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► **To cite this version:**

Cécile Formosa-Dague, Marion Schiavone, Anita Boisrame, Mathias Richard, Raphael Duval, et al.. Multiparametric imaging of adhesive nanodomains at the surface of *Candida albicans* by atomic force microscopy. *Nanomedicine: Nanotechnology, Biology and Medicine*, Elsevier, 2015, 11 (1), pp.57-65. <10.1016/j.nano.2014.07.008>. <hal-01553144>

HAL Id: hal-01553144

<https://hal.laas.fr/hal-01553144>

Submitted on 7 Sep 2017

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1 **Multiparametric imaging of adhesive nanodomains at the surface of**
2 ***Candida albicans* by Atomic Force Microscopy**

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17
18 Abstract word count: 138 words / Manuscript word count: 4451 words / Number of figures: 5 /
19 Number of references: 40

20
21 This work was supported by an ANR young scientist program (AFMYST project ANR-11-JSV5-
22 001-01 n° SD 30024331) to ED. CF and MS are respectively supported by a grant from
23 “Direction Générale de l’Armement” (DGA) and from Lallemand SAS.

25 **Abstract**

26 *Candida albicans* is an opportunistic pathogen. It adheres to mammalian cells through a variety
27 of adhesins that interact with hosts ligands. The spatial organization of these adhesins on the
28 cellular interface is however poorly understood, mainly because of the lack of an instrument able
29 to track single molecules on single cells. In this context, the Atomic Force Microscope (AFM)
30 makes it possible to analyze the force signature of single proteins on single cells. The present
31 study is dedicated to the mapping of the adhesive properties of *C. albicans* cells. We observed
32 that the adhesins at the cell surface were organized in nanodomains composed of free or
33 aggregated mannoproteins. This was demonstrated by the use of functionalized AFM tips and
34 synthetic amyloid forming/disrupting peptides. This direct visualization of amyloids
35 nanodomains will help in understanding the virulence factors of *C. albicans*.

36

37

38 **Background**

39 The yeast *Candida albicans* has emerged as a major public health problem these last two
40 decades. This opportunistic pathogen causes a wide range of infections from surface infections, to
41 mucosal and blood-stream infections¹. Whereas mucosal infections are common and occur in
42 healthy organisms, blood-stream infections are observed only in immunocompromised patients
43 and are life-threatening. This type of infections, also known as candidaemia, can develop into
44 disseminated candidiasis when the infection spreads to internal organs, leading to high mortality
45 rates². In order to colonize and subsequently to disseminate in the blood stream *C. albicans* needs
46 to adhere to different substrates. This first stage of infection³ is mediated by adhesins that are
47 found on the surface of the yeast cell wall. Many of these adhesins are mannoproteins, and
48 among them, the adhesin family identified as having a major role in host cell attachment is the
49 Als (Agglutinin-like Sequences) family⁴.

50 The Als were initially reported as having homologies with the proteins responsible for
51 auto-agglutination in the baker yeast *Saccharomyces cerevisiae*. Eight Als have been identified,
52 they all are primarily involved in host-pathogen interactions⁵. It was found that there were
53 amyloid-forming sequences in the Als adhesins of *Candida albicans*⁶. Amyloids are insoluble
54 fibrillar protein aggregates whose core consists in crystalline arrays of identical sequence in many
55 molecules of the amyloid protein^{7,8}. Cells expressing the Als proteins can rapidly aggregate, and
56 the aggregation has amyloid-like properties. Like amyloid formation, aggregation ability
57 propagates through the adherent cell population and depends on conformational changes of the
58 Als protein. This transition of the conformational state to an aggregative state of the proteins is
59 characterized by the formation of hydrophobic nanodomains on the entire surface of the cell⁹.

60 A few papers written by Lipke's team were dedicated to the direct visualization of these
61 nanodomains using fluorescent dyes such as thioflavin T or 8-anilino-1-naphthalene-sulfonic acid

62 (ANS)^{6,8,9}. Another technique that can be used to visualize these nanodomains is Atomic Force
63 Microscopy (AFM). AFM has recently emerged as a valuable tool to study the surface of living
64 cells¹⁰, and especially pathogenic cells¹¹. This technology has been used by Alsteens *et al.* to
65 image the formation and propagation of nanodomains in living yeast cells¹² and also to unfold
66 amyloid proteins from the yeasts surface using Single Molecule Force Spectroscopy¹³⁻¹⁵. To this
67 end, the authors functionalized AFM tips with antibodies targeted against the Als protein directly
68 or against an epitope tag present in the Als protein. These studies allowed the authors to localize
69 the adhesive nanodomains caused by the aggregation of Als proteins at the surface of living yeast
70 cells, and to unravel the structure of the Als proteins studied by stretching.

71 In our study, we used AFM as an imaging tool to visualize and localize adhesins
72 nanodomains at the surface of living wild-type *Candida albicans* cells. Using recent
73 developments in the AFM technology, we have imaged and quantified at the same time the
74 nanomechanical properties, the adhesiveness (force and nature of the interaction), the size and the
75 thickness of the nanodomains^{16,17}, at high resolution. The data collected showed that these
76 nanodomains are localized differently at the surface of the cell, depending on the structures
77 featured by the cells (bud scars, buds). We also showed that there were degrees of adhesiveness,
78 depending on whether the amyloid proteins had totally aggregated (hydrophobic nanodomains) or
79 not, and that these degrees of aggregation were directly correlated to the stiffness of the yeast cell
80 wall. Finally, using force measurements and amyloid forming or inhibiting peptides, we showed
81 that Als proteins (probably among others) were participating to these nanodomains.

82

83 **Methods**

84 **Yeasts growth conditions**

85 *Candida albicans* (from ABC Platform® Bugs Bank, Nancy, France) was stocked at -80°C,
86 revived on Yeast Peptone Dextrose agar (Difco, 242720-500g) and grown in Yeast Peptone
87 Dextrose broth (Difco, 242820-500g) for 20 hours at 30°C under static conditions.

88

89 **Sample preparation for AFM experiments**

90 Yeast cells were concentrated by centrifugation, washed two times in acetate buffer (18 mM
91 CH₃COONa, 1 mM CaCl₂, 1 mM MnCl₂, pH = 5.2), resuspended in acetate buffer, and
92 immobilized on polydimethylsiloxane (PDMS) stamps prepared as described by Dague *et al*¹⁸.
93 Briefly, freshly oxygen activated microstructured PDMS stamps were covered by a total of 100
94 μL of the solution of cells and allowed to stand for 15 minutes at room temperature. The cells
95 were then deposited into the microstructures of the stamp by convective/capillary assembly.
96 Images were recorded in acetate buffer in Quantitative Imaging™ mode with MLCT AUWH
97 (Bruker) cantilevers (nominal spring constant of 0.01 N/m). The applied force was kept at 1.5 nN
98 for imaging and at 0.5 nN for force spectroscopy experiments. The loading rate for imaging was
99 of 2 500 000 pN/s (acquisition frequency of the force curves is of 25 Hz) and for force
100 spectroscopy of 75 000 pN/s (acquisition frequency of the force curves is of 1.25 Hz). For
101 imaging and force spectroscopy, we used an AFM Nanowizard III (JPK Instruments, Berlin,
102 Germany). The cantilevers spring constants were determined by the thermal noise method¹⁹. For
103 all the results presented in this study, silicon nitride AFM tips were bare, except in the case of
104 figure 4g (lower panel), where a functionalized AFM tip has been used.

105

106 **AFM tips fonctionnalization**

107 The functionalized tips were produced according to a french patent of the authors described later
108 in sensors and actuators²⁰. Briefly, AFM tips were functionalized with dendrimers presenting

109 CHO functions able to covalently link with NH₂ functions of proteins. These dendritips were then
110 incubated with the lectin Concanavalin A (Sigma, L7647-100MG, 100µg/mL) for 1 hour, before
111 being used for force spectroscopy experiments.

112

113 **Results analysis**

114 All results were analyzed using the Data Processing software from JPK Instruments. The stiffness
115 values measured on cells were determined from the slope of the linear portion of the raw
116 deflections versus piezo displacement curves, according to:

$$k_{cell} = k \left(\frac{s}{1-s} \right)$$

117 with s the experimentally accessible slope of the compliance region reached for sufficient loading
118 forces. In this model, the experimental setup can be represented by two linear springs, one is the
119 AFM's cantilever, and the other is the cell envelope exhibiting an effective spring constant. It is
120 then possible to calculate the effective spring constant k_{cell} of the cell envelope from the
121 observed slope s of the force curve and the known spring constant k of the cantilever²¹.

122

123 **Results**

124 *Candida albicans cells display localized adhesiveness*

125 Thanks to our innovative method to immobilize cells into PDMS stamps¹⁸, and using
126 Quantitative Imaging™ mode¹⁶, we were able to image and quantify the adhesive properties of
127 single *C. albicans* cells at the same time. Figure 1a shows a budding yeast cell; on the
128 corresponding adhesion image (Figure 1b), we can see that only the bud, and not the mother-cell,
129 presents adhesives patches. This original result is surprising as non-budding cells are highly
130 adhesive (see below). This result seems to indicate that the mother-cell cell wall changes during

131 the budding process. As for the cell in figure 1c, this cell displays two bud scars, a common
132 feature at the surface of yeast cells, which are not adhesive whereas the rest of the cell is. This
133 type of distribution of the adhesion on yeast cells has already been seen using
134 immunofluorescence with antibodies targeted against surface proteins of *C. albicans*. Coleman *et*
135 *al.* for example showed that the Als1 protein was expressed all over *C. albicans* cells, with the
136 exception of bud scars²². The comparison of our results to these data suggests then that the
137 adhesions probed by AFM might be due to surface proteins, such as Als1 in the case of the cell
138 presenting bud scars, but perhaps also others adhesins.

139

140 ***C. albicans* cell wall adhesins are able to aggregate into nanodomains**

141 As showed before^{9,12}, the proteins expressed at the surface of *C. albicans* cell wall are
142 able to aggregate, and to form nanodomains. However, these nanodomains have not been yet
143 characterized at the nanoscale, nor were imaged at high resolution. In fact, these nanodomains
144 have specific adhesive properties that can be mapped using AFM in the Quantitative ImagingTM
145 mode. High resolution (256 pixels²) adhesive images are presented in Figure 2. It shows adhesive
146 nanodomains, at the surface of a living wild-type *C. albicans* cell. On the cell presented in this
147 figure (Figure 2a), the corresponding adhesion image shows very distinct adhesive nanodomains
148 that were probed with bare AFM tips. These nanodomains are homogeneously distributed all over
149 the cell here, which does not present any morphological features such as buds or bud scars. When
150 zooming into small areas on top of the cell (white squares on figure 2b), we could measure the
151 area of each nanodomain. On this cell and on another one showed in Figure 3a, 60 nanodomains
152 areas were measured; the values obtained plotted on figure 2e shows that nanodomains have an
153 average area of $0.09 \pm 0.03 \mu\text{m}^2$. This corresponds to an average diameter of 170 nm, which
154 confirms the nanoscale of these nanodomains. Some of the nanodomains are also higher than the

155 rest of the cell wall. When the whole cell is imaged, it is not visible; however, specific analysis of
156 the Figure 3d and the graphic 3h representing the topography of the cell surface revealed
157 nanodomains that had a different height compared to the rest of the cell. The cross-sections taken
158 along the blue line showed the height of a nanodomain of 20 nm. Once again, this confirmed the
159 nanoscale of the nanodomains at the surface of *C. albicans*.

160

161 ***Different nanodomains have different nanomechanical properties***

162 Adhesion is measured as the rupture force recorded when retracting the tip from the surface,
163 when approaching and pulling with the tip on the cell wall, thus AFM makes it possible to
164 measure nanomechanical properties of living cells. Here we choose to use an analysis based on
165 the Hooke model which considers the coupled cantilever / cell wall as a spring. The stiffness
166 values measured on cells were determined from the slope of the linear portion of the raw
167 deflections versus piezo displacement curves, according to:

$$k_{cell} = k \left(\frac{s}{1-s} \right)$$

168 with s the experimentally accessible slope of the compliance region reached for sufficient loading
169 forces. Indeed, the most interesting result in this study is the correlation that can be directly made
170 between the adhesiveness of the nanodomain, and its stiffness. Nanodomains on figure 3e
171 (adhesion map) circled in red were found on the stiffness image (figure 3f) circled in black; they
172 correspond to the zones where the stiffness of the cell wall is increased, to 13.4 ± 0.3 nN/ μ m. As
173 for less adhesive nanodomains, they do not present any difference in stiffness from the rest of the
174 cell, and are 12.4 ± 0.2 nN/ μ m. The 3D-view of the adhesion, mapped with the stiffness (figure
175 3g) illustrates this clear correlation; the more adhesive the nanodomain is, the stiffer it is.

176 Another fascinating point is that for the more adhesive nanodomains, the retract force
177 curves present typical hydrophobic adhesions²³⁻²⁵, with adhesions occurring immediately when
178 the tip is retracted from the surface. Force curves from the other, less adhesive, nanodomains
179 presented retract adhesions resembling to proteins unfolding, occurring several nanometers after
180 the tip withdrawal. Therefore it seems that the nanodomains are of 2 different natures. There is a
181 class of nanodomains, hydrophobic, higher and stiff, and another class displaying proteins
182 unfolding properties, as soft as the rest of the cell wall. What is the molecular nature of these 2
183 types of nanodomains, and are they correlated?

184

185 *Understanding the adhesive properties of the 2 nanodomains classes*

186 To answer the previous question, we monitored the retract force curves recorded on the
187 nanodomains (Figure 4). We found the same correlation as in figure 3; the force curves recorded
188 on an adhesive nanodomains presented hydrophobic retract adhesions, whereas the force curves
189 recorded on a less adhesive nanodomain presented protein, unfolding like, profiles. In order to
190 determine the nature of these last unfoldings, we probed the surface of *C. albicans* cells with an
191 AFM tip functionalized with Concanavalin A (ConA), a protein that interacts with yeast
192 mannoproteins, such as surface adhesins. The resulting force curves (figure 4g) showed retract
193 adhesions displaying unfoldings of different lengths, but with a similar profile. We also observed
194 condensed spikes with adhesion forces between 0 and 50 pN. This value was consistent with
195 specific interactions between ConA and mannoproteins²⁶.

196 In a previous study conducted in 2009¹⁴ by Alsteens *et al.*, adhesins (Als5) were unfolded
197 from the surface of live *S. cerevisiae* cells overexpressing this protein. The retract force curves
198 obtained in this study show high similarity with the ones we obtain here with functionalized AFM
199 tips, with the presence of serin-threonin rich segments (condensed pikes on figure 4g). We can

200 therefore, based on this comparison with the data of the literature, conclude that the less adhesive
201 nanodomains at the surface of live *C. albicans* are composed of free adhesins, and maybe of Als
202 proteins. However, since all adhesins (like Als, Hwp1, Eap1, Rbt1 etc) are mannoproteins, we
203 cannot, at this stage, make a statement on which adhesins are unfolded here.

204 As for the second type of nanodomains, the hydrophobic ones, our hypothesis is that they
205 are composed of the same proteins as the less adhesive ones. In fact, adhesins (like Als) display
206 amyloids sequences located on a domain of the protein called T, that enable them to change their
207 conformation⁶ and to aggregate into amyloid nanodomains. And when this phenomena is started,
208 it propagates to the whole cell⁹. We therefore made the hypothesis that the adhesive nanodomains
209 are in fact amyloid nanodomains, made of Als proteins.

210

211 ***From adhesins to amyloid nanodomains: the role of Als proteins***

212 To verify this hypothesis, and according to the literature on Als proteins, we synthesized a
213 peptide exhibiting the same sequence as the one of the T domain of the Als1/3/5 proteins. We
214 then put this peptide in the presence of the cells in order to trigger the amyloids formation. We
215 also synthesized the same peptide, but with a mutation on one amino acid (V326N peptide), in
216 order to obtain a peptide that inhibits the formation of the amyloid nanodomains⁸. Since Als3 is
217 only expressed on the surface of hyphae, we will only be able to generate or destroy the amyloid
218 formation of Als 1 and 5. The results presented in figure 5 showed a cell before and after adding
219 the mutated peptide. We can clearly see on these adhesion images the loss of general adhesion
220 and of two nanodomains at the center of the cell. It seems like the mutated peptide disrupted the
221 amyloids at the surface of the cell. And the other way around, when cells were incubated with the
222 amyloid forming peptide, we could observe the formation of the nanodomains at the surface of
223 the cells, as it is showed on the adhesion images on local areas on top of *C. albicans* cells in

224 figure 5c, d and e. These results allow us to conclude that the proteins at the origin of the
225 nanodomains are then mannoproteins, more specifically Als1 or 5, or both, that form amyloids.

226

227 **Discussion**

228 We show in this study that wild-type live *C. albicans* cells exhibit extraordinary adhesive
229 properties. In the case of budding cells, placed in acetate buffer at 25°C for 2h, we observed that
230 the mother cell is not adhesive and that only the bud presents adhesive nanodomains. On the
231 contrary, we show that non budding cells are covered by adhesive nanodomains, in the same
232 experimental conditions. This illustrates the amazing plasticity of this species^{27,28} able to grow as
233 a commensal or as a pathogen^{29,30}, in all the parts of the intestinal track, but also on the vaginal
234 mucosa, as unicellular budding cells or as filamentous hyphae. Moreover its cell wall is
235 permanently remodeled as a reaction to its environment (temperature, pH, dissolved O₂, ions,
236 interacting surface/cells/bacteria) what makes it challenging to reproduce the experimental
237 conditions inducing a certain cell wall phenotype. We then demonstrate that the molecules at the
238 origin of these adhesions could aggregate into nanodomains, which can be probed at high
239 resolution using a suited AFM mode, QITM. These nanodomains are different in terms of level of
240 adhesiveness, which is a property directly correlated to their stiffness and to the hydrophobic
241 state or not of the molecule at the origin of these nanodomains. We then went further in the study,
242 using functionalized AFM tips, and were able to determine that the less adhesive nanodomains
243 were formed by mannoproteins that can interact specifically with Concanavalin A. These
244 mannoproteins are able to aggregate to form the adhesive nanodomains because they have
245 amyloid properties as we showed in figure5.

246 Amyloid aggregation is a primitive³¹ and very stable³² protein folding and a common
247 structural motif. It is a cross β -sheet quaternary structure that usually auto-aggregates as fibrils. It

248 has been, first, associated with neurodegenerative diseases like Alzheimer, Parkinson, or
249 Creutzfeldt-Jakob diseases. However it is more and more unclear if the amyloids lesions are the
250 cause or a consequence of the disease. Amyloid aggregates are now described as functional
251 proteins assembly and can be found from bacteria to humans³³. In microorganisms, amyloid has
252 been described as a functional coat³⁴. It consists in curli (*E. coli*), chaperons (*Streptomyces*) or
253 hydrophobins (*Aspergillus etc*); all of these proteins are implicated in adhesion to the host and in
254 the invasion, infection process. It is now well known that adhesins (and especially *AIs*) of *C.*
255 *albicans* have amyloid-forming sequences^{6,35} and that these proteins form domains involved in
256 cell aggregation or biofilm formation³⁶. Nevertheless the characterization, structure and
257 properties of the amyloids adhesive nanodomains remain unclear.

258 In this work we measured for the first time the nanoscale size of amyloid domains
259 (average area of $0.09 \mu\text{m}^2$) at the surface of live *C. albicans* cells. The domains are of 2 different
260 classes. Some present the characteristic of individual proteins whereas the others are
261 hydrophobic, stiffer than the rest of the cell ($13.4 \pm 0.3 \text{ nN}/\mu\text{m}$ compared to $12.4 \pm 0.2 \text{ nN}/\mu\text{m}$),
262 and are slightly protruding. It means that there is a state modification from soluble proteins into
263 insoluble proteins, which is a characteristic of amyloid structures. This transformation is
264 dependent on the proteins concentration and can only occur when the protein density exceeds a
265 threshold. The roles of the two classes of domains are probably different. On one hand we could
266 hypothesize that the hydrophobic nanodomains were involved in the cell adhesion to abiotic
267 hydrophobic surfaces or to cell membrane as it is known that membrane binding is an inherent
268 property of amyloid aggregates^{37,38}. Amyloid aggregation is also a way to store proteins, in a
269 limited space and to sort them when required. This has been demonstrated for hormones in
270 secretory granules^{39,40}. Thus *C. albicans* may store some adhesins for the subsequent invasion
271 phases. On the other hand the protein like domains may be responsible for specific adhesion to

272 fibronectin and other extra cellular proteins of the matrix. It seems rational that several adhesins,
273 brought together, would be more efficient in a binding process than a single adhesion. This
274 finding has to be added to *C. albicans* plasticity²⁸ and participate to explain its remarkable
275 adaptation and pathogenicity.

276 However, there are still many things to explore on the cell wall of *C. albicans*, and future
277 work will be dedicated to exploring the changes appearing on the mother cell during the budding
278 process.

279

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374

375 **Figure Caption**

376 **Figure 1. Localization of the adhesive properties of *C. albicans* cells.** (a) Height image (z-
377 range = 1.5 μm) of a budding *C. albicans* cell in a PDMS stamp, and (b) adhesion image
378 corresponding to the height image. On (a), MC stands for Mother cell, BC stands for Budding
379 cell, and the red dotted line represents the demarcation between the two different cells. (c) Height
380 image (z-range = 3.5 μm) of a single *C. albicans* cell exhibiting two bud scars, and (d) adhesion
381 image corresponding to the height image.

382

383 **Figure 2. Imaging of the adhesive domains of *C. albicans* cells in acetate buffer, at 25°C for**
384 **2 hours.** (a) Height image (z-range = 2.5 μm) of a single *C. albicans* cell in a
385 polydimethylsiloxane (PDMS) stamp, and (b) adhesion images corresponding to the height
386 images. (c, d) Adhesion images of small areas on top of the cell, represented by the white squares
387 in b. (e) distribution of the areas values of the domains in c and d.

388

389 **Figure 3. Nanomechanics of the adhesive domains of *C. albicans* cells.** (a) Height image (z-
390 range = 2.5 μm) of a *C. albicans* cell in a PDMS stamp, (b) corresponding adhesion image, and
391 (c) corresponding stiffness image. (d) Height image (z-range = 100 nm) of a small area on top of
392 the cell, represented by the white square on (a), (e) corresponding adhesion image and (f)
393 corresponding stiffness image. Note that the adhesive nanodomains circled in red on (e) are also

394 found on the stiffness image (black circles on f). (g) is a 3D-image of the adhesion mapped with
395 the stiffness. (h) cross-section taken along the blue line on (d), and (i), distribution of the stiffness
396 values corresponding to the yeast cell wall and the less adhesive domains (blue columns) or to the
397 most adhesive domains (yellow columns).

398
399 **Figure 4. Adhesion force curves of *C. albicans* adhesive domains.** (a) Height image (z-range =
400 4.0 μm) of a *C. albicans* cell in a PDMS stamp. (b) Adhesion image of a small area on top of the
401 cell, represented by the white square on (a). (c and d) representative force curves obtained on the
402 zones indicated by the arrows on (c). (e) Height image (z-range = 2.5 μm) of a *C. albicans* cell in
403 a PDMS stamp and (f) corresponding adhesion image recorded with a bare tip. (g) representative
404 force curves obtained in the zone delimited by a white square on (f) with the Con A tip.

405
406 **Figure 5. Imaging of the adhesive domains of *C. albicans* cells treated with Als1, 3, 5p**
407 **amyloid disrupting peptide (V326N peptide) or Als1, 3, 5p amyloid forming peptide (Als**
408 **peptide).** (a) Adhesion image of a single *C. albicans* cell in a PDMS stamp and (b)
409 corresponding adhesion image after adding V326N peptide. (c, d and e) Adhesion images of
410 small areas on top of a *C. albicans* cell after adding the Als peptide.

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