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The volume and hydration of the *Cryptococcus neoformans* polysaccharide capsule

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Abstract

We present a new method to measure capsule size in the human fungal pathogen *Cryptococcus neoformans* that avoids the limitations and biases inherent in India ink measurements. The method is based on the use of γ-radiation, which efficiently releases the capsule from the cell. By comparing the volume of irradiated and non-irradiated cells, one can accurately estimate the relative size of the capsule per cell. This method was also used to obtain an estimate of the capsule weight and water content. The *C. neoformans* capsule is a highly hydrated structure in all the conditions measured. However, after capsule enlargement, the amount of capsular polysaccharide significantly increases, suggesting a that capsule growth has a high energy cost for the cell.

Index Descriptors: *Cryptococcus neoformans*; Capsule; India ink; γ-Radiation; Fungal pathogen; Virulence factor

1. Introduction

Cryptococcus neoformans is an encapsulated fungal pathogen that primarily affects immunocompromised patients, causing chronic pneumonia and meningitis, which is associated with host death unless treated. Several virulence factors have been described for *C. neoformans*, such as melanin production, phospholipase and urease activity, and the ability to grow at 37 °C (Casadevall and Perfect, 1998). However, the dominant virulence factor is the polysaccharide capsule that surrounds the cell body (McClelland et al., 2006; Chang and Kwon-Chung, 1994). This structure is composed primarily of glucuronoxylomannan (GXM, 90–95%); with galactoxylomannan and mannoproteins being minor components (Bose et al., 2003; McFadden and Casadevall, 2001; Doering, 2000; Reiss et al., 1985). The capsule plays an important role during the interaction of the pathogen with the host (Vecchiarelli, 2005; Casadevall and Perfect, 1998; Chang and Kwon-Chung, 1994). Capsular polysaccharide is shed *in vitro* to the medium (Cherniak and Sundstrom, 1994) and during *in vivo* infection into tissue (Goldman et al., 1995). During host infection, the capsular polysaccharide has several immunomodulary effects, such as inhibition of leukocyte migration, complement depletion, and Ab unresponsiveness (Vecchiarelli, 2005; Retini et al., 1998; Vecchiarelli et al., 1996; Macher et al., 1978). It also has antiphagocytic properties (Zaragoza et al., 2003a; Kozel and Gotschlich, 1982). Finally, acapsular mutants show reduced virulence (Chang and Kwon-Chung, 1994). Because of its great importance to *C. neoformans* pathogenesis, much research has focused on investigating the characteristics of the polysaccharide capsule.

One of the remarkable characteristics of the cryptococcal capsule is that it can change dramatically in size, depending on the environmental conditions. There are several factors that induce capsule enlargement, such as

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mammalian serum, low nutrient concentration, basic pH, high CO_2, low iron, and *in vivo* conditions (Zaragoza and Casadevall, 2004; Zaragoza et al., 2003b; Feldmesser et al., 2001; Rivera et al., 1998; Vartivarian et al., 1993; Granger et al., 1985; Love et al., 1985; Anna, 1979; Cruickshank et al., 1973; Bergman, 1965). Growth in rich culture media (Littman, 1958) and high osmotic pressure (Jacobson et al., 1989) produce reduction in capsule size. Increase in capsule size is important for virulence, since there is evidence that mutants that cannot increase capsule size are avirulent (D'Souza et al., 2001; Granger et al., 1985). In addition, mutants that overproduce capsule are hypervirulent (D'Souza et al., 2001). Specifically, capsule enlargement can interfere with complement-mediated phagocytosis (Zaragoza et al., 2003b; Kozel et al., 1996). This provides a plausible explanation for decreased virulence of mutant cells that cannot increase capsule size.

In the cryptococcal field the measurement of capsule size is critically important when comparing strains and ascertaining capsule responsiveness to various environmental conditions. Two methods have been utilized to measure capsule size. The volume of the packed cells in capillary tubes was used as an indicator of capsule size difference between strains or conditions (Granger et al., 1985). However, this method did not give any insight into the size of the capsule itself, as compared to the size of the cell body. A second approach uses light microscopy and the observation that the polysaccharide capsule excludes India ink dye (Anna, 1979). The staining of cells with India ink allows for the visualization of the capsule as a white halo around the cell body, and to measure the size of the capsule manually. The India Ink staining is relatively easy and is available and affordable to every laboratory in the field, being this the main reason why it has been largely used to measure capsule size.

Although the India ink method can give very precise measurements, it has several inherent problems. First, there is no guarantee that the measurement is made along the equatorial plane of the cell and measurements away from the equator could significantly overestimate the size of the capsule relative to that of the cell. Second, the edge of the capsule is assumed to be a layer where India ink particles are excluded. Given that the outer regions of the capsule are more porous (Gates et al., 2004), it is conceivable that India ink exclusion measurement under estimates the size of the capsule. In this paper, we identify new limitations of the India ink method, and propose a new method to measure capsule size, based on the capsular release that occurs after γ-irradiation of the yeast cells. In addition, we demonstrate that this technique can be used to obtain other capsule parameters, such as weight and water content.

2. Material and methods

2.1. Yeast strains and growth conditions

Cryptococcus neoformans strain H99 (serotype A) was used because it has an homogenous capsule size distribution after incubation in capsule growth media (Zaragoza et al., 2003b). In some experiments the acapsular *cap67* mutant was used (Jacobson et al., 1982). To induce an increase in capsule size, cells were inoculated into diluted Sabouraud dextrose media (Sab, 1/10 dilution) in 50 mM MOPS, pH 7.4 (Zaragoza and Casadevall, 2004), at a cell density of 5×10^6 cells/mL, and incubated at 37 °C overnight.

2.2. γ-Irradiation of the yeast cells and cell volume measurement in hematocrit tubes

Cells with enlarged capsule were washed with PBS, and suspended at a cell density of $1–5 \times 10^8$ cells/mL. Two parallel samples were prepared, and one was exposed to γ-radiation emitted from radioisotope ^{137}Cs at a dose rate of 1388 rads/min for 40 min, using a Shepherd Mark I Irradiator. Irradiation has been shown to release more than 95% of the capsular polysaccharide (Bryan et al., 2005). The second sample was kept at room temperature as a non-irradiated control. After the irradiation, cells were washed with PBS to remove the shed polysaccharide, and both samples were then suspended at about 2×10^8 cells/mL, maintaining an equal cell density in both irradiated and non-irradiated samples. To measure the volume of the cells, we placed 50 μL of each cell suspension within hematocrit capillary tubes, sealed the tip with parafilm (to prevent evaporation) and placed them vertically overnight to allow cell packing by gravity. The packed volume of cells was measured for irradiated and non-irradiated samples, and the difference between these two samples calculated. This represents the portion of the packed cell volume of the total population that can be attributed to the capsule.

2.3. Microscopy and capsule size measurement

The cells were suspended in an India Ink suspension and observed under an Olympus AX70 microscope. Pictures were taken using a QImaging Retiga 1300 Digital camera using the QCapture Suite V2.46 software (QImaging, Burnaby BC, Canada). Capsule size was measured in these images using Adobe Photoshop 7.0 for Windows (San Jose, CA). Capsule size was defined as the difference between the diameter of the total cell (capsule included) and the cell body diameter, defined by the cell wall.

2.4. Fluorescence, confocal imaging and 3D reconstruction

To obtain 3D images of the cells between the slide and the coverslip, cells were incubated with 10 μg/ml of mAb to GXM 12A1 [IgM isotype, (Casadevall et al., 1994)] for 1 h at 37 °C. Then the cells were washed with PBS and incubated with calcofluor (50 μg/mL) and GAM-IgM-TRITC conjugated Ab (10 μg/mL, Southern Biotechnologies, Birmingham, AL). After 1 h at 37 °C, the cells were washed, and suspended in mounting medium (50% glycerol and 50 mM *N*-propyl gallate in PBS). *Z*-series from each cell

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were obtained by taking pictures every 0.25 microns with a Leica AOBS Laser Scanning Confocal microscope. 3D images were obtained processing the pictures with ImageJ (NIH software) and Voxx softwares (program owned by the Indiana University).

2.5. Cellular weight and water content calculation

For these calculations, we measured the cell volume and wet/dry weight for 10^{10} cells before and after 40 min of γ-irradiation treatment. These parameters were measured for cells with small capsule, grown in Sabouraud's dextrose medium, and for cells with enlarged capsule, after incubation in 50 mM, MOPS pH 7.4, with 10% Sabouraud medium. For capsule wet weight calculations, 1 mL of the irradiated cell suspensions were placed in pre-weighed 1.5 mL microfuge tubes and pelleted at 13,000 rpm in a benchtop microcentrifuge. Supernatants were carefully removed, pellets washed several times in dH_2O to eliminate the released polysaccharide, and the microfuge tubes weighed using an analytical balance. The wet weight of the capsule was expressed as the weight difference between the non-irradiated and γ-irradiated samples. For capsule dry weight calculations, the wet samples were then lyophilized. The tubes were weighed, and the dry weight of the capsule calculated as above. The amount of water in the capsule was expressed as the weight difference of the capsule between the pre- and post-lyophilized samples.

3. Results and discussion

We have recently observed that the India Ink technique has several significant limitations. When capsular polysaccharide size was measured by India Ink staining for a cell suspension between glass slides, we observed that the cells were not equally distributed as a function of size across the slide (Fig. 1). Cells with the largest capsule, and thus largest size, were concentrated at the slide center. In contrast, cells with smaller capsule became concentrated at the edges of the slide preparation. To quantify this phenomenon, we analyzed 51 pictures taken across one slide, starting at one side and ending at the other. The average total size, cell body and capsule size for all cells per photograph was measured, and plotted according to its slide relative position. A total of 1600 cells were measured. We observed that the distribution throughout the slide correlated with both capsule and cell body size, and thus, total size of the cell (Fig. 2). This result confirmed our initial observation by India ink, and is most likely the result of the downward force, caused by the placement of the coverslip, which is applied initially to the largest cells at the center of the slide (or site of culture droplet placement). Therefore, we hypothesized that the larger cells became trapped in the area where they were placed, while smaller cells retained the ability to move with the preparation as the liquid expands across the slide. This effect of coverslip force on deforming the largest cells was confirmed by confocal microscopy and 3D image reconstruction of cells with large capsule. The capsule was labeled with mAb to GXM 12A1 (IgM), detected with a goat anti-mouse IgM Ab conjugated to rhodamine (TRITC) and the cell wall detected with calcofluor. Then, confocal *z*-series images were taken (0.25 μm separation) and a 3D reconstruction generated using ImageJ software (NIH). We observed the capsule deformation caused by the pressure of the slide coverslip (inset, lower panel of Fig. 2). Hence, India Ink measurements have two additional problems: deformation of large encapsulated cells and fluid transport phenomena where cells of different size localize to different parts of the glass slide. Consequently, for heterogeneous cell preparations, India Ink staining will not give an accurate estimate of capsule size in the population, unless hundreds of cells are measured in many regions of the slide preparation.

We developed a new simple method for the precise measurement of capsule relative size for an entire population using γ-radiation. γ-Radiation releases the capsular polysaccharide from the cell, presumably through the generation of free radicals that attack capsular polysaccharide fibers, which are non-covalently attached to the cell body (Bryan et al., 2005). γ-Radiation is a technique widely used in immunology, because it destroys the bone marrow and suitable equipment for irradiating samples is available in many institutions. The release of the capsular polysaccharide dramatically affects the volume of packed cells. We assumed that the measurement of the volume of packed cells before and after irradiation would allow for an accurate measurement of the capsule volume for the entire cell population. We validated this method by using cells with enlarged capsule, and comparing the measured capsule size obtained through the irradiation technique with the capsule size obtained with the microscope.

After irradiation, the volume of the cells decreased by approximately 85% when compared to the non-irradiated

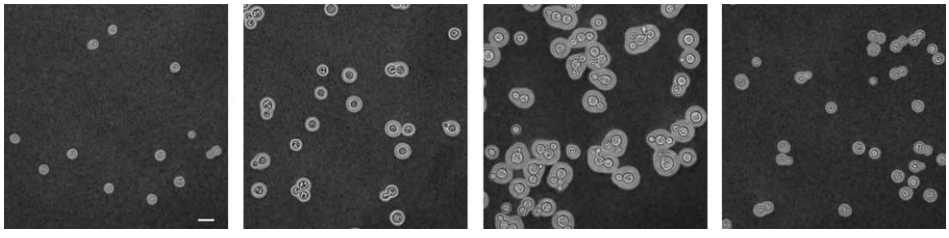

Fig. 1. Light micrographs of different regions of the same slide of *C. neoformans* cells suspended in India Ink. Scale bar, 10 μm. The pictures were taken in the same plane of the slide, and they are consecutive (from left to right). They correspond to position 5, 11, 21, and 36 from Fig. 2, respectively.

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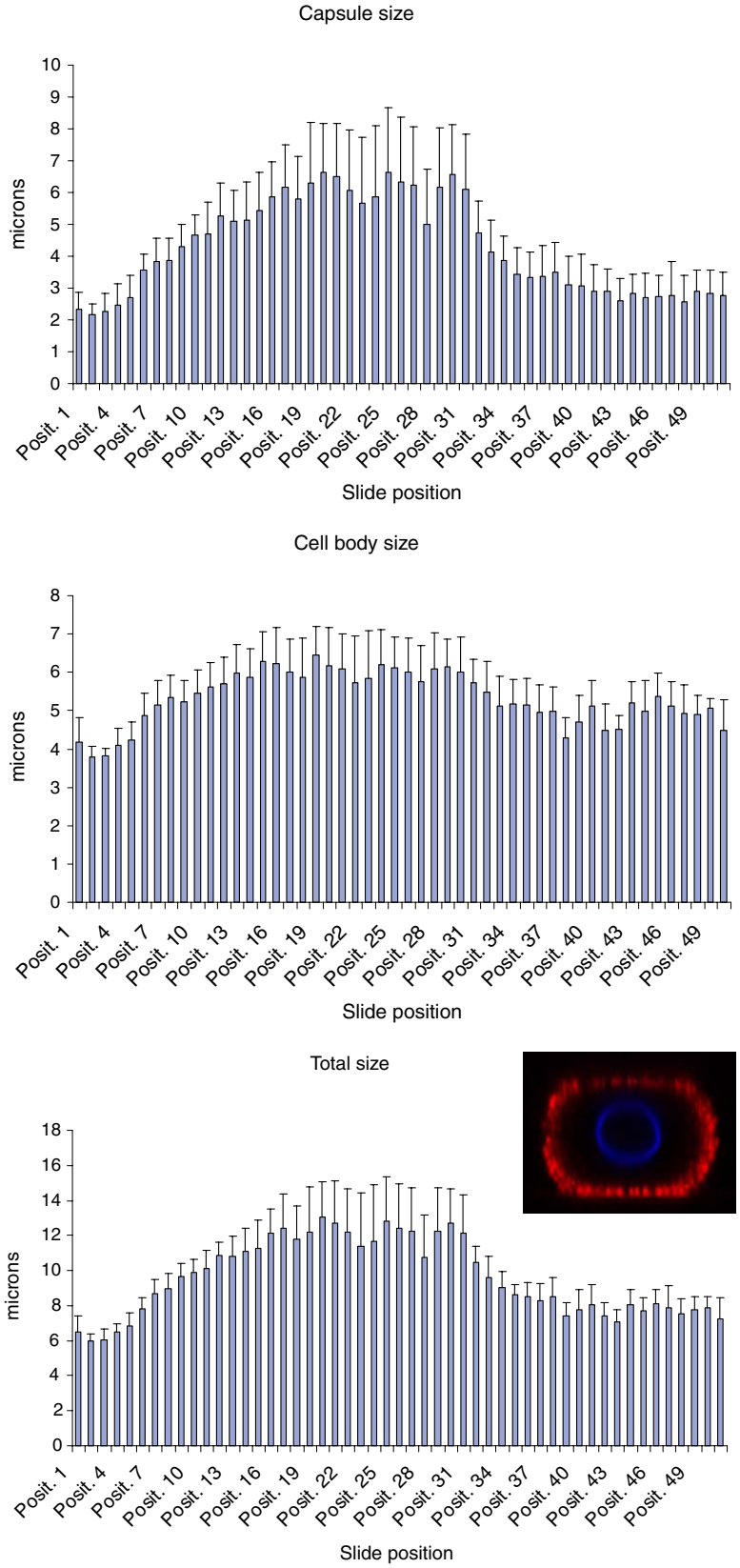

Fig. 2. Cell size distribution across an India ink slide preparation of *C. neoformans* strain H99. Fifty-one different pictures were taken from the same slide. Images are shown in order, starting from the left of the slide (position 1 on *x*-axis) and finishing at the right (position 51). Total cell (capsule + cell body), cell body, and capsule sizes were measured using Adobe Photoshop 7.0. The average and standard deviation for each of these parameters were calculated for each image, and plotted according to its position in the slide. Inset in the bottom panel shows the *z*-slice generated from 3D image reconstruction of one cell where the capsule has been distorted between the slide surface and coverslip, as indicated by the immunofluorescent signal detected from a capsule-binding mAb.

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Table 1
Capsule size measurements after γ-irradiation of *C. neoformans* cells[a]

Strain (conditions)	Volume before irradiation (μL/10^7 cells)	Volume after irradiation (μL/10^7 cells)	Volume reduction (μL/10^7 cells)	Percent volume decrease (%)
H99 (large capsule[b])	27 ± 1.2	4 ± 0.1	23	85
H99 (small capsule[b])	5.3 ± 0.07	3.4 ± 0.05	1.9	36
cap67 (acapsular[b])	4.8 ± 0.14	4.8 ± 0.25	None	None

[a] This experiment was repeated at least twice for each condition. All the experiments gave similar results. The data presented is a representative experiment, in which the cells were placed in three different hematocrit tubes. The standard deviation of these three tubes is shown.
[b] Cultures represent a homogeneous population, grown overnight in either capsule enlarging media (large capsule) or Sab media (small capsule) for approximately 16 h.

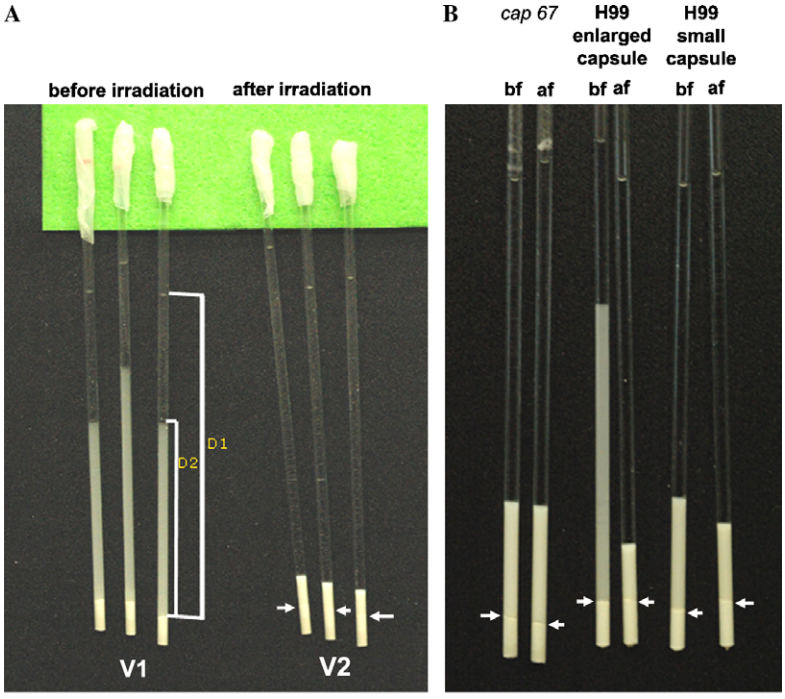

Fig. 3. Demonstration of capsule measurement for non-irradiated and γ-irradiated cells using γ-radiation and cell packing techniques. (A) Cell samples from H99 strains with enlarged capsule were resuspended to the same cell concentration, and equivalent volumes placed into hematocrit tubes. Tubes were placed vertically overnight, to allow for cell settling, and pictures taken of the packed hematocrits. The distance of the total volume placed (D1) and that of the packed cells (D2) was measured using Adobe Photoshop 7.0. Volume and capsule relative size of the packed cells was calculated as indicated in the figure and text. For each sample, triplicate hematocrits were prepared and values averaged, although only one set is shown here. (B) Hematocrit tubes containing *C. neoformans* cells with enlarged capsule (1.5×10^7 cells), small capsule (2.5×10^7 cells), and from the acapsular mutant *cap67* (2.75×10^7 cells), before (bf) and after (af) γ-irradiation of the cells. After the treatment, a 50 μL volume of each cell suspension was placed in hematocrit tubes, for overnight cell packing. Volumes were calculated as indicated in (A). Arrows at the bottom of the capillary tubes in (A) and (B) indicate the bottom of the samples.

sample (Table 1, Figs. 3A, B), which indicates that in these cells the capsule accounts for approximately 85% of the total cell volume. This reduction is similar to that calculated from volume measurements by India ink staining (around 90%, data not shown). In cells where the capsule was not enlarged, the reduction in volume was around a 40%, which is consistent with the relative size of the capsule volume measured by India Ink, which is around 45–50%, (result not shown). As a control, the experiment included the acapsular mutant, *cap67*. As expected, there was no measurable volume decrease after irradiation. Therefore, the irradiation procedure only results in loss of volume from the capsular polysaccharide.

We used this method to measure the capsule loss in a heterogeneous population of cells, where the inherent bias of classical India ink measurements would not give an accurate measurement of average capsule volume in this case. Cells were incubated in capsule enlargement media for four days, and capsule volume measured by hematocrit cell packing. In these conditions, we have observed that new buds typically will remain very small, and will not develop large capsule, due to nutrient depletion. This contributed to

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the increased heterogeneity of the population in cell size. Using this method, we measured a 63% reduction in volume, due to capsule loss (data not shown). This value reflects the average reduction for the entire heterogeneous population, and is less than that observed for the homogeneous population most likely due to the small loss of capsular polysaccharide from the newer buds.

It is tempting to attempt a calculation of the cell volume based on the number of cells and the volume measured by hematocrit cell packing. However, our attempts to calculate volume from considerations of sphere packing have not given reasonable results, and an exploration of this topic reveals that this calculation is not trivial. The packed volume of cells cannot be simply divided by number of cells to yield cell volume. According to Kepler's law of sphere packing, the volume of the packed spherical cells will also include extracellular spaces between spheres, calculated as $(16/3)\pi r^3$. However, random packing of cryptococcal cells is very difficult to predict by the Kepler formula. This formula defines the volume of hard spheres in hexagonal or face-centered cubic packing, but this occurs only in ideal conditions, and requires that spheres have defined boundaries. Given that a cryptococcal cell suspension will include cells of different diameter and irregularly-shaped cells from budding it is very unlikely that ideal packing will occur. Also, it is likely that the edge of the capsule of cryptococcal cells is not a rigid, incompressible boundary. In fact, this study shows that the capsule is compressible when *C. neoformans* cells are suspended between two glass slides. Both factors almost certainly result in non-ideal packing. Consequently, other parameters such as cell diameter and cell volume should not calculated. However, the relative size of the capsule is not affected by these considerations and can be easily calculated. Although capsule deformability effects could conceivably affect packing volume in the capillary tubes we note that our method relies on gentle settling due to gravity and consequently yeast cells are not exposed to very strong forces. Relative size measurements are more relevant for comparisons between different populations and different strains of *C. neoformans* because it is normalized by total cell size. Several cryptococcal species show varying capsule size after enlargement (Zaragoza et al., 2003b). This method could be useful in comparisons of capsule differences between several strains or conditions.

Finally, we used this method to gain insights into two additional physical properties of the capsule, the increase in capsule weight that occurs following capsule enlargement, and the hydration of the capsule. Comparison of the weight measurements between cells grown in Sabouraud or capsule enlarging medium measurements allowed us to estimate the amount of polysaccharide that accumulated in the capsule after capsule enlargement (data not shown). The capsule weight for cells with small capsule was not measurable, presumably because the amount of polysaccharide released by γ-radiation was below the sensitivity of our experimental design. In these experimental conditions, 40% of the total volume of the cell consisted of the capsule (Table 1). Therefore, we can conclude that most of the mass of the capsule is water, and that the capsule is a highly hydrated structure. The water content of the capsule was calculated as over 95% of the total mass and volume. For cells with enlarged capsules, the capsule volume increased to 90% of total cell volume (Table 1). However, the capsule mass was only 10% the dry weight of the cells. Considering that the capsule has enlarged over a period of 16 h (overnight), this result indicates that the cells synthesize a significant amount of capsular polysaccharide within a short time.

The *C. neoformans* capsule is a very acidic structure due to the high content in glucuronic acid, which potentially suggests could retain a high amount of water. We have confirmed this expectation using the method proposed in this paper, and observed that the water could account for more of 95% of the weight of the capsule. From these experiments one can also deduce that the process of capsule enlargement depends on the addition of newly synthesized polysaccharide to the capsule and does not occur by a conformational change that involves stretching of the old polysaccharide fibers, as the weight of the capsule increased appreciably after enlargement. Since the weight of the capsule before induction is not detectable by the method used in this paper, the cell must invest a significant amount of energy in growing and enlarging the capsule.

In summary, we demonstrate that the capsule of *C. neoformans* is compressible and we propose a new method for the measurement of the relative capsular size in *C. neoformans* that avoids deformability and fluid transport effects. This method is based on volume changes following γ-radiation and has the advantage of providing the average capsule relative size for the entire population, without bias. In cases where the population is heterogeneous, classical methods for capsule volume measurement have this limitation. For homogeneous cell populations, this approach complements measurements obtained from classical India ink staining. In addition, the use of γ-radiation allowed us also to address other basic questions about capsule structure, such as capsule weight and water content in relation to that of the cell. These experiments show that the water content of the capsule is very high, over 95% of the total mass and volume. Still, during capsule enlargement conditions, the cell must invest a significant amount of energy in growing and enlarging the capsule, since they accumulate an additional 10% total cell weight in capsule polysaccharide. This deduced fact highlights the importance of this capsule remodeling in the life of the yeast, as well as in host interactions. .

Change in capsule size is an important factor in the pathogenesis of *C. neoformans*, therefore we feel that the increased accuracy and precision of our method in analysis of capsule physical properties will make it an attractive alternative, particularly in studies relevant to *C. neoformans* virulence.

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