Aggregation of yeast cells: direct measurement of discrete lectin–carbohydrate interactions

Ahmed Touhami,1 Barbara Hoffmann,2 Andrea Vasella,2 Frédéric A. Denis1 and Yves F. Dufrêne1

1Unité de Chimie des Interfaces, Université Catholique de Louvain, Croix du Sud 2/18, B-1348 Louvain-la-Neuve, Belgium
2Laboratorium für Organische Chemie, HCI H 317, ETH-Hönggerberg, CH-8093 Zürich, Switzerland

Aggregation of microbial cells mediated by specific interactions plays a pivotal role in the natural environment, in medicine and in biotechnological processes. Here we used atomic force microscopy (AFM) to measure individual lectin–carbohydrate interactions involved in the flocculation of yeast cells, an aggregation event of crucial importance in fermentation technology. AFM probes functionalized with oligoglucose carbohydrates were used to record force-distance curves on living yeast cells at a rate of 0.5 m s⁻¹. Flocculating cells showed adhesion forces of 121 ± 53 pN, reflecting the specific interaction between individual cell-surface lectins and glucose residues. Similar adhesion forces, 117 ± 41 pN, were measured using probes functionalized with the lectin concanavalin A and attributed to specific binding to cell-surface mannose residues. By contrast, specific interaction forces were not observed in non-flocculating conditions, i.e. in the presence of mannose or when using non-flocculating cells, pointing to their involvement in yeast flocculation. The single molecule force spectroscopy measurements presented here provide a means to study a variety of cellular interactions at the molecular level, such as the adhesion of bacteria to animal and plant tissues.

INTRODUCTION

Cellular events such as microbial aggregation, microbial adhesion and biofilm formation are of crucial importance in the natural environment, in medicine and in biotechnological processes (Savage & Fletcher, 1985; Costerton et al., 1999; Watnick & Kolter, 2000). During the past few decades, much progress has been made in understanding the mechanisms underlying cellular interactions. A number of techniques have been used to assess cell adhesion (Busscher & van der Mei, 1995) and cell aggregation (Dengis et al., 1995) behaviours. Relationships have been established between adhesion data and overall cell-surface physicochemical properties and theoretical models have been successfully applied to describe adhesion behaviours, i.e. the so-called DLVO theory, which accounts for electrostatic and van der Waals interactions, and thermodynamic approaches, which consider the balance of interfacial free energies between the different partners (Busscher et al., 1999). Yet, discrepancies have often been reported between experimental data and theoretical predictions that are likely to be due to the fact that the molecular heterogeneity of the cell surface was not taken into account. Indeed, appendages (fimbriae, fibrils) and macromolecules protruding from the cell surface may generate interactions that are not considered in classical models. Furthermore, specific interactions between localized receptor and ligand molecules may play a central role in mediating the adhesion of bacteria to living tissues and the flocculation of yeast cells. Consequently, there is clearly a need in cell adhesion studies to develop methods that can probe molecular interactions at the subcellular level.

Atomic force microscopy (AFM) (Binnig et al., 1986) has emerged as a powerful tool to measure intramolecular and intermolecular forces associated with biological systems (Lee et al., 1994; Florin et al., 1994; Hinterdorfer et al., 1996; Benoit et al., 2000). Recently, AFM force measurements were applied to microbial systems, providing new insight into microbial cell-surface functions (for recent reviews, see Dufrêne, 2002, 2003). In particular, force spectroscopy was used to investigate electrostatic and steric interactions associated with negatively charged bacterial strains (Camesano & Logan, 2000); to measure the interaction forces between Escherichia coli strains and polymer biomaterials (Razatos et al., 1998), between Aspergillus niger spores and mica (Bowen et al., 2000) and between Shewanella oneidensis bacteria and mineral surfaces (Lower et al., 2001); to probe the stiffness of bacterial cell wall components (Xu et al., 1996; Yao et al., 1999); to unzip single proteins from the
hexagonally packed intermediate layer of *Deinococcus radiodurans* (Müller et al., 1999); to map the distribution of yeast cell wall polysaccharides (Gad et al., 1997); and to stretch macromolecules at the surface of fungal (van der Aa et al., 2001) and bacterial (Abu-Lail & Camesano, 2002) cells.

Here, we use AFM force spectroscopy to measure discrete lectin-carbohydrate interactions involved in the reversible aggregation of yeast cells, a process referred to as yeast flocculation which is of particular importance in fermentation processes such as brewing and wine-making (Calleja, 1989; Stratford, 1992; Dengis et al., 1995; Jin & Speers, 1998). While cell flocculation has been examined for over a century, the flocculation mechanisms remain poorly understood at the molecular level. Biochemical studies have revealed the involvement of specific interactions between cell-surface lectins and mannose residues, based on the finding that sugars such as mannose and glucose can specifically inhibit flocculation (Miki et al., 1982). However, direct measurement of these interactions has not been possible up to now.

**METHODS**

**Micro-organisms and culture conditions.** Brewing yeast strains *Saccharomyces carlsbergensis* MUCL 28285 (bottom fermenting strain) and *Saccharomyces cerevisiae* MUCL 38475 (top fermenting strain) were kindly supplied by Professor A. M. Corbisier (Unité de Microbiologie, Université Catholique de Louvain, Belgium). The cells were grown as described by Dengis et al. (1995). Yeast cells were stored in 20% (v/v) glycerol at −20°C. They were plated in Petri dishes on solid medium [1% (w/v) yeast extract, 3% (w/v) glucose, 2.5% (w/v) agar] and incubated at 30°C for 3 days. The plates were stored at 4°C and repitched every 3 weeks by plating one yeast colony on a new solid medium. The culture broth contained 2% (w/v) yeast extract and 5% (w/v) glucose. Two or three colonies from the solid medium plate were used as inoculum for a 10 ml preculture which was grown by shaking overnight at 30°C. Cells of the preculture were used to inoculate 200 ml of culture medium to a concentration of about 2 × 10⁶ cells ml⁻¹. The two strains were incubated on a shaker at 100 r.p.m. either at 10°C (*S. carlsbergensis*) or at 30°C (*S. cerevisiae*). The exponential and stationary growth phases were reached after about 2 and 3 days, respectively, for *S. carlsbergensis*, and after about 10 and 14 h, respectively, for *S. cerevisiae*.

**Flocculation test.** Flocculation was quantified by measuring the optical density of yeast suspensions after agitation and subsequent undisturbed standing to allow floc settling (Dengis et al., 1995). Test tubes (16 × 100 mm) containing 5 ml of the following solutions were used: 10 mM sodium acetate/acetic acid buffer (pH 5.2), 0 or 100 mM D- (+)-mannose, and CaCl₂ at various concentrations. Yeast cells were added as a concentrated suspension under whirl mixing to reach a final concentration of 10⁶ cells ml⁻¹. The tubes were agitated upside down at 25 r.p.m. for 15 min, left undisturbed for 15 min and the OD₆₆₀ was then measured (Spectronic Instruments). Optical micrographs of the cells were obtained using a commercial optical microscope (Olympus BH-2).

**Functionalization of AFM probes.** Self-assembled monolayers (SAMs) of thiol-terminated hexaamylose molecules were used to produce carbohydrate-coated AFM probes. The functionalization strategy and its validation using X-ray photoelectron spectroscopy and AFM imaging were described in a previous paper (Touhami et al., 2003). Briefly, commercial Si₃N₄ AFM cantilevers (Thermo-Microscopes) were coated by electron beam thermal evaporation with a 5 nm thick Cr layer followed by a 30 nm thick Au layer. They were cleaned for 5 min by UV/ozone treatment (Jelig), rinsed in ethanol and immersed for 3 h in 0.05 mM solution of the thiol-terminated hexaamylose in a 1:1 solution of methylene chloride and ethanol. The functionalized cantilevers were then rinsed in three baths of methylene chloride, sonication being briefly applied during the rinsing step to remove loosely bound aggregates, and then dried in a gentle nitrogen stream. Using AFM imaging and force measurements, we recently showed that the carbohydrate-coated probes interact specifically with concanavalin A (Con A), a glucose/mannose-specific lectin (Touhami et al., 2003).

Lectin-coated probes were created using carboxymethyl-amyllose as a spacer to provide mobility to the protein and minimize non-specific adsorption (Johnsson et al., 1991). Gold-coated probes were cleaned for 5 min by UV/ozone treatment (UVO-Cleaner), rinsed in ethanol, immersed for 16 h in 1 mM solution of HS(CH₂)₂NH₂ (Aldrich; used as received) and then rinsed with ethanol. A PBS solution (pH 7.2) of 10 mg carboxymethyl-amyllose ml⁻¹ (Sigma) was activated with 20 mg N-hydroxysuccinimide (NHS) ml⁻¹ (Aldrich) and 50 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) ml⁻¹ (Sigma) for 5 min. Modified probes were then incubated with the NHS-activated amyllose for 10 min, rinsed three times in PBS, incubated with Con A (Sigma) (0.5 mg ml⁻¹ in PBS; pH 7.2) for 30 min and intensively rinsed with water to remove the unbound proteins. Water used in our experiments was HPLC grade produced by a MilliQ plus system from Millipore (MilliQ water).

**AFM measurements.** AFM force-distance curves were obtained at room temperature, using a commercial microscope (Nanoscope III; Digital Instruments). Cells were immobilized by mechanical trapping in a polycarbonate membrane (Millipore) with a pore size similar to the cell size (Dufrêne et al., 1999; Dufrêne, 2002). Measurements were performed in buffered solutions (sodium acetate/acidic acid; pH 5.2) containing 1 mM CaCl₂ (carbohydrate-coated probe) or 1 mM CaCl₂ + 1 mM MnCl₂ (lectin-coated probe). Blocking control experiments were performed by adding 100 mM D- (+)-mannose to the solutions. The spring constants of functionalized cantilevers were found to be 8 ± 0.4 N m⁻¹, as determined by measuring the free resonance frequency in air (Cleveland et al., 1993). Retraction force curves were recorded at a rate of 0.5 μm s⁻¹ (rate of the piezo displacement).

**RESULTS AND DISCUSSION**

**Factors controlling flocculation**

We selected two *Saccharomyces* strains exhibiting marked differences in their flocculation behaviours (Dengis et al., 1995). *S. carlsbergensis* MUCL 28285 is known to flocculate according to a lectin-mediated mechanism in which active lectin receptors appear at the cell surface in the stationary phase and bind mannose residues of adjacent cells in the presence of Ca²⁺ ions (Fig. 1). Yeast cells were submitted to flocculation tests to determine the factors influencing flocculation (Fig. 2). Consistent with previous work (Miki et al., 1982; Dengis et al., 1995), we found that Ca²⁺ was needed to observe flocculation of *S. carlsbergensis*, indicating that lectins require the presence of Ca²⁺ to adopt their active conformation. Cells of the exponential phase never flocculated, suggesting that lectins are only present/active in the stationary phase (Figs 1 and 2). Addition of mannose...
inhibited flocculation pointing to the involvement of mannose-specific lectins in cell–cell interactions. Unlike the *S. carlsbergensis* strain, *S. cerevisiae* MUCL 38475 never flocculated even in the presence of Ca$^{2+}$ (Fig. 2).

### Specific interactions between cell-surface lectins and carbohydrate-coated probes

Yeast cells were immobilized in porous polymer membranes for AFM characterization in aqueous solution. This method does not use drying or chemical fixation, thereby making it possible to investigate living cells while preserving the native macromolecular architecture of the surface (Dufréne et al., 1999; Dufréne, 2002).

Using carbohydrate-coated probes, single cells were first imaged at high resolution. The surface structure was smooth and homogeneous, as demonstrated previously using silicon nitride probes (Ahimou et al., 2003). Multiple force-distance curves were then recorded at various locations at a rate of 0.5 µm s$^{-1}$. For *S. carlsbergensis* in flocculating conditions (stationary phase; 1 mM Ca$^{2+}$), single or multiple unbinding forces were observed in about 50% of a total of 200 force-distance curves (Fig. 3a), the remaining curves exhibiting no adhesion. The occurrence or lack of adhesion forces was found to depend essentially on the spot investigated, suggesting some heterogeneity in the distribution of the cell-surface molecules. The histogram of the largest unbinding forces (Fig. 3c) displayed an asymmetric

![Fig. 1. Flocculation of yeast cells through specific lectin-carbohydrate interactions. (a–d) Optical micrographs of *S. carlsbergensis* cells in exponential phase (a, c) and in stationary phase (b, d). Bars, 20 µm (a, b) and 10 µm (c, d). In the lectin-mediated mechanism, active lectins appear at the cell surface in the stationary phase and specifically bind to mannan carbohydrates of adjacent cells (e, f).](image)

![Fig. 2. Flocculation tests. OD$_{660}$ values of yeast suspensions as a function of the Ca$^{2+}$ concentration in the solution are presented. Open squares, exponential-phase cells of *S. carlsbergensis*; Circles, stationary-phase cells of *S. carlsbergensis* in the absence (filled) or presence (open) of mannose; open triangles, stationary-phase cells of *S. cerevisiae* in the absence of mannose.](image)
distribution centred at $121 \pm 53$ pN ($n=100$). Adhesion histograms from independent experiments showed similar mean values and distribution. To demonstrate that this adhesion force reflects the specific interaction between cell-surface lectins and glucose residues on the probe, the same experiment was carried out in the presence of mannose (Fig. 4a, b). Under these conditions, little or no adhesion was detected, indicating that mannose had blocked the lectin receptor sites. Interestingly, the measured force of 121 pN is in the range of force values (75–200 pN) obtained by Gad et al. (1997) between lectin-coated probes and yeast cells. It is also close to values typically reported for individual receptor–ligand interactions at fairly comparable rupture rates. By way of example, mean adhesion forces of 160, 112 and 96 pN were reported for the specific interaction between avidin–biotin (Florin et al., 1994), antibody–antigen (Dammer et al., 1996) and lectin–carbohydrate (Touhami et al., 2003). Taken together, these observations lead us to believe that the 121 pN force reflects the individual interaction between cell-surface lectins and glucose residues. There are several important questions to address in future research, including assessing the dependence of the unbinding forces on the pulling rate (dynamic force spectroscopy measurements) and determining whether the multiple unbinding events are due to the rupture of multiple receptor–ligand complexes or to some other phenomenon such as protein unfolding.

To relate our force measurements to the flocculation behaviour, we recorded force-distance curves in conditions where yeast cells do not flocculate: S. carlsbergensis in the stationary phase in the presence of mannose (Fig. 4a, b; see above), S. carlsbergensis in the exponential phase (Fig. 4c, d) and S. cerevisiae in the stationary phase (Fig. 4e, f). In these
three conditions, most of the curves showed no adhesion forces regardless of the location investigated, indicating that active cell-surface lectins were not available for interaction with the probe. The correlation between flocculation behaviour and force measurements provides direct evidence that the measured 121 pN adhesion force arises from specific lectin–carbohydrate interactions involved in yeast flocculation. Note that the calcium concentration used in our force measurements (1 mM) was selected in view of the flocculation test data (Fig. 2). In future, it would be interesting to investigate the effect of calcium concentration on the measured forces.

**Specific interactions between cell-surface mannose residues and lectin-coated probes**

Flocculation of *S. carlsbergensis* through the lectin mechanism involves the binding of cell-surface lectins to mannose residues (mannan) of adjacent cells. Consequently, one would expect that AFM probes terminated with mannose-specific lectins should interact specifically with mannose residues of *S. carlsbergensis* cells. To test this hypothesis, force-distance curves were recorded between *S. carlsbergensis* cells in the stationary phase and Con A-coated probes. Note that measurements were performed in 1 mM Ca$^{2+}$ + 1 mM Mn$^{2+}$ solutions, since both ions are required for Con A lectin activity. Most force-distance curves showed single or multiple unbinding forces (Fig. 5a) and the adhesion histogram (Fig. 5c) revealed a mean adhesion force of $117 \pm 41$ pN ($n=100$) which is close to the mean value obtained with the carbohydrate probe. We attribute this adhesion force to the specific binding between Con A and cell-surface mannose residues, which is supported by the finding that this adhesion force was not observed in the presence of mannose (Fig. 5d, e).

In conclusion, using AFM with biofunctionalized probes we have measured individual lectin–carbohydrate interactions involved in the flocculation of *S. carlsbergensis*. In flocculating conditions, adhesion forces of 121 and 117 pN were measured with carbohydrate- and Con A-terminated probes, and interpreted as reflecting the specific interaction with individual cell-surface lectins and mannose residues, respectively. These adhesion forces were not detected in non-flocculating conditions (presence of mannose, exponential phase, *S. cerevisiae* strain), leading us to conclude that the measured specific interactions are involved in the flocculation process. The use of biofunctionalized AFM probes has great potential in microbiology and cell biology for mapping the distribution of specific sites at cell surfaces. For instance, adhesion maps recorded on germinating/dividing microbial cells could reveal important differences between old and newly formed cell-wall material. We also suggest that this approach could lead to the development of new applications in biotechnology and biomedicine, e.g. for the rapid screening of micro-organisms.

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