

Full Paper

Elemental biochemical analysis of the polysaccharides in the extracellular matrix of the yeast *Saccharomyces cerevisiae*

Fábio Faria-Oliveira¹, Joana Carvalho¹, Celso LR Belmiro^{2,3}, Gustavo Ramalho³, Mauro Pavão³, Cândida Lucas¹ and Célia Ferreira¹

¹ Centre of Molecular and Environmental Biology (CBMA), Department of Biology, University of Minho, Portugal

² Laboratory of Glycoconjugates Biochemistry and Cellular Biology, Federal University of Rio de Janeiro, Campus of Macaé, RJ, Brazil

³ Laboratory of Glycoconjugates Biochemistry and Cellular Biology, Institute of Medical Biochemistry, Federal University of Rio de Janeiro, RJ, Brazil

In yeast multicellular aggregates, such as biofilms and colonies, cells are supported by a yeast extracellular matrix (yECM) of glycosidic nature, the composition of which is mostly unknown. *Saccharomyces cerevisiae* ECM was produced, extracted and partitioned. An analytical-grade pure glycoside fraction was obtained, fractionated by anionic exchange liquid chromatography and analyzed by gas chromatography–mass spectrometry and polyacrylamide gel electrophoresis. Two different molecular weight polysaccharides were found, composed of glucose, mannose and small relative amounts of galactose. One of the polysaccharides had a low molecular weight, compatible with the association with glycoproteins abundantly occurring in yECM. In addition, these polysaccharide species were separated by diamminopropane agarose gel electrophoresis and induced metachromatic shift, suggesting chemical substitution, which was corroborated by anticoagulation activity. This was shown to be associated with the double deletion of the yeast homologues of the mammalian Hedgehog modulators Hhat1 and Hhat, respectively yeast Gup1 and Gup2. These results pioneer the study of the molecular biology of the ECM supporting *S. cerevisiae* multicellular aggregates such as biofilms.

Keywords: Yeast / Extracellular matrix / Polysaccharides

Received: October 9, 2014; accepted: December 8, 2014

DOI 10.1002/jobm.201400781

Introduction

In a multicellular organism, the maintenance of the homeostatic balance required for regular function ultimately depends on the extracellular matrix (ECM) where cells are embedded. The ECM provides a cell-supporting scaffold and helps maintaining the biophysical integrity of tissues and organs. ECM is under constant remodeling, changing rigidity, porosity and spatial disposition, therefore orienting cell movement and tissue growing [1, 2]. Moreover, the diffusion of molecules from cell to cell depends on ECM, that in this way influences directly the availability of signalling effectors of all kinds,

including growth factors and hormones [3, 4], playing crucial roles in all tissue rearrangements [5].

Mammalian ECM presents a great number of functional molecules, biochemically and biophysically diverse, including proteins, glycoproteins, glycosaminoglycans (GAGs) and proteoglycans (PGs). PGs are vital structural and signalling molecules composed of a core protein with one or more covalently attached GAGs [6, 7], which can be a single GAG, as decorin [8], or several units of different GAGs, as versican and aggrecan [9, 10]. GAGs are linear heteropolysaccharides consisting of repeating disaccharide units, composed of a hexosamine (glucosamine or galactosamine, frequently *N*-substituted) and a hexuronic acid (glucuronic acid or iduronic acid). Exceptionally, in keratan sulphate, *N*-acetyl-glucosamine associates with galactose instead. GAGs are generally multi-sulphated, with the exception of hyaluronan [6, 7, 11–13]. In mammals, a few PGs are secreted to the

Correspondence: Cândida Lucas, CBMA, Department of Biology, University of Minho, Campus de Gualtar, 4710-054 Braga, Portugal
E-mail: clucas@bio.uminho.pt
Phone: +351253604310
Fax: +351253678980

extracellular space, like the large molecular weight aggrecan, or the small leucine-rich PGs [14–16]. Some are membrane tethered, through a glycosylphosphatidylinositol anchor, like glypican, or by a membrane spanning protein core, as syndecan [7, 17]. A high molecular diversity arises from the different combinations of PGs protein cores with one or more types of GAGs, eventually leading to a wide variety of biological roles. As such, disturbances in PGs synthesis and turnover lead to severe pathologies [5]. Moreover, important medical properties were also reported for some ECM components. Heparin, for example, presents anti-thrombotic, anti-coagulant, anti-inflammatory and anti-metastatic properties [18–20].

The presence of ECM-like extracellular polymeric substances is known to support and protect multicellular bacterial and fungal aggregates [21–27]. Cells of the yeast *Saccharomyces cerevisiae* are able to form numerous multicellular aggregates: colonies [28], stalks [29, 30] and mats/biofilms [31, 32]. Colonies display complex organization, being cells embedded in an ECM-like extracellular polymeric substance with glycosidic nature [27, 33]. This substance promotes the conduction of nutrients and water to the top layers of cells in the colony [27], preventing desiccation [34] and providing protection against xenobiotics [33]. A colony contains subpopulations of cells in different physiological states [35], a substantial part of which in a non-dividing/quiescent state [36]. In accordance with diverse physiological states, differently positioned cells in a colony express different genes [37–39]. Identically, the biofilms formed by the opportunistic human pathogen *Candida albicans* also present multicellular spatial organization, supported by an extracellular matrix in which genetically different cells are embedded [40–43].

The chemical nature of microbial ECM has been poorly assessed [40–42]. In biofilms of *C. albicans*, ECM was shown to contain proteins, polysaccharides and DNA [42, 44, 45]. In colonies of *S. cerevisiae*, ECM was shown to also contain proteins – glycoproteins – and to be highly glycosylated [27, 33]. In flocs produced during fermentation of *S. cerevisiae* strains overexpressing the flocculin gene *FLO1*, ECM was reported to contain mono- and polysaccharides of glucose and mannose, the latter in the form of highly branched mannan [46]. The lack of detailed biochemical and structural information on the sugar scaffold of yeast ECM, be it in colonies, flocs or biofilms, derives from the difficulty in producing amounts large enough to apply heavy chromatographic procedures. This problem has been overcome applying a new methodology for mimicking biofilms through the production of homogenous mats of cells from which

large amounts of yeast ECM can be easily extracted, allowing straightforward manipulation of strains, media and growth conditions [47]. In the present work, abundant ECM from biofilm-like mats of *S. cerevisiae* was extracted and partitioned to obtain an analytical-grade glycoside fraction. This was analyzed by mass spectrometry, anion exchange liquid chromatography, polyacrylamide gel electrophoresis and diaminopropane agarose gel electrophoresis. Two polysaccharide species were identified, which were composed of glucose, mannose, galactose and putatively uronic acid. In addition, chemical substitution was evidenced by (1) induced metachromasia in toluidine blue and dimethylmethylene blue dyes and (2) the degree of the activated partial thromboplastin time observed in samples that originated from different yeast strains. Although the chemical nature of the glycoside bonds and the macromolecular structure of the polysaccharides require other in-depth analytical approaches, this work pioneers the acknowledgement of the polysaccharide nature and complexity of *S. cerevisiae* ECM.

Materials and methods

Strains, media and culture conditions

The *S. cerevisiae* strains W303–1A (*MATa leu2–3 leu2–112 ura3–1 trp1–1 his3–11 his3–15 ade2–1 can1–100*), BHY54, Cly5 and Cly3 (isogenic to W303–1A but *gup1::His5⁺, gup2::KanMX* or *gup1::His5⁺gup2::KanMX*, respectively) [48] were used. Batch cultures were performed in YPD (Himedia, India) [1% yeast extract (w/v), 2% peptone (w/v), 2% glucose (w/v), 0.005% adenine hemisulphate (w/v)] at 200 rpm, 30 °C and the air to liquid ratio of 2:1. Growth was monitored by measuring OD₆₀₀. Soluble yeast ECM (yECM) from biofilm-like mat was prepared as described before [47].

yECM protein fraction collection and western blotting

The yECM protein fraction was obtained supplementing the culture homogenisation buffer [47] with a protease inhibitor cocktail (PMSF, 0.2 µg ml⁻¹, Aprotinin, 0.32 µg ml⁻¹, Pepstatin, 1 µg ml⁻¹, Leupeptin, 1 µg ml⁻¹). All enzymes were purchased from Sigma (EUA). The suspension was centrifuged (10 min, 15,000 rpm, 4 °C) (Sigma 4–16K). The supernatant was filtered through a 0.45-µm membrane and freeze-dried (Christ Alpha 2–4 Christ LDC-1 m, B. Braun). Proteins were separated in 10% PAGE, blotted in a PVDF membrane (BioRad, DE) and probed with concanavalin A-HRP (Horse Radish Peroxidase) conjugate (Sigma, EUA), as previously described [27].

yECM glycoside fraction collection, polysaccharide precipitation and separation

Freeze dried yECM [47] was resuspended in digesting buffer (0.1 M sodium acetate (Merck, DE), 5 mM EDTA (Sigma, EUA), 5 mM cysteine (Merck, DE), pH 5.5) at 1 g 20 ml⁻¹. Double-crystallized papain (Sigma, EUA) was added to the mixture (10 mg ml⁻¹) and incubated at 60 °C overnight to degrade proteins in suspension. The mixture was centrifuged (3000 rpm, 10 min, 4 °C) (Sigma 4–16K), and the clear supernatant was collected and subjected to ethanol precipitation. Three volumes of ethanol (Sigma, EUA) 95–99% (v/v) were added to the supernatant and incubated overnight at 4 °C. The precipitate was collected by centrifuging (3000 rpm, 10 min, 4 °C) and left to evaporate the residual ethanol. An aliquot of the resulting pellet was resuspended in deionized water, stored at 4 °C and used for (1) electrophoretic analysis, (2) evaluation of total sugar using the phenol–sulphuric acid method [49], (3) probing the presence of hexuronic acids by the carbazole method [50] and (4) testing metachromatic shift of 1,9-dimethyl-methylene blue (DMMB) [51, 52]. Another aliquot of the ethanol-precipitated pellet (20 mg) was resuspended in 1 ml of MilliQ water, filtered through a 0.22-µm syringe filter and applied to a Hitrap Q-XL-FPLC column for anionic exchange liquid chromatography (FPLC) (Pharmacia Biotech). The column was equilibrated with 20 mM Tris-HCl (Merck, DE) (pH 8.6). The polysaccharides were eluted by a linear gradient of 0–3.0 M NaCl (Merck, DE) (10 ml) at a flow rate of 0.50 ml min⁻¹, fractions of 0.5 ml were collected. Hexuronic acids [50], total sugars [49] and sulphated polysaccharides were assessed [52]. The fractions with detected peaks were collected, dialysed against MilliQ water for 48 h and freeze-dried.

Chemical analysis of yECM glycoside fraction

Gas chromatography–mass spectrometry (GC–MS). The monosaccharide ratio was determined by GC–MS, analyzing the corresponding alditol acetate derivatives, as previously described [53]. Briefly, the samples were fast hydrolysed with trifluoroacetic acid (Fisher, EUA) at 100 °C for 4 h. The monosaccharides released were converted into alditol acetate derivatives by successive reduction with NaBH₄ (Fisher, EUA) and acetylation with Ac₂O pyridine (Merck, DE), allowing identification by GC–MS. The samples were analyzed by a GC–MS system (Shimadzu QP2010 Plus) equipped with a Restek RTX-5MS column. Ultrapure helium (99.999%) was used as the carrier at a constant flow rate of 1.0 ml min⁻¹. The oven temperature was programmed to increase from 110 to 260 °C in a 70-min period. The ion source and interface temperatures were 200 and 230 °C, respectively.

Glycosaminoglycan–polyacrylamide gel electrophoresis (GAG–PAGE). Each sample (10 µg) of yECM glycosides was applied to a 1-mm-thick 6% polyacrylamide (BioRad, DE) gel in 3X polyacrylamide gel electrophoresis (PAGE) running buffer (60 mM Tris-HCl, pH 8.6). Electrophoresis was run at 100 V. The gel was stained with 0.1% toluidine blue (Merck, DE) in 1% acetic acid (Sigma, EUA). After staining, the gel was washed overnight in 1% acetic acid. The markers of molecular mass (Sigma, EUA) were dextran sulphate 8 (average MW: 8000 Da), chondroitin 4-sulphate (average MW: 36,000 Da) and dextran sulphate 100 (average MW: 100,000 Da).

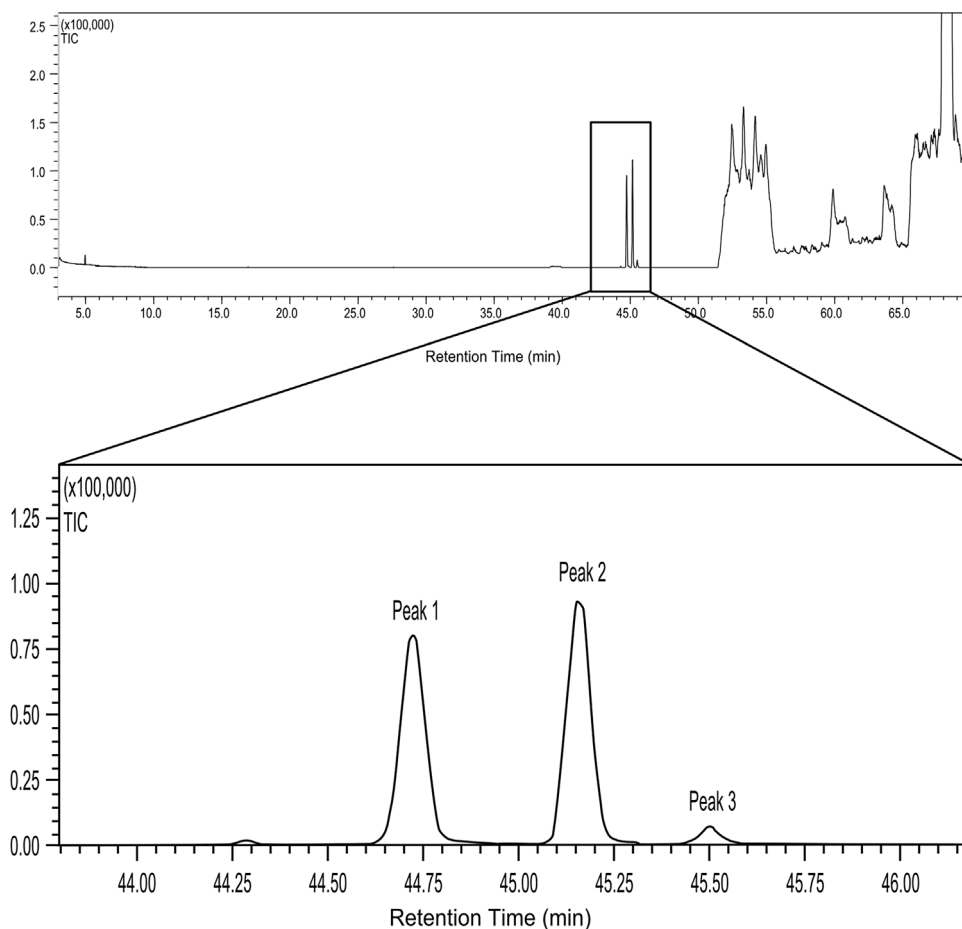
Diaminopropane agarose gel electrophoresis (DAE). Diaminopropane agarose gel electrophoresis (DAE) was performed as described previously [13]. Briefly, app. 1.5 µg of the sample was applied to a 0.5% agarose (Sigma, EUA) gel in a buffer composed of 50 mM 1,3-diaminopropane acetate (Sigma, EUA) (pH 9.0), and electrophoresis was run for 1 h at 100 V. As a standard, a mixture of glycosaminoglycans (GAGs) (Sigma, EUA), containing chondroitin sulphate (CS), dermatan sulphate (DS) and heparan sulphate (HS) (1.5 µg of each), was used. GAGs were fixed with aqueous 0.1% Cetavlon[®] (Sigma, EUA), air dried and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v/v).

Anticoagulant effect measured by activated partial thromboplastin time (aPTT). Assays of activated partial thromboplastin time (aPTT) were performed by incubating 100 µl of human plasma with 10 µl of the yECM polysaccharide sample at 37 °C for 1 min. As a control, incubation was performed with a solution standard of porcine intestinal mucosa heparin (50 µg ml⁻¹) (Sigma, EUA). Subsequently, 100 µl of cephalin (Fisher, EUA) were added, and the mixtures were re-incubated for 2 min, after which 100 µl of CaCl₂ (Sigma, EUA) (0.25 M) were added and the clotting time recorded in a coagulometer (KC4A, Amelung) [54]. The experiments using human blood samples were performed in accordance with the Declaration of Helsinki (2008) of the World Medical Association and were approved by the Ethics Committee of the Federal University of Rio de Janeiro, Brazil.

Results

S. cerevisiae ECM glycoside composition

A well-known laboratory wild-type strain of *S. cerevisiae* (W303) was used to produce and extract yECM and purify the correspondent glycoside fraction. The approach yielded an analytical-grade pure fraction that was used to identify the components of yECM polysaccharides. GC–MS results (Fig. 1) indicated the presence of glucose



Peak number	Retention time (tR - min)	Proportion (% of total)	Sugar	<i>m/z</i> (GC-MS)
1	44,7	44	Mannose	43, 85, 115, 127, 145, 187, 217
2	45,1	52	Glucose	43, 85, 115, 127, 145, 187, 217
3	45,5	4	Galactose	43, 85, 115, 127, 145, 187, 217

Figure 1. Mass spectrometry of *S. cerevisiae* ECM glycoside fraction. GC-MS of yECM glycoside constituents showed the presence of glucose, mannose and galactose. The retention times and main mass-to-charge ratios for each monomer are included in the table.

(52%), mannose (44%) and galactose (4%). Furthermore, considering that polysaccharides containing uronic acids, common in ECM of higher eukaryotes [7], have also been reported in bacterial ECM [24, 55], the presence of uronic acids was firstly tested by the carbazole method using the yECM sample before separation of protein/sugar fractions. Results were positive for the presence of this type of sugar (not shown).

The yECM glycoside sample was subsequently analyzed by GAG-PAGE. Two different polydisperse bands were observed (Fig. 2A, arrows). The polydispersion obtained during the electrophoretic migration of polysaccharides derives from the possible existence of different numbers of repeating saccharide units in a same glycoside species. Their average size of app. 35–40 kDa and <8 kDa was estimated by comparison

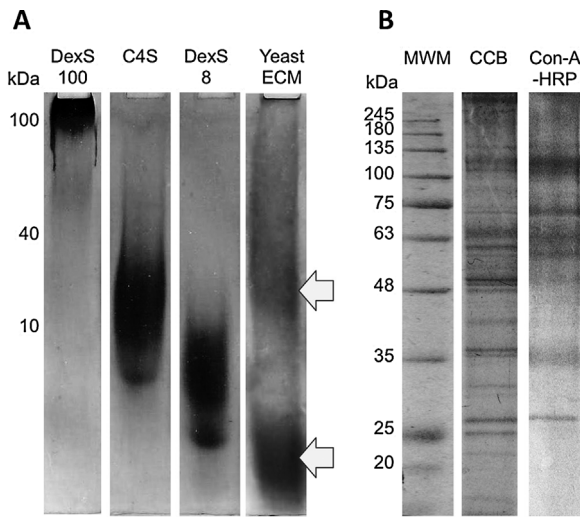


Figure 2. Electrophoretic analysis of *S. cerevisiae* ECM polysaccharides (A) and proteins (B). (A) GAG-PAGE of the yECM glycoside fraction revealed two components (arrows), one with ≤ 8 kDa and another in considerably smaller amounts with app. 35–40 kDa. Molecular weight standards (STD): DexS100, dextran sulphate (MW: 100,000 Da), CS4, chondroitin 4-sulphate (MW: 36,000 Da) and DexS8, dextran sulphate (MW: 8000 Da). (B) SDS-PAGE analysis of the yECM protein fraction, and further blotting with concanavalin A-HRP (Horse Radish Peroxidase) conjugate for the presence of glycoproteins. (CCB, Colloidal Coomassie Blue staining).

with the known molecular weight (MW) of the GAGs in control lanes. The higher MW band (app. 35–40 kDa) was present in much lesser amounts than the lower MW band (< 8 kDa). This last band could consist of oligosaccharides and/or disaccharide units, likely associated with glycoproteins in yECM. The putative existence of this type of proteins was probed in the yECM sample prior to

glycoside/protein fractionation using concanavalin A, which recognizes glycosyl and mannosyl groups. Intense bands were detected, most of which were easily attributable to protein bands on the correspondent SDS-PAGE (Fig. 2B). These results concur with previous suggestions from the literature in which *S. cerevisiae* ECM contains glycoproteins and displays a highly glycosylated pattern [27, 33] further showing the correspondent sugar composition.

Putative chemical substitution of extracellular matrix (ECM) polysaccharides in *S. cerevisiae*

The induction of metachromasia is frequent in chemically substituted polysaccharides, namely highly sulphated GAGs from ECM of higher eukaryotes [56]. A first assay using the yECM polysaccharide extract induced metachromasia in DMMB and toluidine blue dyes (not shown). Subsequently, the yECM glycoside sample was fractionated by FPLC (Fig. 3A). Fractions (0.5 ml) were collected, and each one was tested for metachromasia and uronic acids. As a control, total sugars in each fraction were quantified. According to the above-mentioned results from GAG-PAGE, fractionation of yECM yielded two major sugar peaks (P1 and P2) (Fig. 3A). Both induced metachromasia in DMMB (Fig. 3A). In addition, P1 and P2 were also tested for uronic acids and yielded positive results (Fig. 3A), consistent with the above-mentioned results using the unfractionated yECM sample.

The P1 and P2 fractions were further analyzed by DAE (Fig. 3B). DAE is a powerful tool to separate compounds with different degrees of chemical substitution, usually sulphated polysaccharides [57]. As a control, the unfractionated yECM sample – PC (pre-chromatographic) fraction – was used. This sample presented two polydisperse

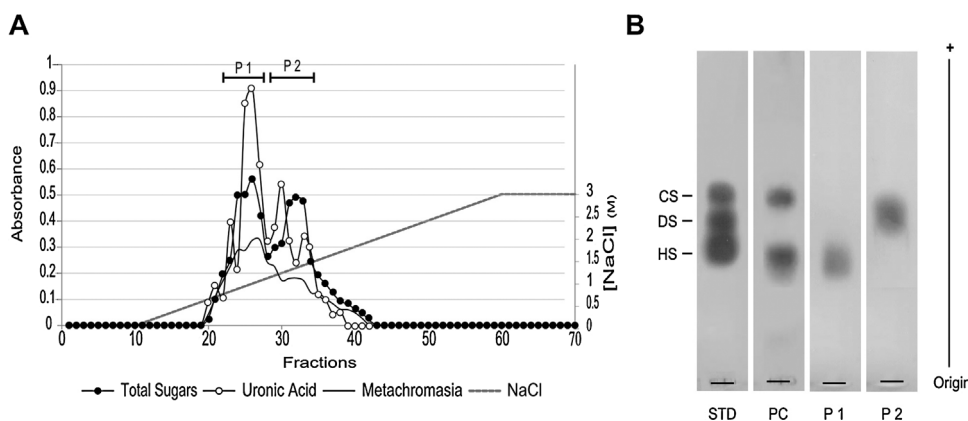


Figure 3. Chromatographic fractionation and electrophoretic profiles of *S. cerevisiae* ECM polysaccharides. (A) FPLC of the yECM glycoside fraction yielded two main compounds, P1 and P2. (B) DAE of P1 and P2 was compared with that of the sample prior to FPLC fractionation (PC). GAG standards (STD): CS, chondroitin sulphate; DS, dermatan sulphate; HS, heparan sulphate.

bands of two metachromatic compounds that migrated in a manner similar to CS and HS. Each corresponded to one of the P1 and P2 fractions (Fig. 3B). Similar to other reports using both FPLC fractionation and DAE [56], the first eluted ECM component (P1) matched the shortest migration in DAE. Combining these results with the results in Fig. 2A, these two compounds are likely to be different molecules.

S. cerevisiae extracellular matrix (ECM) polysaccharides display anticoagulant activity

Several studies showed the importance of sulphate substitution for the anticoagulant effect of heparin [58], DS [59] or their analogues [13, 18, 54]. In the literature concerning yeast, there is no reference to polysaccharide sulphation processes. Therefore, yECM components were further assessed for anticoagulant properties as a means to confirm the existence of this type of chemical substitution in yeast. The yECM PC fraction (above) was tested *in vitro* for anticoagulant properties by measuring aPTT [54]. For this assay, the *S. cerevisiae* wild type strain was used in parallel with the mutants defective in *GUP1* and/or *GUP2*, the genes encoding the yeast counterparts of the mammalian Hedgehog pathway modulators, HHATL and HHAT [60]. In mammals, ECM is the major vehicle and modulator of Hedgehog signalling, to which it responds with ever changing molecular structure and composition [3, 4]. The PC fractions from the *S. cerevisiae* wild type strain, the single deletion strains, *gup1* Δ and *gup2* Δ and the double deletion strain, *gup1* Δ *gup2* Δ were used to test the anticoagulant effect. Heparin was used as a control. Results in Fig. 4 show that yECM from the wild type strain and *gup1* Δ and *gup2* Δ strains did not display

a detectable anticoagulant effect, while in contrast, the yECM extract from the *gup1* Δ *gup2* Δ mutant strain displayed a significant effect.

Discussion

The present study was devised to determine the chemical composition of the glycoside components in ECM of *S. cerevisiae* biofilms. To achieve this and considering that *S. cerevisiae* does not form biofilms on inert surfaces as easily as other well-known yeasts such as *C. albicans* or *C. glabrata* [31], we devised a method to produce large biofilm-like homogenous overlays of cells mats from which generous amounts of ECM could be reproducibly extracted fuelling chemical/molecular analysis [47].

Previous results for ECM of *C. albicans* biofilms revealed the presence of β -1,3 glucan and a polysaccharide composed of α -D- and β -D-glucose, α -D-mannose, α -L-rhamnose and *N*-acetylglucosamine [25]. On the other hand, in *S. cerevisiae* ECM, the presence of glycoproteins was reported for colonies [27, 33]. Floccs from *S. cerevisiae* overexpressing *FLO1* secreted glucose and mannose, most of which organized into highly branched polysaccharides [46]. These authors also reported the presence of galactose, but attributed it to plasmid induction requests used during cells culture. Our results using a wild type strain of *S. cerevisiae* cultivated to form biofilm-like mats showed the yECM glycoside fraction to be composed of glucose, mannose and galactose. The relative amounts of galactose predicted by this methodology are very small, therefore, its true existence

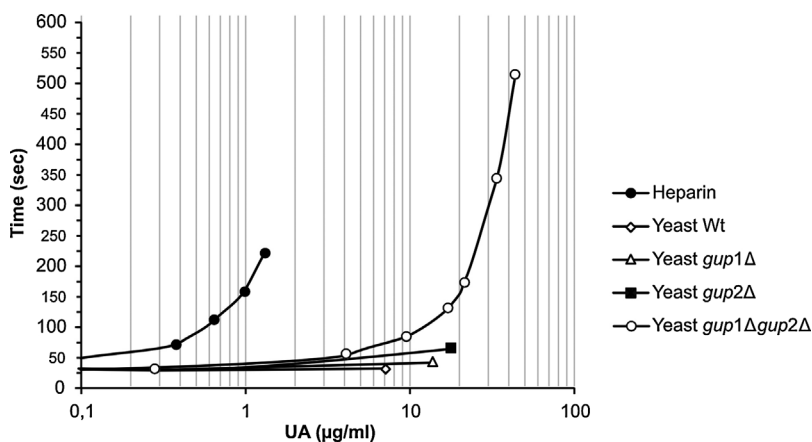


Figure 4. Anticoagulant effect of *S. cerevisiae* ECM extracts from several yeast strains. The anticoagulant effect of yECM polysaccharides originating from wild type and mutant yeast strains was tested measuring the activated partial thromboplastin time (aPTT) assay. Differences in the anticoagulant effect represent an indirect measurement of the presence of sulphate groups. Porcine intestinal mucosa heparin ($50 \mu\text{g ml}^{-1}$) was used as a control. UA stands for uronic acid.

requires further confirmation. Still, filamentous fungi have been reported to produce galactoglycans, such as the galactomannan found in *Aspergillus fumigatus* biofilms [61] or the galactoxylomannan found in the capsule of the yeast *Cryptococcus neoformans* [62]. On the other hand, chemical staining revealed the putative presence of uronic acids, which were undetected by GC-MS. Although the presence of uronic acids has been reported in other microbes [24, 55], this requires confirmation by in-depth analytical methods.

GAG-PAGE of the yECM glycoside fraction showed the presence of two glycoside species with different MW, which were subsequently separated by FPLC. The one with the lower MW (<8 kDa), consistent with the highly glycosylated nature of the SDS-PAGE profile as revealed by the association with concanavalin A, could comprise oligosaccharides and/or disaccharide units belonging to glycoproteins *in vivo*. Both glycoside species tested positive for uronic acids by chemical staining. In addition, they also tested positive for chemical substitution by metachromasia, which was confirmed in the unfractionated yECM sample composed of sugars and proteins and by DAE. The induction of metachromasia is frequent in chemically substituted polysaccharides, namely highly sulphated GAGs from ECM of higher eukaryotes [56] and is mostly associated with the presence of sulphate groups [52]. However, metachromasia may also be induced by a wide variety of polyanions [51, 63, 64], such as polyphosphates, polyacrylates, polysulphates, carboxylated polysaccharides and some proteins and nucleic acids [51]. The methodologies used to separate yECM protein and glycoside fractions ensure a glycoside protein-free fraction but do not avoid putative very low mass contaminants such as amino acids or nucleotides. Nevertheless, the clearness of the DAE electrophoretic profile strongly suggests the absence of these contaminants, or their putative presence in amounts below the level of detection.

The sulphation pattern is considered to be responsible for the degree of anticoagulation ability of mammalian heparin and other polysaccharides [18]. Considering the absence of references in the literature to polysaccharide sulphation in yeast, the results obtained with metachromasia and DAE were challenged through an assay of anticoagulation activity against human plasma – aPTT. For this purpose, several yeast genetic backgrounds were used. The rationale behind their choice is the fact that mammalian ECM governs and is governed by the Hedgehog signalling pathway [3, 4], for which Hhat and Hhatl modulators [60] were first described as yeast proteins, Gup1p and Gup2p [65, 66]. Gup1p and Gup2p are extremely pleiotropic, interfering with namely cell

wall and plasma membrane composition and function, including wall polysaccharide assembly, lipid metabolism and rafts and GPI anchors integrity [48, 67, 68]. The anticoagulation activity displayed by the *gup1Δgup2Δ* mutant suggests that the disruption of these genes triggers a process of chemical substitution of yECM polysaccharides absent or residual in the other strains, including the wild type strain. As mentioned above, there is no evidence in yeast of a molecular apparatus that may be responsible for sulphation of polysaccharides or other compounds. Anticoagulation activity suggests that sulphate substitution may actually be possible in yeast. Ultimately, the differences observed between mutant strains defective in either or both GUP genes also suggest that it is dynamically regulated, in which the two Gup proteins may have a role.

Final remarks

This work reports the chemical nature of *S. cerevisiae* ECM polysaccharides. Indications are provided of the presence of two different MW polysaccharides composed of glucose, mannose and galactose and putatively also uronic acids. Moreover, results also point to chemical substitution of yECM polysaccharides, compatible with the results obtained with metachromasia by DMMB and toluidine blue, DAE and aPTT. The fact that the observed yECM-derived anticoagulant effect was restricted to yECM from a mutant lacking both yeast Gup1 and Gup2 proteins suggests the involvement of both proteins in the process of chemical modification of yECM polysaccharides. The fact that this effect was not detected in the wild type strain and in the correspondent single mutant further suggests dynamic regulation of the underlying processes, which may be absent or simply below detection levels in these strains. Finally, considering that sulphation governs anticoagulation properties of mammalian, algae and ascidian polysaccharides, results suggest an equivalent nature of chemical substitution of yECM polysaccharides. These results pioneer the study of yeast cell surface glycomics beyond the cell wall in the context of biofilms or colonies as multicellular aggregates, opening the way to a new understanding of yeast in a societal context of large multicellular aggregates, analogous to higher eukaryotic tissues.

Acknowledgments

The polysaccharide analysis was performed at the Laboratory of Glycoconjugates Biochemistry and Cellular Biology, UFRJ, Brazil. Fábio Faria-Oliveira was supported by a PhD scholarship (SFRH/BD/45368/2008)

from FCT (Fundação para a Ciência e a Tecnologia). This work was funded by Marie Curie Initial Training Network GLYCOPHARM (PITN-GA-2012-317297), and FCT/MEC through Portuguese funds (PIDDAC)–PEst-OE/BIA/UI4050/2014.

Conflict of interests

The authors declare that there are no conflicts of interests relating the present work.

References

- [1] Condeelis, J., Segall, J.E., 2003. Intravital imaging of cell movement in tumours. *Nat. Rev. Cancer*, **3**, 921–930.
- [2] Lu, P., Weaver, V.M., Werb, Z., 2012. The extracellular matrix: A dynamic niche in cancer progression. *J. Cell Biol.*, **196**, 395–406.
- [3] Rozario, T., DeSimone, D.W., 2010. The extracellular matrix in development and morphogenesis: A dynamic view. *Dev. Biol.*, **341**, 126–140.
- [4] Lu, P., Takai, K., Weaver, V.M., Werb, Z., 2011. Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harb. Perspect. Biol.*, **3**, a005058.
- [5] Iozzo, R.V., Karamanos, N., 2010. Proteoglycans in health and disease: Emerging concepts and future directions. *FEBS J.*, **277**, 3863.
- [6] Afratis, N., Gialeli, C., Nikitovic, D., Tsegenidis, T., et al., 2012. Glycosaminoglycans: Key players in cancer cell biology and treatment. *FEBS J.*, **279**, 1177–1197.
- [7] Esko, J.D., Kimata, K., Lindahl, U., 2009. Proteoglycans and sulfated glycosaminoglycans, in: Varki, A., Cummings, R. D., Esko, J.D., Freeze, H.H., et al. (Eds.), *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press, New York, 229–248.
- [8] Sawhney, R., Hering, T., Sandell, L., 1991. Biosynthesis of small proteoglycan II (decorin) by chondrocytes and evidence for a procure protein. *J. Biol. Chem.*, **266**, 9231–9240.
- [9] Dudhia, J., 2005. Aggrecan, aging and assembly in articular cartilage. *Cell. Mol. Life Sci.*, **62**, 2241–2256.
- [10] Zimmermann, D.R., Ruoslahti, E., 1989. Multiple domains of the large fibroblast proteoglycan, versican. *EMBO J.*, **8**, 2975.
- [11] Bülow, H.E., Hobert, O., 2006. The molecular diversity of glycosaminoglycans shapes animal development. *Annu. Rev. Cell. Dev. Biol.*, **22**, 375–407.
- [12] Gandhi, N.S., Mancera, R.L., 2008. The structure of glycosaminoglycans and their interactions with proteins. *Chem. Biol. Drug Des.*, **72**, 455–482.
- [13] Pavão, M.S.G., Aiello, K.R.M., Werneck, C.C., Silva, L.C.F., et al., 1998. Highly sulfated dermatan sulfates from ascidians. *J. Biol. Chem.*, **273**, 27848–27857.
- [14] Schaefer, L., Iozzo, R.V., 2008. Biological functions of the small leucine-rich proteoglycans: From genetics to signal transduction. *J. Biol. Chem.*, **283**, 21305–21309.
- [15] Schaefer, L., Schaefer, R.M., 2010. Proteoglycans: From structural compounds to signaling molecules. *Cell Tissue Res*, **339**, 237–246.
- [16] Kiani, C., Chen, L., Wu, Y.J., Yee, A.J., et al., 2002. Structure and function of aggrecan. *Cell Res.*, **12**, 19–32.
- [17] Bishop, J.R., Schuksz, M., Esko, J.D., 2007. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature*, **446**, 1030–1037.
- [18] Belmiro, C.L., Castelo-Branco, M.T., Melim, L.M., Schnaider, A., et al., 2009. Unfractionated heparin and new heparin analogues from ascidians (chordate-tunicate) ameliorate colitis in rats. *J. Biol. Chem.*, **284**, 11267–11278.
- [19] Borsig, L., Wong, R., Feramisco, J., Nadeau, D.R., et al., 2001. Heparin and cancer revisited: Mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. *Proc. Natl. Acad. Sci. USA*, **98**, 3352–3357.
- [20] Kozłowski, E., Pavao, M., 2011. Effect of sulfated glycosaminoglycans on tumor invasion and metastasis. *Front. Biosci.*, **3**, 1541–1551.
- [21] Roberson, E.B., Firestone, M.K., 1992. Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. *Appl. Environ. Microbiol.*, **58**, 1284–1291.
- [22] von Bodman, S.B., Majerczak, D.R., Coplin, D.L., 1998. A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. *stewartii*. *Proc. Natl. Acad. Sci. USA*, **95**, 7687–7692.
- [23] Sutherland, I., 2001. Biofilm exopolysaccharides: A strong and sticky framework. *Microbiology*, **147**, 3–9.
- [24] Tielen, P., Strathmann, M., Jaeger, K.E., Flemming, H.C., et al., 2005. Alginate acetylation influences initial surface colonization by mucoid *Pseudomonas aeruginosa*. *Microbiol. Res.*, **160**, 165–176.
- [25] Lal, P., Sharma, D., Pruthi, P., Pruthi, V., 2010. Exopolysaccharide analysis of biofilm-forming *Candida albicans*. *J. Appl. Microbiol.*, **109**, 128–136.
- [26] Romero, D., Aguilar, C., Losick, R., Kolter, R., 2010. Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms. *Proc. Natl. Acad. Sci. USA*, **107**, 2230–2234.
- [27] Kuthan, M., Devaux, F., Janderova, B., Slaninova, I., et al., 2003. Domestication of wild *Saccharomyces cerevisiae* is accompanied by changes in gene expression and colony morphology. *Mol. Microbiol.*, **47**, 745–754.
- [28] Palková, Z., Váchová, L., 2006. Life within a community: Benefit to yeast long-term survival. *FEMS Microbiol. Rev.*, **30**, 806–824.
- [29] Engelberg, D., Mimran, A., Martinetto, H., Otto, J., et al., 1998. Multicellular stalk-like structures in *Saccharomyces cerevisiae*. *J. Bacteriol.*, **180**, 3992–3996.
- [30] Scherz, R., Shinder, V., Engelberg, D., 2001. Anatomical analysis of *Saccharomyces cerevisiae* stalk-like structures reveals spatial organization and cell specialization. *J. Bacteriol.*, **183**, 5402–5413.
- [31] Reynolds, T.B., Fink, G.R., 2001. Bakers' yeast, a model for fungal biofilm formation. *Science*, **291**, 878–881.

- [32] Reynolds, T.B., Jansen, A., Peng, X., Fink, G.R., 2008. Mat formation in *Saccharomyces cerevisiae* requires nutrient and pH gradients. *Eukaryot. Cell*, **7**, 122–130.
- [33] Váchová, L., Štoviče, V., Hlaváče, O., Chernyavskiy, O., et al., 2011. Flo11p, drug efflux pumps, and the extracellular matrix cooperate to form biofilm yeast colonies. *J. Cell. Biol.*, **194**, 679–687.
- [34] Štoviček, V., Váchová, L., Kuthan, M., Palková, Z., 2010. General factors important for the formation of structured biofilm-like yeast colonies. *Fungal Genet. Biol.*, **47**, 1012–1022.
- [35] Čáp, M., Štěpánek, L., Harant, K., Váchová, L., et al., 2012. Cell differentiation within a yeast colony: Metabolic and regulatory parallels with a tumor-affected organism. *Mol. Cell.*, **46**, 436–448.
- [36] Palková, Z., Devaux, F., Ricicová, M., Mináriková, L., et al., 2002. Ammonia pulses and metabolic oscillations guide yeast colony development. *Mol. Biol. Cell.*, **13**, 3901–3914.
- [37] Váchová, L., Palková, Z., 2005. Physiological regulation of yeast cell death in multicellular colonies is triggered by ammonia. *J. Cell. Biol.*, **169**, 711–717.
- [38] Vopálenská, I., Štoviček, V., Jandrová, B., Váchová, L., et al., 2010. Role of distinct dimorphic transitions in territory colonizing and formation of yeast colony architecture. *Environ. Microbiol.*, **12**, 264–277.
- [39] Váchová, L., Chernyavskiy, O., Strachotová, D., Bianchini, P., et al., 2009. Architecture of developing multicellular yeast colony: Spatio-temporal expression of Ato1p ammonium exporter. *Environ. Microbiol.*, **11**, 1866–1877.
- [40] Nobile, C.J., Mitchell, A.P., 2006. Genetics and genomics of *Candida albicans* biofilm formation. *Cell. Microbiol.*, **8**, 1382–1391.
- [41] Fanning, S., Mitchell, A.P., 2012. Fungal biofilms. *PLoS Pathog.*, **8**, e1002585.
- [42] De Beer, D., Stoodley, P., 2013. Microbial biofilms, in: Rosenberg, E., DeLong, E., Lory, S., Stackebrandt, E., et al. (Eds.), *The Prokaryotes*, Springer, Berlin, 343–372.
- [43] Chandra, J., Kuhn, D.M., Mukherjee, P.K., Hoyer, L.L., et al., 2001. Biofilm formation by the fungal pathogen *Candida albicans*: Development, architecture, and drug resistance. *J. Bacteriol.*, **183**, 5385–5394.
- [44] Al-Fattani, M.A., Douglas, L.J., 2006. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J. Med. Microbiol.*, **55**, 999–1008.
- [45] Martins, M., Uppuluri, P., Thomas, D.P., Cleary, I.A., et al., 2010. Presence of extracellular DNA in the *Candida albicans* biofilm matrix and its contribution to biofilms. *Mycopathologia*, **169**, 323–331.
- [46] Beauvais, A., Loussert, C., Prevost, M.C., Verstrepen, K., et al., 2009. Characterization of a biofilm-like extracellular matrix in *FLO1*-expressing *Saccharomyces cerevisiae* cells. *FEMS Yeast Res.*, **9**, 411–419.
- [47] Faria-Oliveira, F., Carvalho, J., Belmiro, C.L.R., Martinez-Gomariz, M., et al., 2014. Methodologies to generate, extract, purify and fractionate yeast ECM for analytical use in proteomics and glycomics. *BMC Microbiol.*, **14**, 144.
- [48] Ferreira, C., Silva, S., van Voorst, F., Aguiar, C., et al., 2006. Absence of Gup1p in *Saccharomyces cerevisiae* results in defective cell wall composition, assembly, stability and morphology. *FEMS Yeast Res.*, **6**, 1027–1038.
- [49] Masuko, T., Minami, A., Iwasaki, N., Majima, T., et al., 2005. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Anal. Biochem.*, **339**, 69–72.
- [50] Bitter, T., Muir, H.M., 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.*, **4**, 330–334.
- [51] Kugel, R.W., 1993. Metachromasy: The interactions between dyes and polyelectrolytes in aqueous solution, in: Urban, M.W., Craver, C.D. (Eds.), *Structure-Property Relations in Polymers-Spectroscopy and Performance*, American Chemical Society, New York, 507–533.
- [52] Farnsdale, R.W., Buttle, D.J., Barrett, A.J., 1986. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim. Biophys. Acta*, **883**, 173–177.
- [53] Ruiz-Matute, A.I., Hernandez-Hernandez, O., Rodriguez-Sanchez, S., Sanz, M.L., et al., 2011. Derivatization of carbohydrates for GC and GC-MS analyses. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **879**, 1226–1240.
- [54] Gomes, A.M., Kozłowski, E.O., Pomin, V.H., de Barros, C.M., et al., 2010. Unique extracellular matrix heparan sulfate from the bivalve *Nodipecten nodosus* (Linnaeus 1758), safely inhibits arterial thrombosis after 7 photochemically induced endothelial lesion. *J. Biol. Chem.*, **285**, 7312–7323.
- [55] Wozniak, D.J., Wyckoff, T.J.O., Starkey, M., Keyser, R., et al., 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proc. Natl. Acad. Sci. USA*, **100**, 7907–7912.
- [56] Pavão, M.S.G., Mourão, P.A.S., Mulloy, B., Tollefsen, D.M., 1995. A unique dermatan sulfate-like glycosaminoglycan from ascidian. *J. Biol. Chem.*, **270**, 31027–31036.
- [57] Cohen, D.M., Mourão, P.A.S., Dietrich, C.P., 1977. Differentiation of mucopolysaccharidoses by analyses of the excreted sulfated mucopolysaccharides. *Clin. Chim. Acta*, **80**, 555–562.
- [58] Jin, L., Abrahams, J.P., Skinner, R., Petitou, M., et al., 1997. The anticoagulant activation of antithrombin by heparin. *Proc. Natl. Acad. Sci. USA*, **94**, 14683–14688.
- [59] Trowbridge, J.M., Gallo, R.L., 2002. Dermatan sulfate: New functions from an old glycosaminoglycan. *Glycobiology*, **12**, 117R–125R.
- [60] Abe, Y., Kita, Y., Niikura, T., 2008. Mammalian Gup1, a homolog of *Saccharomyces cerevisiae* glycerol uptake/transporter 1, acts as a negative regulator for N-terminal palmitoylation of Sonic hedgehog. *FEBS J.*, **275**, 318–331.
- [61] Papi, M., Maiorana, A., Bugli, F., Torelli, R., et al., 2012. Detection of biofilm-grown *Aspergillus fumigatus* by means of atomic force spectroscopy: Ultrastructural effects of alginate lyase. *Microsc. Microanal.*, **18**, 1088–1094.
- [62] Vecchiarelli, A., Pericolini, E., Gabrielli, E., Chow, S.K., et al., 2011. *Cryptococcus neoformans* galactoxylomannan is a potent negative immunomodulator, inspiring new approaches in anti-inflammatory immunotherapy. *Immunotherapy*, **3**, 997–1005.
- [63] Nandini, R., Vishalakshi, B., 2010. A study of interaction of cationic dyes with anionic polyelectrolytes. *Spectrochim. Acta A*, **75**, 14–20.

- [64] Nandini, R., Vishalakshi, B., 2011. A comparative study of polyelectrolyte induced metachromasy. *J. Polym. Eng.*, **31**, 191.
- [65] Holst, B., Lunde, C., Lages, F., Oliveira, R., *et al.*, 2000. *GUP1* and its close homologue *GUP2*, encoding multimembrane-spanning proteins involved in active glycerol uptake in *Saccharomyces cerevisiae*. *Mol. Microbiol.*, **37**, 108–124.
- [66] Hofmann, K., 2000. A superfamily of membrane-bound *O*-acyltransferases with implications for wnt signaling. *Trends Biochem. Sci.*, **25**, 111–112.
- [67] Ferreira, C., Lucas, C., 2008. The yeast *O*-acyltransferase Gup1p interferes in lipid metabolism with direct consequences on the sphingolipid-sterol-ordered domains integrity/assembly. *Biochim. Biophys. Acta*, **1778**, 2648–2653.
- [68] Ferreira, C., Silva, S., Faria-Oliveira, F., Pinho, E., *et al.*, 2010. *Candida albicans* virulence and drug-resistance requires the *O*-acyltransferase Gup1p. *BMC Microbiol.*, **10**, 238.