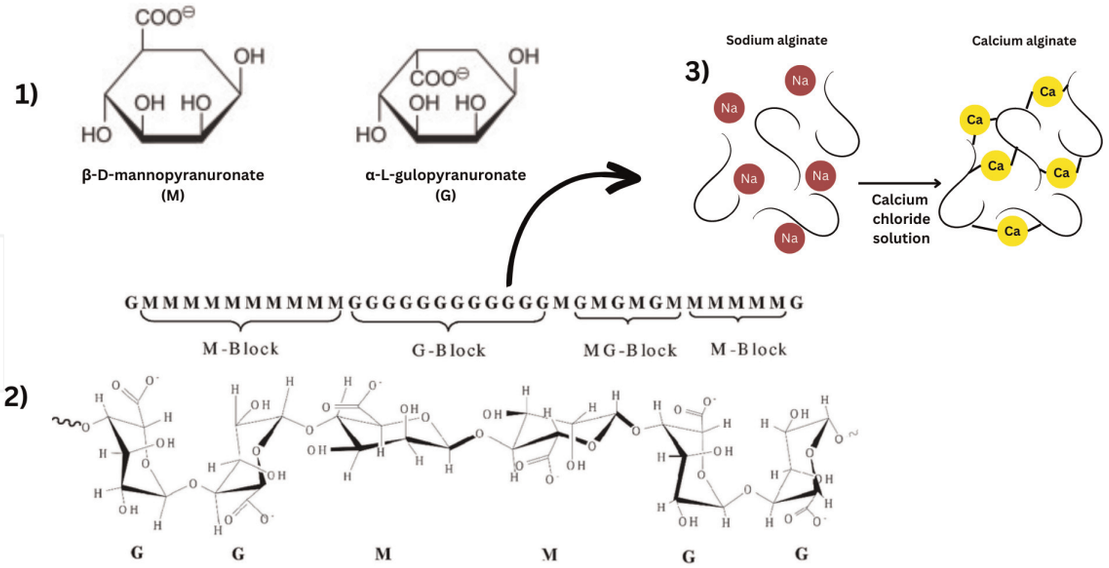

Figure 1.
Structural properties of alginate. (1) polysaccharide subunits of alginate. (2) Homopolymeric and heterogeneous sequences.

compositions (M/G ratio) and by the distribution of the different blocks along the chains [2]. The molecular proportions of G and M subunits in any given alginate vary depending on the source from which it is extracted as well as on the season and the ecological conditions of algal growth [3]. More specifically, the proportions of MM, MG, and GG blocks modulate the physical properties of alginate, where alginates with higher G contents tend to have higher gelling properties. In contrast, those with higher M are associated with higher viscosity [4]. Additionally, alginates with high M/G ratios generate elastic gels, while brittle gels result from alginates with low M/G ratios [1, 5]. Thus, the chemical composition of alginate is fundamental to its intended applications. Alginates have been extracted and purified from different sources including cell walls of brown algae (also known as brown seaweeds), bacterial capsules of *Azotobacter sp*. and *Pseudomonas sp*. They provide flexibility and strong structure to algae to buffer the impact of strong water waves, and support bacteria in biofilm formation, adherence, and colonisation [6] (**Figure 2**).

1.2 Physical properties of alginates

1.2.1 Solubility

Being a polymer of acidic sugar residues, alginate exists in solution as a negatively charged viscous solution and as metal salts (such as sodium, magnesium potassium salts of alginate) in solid states. Among these, sodium alginates are water soluble, resulting in solutions of varying viscosity, depending on the M/G ratio and the alginate molecular weight (MW). Although high MW alginate exhibits greater gel strength, especially when it has more significant G-block content, they are challenging to handle because of the high viscosity of its resulting solution [7]. The water-soluble salt of alginate has an excellent capacity to retain a large amount of water and some amounts of organic matter [8], which is characteristic of hydrogels. Due to its polydispersity, MW of alginate is usually taken as average over the wide distribution of MW ranging from 32,000 to 400,000 g/mol, depending on the source from which the

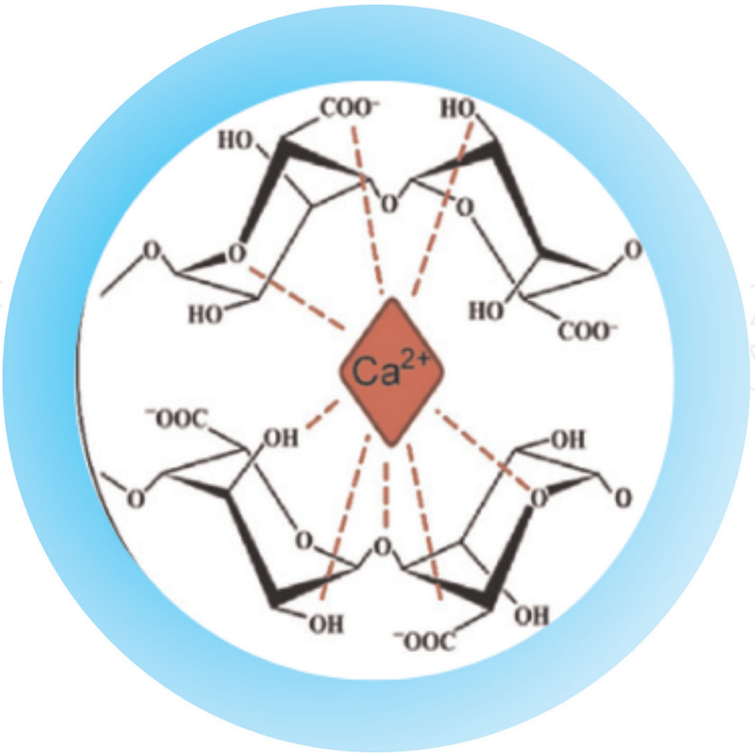

Figure 2.
Illustration of alginate polymer.

alginates were extracted [9]. The MW distribution of alginate has been shown to modulate the viscosity of the pre-gel solution and the rigidity of the gel, thus it influences the choice of alginate for specific applications.

1.2.2 Hydrophilicity

One of the most attractive physical properties of alginate employed in substance immobilisation in industrial and biotechnological applications is its hydrophilicity which controls the swelling behaviour of the alginate gel. Thanks to the early works of Haug *et al.* [10], who reported the pKa values of M-blocks and G-blocks to be 3.38 and 3.65, respectively, above which values the carboxyl groups become deprotonated leading to electrostatic repulsion, thus conferring high hydrophilicity to the alginate polymer [7]. By the same token, in an acidic solution, the alginate polymer chain tends to aggregate because of the decrease in hydrophilicity brought about by the protonation of the carboxyl groups [11]. This phenomenon of aggregation/precipitation of alginate at lower pH values has been investigated concerning the structural composition. For instance, alginates with higher amounts of alternating heterogeneous blocks (MG and GM) tend to precipitate at lower pH values than those that are composed of homogenous blocks (MM and GG) [12]. This pH-dependent change in the hydrophilicity of alginate polymers and the consequent reversible swelling has been exploited in encapsulation studies in targeted substance delivery. To exert robust control over the swelling property of alginate, an innovative double-layer hydrogel based on alginate-carboxymethyl cellulose and synthetic polymer (polyacrylamide) was fabricated for a sustained drug delivery system by a simple and mild method [13], which was prepared by ionic crosslinking (pH-sensitive in a weak alkaline environment), while the outer layer was fabricated by chemical crosslinking having physicochemical

stability, to prevent inner hydrogel expansion [13]. Thus, modulating the physical properties of alginate polymers enables differential manipulation thereof.

1.3 Physical crosslinking of alginate (ionic crosslinking)

In the presence of polyvalent positive ions (such as Mg^{2+}, Ca^{2+}, and Ba^{2+}), alginate can undergo intra and inter-chain crosslinking, forming a stable 3D, thermally irreversible, and water-insoluble network called hydrogel [2], in a process called gelation. Gelation entails a simple substitution of sodium ions with divalent cations of alginate. The type of gel formed, and the rate of gel formation depend on the type and concentration of cation as well as their binding kinetics ($Mg^{2+} < < Ca^{2+} < Zn^{2+} < Sr^{2+} < Ba^{2+}$). Proper control of cation addition can lead to gel formation with controllable homogeneity. Calcium cation-mediated gelation has been widely reported and preferred method. Alginate can form a stable gel with as low a concentration as 1% (w/v) of the polymer in a relatively simple aqueous process at room temperature providing a reticulated matrix that is biocompatible with slow. Degradation rates [14, 15], which have thus led to its extensive use in the immobilisation of cells and biomolecules, retaining their biological activity. The apparent limitation of calcium alginate gel is the burst effect and fast release due to their high porosity [16], explaining the need to develop alginate-composites, and introduce reactive functionalities to minimise the undesirable loss of the immobilised biomolecules.

1.4 De-crosslinking of alginate

The reversibility of cation-mediated crosslinking of alginate is a limitation when the application requires gels integrity, while on the other hand, reversibility brings flexibility in some applications. The commonly used alginate matrices crosslinked with Ca^{2+} ions are unstable in the physiological environment or in standard buffer solutions with a high concentration of counterions (such as phosphate and citrate ions) and chelators that can extract Ca^{2+} from the alginate and liquefy the system [17]. In addition, alginate monomer linkages can be cleaved through free radical oxidation (by oxidative-reductive depolymerisation reactions) [18] and a pH degradation approach. Generally, alginate tends to be more stable around neutral pH values and undergo proton-induced hydrolysis in pH values below 5, leading to the shrinkage of the gel. In contrast, higher pH than 10 initiates degradation via β-alkoxy elimination, leading to alginate gel dissolution [19]. Alginate gel degradation can be achieved by a combination of monovalent cation and adjustment of ionic strength and acidity of the media [20]. The rate of dissolution of alginate can thus be controlled by oxidation [21], reduction of molecular weight of alginate [22], and the use of more vital divalent metals such as barium [17]. Leaching of polymers out of the gel will occur when exposed to a continuous flow of divalent-poor cation medium.

1.5 Chapter overview

Apart from crosslinking, the carboxyl functional group on this polymer can be modified or activated to become reactive towards other functional groups (such as -NH_2) linked to biomolecules, thus, serving as a conjugational point. However, because excessive modification of the carboxyl groups with other (bio)molecules makes them unavailable for the polyvalent cations required for the gelation process, alternative

efforts are being made to incorporate other molecules (such as natural and synthetic polymers, nanoparticles, etc.) into alginate to form alginate composites.

In this review paper, we describe the preparations of alginate composites and discuss their biotechnological applications in the immobilisation of biomolecules such as enzymes, cells, and microbes. The application of alginate as a matrix for the functionalisation of biosensors was also highlighted. Finally, future research courses were provided in using alginate composites for immobilisation technology.

2. Advances in tailored hybrid alginate compositions in biotechnology applications

The alginate and its derived composites have widespread uses from chromatography [23] and wound healing [24] to tissue engineering, drug delivery [25], enzyme immobilisation, and cell encapsulation (**Figure 3**) [26].

Alginate exhibits instantaneous gelation in the presence of multivalent cations such as divalent cations (Ca^{2+}, Ba^{2+}) [17, 27] and cationic polymers such as poly-L-lysine and chitosan [28, 29]. Due to its excellent gelation feature and biocompatibility, alginate has encapsulated a variety of entities as macroscopic alginate-based spheres such as beads and microcapsules [30]. There are two major classical strategies for encapsulating biomolecules in macroscopic alginate spheres. First is the general approach of dripping an alginate solution containing the biomolecules or cells of interest into a crosslinker solution, producing alginate beads instantaneously. In contrast, an inverse approach in which the viscous solution containing a crosslinker and biomolecules are added in drops into an alginate solution, yielding microcapsules having a viscous core and an alginate shell as shown in **Figure 4** [31]. These approaches are seamless and beneficial for active biomolecule immobilisation, but the associated undesirable leakage of the enclosed entity from the matrix limits their more comprehensive application [32]. Many attempts have continuously been made to address the leakage limitation associated with encapsulation approach. Such attempts

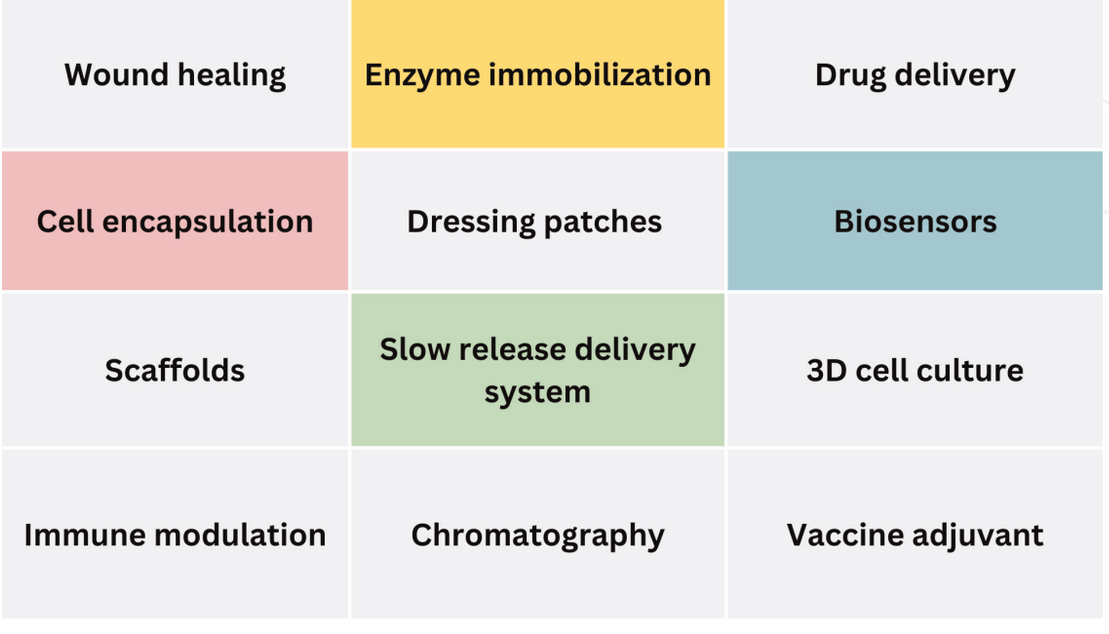

Figure 3.
Biotechnology applications of hybrid-alginate based systems.

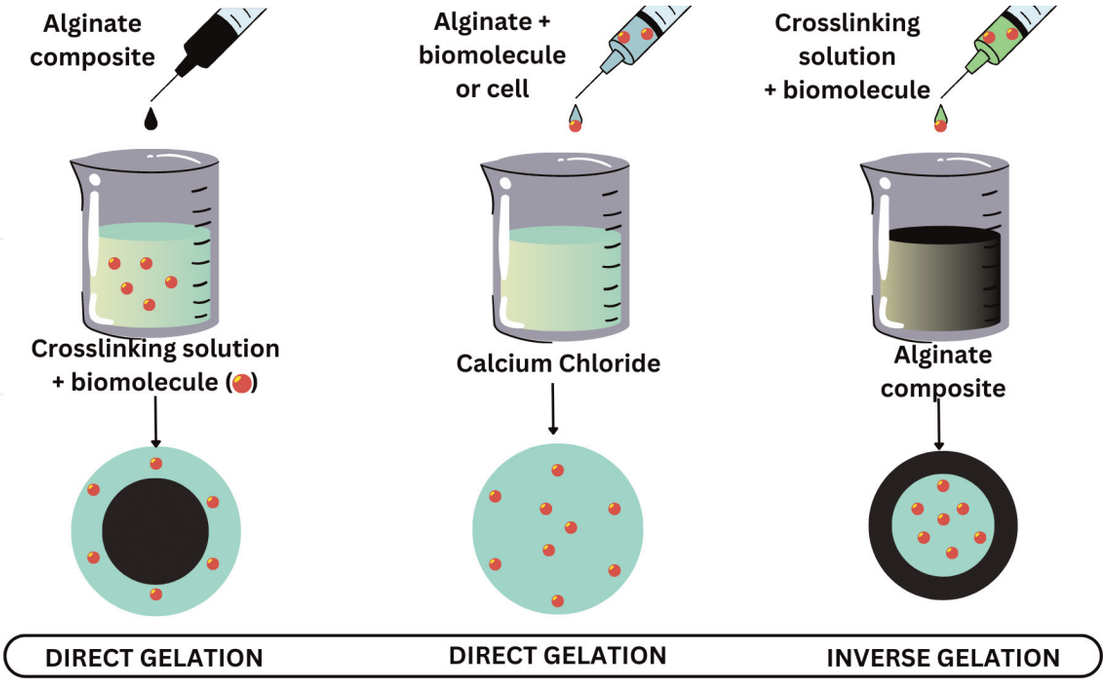

Figure 4.
Two classical approaches of encapsulating biomolecules within macro/microscopic alginate spheres.

include the use of nanoparticles to immobilise the enzyme followed by entrapment within the hydrogel [33], covalent immobilisation [34], covalent affinity immobilisation [35, 36], modulation of the pore of the alginate matrix with other materials such as polycations synthetic polymers [37], natural polymers [38], crosslinking agents [39], inorganics [40] among others.

3. Composites of alginate

Due to the inherent limitation associated with the use of alginate, a wide range of materials have been added to alginate, producing functional composite materials (**Figure 5**) with desirable features for a wide range of applications [41–44]. Depending on the intended application, alginate composites are produced either via covalent or noncovalent linkage. Different materials have been added to dope alginate and impart exciting additional features such as improved physical strength (thermal stability) [45], magnetic responsiveness, electrical conductivity, cell adhesion, 3D printability [46], and supplementary conjugation chemistries, among others.

4. Methods of composite preparation

There are broad means of preparing alginate composites – both physical or chemical preparations. Before gel formation, the latter entails a chemical reaction between alginate and other materials, such as monomers, polymers, and nanoparticles. For example, alginate-pyrrole [47] and alginate-biotin [48] composites have been prepared by covalent conjugation to obtain electrically conductive and affinity-labelled hydrogels. When the composite is prepared from two polymers, the polymers are mixed and then crosslinked sequentially via crosslinkers or other treatments (**Figure 6**).

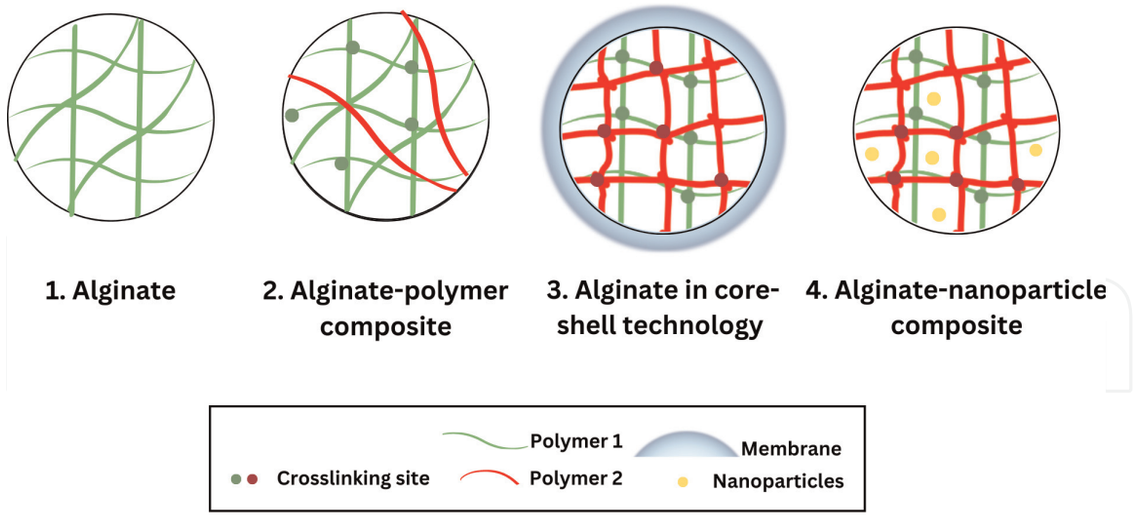

Figure 5.
Composites of alginate.

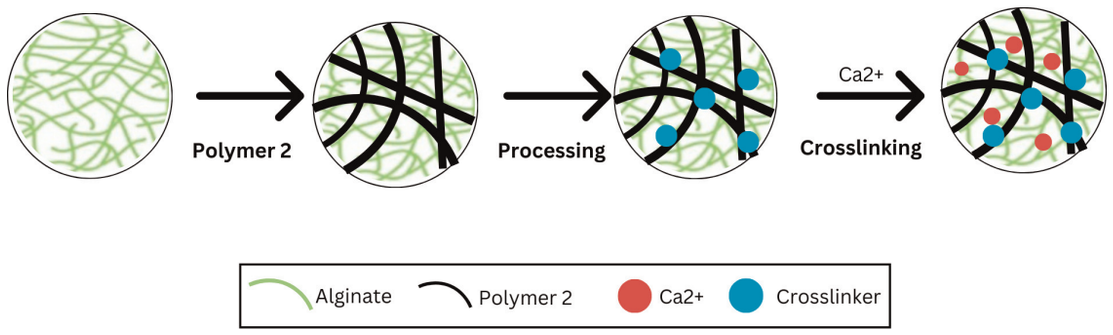

Figure 6.
Methods of composite preparation.

In an event where the composite is between alginate and nanomaterials, the nanomaterials are incorporated within the bulk hydrogel framework by any of three broad methods, blending, *in situ* precipitation, or grafting-onto [49]. The blending method involves mixing the nanomaterial with a hydrogel precursor solution at an optimised molar ratio, followed by a crosslink of the hydrogels to entrap the nanomaterial. In contrast, in the case of *in situ* precipitation, the hydrogel network is prepared via crosslinking, after which the nanomaterial is synthesised by precipitation into the polymer hydrogels after the crosslinking reaction. Grafting several functional groups onto the surface of the nanomaterials as nano-crosslinkers eventually leads to the crosslinking reaction [49].

The preparation of alginate composites has, over the years, evolved, taking different physical formats starting with beads, and microbeads formation [50], to nanoscale preparations such as nanoparticles [51], nanogel [52], thin film [53], and nanofibers [54], variations which relate to different biotechnological applications (**Figure 7**) such as in diagnostics and therapeutics. Beads of alginate are formed by extruding alginate droplets into a crosslinking bath and are found in medical applications in cell immobilisation and scaffolding for tissue regeneration.

Alginate nanoparticles (NPs) (typically between 150 and 250 nm) [55] were anticipated to gain popularity in biomedical applications due to the significant reduction in particle size that has been achieved, thanks to rapid technological advancement.

Image					
Name	**Bead**	**Microbead**	**Nanoparticle**	**Nanofibers**	**Thin film**
Function	• Drug delivery • Biocatalysis	Cell encapsulation	Molecular delivery	Immobilization	Electrochemical bionsesor

Figure 7.
Applications of different formats of alginate composites.

However, unlike other mucoadhesive polysaccharides such as chitosan, which has been chiefly employed for the development of NPs, the alginate-based nanoparticles are only sporadically reported because of the difficulty of achieving nanoscale size ranges using only alginate [56]. The conventional synthetic approach entails the modification [57] of Ca^{2+}-mediated ionotropic crosslinking and/or replacement of the crosslinking with water-in-oil nano-emulsions [58, 59] and, in some cases, using polyelectrolytes such as chitosan [60, 61]. An advancement of the conventional method was the incorporation of nanocarriers such as liposomes into the alginate, exhibiting a mucoadhesive property. Haidar and colleagues exemplified this approach in encapsulating and delivering growth factors using core-shell hybrid NPs formed by the layer-by-layer assembly of alginate and chitosan on liposomes [62]. Furthermore, alginate NPs were prepared using liposomal cores with a high melting point (MP) as reaction vessels to template the alginate NPs assembly. The alginate was encapsulated within the liposome core and then exposed to Ca^{2+} at a temperature higher than the MP of the bilayer such that the Ca^{2+} could diffuse into the core and initiate a gradual

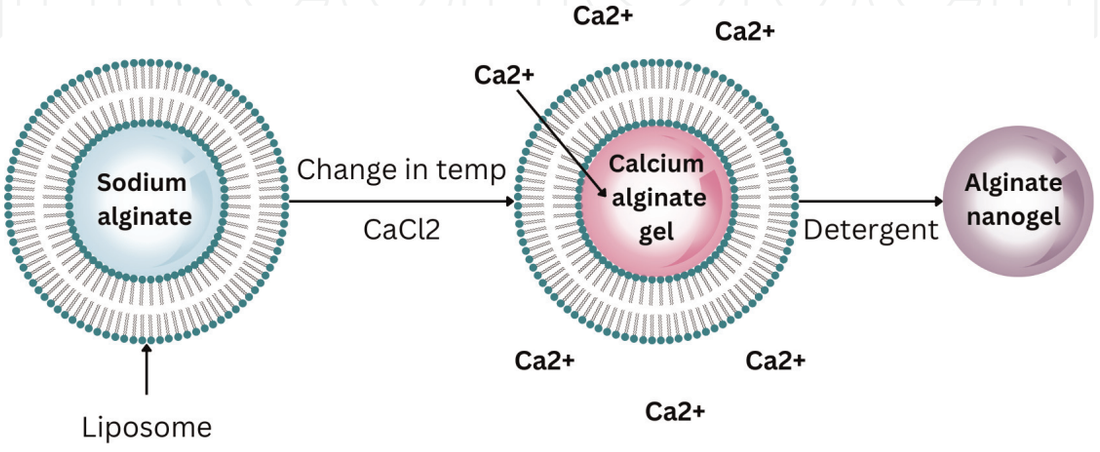

Figure 8.
Preparation of alginate nanoparticles using liposomal templates.

gelation of the alginate, after which the liposome was removed by treatment with detergent to obtain nanoparticles of alginate as shown in **Figure 8** [63, 62].

Alginate nanoparticles have been used in immunotherapy immobilisation (by encapsulation) of antigens [64, 65]. In a particular experiment aimed at the targeted delivery of antigens to dendritic cells, Zhang *et al*. prepared mannose-functionalised alginate (MAN-ALG) NPs using a Ca^{2+} external gelation approach [65]. MAN-ALG was used for dendritic cell targeting while the model antigen, ovalbumin, was conjugated to the partially oxidised alginate (ALG-OVA) separately by a pH-sensitive Schiff base bond conjugation. MAN-ALG and ALG-OVA were used to prepare the NPs used for an *in vitro* study, where the NPs were found to enhance the dendritic antigen uptake and cytosolic release. More recently a composite of alginate was used to prepare NPs for the immobilisation of glucose oxidase used for glucose-sensitive insulin delivery systems in mice [66] (**Table 1**).

Additive Materials	Type of immobilisation	Enhanced features	Application	Ref
$NiFe_2O_4$ Nanoparticles	Entrapment	Impartation of electrical conductivity to the composite cryogel	Immobilisation of Glucose oxidase for the development of an electrochemical glucose sensor	[67]
Polyvinyl alcohol	Encapsulation	Bacteria immobilised by PVA-sodium alginate showed superiority in pH resistance, reuses, material cost, heat resistance, and overall performance	Immobilisation ammonia-oxidising bacteria	[68]
Straw (lignin and cellulose)	Encapsulation	Immobilisation property	Immobilisation of biosurfactant-producing bacteria in bioremediation	[69]
Pyrrole	Entrapment	Electrical conductivity	Cell immobilisation and amperometric algal *Chlorella vulgaris* cell biosensors development	[70]
Polystyrene	Adsorption	Binding capacity	Cell immobilisation on electrospun alginate bio-composite	[71]
Solid lipid nanoparticles (SLN)	Encapsulation	Immunogenicity	Evaluation of the immunogenicity of alginate-SLN for immunisation against *Pseudomonas aeruginosa*	[72]
Chitosan and trimethyl chitosan nanoparticles		Increased stability and immunostimulatory effect	Encapsulation of inactivated PR8 influenza virus for immunisation	[73]
Polypyrrole	Physical entrapment	Electrical conductivity of the composite	Immobilisation of polyphenol oxidase for the construction of an amperometric catechol sensor	[47, 74]
Poly (lactic-co-glycolic acid) (PLGA)	Encapsulation	Physical stabilisation	Microsphere encapsulation and targeted delivery of bovine serum albumin and anti-laminin antibody protein	[75]

Additive Materials	Type of immobilisation	Enhanced features	Application	Ref
Spider silk protein fibres	Adsorption	Adsorbent property	Purification of human serum IgG	[76]
Mannose	Covalent (Schiff base)	Cell targeting (Dendritic cell)	Antigen immobilisation (ovalbumin). For dendritic antigen uptake and pH-sensitive cytosolic release	[65]
Poly (phenylboronic acrylamide acid		Nanoparticles formation	Glucose oxidase immobilisation. For enhanced glucose-triggered insulin delivery in diabetic mice	[66]
Biotin and Pyrrole	Affinity interaction	Electrical conductivity	Immobilisation of glucose oxidase and construction of amperometric glucose sensor	[77]
Arginine-glutamine-aspartic (tripeptide)	Microencapsulation	Cell adhesion	Co-encapsulation of anti-BMP2 monoclonal antibody and mesenchymal stem cells for bone tissue engineering	[78]

Table 1.
Alginate composites with functional properties or enhanced immobilisation systems.

5. General immobilisation approaches

There are broadly two categories of biomolecules immobilisation – physical and chemical methods. Physical methods include entrapment, adsorption, and microencapsulation, the chemical ones are covalent attachment, crosslinking, ionic bonding, and conjugation by affinity interactions (**Figure 9**).

Adsorption on insoluble matrices: this immobilisation involves the biomolecules' attachment to an insoluble support material via noncovalent linkages such as hydrogen bonding, electrostatic or hydrophobic interactions, and van der Waals forces without any need for pre-activation of the support. Alginate and its composites are among the commonly used immobilisation matrices. This simple and mild approach ensures that biomolecules added directly to the surface of active hydrogel adsorbents are adsorbed without considerable conformational/activity perturbation. Parameters influencing this approach include the pH, nature of the solvent, ionic strength, concentration of the biomolecules, and its adsorbents [79–81]. For instance, when the biomolecule(s) to be immobilised are proteinaceous, the pH and ionic strength control becomes critical, as the net charge of proteins changes according to the pH of the solution, thereby altering the kind of electrostatic interactions (**Figure 10**).

5.1 Covalent immobilisation to insoluble matrices

Covalent immobilisation is a chemical means of immobilising biomolecules onto the insoluble matrix by means of covalent bonds such as peptide and disulphide bonds, and Schiff base. The biomolecules get attached to the reactive groups (e.g., hydroxyl, amide, amino, carboxyl groups) present on the hydrogel matrix or via the spacer arm, which is attached to the matrix [82]. While this immobilisation method benefits from the non-leakage of the immobilised species, it is associated with a higher

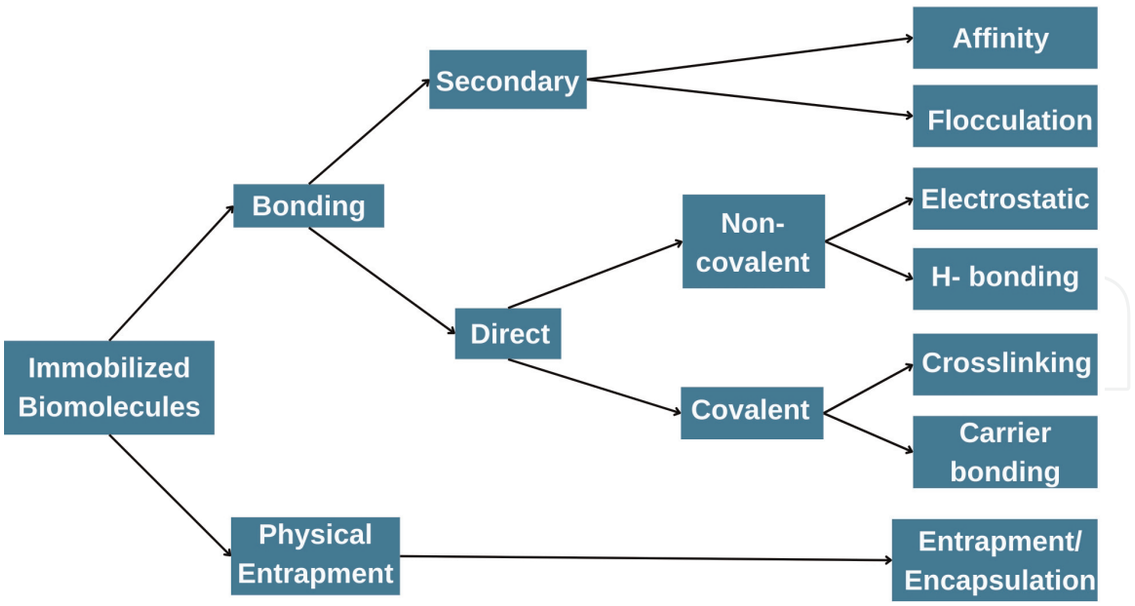

Figure 9.
Categorisation of immobilisation methods based on the kind of interaction between the biomolecules and the immobilisation matrix.

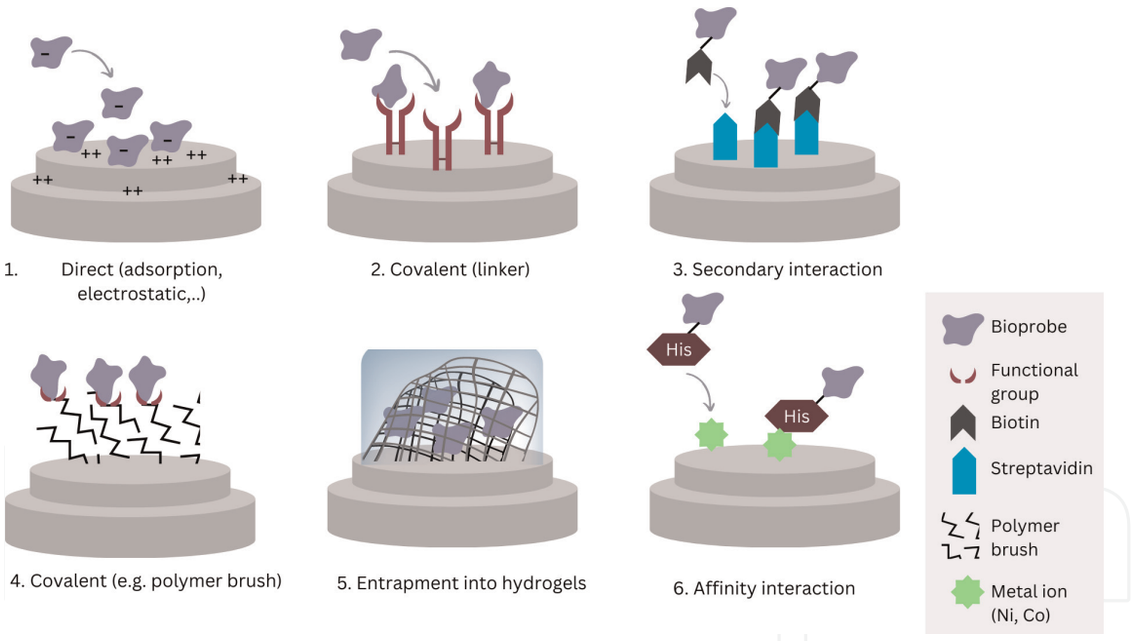

Figure 10.
Pictorial representation of common immobilisation strategies.

tendency to modify the activity of the immobilised biomolecules depending on the choice of immobilisation reagents and conditions [83]. Unreacted reagents used in this approach must be removed (by filtration, centrifugation, or dialysis) or quenched by another reagent. For instance, the unreacted 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) used in the activation of carboxyl groups of alginates for covalent conjugation with amino-containing biomolecules must be quenched by 2-mercaptoethanol [84, 85] or removed by either dialysis [47], centrifugation [86] or centrifugal ultrafilter [87]. Some of these immobilisation treatments are incompatible with alginate in terms of stability to solvent and chemicals, coupled with the limited

availability of surface chemistries on alginate, that reactive towards the common functional groups found in biomolecules.

Specifically, because the carboxyl functional group on alginate is involved in both the formation of hydrogel (in the presence of divalent cations) and bioconjugation reactions, additional efforts were made to compose alginate with anchor points using other polymers, such as chitosan, gelatine, and polyvinyl alcohol, as well as between surface-functionalized nanoparticles and quantum dots. Also, fully, or partially oxidised alginate can be used for the covalent immobilisation of biomolecules via Schiff base formation. In 2015, Hou and colleagues reported the covalent immobilisation of *Candida rugosa* lipase onto the magnetic bio-composite of polydopamine/alginate [88]. In this work, the oxidised form of alginate – alginate dialdehyde (ADA) was used in conjunction with polydopamine-coated magnetic nanoparticles as the immobilisation support, where the enzyme is covalently bonded to the ADA via Schiff base formation. While the research benefited from the ease of separation because of the magnetic responsiveness, a significant finding was the enhancement of temperature and pH stability of the immobilised lipase [88]. Another important immobilisation strategy was evaluated by Abd El-Ghaffar and Hasmem using a composite of chitosan grafted with polymethyl methacrylate (PMMA-g-CS) and calcium alginate to immobilise chymotrypsin [33]. Firstly, the enzyme chymotrypsin was bonded to the PPMA-g-CS by covalence and then encapsulated within calcium alginate [33]. The advantage of this approach is the freedom to immobilise as many molecules as possible onto the support since alginate is not directly involved in any chemical binding with the biomolecules, thereby retaining the hydrogelation capability of the alginate. Here, the alginate serves to provide insoluble porous aqueous support for the immobilised enzyme.

More recently, the amino silane-alginate hybrid hydrogel was prepared by Kurayama *et al.* for enzyme immobilisation [89] as an improvement over the previous attempts of preparing alginate microcapsule and then reacting with 3-aminopropyltriethoxysilane (APTES) via electrostatic interaction between the negatively charged carboxyl group of alginate and the positively charged amino group of the APTES [90, 91]. Kurayama *et al.* reported a facile one-step method of immobilising an enzyme on APTES-alginate hybrid beads by simply dripping a solution of sodium alginate containing the enzyme into a crosslinker solution containing CaCl2 and APTES [89]. The hybrid bead was used to immobilise formate dehydrogenase as a model enzyme resulting in an immobilisation yield of 100% and nine cycles of reuse without loss of enzyme activity. This approach is desirable for enzyme immobilisation for its simplicity and efficiency. The presence of APTES in the hybrid beads facilitates electrostatic interactions between the hydrogel and the enzyme, thereby enhancing the retention of the entrapped enzyme within the gel matrix, as evidenced by the many cycles of enzyme reuse. APTES has been used to functionalised magnetic nanoparticles to facilitate the surface reactivity of the nanoparticles towards carboxyl or amine-containing biomolecules using carbodiimide coupling or glutaraldehyde crosslinking, respectively. The new hybrid APTES-alginate can be a platform for immobilising two or more biomolecules having either carboxyl or amino functional groups by selective bonding properties. In another experiment, alginate-montmorillonite composite beads were prepared as an efficient carrier for pectinase immobilisation by Mohammadi *et al*. [92]. Being reputed for their high surface area, high ion exchange, and high adsorption ability, montmorillonite (MMT) fillers have been applied in various nanocomposite systems [93]. Therefore, the authors expected that incorporating MMT into alginate could offer a better immobilisation platform for

an industrial enzyme – pectinase. The alginate-MMT beads were crosslinked with glutaraldehyde, after which pectinase was covalently immobilised via glutaraldehyde-mediated coupling on the beads, displaying a characteristically higher activity than the free enzyme [92].

5.2 Immobilisation by microencapsulation and entrapment

Encapsulation and entrapment are terms that are in most cases broadly used interchangeably to refer to the act of enclosing substances with semi-permeable structures. However, they are technically not the same. Entrapment involves crosslinking the biomolecules to a polymer, such as an alginate, to cover the biomolecule within the porous polymer lattice. The distinguishing principle behind this technique is the formation of a cross-linked polymeric network around the material to be trapped, which is usually performed by mixing the monomers, a cross-linking agent, and the material to be entrapped in a buffered solution and then adding a catalyst system, to initiate the polymerisation [94]. The entrapment allows for the permeation of appropriately sized substrate and release of products in an enzyme study while the porosity can also be adjusted to selectively retain other biomolecules of interest. Encapsulation involves enveloping the cell suspension (or other biological species) within a membrane system in such a way that the membrane creates an intracellular environment for the encapsulated entities, preventing them from leaking out or coming into direct contact with the external environment [95]. Thus, encapsulation offers a flexibility of enclosing any concentration, or volume of cells or biomolecules within membrane envelopes of different configurations. For this reason, encapsulation has been fondly applied in targeted and controlled substance release (**Figure 11**) and the immobilisation of biocatalysts in industrial processes and bioremediation.

This immobilisation approach benefits from the simplicity of the process. Major setbacks that continue to motivate additional research interest are the diffusional constraints where there could be undesirable leakage of the entrapped entity in the

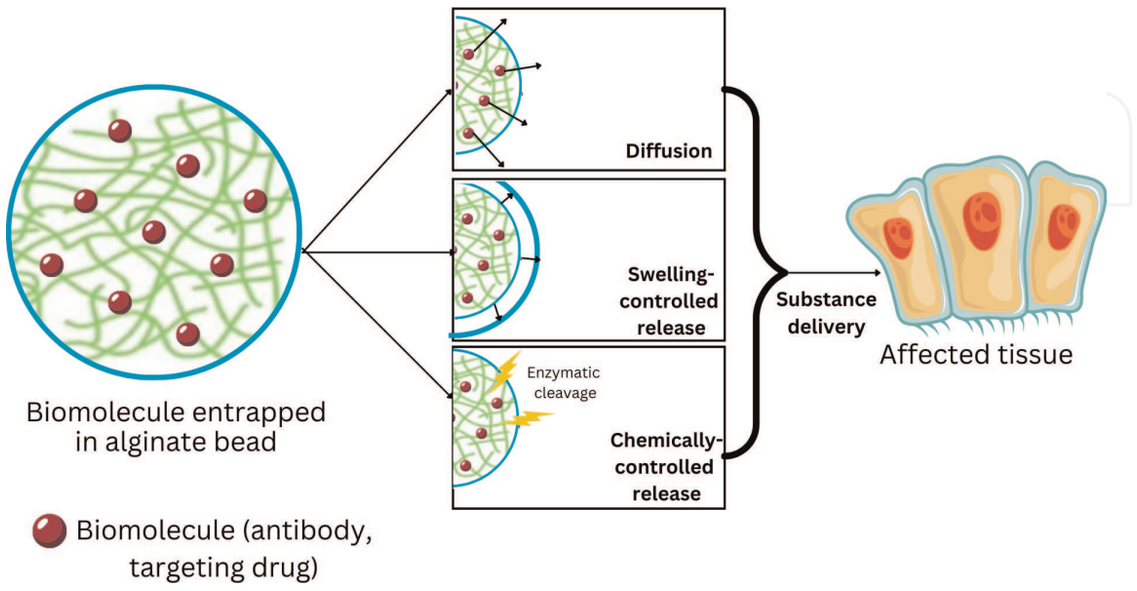

Figure 11.
Application of alginate-based encapsulation system in the immobilisation of biomolecules for targeted substance delivery in biomedical application.

event of changing mechanical properties of the matrix; also, only small-sized substrates/products can be used [82, 96]. Alginate composites have been shown thus far to address the significant setbacks associated with immobilisation by encapsulation.

Alginate-based supports are usually prepared in a gel form by crosslinking between the carboxyl group of the α-l-guluronic acid with a solution of divalent cation crosslinkers such as calcium chloride, barium chloride, or poly(l-lysine). Because of the instability of calcium alginate gel in the presence of high concentrations of phosphate and citrate ions as well as ethylenediaminetetraacetic acid (EDTA), typically found in standard buffer solutions and enzyme reaction medium, composites of alginate became attractive alternatives to overcome such limitations. Taqieddin *et al*. prepared a composite of alginate/chitosan for immobilising β-galactosidase by core-shell microcapsule technology, where alginate was used to encapsulate the enzyme, serving as the core, and chitosan as the semipermeable shell [17]. In this study, using different divalent cations, Ca^{2+} and Ba^{2+} liquid and solid alginate cores were obtained, with 60 and 100% loading efficiencies, respectively. One advantage of this approach is the freedom to control the transport of substrates, products, and cofactors by controlling the outer chitosan shell while the biomolecules are stably immobilised in the inner core. This alginate/chitosan core-shell technology was revisited in 2021 by Mirdamadian and colleagues in a slightly different configuration where chitosan served as the core and alginate, the permeable reactive barrier [97]. In their study, the microcapsule core of chitosan was prepared by crosslinking with sodium tripolyphosphate, encapsulating the calcium peroxide (CaO_2) nanoparticles, and coating the horseradish peroxidase (HRP)-containing alginate layer crosslinked with calcium [97]. The novelty in this approach has to do with microcapsule immobilisation of the enzyme and oxygen-releasing nanoparticles together but at different layers to produce permeable barriers for the bioremediation of phenol in contaminated waters.

Additionally, this technology addressed the low level of dissolved oxygen limitation associated with the aerobic treatment of phenol-polluted groundwater by encapsulating oxygen-releasing nanoparticles within the core to ensure a continuous *in situ* supply of hydrogen peroxide needed for the HRP reaction. Farias *et al*. also immobilised HRP on calcium alginate beads to remove reactive dyes [98]. A one-step chitosan/alginate core-shell matrix has also been reported, taking advantage of chitosan's pH-responsive sol-gel transition property and the calcium-responsive sol-gel transition property of alginate [99]. Apart from the simplicity of methodology, environmental friendliness, and mild condition of this approach, this study demonstrated the pH-responsive reversible sol-gel transition of the crosslinked chitosan core, suggesting the possibility to change the core state (liquid or solid) via pH adjustment. It also showed that the alginate thickness could be modulated easily, making the entire technology suitable for controlled substance release through pH and shell thickness controls. Also, monodisperse core-shell alginate (micro)-capsules with oil core generated from droplets millifluidic was published by Martins and colleagues in 2017 using the original alginate inverse gelation method [31]. In this inverse gelation, oil and $CaCl_2$ solution are emulsified and added into the alginate solution so that the Ca^{2+} ions diffuse from the emulsion drop to the alginate bath, crosslinking the surrounding alginate molecules resulting in core-shell microcapsules. Direct gelation method involves the preparation of alginate and the (bio)molecules to be encapsulated and then dropping the mixture into the bath containing the crosslinker thereby forming alginate beads (**Figure 3**) This approach can be suitable for the immobilisation of enzymes such as lipase that catalyses reaction at the oil-water interfaces.

A composite of alginate-grafted-β-cyclodextrin has been used to immobilise β-mannanase, an enzyme popularly used to treat coffee and tea waste in the food sector [100]. The choice of β-cyclodextrin, a seven-sugar unit cyclic oligosaccharide was due to its ability to form additional complexes with a wide variety of macromolecules, leading to an enhanced overall stability and adsorption capacity of the resulting matrix. The grafting of cyclodextrin to the alginate resulted in increased pH and temperature optima (typically from 6.0 to 7.0 and 50–55°C, respectively), thermostability, and extended reusability. More studies are needed on the possible interaction between alginate and cyclodextrin and the immobilised species having different net charges. The effect of cyclodextrin on the porosity of the composite gel and the crosslinking is an interesting research aspect to peer into in the future.

5.3 Immobilisation by bio-affinity interactions

Protein-protein and protein-small molecule binding interactions are among the widely employed immobilisation strategies that have continued to gain popularity in biomedical and biotechnological applications leveraging the selectivity of such interactions. The immobilisation by bio-affinity interaction demonstrates a characteristically high specificity with respect to the identity of the binding partners and the precise location on the matrix/molecules on which the binding takes place. In this context, the binding of the biomolecules to the matrix is by specific ligands such as his-tag on biomolecule to a metal ion-containing matrix, lectin-containing domain to carbohydrate moieties present on the matrix or biotin on the biomolecules to avidin on the matrix (or vice versa) [82]. The ligands can be naturally present on the biomolecules [101] or attached artificially by fusing the nucleotide sequence corresponding to the tag with the gene coding for the protein of interest. Polyhistidine tag is the most well-known genetically encoded affinity tag. His-tag is a sequential hexahistidine residue that can chelate metal ions such as Ni (II), Co (II), Zn (II), and Cu (II). These metal ions can be prepared for immobilisation by treatment with a chelating moiety such as nitrilotriacetic acid [102, 103] or iminodiacetic acid and can be used alone. For example, because alginate is polyanionic, several alginate nickel composites have been prepared from alginate and $NiCl_2$ [104, 105]. Other affinity tags such as biotin and avidin can be attached to the biomolecules by selected chemistries [106]. This selective approach induces minimal conformational changes to the immobilised entities such as cytokines, growth factors, enzymes, mammalian cell lines, and antibodies [107].

5.4 Immobilisation and multipoint stabilisation

Polyvinyl alcohol/alginate and polyethylene oxide/alginate nanofibers were prepared by electrospinning for the immobilisation of lipase by Doğaç *et al*. [108]. Lipase immobilised on both composite alginate nanofibers showed high enzyme loading, and remarkable thermal, operational, and pH stability properties being stabilised at two levels, first, immobilisation by adsorption followed by glutaraldehyde crosslinking methods.

6. Microbial cells immobilisation

Among the commonly immobilised biomolecules are enzymes and microbial cells. Enzymes are biomolecules capable of accelerating the rate of chemical reactions by

acting as biological catalysts. The molecules upon which an enzyme might act is called substrate, which is transformed into products. As biocatalysts, enzymes participate in chemical reactions but are not consumed. Therefore, a particular enzyme can be reused repeatedly under optimum assay conditions and a sufficiently high substrate concentration, provided the products are continuously removed. Immobilisation of an enzyme onto solid support appeared to be an effective means of achieving improved recovery of the enzyme for reuse, better operational and storage stability, enhanced pH and thermal resistance, and product separation and purification [109–111].

According to the definition of immobilised enzyme given at the first enzyme engineering conference in 1971, "immobilised enzymes are physically confined or localised in a certain defined region of space with the retention of their catalytic activity, which can be used repeatedly and continuously" [112]. A significant aspect of this definition involves the retention of enzymatic activity, which need not be complete but should be high enough to be of practical interest. Typically, a residual activity of about 50% is typical, whereas a residual activity below 25% may be unacceptable [113]. Enzyme immobilisation has therefore continued to attract research interest from fundamental academic research to various industrial applications, inspiring more innovative immobilisation approaches in terms of simplicity of approach and enhanced stabilisation and performance.

The choice of using immobilised or soluble biomolecules (enzymes and cells) in industrial processes is driven by the cost of the biomolecules and the application. However, immobilised species are mostly preferred because of their reusability and adaptability to different process formats [114]. Basso and Serban summarised the factors that affect enzyme immobilisation which must be considered in the study (**Figure 12**) [115]. In a typical immobilisation process, the selectivity, stability, and kinetics of enzyme are carefully considered alongside the immobilisation matrix's physical, chemical, and mechanical properties to maximise the process's productivity (kg product/unit of the immobilised entities) [115].

The basic idea behind enzyme immobilisation started with the entrapment of enzymes within semipermeable materials that would allow the substrate and cofactors

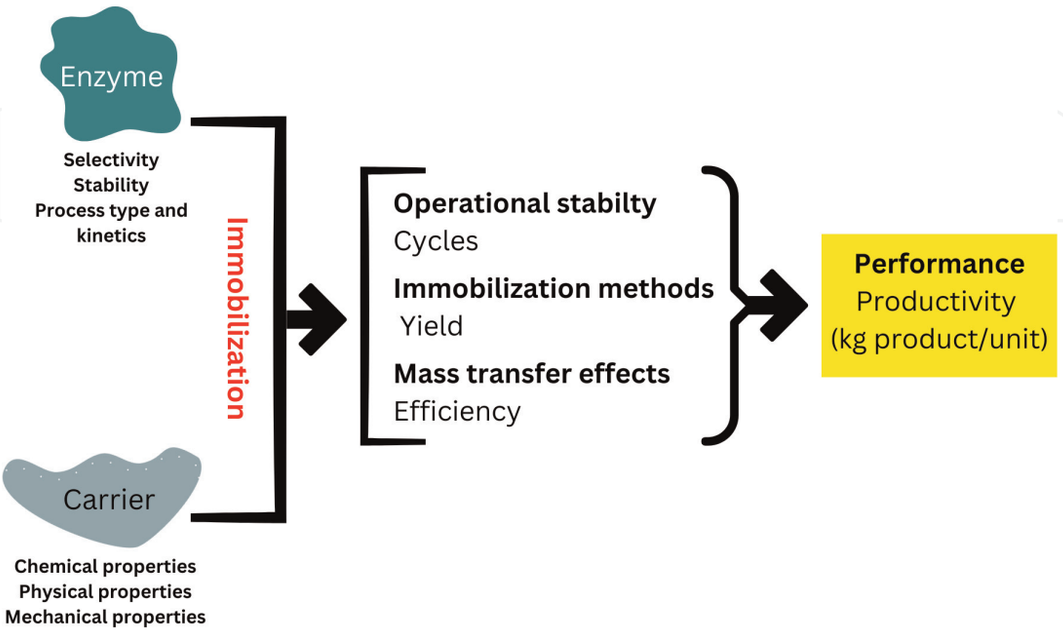

Figure 12.
Factors affecting biomolecules immobilisation.

to pass through them while the enzyme is retained within the matrix [17]. Thus, the control of the porosity of the matrix became a critical criterion. Depending on the type of enzyme and intended application, the material should be at least non-degradable and compatible with the enzyme's optimum assay condition. Also, the immobilisation process should be simple and mild enough in order not to denature the enzyme in the process, and in the case of *in vivo* application, the material must not be immunogenic. Given these requirements for the immobilised enzymes, alginate hydrogels fulfil these requirements and have thus continued to gain popularity in many enzyme and cell immobilisation studies.

7. Cell immobilisation

The process of localising intact cells onto a surface without compromising their essential biological function is known as cell immobilisation. This technique allows the cell system to be reused multiple times and eliminates the negative feedback inhibition that metabolic products have on cells [116]. Cell cultures enable scientists to understand the mechanism behind the disease, the action of drugs, tissue morphology, cell biology, protein synthesis, and tissue engineering [117].

In 1906, for the first time, Harrison cultured cells as part of his investigation into the development of nerve fibres [118]. Since then, cells are mainly cultivated in two dimensions (2D). In 2D cultures, cells develop as a monolayer adhering to a plastic or glass surface in a culture flask or flat petri dish [116]. Although 2D adherent cultures are simple and cost-effective, they have many drawbacks, including the inability to mimic the native structures of tissues in both health and disease. In the 1970s, Hamburg and Salmon conducted one of the earliest three-dimensional (3D) cultures [119]. The 3D systems sustain cell development, organisation, and differentiation like what is found in the human body. A variety of materials enable the 3D cell culture. Among these materials, alginate hydrogels are practical as a framework for immobilising cells in 3D cell culture [120]. In 1980, alginate was first used as an artificial semipermeable membrane enclosing viable islets [121]. Since then, alginate microbeads have been employed with many cell types *in vivo* and *in vitro* [120]. Alginate offers a fantastic toolset for design and optimisation, even though one system is likely to fit only some research or cell types [120]. The hydrogels used for *in vitro* 3D cell culturing have specific physicochemical characteristics, such as hardness, water holding capacity (WHC), swelling-erosion ratio, and swelling rate, to mirror the natural extracellular matrices (ECMs) found in living things [122].

7.1 Encapsulation in beads

Lim and Sun were the first to develop the encapsulation method for immobilising cells [49]. The researchers encapsulated pancreatic islet cells in calcium alginate matrices (**Figure 13**). Alginates have relatively little natural cell attachment and cellular contact, which is a crucial property [123]. This can benefit cell encapsulation applications but may be a drawback for other applications in tissue engineering. Alginate can be altered by including peptides for cell adhesion [124] or other bioactive components [120]. Also, the strength of the surface coating and the capsule porosity can be regulated by wrapping the alginate gel matrix with polycations such as poly-L-ornithine, poly-L-lysine, or chitosan [120, 125].

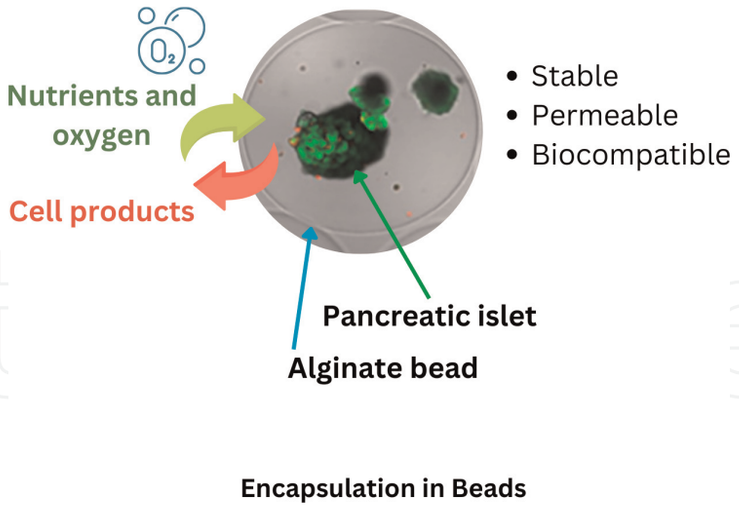

Figure 13.
Encapsulation within the alginate composite beads.

Encapsulating cells in an alginate gel is a safe, and adaptable approach for immobilising cells [125]. Alginate and cells are combined once the osmolality is regulated, and the mixture is then ejected (extruded or dripped) into a calcium chloride bath [120]. The instantaneous ionic crosslinking reaction traps live cells within an alginate hydrogel bead. The development of artificial organs via cell encapsulation is being researched to treat many different ailments [126]. The artificial pancreas used to treat diabetes is perhaps the best-known example (encapsulated pancreatic islets) [127]. By injecting encapsulated canine islet allografts intraperitoneally, Soon-Shiong and colleagues formed mechanically stable microcapsules with alginate, high in guluronic acid, and reported extended remission of diabetes in the diabetic dog model [128]. In other reports, the brains of dogs receiving treatment for spontaneous brain tumours were transplanted with alginate-encapsulated cells that produce the anti-angiogenic protein endostatin [129, 130]. Alginate has been used to immobilise a wide variety of other cell types, including chondrocytes [131, 16], mesenchymal stem cells [124, 132], and adipose-derived stem cells [133, 134], as summarised in **Table 2**.

According to the cell encapsulation approach, cells are enclosed within an artificial enclosure and separated from the host immune system by a semipermeable barrier that protects the transplanted cells from the host immunological response [137]. However, the membrane allows for the flow of small molecules such as glucose, oxygen, therapeutic molecules, and waste materials while isolating cells from the immune reaction [138]. The encapsulation technique eliminates the need for harmful immunosuppressant drugs after transplantation [128] and overcomes the shortage of available donors by enabling allogeneic and xenogeneic transplants [126, 137]. Most techniques for encapsulation cells in alginate consist of two phases. The first step is the development of an internal phase, during which the alginate or composite is divided into tiny droplets. The droplets are solidified in the second step, either by gelling or creating a membrane at the surface of the droplets [120].

7.2 Cell entrapment by self-gelling

Systems for self-gelling (or delayed gelation) is the one in which the gelling of the gels happens inside the body (*in situ*) as shown in **Figure 14**. This method enables

Alginate-Based Applications in Biotechnology with a Special Mention to Biosensors
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Immobilisation techniques	**Encapsulation in Beads**					**Cell entrapment by Self-gelling**	**Electro static Droplet Generation**
Example of Immobilised Cell type	Pancreatic islet cells	Engineered human embryonal kidney 293 cells	Chondrocytes	Adipose-derived stem cells	Mesenchymal stem cells	Human mesenchymal stem cells	Hybridoma and Langerhans islet
Reference	[121, 128]	[129, 130]	[131]	[133, 134]	[124, 132]	[135]	[136]

Table 2.
Different cell immobilisation techniques and their applications.

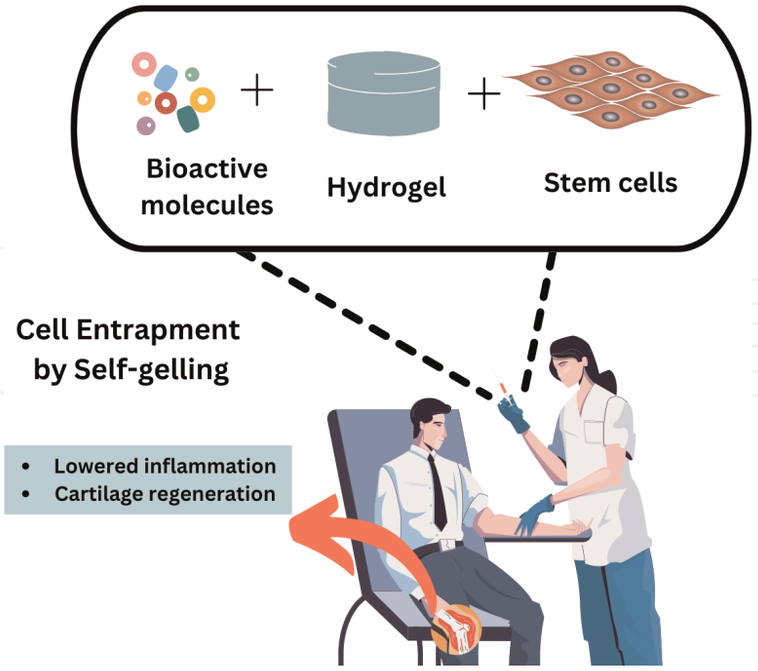

Figure 14.
Cell entrapment by self-gelling.

implantation with less invasive surgical procedures, thus making delivery easier since they precisely occupy tissue spaces and defects [120]. Herlofsen *et al*. in their study of human mesenchymal stem cells (hMSCs) used the self-gelling system [135]. In their study, calcium ions from the calcium alginate particles diffused into the sodium alginate solution, forming an alginate hydrogel that entraps the cells. Self-gelling alginate hydrogel enables the homogenous distribution of the cells within a hydrogel with specific dimensions and shapes. Herlofsen *et al*.'s study showed how the hMSC differentiation led to the upregulation of many genes related to hyaline chondrogenesis, which might be exploited to repair possible lesions of hyaline cartilage. Available data also indicate that the self-gelling approach might get around some of the drawbacks of the 3D scaffolds that are now available, including retrieval of cells and the staining and imaging of cells in situ [139]. Andersen and colleagues [139] used dried calcium alginate foams as a scaffold, which supplies the gelling ions for the alginate solution that occupies the foam's pores and subsequently forms a gel. Cells were evenly distributed throughout the scaffold and entrapped by *in situ* gelations initiated by calcium ions that diffuse from the foam while the alginate solution is rehydrating it.

The formation of tiny microbeads to reduce the mass transfer resistance problem associated with big-diameter beads has been a critical concern in cell immobilisation [140]. The conventional method used for a long time includes swiftly passing the cell/gel solution through a nozzle with compressed air to produce alginate beads [141]. Different techniques for forming droplets have been described, such as extrusion through a needle, Coaxial air (or liquid flow), electrostatic potential, vibrating capillary jet breakage, a pressurised-vessel generated droplets from a vibrating nozzle, and rotating capillary jet breakage [142].

Attempts to use electric fields to form cell immobilisation beads have been successful [136, 140]. For example, electrostatic droplet generation can produce significantly smaller beads than an air jet extruder [140]. Additionally, bead size can be

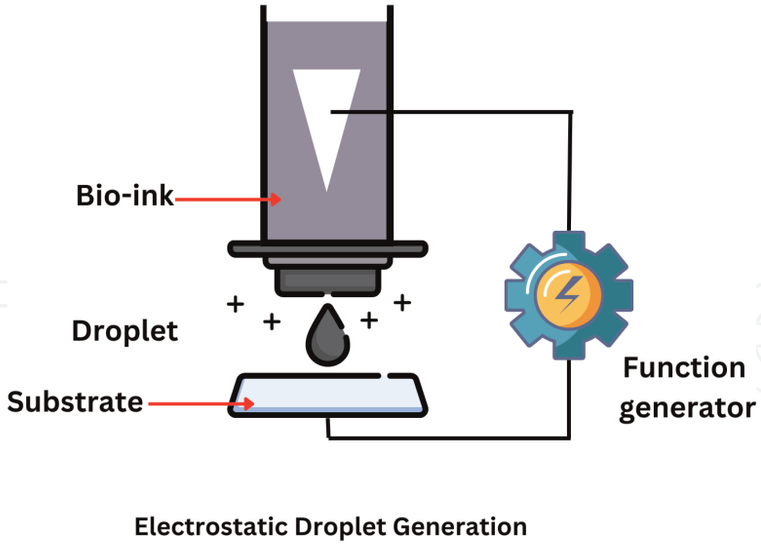

Figure 15.
Electrostatic droplet generation.

easily controlled by adjusting the applied potential. This application's fundamental idea is the electrostatic force that disturbs a liquid surface to generate a charged stream of tiny droplets [136]. Lord Rayleigh was the first to thoroughly investigate the impact of electrostatic forces on atomised liquid droplets as he looked at the stability of a jet of liquid both with and without an applied electric field [143]. When a liquid is exposed to an electric field, a charge is generated on its surface, and due to mutual charge repulsion, a force that pushes outward is produced [140]. The electrostatic surface pressure can drive a drop of liquid into a conical shape under the right circumstances, such as when a liquid is forced through a needle [136], **Figure 15**. The discharge of charged droplets from the liquid's tip causes excess charge to be discharged [136, 140]. The electrode geometry, applied voltage, collecting solution distance, and needle diameter all affect the emission process [116]. After being exposed to strong electrostatic potentials, there was no discernible reduction in the survival of immobilised cell cultures [136].

8. Microbial immobilisation

Immobilised microbial systems are becoming increasingly popular in various fermentation processes [144, 145].

The benefits of immobilised microbes over free-cell batch methods come from the ability to use immobilised microbes in continuous operations. Additionally, immobilised microbial cells maintain high cell densities per unit of bioreactor volume long after the nominal washout rate and produce incredibly high fermentation rates [146]. The immobilisation process imitates the phenomenon of microorganisms naturally attaching to various surfaces in nature [147]. Most ethanol production methods are constrained by low ethanol production rates and issues with recycling and separation depending on the microorganism employed [148]. Immobilised microbial cells used in continuous fermentation operations have the potential to boost ethanol output while cutting costs [149].

In contrast to batch processes, continuous systems allow for higher cell densities inside the bioreactor and smaller reaction volumes [146, 3]. Whole-microbial cell immobilisation has received attention from numerous research teams as a potential replacement for traditional microbial fermentation techniques [148]. In particular, microbial cell immobilisation is also simple to apply in sterile circumstances. The production procedure can be performed under minimally stressful circumstances without applying chemical reagents that could seriously harm the environment and hinder cell activity [150].

8.1 Microbial encapsulation

As the demand for biorefineries increases, it becomes increasingly important to efficiently use all sugars generated from biomass materials [151]. One of the significant obstacles to the viability of the bioprocess is the presence of inhibitory agents in biomass hydrolysates [150]. While First-generation (1G) bioethanol is produced from fermentable sugars directly extracted from food, second-generation (2G) bioethanol is made from lignocellulosic biomass [152]. One of the advantages of 2G is that it is not in competition with food production. However, there are still some challenges in producing 2G ethanol because of the challenge of releasing fermentable sugars completely, the need for effective biomass pretreatments, and low conversion efficiency and yield [153, 154]. Amutha and Gunasekaran [155] found that a higher ethanol yield from liquefied cassava starch was obtained with co-immobilised *Zymomonas mobilis* and *Saccharomyces diastatitus* cultures than with free-state cells. Notably, the immobilised cells caused fermentation processes to end earlier due to the sizeable cellular biomass within the support material, implying reduced processing time.

Additionally, Amutha and Gunasekaran observed that the microbial cells maintain their activity throughout numerous successive batches. Compared to pure-alginate beads, the hybrid alginate-chitosan gel produced improved yeast activity at crude hydrolysate of sugarcane bagasse hemicellulose [150]. Soares *et al.*'s finding showed the possibility of a hybrid gel boosting Second-generation (2G) bioethanol output and prolonging microbial recycling.

Because of its toxic effect and nonbiodegradability, heavy metal pollution poses a significant threat to both human health and the integrity of the ecological system [156]. The use of microorganisms to clean up hazardous metal wastes has already attracted the considerable interest of scientists due to its excellent benefits, which include high efficiency, low cost, and environment friendliness [156]. Utilising Polyvinyl alcohol, sodium alginate, and multiwalled carbon nanotubes, Pang *et al.* immobilise *P. aeruginosa* for hexavalent chromium Cr(VI) detoxification [157]. The beads Pang *et al.* used were immobilised, frozen, and thawed to increase their mechanical strength. The immobilised *P. aeruginosa* bacteria were able to decrease 80 mg/L Cr (VI) in 84 hours, but the free cells were rendered inactive at that concentration of the heavy metal. Also, *P. aeruginosa,* immobilised using alginate and biochar as composite carriers, was used in removing the contaminant acenaphthene from wastewater [158]. According to Lu *et al.*, the immobilised system was promising and thus can be applied to many sewage treatment reactors and the on-site clean-up of contaminated water. Guo *et al.* [159] immobilised *Bacillus subtilis* to remove ammonia nitrogen from swine effluent using chitosan-sodium alginate composite carriers. The immobilised *B. subtilis* was tolerant to high pollutant concentrations, with promising potential application for removing ammonia nitrogen from wastewater.

Immobilisation techniques	Immobilised microbial type	Application	Reference
Microbial Encapsulation	Yeast	Production of 2G bioethanol	[150]
	Bacteria	Bioremediation	[157]
	Bacteria	Wastewater treatment	[158] [159].
	Bacteria	Biohydrogen production	[161]
	Algae	Wastewater treatment	[162]
	Bacteria	Dye decolourisation and industrial wastewater treatment	[163]
Microbial Entrapment	Yeast	Bioethanol Production	[164–166]
Electrostatic Droplet Generation	Yeast	Fermentation of wine, beer, and cider; and production of bioethanol	[146, 167]
	Bacteria	Lactic acid production	[168]

Table 3.
Microbial immobilisation techniques and applications.

The production of renewable hydrogen from biological means is promising. Governments, researchers, and businesses have all noticed the use of biohydrogen gas as an alternative to traditional fossil fuels since it is seen as a green answer to environmental problems [160]. *Clostridium intestinale* immobilised inside 2% calcium-alginate beads were used to produce hydrogen in strictly anaerobic circumstances [161]. Güngörmüşler *et al.* data indicate that although the bacteria inside hydrogel beads experienced a lag at the start of the fermentation process, the immobilised cells outperformed suspended cultures in terms of volumetric rate of production and molar yields of hydrogen. *Chlamydomonas reinhardtii* and *C. vulgaris*, two different microalgae species, were used to assess the effectiveness of nutrient removal [162]. According to Lee *et al.*, the microalgae species removed the nutrients efficiently. Specifically, the photo-bioreactors with 20% algal bead volume fractions removed 95% of total Nitrogen and completely reduced total *phosphorus* in 3 phases of treatment. In another study conducted using immobilised yeast cells (that expressed Laccase from *Streptomyces cyaneus)*, Popović *et al.* completely decoloured Reactive Black 5, Amido Black 10B, Remazol Brilliant Blue, and Evans Blue [163]. Popović *et al.*'s findings suggest that dye decolourisation could be carried out using laccase-coated yeast cell walls encapsulated within dopamine-alginate beads (**Table 3**).

8.2 Microbial entrapment

S. Cerevisiae, immobilised by entrapment in calcium alginate, was shown to maximise ethanol generation at different alginic acid content, size of the bead, concentration of glucose, temperature, and hardening time [164]. Mishra *et al.* employed lignocellulosic hydrolysate from rice straw in a packed bed reactor. The use of rice straw enzymatic hydrolysate makes Mishra *et al.*'s procedure economical and environmentally beneficial since no antibiotics were used and no detoxification was needed. Matthew *et al.* [165] compared the bioethanol production capacity of free-living or immobilised *Saccharomyces cerevisiae* from oilseed rape straw hydrolysate. The yeast cells were either immobilised as a biofilm on grains, Leca, or reticulated foam or entrapped in alginate

beads or Lentikat® discs. Overall, the research's objectives were to evaluate the bioethanol yields produced by free and immobilised systems and to determine the most effective method of immobilisation in terms of bioethanol production and durability of the immobilised cell system. Compared to the free-living cells and immobilised as a biofilm, cell entrapment in alginate beads and Lentikat® discs produced noticeably greater bioethanol yields. Essentially, yeast immobilised on alginate films generated a larger ethanol yield than free yeast cells under the same conditions [166].

8.3 Microbial electrostatic droplet generation

Electrostatic extrusion is an innovative and effective method for immobilising microbial cells. The specific need to use tiny beads for many different fermentation processes, such as beer, wine, and cider fermentation, makes electrostatic extrusion attractive. To overcome diffusion constraints of metabolic products and nutrients inside the carrier matrix, small immobilisation beads are needed for fermentation [144–146]. A considerable decrease in droplet size is often achieved using electrostatic extrusion. Nevertheless, the presence of microbial cells often slows network formation and reduces the Ca-alginate hydrogel's strength properties [169]. As opposed to emulsion procedures, electrostatic extrusion yields homogeneous and small beads, as small as 50 μm in diameter [170].

In electrostatic droplet generation, the diameter of the microbeads typically increases when microbial cells are present [146]. The microbial cell concentration may be a crucial element in electrostatic droplet generation, which is determined by the microbe type's growth characteristics or the immobilised system's desired functionality [146, 170]. Microbial electrostatic droplet generation relies on the utilisation of electrostatic forces to disrupt a liquid of a needle tip and generate a charged stream of tiny droplets (**Figure 15**) [170]. Nikolić *et al.* examined how immobilisation affected the conversion of corn meal hydrolyzates into bioethanol [167]. The authors immobilised yeast cells in Ca-alginate using the electrostatic droplet generation technique. According to their findings, diffusion and reduced levels in the bead core caused the yeast cells to have a greater tolerance to an increased substrate and product contents than free cells did.

The electrostatic droplet approach was also used to immobilise *Lactobacillus rhamnosus* in a poly(vinyl alcohol)/calcium alginate (PVA/Ca-alginate) composite for use in lactic acid fermentation [168]. Mechanical characterisation revealed that the PVA/Ca-alginate beads had a significant elastic character. *L. rhamnosus* showed remarkable survival in addition to withstanding a relatively abrupt immobilisation treatment involving "freezing-thawing." Furthermore, the immobilised biocatalyst outperformed the free cell fermentation system by 37.1% because of its excellent operational and mechanical stability and capacity to withstand the potentially stressful "freezing-thawing" approach.

9. Application of alginate composites in the development of biosensors

The driving force in developing biosensors has remained the need to increase the sensitivity, selectivity, and stability or reduce the production costs of the biosensors [67]. Moreover, such development strategies could range from the biological compound exploration of biological sensing elements such as enzymes, DNA, antibodies, cells, and supporting materials for biological compound immobilisation to detector

improvisation. A biosensor is a self-contained analytical device that uses a specific interaction between analytes and their biological recognition to provide qualitative, quantitative, and semiquantitative information about the analyte(s) being probed. It consists of 3 main components – the sensing element, the transducer, and the detection system (**Figure 16**). The sensing element is made of biomolecules (proteins and nucleic acids), that are immobilised on matrix/support and can interact specifically with the analyte of interest leading to a measurable biochemical response. The biomolecular recognition element (BRE) of a biosensor determines the selectivity and specificity of that biosensor, and it has been a subject of intense research. Specifically, more attention is being paid to the functionalisation – the art of immobilising the biological material onto the support, because it constitutes a critical step in optimising the sensor's performance. Functionalisation must ensure that the structure and activity of the immobilised material are at least preserved or enhanced. Thus, simple and efficient immobilisation techniques are continuously sought.

$NiFe_2O_4$ nanoparticles-modified alginate cryogel has been used to develop an electrochemical glucose sensor by entrapping a glucose oxidase within the NPs-alginate composite gels [67]. The NPs were added to impart electrical conductivity to the alginate so that the oxidation-reduction events at the working electrode could be efficiently detected and thereby increase the sensitivity of the biosensor. When the oxidation and reduction peaks at the enzymatic electrodes prepared by only alginate and NPs-modified alginate were compared, the latter showed higher oxidation and reduction peaks because of the large surface area of the porous cryogel combined with the nickel-ferrite NPs [67]. This biosensor showed a limit of detection (LOD) of 0.32 mM and a limit of quantification of 1.06 mM, which was a landslide sensitivity over a colourimetric alginate-based glucose biosensor [171], and near-infrared alginate-based glucose biosensor [172]. A separate study developed a sensitive amperometric electrochemical glucose sensor by electro-copolymerisation of covalently coupled biotin-pyrrole and alginate pyrrole to immobilise glucose oxidase [77]. The sensor construction consisted of the conjugation of biotinylated-glucose oxidase (B-GOx) to B-Py through avidin (Av) bridges, followed by copolymerisation with Alginate-Pyrrole. When the set-up did not include the pyrrole-modified alginate but

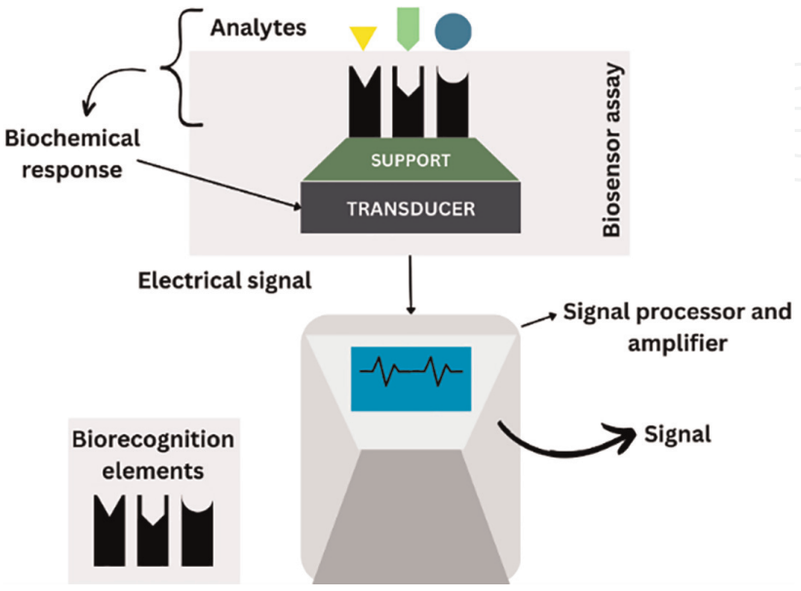

Figure 16.
Components of a typical biosensor.

unmodified alginate, its performance values were significantly less [77]. Another electrically conductive alginate-polypyrrole composite has been investigated for biosensor development. In 2005, Abu-Rabeah *et al*. synthesised alginate-pyrrole conjugate to develop an amperometric sensor. The electrochemical polymerisation of pyrrole monomers generated alginate-polypyrrole. During the electrochemical synthesis of alginate-polypyrrole, the polyphenol oxidase (PPO) enzyme became physically entrapped within the alginate composite matrix. The entrapped enzyme was used to examine its amperometric determination of catechol, providing a sensor sensitivity of 350 and 80 μA M^{-1} cm^{-2}, respectively, for polypyrrole–alginate and alginate biosensors [47]. The pyrrole-based electroconductive alginate gel has also been used in the entrapment of algal cells of *C. vulgaris* to develop amperometric sensors [70]. The same research group investigated the enzyme retention capacity of an electropolymerised polypyrrole-alginate matrix used for glucose oxidase-based biosensor construction. Like other reports on alginate-pyrrole enzyme immobilisation, this study showed an improvement in enzyme retention compared to the preparations involving only alginate. Electropolymerised alginate-polypyrrole protected the gel from the destructive effects of phosphate anions that could otherwise have competed for the Ca^{2+} used for the gelation of the composite [74]. Alginate composites exhibiting electrical conductivity are continuously investigated in developing highly sensitive biosensors. Antibodies immobilised on solid surfaces continue to find wide applications in immunosensors, affinity chromatography and diagnostic immune assays [173]. Alginate is among the solid surfaces used for immobilising antibodies and proteins due to its non-toxicity and gel-forming properties. Moreso, alginate derived composites have found extensive application in optical sensors development. For instance, covalently linked biotin-alginate was used for the encapsulation of genetically modified bioluminescent reporter cells into microspheres for determination of a model toxin, mitomycin [48]. The biosensor was fabricated by carbodiimide mediated covalent conjugation of biotin to the alginate resulting in a composite which was used to encapsulate the bioreporter within the microsphere (**Figures 17** and **18**).

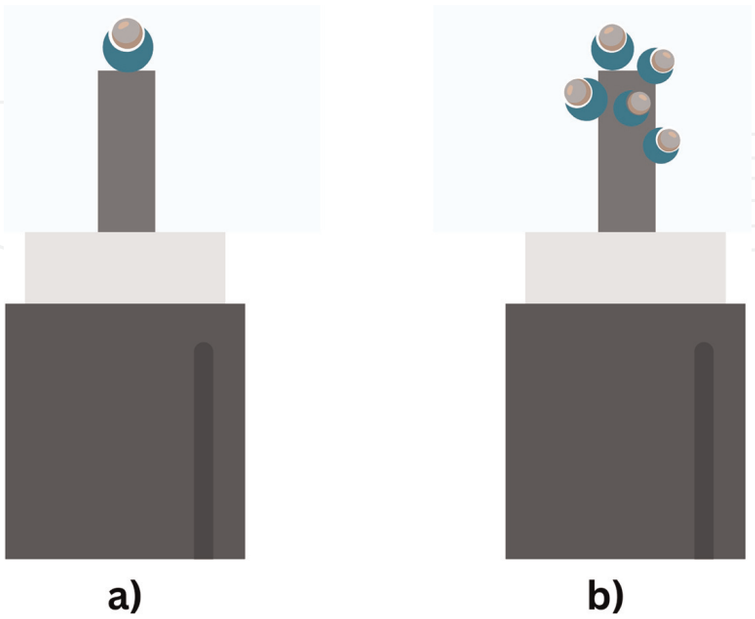

Figure 17.
Biotin-alginate microspheres conjugated to an optical fibre via avidin–biotin affinity interactions: (a) attachment of a lone bead to the end face of the fibre and (b) coating of the fibre with a number of microspheres [48].

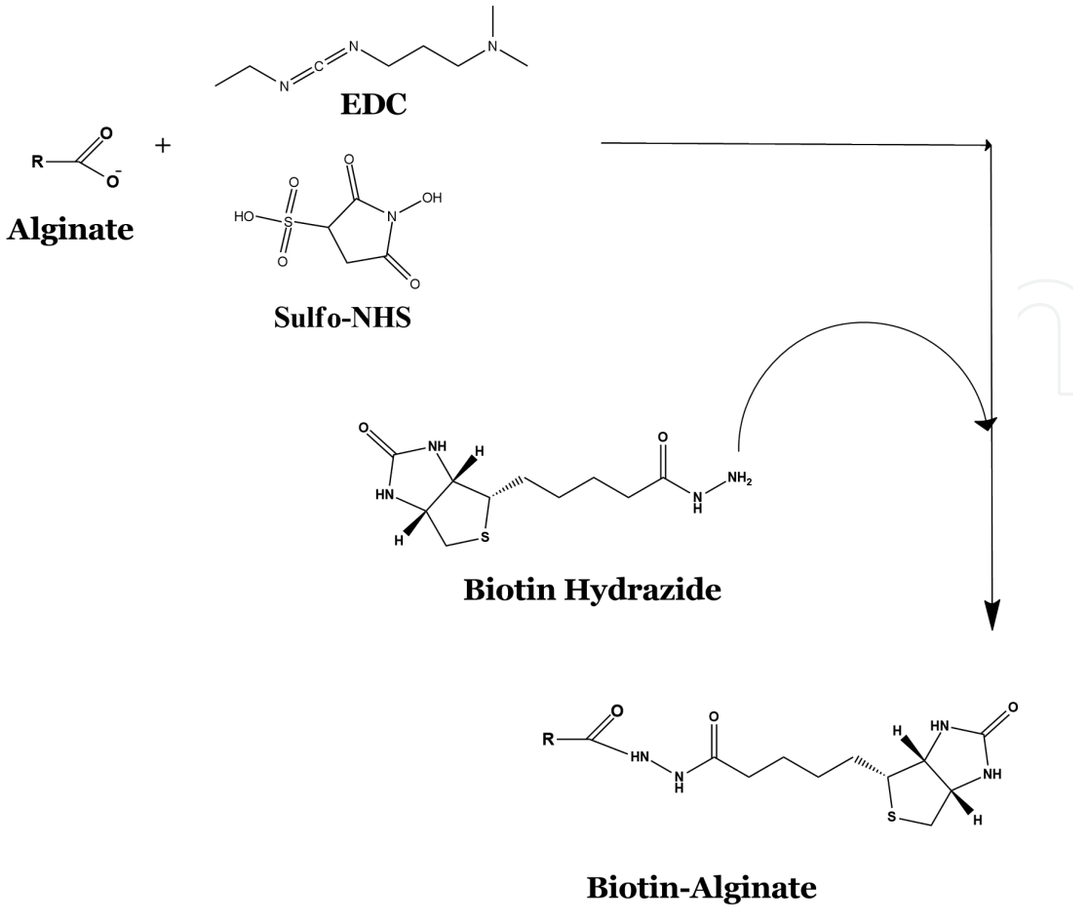

Figure 18.
Biotin coupling to alginate via carbodiimide chemistry [48]. Where EDC represents 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide), Sulfo-NHS is (N-hydroxysulfosuccinimide) and R represents the alginate polymer bearing carboxyl functional groups.

The biotinylated microspheres were conjugated to the surface of a streptavidin-coated multimode optical fibre which served as a transducer for the generated light. It was possible to attach both lone and multiple microspheres to the end of the optical fibre via avidin-biotin affinity [48]. The biosensors performance of the composite showed that biotin-alginate microsphere prevented diffusional loss of the encapsulated bioreporter that would have occurred using alginate alone. In 2017, Li et al. prepared an alginate-methacrylate based whole cell biosensor for the detection of quorum sensing molecules [174]. The biosensor development involved the encapsulation of the genetically reporter bacteria within the double crosslinked alginate-methacrylate microbeads. The entrapped bioreporter produces fluorescence by a dose-dependent expression of green fluorescent protein in response to the *P. aeruginosa* secreted autoinducer signalling molecule. The resulting biosensor unit is facile, as the combination of ionic cross-linking and photo-cross-linking affords the formation of stable and robust alginate-based microbeads with decreased swelling ratio, increased stability, and good permeability of dye-labelled autoinducers [174]. The encapsulation efficiency and the viability of the encapsulated reporter bacteria were remarkable, while the increased bead stability reportedly led to 10 times decrease in bacteria leaching from the beads. Alginate and its derived composites have been continuously evaluated for sensors and biosensors applications.

10. Conclusion and future perspective

The exploitation of alginate and its composites as immobilisation support matrices remains a promising research field with limitless potentials of creating innovative and advanced functional materials from the sustainable natural resources on earth. Thanks to their attractive features, including non-toxicity, ease of preparation, excellent biocompatibility, biodegradability, and amenability to chemical functionalisation, alginate and its composites have continued to find widespread biotechnological and biomedical applications. Incorporating other substances (such as natural or synthetic polymers and nanoparticles) into alginate results in alginate composite materials with enhanced or novel physicochemical properties. Thus, the preparation and characterisation of various alginate composites have become increasingly attractive to most biomaterial engineers.

Alginate composite as an immobilisation matrix has witnessed tremendous advancements in the past few decades ranging from the essential encapsulation of molecules to a more stable immobilisation, engaging two or more strategies. The concept of composite formation of alginates derives from the need to overcome the apparent limitation associated with the alginate in terms of physicochemical parameters such as enhanced physical strength, controlled porosity, improved interaction between the alginate support and the biomolecules as well as the impartation of other features such as electrical and magnetic responsiveness among others. So far, better immobilisation performance in terms of porosity and chemical reactivities has been achieved.

Any given immobilisation approach should be simple and able to maintain the integrity and activity of the immobilised entity. The facile nature of immobilisation by encapsulation has drawn much interest in most biotechnological applications, directing enormous research efforts towards improving the encapsulation performance of alginate. The concept of composite formation has led to a tremendous advance in the immobilisation efficiency of alginate hydrogels, one of which is the emergence of the core-shell technology, widely used in targeted delivery and controlled substance release. Depending on the configuration, alginate (or its composite material) could be either the core or the outer shell, as discussed above. The advent of core-shell technology was a breakthrough in immobilisation studies. Furthermore, alginate composites demonstrate different stabilities as well as swelling behaviours in different ionic environments. With a careful choice of dopant in composite alginate formation, one can have the freedom to exert control over alginate.

Hu *et al*. prepared a dual layer of alginate-carboxymethyl cellulose (Alg-CMC) and polyacrylamide (outer layer) for the encapsulation of protein intended for targeted delivery application [13]. In their study, the swelling behaviour of the inner layer (Alg-CMC) was regulated by the outer layer (synthetic polymer) with negligible swelling capacity under the experimental condition. This concept could be expanded for the immobilisation of catalytic biomolecules and cells where the outer layer could serve as a selective permeability barrier with controllable porosity to allow for material exchange and protect the inner alginate layer against degradation. Another aspect of interest is the possibility of immobilising the biomolecule on the dopants and subsequent encapsulation within the alginate matrix. This approach could address the unpredictable diffusional loss of the encapsulated materials.

Chemical (covalent/affinity) immobilisation does not suffer diffusional loss. However, the immobilisation chemistry must be carefully selected to have a negligible effect on the structure and activity of the immobilised species. The chemistry must be simple and interact with a site other than the catalytic site (in the case of enzymes),

preserving the structure and conformation of the enzyme. These requirements have led to the development a hetero-functional immobilisation matrix, which can offer multi-point stabilisation of the immobilised species. Alginate contains carboxyl and hydroxyl functional groups, which can facilitate the conjugation and stabilisation of biomolecules. Moreso, via the hybrid approach, more functionalities can be introduced to the alginate for additional stabilisation.

Thiol, epoxy, and glyoxal group-containing immobilisation support matrices are documented to exhibit excellent biomolecular immobilisation and stabilisation capacity. Preparation of alginate composite with such hetero functionalities holds the prospect for exponential advancement of the application of alginate in industrial, biotechnological, and medical contexts. Thus, alginate and its derived composites hold a high prospect for the co-immobilisation and co-localisation of cells [175] and other biological payloads with emerging biotechnological uses.

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