Probing (macro)molecular transport through cell walls†

Giona Kilcher,[a] Daniela Delneri,[b] Craig Duckham[c] and Nicola Tirelli*[a]

Received 19th November 2007, Accepted 23rd January 2008
First published as an Advance Article on the web 29th April 2008
DOI: 10.1039/b717840a

We here report a study on the passive permeability of hydrophobic probes through the cell wall of *Saccharomyces cerevisiae*. In this study we have prepared a series of fluorescent probes with similar chemical composition and molecular weight ranging from a few hundreds to a few thousands of g mol^{-1}. Their permeation into the cell body exhibits a clear MW cut-off and the underlying mechanism is governed by the permeation of individual molecules rather than aggregates. We also show that it is possible to reversibly alter the cell wall permeation properties without compromising the essence of its structure, by modifying the polarity/dielectric constant of the wall through solvent exchange.

Introduction

Background

Yeast cells, as well as other microbial organisms provided with thick cell walls, are able to internalise conspicuous quantities (up and above 30% of their own weight) of hydrophobic compounds through a mechanism of passive internalisation.[1–4] The passive character of this phenomenon is confirmed by the fact that permeation and encapsulation takes place equally for both live and dead cells[1] and even for cells with their cytoplasm completely removed.[4] To date, a large number of structurally-different low molecular weight compounds have been encapsulated: the process is performed by simply exposing yeast cells to water suspensions of hydrophobes,[1]

This discovery has opened the way to a new mechanism of encapsulation that exploits cells as microcontainers. The enveloping cell wall acts as the main barrier, entrapping and protecting the active components. The large availability at low cost, the complete biodegradability of the capsular material (the yeast cell body) and the size in a micron range (fairly large surface area but still restricted mobility) makes of the loaded yeast cells very interesting containers for a number of applications, ranging from food to agriculture.[1,3,5,6]

[a]*School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Oxford Road, Manchester, United Kingdom M13 9PT*
[b]*Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester, United Kingdom M13 9PT*
[c]*Micap PLC, Pemberton Business Centre, Richmond Hill, Wigan, Pemberton, United Kingdom WN5 8AA*

† Electronic supplementary information (ESI) available: Synthesis of fluorescent probes. See DOI: 10.1039/b717840a

Yeast cell walls

Yeasts, being fungi, are eukaryotic organisms that, similarly to prokaryotes, possess a robust cell wall. This is a 100–200 nm thick structure, which generally accounts for up to 25–30% of the dry weight of the cell. In terms of composition and morphology, the cell wall is known to be mostly composed of a network of fibre-forming, linear and high molecular weight (MW) β-1,3-glucans dispersed in a matrix of low MW and branched β-1,6-glucans. This matrix is also connected to mannoproteins on the external side of the wall and is stiffened by the presence of chitin rich domains:[7–11] mannoproteins are mostly linked to β-1,6-glucans, which connect them to the stiffer components (β-1,3-glucans and chitin), although some proteins (the alkali-sensitive linkage cell wall proterins) are also directly linked to β-1,3-glucans. This heterogeneous network (sketched in Fig. 1) provides mechanical support to the cell; however, through its mesh size and composition, it can also control the diffusion of components from and to the cytoplasm.

In particular, the complex mesh-like structure of the cell wall should determine a control on permeation that is, reportedly, size-selective: a large body of experiments show that a number of hydrophilic molecules passively permeate through the yeast cell wall and early works suggested a molecular weight cut-off in the proximity of 1000 g mol^{-1}.[12] This threshold has in many cases been questioned, since it is largely dependent on the state of yeast, for example fast-growing cells are more permeable than slow-growing ones and even more than non-growing (stationary phase) ones; in addition large molecules (*e.g.* cytochrome C) may also permeate when the wall is probably "stretched" due to high internal osmotic pressure.[13–15] However, the above cut-off value is likely to be valid at least for cells in the stationary phase, whose wall is stiffer due to increased thickness (while the density of elastically active cross-links (= the glucan layer cross-link density) seems not to increase) and less permeable, reportedly due to changes in the mannoprotein layer: increase in phosphorylation of mannose residues (more negative charges) and in intermolecular disulfide bridges. Mannoproteins would therefore form a kind of highly cross-linked corona, which could have a profound influence on diffusion, but only a marginal one on the wall elastic modulus.

On the other hand, few hypotheses have yet been formulated regarding the diffusion of hydrophobes. In this case, the permeation through a polar structure (when considering yeast in a hydrated state) depends strongly on the solubility of the hydrophobe in the wall material and not only on its molecular size (↔ diffusion coefficient). Additionally, the size cut-off for hydrophobes may be different from that for hydrophiles. More precisely, it is possible to see three limiting cases:

(a) Hydrophobic compounds permeate as aggregates (droplets) through pores of the cell wall network; if there is a MW cut-off, it is because the aggregates of high MW are too viscous and move much slower than those of low MW compounds.

(b) Hydrophobic compounds diffuse as individual molecules through pores of the cell wall network; if there is a MW cut-off, it is because high MW molecules exceed the pore dimensions.

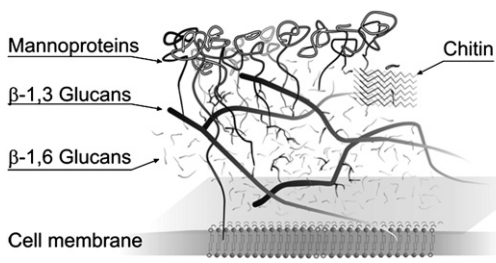

Fig. 1 Scheme of the structure of the yeast cell wall.

(c) Hydrophobic compounds diffuse as individual molecules through a substantially homogeneous matrix (the mesh size is in this case not relevant: either very large or modifiable); if there is a MW cut-off, it is because high MW molecules have a reduced solubility in the cell wall: in the absence of strong enthalpic interactions, solubility (entropy of mixing) decreases with increasing MW (naively, we adopt a simple Flory–Huggins conceptual approach where we consider the solvent-swollen cell wall as a solution, albeit of infinite viscosity, reducing the complex situation of a more than ternary system (solvent, hydrophobic compound, network with heterogeneous structure) down to a binary one).

Methodology

We have produced a series of hydrophobic, fluorescently-labelled and oligomeric probes with variable molecular weight (and polydispersity index <1.15) but identical chemical structure, which is similar to that of hydrophobes known to permeate very well through yeast cell walls (organic sulfides). Specifically, we have employed episulfide anionic ring-opening polymerisation, a technique we have a considerable experience with.[16–19] These probes, together with two other smaller probes (all structures listed in Fig. 1) were used to:

(1) Unequivocally demonstrate the existence of a molecular weight cut-off in hydrophobe permeation through intact cell walls. We have employed yeast cells cultured in a stationary state (lower proliferation, older cells, thicker cell walls), since their cell walls have a more reproducible thickness and composition (results obtained through the study of cell wall enzymatic degradation with zymolyase, following a literature approach,[11] data not shown) (Fig. 2).

(2) Verify hypothesis (a), *i.e.* whether viscosity of the hydrophobic suspensions plays a role independently on the molecular weight of the component: a low MW

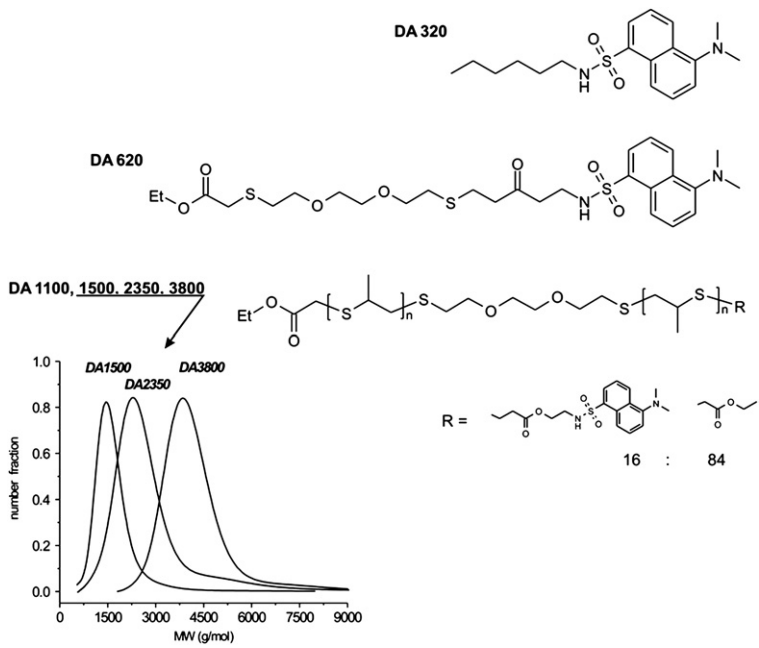

Fig. 2 Overview of the fluorescent probes synthesised in this study. The dansyl group was chosen for its high stability both to chemical agents and to photobleaching (λ_{exc} = 343 nm; λ_{em} = 494 nm). The introduction of dansyl or 2-acetoxyethyl groups was accomplished by end-capping thiolate groups (for DA1100 to DA3800 at the termini of polysulfide chains) with dansyl acrylate (2-dansylamino ethyl acrylate) and ethyl 2-bromoacetate, respectively.

hydrophobic component that, at the same time, is a good solvent for the probes, lowers the viscosity of their suspensions and permeates well into yeast cells was used as a diluent. If a differential penetration is still observed for probes with different MW, a sort of molecular "sieving", this would indicate the MW threshold to be related directly to the probe molecular dimensions and, therefore:

(3) Verify hypothesis (b), *i.e.* whether there are pores of limited dimensions that "sieve off" large molecules. The external mannoprotein layer is heavily disulfide-cross-linked when yeast cells are in a stationary state and is the most likely site for the presence of size-selective pores. If, however, after treatment with reducing agents differential penetration is still observed for probes with different MW, this would suggest the MW threshold not to be mainly dependent on the porosity of the mannoprotein layer.

4) Verify hypothesis (c), *i.e.* whether solubility in the cell wall (a kind of hydrogel) is the limiting factor for permeation. These experiments were accomplished by replacing water with a polar but not protic solvent that can swell the cell wall to a similar extent to water and without compromising its integrity and its composition. The solvent exchange should provide a less polar environment and therefore possibly a higher solubility for probes with larger MW (a less positive ΔH, which can be compensated by the mixing entropy also for compounds with a larger MW).

Results and discussions

Existence of a molecular weight cut-off for hydrophobes

First, it can be noted (from confocal microscopy, Fig. 3) that low MW hydrophobes penetrate into the yeast cytoplasm, *i.e.* they are not stopped at the level of the cell wall or cell membrane.

Regarding the mode of permeation, it seems unlikely that the round bodies found in the cytoplasm permeate intact through the cell wall. In addition, no strongly fluorescent body was found in close association with the wall. We can therefore cautiously exclude the permeation to happen through migration of such "large" (>500 nm) aggregates.

Yeast cells were then exposed to suspensions of hydrophobes with different MW, with the final result that no or very weak fluorescence was recorded for MW $\geq$ 1500 g mol^{-1} and MW =1100 g mol^{-1}, respectively (Fig. 4).

This indicates a generic MW cut-off in the region comprised between 620 and 1100 g mol^{-1}.

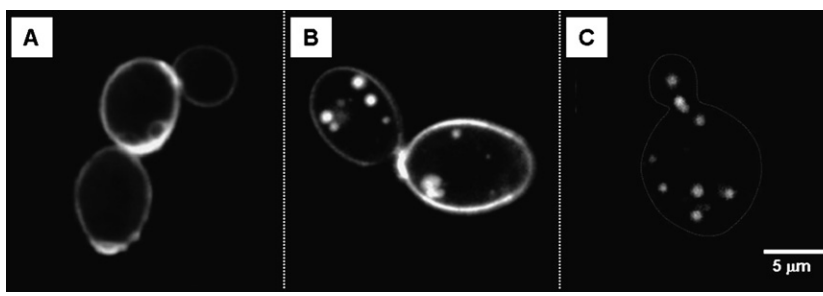

Fig. 3 Confocal laser scanning microscopy pictures focused on the equatorial plane of (A) yeast cells stained with Calcofluor White, a stain specific for chitin (more specifically, we presume it is for chitin in amorphous form); (B) yeast cells exposed to 0.5 mM DA-320 and then stained with Calcofluor White and (C) yeast cells exposed only to DA-320, 0.5 mM (the contour is highlighted for ease of reading). Fluorescence collected in a single channel in the range 420–600 nm.

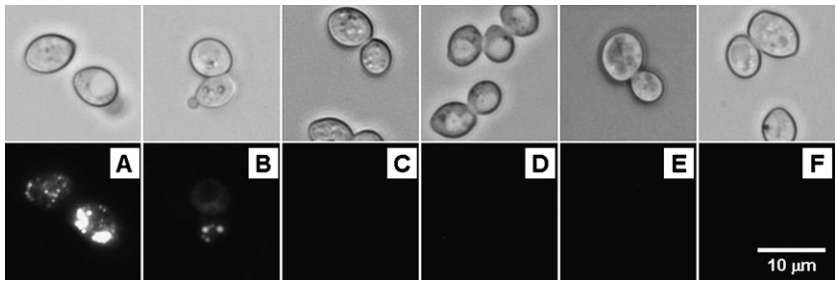

Fig. 4 Bright field and fluorescence microscopy pictures of *S. cerevisiae* exposed to 0.5 mM probe dispersions in water (A: DA-320; B: DA-620; C: DA-1100, D: DA-1500, E: DA-2400, F: DA-3800 g mol^{-1}).The addition of a hydrophobic, low MW diluent would decrease the overall viscosity of the aggregates, without affecting the MW of the larger components.

Effect of changes in hydrophobe viscosity

Limonene is a good solvent for all probes and is also well-encapsulated in yeast (>26 wt%[4]); if the viscosity of the hydrophobic phase plays a major role, the addition of limonene (as a low viscosity diluent: 0.9 mPa s^{-1} compared to 1 mPa s^{-1} for DA620, 3 mPa s^{-1} for DA1100 and 5 mPa s^{-1} for DA3800 ($\pm$0.3 mPa s^{-1})) should increase the incorporation of a high MW probe.

Limonene addition, however, did not provide significant changes: encapsulation was again recorded only for the low MW probes (DA-320 and DA-620) without any sharp dependence on probe concentration (varied in the range 0.05–2 mM) or limonene concentration (0, 5, 10 and 25 wt%) in the water dispersion; limonene, on the other hand and as expected, is massively incorporated. These experiments are summarised in a semi-quantitative fashion in Fig. 5, where we have evaluated the probe cellular concentration from its fluorescence emission, expressing in a scale 0–5 where 5 corresponds to the encapsulation of DA-320 in the absence of limonene from a 2 mM water dispersion. The probes are localised in round bodies throughout the cytoplasm, whose dimensions are comparable to those obtained in the absence of limonene (Fig. 3).

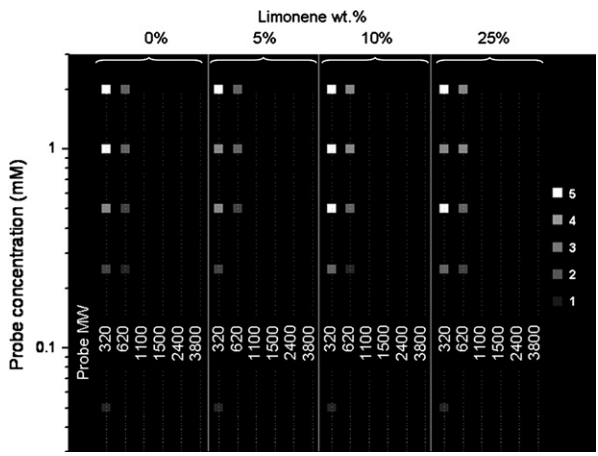

Fig. 5 Average fluorescent intensity (classified in a 1–5 scale from the 0–256 gray-value analysis of probe fluorescence) as a function of the probe and limonene concentration (0, 0.05, 0.25, 1 and 2 mM and 0, 5, 10, 25 wt%, respectively).

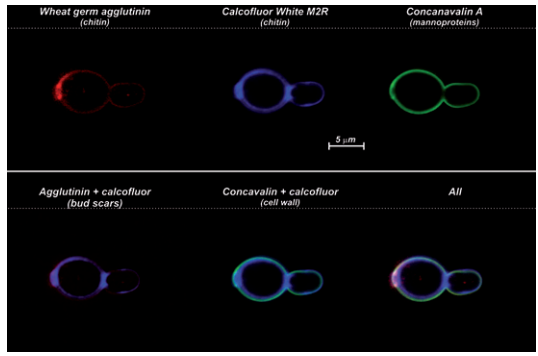

Fig. 6 Triple stain confocal pictures of yeast treated with DTT. Agglutinin stains for strongly aggregated ("crystalline") chitin, Calcofluor White for "nascent" ("amorphous") chitin, concanavalin A for mannoproteins. Analogous pictures are obtained for untreated yeast, showing therefore no effect of DTT treatment. Glucans, the main components of the cell wall, are not stained, but optical microscopy pictures reveal no change in cell wall thickness and refractive index, suggesting the glucans not to undergo any reorganisation.

Effect of changes in cell wall structure: reduction of disulfide cross-links

A popular disulfide reducing agent (DTT), employed in concentrations up to 20 mM of DTT, did not produce any sound effect in the cell wall general composition (Fig. 6), but no effect on the internalisation behaviour was apparent too.

Effect of changes in cell wall structure: swelling in a polar solvent

We hypothesise that if the cell is exposed to an appropriate organic solvent, which neither solubilises, nor swells the cell wall to an extent to dramatically change its thickness, the main effect would be a change in its polarity (*i.e.*, its dielectric constant, its Hildebrand parameter) with little effect on its mesh size. This is a coarse approximation: we suppose an affine-like behaviour of the cell wall macromolecular network, with the mesh size being linearly related to the linear swelling.

If we, therefore, obtain a relatively small (say <20%) variation in cell wall thickness and we assume a corresponding variation in the mesh size, we expect not to see any sound difference in size exclusion effects for probes whose size differ up to one order of magnitude.

The approximation of the cell wall to an affine network is coarse in that it does not take into account that replacement of water with an organic solvent may cause drastic changes in macromolecular aggregation, which would not allow us to use an affine-like model.

We have selected a very polar solvent, dimethylsulfoxide (DMSO), which has the highest dielectric constant (48, compared *e.g.* to 39 of DMF) among the water-compatible organic solvents. DMSO is known to have a toxic effect on yeast, but, remarkably, it is tolerated up to high concentrations: 30% viability is maintained after 5 min incubation in a 40% DMSO–H_2O solution dropping to 10% only after 90 minutes,[20] immediate viability is lost only for solutions with DMSO >50%. Most importantly, the hydrophobic probes used in this study are all soluble in DMSO.

Effects of solvent exchange on the cell wall structure

Overall cell shape. Even if dead, yeast cells maintain a structural integrity even in 100% DMSO (Fig. 7B1) and this is retained also after transferring the cells back to water milieu (Fig. 7C1). The cytoplasm organisation is, however, completely destroyed.

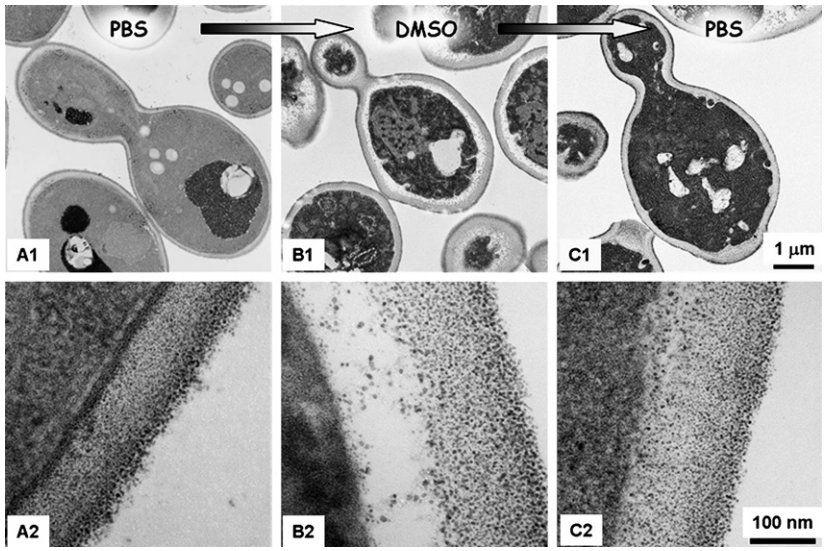

Fig. 7 Transmission electron microscopy pictures of uranyl acetate stained *S. cerevisiae* previously fixed with 1% glutaraldehyde solutions in PBS (A), DMSO (B) and in PBS after exposure in DMSO (C).

Cell membrane. This 20 nm thick layer between the cytoplasm and cell wall clearly visible in Fig. 7A2) seems to disappear (Fig. 7B2), similarly to the membranes of internal organelles. These membranes are not restored when cells are transferred back to water milieu (Fig. 7C2).

Whole cell wall. The complete exchange of water with DMSO remarkably does not seem to dramatically compromise the structure and dimension of the cell wall. The wall appears (Fig. 7B2) slightly thicker in DMSO (initially $\approx$ 100 nm, in DMSO $\approx$ 130 nm), and this increase is retained after transferring the cells back to water (Fig. 7C2). This dimensional change may be, however, more apparent than real: the distance from the outer border of the wall from the cytoplasm is substantially not altered, possibly suggesting that a layer of cell wall-linked membrane components (*e.g.* membrane proteins) may be seen as part of membrane before the solvent exchange and as part of wall afterwards. The staining pattern (uranyl acetate) becomes more disperse in DMSO and is largely unaffected by the final DMSO–water change. It is, however, always possible to recognise an external darker region, that we assume to be still associated to mannoproteins.

Cell wall individual components. (A) *Glucans*. It is known that cross-linked glucans are not extracted by DMSO, unless cells are first treated with sodium hypochlorite to degrade their structure;[21] on the other hand, DMSO is a good solvent for most neutral sugars, as much as water is; therefore we assume the glucan network swelling degree in DMSO not to be largely different from that in water.

(B) *Chitin*. Differently to the other components, chitin distribution is largely affected by the solvent exchange. However, the two stains used for chitin, indicate partially different outcomes (Fig. 8): Calcofluor White, which preferentially stains to less aggregated chitin, shows an almost completely homogeneous dispersion of chitin throughout the cell body, with little or no preference towards the cell wall and some preference only for incipient bud scars, but not for old ones (where one generally has strongly aggregated chitin). Wheat germ agglutinin (which, on the contrary, stains preferentially for aggregated chitin) still indicates that cell wall and bud scars are still preferential locations. DMSO seems therefore to alter the distribution

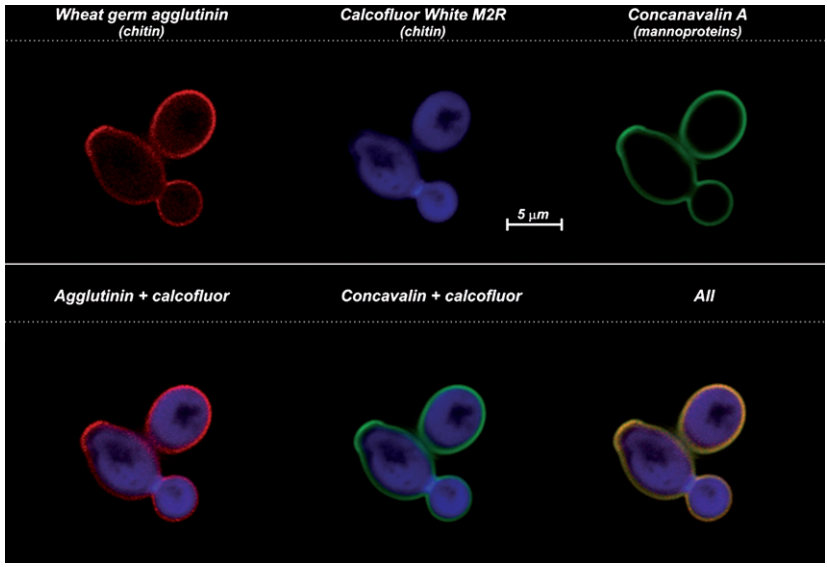

Fig. 8 Confocal laser scanning microscopy pictures for yeast cells exposed to DMSO for 120′ and then re-conditioned in a water environment. Agglutinin and Calcofluor White stain chitin (respectively more or less aggregated), while concanavalin stains the mannoproteins.

primarily of the less aggregated (nascent) chitin. Again, no dramatic difference is recorded employing prolonged exposure times (*e.g.* no significant difference between 15′, 60′ or 120′).

(C) *Mannoproteins*. It is known that extraction even with powerful surfactants (SDS) does not remove significant amounts of mannoproteins,[22] while it mostly affects membrane proteins. Furthermore, confocal microscopy of yeast cells after the water–DMSO–water sequence (Fig. 8) shows the signals of concanavalin A still associated to the cell wall, indicating therefore that the localisation of mannoproteins is largely unaffected by solvent exchange, even after exposure times as long as 120′.

An additional treatment with a reducing agent (DTT) provided no significant change, indicating that, even in the absence of disulfide bonding, the treatment with DMSO does not mobilise mannoproteins, *i.e.*, DMSO does not apparently affect their covalent linkages to the underlying glucan network.

Effects of solvent exchange on the cell wall permeability

Transferring back to water the cells previously exposed to DMSO (*i.e.*, performing internalisation experiments in a water environment), one observes a MW cut-off similar to that recorded on intact cells (Fig. 9). It is therefore possible to infer that (a) mannoproteins do not play a major role in hydrophobe passive transport, since this is not sensitive to whether mannoproteins are or not cross-linked, (b) chitin and cell membrane are largely affected by solvent exchange, but this does not influence the MW dependence of permeation, (c) glucans seem to be left largely unaltered in the solvent exchange process and it is therefore reasonable to assume the glucan network to be responsible of the MW-dependent permeation.

On the other hand, performing the encapsulation experiments in DMSO, even probes with high MW (up to 3400 g mol^{-1}) were efficiently encapsulated, with a small influence of molecular weight (Fig. 10). Once back in water again, the probes remain entrapped in the cells. Confocal microscopy clearly shows that all probes to be present in aggregates distributed throughout the cytoplasm (Fig. 11), similarly to what recorded to low MW hydrophobes after loading in water.

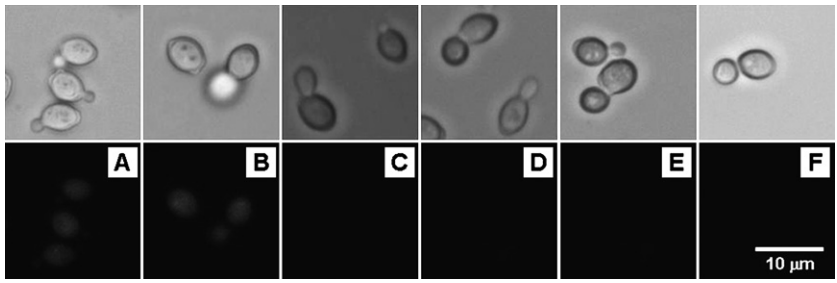

Fig. 9 Bright field and fluorescence microscopy pictures of *S. cerevisiae* exposed to 0.5 mM probe dispersions in PBS after previous incubation for 2 h in DMSO (A: DA-320; B: DA-620; C: DA-1100, D: DA-1500, E: DA-2400, F: DA-3800 g mol^{-1}).

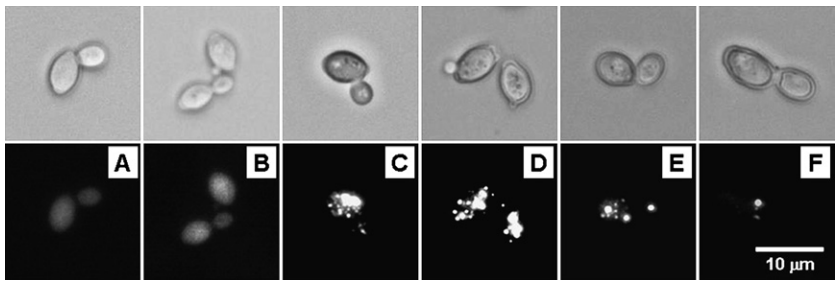

Fig. 10 Bright field and fluorescence microscopy pictures of *S. cerevisiae* exposed to a DMSO solution containing 20 wt% of probes (A: DA-320; B: DA-620; C: DA-1100, D: DA-1500, E: DA-2400, F: DA-3800 g mol^{-1}) and finally transferred back to water milieu.

It cannot be completely excluded that the internalisation of high MW hydrophobes could be caused by an increase in pore dimensions of the DMSO-swollen cell wall. However, (a) no significant morphological change is recorded in the DMSO-to-water exchange and therefore, in the hypothesis of an affine-like behaviour, the mesh size should not vary dramatically, and (b) when back in water the high MW hydrophobes do not permeate; we are therefore inclined to mostly relate their internalisation in the presence of DMSO to an increased polymer solubility in the solvent-swollen walls.

Experimental

Synthetic procedures

The preparation of the polysulfide fluorescent probes was accomplished according to literature procedures based on episulfide polymerisation and end-capping through Michael-type addition or nucleophilic substitution. A complete description of the procedures is provided in the supporting information.†

Cell preparation

S. cerevisiae (wild type diploid BY4743) was routinely batch-grown under sterile condition in YPD (1% yeast extract, 2% bacteriological peptone and 2% glucose): 10^6–10^7 cells were inoculated in 50 ml growing medium and incubated at 29 °C (170 rpm) in an orbital shaking incubator (MODEL G25, New Brunswick Scientific CO.INC, Edison, N.J., USA). Cells were harvested at desired concentration, centrifuged at 3000 rpm for 3 min and after removal of the supernatant rinsed in deionised water. The suspension was then centrifuged and the pellets suspended at the desired

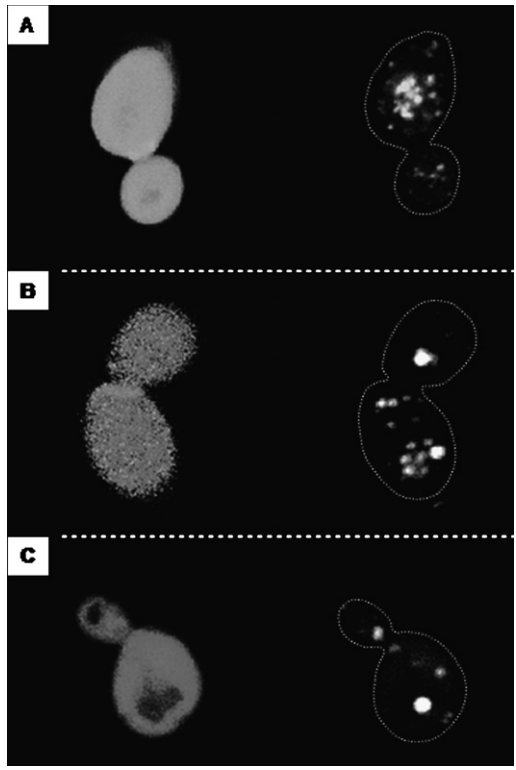

Fig. 11 Confocal laser scanning microscopy picture focusing the equatorial plane of *S. cerevisiae* exposed to DMSO solutions of probes of increasing molar mass (A: 1100, B: 1500, C: 2400 g mol^{-1}), transferred in PBS solution and finally stained with Calcofluor White. The fluorescence of the probes and of Calcofluor White could be collected separately; the former one was acquired in the range 540–620 nm and the latter one at 400–450 nm.

concentration in the working medium: PBS buffer (Dulbecco A, PH 7.3), TE-Buffer (50 mM Tris-HCl, 0.15 M NaCl, 5 mM EDTA, PH 7.5), TRIS buffer (10 mM Tris-HCl, PH 7.4) or DMSO.

Internalisation essays in aqueous dispersion

The fluorescent probes were dissolved in DMSO to prepare stock solutions that were 100, 50, 25, 12.5 and 2.5 mM in fluorescent groups (concentrations calculated numerical average molecular weights: a 25 mM solution corresponds to: 9 mg g^{-1} (DA-320), 17 mg g^{-1} (DA-620), 146 mg g^{-1} (DA-1100), 87 mg g^{-1} (DA-1500), 140 mg g^{-1} (DA-2500), 202 mg g^{-1} (DA-3800)). The solutions were then stored at −20 °C and protected from light prior to use.

Cells were harvested in the late stationary phase and, following preparation as previously described, were finally suspended in PBS buffer (5×10^7 cells per ml). The internalisation essay was carried out in pure aqueous solution for case (a), or in heterogeneous phase with the addition of limonene for case (b).

Case (a): 490 µl of suspension were transferred to a 1.5 ml centrifuge tube and 10 µl of probes solution in DMSO were added yielding a final concentration of the fluorescent dye of 2, 1, 0.5, 0.25 and 0.05 mM.

Case (b): variable volumes of the cellular suspension (465, 440 or 365 µl) were transferred to a 1.5 ml centrifuge tube and then limonene (25, 50 or 125 µl) and 10 µl of probes solution in DMSO were added yielding heterogeneous mixtures of

5, 10 and 25% wt limonene and a final concentration of the fluorescent dye of 2, 1, 0.5, 0.25 and 0.05 mM.

The dispersions were incubated in an orbital shaker (Eppendorf Thermomixer) at 40 °C for 30 minutes (1500 rpm) protected from light. Afterwards, cells were separated from the remaining hydrophobic phase repeating three times the following rinsing procedure: tubes were centrifuged at 3000 rpm for 3 min, the supernatant carefully completely removed; pellets were then suspended in fresh PBS and transferred each time in a new tube prior to repeat the cycle.

Reduction of mannoproteins

Yeast cells, harvested and prepared as previously described, were suspended in TRIS Buffer (10 mM Tris-HCl, PH 7.4) at a concentration of 5×10^7 cells per ml. Aliquots of 500 μl were centrifuged, and after removal of the supernatant, the pellets were suspended in 500 μl TRIS Buffer with or without 10 or 20 mM of the disulfide reducing agent dithiothreitol (DTT) and incubated in a incubated in an orbital shaker (Eppendorf Thermomixer) at 4 °C for 30 min under gentle agitation (500 rpm). Cells were subsequently washed three times with TRIS Buffer and finally suspended either in the buffer/solvent for the encapsulation of staining procedure.

Cell treatment with DMSO and resulting internalisation essays

Yeast cells, harvested and prepared as previously described, were suspended in DMSO and allowed for 10 minutes at 30 °C under gentle shaking to facilitate the diffusion of the organic solvent in the cell body. Cells were then centrifuged at 3000 rpm for 5 min and suspended in an equal volume of fresh DMSO yielding a final cellular concentration of 5×10^7 cells per ml.

Internalisation in DMSO. The fluorescent probes were dissolved in DMSO to prepare stock solutions of 20 wt% solid content and were stored at −20 °C and protected from light prior to being used. 500 μl of cellular suspension in DMSO (5×10^7 cells per ml) were centrifuged for 3 min at 5000 rpm and pellets were then separated from the solvent. The pellets were added of 50 μl of the probe solution in DMSO (20 wt%) and incubated at 40 °C for 2 hours (1500 rpm), always protected from light. The tubes were centrifuged for 3 min at 5000 rpm to remove the supernatant. The resulting pellets were rinsed and gradually transferred to an aqueous environment by progressively increasing the water fraction (0, 5, 10, 25, 50, 75 and 100%) after each rinsing cycle, which was composed of centrifugation at 5000 rpm (3 min), complete removal of supernatant, re-suspension in fresh solution, transfer in new tube and shaking for 5 min (30 °C, 1000 rpm). The cellular suspension in water was stained with Calcofluor White as previously described and finally prepared for optical microscopy.

Internalisation in aqueous environment after DMSO treatment. Cells were incubated and processed as previously described but without the presence of a fluorescent probe. After finally transferring cells in water, the internalisation essay was performed as described for the internalisation in aqueous dispersion

Cell wall staining

Stains stock solutions were prepared and stored according the manufacturer recommendations: dry Calcofluor White was dissolved in deionised water to yield a 5 mM solution (aliquots of 10 μl were separately frozen at −20 °C and thawed prior to be used); dry Concanavalin A was dissolved in sodium bicarbonate buffer (0.1 M, PH 8.3) to yield a stock solution of 5 mg ml^{-1} (aliquots of 20 μl were separately frozen at −20 °C and thawed prior to use); dry wheat germ agglutinin was dissolved in sodium

bicarbonate buffer (0.1 M, PH 8.3) to yield a stock solution of 5 mg ml^{-1} (aliquots of 20 μl were separately frozen at −20 °C and thawed prior to use).

Cell wall staining procedure: a cellular suspension in the aqueous medium (5×10^7 cell per ml) was transferred into sodium bicarbonate buffer (0.1 M, PH 8.3); 20 μl of the cellular dispersion were then transferred to a 500 μl centrifuge tube and stained with one or more dyes: (a) 20 μl of a Calcofluor white 0.5 mM aqueous solution (separately prepared by diluting the 5.0 mM stock solution with sodium carbonate buffer); (b) 20 μl of Concanavalin A 250 μg ml^{-1} staining solution (prepared by diluting with sodium bicarbonate of a 5 mg ml^{-1} stock solution); (c) 20 μl of wheat agglutinin 125 μg ml^{-1} staining solution (prepared by diluting with sodium bicarbonate of the 5 mg ml^{-1} stock solution). Sodium bicarbonate buffer was always added up to a solution total volume of 100 μl, so that the final cellular concentration was always 1×10^7 cell per ml and the dye final concentrations were 0.1 mM for Calcofluor White, 50 μg ml^{-1} for concanavalin A and 25 μg ml^{-1} for wheat germ agglutinin. The suspension was then incubated protected from light for 20 min at 25 °C under gentle orbital agitation (Eppendorf Thermoxer). After centrifugation for 3 min at 3000 and removal of the supernatant the cells were suspended in 20 μl fresh sodium bicarbonate (final cellular concentration 5×10^7 cell per ml) and prepared for the optical microscopy.

Optical microscopy

Fluorescent images were acquired on a Leica DMI6000 equipped with a mercury arc lamps and fluorescent A4 Leica filter cube (excitation filter 360/40 nm, dichromatic mirror 400 nm and suppression filter 470/40 nm). Magnification, light intensity and shutter time were the same for all the fluorescent images (Objective 63×, Fluorescence Intensity Manager (FIM) 55%, exposure time 90 ms).

Confocal laser scanning microscopy images were acquired on a Leica SP2 AOBS confocal microscope with a 63.0× oil immersion objective; the 405 nm blue diode laser line was used to excite the Calcofluor White and the polymeric probes, the visible laser at 488 nm for concanavalin A and at 633 nm for wheat germ agglutinin.

In order to immobilise the cells without altering the wall structure, cells were embedded in a matrix formed on the microscope slide by first depositing 3 μl cellular dispersion in 6 μl of 2.0 wt% purified alginate solution (kindly donated by Medipol SA, Lausanne, Switzerland) next to 3 μl of a 0.1 M $CaCl_2$ solution; the two drops were mixed by placing the cover slip, which triggered the *in situ* formation of the matrix. Slides were then sealed and stored for 2 hours protected from light prior to use.

Semi-quantitative image analysis. Color fluorescence images (acquired with the Leica DMI6000 under the same conditions as previously reported) were converted to 8-bit images and a threshold was applied to eliminate any background signal. ImageJ images analysis software was applied for a semi-quantitative image-based analysis of the cellular fluorescence signal intensity: cell areas were selected with multiple region of interest (RoI); the average gray-scale intensity (in a range between 0–256) over the RoI was derived from the gray-scale intensity distribution over all the pixels located in the RoI. We have always assumed this value to be linearly related to the quantity of internalized probe; however, we do not presume these measurements to be strictly quantitative. The average gray-scale intensity was then expressed in a scale from 0 to 6.

Transmission electron microscopy (TEM)

Cells dispersed in the two different suspension media (water or DMSO) were fixed with glutaraldehyde according to the following procedures, respectively: (a) to a 500 μl dispersion of yeast (10.10^7 cell per ml) in deionised water were added

500 μl of 2% glutaraldehyde solution in 0.2 M PHEM buffer (120 mM PIPES, 50 mM HEPES, 4 mM MgCl2, 20 mM EGTA, pH 7.2); (b) to a 500 μl dispersion of yeast (10.10^7 cell per ml) in DMSO were added 500 μl of a 2% glutaraldehyde solution in DMSO. In both fixing procedures the reaction was allowed to proceed for 24 hours and then cells were rinsed and transferred in cacodylate buffer (0.1 M) solution and post-fixed with 1% w/w osmium tetroxide (OsO_4) for 2 h. Cells were rinsed in cacodylate buffer (0.1 M) and subsequently progressively dehydrated using aqueous ethanol solutions of increasing concentration (1 hour in 50, 70, 90 and 100% solutions) and finally acetone. The organic dispersion was mixed with acetone–epoxy-resin (3 : 1 mixture) and allowed for 1 h then the resin concentration was progressively increased to 100% (1 hour in acetone–resin 1 : 1, 1 : 3 and 100% resin) and polymerized at 60 °C in an oven for 24 h. Section of 70 nm were cut using a Reichert Jung Ultracut Ultramicrotomes × 3 and then stained first with 1% w/w uranyl acetate water solution for 1 min and the with 0.3% w/w lead citrate solution for 1 min. The sections were rinsed with fresh water, dried and finally observed using a Philips FEI Tecnai 12.

Conclusions

Cell walls of *S. cerevisiae* present a molecular weight (MW) cut-off in the range between 700 and 1000 g mol^{-1}. Probes with a lower-than-threshold MW can penetrate into the cell and form round bodies of a submicron size (200–1000 nm), possibly stabilized inside organelles or by other cellular material of amphiphilic character.

The MW cut-off does not depend on the increased viscosity of the aggregates formed by the hydrophobes with increasing MW: dilution with a low MW hydrophobe leads to a "sieving off" of large molecules.

The exposure of yeast to a polar organic solvent (DMSO), although producing dramatic effects on cell ultrastructure, does not seriously affect the MW-dependent behaviour of the cell wall permeability, when cells are back in water. We have tentatively associated this effect mostly to the glucan network as the only element regulating the hydrophobe permeability through cell wall, which is not largely influenced by DMSO: cell membrane and chitin were, at various degrees, irreversibly affected by the treatment, whereas mannoproteins seem not to play a major role in hydrophobe permeation.

In DMSO, permeation occurs at least up to molecular weights of a few thousands g mol^{-1}. We have therefore concluded the original MW cut-off to be related to the increasingly poorer solubility in a water-swollen environment for hydrophobic probes with increasing MW.

This finding offers a possibly new way to read the passive (spontaneous) permeation of hydrophobes (and maybe amphiphiles too) through cell walls, in that it relates it mostly to the solubility in a water-swollen network more than to the mesh size of that network.

The result is very relevant to understand the mechanism of this successful microencapsulation technology, a simple technique based on sustainable, biodegradable and cost-effective raw materials.

Acknowledgements

The authors want to thank Micap PLC (Wigan, UK) for the fundamental financial support, the enthusiasm and the freedom of research. The authors also gratefully acknowledge the EM Unit, Faculty of Life Sciences at University of Manchester, for the use of the transmission electron microscope, and Dr Aleksandr Mironov for the preparation of TEM samples and for helpful discussions. BBSRC is gratefully acknowledged for financial support (grant No. BBS/B/01537). NT is indebted to EPSRC for an Advanced Research Fellowship.

References

1 G. Nelson, Patent WO2004037232, 2005.
2 G. Nelson, J. R. P. Bishop and J. Lamb, *J. Microencapsulation*, 1998, **15**, 761–773.
3 G. Nelson, C. Duckham and E. Round, Patent GB2424408, 2003.
4 V. Normand, G. Dardelle, P.-E. Bourquerand, L. Nicolas and D. J. Johnston, *J. Agric. Food Chem.*, 2005, **53**, 7532–7543.
5 M. Crothers, C. Duckham and G. Nelson, Patent WO2004084947, 2004.
6 M. Crothers and G. Nelson, Patent WO2004037232, 2004.
7 E. Cabib, R. Kollar, B. B. Reinhold, E. Petrakova, H. J. C. Yehi, G. Ashwell, J. Drgonova, J. C. Kapteyn and F. Klis, *J. Biol. Chem.*, 1997, **272**, 17762–17775.
8 F. Klis, *Yeast*, 1994, **10**, 851–869.
9 R. Kollár, E. Petráková, G. Ashwell, P. W. Robbins and E. Cabib, *J. Biol. Chem.*, 1995, **270**, 1170–1178.
10 R. Kollar, B. B. Reinhold, E. Petrakova, H. J. Yeh, G. Ashwell, J. Drgonova, J. C. Kapteyn, F. M. Klis and E. Cabib, *J. Biol. Chem.*, 1997, **272**, 17762–17775.
11 P. N. Lipke and R. Ovalle, *J. Bacteriol.*, 1998, **180**, 3735–3740.
12 R. L. Scherrer, L. Louden and P. Gerhardt, *J. Bacteriol.*, 1974, **118**, 534–540.
13 J. G. De Nobel, F. M. Klis, T. Munnik, J. Priem and H. van den Ende, *Yeast*, 1990, **6**, 483–490.
14 J. G. Denobel and J. A. Barnett, *Yeast*, 1991, **7**, 313–323.
15 J. G. Denobel, F. M. Klis, J. Priem, T. Munnik and H. Vandenende, *Yeast*, 1990, **6**, 491–499.
16 G. Kilcher, L. Wang, C. Duckham and N. Tirelli, *Macromolecules*, 2007, **40**, 5141–5149.
17 A. Rehor, J. A. Hubbell and N. Tirelli, *Langmuir*, 2005, **21**, 411–417.
18 A. Napoli, M. Valentini, N. Tirelli, M. Muller and J. A. Hubbell, *Nat. Mater.*, 2004, **3**, 183–189.
19 A. Napoli, N. Tirelli, G. Kilcher and J. A. Hubbell, *Macromolecules*, 2001, **34**, 8913–8917.
20 B. Yee, S. Tsuyumu and B. G. Adams, *Biochem. Biophys. Res. Commun.*, 1972, **49**, 1336-&.
21 N. Ohno, M. Uchiyama, A. Tsuzuki, K. Tokunaka, N. N. Miura, Y. Adachi, M. W. Aizawa, H. Tamura, S. Tanaka and T. Yadomae, *Carbohydr. Res.*, 1999, **316**, 161–172.
22 F. M. Klis, P. Mol, K. Hellingwerf and S. Brul, *FEMS Microbiol. Rev.*, 2002, **26**, 239–256.

