Brewer's Yeast Polysaccharides – a review of their exquisite structural features and biomedical applications

Rita Bastos[a], Patrícia G. Oliveira[a,b], Vítor M. Gaspar[b], João F. Mano[b], Manuel A. Coimbra[a], Elisabete Coelho[a]*

[a] LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[b] CICECO–Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

*Corresponding author.

Email address: ecoelho@ua.pt

ABSTRACT

Recent advances on brewer's yeast cell wall polysaccharides have unraveled exquisite structural features and diverse composition with (β1→3), (β1→6), (α1→4), (β1→4)-mix-linked glucans that are recognized to interact with different cell receptors and trigger specific biological responses. Herein, a comprehensive showcase of structure-biofunctional relationships between yeast polysaccharides and their biological targets is highlighted, with a focus on polysaccharide features that govern the biomedical activity. The insolubility of β-glucans is a crucial factor for binding and activation of Dectin-1 receptor, operating as adjuvants of immune responses. Contrarily, soluble low molecular weight β-glucans have a strong inhibition of reactive oxygen species production, acting as antagonists of Dectin-1 mediated signaling. Soluble glucan-protein moieties can also act as antitumoral agents. The balance between mannoproteins-TLR2 and β-glucans-Dectin-1 receptors-activation is crucial for osteogenesis. Biomedical applications value can also be obtained from yeast microcapsules as oral delivery systems, where highly branched (β1→6)-glucans lead to higher receptor affinity.

Keywords: Brewer's spent yeast; beta-glucan, mannoprotein, microcapsule, regenerative medicine, drug delivery

1. Introduction

Saccharomyces sensu stricto, including *S. cerevisiae*, *S. bayanus*, and *S. pastorianus* (the latter also known as *carlsbergensis*), are by far the most used yeasts in the brewing industry. Industrial handling of *Saccharomyces* yeasts is mainly due to their fast growth, good capacity to produce ethanol and a great tolerance against environmental *stress*, including high ethanol concentration and low oxygen levels **(Ferreira et al., 2010)**. Brewer's yeast is conventionally divided into two main classes: the top-fermenting (*ale*) and the bottom-fermenting (*lager*) yeasts. Lager beers produced by bottom-fermenting *S. pastorianus* yeasts are the most widespread beer types throughout the world and contribute to more than 90 % of the total beer market **(Dequin & Casaregola, 2011)**. More than the crucial role of yeast in beer production, the brewer spent yeast (BSY) represents a promising source of value-added compounds and low cost raw material **(Ferreira et al., 2010)**. Yeast cell wall polysaccharides, namely glucans and mannoproteins, have been widely studied and gained commercial interest for a broad spectrum of industrial applications. Although the initial focus for yeast polysaccharides valuation has been placed on food and feed industry as dietary fibres, emulsifying agents or fat replacers, currently there is an increasing interest for numerous pharmaceutical and biomedical applications, with an emerging focus in immunology, tissue engineering, vaccines, and drug delivery systems being observed in recent years **(Freitas et al., 2015)**.

This review exploits the brewer's yeast cell wall polysaccharides and the most recent information on the structure, including new exquisite structural features that could potentiate BSY valorization for biomedical applications. Herein properties as immune response modifiers and valuation perspectives for the biomedical field, including antitumoral activity, bone regeneration, antioxidant activity, and drug delivery were described for the yeast cell wall polysaccharides.

2. Brewer's yeast polysaccharides – focus on structural features

Saccharomyces cell wall represents 15–25 % of the cell dry weight and has a complex structure, composed by up to 90 % polysaccharides, generally organized in a cell wall system of layers (Figure 1): the outer amorphous layer of phosphorylated mannoproteins; the middle layer of alkali-soluble glucans; and an inner rigid layer of alkali insoluble glucans and chitin, which contributes to the cell wall shape and rigidity **(Aguilar-Uscanga & Francois, 2003; Fleet & Manners, 1976; Klis et al., 2006; Kollár et al., 1997)**. Its three-dimensional (3D) shape is preserved upon alkali extraction **(Bastos et al., 2015; Pinto et al., 2015)** but is destroyed after chlorite oxidation, allowing one to hypothesise that the three-dimensional shape of the yeast cell wall is preserved by a network of glucans and proteins **(Coelho et al., 2015)**. Beyond the maintenance of the cell shape, the yeast cell wall stabilizes the internal osmotic conditions, protects against physical stress, acts as a molecular sieve, a scaffold of proteins, and controls molecular recognition and cell adhesion **(Alsteens et al., 2008)**. The cell wall was considered for a long time as an inert exoskeleton. However, it is well established nowadays that yeast cell wall has as a dynamic structure that is continuously changing as a result of environmental modifications **(Latgé, 2007).** In particular, polysaccharides that constitute the cell walls of brewer's spent yeast are structurally different from those of the yeast before the industrial process due to modifications promoted by its adaptation to the stress of fermentation conditions **(Bastos et al., 2015; Latgé, 2007)**.

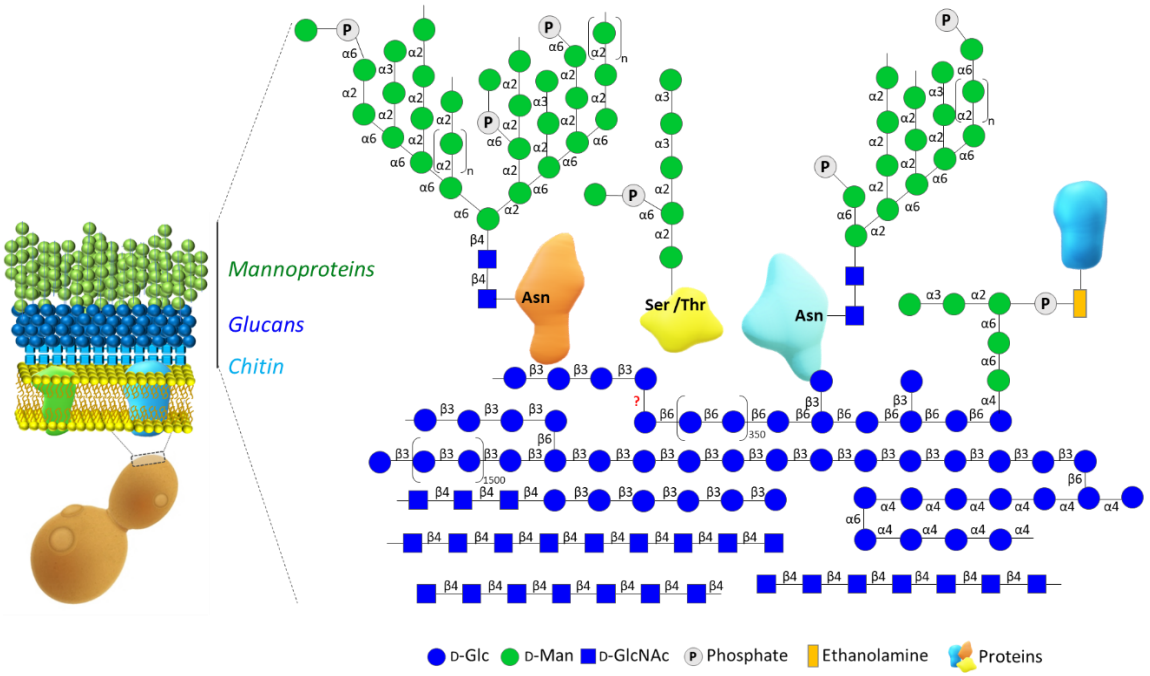


Figure 1: Schematic overview of yeast cell wall structure and composition. The polysaccharide structures were built according the motifs reported by **Arvindekar and Patil (2002); Klis et al. (2002); Kollár et al. (1997); Latgé (2007); Lipke and Ovalle (1998); Manners, Masson, and Patterson (1973)**. Abbreviations: Glc – Glucose, Man – Mannose, GlcNAc – N-Acetylglucosamine, Asn- Asparagine, Ser- Serine, Thr – Threonine.

2.1. β-Glucans

Saccharomyces cell wall is generally described as being comprised by *β*-glucans (55-65 % dry basis) linked through (*β*1→3)- and (*β*1→6)-D-Glc linkages (Glc - Glucose, Figure 2A and 2B). The (*β*1→3)-D-glucans, branched through (*β*1→6)-D-Glc linkages, constitute 80-90 % of the cell wall inner layer, establishing a fibrous network with an average degree of polymerization (DP) of ~1500 residues and a molecular weight of 240 kDa **(Klis et al., 2006)**. The fibre length of (*β*1→3)-glucans can reach about three to six times the average wall thickness with a maximum of 600 nm **(Lipke & Ovalle, 1998)**. Most (*β*1→3)-D-glucans have a helical conformation formed by a single or three hydrogen-bonded polysaccharide chains (triple helix). These helix structures confer elasticity to yeast cell wall **(Kwiatkowski et al., 2009)**. The (*β*1→3)-glucans function as

covalent attachment site to the other wall components , Chitin chains can be cross-linked through a (β1→4)-linkage to (β1→3)-D-glucans non-reducing end (Figure 2C) **(Lesage & Bussey, 2006; Manners, Masson, & Patterson, 1973)**. Cell wall mannoproteins, such as Pir proteins (**P**roteins with **i**nternal **r**epeats), can directly connect to (β1→3)-D-glucans through an ester linkage between a hydroxyl group of the glucan and the γ-carboxylic group of a glutamic acid residue from Pir repeated sequences (Figure 2E). Because most of Pir proteins can contain several repeat sequences, they may provide sites for cross-linking of multiple (β1→3)-D-glucans **(Ecker et al., 2006; Levin, 2011)**.

The (β1→6)-D-glucans are highly branched polysaccharides shorter than (β1→3)-D-glucans, with an average degree of polymerization of ~150-200 residues and molecular weight of 24 kDa (Figure 2B). These glucans account for 8-18 % of the remainder cell wall inner layer and have an adhesive role in cell wall organization. The (β1→6)-D-glucans connect the lipidless glycosylphosphatidylinositol dependent cell wall mannoproteins (GPIr-CWP) to the (β1→3)-D-glucans network (Figure 2F) and may also function as acceptor site for chitin, particularly in case of cell wall stress **(Klis et al., 2002; Manners, Masson, Patterson, et al., 1973)**.

The yeast cell wall glucans are generally classified in three classes based on their solubility in aqueous solutions, namely water-soluble, alkali soluble, and alkali-insoluble glucans. The major fraction, that accounts for ~35 % of cell wall dry weight, is the alkali-insoluble (β1→3)-D-glucans and (β1→3)-linked side chains (3-6 %) linked through (β1→6)-D-linked Glc residues (Figure 2A). The bond between the reducing end of (β1→6)- and (β1→3)-D-glucans still remains an uncharacterized linkage **(Lesage & Bussey, 2006; Manners, Masson, & Patterson, 1973)**. Alkali-insoluble (β1→3)-D-glucans establish linkages to chitin chains, rendering the structure insoluble (Figure 2C). These glucans contribute to the maintenance of cell wall mechanical strength and shape **(Fleet & Manners, 1976; Kollár et al., 1997)**. The second fraction comprises the alkali-

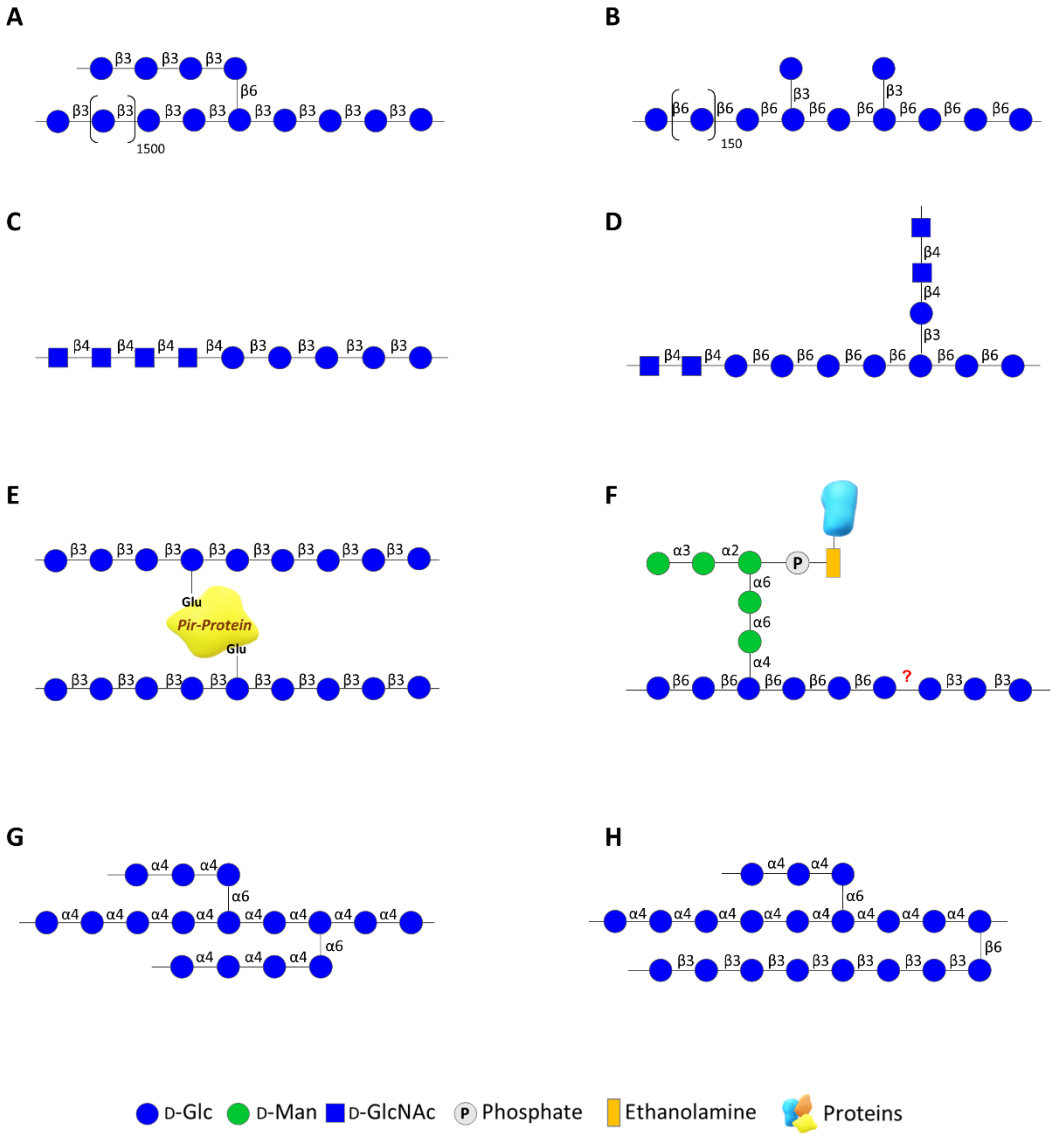
soluble (*β*1→3)-D-glucans (~20 % of cell wall dry weight) with higher branched structure than the insoluble ones, which confers flexibility to the cell wall. The alkali-soluble (*β*1→3)-D-glucans seem not to establish cross-linkages with chitin **(Hartland et al., 1994)**. The third fraction is composed of water-soluble glucans and comprises (*β*1→6)-D-glucans that are short polymers and highly branched structures, with around 14 % of side-branching with a (*β*1→3)-D-Glc residue (Figure 2B) **(Manners, Masson, Patterson, et al., 1973)**.

2.2. Glycogen

Glycogen is an *α*-glucan linearly linked by (*α*1→4)-D-Glc and highly branched via (*α*1→6)-D-Glc linkages (Figure 2G). Glycogen is an important energy reservoir that impacts *Saccharomyces* fermentation performance, promotes viability during storage, and provides energy for yeast metabolic activities in times of starvation **(Stewart & Russell, 1986)**. In *Saccharomyces*, glycogen occurs in two pools: the soluble glycogen located in cytosol, and the insoluble glycogen covalently linked to the cell wall (*β*1→3)-D-glucans through a (*β*1→6)-linkage (Figure 2H) **(Arvindekar & Patil, 2002)**. A third pool of glycogen on the cell surface of bottom fermenting yeast *Saccharomyces* was also described. When the cell surface glycogen is removed through enzymatic treatment, the resultant cells lost the capacity of flocculation and form poor aggregates, compromising yeast cell performance and viability **(Dake et al., 2009)**. On yeast cell wall, the glycogen content can vary from 1 % to 29 % (dry basis), depending upon the nutritional status of the cells, the method of isolation, the method of analysis, and the phase of growth during which the cells were harvested **(Lillie & Pringle, 1980)**

2.3. Chitin

Chitin, a linear polymer of (β1→4)-linked GlcNAc (*N*-acetylglucosamine), is the minor constituent of *Saccharomyces* wall (1 to 2 % cell wall dry weight). In the cell wall, chitin chains occur in three forms: (i) free chains (40 %), (ii) linked to the nonreducing end of (β1→3)-D-glucans via (β1→4)-linkage of chitin reducing end (40-45 %) (Figure 2C) or (iii) linked to (β1→6)-D-glucans non reducing end or to a side-branch (β1→3)-linked Glc (15-20 %) (Figure 2D) **(Cabib, 2009)**. Chitin levels can vary according to the cell division stage and increase as a response to environmental stress. Although D-GlcNAc quantification is conditioned by the cell walls extraction method, the average length of chitin chains is estimated as being in the range of 110-190 D-GlcNAc residues. Chitin is concentrated at the budding neck of the yeast cell wall where its crystalline structure confers stretching resistance to the cell wall **(Klis et al., 2002; Lesage & Bussey, 2006)**.



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148 **Figure 2:** Structure of different glucans that occurs in yeast cell wall. A-B: (*β*1→3)- and
149 (*β*1→6)-D-glucans; C-D: (*β*1→3)- and (*β*1→6)-D-glucans linked to chitin; E: (*β*1→3)-D-
150 glucans crosslinked through Pir–proteins; F: Complex between a GPIr–CWP, (*β*1→6)-
151 and (*β*1→3)-D-glucans; G: Glycogen; and H: Glycogen linked to (*β*1→3)-D-Glucans.
152 Abbreviations: Glc – glucose, Man – mannose, GlcNAc – *N*-acetylglucosamine, Glu –
153 glutamic acid

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2.4. Mannoproteins

The chemical structure of the cell wall outer mannan layer is characteristic of the yeast species and can be used as a taxonomic aid. Yeast wall mannoproteins are highly glycosylated polypeptides, often containing 50 to 95 % carbohydrate by weight. Cell wall mannoproteins of Saccharomyces species can be divided in two classes (Figure 3). The *N*-linked mannoproteins are comprised of 10 % of protein and 90 % of carbohydrate moiety, with 50 to 200 α-linked mannose units. These mannans have a long (α1→6) linked backbone branched with short (α1→2) and (α1→3)-Man (Man – mannose) linked side chains and are attached to proteins through an *N*-glycosidic bond between two D-GlcNAc residues and the asparagine amide nitrogen **(Lesage & Bussey, 2006)**. The *O*-linked mannoproteins have higher content of protein moiety (50 %) and are characterized by short chains of up to five mannose units, with the first two residues (α1→2)-linked and the subsequent ones (α1→3)-linked. The linkage to protein moiety is established *via* the hydroxyl side chains of serine or threonine residues. Despite the small size of the *O*-linked chains, many cell wall proteins have serine/threonine-rich domains, resulting in a high number of *O*-chains per protein and a consequent high amount of *O*-linked mannose in the cell walls **(Lesage & Bussey, 2006; Loibl & Strahl, 2013)**. An important structural feature of yeast cell wall mannoproteins is the presence of phosphorylated mannose residues. Mannosylphosphate gives a net negative charge to cell wall mannoproteins, relevant for water retention and yeast flocculation and protection **(Jigami & Odani, 1999)**.

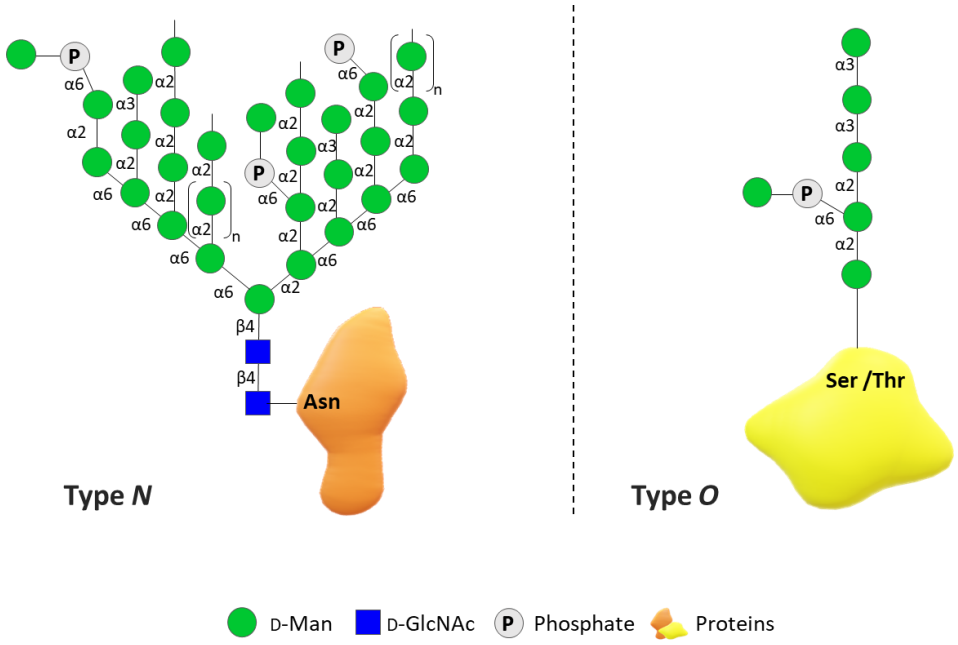


Figure 3: Schematic structure of *N*-linked and *O*-linked mannoproteins that naturally occur in Saccharomyces yeast cell wall. Illustration built according to **Jigami and Odani (1999)**. Abbreviations: Man – mannose, GlcNAc – *N*-acetylglucosamine; Asn – asparagine; Ser – serine; Thr – threonine.

2.5. The exquisite structural features of spent yeast polysaccharides arising from brewing process

Although hundreds of papers still look to *Saccharomyces* yeast cell wall as a well-defined system of layers comprised mainly *β*-glucans linked by (*β*1→3)- and (*β*1→6)-D-Glc **(Avramia & Amariei, 2021)**, the presence of (*β*1→4)-D-Glc linkages as components of cell wall alkali insoluble glucans was already reported for the brewer's *Saccharomyces* yeast **(Bastos et al., 2015; Pinto et al., 2015)**. The structural analysis of yeast cell wall glucans is highly challenging and generally hampered by their insoluble nature, being one of the main reasons why researches investigating yeast cell wall structure usually focus on the soluble material that is recovered **(Fleet & Manners, 1976; Huang, 2008; Shokri et al., 2008)**. Also, a structurally detailed characterization of glucans usually requires their prior solubilization. However, an alkali-insoluble glucan rich residue still remains after organic, acid, or alkali extraction, which can mask important structural details, as shown

for *Saccharomyces pastorianus.* Even after an extensively organic, water and alkali extraction sequential procedure, the alkali-insoluble residue was still composed by around 50 % of (1→4)-D-Glc linkages. The insoluble glucans of this residue, when hydrolysed with α-amylase (endo (α1→4)-D-glucanase) and cellulase (complex of three different (β1→4)-D-Glc*p* hydrolases: endoglucanase, exoglucanase, and β-glucosidase), showed that (1→4)-linked glucans accounted for 60 % (α1→4)-linked residues and 40 % were (β1→4)-linked, respectively **(Pinto et al., 2015)**. (β1→4)-D-Glc linked glucans are constituents of cellulose of plant cell walls or cereal mixed-linked glucans but were not described before for *Saccharomyces* cell wall components.

The report of both (α1→4)- and (β1→4)-linked cell wall glucans open new questions to the state of the art about *Saccharomyces* cell wall (1→4)-D-glucans, when described as insoluble glycogen, and brings new perspectives to understand the distribution of these new exquisite *Saccharomyces* cell wall linkages. Do they occur as cellulose-like structures, as mixed-linkages or interconnected with other cell wall components?

Brewer's yeast cell wall polysaccharide composition and structure are highly influenced by environmental conditions during brewing process. Industrial fermentation imposes numerous environmental stresses to yeast cells, which ultimately affect their viability and bioactivity (i.e., temperature shock, osmotic stress, oxidative stress, oxygen availability, hydrostatic pressure, ethanol concentration, internal acidification, and nutrient limitation) **(Ekberg et al., 2013; Gibson et al., 2007)**. Furthermore, a single batch of a lager yeast such as *S. pastorianus* may experience stressful conditions several times, as it is commonly recycled in multiple fermentations, until a maximum of 17-20 repitchings before being discarded **(Bühligen et al., 2014; Bühligen et al., 2013)**. The brewing yeasts are generally considered resistant to stress mainly due to their rigid cell wall that acts as a primary physical barrier. Still, the cell wall architecture is highly

dynamic in nature, modifying its composition and functionality in response to external stress and stimuli **(Klis et al., 2002)**. The cell wall polysaccharides of a BSY can reveal different structural features. The analysis of insoluble glucans from *S. pastorianus* inoculum yeast (before the brewing process) showed that the inoculum presented the same ratio of (α1→4)-linked and (β1→4)-linked glucans as observed for BSY. However, throughout the brewing process, *S. pastorianus* increases both (α1→4)- and (β1→4)-linked cell wall glucans, with the concomitant decrease of the relative percentage of (β1→3)-D-glucans **(Bastos et al., 2015)**. During an alcoholic fermentation where 8 % (v/v) of ethanol production is achieved, the cell wall glycogen can exponentially increase 3-fold more than intracellular glycogen **(Dake et al., 2011).** The presence of a higher glycogen content in cell wall seems to be important for yeast osmotic tolerance during the recycling between fermentative processes **(Lillie & Pringle, 1980).** The increase in cellulose-like (β1→4)-D-Glc linkages was suggested as a requirement for yeast to reinforce the cell wall rigidity and strength to prevent the cellular rupture **(Bastos et al., 2015)**. During brewing process, (β1→6)-D-glucans also increase with temperature and ethanol stress, leading to an agglomeration of their linked mannoproteins and a reinforcement of fibrillar glucan layer, protecting the wall from the action of lytic enzymes. Chitin content is one of the major outcomes of yeast cell wall response to stress, with a strong increase from 1-2 % up 20 % of the cell wall dry mass **(Smits et al., 1999)**. The increase in chitin availability is also consistent with alternative mechanisms for incorporation of cell wall proteins. When (β1→3)-D-glucans decrease, about 40 % of GPI-dependent cell wall proteins become resistant to (β1→3)-glucanase as a result of an increased formation of chitin→(β1→6)-glucan linkages. **(Kapteyn et al., 1999)**. Also, cell wall mannoproteins undergo remodelling during the brewing process, favouring yeast flocculation *via* the inter-association of cell wall lectins with α-mannan receptors **(Gibson et al., 2007; Powell et al., 2003)**. Moreover, *S. pastorianus* cell wall mannoproteins

increase their branching degree with the brewing process, which is important for a more efficient yeast flocculation and protection of glucans against lytic enzymes. Under stress environments, yeast flocculation is also favoured by an increase in mannoproteins phosphorylation. As mannosylphosphates give a net negative charge to cell wall surface, they may provide a more hydrophilic and rigid structure to the yeasts, increasing their tolerance against osmotic stress and lytic enzymes **(Bastos et al., 2015; Jigami & Odani, 1999)**.

3. Yeast polysaccharides for biomedical applications: relevance and advances

Leveraging yeast unique biological features for biomedical applications is a highly valuable approach considering its wide availability and possible bioactivity of its cell wall polysaccharides, namely mannoproteins and β-glucans. While there are many published studies reporting their biological effects, mostly for β-glucans, rarely a detailed structure characterization of these polysaccharides has been made. The structural variability of β-glucans, namely their molecular weight (MW), branching degree (BD), tertiary structure, purity, solubility, and linkage patterns is recognized to affect their biological activity **(Han et al., 2020; Walachowski et al., 2016)**. Therefore, the lack of a detailed description of the extraction protocol and structural characterization of these components hampers the comparison of different results and can lead to misleading conclusions of their structure-activity relationship, and consequent incorrect extrapolations to all β-glucans. Gathering on this, in the following sections only the most relevant studies where yeast polysaccharides were structurally characterized are presented to better shed a light on their realistic potential for medical applications (Figure 4).

3.1. Immunomodulatory properties

β-Glucans have potential for biomedical applications due to their modulation of inflammatory responses and metabolic activity, anti-infectious properties, and anti-cancer features **(Aizawa et al., 2018; Silva et al., 2015; Yodthong et al., 2020)**. On this focus, conserved structural features of *β*-glucans can trigger defenses against fungi and bacteria through pathogen-associated molecular patterns (PAMPs), naturally existing in humans **(Han et al., 2020)**. Regarding immunomodulation, human immune system cells have specific surface receptors that are responsible for the recognition of *β*-glucans, namely: (i) Dectin-1 and (ii) Complement Receptor type 3 (CR3) (Figure 4) **(Batbayar et al., 2012; De Marco Castro et al., 2020)**. Dectin-1 is a member of C-type lectin receptors expressed in monocytes, macrophages, neutrophils, and dendritic cells **(Jin et al., 2018)** that recognizes (*β*1→3)- and (*β*1→6)-linked glucans **(Vannucci et al., 2013)**. (*β*1→3)-Glucans with higher branching degree with (*β*1→6)-Glc side chains exhibit higher receptor affinity **(Jamas et al., 1996)**. CR3 has a lectin site for *β*-glucans binding and another binding site for the complement activation molecule (iC3), expressed in several leukocytes **(De Marco Castro et al., 2020)**. The binding of brewer yeasts (*β*1→3)- and (*β*1→6)-linked glucans to their specific receptors activates the downstream Dectin-1/Syk/NF-κB signaling pathway, triggering an immunostimulatory event **(Zhang et al., 2019)**.

The signaling via Dectin-1 and CR3 is dependent on the polysaccharide solubility **(Batbayar et al., 2012; Qi et al., 2011)**. The soluble *β*-glucans bind to CR3 receptor for immune system stimulation, while insoluble *β*-glucans (particulates) have higher affinity to Dectin-1 receptor. Nevertheless, CR3 signaling can also occur after insoluble particles' phagocytosis and its subsequent degradation, which results in soluble fragmented *β*-glucans capable of activating CR3 receptor **(Barsanti et al., 2011; Jin et al., 2018; Pan**

et al., 2019). *β*-Glucans also interact with other Pattern Recognition Receptors (PRRs), as Toll-like Receptors (TLR), scavenger receptors, and lactosylceramide receptors **(Jin et al., 2018; Pan et al., 2019)**. For instance, while a higher secretion of inflammatory cytokines, such as TNF-*α*, is induced by insoluble glucans via Dectin-1 receptor, soluble *β*-glucans also stimulates the production of this cytokine, although in a lower extent, probably through scavenger receptors **(Berven et al., 2015)**.

The relevance of *β*-glucans binding and activation of Dectin-1 mediated signaling was shown for brewer's yeast, where insoluble and partly soluble *β*-glucans with similar amounts of (*β*1→3)-D-Glc, (*β*1→6)-D-Glc, (1→4)-D-Glc and (*β*1→3,6)-D-Glc linkages showed only to elicit a inhibitory activity of legumain, a proteolytic enzyme expressed in macrophages and involved in inflammatory conditions, by the insoluble polysaccharides **(Berven et al., 2015)**.

Despite being more bioactive, insoluble *β*-glucans are challenging to handle and process in laboratory conditions. Thus, some attempts have been made to improve the water solubility of *β*-glucans, which include their chemical modification with the introduction of hydrophilic groups in the polysaccharide chain. Insoluble *β*-glucans with 1.5 % of protein and 94 % of *β*-glucans (80 % corresponding to (*β*1→3)-D-glucans and 20 % to (*β*1→6)-D-glucans) solubilized via phosphorylation, resulted in water-soluble *β*-glucans with MW 6.6-10.0 kDa, degree of substitution (DS) 0.77-2.09, and degree of polymerization (DP) 17.6-33.7, whereas the glucans with 8.8 kDa, DS 1.24 and DP 30.5 were the most biological active, with an immunomodulation similar to insoluble *β*-glucans **(Shi et al., 2018)**. Additionally, the solubility attained by carboxymethylation of yeast polysaccharides with a substitution degree of 0.8 was shown to improve polysaccharides immunogenic activity, with increased TNF-*α* expression **(Liepins et al., 2015)**. The solubility of yeast *β*-glucans can also be improved by increasing their charge number via oxidation of primary alcohols into carboxylic groups **(Ma et al., 2020)**.

Insoluble β-glucans can also be rendered soluble by depolymerization through heat degradation, yielding soluble β-glucans, mainly (β1→3)-linked glucose residues with (β1→6)-linked side chains, mostly in a triple helix conformation **(Ishimoto et al., 2017)**. β-Glucans can exhibit single-helix, triple-helix, random coils or spherical conformation, according to their minimum conformational energy in a given solution . The different conformations are related to the hydrogen bonding between intra and/or inter-chains **(Ferreira et al., 2015; Han et al., 2020)**. It is suggested that β-glucans with triple-helix conformation, as (β1→3)-D-glucans found in brewer's yeast cell walls **(Kwiatkowski et al., 2009)**, are biologically more active (Figure 4). Although not entirely understood, the higher interaction of triple-helix glucans with immune cells may be related to a higher stability and a higher receptor affinity when compared to single-helix conformers **(Meng et al., 2020)**. Moreover, spherical β-glucans conformation also exhibited high receptor affinity with Dectin-1 **(Zheng et al., 2021)**.

Generally, high MW β-glucans show considerably higher activity, being able to induce immune responses more efficiently. The water-soluble brewer yeast β-glucans with 2496 kDa showed higher macrophage RAW 264.7 cell proliferation among five different MW β-glucans populations ranging from 43.5 to 3398 kDa, which was followed by the glucans with 3398 kDa, 1335 kDa, 97.5 kDa, and 43.5 kDa **(Zheng et al., 2019)**. Shorter β-glucans (< 10 kDa) are inactive or present lower immunostimulatory activity **(Barsanti et al., 2011; Ferreira et al., 2015; Han et al., 2020)**. The size dependent activity may be explained by an increased structure stability and required binding sequences that will allow a greater affinity for cellular receptors **(Han et al., 2020)**. Nonetheless, soluble β-glucans with low MW have a stronger inhibition of reactive oxygen species (ROS) production, acting as an antagonist of Dectin-1 mediated signaling (Figure 4) **(Ishimoto et al., 2017)**. When soluble β-glucans have MW < 5 kDa, the Dectin-1 binding affinity is even weaker, resulting suppressive effect of ROS and tumor necrosis

354 factor alpha (TNF-α) production via antagonistic activity. A DP of 16 seems to be
355 essential for Dectin-1 receptor binding **(Ishimoto et al., 2018)**.

356 Beyond the MW, a high degree of branching (DB), between 0.2 (1 branched residue
357 in 5 Glc residues) and 0.33 (1 branched residue in 3 Glc residues), has shown to be an
358 important contributing factor to β-glucans immunomodulatory activity (Figure 4)
359 **(Barsanti et al., 2011; Ferreira et al., 2015; Han et al., 2020)**.

360 *S. cerevisiae* och1Δ mutant has a cell wall with lower levels of highly entangled *N*-linked
361 mannans, exposing the underlying β-glucans and chitin, and increasing the cell wall
362 porosity. The exposure of these mutant yeasts to RAW 264.7 macrophages increases
363 TNF-α secretion via Dectin-1 and other PRRs **(Yadav et al., 2020)**. However, the
364 immunomodulatory activity induced by extracts of mutant yeast varies according to the
365 stimulated cell. In fact, human peripheral blood mononuclear cells, when exposed to
366 och1Δ mutants, exhibit a decreased cytokine secretion (i.e., TNF-α, IL-6 and IL-10),
367 independently from Dectin-1 recognition. This suggests that the activity of these cells is
368 modulated via *N*-mannosylation recognition, instead of β-glucans. Och1 is responsible
369 for the addition of (α1→6)-D-Man to the *N*-glycan core. When this activity is impaired,
370 the yeast cell wall presents reduced levels of highly entangled *N*-linked mannans,
371 exposing the underlying β-glucans and chitin, and increasing the cell wall porosity. The
372 increased branching degree caused by the brewing process **(Bastos et al., 2015)** allows to
373 infer that these highly branched mannoproteins will hinder the recognition of β-glucans
374 by the immune system.

375 **3.2. Anti-tumoral activity**

376 The binding of β-glucans to the lectin domain of neutrophils' CR3 stimulate the
377 cytotoxicity against iC3b-opsonized tumor cells, leading to an innate immune system
378 response **(Li et al., 2006)**. This relates the antitumoral action of these polysaccharides

379 with their immunomodulation activity. Following the innate immune system role,
380 adaptative immune responses have been also described, where T-cell activity is
381 stimulated with the increased production of anti-inflammatory cytokines in the tumor
382 microenvironment **(Li et al., 2010)**. Moreover, by interacting with Dectin-1 receptors,
383 yeast-derived (β1→3)- branched with (β1→6)-D-glucans are able to inhibit the
384 accumulation of regulatory myeloid-derived suppressor cells (MDSC) in the tumor site
385 and increase anti-tumoral immune response through the promotion of macrophages and
386 dendritic cells' infiltration **(Tian et al., 2013)**. Furthermore, whole β-glucan particles
387 (insoluble (β1→3)-glucan particles prepared from *S. cerevisiae*) induce apoptosis and
388 respiratory burst by direct interaction with Dectin-1 of polymorphonuclear MDSC
389 **(Albeituni et al., 2016)**. Both these pathways have shown to act synergistically and to
390 delay tumor progression in Lewis lung carcinoma mice models **(Albeituni et al., 2016;**
391 **Tian et al., 2013)**.
392 The tumor microenvironment is generally characterized by immune-suppressive
393 conditions in which M2 polarized macrophages (anti-inflammatory phenotype) or tumor
394 associated macrophages (TAM) are predominant in relation to M1 polarized macrophages
395 (pro-inflammatory phenotype) **(Duan & Luo, 2021; Parisi et al., 2018)**. Attempts to
396 repolarize macrophages toward an M1 phenotype in the tumor niche and to screen anti-
397 TAM treatments have been made to stimulate the invasion of tumor by immune cells
398 (*e.g.*, dendritic, macrophages, T cells). Up-to-date several β-glucans from yeast were
399 evaluated for their capability to alter IL-4 mediated M2 human monocyte-derived
400 macrophages polarization toward a more M1 phenotype **(De Graaff et al., 2020)**. Despite
401 being able to maintain anti-inflammatory gene biomarkers expression, zymosan, an
402 commercial insoluble preparation of *S. cerevisiae* yeast cell walls, composed by (β1→3)-
403 D-glucans backbone and Glc residues (β1→6)-linked as side chains (48 % carbohydrates

– 87 % Glc, 11 % Man, and 1 % Rha, and 18 % proteins), and a second yeast derived β-glucan fraction (75 % carbohydrates – 97 % Glc and 12 % proteins), significantly increased the expression of genes related to pro-inflammatory macrophages. This was demonstrated by inducing inflammatory cytokine expression (*i.e.*, IL-6, TNF-α and IL-10) and enhancing the secretion of chemo-attractants that contribute for recruiting immune cells (e.g., neutrophils, T cells and natural killer cells) to the tumor site **(De Graaff et al., 2020)**. These findings support β-glucans, allowing their use as mediators of tumor immunotherapy.

Although soluble (β1→3)/(β1→6)-D-glucans also demonstrated anti-tumoral effect in S180 tumor-bearing mice via specific recognition by PRRs located on immune cells **(Mo et al., 2017)**, they can have a direct action in tumor cells, leading to changes in cell cycle and cellular apoptosis. This was verified for soluble (β1→3)/(β1→6)-linked glucans (MW: 17 kDa; DB: 0.03-0.2) **(Mo et al., 2017)**, which reduces tumor growth and decreases its invasive capacity **(Wang et al., 2020)**. Even so, the underlying mechanism underneath the structure of yeast polysaccharides and their anti-tumor activity, remains to be clarified.

Alkali-insoluble β-glucans of *S. cerevisiae* (78 % of carbohydrates, 896 kDa), upon modification by sulfation in acidic medium, resulted in three low molecular weight soluble fractions (i) 13 kDa, degree of substitution (DS) 0.16; ii) 17 kDa, DS 0.24; iii) 19 kDa, DS 0.27), where the lower MW and DS demonstrated stronger immunological (IL-2 and IFN-γ secretion) and antioxidant activities **(Lei et al., 2015)**. The production of IFN-γ is associated to tumour inhibition and inductions of its regression, hence triggering its secretion can yield a valuable therapeutic outcome.

3.3. Immunomodulation of host response: Applications in bone implants and tissue repair

A successful bone-implant integration depends on an exquisite balance between the inflammation, because of the immune response to biomaterials and/or the implantation process itself, and tissue regeneration (calcium and collagen deposition). This balance is modulated by immune cells, particularly by macrophages. Hence, the use of biocompatible implant-coating biomaterials, especially polysaccharides with immunomodulatory activities, may be beneficial to osteointegration (Figure 4). Zymosan grafted onto titanium implants' surface directly interacts with macrophages and triggers M2 polarization, essential for the regeneration process **(Shi et al., 2018)**

On the other hand, the administration of *β*-glucan from *Saccharomyces cerevisiae* reduces alveolar bone loss in periodontitis models **(Breivik et al., 2005; Silva et al., 2015; Silva et al., 2017)**. This reduction may be related to modulation of immune system with decreased proinflammatory cytokines.

Zymosan can activate both TLR2 and Dectin-1 receptors and subsequent signaling pathways, enhancing osseointegration with the expression of prohealing factors, such as osteogenic inducers (i.e., oncostatin M), pro-angiogenic factors (e.g., vascular endothelial growth factor – VEGF), proliferative inducers (e.g., platelet-derived growth factor PDGF), and immunosuppressive cytokines (e.g., IL-10, etc. by macrophages. Through the direct interaction between the zymosan coating and macrophages, a pro-regenerative environment is created, stimulating osteoblast differentiation and new bone growth in implant-bone interface **(Shi et al., 2018)**. However, there seems to be a thin line between benefits and negative outcomes of zymosan biological activity. Higher doses can be detrimental by promoting an uncontrollable inflammation and increasing the production of free oxygen radicals which promotes tissue necrosis, pseudo-arthritis and stimulates

bone resorption, demonstrating an inhibitory action on fracture healing **(Duygulu et al., 2007)**. Hydroxyl radical and superoxide anion scavenging activity has also been associated to *S. cerevisiae* cell wall alkali-insoluble linear (β1→3)-D-glucans modified by carboxymethylation, phosphorylation and sulfation **(Tang et al., 2017)**. Deproteinated mannans from *S. cerevisiae* cell wall (84 kDa) may be useful for bone regeneration due to their scavenging effects, scavenging deleterious superoxide anions (O_2^-) and hydroxyl radicals (OH^-) **(Domazetovic et al., 2017; Duygulu et al., 2007; Liu et al., 2018)**. Similarly, a complex of mannan (70 kDa)/β-glucans (50 kDa) (51 %/49 %) able to protect intestinal porcine epithelial cells (IPEC-J2) from oxidative stress can also function as an antioxidant system, acting on suppression of autophagy and apoptosis **(Guo et al., 2019)**.

Zymosan inflammatory effects may be attenuated by amination in the C_6 of Glc residues without compromising its cellular uptake **(Doble et al., 2020)**. However, less purified zymosan extracts, with high amount of mannans, are on the other hand potent inflammatory inducers, promoting neutrophil infiltration and pro-inflammatory cytokines and chemokines production **(Walachowski et al., 2016)**. Increased β-glucans purity (≥ 65 %) generally induces a weaker activation of NF-κB/AP-1 signaling, while unpurified *S. cerevisiae* cell wall extracts strongly activate this signaling pathway. Furthermore, with the purification of β-glucans TLR2/4 ligands are removed while Dectin-1 ligands are maintained. Contrarily to extracts with lower β-glucans content, which strongly activate TLR2 and TLR4, the enriched extracts stimulate Dectin-1 activation **(Walachowski et al., 2016)**. The heterogeneity of β-glucan structures, including zymosan, may result in samples containing soluble β-glucans that can influence the biological responses and the activation of distinct signaling pathways.

The co-stimulation of osteoclast progenitor cells by receptor activator of NF-κB ligand (RANKL) and TLRs agonists suppresses osteoclast differentiation **(Souza & Lerner, 2019)**. The capability to abolish osteoclast differentiation with low molecular (β1→3)-

glucans (3 kDa) in a co-culture of mouse osteoblasts and bone marrow macrophages was attained through the interaction with primary osteoblast (via decreased expression of RANKL) and with the TLR2/TLR6 complex (via inhibited differentiation of osteoclasts precursor cells). Laminaritetraose, laminarihexaose, and laminariheptaose did not exert an antiosteoclastogenic effect **(Aizawa et al., 2018)**, suggesting that a MW superior to DP7 may be required for bone resorption repression. The inhibition of osteoclasts activity was also observed using soluble (β1→3)-glucan branched with (β1→6)-glucan from *S. cerevisiae* with a MW of 12-16 kDa **(Sorgente et al., 2003)**. Contrarily, when using soluble linear (β1→3)-glucan the inhibition of RANKL-induced osteoclast differentiation was only possible with a MW > 50 kDa **(Jang et al., 2013)**. The presence of (β1→6) side chains may also enhance the activity of low MW β-glucans.

3.4. Drug delivery system formulations

Yeast microcapsules can be formulated by submitting yeasts to a combination of chemical treatments with bases (e.g., 1 M NaOH), acids (e.g., HCl at pH 4-5), and organic solvents (e.g., isopropanol and acetone) to remove the cytoplasmatic content and preserve the structure formed by water insoluble polysaccharides of the yeast cell wall **(Sabu et al., 2018)**. This results in hollow and porous particles (pore size: ~600 nm) with an average diameter of 2-4 µm (Figure 4) **(Lee et al., 2019; Zhang et al., 2017)**. These yeast cell wall microcapsules consist essentially of (β1→3)-linked glucans (80 %), and in a lower amount mannoproteins (< 1 %) and chitin (2-4 %) **(Lee et al., 2019; Soto & Ostroff, 2008)**. Glycogen can account for the remaining material as shown for brewers´ spent yeast **(Bastos et al., 2015)**. The microstructure of yeast glucan particles can be affected by the drying method applied after particles preparation. Comparison between solvent exchange, lyophilisation and spray drying methodologies revealed that spray dried particles preserved most the ellipsoid native yeast structure, exhibited the lowest

extent of interparticle hydrogen bonds and presented twice higher immunomodulatory activity. Thus, the interaction and diffusion process of yeast particles with biological systems can be positively affected using spray dried method by reducing structures association and formation of larger particles that sediment faster **(Hromádková et al., 2003)**.

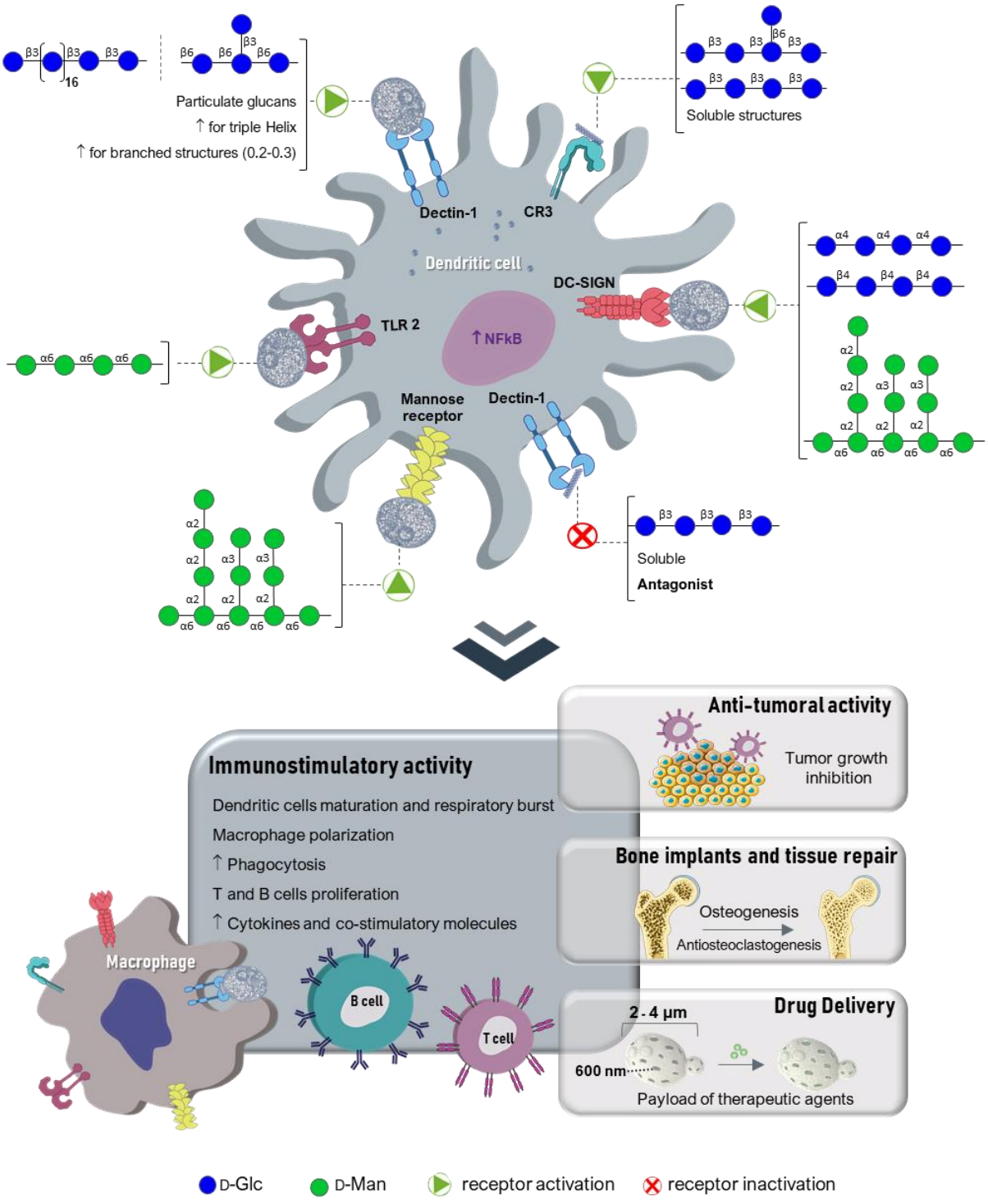
The structural composition of yeast microcapsules renders them suitable for drug encapsulation and delivery. The encapsulation of bioactive molecules is generally performed by natural diffusion, but other methods such as electrostatic adsorption **(Zhang et al., 2020)**, slurry evaporation **(Rotrekl et al., 2020)**, spray drying **(Šalamúnová et al., 2021)**, and dissolving-precipitation **(Sun et al., 2020)** have already been applied. The incorporation of drugs in yeast microcapsules is commonly accompanied by surface functionalization with a hydrogel layering of alginate and/or chitosan to entrap the drug inside the microcapsule, allowing a more spatiotemporally controlled release kinetics of the therapeutic agent **(Soto et al., 2010)**. Yeast-derived microcapsules has been reported as carriers for nucleic acids, drugs **(Sun et al., 2020)**, nanoparticles **(Ren et al., 2018)**, liposomes **(Li et al., 2020)**, vaccines **(Huang et al., 2010)**, and diagnostic agents **(Garello et al., 2015)**.

The release kinetics varies as a function of: (i) the biomolecule MW (higher MW have higher retention times) **(Jamas et al., 1989)**, (ii) their solubility in the surrounding environment (higher solubility, higher release rate) **(Jamas et al., 1989)**, (iii) the degree of branching associated with yeast microcapsules permeability (higher branching decrease the release rate) **(Jamas et al., 1989)**, (iv) microcapsule porosity that may vary according to the ratio of (β1→3)/(β1→6)-D-glucans (higher porosity lead to a higher release rate) **(Ostroff et al., 1991)**, and (v) the presence of yeast microcapsules covering with hydrogel layers, decreasing the payload burst release **(Soto et al., 2010)**.

529 The safety, lack of toxicity and resistance in large extent to digestive degradation of
530 yeast microcapsules, also referred as *β*-glucan particles, supports their extensive use in
531 oral administration. The application of yeast microcapsules as oral delivery system is
532 possible since *β*-glucan particles are recognized as safe (GRAS) by the U.S. Food and
533 Drug Administration **(Adams, 2017)**. Oral delivery, compared to systemic delivery, has
534 been considered the most desired form of administration owing to its convenience, higher
535 compliance by the patient and easy modulation of the dose to be administrated **(Zhang et**
536 **al., 2020)**. Notwithstanding, oral uptake has several limitations related to the low
537 solubility, low permeability, sensitivity to human P-glycoprotein (a protein that mediates
538 drugs efflux at intestinal epithelia), and gastrointestinal environment **(Foley et al., 2021;**
539 **Li et al., 2020; Ren et al., 2018; Sabu et al., 2019)**. Exposure during 4 h to simulated
540 gastric fluid followed by 8 h exposure to simulated intestinal fluid resulted in lysis of part
541 of microcapsules-derived from *S. cerevisiae* SAF-Mannan **(Zhang et al., 2020)**. The
542 degradation rate differs according to the microcapsules structural composition **(Legentil**
543 **et al., 2015)**. Digestive enzymatic activity on glycogen may explain the sensitivity of
544 microcapsules of gastrointestinal environment **(Bastos et al., 2015)**. Yeast microcapsules
545 are biodegradable and their disintegration may result from macrophages digestion (> 13
546 days) **(Hong et al., 2004)**.
547 When reaching the intestinal track, yeast microcapsules are internalized by intestinal
548 M cells, which are found mainly in the lymphoid tissues of the mucosa, associated with
549 Peyer's patch, and phagocytised by macrophages after recognition of *β*-glucans by dectin-
550 1 and CR3 receptors **(Ren et al., 2018; Zhou et al., 2017)**. Microcapsules can also be
551 accumulated in $CD11c^{+}$ dendritic cells in subepithelial dome regions **(De Jesus et al.,**
552 **2014)**. This enables an efficient and specific transport of yeast microcapsules through
553 systemic circulation and lymphatic system, towards inflammable and tumour sites,
554 increasing the biocompatibility and safety of the administered drugs, without affecting

555 macrophages migration rate, conserving their physiological function **(Zhou et al., 2017)**.
556 Furthermore, the yeast microcapsules surface modification may improve its uptake in the
557 Payer's patch, as observed by a poly-L-Dopa coating, assigning negative charges to yeast
558 microcapsules **(Soto et al., 2019)**. It should be noted that although authors refer in the
559 title and graphical abstract the use of polydopamine, which does not confer negative
560 charges, poly-L-Dopa was in fact used.

561 Although research related to yeast microcapsules application in the biomedical field
562 has mainly focused on their oral administration, other forms of application, as injection
563 in injured tissue or tumour, may be explored. Moreover, the vast potential of yeast
564 microcapsules can be further improved by surface chemical modifications, possible due
565 to the high number of hydroxyl groups. Chemical conjugation of A/G protein (a
566 recombinant fusion protein that combines both Protein A and Protein G binding domains
567 for human immunoglobulin G - IgG) to yeast microcapsules via 1-ethyl-3-(3-
568 dimethylaminopropyl)carbodiimide hydrochloride (EDC) linker was already performed
569 **(Yang et al., 2020)**, showing that yeast microcapsules can be tailored to different
570 purposes, thus creating new opportunities in the biomedical field.



571

572 **Figure 4:** Illustration of immune system modulation by yeast glucan and mannan
573 structures, through mediation of immune system cells (*e.g.* dendritic cells and
574 macrophages) surface receptors (Dectin-1, CR3 – Complement Receptor 3; DC-SIGN -
575 Dendritic cell-specific ICAM-3-grabbing nonintegrin; Mannose receptor; TLR – Toll like
576 receptor). Schematic overview of immunostimulatory response (signalling, cells
577 activation and proliferation) and related biomedical applications (anti-tumoral, bone
578 implant and regeneration field, drug delivery systems). Parts of the figure (tumor cells

and bones) were drawn using pictures from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

4. Perspectives for the future use of brewer's spent yeast (BSY)

The yeast cell wall polysaccharide dynamic modifications that are inherently promoted with the brewing process opens new perspectives for BSY valuation in several biomedical applications that benefit from such added functionalities as discussed. As all yeast microcapsules, BSY cell wall three-dimensional structure is preserved after autolysis. The BSY mesh is preserved even after the sequential extraction of polysaccharides. The microcapsule is comprised mainly by glucans with (α1→4)-, (β1→4)-, and (β1→3)-linkages, resistant to alkali extraction until 8 M KOH **(Pinto et al., 2015)**. The polysaccharides of BSY, when compared with the polysaccharides of *Saccharomyces* cell walls, have an exquisite presence of (β1→4)-D-glucose linkages, as well as (α1→4)- and (β1→4)-D-glucose linkages connected to the (β1→3)- and (β1→6)-D-glucose linkages. The structure can be partially destroyed by cellulase and α-amylase hydrolysis **(Bastos et al., 2015)** or destroyed by hypochlorite oxidation **(Coelho et al., 2015)**. The complexity of BSY glucans can open new applications beyond the ones already reported for yeast polysaccharides.

The structural features of BSY glucan network containing (α1→4)-, (β1→4)-, and (β1→3)-linkages unlocks new avenues aiming at the recognition by another key cell receptors. Indeed, beyond the recognition by immune-receptors of (β1→3)-D-glucans, promoting yeast microcapsules internalization **(Huang et al., 2012)**, the (α1→4)-D-glucans can also interact with key immune-receptors, such as dendritic cell-specific ICAM-3-grabbing nonintegrin - DC-SIGN **(Souza & Lerner, 2019)** acting as immune modulator, by promoting immune system homeostasis regulation (Figure 4) **(Geurtsen et al., 2009)**. The DC-SIGN present in dendritic cells and macrophages showed a broad

binding profile to glucans with preference for (α1→4)-D-Glc, including also (β1→4)-D-Glc linkages **(Li & Feizi, 2018)**. The microcapsules containing these specific polysaccharides can be internalized by Dectin-1 **(De Jesus et al., 2014)** and DC-SIGN **(Valera et al., 2008)**. To take advantage of the unique mix-linked (α1→4)-, (β1→4)-, (β1→6)- and (β1→3)-linkages of yeast glucans with receptors interaction, the action mode required for the desired application could be tuned.

Recent advances in chemical engineering toolboxes that enable rapid and biocompatible polysaccharides chemical modification with numerous functional moieties (e.g., photocrosslinkable groups, enzyme substrates, guest-host moieties, etc.) may further transfix their functionality and processability. These strategies will pave the way for hybrid biomaterial-yeast bioconjugates with increased bioactivity and value. The functionalization of yeast cell walls and cell-surface initiated polymerization on-demand are highly exciting advances that may further boost BSY bioactivity and grant an added-value, regarding the final therapeutic outcome **(Niu et al., 2017)**. Emerging advances in exploiting yeasts as engineered living materials through modification with light- and chemical responsive receptors for sensing applications, are also envisioned to provide new applications of these organisms. It is expected that the growing development of synthetic biology tools will contribute to upcoming advances also in this field **(Burgos-Morales et al., 2021)**.

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