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Article

Yeast and fungal cell-wall polysaccharides can self-assemble *in vitro* into an ultrastructure resembling *in vivo* yeast cell walls

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Abstract Polysaccharides account for more than 90% of the content of fungal cell walls, but the mechanism underlying the formation of the architecture of the cell walls, which consist of microfibrils embedded in an amorphous wall matrix, remains unknown. We used electron microscopy to investigate ten different fungal cell-wall polysaccharides to determine whether they could self-assemble into the fibrillar or amorphous component of fungal cell walls in a test tube without enzymes. The ultrastructures formed by precipitating β-1,3-glucan and β-1,6-glucan are different depending on the existence of branching in the molecule. Linear β-1,3-glucan and linear β-1,6-glucan precipitate into a fibrillar ultrastructure. Branched β-1,6-glucan, mannan and glycogen precipitates are amorphous. Branched β-1,3-glucan forms a fibrillar plus amorphous ultrastructure. Self-assembly among combinations of different linear and branched cell-wall polysaccharides results in an ultrastructure that resembles that of a yeast cell wall, which suggests that self-assembly of polysaccharides may participate in the development of the three-dimensional architecture of the yeast cell wall.

Keywords cell-wall polysaccharides, self-assembly, yeast, fungi, *Saccharomyces cerevisiae*

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Introduction

The fungal cell wall is an extracytoplasmic exoskeleton that contains a three-dimensional network of polysaccharides constituting more than 90% of the cell wall. The structural core of the wall is generally thought to be fibrillar and is embedded in amorphous cement [1–3]. However, the mechanism underlying polysaccharide assembly into cell-wall components is unknown. One of the best-studied types of fungal wall is that of the yeast *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* cell walls are constructed from α-mannoproteins (28%), 3 types of β-glucans (30–60%), including alkali-insoluble branched β-1,3-D-glucan [4], acid-soluble branched β-1,6-D-glucan [5] and alkali-soluble branched β-1,3-D-glucan [6], chitin (1–2%), proteins (2–3%) and glycogen [7–12]. While glucans and mannans are evenly distributed in the cell walls, most of the chitin is concentrated in bud scars [13–16]. The function of glycogen in the yeast cell wall remains unclear [7].

Electron micrographs of untreated yeast have shown an amorphous cell wall surface [17,18]. These native walls produce a diffuse X-ray diffraction diagram, testifying against the presence of fibrils in the cell wall [18]. Cells boiled in 2% HCl and 3% NaOH for 3 h lose this amorphous wall surface material. Under the amorphous surface, microfibrils crystallize *in vitro* from a partially hydrolyzed

'hydroglucan' that exhibits a sharp X-ray diffraction pattern corresponding to that of linear crystalline β-1,3-glucan paramylon [18]. Other studies have claimed that there are no microfibrils in the yeast cell wall [19]. In contrast, naked yeast protoplasts in liquid media form wall microfibrils *de novo* [20–22] from linear β-1,3-glucan [22–24]. The observation of these protoplast microfibrils encouraged us to look for microfibrils in the yeast cell wall. After selectively digesting amorphous wall β-1,6-glucan and β-1,3-glucan with purified bacterial endo-β-1,6-glucanase and bacterial endo-β-1,3-glucanase, we observed the cell wall using electron microscopy and discovered a continuous fibrillar skeletal component under the external amorphous wall surface of *S. cerevisiae* [2]. In addition, selective digestion of mannoproteins by pronase demonstrated that the fibrillar component is embedded in the amorphous glucan matrix and indicated that amorphous β-1,6-glucan and β-1,3-glucan may act as linkers between the inner fibrillar cell-wall skeleton and the outer cell-wall mannoproteins [2]. To identify the nature of the wall microfibrils, we used yeast endo-β-1,3-glucanase to show that β-1,3-glucan microfibrils are in the yeast cell wall. Treatment with yeast endo-β-1,3-glucanase caused complete disassembly of the wall architecture, except in the bud scars [2]. Furthermore, we fragmented cell walls through prolonged mechanical breakage and applied negative staining, which led us to conclude that there is a fibrillar component in native, enzymatically untreated yeast cell walls [2,3].

Although the chemical composition and ultrastructure of yeast cell walls have been thoroughly studied, the mechanism underlying the development of wall architecture is still unknown. In contrast to what is observed in yeasts, the unicellular green alga *Chlamydomonas reinhardii* has cell walls that self-assemble *in vitro* from dissociated wall glycoprotein subunits in the presence of wall 'nucleation centres' [25,26]. Previously, we observed that yeast protoplasts that are devoid of their original cell walls can partially regenerate a new cell wall when grown on agar [27] or can completely regenerate a new cell wall when grown inside gelatine [28–31]. Our analyses of naked yeast protoplasts led us to hypothesize that the wall polymers synthesized and secreted to the external surface of naked protoplasts self-assemble *de novo* into cell walls around the regenerating protoplasts.

Previously, we studied the self-assembly of linear β-1,3-glucan from *S. cerevisiae* protoplast fibrillar nets that had been dissolved in 1 M NaOH at 4°C and passed through ultrafilters with a pore size of 10 nm. We found that after neutralization or dialysis, molecularly dispersed glucan self-assembled *in vitro* into microfibrils [32]. Similarly, β-1,3-glucan microfibrils and α-1,3-glucan microfibrils self-assembled *in vitro* from purified and neutralized glucans from *Schizosaccharomyces pombe* protoplast fibrillar nets dissolved in 1 M NaOH [33]. Additionally, chitin extracted from *S. cerevisiae* cell walls and protoplast fibrillar nets using 30% HCl at 4°C and then neutralized self-assembled *in vitro* into the fine granular amorphous component of cell walls due to its short molecules [14]. However, complete yeast cell-wall assembly has not yet been experimentally investigated. The objective of this work was to determine whether purified yeast and fungal cell-wall polysaccharides, dissolved in a test tube and neutralized, could self-assemble *in vitro* into cell wall-like structures containing microfibrils embedded in an amorphous matrix that resemble the native yeast cell wall of regenerating protoplasts.

Materials and methods

Cultivation of *Saccharomyces cerevisiae* wild-type cells

Saccharomyces cerevisiae (CCY21-4-59) cells were cultivated in malt extract medium at pH 5.4 at 23°C for ~10–15 h. To prepare protoplasts, the cells were diluted with fresh medium and cultivated on a shaker for 5 h [32].

Formation of protoplasts and isolation of their fibrillar nets from liquid medium

Protoplasts were prepared from cells using snail enzymes in an osmotic stabilizer and cultivated in liquid N1 medium for ~20 h. Isolation of fibrillar nets from protoplasts was performed *via* osmotic lysis with distilled water, and purification was performed by treatment with 0.5% hot lauryl sulphate [32].

Protoplast cell-wall regeneration and electron microscopy of these cell walls

Protoplasts were regenerated in 30% gelatine-containing N1 medium. Their cell walls were isolated, washed and studied under transmission electron microscopy after metal shadowing [30,31].

We used the following polysaccharides isolated from the cell walls of *Saccharomyces cerevisiae* for *in vitro* reaggregation studies

Cell-wall mannan [10], isolated by Slovakofarma Bratislava, Slovakia, was kindly provided by Doc. Ing. V. Farkaš, DrSc., of the Chemical Institute of SAV, Bratislava, Slovakia. Electron micrographs of native mannan dissolved in redistilled water have previously been reported [34]. **A2 Glucan** was kindly provided by Prof. D.J. Manners of the University of Edinburgh, Scotland. Prof. Manners used the A2 Glucan from *S. cerevisiae* cell walls to describe branched β-1,3-glucan [4]. Electron microscopy analyses of native A2 glucan before and after NaOH and acetic acid treatment have previously been reported [34]. **β-1,6-D-Glucan** was kindly provided by Prof. D.J. Manners of the University of Edinburgh, Scotland. Prof. Manners isolated the β-1,6-D-Glucan and described it as branched β-1,6-glucan [5]. Electron microscopy analyses of native β-1,6-D-glucan dissolved in redistilled water have been presented previously [34]. Finally, **Glycogen** (α-1,4-,α-1,6-branched glucan) [7] was kindly provided by Dr P. Biely, DrSc., from the Chemical Institute of SAV, Bratislava, Slovakia.

We used the following fungal cell-wall polysaccharides and other polysaccharides for *in vitro* reaggregation studies

Pachyman, a linear β-1,3-glucan from the basidiomycete *Poria cocos* [35], was dissolved in DMSO, and the insoluble sediment was removed by centrifugation. Lipids were extracted and kindly provided by Dr D.R. Kreger of the University Groningen, the Netherlands. **Pustulan**, a linear β-1,6-D-glucan from the lichen fungus *Umbilicaria pustulata* [36], was kindly provided by Dr P. Biely of the Chemical institute of SAV Bratislava, Slovakia. **Pseudonigeran**, a linear α-1,3-glucan with α-1,4-linkages [37], isolated from the mycelium of *Aspergillus niger* NRRL 326, was kindly provided by Prof. H. J. Phaff of the University of California, Davis, USA. **Paramylon**, a linear β-1,3-glucan from the protozoan *Astasia longa* [38], was kindly provided by Dr B. J. D. Meeuse of the University of Washington, Seattle, USA. **Laminarin**, a linear β-1,3-,β-1,6-glucan from brown algae [38], was kindly provided by Prof. H. J. Phaff of the University of California, Davis, USA.

Dissolution of alkali-soluble linear β-1,3-glucan from the fibrillar nets of *S. cerevisiae* protoplasts was performed in 1 M NaOH at 4°C for 5 days. The insoluble components of the fibrillar nets, containing branched insoluble β-1,3-glucan and chitin, were separated from the alkali-soluble glucans *via* repeated centrifugation for 30 and 60 min at 15 000*g* and ultrafiltration through collodium ultrafilters with a pore size of 10 nm [23,24,32].

In vitro reaggregation of alkali-soluble linear β-(1 → 3)-glucan from *S. cerevisiae* protoplasts was performed *via* neutralization of 1 M NaOH containing the dissolved glucan with 1 M acetic acid to a pH of 6.5 [32,39].

Solubilization and *in vitro* reaggregation of polysaccharides

All β-1,3-glucans, α-1,3-glucan and the linear β-1,6-glucan pustulan were dissolved to produce a 1% solution of each glucan in a 1 M NaOH solution at 4°C for 24 h. After repeated centrifugation for 30 and 60 min at 15 000*g* to remove insoluble material, the supernatant was removed and used for *in vitro* reaggregation studies after neutralization with 1 M acetic acid.

Branched β-1,6-glucan, glycogen and mannan from the *S. cerevisiae* cell wall were dissolved in 1 M acetic acid [5,8]. After repeated centrifugation for 30 and 60 min at 15 000*g*, the supernatant was used for *in vitro* reaggregation *via* neutralization with 1 M NaOH.

Combined reaggregation of polysaccharides dissolved in 1 M NaOH

To analyse the combined reaggregation of different polysaccharides dissolved in 1 M NaOH, the same volume (usually 200 µl) of each polysaccharide solution was combined. Simultaneous neutralization of the combined polysaccharides was then accomplished using a corresponding volume of 1 M acetic acid.

Combined reaggregation of polysaccharides dissolved in 1 M acetic acid

To examine the combined reaggregation of different polysaccharides dissolved in 1 M acetic acid, the same volume (usually 200 µl) of each polysaccharide solution was combined. Simultaneous neutralization of the combined polysaccharides was then accomplished using a corresponding volume of 1 M NaOH.

Combined reaggregation of polysaccharides dissolved in 1 M acetic acid and in 1 M NaOH

To examine the combined reaggregation of different polysaccharides, the same volume (usually 200 µl) of each polysaccharide solution was combined. Neutralization was performed by mixing the combined solutions of the dissolved polysaccharides in 1 M acetic acid and 1 M NaOH with the same final volumes of 1 M NaOH and 1 M acetic acid.

Electron microscopy analysis of the *in vitro*-reaggregated polysaccharides was performed. The samples were washed three times *via* centrifugation with redistilled water and applied to electron microscope grids with formvar membranes. Dried samples were then metal-shadowed using Pt at a 15° angle. All preparations were observed and photographed with a Tesla BS 500 transmission electron microscope (Tesla Brno, Czech Republic).

Image-processing software

Adobe Photoshop 8.0 for Windows was used for all figures.

Results

One experimental approach for studying the mechanism underlying the assembly of the architecture of fungal cell walls *in vitro* involves investigation of three possibilities: (i) whether some cell-wall polysaccharides can self-assemble into microfibrils *in vitro*, (ii) whether some cell-wall polysaccharides can self-assemble into the amorphous component of cell walls *in vitro* and (iii) whether *in vitro* self-assembly of combinations of different cell-wall polysaccharides results in an ultrastructure similar to that of the native cell walls formed by regenerating yeast protoplasts *in vivo*. To address these possibilities, we designed several experiments. First, we investigated the ultrastructure of single yeast and fungal polysaccharides that self-assemble *in vitro*. Then, we investigated mixtures of two, three, four and five polysaccharides that self-assemble together *in vitro*. Finally, we examined the ultrastructure of the structures that self-assembled *in vitro* from the combined polysaccharides and compared it with the ultrastructure of *de novo*-assembled native cell walls from regenerated yeast protoplasts.

In vitro self-assembly of single polysaccharides from cell walls ('one-step' self-assembly) (Table 1A)

(1) Linear protoplast β-1,3-glucan precipitated into microfibrils (Fig. 1).
(2) Branched wall β-1,3-glucan precipitated into both the microfibrillar and the amorphous components (Fig. 2).
(3) Linear wall β-1,6-glucan pustulan precipitated into microfibrils (Fig. 3).
(4) Branched wall β-1,6-glucan precipitated into the amorphous component (Fig. 4).
(5) Branched wall mannans (α-1,6-, α-1,2-, α-1,3-linked polymer) precipitated into the amorphous component (see the supplementary data online, Fig. S1).
(6) Branched wall glycogen (α-1,4-, α-1,6-glucan) precipitated into the amorphous component (see the supplementary data online, Fig. S2).
(7) Wall pseudonigeran (α-1,3-, α-1,4-glucan) precipitated into the fine granular amorphous component (see the supplementary data online, Fig. S3).
(8) Paramylon, linear β-1,3-glucan precipitated into the fibrillar component (see the supplementary data online, Fig. S4).
(9) Pachyman, linear β-1,3-glucan precipitated into the fibrillar component (see the supplementary data online, Fig. S5).
(10) Laminarin, linear β-1,3-, β-1,6 glucan precipitated into the fibrillar component (see the supplementary data online, Fig. S6).

Conclusions: (i) All the linear polysaccharides (β-1,3-glucan from protoplasts, paramylon, pachyman and laminarin, and β-1,6-glucan pustulan) self-assemble *in vitro* into the microfibrillar component of cell walls. (ii) Branched wall β-1,6-glucan,

Table 1. Analysis under electron microscopy of in vitro self-assembly of purified yeast and fungal-cell-wall polysaccharides to form supramolecular complexes and cell-wall-like structures

Polysaccharide used	Detected under the electron microscope as	Figure number
A. Single polysaccharides ('one-step' self-assembly):		
1. Linear β-1,3-glucan from *S. cerevisiae* protoplasts	Microfibrils	(Fig. 1)
2. Branched β-1,3-glucan from *S. cerevisiae* walls	Microfibrils + amorphous comp	(Fig. 2)
3. Linear β-1,6-glucan pustulan	Microfibrils	(Fig. 3)
4. Branched β-1,6-glucan from *S. cerevisiae* walls	Amorphous comp	(Fig. 4)
5. Mannan from *S. cerevisiae* walls	Amorphous comp (see the supplementary data online, Fig. S1)	
6. Glycogen from *S. cerevisiae*	Amorphous comp (see the supplementary data online, Fig. S2)	
7. α-1,3-glucan (pseudonigeran) from the cell walls of *A. niger*	Amorphous comp (see the supplementary data online, Fig. S3)	
8. Paramylon	Microfibrils (see the supplementary data online, Fig. S4)	
9. Pachyman	Microfibrils (see the supplementary data online, Fig. S5)	
10. Laminarin	Microfibrils (see the supplementary data online, Fig. S6)	
B. 'Two-step' self-assembly:		
1. Linear protoplast β-1,3-glucan + branched wall β-1,3-glucan	Microfibrils + amorphous comp	(Fig. 5)
2. Linear protoplast β-1,3-glucan + laminarin	microfibrils (see the supplementary data online, Fig. S7)	
3. Linear protoplast β-1,3-glucan + pachyman	Microfibrils (see the supplementary data online, Fig. S8)	
4. Linear protoplast β-1,3-glucan + paramylon	Microfibrils (see the supplementary data online, Fig. S9)	
5. Linear protoplast β-1,3-glucan + pustulan	Microfibrils (see the supplementary data online, Fig. S10)	
6. Linear protoplast β-1,3-glucan + branched wall β-1,6-glucan	Microfibrils (see the supplementary data online, Fig. S11)	
7. Linear protoplast β-1,3-glucan + branched glycogen	Microfibrils (see the supplementary data online, Fig. S12)	
8. Linear protoplast β-1,3-glucan + branched wall mannan	Microfibrils (see the supplementary data online, Fig. S13)	
9. Linear protoplast β-1,3-glucan + wall α-1,3-glucan	Microfibrils + amorphous comp (see the supplementary data online, Fig. S14)	
C. 'Three-step' self-assembly:		
1. Linear protoplast β-1,3-glucan + branched wall β-1,3-glucan + branched wall β-1,6-glucan	Microfibrils + amorphous comp	(Fig. 6)
2. Linear protoplast β-1,3-glucan + branched wall β-1,6-glucan + mannan	Microfibrils + amorphous comp (see the supplementary data online, Fig. S15)	
3. Linear protoplast β-1,3-glucan + branched wall β-1,6-glucan + glycogen	Microfibrils + amorphous comp (see the supplementary data online, Fig. S16)	
4. Linear protoplast β-1,3-glucan + mannan + glycogen	Microfibrils + amorphous comp (see the supplementary data online, Fig. S17)	
5. Linear protoplast β-1,3-glucan + branched wall β-1,3-glucan + mannan	Microfibrils + amorphous comp (see the supplementary data online, Fig. S18)	
D 'Four-step' self-assembly:		
1. Linear protoplast β-1,3-glucan + branched wall β-1,3-glucan + branched wall β-1,6-glucan + mannan	Microfibrils + amorphous comp	(Fig. 7)

Continued

Table 1. *Continued*

Polysaccharide used	Detected under the electron microscope as	Figure number
2. Linear protoplast β-1,3-glucan + branched wall β-1,6-glucan + mannan + glycogen	Microfibrils + amorphous comp (see the supplementary data online, Fig. S19)	
E. 'Five-step' self-assembly: 1. linear protoplast β-1,3-glucan + branched wall β-1,3-glucan + branched wall + β-1,6-glucan + mannan + glycogen	Microfibrils + amorphous comp	(Fig. 8)
F. Native cell wall formed by a regenerating yeast protoplast *in vivo*: Native wall formed by a *S. cerevisiae* protoplast in 30% gelatine medium at 23°C. Components assembled *in vivo*. The fibrillar component is composed of β-1,3-D-glucan and is partially masked by the amorphous component	Microfibrils + amorphous matrix	(Fig. 9)

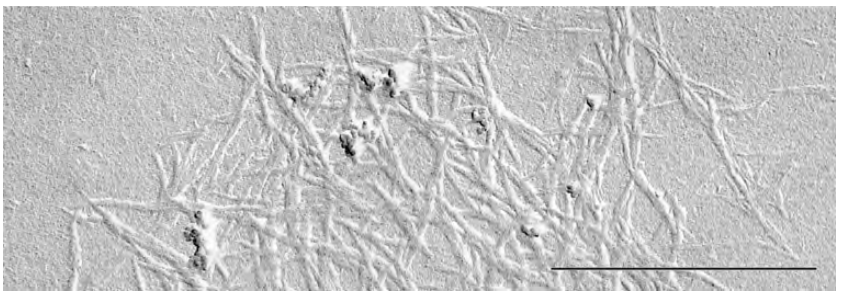

Fig. 1. Electron micrograph of microfibrils self-assembled *in vitro* from linear β-1,3-glucan from the fibrillar nets of *S. cerevisiae* protoplasts from liquid medium. Magnification ×32 000.

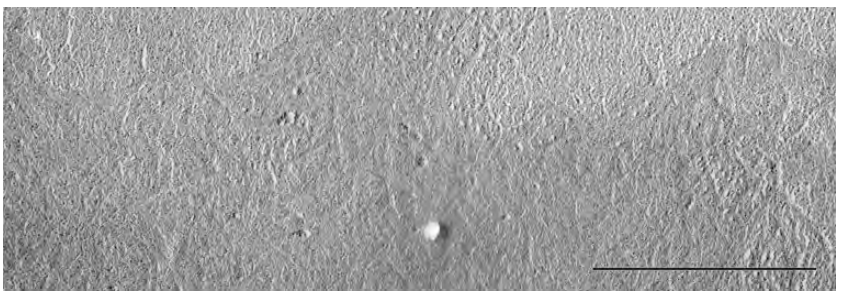

Fig. 2. Electron micrograph of microfibrils and the amorphous component self-assembled *in vitro* from branched β-1,3-glucan from the cell walls of *S. cerevisiae*. Magnification ×27 000.

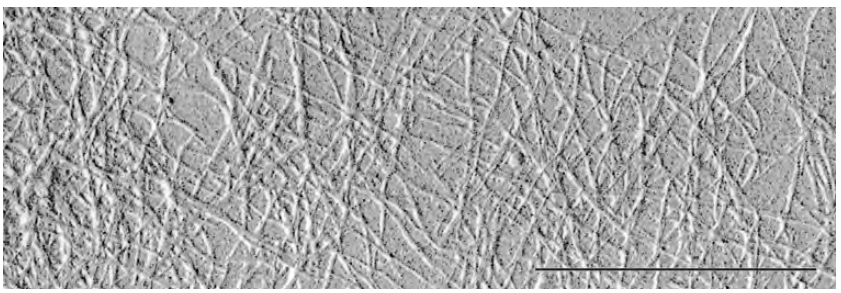

Fig. 3. Electron micrograph of microfibrils self-assembled *in vitro* from the linear β-1,6-glucan pustulan. Magnification ×34 000.

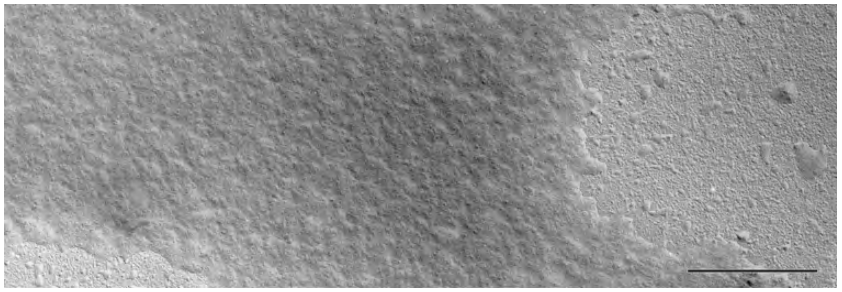

Fig. 4. Electron micrograph of *in vitro* self-assembled branched β-1,6-glucan from the cell walls of *S. cerevisiae*. Magnification ×16 000 [34].

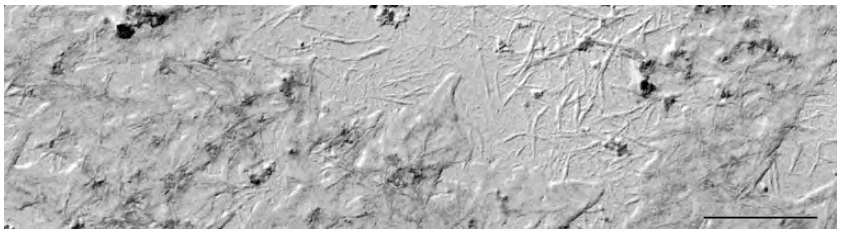

Fig. 5. Electron micrograph of microfibrils and the amorphous component self-assembled *in vitro* from linear β-1, 3-glucan from *S. cerevisiae* protoplasts and branched β-1,3-glucan from *S. cerevisiae* cell walls. Magnification ×14 000.

mannan and glycogen self-assemble *in vitro* into the amorphous component of cell walls. (iii) Branched wall β-1,3-glucan self-assembles *in vitro* into both the microfibrillar and the amorphous component of cell walls.

Analysis of the *in vitro* self-assembly of combinations of two different cell-wall polysaccharides ('two-step' self-assembly) (Table 1B)

(1) Linear protoplast β-1,3-glucan + branched wall β-1,3-glucan precipitated into the fibrillar and amorphous components (Fig. 5).
(2) Linear protoplast β-1,3-glucan + laminarin precipitated into microfibrils (see the supplementary data online, Fig. S7).
(3) Linear protoplast β-1,3-glucan + pachyman precipitated into microfibrils (see the supplementary data online, Fig. S8).
(4) Linear protoplast β-1,3-glucan + paramylon precipitated into microfibrils (see the supplementary data online, Fig. S9).
(5) Linear protoplast β-1,3-glucan + pustulan precipitated into microfibrils (see the supplementary data online, Fig. S10).
(6) Linear protoplast β-1,3-glucan + branched wall β-1,6-glucan precipitated into microfibrils (see the supplementary data online, Fig. S11).
(7) Linear protoplast β-1,3-glucan + branched wall glycogen precipitated into microfibrils (see the supplementary data online, Fig. S12).
(8) Linear protoplast β-1,3-glucan + branched wall mannan precipitated into microfibrils (see the supplementary data online, Fig. S13).
(9) Linear protoplast β-1,3-glucan + wall α-1,3-glucan precipitated into microfibrils and the fine granular amorphous component (see the supplementary data online, Fig. S14).

Conclusions: (i) When linear protoplast β-1,3-glucan + branched wall β-1,3-glucan, or linear protoplast β-1,3-glucan + α-1,3-glucan were combined, they precipitated into both the fibrillar and amorphous cell-wall components. (ii) All other combinations of two different polysaccharides precipitated into the fibrillar component of cell walls. No other combination produced the amorphous component of cell walls.

Analysis of the *in vitro* self-assembly of combinations of three different cell-wall polysaccharides ('three-step' self-assembly) (Table 1C)

(1) Linear protoplast β-1,3-glucan + branched wall β-1,3-glucan + branched wall β-1,6-glucan precipitated into the fibrillar component of cell

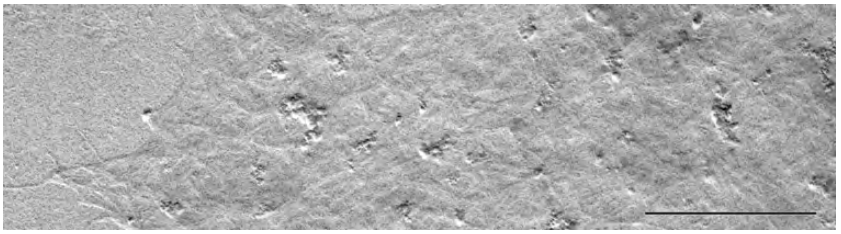

Fig. 6. Electron micrograph of microfibrils and the amorphous component self-assembled *in vitro* from linear β-1,3-glucan from *S. cerevisiae* protoplasts and branched wall β-1,3-glucan and branched wall β-1,6-glucan from *S. cerevisiae* cells. Magnification ×21 000.

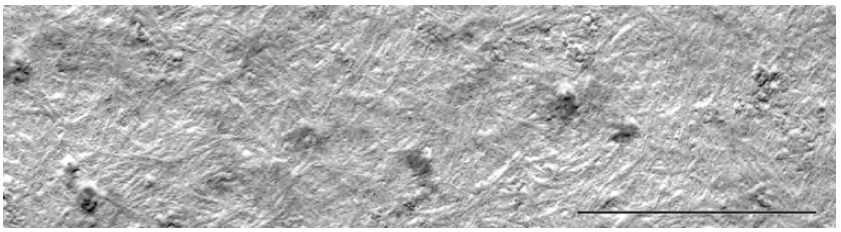

Fig. 7. Electron micrograph of microfibrils and the amorphous component self-assembled *in vitro* from linear β-1,3-glucan from *S. cerevisiae* protoplasts and branched wall β-1,3-glucan and branched wall β-1,6-glucan and branched wall mannan from *S. cerevisiae* cells. Magnification ×30 000.

walls joined together and masked by the amorphous component (Fig. 6).

(2) Linear protoplast β-1,3-glucan + branched β-1,6-glucan + branched mannan from *S. cerevisiae* walls precipitated into a compact fibrillar component densely glued together by the amorphous matrix (see the supplementary data online, Fig. S15).

(3) Linear protoplast β-1,3-glucan + branched β-1,6-glucan + branched glycogen from *S. cerevisiae* walls precipitated into the fibrillar component glued to wide bundles of microfibrils by the amorphous matrix (see the supplementary data online, Fig. S16).

(4) Linear protoplast β-1,3-glucan + branched mannan + branched glycogen from *S. cerevisiae* walls precipitated into the fibrillar component embedded within a dense continuous amorphous component that almost completely covered the microfibrils (see the supplementary data online, Fig. S17).

(5) Linear protoplast β-1,3-glucan + branched β-1,3-glucan + branched mannan from *S. cerevisiae* walls precipitated into the fibrillar component glued together and partially masked by the amorphous component (see the supplementary data online, Fig. S18).

Conclusion: All the examined combinations of three different polysaccharides self-assembled *in vitro* into a complex of fibrillar and amorphous components resembling that of the cell wall regenerated *de novo* by yeast protoplasts.

Analysis of the *in vitro* self-assembly of combinations of four different cell-wall polysaccharides ('four-step' self-assembly) (Table 1D)

(1) Linear protoplast β-1,3-glucan + branched β-1,3-glucan + branched β-1,6-glucan + branched mannan from *S. cerevisiae* walls precipitated into the compact fibrillar component densely glued together by the amorphous matrix (Fig. 7).

(2) Linear protoplast β-1,3-glucan + branched β-1,6-glucan + branched mannan + branched glycogen from *S. cerevisiae* walls precipitated into the fibrillar component densely glued together and masked by the amorphous matrix (see the supplementary data online, Fig. S19).

Conclusion: Both combinations of four different polysaccharides self-assembled *in vitro* into a complex of fibrillar and amorphous cell-wall components that resembled that of the cell wall regenerated *de novo* by yeast protoplasts.

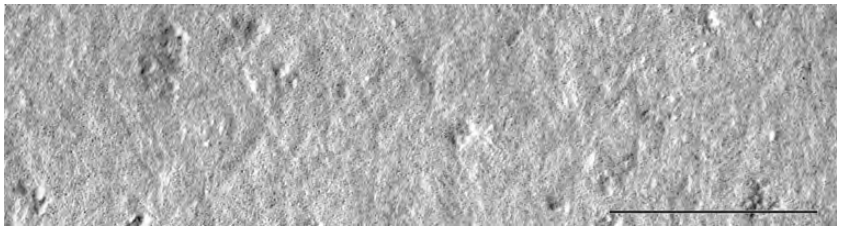

Fig. 8. Electron micrograph of microfibrils and the amorphous component self-assembled *in vitro* from linear β-1,3-glucan from *S. cerevisiae* protoplasts and branched wall β-1,3-glucan, branched wall β-1,6-glucan and branched wall mannan and glycogen from *S. cerevisiae* cells. Magnification ×25 000.

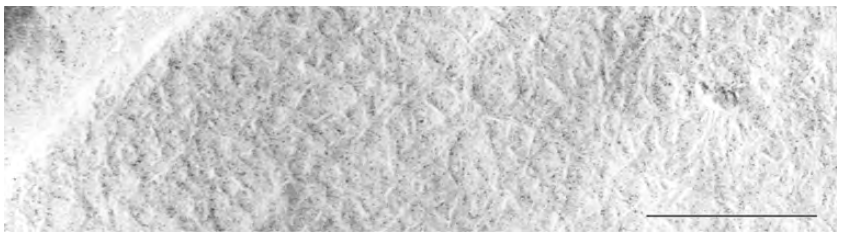

Fig. 9. Electron micrograph of the native β-1,3-D-glucan fibrillar wall component partially masked by the amorphous wall component, formed by *S. cerevisiae* protoplasts after 2 h of regeneration in 30% gelatine medium at 23°C. Magnification ×20 000.

Analysis of the *in vitro* self-assembly of a combination of five different cell-wall polysaccharides ('five-step' self-assembly) (Table 1E)

(1) Linear protoplast β-1,3-glucan + branched β-1,3-glucan + branched β-1,6-glucan + branched mannan + branched wall glycogen from *S. cerevisiae* walls precipitated into the fibrillar component embedded in the amorphous matrix (Fig. 8).

Conclusion: The combination of five different cell-wall polysaccharides self-assembled *in vitro* into a complex of the fibrillar and amorphous cell-wall components that resembled that of the cell wall regenerated *de novo* by yeast protoplasts.

In vivo assembly of the native yeast cell wall regenerated *de novo* by *Saccharomyces cerevisiae* protoplasts (Table 1F)

For the comparison with native yeast cell walls, we present an electron micrograph of the native yeast cell wall from a partially regenerated *S. cerevisiae* protoplast that shows both the fibrillar and amorphous wall components (Fig. 9).

Conclusion: The ultrastructure of the native cell wall of yeast protoplasts, regenerated both *in vivo* and *de novo*, contained a complex of the fibrillar and the amorphous components.

Discussion

Self-assembly is the spontaneous self-organization of molecules at a higher level of order, following the laws of thermodynamics and proceeding without enzymes, energy or additional information [40]. Weak physical–chemical interactions are the cause of self-assembly. If these interactions are sufficiently strong to overcome thermal diffusion, then self-assembly may lead to molecular associations stabilized by Van der Waals bonds, hydrogen bonds or electrostatic interactions. The shapes of molecules play an important role in self-assembly because they limit the number of molecules that can form secondary bonds with one another. Strong molecular interactions require complementary molecular shapes [40–42]. Self-assembly plays key roles in many biological processes, such as the initiation of the production of biomembranes, ribosomes, microtubules and microfilaments and the assembly of complexes initiating transcription, translation, and DNA replication as well as scientific techniques such as FISH to examine RNA-DNA or DNA-DNA hybridizations, PCR, DNA sequencing, and Southern blotting. Therefore, we hypothesized that self-assembly is not limited to nucleic acids and proteins and that polysaccharides can also self-assemble. Here, we investigated the role of self-assembly in the development of cell-wall architecture, using 10 different cell-wall polysaccharides:

the branched polysaccharides branched β-1,3-D-glucan, β-1,6-glucan, mannan and glycogen from the cell walls of *S. cerevisiae*; and linear polysaccharides β-1,3-D-glucan from yeast protoplasts, pachyman from *P. cocos*, pustulan from *U. pustulata*, paramylon from *A. longa* and laminarin from *Laminaria digitata*. In test tubes, all the linear polysaccharides self-assembled into the fibrillar component of cell walls. Branched wall β-1,6-D-glucan, mannan and glycogen self-assembled *in vitro* into the amorphous cell-wall component. When linear and branched polymers were self-assembled *in vitro* together, fibrillar-amorphous wall-like complexes originated with an ultrastructure that resembled the regenerating wall of yeast protoplasts. These results show that self-assembly of polysaccharides can participate in the *in vivo* development of yeast-cell-wall architecture.

In vitro self-assembly of polysaccharides into microfibrils

Linear protoplast β-1,3-glucan [32] as well as all the linear β-1,3-glucans investigated in this study self-assembled to form microfibrils *in vitro*. Dissolution of polysaccharides in 1 M NaOH occurs through disruption of the hydrogen bonds between crystallized chains [38,43]. The linear β-1,3-glucan curdlan with a degree of polymerization (DP) of 450 forms regular helices when dissolved at concentrations of NaOH lower than 0.19 M. In 0.19–0.24 M NaOH, dissolved glucan molecules transition into random coils, and at concentrations higher than 0.24 M NaOH, only random coils are formed [44,45]. We dissolved all the glucans examined in this study in 1 M NaOH. Therefore, it is most likely that they took the form of random coils. Dissolved protoplast glucans easily pass through ultrafilters with a pore size of 10 nm and did not sediment after 60 min of centrifugation at 15 000*g*. This finding indirectly demonstrates that the glucans were molecularly dispersed in 'random coils' [32], which agrees with other studies [44,45]. After the neutralization of NaOH, the decrease in the ionization of OH groups and of their electrostatic interactions leads to a conformational transition from random coils to cylindrical helices. Owing to the decrease in repulsive forces, segments of individual glucan chains associate, and their interchain interactions lead to the formation of triple helices [43,46,47]. It is likely that hydrogen bonds stabilize the triple-helices, similar to the helical arrangement of single chains [48]. Furthermore, linear β-1,3-glucan chains with both ends free can randomly associate because of the absence of side branches and form the triple helices that we identified by both X-ray diffraction and electron microscopy as elementary fibrils laterally aggregated with microfibrils [31,32,49]. Free molecular ends may also associate with other molecules, resulting in the generation of a three-dimensional network of microfibrils through joint zones between single-molecular chains.

Self-assembly of polysaccharides into microfibrils requires long linear molecules that firmly and tightly associate because of their exhibiting complementary molecular shapes. A low degree of branching [4] also permits the generation of microfibrils, as shown by the ability of branched wall β-1,3-glucan to form microfibrils in addition to the amorphous component (Fig. 2) (discussed below).

In vitro self-assembly of polysaccharides into the amorphous component of cell walls

The amorphous component of cell walls originates from branched polysaccharides because side branches do not allow the tight association of molecular chains that is necessary for the formation of microfibrils. This phenomenon explains why alkali-soluble glucan from the cell walls of yeast cells [6], branched β-1,6-glucan, branched mannan and branched glycogen only form amorphous aggregates *in vitro*. However, the short linear chains of chitin [14], α-1,3-glucan pseudonigeran [37], and curdlan with a DP of 35 [50] also form the amorphous component because the lengths of their chains are insufficient for microfibril formation, but in other organisms, chitin microfibrils [1] and α-1,3-glucan microfibrils [3,33] likely originate from longer molecules.

The generation of the amorphous component of cell walls requires a high degree of branching and lower degree of polymerization among polysaccharide molecules.

In vitro self-assembly of polysaccharides into both the fibrillar and amorphous components

Linear β-1,3-glucan precipitated in the presence of an amorphous polymer (mannan, or β-1,6-glucan, or

glycogen) does not generate fibrillar-amorphous complexes and produces only the fibrillar component. Mannan, β-1,6-glucan and glycogen, which are soluble in water, are most likely unable to bind the fibrillar component because they do not have complementary shapes and are washed out during the preparation of samples for electron microscopy. The solubility of these molecules may explain why *S. cerevisiae* protoplasts in liquid media form 'fibrillar nets' without any amorphous matrix. Stable amorphous aggregates originate *in vitro* from the precipitation of a combination of at least two different branched polymers (β-1,6-glucan + mannan, β-1,6-glucan + glycogen, or glycogen + mannan). They most likely form a three-dimensional polysaccharide network that is insoluble in water and includes a fibrillar component inside of the amorphous component.

The formation of microfibrils with an amorphous component requires at least one linear polysaccharide and two different branched wall polysaccharides.

Surprisingly, branched β-1,3-glucan extracted from the yeast cell wall with 1 M NaOH at 4°C [4] self-assembled *in vitro* not only into the amorphous component, as we expected, but also into microfibrils. Eddy and Woodhead [51] precipitated alkali-soluble glucan from *Saccharomyces carlsbergensis in vitro* and observed that it presented an amorphous appearance, but the glucan from protoplasts precipitated into fibrils in two out of five experiments. Why might this difference occur? In *S. cerevisiae* protoplasts, we observed only glucan microfibrils [32], which is also the case for linear curdlan with a DP of 450 [52]. However, short chains of curdlan with a DP of 35 only form amorphous precipitates *in vitro*. Their length was apparently insufficient for the generation of microfibrils [50]. Thus, the branched wall β-1,3-D-glucan studied here most likely contains two different types of molecules: (i) longer and less branched glucan, which forms fibrils; and (ii) shorter and more branched glucan, which forms the amorphous component.

Self-assembly of purified wall polysaccharides results in the formation of wall microfibrils and the amorphous wall component. Self-assembly can also result in complexes of both components that resemble the ultrastructure of the native wall formed by regenerating yeast protoplasts *in vivo*.

Conclusions

The fibrillar component of cell walls can originate *in vitro* through self-assembly of any of the linear β-1, 3-glucans from protoplast fibrillar nets, paramylon, laminarin, pachyman and the linear β-1, 6-glucan pustulan. The amorphous component of cell walls can originate *in vitro* through self-assembly of branched β-1,6-glucan, mannan and glycogen from yeast cell walls. Fibrillar and amorphous complexes that have an ultrastructure similar to that of the *in vivo* yeast cell wall can originate *in vitro* through self-assembly from a mixture of linear protoplast β-1,3-glucan, cell wall branched β-1,6-glucan, mannan and glycogen. Self-assembly of polysaccharides can participate in the development of the three-dimensional architecture of yeast and fungal cell walls.

Supplementary data

Supplementary data are available at http://jmicro.oxfordjournals.org/.

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