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Cell encapsulation using chitosan: chemical aspects and applications

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Abstract

In this work, the main approaches for the preparation of encapsulating matrices using chitosan-containing formulations have been reviewed. Various methodologies have been considered, such as physical intermolecular bonds and chemical cross-linking reactions, including the click reactions which have become novel in the cross-linking of systems containing this biopolymer. Likewise, the formation of different macroscopic assemblies such as spheroids, vesicles, layer by layer polycomplexes, etc., has been addressed. In the final part of the work, the main achievements reported with these matrices in the encapsulation of cells, both eukaryotic and prokaryotic, are discussed, emphasizing their potential applications and perspectives in different fields as medicine (treatment of traumatic diseases, diabetes, venous diseases, tissue regeneration, transplantation and tolerance); food (administration of probiotics); industrial applications (bioethanol production); etc.

Keywords: Cell encapsulating matrices; Click reaction; chemical crosslinking; ionotropic gelation

Resumen

Encapsulación de células usando quitosano: aspectos químicos y aplicaciones. En este trabajo se han revisado los principales enfoques para la preparación de matrices encapsulantes utilizando formulaciones que contienen quitosano. Se han considerado diversas metodologías, como las uniones intermoleculares físicas y las reacciones químicas de entrecruzamiento, incluidas las reacciones *click*, las cuales se han vuelto una novedad en la reticulación de sistemas que contienen este biopolímero. Asimismo, se ha abordado la formación de diferentes ensamblajes macroscópicos como esferoides, vesículas, policomplejos capa a capa, etc. En la parte final del trabajo se discuten los principales logros reportados con estas matrices en el encapsulado de células, tanto eucariotas como procariotas, enfatizando sus potenciales aplicaciones y perspectivas en diferentes campos como la medicina (tratamiento de enfermedades traumáticas, diabetes, enfermedades venosas, regeneración de tejidos, trasplante y tolerancia); en la industria alimentaria (administración de probióticos); aplicaciones industriales (producción de bioetanol); etc.

Palabras claves: Matrices encapsulantes de células; reacción click; reacciones de entrecruzamiento; gelación inotrópica

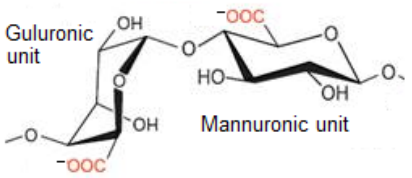
Introduction

Cell encapsulation basically consists of confining living cells within non-living matrices in order to protect their physical integrity, preserving also their normal metabolic activities, for their subsequent transit or use in risky environments for them. The method was proposed for the first time by Chang in the 60s of the previous century, showing different experimental approaches that allow it to be achived[1].

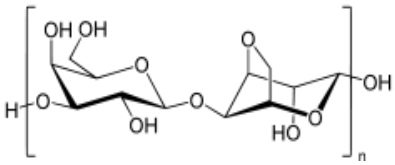
One of the main reasons for the encapsulation of cells is the protection that the encapsulating coating gives them, which is usually formed by a partially permeable polymeric membrane artificially created. Thus, in the case of transplanted cells, encapsulation could prevent their rejection if it manages to "hide" them from the host's immune system (a process known as immunoisolation), without the need to use immunosuppressants[2].

Research on new systems for cell encapsulation, or the improvement of already known systems, will always be very topical because the results are potentially applicable in the treatment of disorders associated with various diseases such as diabetes, neurological degeneration, hemophilia, cancer, kidney failure, etc.[3-5]. In a broader sense, the search for new matrices for the encapsulation of proteins, peptides, DNA, cells, and even microorganisms, has been oriented towards

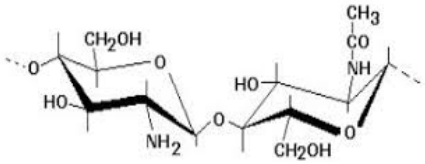
the use of biomaterials such as polysaccharides, i.e., alginates[6], agarose[7], chitosan[8] (see chemical structures in figure 1); proteins, i.e., gelatin[9], collagen[10], silk fiber[11]; polynucleotides (RNA and DNA[12]) and some biodegradable polymers such as polylactic and polyglycolic acids and their copolymers[13] and polyhydroxyalkanoates[14] (see chemical structures in figure 2). Among the current most important reasons for the preference of these materials is their biodegradability, since it is intended that they not only be able to transport cells but also allow the design of controlled release systems towards well pre-established therapeutic targets.



(a) Alginate

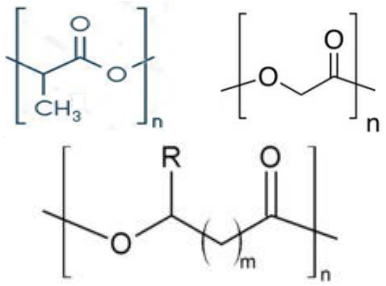


(b) Agarose



(c) Chitosan

Fig. 1: Chemical structure of some polysaccharides employed in the cell encapsulation.



(a) Polylactic acid (b) Polyglycolic acid (c) Polyhydroxyalcanoate

Fig. 2: Chemical structure of some biodegradable polymers employed in the cell encapsulation.

In this work, the main approaches using chitosan-containing formulations for the encapsulation of cells are reviewed. Different methodologies have been considered for the formation of the encapsulating matrix, such as physical and chemical cross-linking reactions, including click reactions. Likewise, the formation of different macroscopic structures such as spheroids, vesicles, layer-by-layer polycomplexes, etc., has been addressed. On the other hand, a brief discussion of the main achievements reported for the chitosan-containing matrices obtained during encapsulation of both, eukaryotic and prokaryotic cells, is also presented, emphasizing their potential applications.

Chemical aspects of the encapsulation of cells using chitosan

Chitosan is a highly versatile polysaccharide which is usually obtained by deacetylation of chitin, a relatively inexpensive material routinely extracted from industrial crustacean processing wastes. However, for applications in the health field, in recent years there has been a tendency to produce it from fungi to minimize the intoxication risks associated to marine product derivatives[15]. It is considered a prominent candidate for the encapsulation of a diversity of materials[16], including cells, to be used in living systems because it has adequate properties for these purposes, such as its non-toxicity, biodegradability, and biocompatibility[17,18]. However, it is essential to consider that for such uses it is necessary to work with materials of high degrees of purity.

From a chemical point of view, some relevant chitosan-characteristic reactions can be established in this kind of applications. Thus, encapsulation of materials within envelopes or matrices containing chitosan in their composition can be achieved using various experimental approaches, such as:

- Formation of three-dimensional networks generated by intermolecular crosslinking due to physical interactions, which can be of various nature (hydrophobic[19], hydrogen bonds[19], molecular entanglement[20], ionic interactions[21], etc.).
- Three-dimensional networks formation caused by covalent bonds linking different polymer chains, which can be achieved through chemical reactions that do not include crosslinking agents[22] or that require their presence, whether they are low or high molecular weight[23]. Among these reactions have recently been included the so-called "click reactions", also known as orthogonal reactions[24], based on chitosan derivatives which are specially prepared for such purposes.

One of the most exploited characteristics of chitosan for this type of application is its cationic nature in aqueous acidic medium, which is enhanced in some derivatives such as quaternary ammonium salts in a wide pH range. This cationic character allows its electrostatic interaction with materials carrying anionic residues, as it has been shown in the preparation of microspheres encapsulating solutions of an anionic polyelectrolyte obtained by oxidation of the polysaccharide scleroglucan (generating pendant carboxylate residues along its chain) within a skin formed by the chitosan/scleroglycan polyelectrolyte complex[25]. Spheres are formed by simply dropping a polyanion solution into a chitosan solution in an acid medium. Similarly, the encapsulation of chitosan solutions within the skin formed by the polyelectrolyte complex of both biopolymers is also possible. An idealized picture of this type of sphere is shown in figure 3.

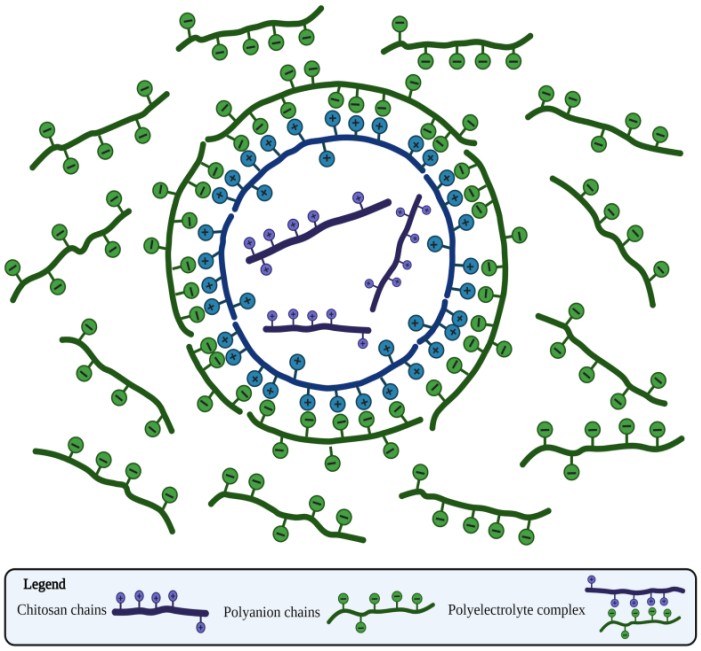


Fig. 3: Idealized structure of a sphere formed by interfacial polycomplexation of chitosan and a polyanion when a drop of chitosan solution is dropped into the polyanion solution.

Some of the most common experimental methods of encapsulation that have been reported using containing-chitosan formulations are: spheres formation by ionotropic crosslinking, i.e., a suspension of cells in a aqueous chitosan solution is dropped over an aqueous solution of sodium tripolyphosphate (STPP) under agitation[26]; cell assemblies confined between layers of chitosan (built layer by layer)[27]; preparation of the gelling mixture containing the cells and its subsequent covalent crosslinking by various chemical routes, i.e., polymerization reactions with thermal initiation[28]; bioorthogonal reactions, in which the experimental conditions must be refined in order to achieve functional materials (considering the complex biological mixtures employed and the presence of living cells)[29]; etc.

Chitosan cross-linking processes in the formation of encapsulating matrices

A great variety of cross-linking processes using chitosan-containing formulations have been reported in the formation of encapsulating matrices. A summary of the most common ones is presented in the following sections.

Cross-linking by physical interactions:

- Precipitation due to pH change: neutralization of an acidic aqueous solution of chitosan ($Q\text{-}NH_3^+ A^-_{(ac)}$) with a base (NaOH) leads to decreasing of chitosan cationic groups, favoring hydrogen bonding and/or hydrophobic interactions and causing its precipitation according to the following reactions:

$Q\text{-}NH_2$ (chitosan) + $HA_{(ac)} \rightarrow Q\text{-}NH_3^+A^-_{(ac)}$
(preparation of a chitosan acidic aqueous solution)

$Q\text{-}NH_3^+A^-_{(ac)} + NaOH_{(ac)} \rightarrow \mathbf{Q\text{-}NH_2}\downarrow + Na^+A^-_{(ac)} + H_2O$
(neutralization and precipitation)

- Aggregation by changing the solvent properties: addition of a miscible solvent (but less polar than water such as 1,2-propanediol) to an aqueous solution of chitosan, in acid medium, causes changes in the properties of the solvent, whose dielectric constant becomes lower[19], unfailingly leading to gelation if right conditions are reached. As in the previous case, hydrogen bonding and hydrophobic interactions will be favored under the new conditions.

- Aggregation due to temperature changes: when the temperature of aqueous solutions of specific chitosan derivatives is increased, hydrophobic aggregates are formed due to the occurrence of a conformational transition which causes gelling of the system, i.e., aqueous solutions of poly(isopropylacrylamide)-grafted chitosan undergo gelling around 29.5 °C[30].

- Ionotropic cross-linking: neutralization of the cationic charges of chitosan with low molecular weight polyanions, such as STPP, generates spherically assembled hydrogels with controllable size. The occurrence of this kind of process has also been reported with other similar polyanions, i.e., adenosine triphosphate (ATP), trisodium citrate, and sodium sulfate[31] (see structures in figure 4).

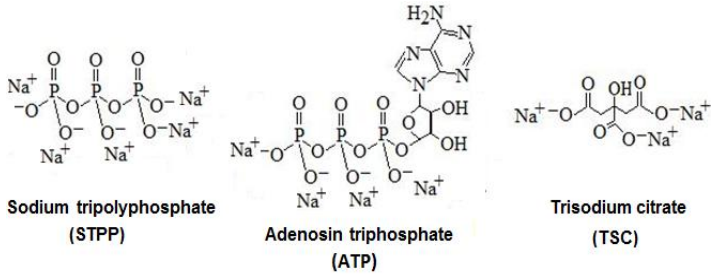


Fig. 4: Chemical structure of some low molecular weight polyanions used as crosslinking agents in ionotropic hydrogelation.

- Formation of polyelectrolyte complexes (PEC): neutralization of electrical charges of opposite sign (positive in chitosan and negative in polyanions) generates composite materials known as chitosan-based polyelectrolyte complexes[32,33], which are also denominated as "chitoplexes"[34]. An important group of these materials, mainly due to their natural origin, are the so-called polyplexes, in which the polyanionic part would be made up of nucleic acids, i.e., plasmidic DNA[35].

Chitosan chemical cross-linking:

- Through hydroxyl groups: a specific example of this type of reaction is its cross-linking with epichlorohydrin[36]; however, it is important to consider that to achieve the selective reaction of the -OH groups in chitosan, usually the primary hydroxyls of the C6 carbon, the amine groups must be previously protected by reactions such as phthaloylation[37] and formation of Schiff bases with aryl-aldehydes[38], which allow their subsequent regeneration. The use of methanesulfonic acid as a solvent has also been reported as a method of protecting amine groups[39]. Other reactions that can lead to cross-linking through the -OH groups, after protection of the amine groups, are reactions with diacyl halides, i.e., adipoyl chloride[39].

- Through amine groups: the most frequently reported covalent cross-linking reaction of chitosan, through the amine groups present on carbon C2, is the formation of Schiff bases with dialdehydes. In this regard, cross-linking using glutaraldehyde has been one of the most studied reactions (see simplified scheme in figure 5), although it has not yet been fully understood due to the complexity involved in this multifactorial process[40]. Although other dialdehydes have also been used for this purpose, such as glyoxal (figure 6a)[41], the current emphasis has been moving to some related compounds, especially those of natural origin, such as genipin (figure 6b)[42] and vanillin (figure 6c)[43], seeking to reduce toxic effects of aldehydes, among other things; nevertheless, it should be noted that cross-linking with this kind of compounds also proceeds through complex mechanisms. Macromolecular dialdehydes has also been assayed to chemical cross-linking of chitosan, i.e., scleroglucan-dialdehyde (figure 6d) obtained by the Malaprade reaction of scleroglucan (oxidation with potassium periodate of the polysaccharide produced by fungi of the genus Sclerotium)[25]. Similarly, a very interesting cross-linking reaction has been achieved using a chitosan-dialdehyde (figure 6e) generated by this same reaction to obtain a cross-linking material containing only chitosan and its dialdehyde derivative[44].

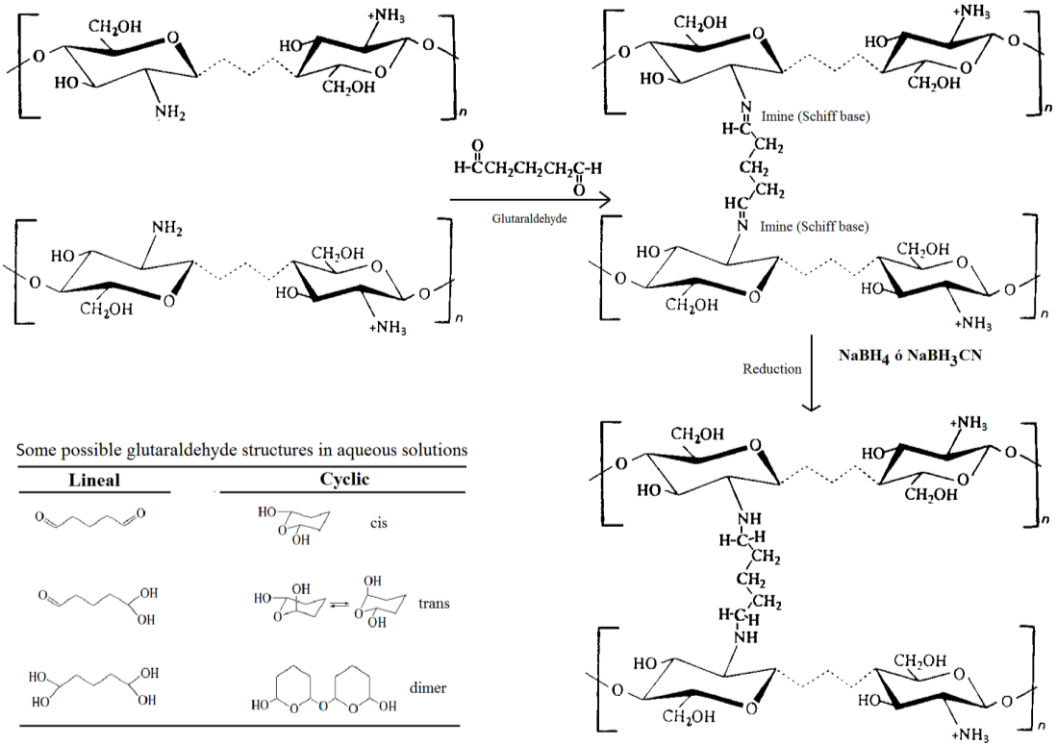


Fig. 5: Simplified scheme of the cross-linking reaction of chitosan with glutaraldehyde via Schiff base formation. The subsequent reduction of the imines and glutaraldehyde structures that can coexist in aqueous solutions are also shown.

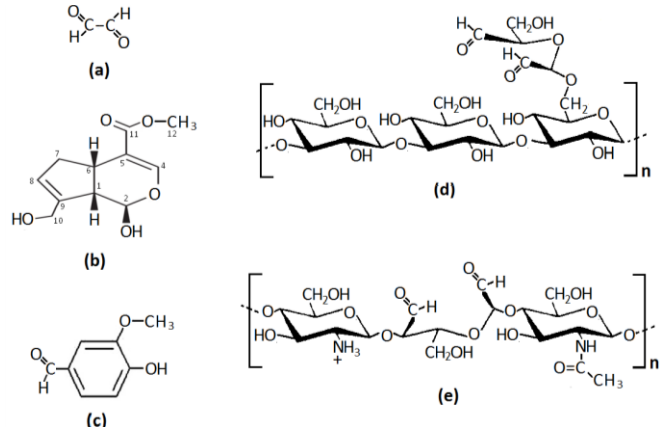


Fig. 6: Chemical structure of some compounds used in the chemical cross-linking of chitosan through the amine group at the C2 carbon: (a) glyoxal, (b) genipin and (c) vanillin, (d) scleroglycan-dialdehyde and (e) chitosan-dialdehyde.

- Through pendant groups added by derivatization: addition of new pendant groups to the chitosan polymer chain can lead to new cross-linking reactions, which allow to obtain novel materials and open new horizons to the versatility of chitosan as a material for use in bioapplications. Generation of these pendant groups can be achieved through a wide variety of chitosan modification reactions, many of which can already be considered routine reactions, through both: amine group at the C2 carbon (acylation, alkylation, quaternization, phosphorylation, sulfation, etc.) as well as hydroxyl groups at C3 and C6 carbons (acylation, alkylation, silylation, halogenation, azidation, etc.)[45]. These derivatives can be subsequently manipulated to establish new processes for cell encapsulation, i.e., coupling of the derivative from 5-azido pentanoic acid and chitosan with ethoxylated glycerol tripropiolate through a click reaction (figure 7), whose product has been assayed with good results in mesenchymal cell encapsulation[24]. Thus, click reactions have increased the prospects for chitosan as promising materials for such applications[29,46].

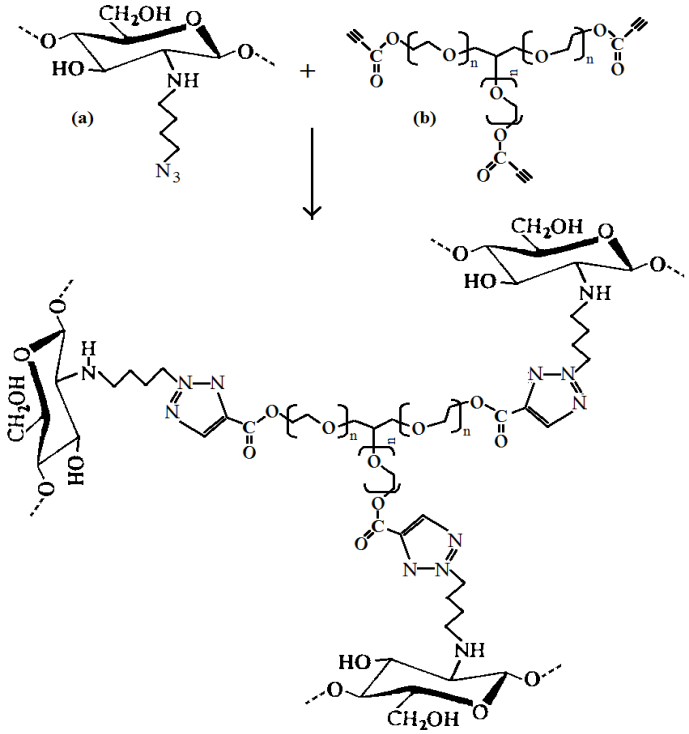


Fig. 7: Chemical cross-linking via click reaction between the derivative from 5-azido pentanoic acid/chitosan (a) and the ethoxylated glycerol tripropiolate (b).

Cell encapsulation using chitosan

Chitosan derivatives, and their combinations with other natural and synthetic polymers, are among the most studied polymeric materials for cell encapsulation[47–52]. Various types of eukaryotic and prokaryotic cells have been used in numerous studies of cell encapsulation with these biopolymers (figures 8-10). Encapsulation of some eukaryotic cells such as chondrocytes[53,54], fibroblasts[47,55], stem cells[56], mesenchymal cells[57], hepatocytes[58], erythrocytes[59], pancreatic β-cells[60], cardiomyocytes[61], etc., have served as the basis for studies focused on cell therapy for the treatment of certain pathologies[48,49,62–65], transplantation and immune tolerance[50,59,65–68], tissue regeneration[47,68] and industrial applications[69]. On the other hand, the encapsulation of bacteria[70–76] has been focused mainly on the oral administration of probiotics[70] and the treatment of some diseases[53]. Each of these topics will be briefly discussed in the following sections.

Eukaryote cells encapsulation

Pathology treatments: chitosan has been used as encapsulating material for mesenchymal stem cells (MSCs) in the treatment of traumatic diseases in which a traumatic injury has occurred, i.e., in the spinal cord (figure 8A); chitosan not only maintains the cellular viability of MSCs but also allows these cells to release vesicles and extracellular trophic factors (growth factors, chemokines, and cytokines), as well as maintain their antioxidant characteristics[48]. MSCs appear to exert a paracrine action that can therapeutically enhance spinal cord regeneration, limiting glial cicatrization[76], reducing cell death at the injured site[77], and acting as

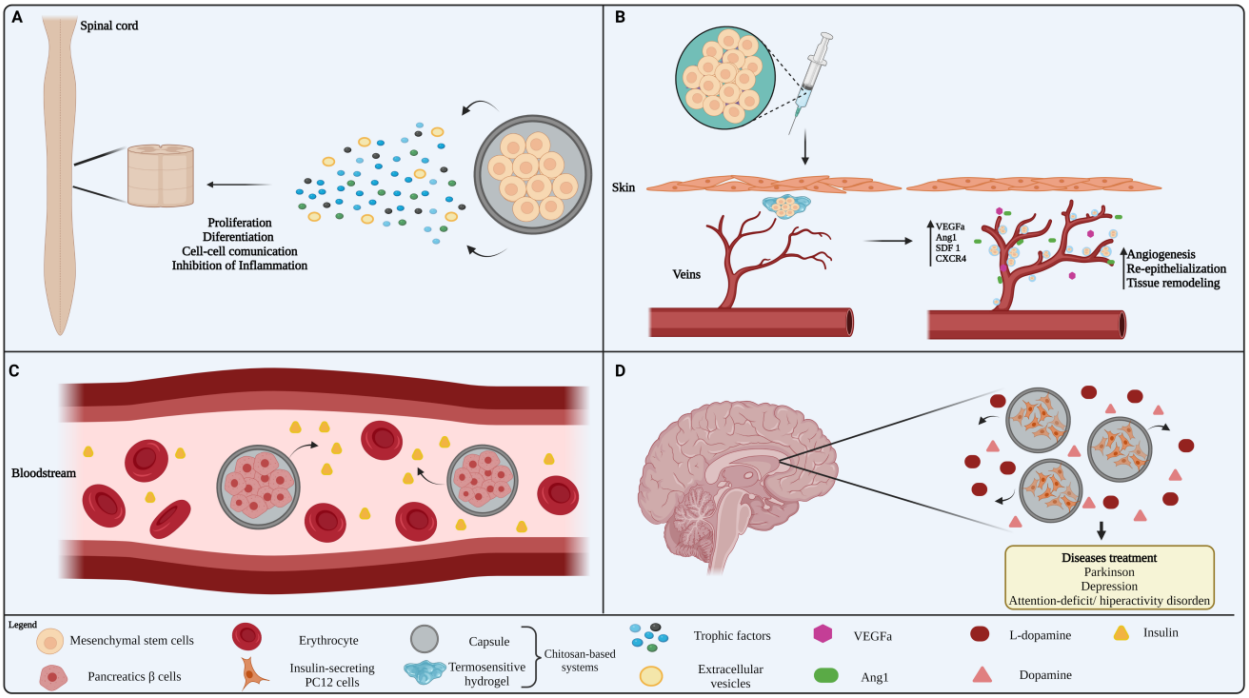


Fig. 8: Encapsulation of eukaryotic cells in chitosan-based systems for treatment of some pathologies. **A.** Traumatic diseases: the release of trophic factors and extracellular vesicles by MSCs promotes the regeneration of the nervous tissue; **B.** Venous diseases: encapsulated MSCs release paracrine factors that modulate inflammation, angiogenesis, and tissue remodeling; **C.** Metabolic diseases such as diabetes: encapsulated pancreatic β-cells could be used as a controlled insulin delivery system for the control of blood glucose; **D.** Neurodegenerative diseases: encapsulation of some neurotransmitter-secreting cells, such as PC12 cells, would be used as a strategy for the treatment of diseases associated with neurotransmitter deficiency or secretory cell dysfunction.

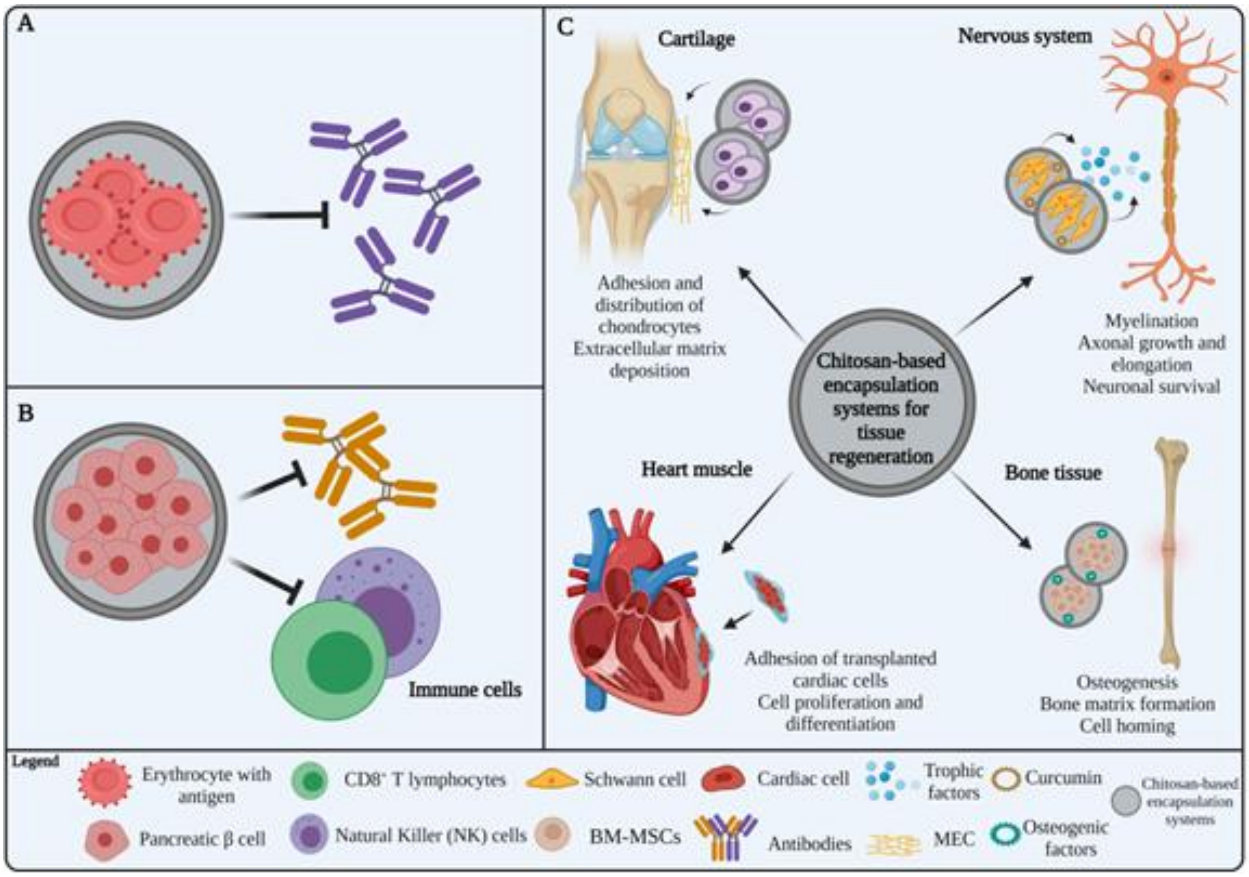


Fig. 9: Encapsulation of eukaryotic cells in chitosan-based systems for tissue transplantation and regeneration: **A.** Erythrocyte transfusion: encapsulation of erythrocytes expressing surface antigens could prevent the antibodies binding to them and, consequently, attenuate recognition of the system host immune; **B.** Pancreatic cell implantation: encapsulation of β-cells would inhibit the adhesion of antibodies to these cells, preventing citotoxicity mediated by natural killer (NK) and CD8 + T cells; **C.** Tissue regeneration: the encapsulation of different types of cells could be a strategy for the regeneration of various tissues (cartilage, nervous system, bone, heart muscle, etc.).

a carrier of signal molecules that regulate cell-to-cell and cell-extracellular matrix communications[48]. Together with MSCs, chitosan could orchestrate the modulation of inflammation, promoting the establishment of a less hostile environment after traumatic injury and, subsequently, the survival of transplanted cells[48].

In other cases, such as diabetes and venous diseases, the injection of heat-sensitive hydrogels of chitosan/collagen/β-glycerophosphate (β-GP) containing three-dimensional spheroidal mesenchymal stem cells (3D MSC) has been studied to accelerate the healing of chronic wounds[62] (figure 8B). The combination of these polymers promotes a conducive environment for encapsulated MSCs, especially accelerating the adhesion, proliferation, secretion, and expression of paracrine factors, such as vascular endothelial growth factor A (VEGFa), angiopoietin 1 (Ang1), factor 1 derived from stromal cells (SDF1) and its chemokine receptor 4 with CXC motif (CXCR4) which, in addition, to reduce inflammation, also promote angiogenesis, re-epithelializa-tion and tissue remodeling in the wound[78].

Besides being proposed for the treatment of venous insufficiency linked to diabetes, encapsulation of pancreatic β-cells in microcapsules of alginate/chitosan (AC) and alginate/chitosan/PEG (ACPEG) could be used as a delivery system for insulin-controlled release for blood glucose control (figure 8C)[49]. These materials could represent a suitable system for pancreatic cell support and insulin secretion. Its permeable-selective nature allows the diffusion of nutrients ant the production and release of insulin[49], offering a therapeutic alternative to traditional treatments of insulin injections and diet. Encapsulation of PC12 cells with chitosan has been evaluated (figure 8D) as a therapeutic strategy for neurodegenerative diseases associated with the loss of dopamine in the cerebral striatum, i.e., Parkinson's disease[79]. PC12 is a dopamine-secreting cell line of great interest in studies of neuroprotective models for Parkinson's disease[80,81]. Besides promoting the viability of PC12 cells, its encapsulation with chitosan stimulates them to produce and release catecholamines and their precursors, such as L-dopa and dopamine, even four weeks after encapsulation[80]. The difference in the secretory capacities of these encapsulated cells is attributed to a possible chitosan interaction with some adhesion molecules present on the cell surface[80]. Therefore, the use of dopamine-secreting cells can be considered as a strategy for treatments of Parkinson's and other diseases associated with dopamine deficiency or secretory cell dysfunction[82,83]. Transplantation and tolerance: microencapsulation is considered a very

promising tool for immuno-isolation in transplantation and immune tolerance studies[84]. In addition to re-presenting an alternative to the chronic suppression of the patient's immune system, which makes these patients vulnerable to other diseases, the encapsulation of living cells serves as an immuno-permeable barrier, increasing cell viability after transplantation. Additionally, these encapsulation systems act as selectively permeable barriers, allowing the free diffusion of nutrients and metabolic waste, and improving cell survival[84].

Some studies have suggested that cell encapsulation with this polymer is a novel and effective strategy in tissue engineering[50,59,65-68,79] (figure 9). Cell transplantation has been proposed as a strategy for the immuno-camouflage of living and functional red blood cells[69]. Encapsulation of erythrocytes in ACPEG capsules could be used to prevent the binding of antibodies to red blood cells and, consequently, to attenuate the recognition of the host's immune system[69] (figure 9A) This strategy would be a great advance in transfusion therapies, since it would allow the production of universal red blood cells, without the use of specific enzymes for the elimination of surface antigens[85,86]. Furthermore, it would be a great advantage in transfusion therapies, especially for rare blood groups[86] or in regions where the frequency of certain blood groups is very low[11]. Transplantation of encapsulated pancreatic β-cells in chitosan-based systems in the treatment of diabetes, additionally to being an alternative for the production of insulin, would function as a barrier minimizing the damage induced by the inflammatory responses to the transplanted cells[49] (figure 9A), contributing to longer life and function during a xenogeneic transplantation[53]. A similar situation can occur for Parkinson's disease, where encapsulation of cells such as PC12 will not only allow the controlled release of dopamine but would also be a method to safely confine these tumor cells and isolate them from the immune system[79].

It should be noted that the immuno-isolating capacity of chitosan microencapsulation is not only attributed to the ability to inhibit the adhesion of antibodies (including IgG) to the transplanted cells[55], but also to the prevention of cytotoxicity mediated by natural T killer cells (NK) and $CD8^{+}$[50] (figure 9B). These cells are crucial in the vertebrate immune system because they act as regulatory agents of the alloimmune response in transplanted patients[89-91]. Notably, $CD8^{+}$ cells can escape to the immunosuppressive effects of drugs such as cyclosporin and rapamycin[91], whereby cell encapsulation with polymers such as chitosan could be an alternative for immune suppression therapy in transplanted patients because of an attenuating effect on immune cells escaping of immunosuppressive drugs effects could be additionally obtained.

Tissue regeneration: due to its biological properties, chitosan has been widely studied as a very promising material in regenerative medicine, being used as scaffolds or platforms for the repair and/or regeneration of various tissues, including skin, bone, liver, cartilage, nerves, and muscle[81] (figure 8C).

Cartilage regeneration: encapsulation of chondrocytes with chitosan-containing systems is considered a great tool in tissue engineering and orthopedics[53,92-95]. The covering obtained with chitosan/hyaluronic acid (HA) fulfilling a temporary function of extracellular matrix (ECM) and creates a favorable chondrogenic microenvironment due to the promotion of deposition of cartilaginous extracellular matrix (CCEM) components by encapsulated chondrocytes[93], facilitating adhesion and uniform distribution of chondrocytes at the implant site[53,94] (figure 9C.1). Furthermore, proliferative activity and differentiation of chondrocytes are stimulated by the presence of these polymers[93]. It should be noted that the encapsulation of adipose tissue-derived stromal cells (ADSC) with chitosan/β-glycerophosphate/starch has been considered as an alternative for the regeneration of cartilage tissue; encapsulation of these cells with these polymers promotes chondrocytic differentiation and CEM accumulation[95].

Nervous system regeneration: several studies have evaluated the encapsulation of neuronal stem cells (NMCs) with chitosan derivatives as a strategy for the repair of nervous tissue[96,97] (figure 9C.1). In murine nerve cells encapsulation studies and injection of neural progenitors-spheroid-type aggregates with self-healing hydrogels (SH-H) of glycol-chitosan and benzaldehyde-difunc-tionalized PEG, at both ends (DF-PEG), induced proliferation and differentiation to neuron-like cells was observed. In addition, cells encapsulated with SH-Hs had the ability to regenerate and rescue neural function in the central nervous system (CNS) of a zebrafish embryo neural injury model (*Danio rerio*), caused by exposure to ethanol[96]. Similarly, the SH-Hs treatment loaded with spheroid neural stem cells (NSCs), additionally to restoring neuronal functions, had a positive influence on the development and hatching rate of treated embryos. The advantage of these neural progenitors encapsulated with SH-Hs could be attributed to their ability to fill physical spaces associated with injurie[97] and facilitate metabolism, oxygen availability, migration and cell-cell communication, creating an adequate microenvironment for the proliferation of encapsulated NSCs[96,97]. On the other hand, the encapsulation of Schwann cells (SCs) with chitosan has also been studied[98] (figure 9C.1). SCs are glial cells that play an important role in the regeneration of the injured peripheral nervous system (PNS)[99]. In this study, the sciatic nerve regeneration was evaluated *in vivo* using artificial neural guide channels of poly-L-lactic acid contained with SCs and curcumin encapsulated in chitosan nanoparticles[98]. Treatment with these nanoparticles induced a significant increase in the number of axons in the injured sciatic nerve, as well as a restoration of motor and sensory function[99].

In these systems, SCs would play an important role in nerve regeneration through the release of neurotrophic factors, i.e., neurotrophic factor derived from the glial ell line (GDNF)[100] and growth factors such as nerve growth factor (NGF)[101], which contribute to the myelination process, promotion of growth and axonal elongation, as well as survival of neurons[100,101] (figure 9C.1). On the other hand, curcumin would act as a factor to decrease apoptosis[98] and stimulate the proliferation of SCs[102] and, consequently, improve the regeneration and functional recovery of injured nerve. Encapsulation and transplantation of SCs together with compounds that facilitate their activity could have a great influence on the therapeutic activity of these cells, notably improving neuronal regeneration therapy.

Bone regeneration: the encapsulation of osteoblasts with chitosan hydrogels has been proposed as a method to transport osteoblast cells in bone disorders treatments[103] (figure C.3). A greater adhesion, proliferation, and expression of type 1 collagen (collagen more abundant in the vertebrate ECM) was achieved through the manufacture of a 3D tracing system to make tissue scaffolds based on pure chitosan and chitosan cross-linked with pectin and genipin, as well as a higher mineralization activity in osteoblast cells *in vitro*[103]. Likewise, some reports based on the encapsulation of stromal MSCs derived from human bone marrow (BM-MSCs), which can self-renew and differentiate into multiple cell lines, demonstrated that its encapsulation in chitosan/dextran hydrogels not only maintained their viability but could also differentiate into adipocytes and osteocytes[104]. Similarly, encapsulation of BM-MSCs together with osteogenic factors, such as bone morphogenic protein-2 (BMP2), in chitosan/poly (ε-caprolactone) heat-sensitive gels have a positive effect on osteogenesis and bone matrix formation[105] (figure 9C.3). More importantly, the encapsulation of these MSCs not only influences their proliferation and differentiation, but they could also serve as an alternative to take advantage of some signaling pathway, such as the stromal cell-derived factor-1 (SDF-1)/CXC receptor 4 (CXCR4) route, very important in the process of mobilization and relocation or "homing" of MSCs[106,107]. Studies focused on MSCs derived from human adipose tissue (hASCs) revealed that after being injected and promoted the over-expression of their chemokine receptor CXC type 4 (CXCR4) these cells had the ability to respond and migrate towards the derived stromal cell factor (SDF-1a), which was released from a injectable thermosensitive hydrogels of chitosan/ glycerolphosphate/ hydroxylethylcellulose (CH/GP/ HEC)[107]. The expression of CXCR4 in cells and the concomitant release of its ligand SDF-1a from CH/GP/HEC hydrogels led to increased localization/retention of hASCs[107]. In addition to the massive infiltration of hASCs, in response to SDF-1a, a process of close vascularization was observed, which could indicate that these hydrogels would act as optimal supports for the migration of endogenous cells, which could facilitate repair and regeneration of tissues.

Regeneration of cardiac muscle tissue: options for the treatment of myocardial infarction are very limited[62] due to the inability of the mature myocardium to regenerate[109]. However, encapsulation of cardiac cells (cardiomyocytes and myoblasts) in photo-crosslinkable hydrogels, obtained from azidobenzoic acid-chitosan- and acryloyl-poly(ethylene glycol)-RGDS (Az-chitosan/Acr-PEG-RGDS), was evaluated as an alternative for regeneration of cardiac tissue (figure 9C.4), obtaining evidence of adhesion, proliferation and differentiation of encapsulated C2C12 myoblasts[62]. Likewise, a high viability of neonatal rat cardiomyocytes encapsulated in these photo-crosslinkable hydrogels was observed. Importantly, when adhesion of these hydrogels in the cardiac tissue was evaluated, it was evident that they remained adhered in the different parts of the heart where were applied, both on the surface (epicardium) and within the ventricle, a relevant fact for the treatment of myocardial infarction[62].

Other applications: chitosan microencapsulation of some yeasts has also been studied for therapeutic and industrial purposes[76,110,111]. Encapsulation of the probiotic *Saccharomyces boulardii* in alginate/chitosan (AC) microspheres showed to have positive effects on its survival, protecting it from acid degradation and accelerating its transit through the gastrointestinal tract[76]; the use of this yeast with similar microencapsulation systems could be of great application not only for the therapies of inflammatory bowel diseases[112] but also for infectious enteritis[113] and enterocolopathies associated with *Clostridium difficile*[114]. On the other hand, the use of alginate/chitosan/alginate (ACA) and genipin/alginate/chitosan (GAC) has been proposed for industrial applications as an attractive method for the encapsulation of yeasts in the production of bioethanol[111]; these systems would improve the stability of the cells and the tolerance to the inhibitors, increasing the amount of biomass inside the reactor and decreasing the cost of recovery, as well as recycling and subsequent processing of the cells. Apparently, encapsulation with systems such as ACA and GAC attenuates the effect of ethanol concentration on yeast growth, which would imply a protective action related to tolerance to stress conditions in the culture.

Encapsulation of bacteria

Administration of probiotics: one of the main challenges in supplementing food with probiotics is that these can remain active in different environmental conditions. In addition to resisting oxygen exposure while functional food products are in storage, probiotics must face up to the host's harsh gastrointestinal conditions (such as gastric pH, bile salts, and enzymes) once ingested[115,116]. Thus, microencapsulation is classified as one of the main solutions for the preservation of probiotics, especially that based on some polymers such as chitosan[71,116-119]. Chitosan has been used in the protection of probiotic cells mainly as a coating/covering, and not as the capsule itself[72,73,120]. Some studies carried out with different bacterial strains have shown that the use of alginate microcapsules

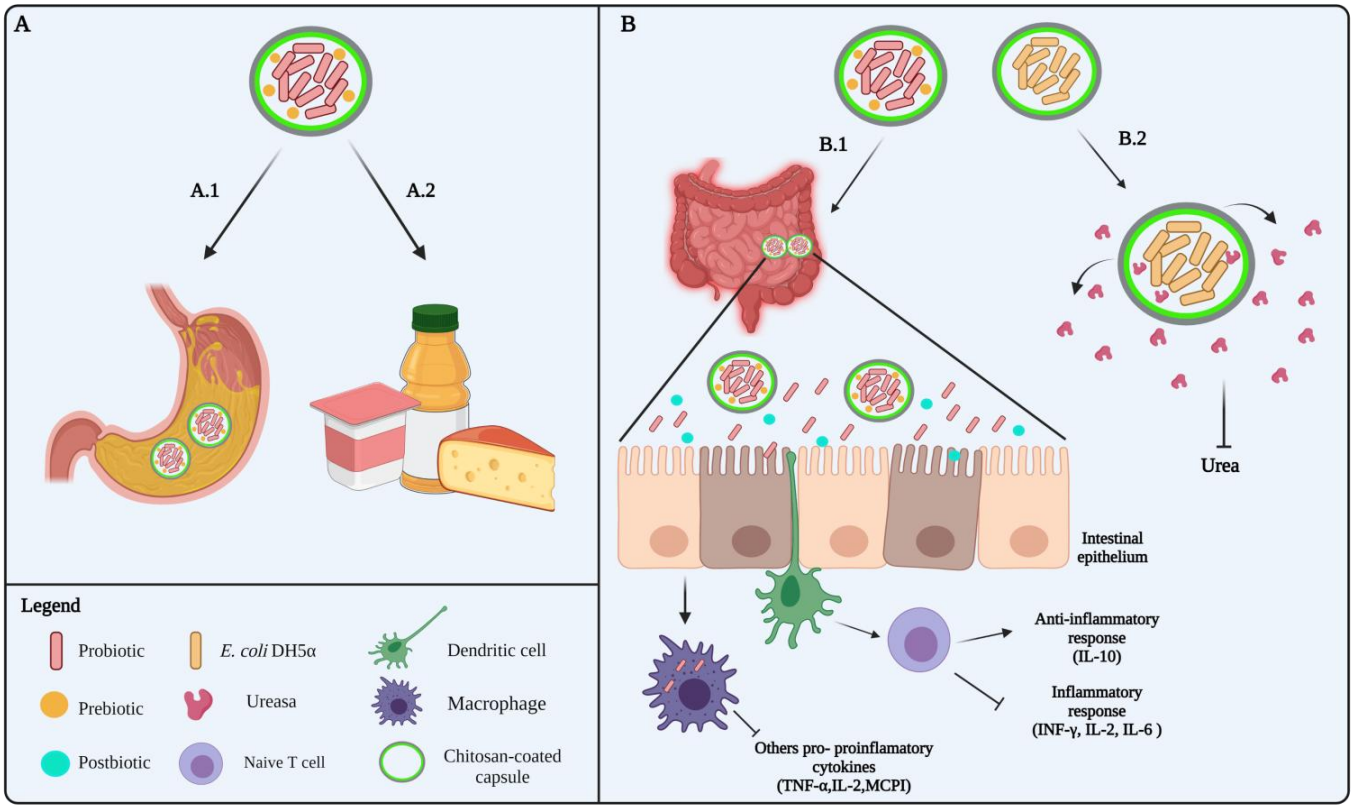


Fig. 10: Encapsulation of prokaryotic cells in systems containing chitosan. **A.** Probiotic protection would allow the storage and protection of the organism in different environmental conditions: **A.1** Efficient protection of probiotics in extreme conditions of stomach pH, bile and digestive enzymes, resulting in a greater number of viable cells in the intestine, **A.2** Confinement of probiotics could contribute to the stability of the microorganism in food matrices; **B.** Encapsulation of probiotics could be used in the treatment of some pathologies such as: **B.1** Bowel inflammatory diseases taking advantage of its anti-inflammatory effect, **B.2** Disorders associated with chronic kidney diseases, i.e., uremia, through overexpression and release of recombinant urease in genetically modified bacteria.

coated with chitosan is the best option for the storage and protection of probiotic bacteria, such as Lactobacillus and Bifidobacterium spp., under different experimental conditions[64,65,67]. Furthermore, chitosan-coated pectin capsules have been reported to efficiently protect Lactobacillus casei CIMB 30185 from extreme stomach pH conditions, resulting in increased numbers of viable cells in the intestine[68].

In addition to protecting or improving the efficiency of the probiotic, some symbiotic encapsulation systems based on chitosan have been developed[71]. In these systems, contrary to others, a prebiotic or a specific carbon source of this is added[120] (figures 10A and 10B) which, in addition to serving as a substrate, can contribute to the stability and survival of the probiotic. A study using symbiotic systems based on AC/*L. casei*/selenium-enriched green tea (TVS) showed that the presence of TVS increases the probiotic survival at a storage temperature of 4 °C, under experimentally simulated gastric and bile solution conditions[71]. Similarly, the co-encapsulation of anthocyanins with *L. casei*, in addition to having a positive effect on the survival of the probiotic in simulated gastric conditions, improves the stability of the microorganism in food matrices such as yogurt[121]. Furthermore, the use of other prebiotics such as inulin and starch has been reported in the co-encapsulation of lactic bacteria such as *Lactobacillus acidophilus*[122,123]. Comprehensively considered, these studies prompt that chitosan encapsulation and/or coating systems can lead to remarkable advances in the development of food and nutraceutical ingredients with markedly improved functionalities.

Treatment of diseases: the encapsulation of bacterial cells in AC gels has been proposed as an oral therapy strategy for some disorders such as inflammatory bowel diseases (Crohn's disease and ulcerative colitis) and uremia[63,64] (figures 10B.1 and 10B.2, respectively). Encapsulation of bacteria such as *Escherichia coli* strain Nissle 1917 (EcN), an organism with probiotic properties, was shown to have an anti-inflammatory and immunomodulatory effect in a colitis rat model[64]. The anti-inflammatory effect of probiotics is attributed to the modulation of the immune system in the intestinal micro-environment[124], specifically through the modulation of the function of some immune cells, such as dendritic cells (DCs) and macrophages, and intestinal epithelial cells, mediating the activation of pattern recognition receptors (PRR) such as Toll-like receptors (TLR) expressed on cell surfaces[125]. Probiotic binding to some of the

TLRs, i.e., TLR2, can inhibit the secretion of cytokines and pro-inflammatory mediators, such as monocyte chemoattractant protein 1 (MCP1), tumor necrosis factor-alpha (TNF-α), interleukins (IL-6, IL-2), but in turn promotes an increased expression of anti-inflammatory cytokines (IL-10)[64,125,126] (figure 10B.1) through the regulation of some signaling pathways, such as the NF-κB pathway and others such as that one triggered by mitogen-activated protein kinases (MAP kinases)[127]. Furthermore, some molecules produced and released by organisms such as bifidobacilli and lactobacilli, also known as postbiotics, can contribute to the anti-inflammatory effect of these organisms. These molecules, which are mainly short-chain fatty acids (SCFA), in particular propionate, acetate and butyrate, apparently exert their action by binding to specific receptors on intestinal epithelial cells (figure 10B.1). Association with these receptors induces the inhibition of the NF-κB signaling pathway and the production of pro-inflammatory cytokines by macrophages[128,129]. Similarly, these fatty acids can promote the induction of differentiation and expansion of regulatory T cells[130]. The encapsulation of some postbiotics with chitosan would be an alternative for the therapy of inflammatory diseases in immune-deficient patients, which could be affected by the administration of bacteria. This could become an interesting topic of study in the very near future.

In the treatment of uremia, a disorder associated with chronic kidney diseases, a genetically manipulated strain of a *Escherichia coli* DH5 harboring the gene encoding urease was used as a model for *in vitro* and *in vivo* evaluation of the ACA microcapsules in oral therapy of this disease; these studies revealed that encapsulation not only had a protective effect on the survival of cells in the gastric environment but also that encapsulated cells could remove urea from the medium[63] (figure 10B.2), suggesting that microencapsulation could allow safe and effective oral administration of live bacterial cells for various clinical applications (figure 10B.2).

Concluding Remarks

Cell encapsulation has become a remarkably successful tool whose utilization seems to extend into different biotechnological fields given its potential to improve key aspects of *in vitro* and *in vivo* cell cultures, including proliferation and differentiation processes, especially in terms of providing greater protection to cells and avoid its recognition by the defense mechanism of the hosts. After 70 years of its initial implementation, it can be said that cell encapsulation is here to stay. Moreover, the development of new and exciting biomaterials over time, which has accelerated dramatically in recent years, seems to guarantee new successes in the years to come.

The valuable biological properties of chitosan, derived from its natural origin, have allowed its approval as an excipient by the European and American pharmacopoeia (chitosan hydrochloride[131] and chitosan[132], respectively). Thus, being chitosan a biomaterial so widely studied for promising applications in areas related to biotechnology such as biomedicine, food, agriculture, etc., it is believed that there will be a significant growth in research on new processes for obtaining it with higher purity indices and from new sources, as well as also in the preparation of derivatives specially designed to achieve specific objectives in cell encapsulation. In this context, click reactions can be seen as one the most logical routes to obtain new encapsulation methods using chitosan derivatives, although this field remains practically virgin due to the existence of a wide variety of others chemical reactions that could theoretically be incorporated into this scheme but they are still awaiting their experimental trial.

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