Candida albicans biofilms: comparative analysis of room-temperature and cryofixation for scanning electron microscopy


*Instituto de Biodiversidade Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil
†Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil
∥Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil
#Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brasil

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Summary

Biofilms are frequently related to invasive fungal infections and are reported to be more resistant to antifungal drugs than planktonic cells. The structural complexity of the biofilm as well as the presence of a polymeric extracellular matrix (ECM) is thought to be associated with this resistant behavior. Scanning electron microscopy (SEM) after room temperature glutaraldehyde-based fixation, have been used to study fungal biofilm structure and drug susceptibility but they usually fail to preserve the ECM and, therefore, are not an optimised methodology to understand the complexity of the fungal biofilm. Thus, in this work, we propose a comparative analysis of room-temperature and cryofixation/freeze substitution of Candida albicans biofilms for SEM observation. Our experiments showed that room-temperature fixative protocols using glutaraldehyde and osmium tetroxide prior to alcohol dehydration led to a complete extraction of the polymeric ECM of biofilms. ECM from fixative and alcohol solutions were recovered after all processing steps and these structures were characterised by biochemistry assays, transmission electron microscopy and mass spectrometry. Cryofixation techniques followed by freeze-substitution lead to a great preservation of both ECM structure and C. albicans biofilm cells, allowing the visualisation of a more reliable biofilm structure. These findings reinforce that cryofixation should be the indicated method for SEM sample preparation to study fungal biofilms as it allows the visualisation of the EMC and the exploration of the biofilm structure to its fullest, as its structural/functional role in interaction with host cells, other pathogens and for drug resistance assays.

Introduction

Biofilms are heterogeneous microbial communities, composed by cells adhered to biotic or abiotic surfaces, that are embedded in a polymeric extracellular matrix (ECM) produced by themselves, and with an altered phenotype when compared to planktonic cells (Donlan & Costerton, 2002). The ECM of the fungal biofilms is composed by a complex mixture of polysaccharides, proteins and nucleic acids (Zarnowski et al., 2014) and may represent a physical barrier, preventing the access of antimicrobial agents into the cells of the biofilm community, which contribute to the increase of drug resistance (Flemming & Wingender, 2010; Chandra & Mukherjee, 2015). This obstacle appears to depend on the amount and nature of the matrix, as well as physicochemical properties of the drug (Nett et al., 2007, Nett et al., 2010a; Taff et al., 2012). Among all medically important fungi, Candida bloodstream infection is the third most common cause of nosocomial infection in patients requiring intensive care and the most common etiologic agent of fungal-related biofilm infection (Ramage et al., 2012). C. albicans, an opportunistic pathogen in immunocompromised patients, is most frequently associated with biofilm formation. Indwelling medical devices, such as intravascular...
catheters, can become colonised with *Candida* spp. allowing
the development of adherent biofilm structures from which
cells can then detach and cause an acute fungemia and/or
disseminated infection (Ramage *et al.*, 2012).

Imaging techniques are an important research tool used
to investigate complex structures in various scientific disci-
plines. Scanning electron microscopy (SEM) has been widely
used to study biofilm structure and drug susceptibility/effect
on biofilm cells. Preparation of biological material for SEM
requires extensive manipulation, including fixation, dehydra-
tion and either air drying or critical-point drying because the
microscope operates at high vacuum (Little *et al.*, 1991). Still,
when room-temperature preparation protocols are applied to
biofilms, little or no ECM can be visualised at the electron mi-
croscope (Tsang *et al.*, 2012; Nieminen *et al.*, 2014; Walraven
*et al.*, 2014; Souza *et al.*, 2016), compromising the under-
standing of full biofilm structure. Therefore, sample preparation
is the biggest challenge concerning the utilisation of SEM for
biofilm studies.

Stabilisation of biological structure by the physical process
of freezing (cryofixation) has been used since 1960 to study the
cell biology of yeasts, including Saccharomyces spp. (Fernandez-
Moran, 1960; Yamaguchi *et al.*, 2009), *Candida* spp. (Osumi,
2006) and the filamentous fungi Aspergillus (OSUMI *et al*.,
techniques (cryofixation), including plunge (PF) and high-
pressure freezing (HPF), constitute better approaches than
chemical fixation for biological samples. The combination
of HPF with subsequent dehydration of the frozen specimen
(freeze substitution, FS) was successfully used to visualise both
cells and the ECM of bacterial biofilms (Dahl & Staehelin, 1989;
Webster *et al.*, 2004). In this work, we propose a protocol based
on cryosample-handling of *C. albicans* biofilms for SEM obser-
vation. Comparative analysis of room-temperature and cry-
operating demonstrated that room-temperature processing
is responsible for the loss of the ECM of *C. albicans* biofilms.
Alternatively, unfixed biofilms were observed by scanning
electron microscope using the environmental mode.

**Materials and methods**

**Microorganism**

All experiments were performed using a *Candida albicans* clinical
isolate (44A) that forms dense biofilms in vitro [64], and
was originally obtained from gastric lavage at the Microbi-
ology/Mycology Laboratory from ‘Arthur de Siqueira Cava-
lanti State Institute of Hematology’ (Hemorio – Rio de
Janeiro, RJ, Brazil).

**Biofilm formation**

Biofilms were formed on small sections of longitudinally
cut sterile central venous catheters (CVCs) (BD™ Intracath
Vialon™, Franklin Lakes, NJ, USA) or directly on the bottom
of 24-well microplates (for the Gas-chromatography/Mass-
spectrometry (GC/MS) experiments), as described previously
(Vila *et al.*, 2013). Briefly, 100 µL of a standardised cell sus-
pension (10⁷ CFU mL⁻¹) was transferred into each well of a
96-well microtiter plate containing the CVC section, and the
plate was incubated for 1.5 h (adhesion phase) at 36 °C in
a platform shaker. After the adhesion phase, cell suspensions
were gently aspirated and, 100 µL of freshly prepared RPMI
1640 medium buffered with MOPS (Sigma Chemical Co.,
Missouri, USA) supplemented with 2% of glucose and 20%
of FBS (Fetal Bovine Serum, Gibco, Grand Island, USA) was
added to each well and the plates were incubated for 24 h at
36 °C in a platform shaker. For GC/MS experiments, biofilms
were grown directly on the bottom surface of 24-well mi-
croplates following the same protocol and volumes were ad-
justed to 500 µL.

**Morphological and chemical analysis of ECM**

**Production of ECM extracts.** To produce control ECM ex-
tracts to be used for negative staining, carbohydrate and pro-
tein content determination and GC/MS analysis, the ECM
of biofilms formed for 24 h on the surface of 24-well mi-
croplates was extracted as previously described (Nett *et al*.,
2007) Briefly, after the biofilm formation for 24 h, the super-
natant was discarded and the biofilm was scrapped from the
microplate surface. The biofilm was sonicated in a microtube
for 10 min, immediately vortexed for 2 min and centrifuged at
4 °C in three cycles of 20 min. Then, the supernatant contain-
ing the ECM was collected (referred to as ‘extracted control
ECM’).

**Negative staining method.** Aliquots of 5 µL of each SEM
sample processing steps and from the ‘extracted ECM control’
were loaded on Formvar-coated copper grids (300 mesh) air-dried,
and then negatively stained with 1% ammonium molybdate.
Grids were air-dried after staining and observed under a FEI-
Tecnai transmission electron microscope (FEI, the Nether-
lands) at 120 kV.

**Total carbohydrate and protein quantification.** The total
amount of carbohydrates was quantified using the phenol/sulfuric
acid method (Dubois *et al.*, 1956), with some modifi-
cations. Aliquots of 25 µL of SEM sample processing
supernatants and of control ECM extracts were added to wells
of 96-well microplates, 25 µL of a 5% phenol and 125 µL
of concentrate sulfuric acid were added to wells, and the samples
were incubated for 30 min. The absorbance was measured at
490 nm using a Spectra-MAX 340 tunable microplate reader
(Molecular Devices Ltd., Sunnyvale, CA, USA).

The total amount of protein was quantified using a protein
assay kit based on the Bradford method (Bio-Rad Protein Ass-
say Kit II, Bio-Rad, USA), using 10-µL aliquots of SEM sample
samples.

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processing supernatants and of control ECM extracts, according to the manufacturer’s instructions. Sample absorbance was measured at 595 nm using a Spectra-MAX 340 Tunable microplate reader (Molecular Devices Ltd.).

Gas–liquid chromatography/mass spectrometry (GC/MS). For carbohydrate analysis, SEM sample processing supernatants and control ECM extracts were hydrolysed with 5 M trifluoroacetic acid for 4 h at 100 °C, reduced with sodium borohydride, and the alditols were acetylated with acetic anhydride:pyridine (1:1, v/v). The acetylated alditols were dissolved in chloroform and analysed in a QP2010 gas–liquid chromatograph mass spectrometer (Shimadzu, Japan) using an Agilent HP-ULTRA2 column, as described previously (Kircher, 1960). Data acquisition was performed in the m/z range of 40–440 in the scanning mode and, in the selected ion monitoring (SIM) mode, at m/z 289 for hexoses, m/z 318 for hexosamines and also at m/z 239 and 259, since both are present in hexoses and hexosamines. The use of the SIM mode allowed the selection of ions that are characteristic for the compounds of interest, preventing superestimation of signals due to coelution of contaminants during peaks integration. Relative concentrations of each monosaccharide were calculated by integrating the areas under the peaks.

Environmental scanning electron microscopy (ESEM)

Mature biofilms formed on CVCs as described above were washed in 0.01 M Phosphate-buffered saline (PBS) and immediately examined under a FEI Quanta 250 environmental SEM (FEI). Samples were analysed at 20 kV, with the temperature maintained at 5–8 °C and humidity of 80–100% (environmental mode).

Room temperature (RT) sample processing

CVCs containing biofilms were processed for SEM-RT as previously described (Vila et al., 2013). Briefly, CVCs were washed in 0.01 M PBS, pH 7.2, and fixed in 2.5% glutaraldehyde and 4% formaldehyde, in 0.1 M cacodylate buffer, for 1 h at room temperature. Subsequently, samples were washed in the same buffer, postfixed in 1% osmium tetroxide (OsO₄) and 1.25% potassium ferrocyanide for 30 min and then dehydrated in a series of increasing ethanol concentrations (30%, 50%, 70%, 90% and 100%) for 30 min at each dehydration step. Samples were critical point dried in CO₂, coated with gold and observed under a FEI Quanta 250 scanning electron microscope (FEI).

For negative staining, carbohydrate and protein content determination and GC/MS analyses, biofilms were formed in CVCs or on the bottom of polystyrene microplate wells and then fixed and dehydrated as described above. The supernatants of chemical fixation and each ethanol dehydration steps were collected and processed for biochemical analyses as it will be describe below.

Plunge-freezing (PF) cryofixation

Plunge-freezing was performed using a Leica EM CPC (Leica, Germany). CVCs containing biofilms were rapidly frozen in liquid ethane at −179 °C and stored in liquid nitrogen.

High-pressure freezing (HPF) cryofixation

Biofilms grown on CVCs were cut into 2-mm sections and placed onto aluminium planchettes containing a thin layer of hexadecane, with the biofilm side facing the planchette surface (Ted Pella Inc, Redwood, USA). A second aluminium planchette was placed over each original planchette containing CVCs, and the specimen was immediately frozen in a HPM 010 high pressure freezer (Bal-Tec, Liechtenstein). Frozen samples were stored in liquid nitrogen.

Freeze-substitution (FS) and SEM observation

Specimens cryofixed using PF or HPF were transferred to a mixture of 1% OsO₄ in acetone and maintained at −90 °C in an AF52 freeze-substitution machine (Leica Inc., Deerfield, IL, USA). The FS program included a 48 h incubation step at −90 °C, after which samples were gradually warmed to −20 °C over a 12 h period, and incubated at −20 °C for 3 h. Then, samples were gradually warmed to 4 °C over a 4 h period. Critical point dried in CO₂, attached to aluminium stubs and coated with gold for observation on a FEI Quanta 250 scanning electron microscope or a FEI Magellan microscope (both from FEI). The measurement of ECM filaments were performed using Image J (Abramoff et al., 2004).

Confocal laser scanning microscopy (CLSM)

For CLSM analysis, biofilms were allowed to form for 24 h on the surface of round coverslips placed inside wells of 24-well microplates. Biofilms were fixed with 4% formaldehyde in 0.01 M PBS (30 min), stained with concanavalin A (ConA) (Invitrogen – Thermo Fisher Scientifics, Waltham, MA, USA) at a final concentration of 25 μg mL⁻¹, for 45 min and with FilmTracer® (Invitrogen – Thermo Fisher Scientifics), for 30 min. Samples were observed in a Zeiss LSM-710 confocal laser scanning microscope (Zeiss, Germany).

Statistical analysis

Nonparametric student’s t-test and one-way ANOVA tests were performed using Prism 5.0 Software (Graphpad, La Jolla, CA, USA) and a p value of <0.05 was considered statistically significant.

Results

Unfixed biofilms observed by environmental scanning electron microscopy (ESEM) are rich in ECM

To evaluate the effects of different sample processing methods on the preservation of biofilm components, our initial
approach was to observe biofilms grown for 24 h on CVCs by ESEM. The advantage of the ESEM is to allow the examination of fully hydrated-unprocessed samples. ESEM images showed a dense structure attached to the CVCs, composed by a complex framework of fungal cells enwrapped by an amorphous ECM (Figs. 1A and B). Higher magnification was difficult to obtain due to charging effects and the continuous drying of the samples, a limitation of the ESEM.

Room temperature sample processing of C. albicans biofilms led to biofilm ECM extraction

The amorphous ECM covering fungal cells observed by ESEM (Figs. 1A and B) was not observed by room temperature sample processing to conventional SEM (Figs. 1 C and D). Chemically-fixed biofilms showed well-preserved hyphae and budding yeast cells with apparently gaps between cells. No ECM material could be observed (Figs. 1 C and D) indicating that the ECM could be lost during room temperature processing for SEM.

ECM is extracted from biofilms during room temperature sample preparation for SEM (SEM-RT)

Both ECM extracted by sonication and recovered during SEM-RT sample processing showed a fibrilar pattern, forming a filamentous ‘webs’, as observed by TEM (Fig. 2). Control samples containing ECM extracted by sonication followed by vortexing presented structures (Fig. 2A) similar to the fibrilar material found in the supernatant of chemical-fixation (Fig. 2B) and from the dehydration processing steps (Figs. 2C and D).

Fiber thickness of control and ethanol dehydration samples varied from 8 to 55 nm (Figs. 2A, C and D). Whereas fibers
Fig. 2. Negative staining of fibers released during routine processing of *Candida albicans* biofilms for scanning electron microscopy (SEM). Extracted extracellular matrix ECM (used as control) (A), and the contents of supernatants from chemical fixation (B) and from ethanol dehydration steps (30% and 70% ethanol, in C and D, respectively) were attached to formvar-coated grids and negatively stained using ammonium molybdate, prior to observation by transmission electron microscopy. Fibers were found in all samples, and were similar in structure to those observed in ECM controls, suggesting that all solutions used for routine, room temperature sample processing for SEM led to ECM extraction. (E) Fiber length measurements showed that ECM extracts and supernatants of SEM sample processing steps contained fibers of similar thickness, suggesting that these fibers also have similar composition. Scale bars, 500 nm.

from fixative supernatant were thicker than the others (15–226 nm, *p* < 0.0001) suggesting an aggregation into bundles, possibly due to fixation (Fig. 2B).

Similar carbohydrate profile was found in biofilm ECM control extracts and in SEM processing supernatants

To confirm that the material released from biofilms during routine room temperature sample preparation was part of the ECM, the carbohydrate profile of the supernatants of sample processing steps was determined by the phenol/sulphuric acid reaction and identified by gas chromatography associated to mass spectrometry (GC/MS) (Figs. 3A and B). Proteins were detected in all fraction supernatants (Fig. 3A). Carbohydrate/protein ratio in room-temperature sample processing supernatants indicated that protein extraction was relatively limited and that carbohydrates were the main components extracted during sample preparation for SEM (Fig. 3A).

GC/MS analysis showed that ECM control extracts have mannose (39%), glucose (30%), galactose (19%) and glucosamine (12%) content (Figs. 3B, S1 and S2). The distribution of monosaccharides in the supernatant of the chemical fixation step was similar to those observed in ECM control extract and the hexose concentrations (i.e. mannose, galactose, glucose and glucosamine) were almost identical (Fig. 3B). The supernatants from dehydration steps (30%, 50%, 70%, 90% and 100% ethanol) displayed a similar monosaccharide profile but in different proportions (Figs. 3B, S1 and S2). Glucose was the major monosaccharide component in dehydration supernatants (74–82%), followed by mannose (15–19%) and small amounts of galactose and glucosamine (1–5%) (Fig. 3B). Finally, we used CLSM of unfixed and nonextracted biofilms to demonstrate that the same ECM components were present after *in vitro* biofilm growth. Biofilms were stained with the lectin concanavalin A (ConA), which binds to glucose and mannose residues and with Filmtracer® Sypro ruby, a biofilm-specific dye that binds to ECM glycoproteins. Reconstruction images showed ConA labeling on biofilm cell walls (Fig. 3D) and a somewhat ‘fuzzy’ component dispersed around biofilm cells, likely corresponding to the presence of mannose and glucose residues in the ECM (Fig. 3D). The extracellular material was also strongly labelled with Filmtracer® (Fig. 3D) due to their high glycoprotein content (Fig. 3D).

Cryofixation followed by freeze substitution preserves the extracellular matrix in *C. albicans* biofilms

When samples were prepared for SEM using cryotechniques (HPF/FS and PF/FS), the entire biofilm structure was preserved and an abundant ECM was observed often completely embedding biofilm cells (Figs. 4 and 5). A mesh of ECM fibers surrounding the biofilm cells and connecting these cells to each other could clearly be observed (Figs. 4 and 5).

PF/FS fixation (Fig. 4) revealed the presence of two or three different layers of ECM surrounding biofilms cells: (i) an extremely thin layer similar to a film, wrapping the cells, (inset in Fig. 4C); (ii) a fibrillar layer in the intercellular space
Fig. 3. Biochemical analysis of *Candida albicans* biofilm components extracted during routine sample processing for scanning electron microscopy (SEM). *C. albicans* biofilms were processed for SEM and the supernatants of sample processing steps (chemical fixation and ethanol dehydration) were collected for biochemical analysis. As a control, the biofilm extracellular matrix (ECM) was extracted by sonication and included in the analysis. (A) Total carbohydrates were quantified using the phenol/sulfuric acid method, and total protein was quantified using a Bradford-based method. All SEM sample processing supernatants contained carbohydrates and proteins, although the ratio of carbohydrate/protein in each sample indicates that there is considerably less protein than carbohydrate extraction from biofilms during SEM sample processing. (B) Monosaccharide composition of extracted biofilm ECM (control sample) and of SEM sample processing supernatants as determined by gas-liquid chromatography/mass spectrometry (GC/MS). Glucose was the main monosaccharide extracted throughout sample processing for SEM. (C) Monosaccharide composition of the remaining supernatant liquid after the freeze substitution of *C. albicans* control biofilms as determined by gas-liquid chromatography/mass spectrometry (GC/MS). (D) Confocal laser scanning microscopy of *Candida albicans* biofilms. Biofilms were stained with concanavalin A (Con-A, green) and Filmtracer® (Sypro Ruby, red) and observed unfixed under a confocal microscope. The detection of both dyes spread throughout the biofilm volume confirm the presence of glucose/mannose residues (green) and glycoproteins (red) in the biofilm.

(arrows in Fig. 4) and (iii) a continuous ‘blanket-like’ cover as an outermost layer (arrowheads in Figs. 4A and D).

High magnification images of HPF/FS fixed biofilm showed a number of fractured cells in which part of the cell wall surface was removed (‘cw’ on Figs. 5A–D) exposing the plasma membrane (PM) of hyphae (Figs. 5A and C) and yeasts (Figs. 5B and D). These fractures were not observed in images of biofilms fixed using PF/FS (Fig. 4). After HPF/FS, it was not possible to distinguish between the different ECM layers and the entire ECM structure is composed by a web of filaments connecting biofilm cells (Fig. 5, white arrows). A similar fibrillar material covered the surface of the cells and filled the intercellular space (white arrows in Figs. 5A–C).

Thickness of ECM fibrils in PF/FS or HPF/FS fixed samples showed similar values (27–93 nm and 24–98 nm, for filaments observed by PF and HPF, respectively; Fig. 6C), however, statistical analysis showed that the fibers seen in PF/FS samples are slightly thicker ($p = 0.0058$) than those observed in HPF/FS samples (Fig. 6C). These fibers are thicker than those observed by negative staining (12–55 nm; Fig. 2), probably due to the gold coating (of approximately 5 nm) used to protect the samples against beam damage and provide
Fig. 4. Scanning electron microscopy (SEM) of *Candida albicans* biofilms fixed by Plunge Freezing (PF). *C. albicans* biofilms were formed on the surface of central venous catheters (CVCs) and fixed using PF, and then subjected to freeze substitution (FS), before SEM observation. (A) The biofilm structure appeared well preserved, showing several interconnected cells embedded in abundant extracellular matrix (ECM) (white arrow). (B) The ECM forms a mesh of fibers and seems to mediate cell-to-cell adhesion (white arrows). (C) Yeast cells were covered by a thin ECM film tightly adhered to the surface of the cells (inset). (D) Biofilm cells were covered by ECM forming a ‘blanket-like’ layer (white arrowheads), and a mesh of fibers was observed in the space between the ‘blanket-like’ cover and the cell surfaces (white arrows). Scale bars, 10 µm (A) and 5 µm (B, C and D).

Discussion

In most biofilms, the microorganism population corresponds to only 10% of the total biomass, the ECM corresponds to the remaining 90% (Flemming & Wingender, 2010). ECM has several roles in the biofilm structure, it maintains the architectural integrity, immobilises water and nutrients inside the biofilm and contributes to antifungal tolerance (Hawser & Douglas, 1995; Flemming & Wingender, 2010; Chandra & Mukherjee, 2015). Thus, the ECM is a key component of the biofilm structure that may lead us to amplify our understanding of the biofilm environment. Electron microscopy is an important technique to observe and study cellular and tissue organisation, however the study of biofilm ECM was arduous since there is an anecdotal evidence of biofilm sensibility to sample preparation at room temperature using chemical fixatives, most of the electron micrographs of *in vitro* biofilms only shows fungal cells with no ECM (Tsang et al., 2012; Nieminen et al., 2014; Walraven et al., 2014; Souza et al., 2016). Although the concept that biofilm ECM is lost during sample processing is widely recognised, our work is the first to prove through combined approaches (biochemistry, morphology and mass spectrometry) that the ECM from *C. albicans* biofilms is actually extracted during the several steps of room-temperature sample preparation for SEM.

Fungal biofilm ECM is composed mainly by proteins and polysaccharides (Baillie & Douglas, 2000; Al-Fattani, 2006; Beauvais et al., 2007; Silva et al., 2009; Singh et al., 2011; Zarnowski et al., 2014). Among the polysaccharides, α-1,2 branched- α-1,6-mannans (87%) associated with unbranched β-1,6-glucans (13%) were the most abundant...
and seems to aggregate, forming a mannan-glucan complex (Zarnowski et al., 2014). For comparison purposes, the estimated diameter of an elemental fibril of β-1,3-glucan is 2.8 nm, and a network of β-1,3-glucan fibers ranging in thickness from approximately 0.4 to 1.2 μm is formed in *Candida* cell walls [61]. In our work, extracted ECM material from *C. albicans* biofilms was used as a control and when observed by negative staining TEM appeared as thin fibers of ~20–30 nm in diameter (Fig. 2A). These fibers might represent bundles of the mannan-glucan complex described recently (Zarnowski et al., 2014), as well as thin bundles of β-1,3-glucan fibrils. Similar fibers were observed in the supernatant from room temperature preparation, confirming that the ECM components are being extracted during the process. This theory is further confirmed by GC-MS, where the results showed that the profile of ECM sugars in the supernatants collected after chemical fixation was similar to that found in ECM control samples (Fig. 3). Yet, mannose and glucose were the major monosaccharides observed in all supernatant samples (Fig. 3B), corroborating the hypothesis that the fibers observed by TEM were bundles of mannan-glucan complex.

The carbohydrate-extraction during room-temperature processing may be due to their high affinity for water and the sample processing for electron microscopy involves several aqueous solutions. Zarnowski et al. (2014) showed previously that proteins are the most abundant components in *C. albicans* ECM, accounting for 55%, followed by carbohydrates (25%), lipids (15%) and nucleic acids (5%).

Here, the strong label of the biofilm ECM with Filmtracer® (Fig. 3C) highlights the presence of glycosylated proteins. Glycoproteins have poor affinity with aqueous solutions and are successfully fixed by glutaraldehyde therefore they are less likely to be extracted during SEM sample processing. This might explain the lower protein content of the supernatants collected here during sample processing in comparison to the control extracted sample (Fig. 3A).

Cryofixation techniques have been reported for decades as a better procedure for sample preservation in the study of
various cell types and organisms. However, to our knowledge, this is the first report on the use of cryofixation techniques to study yeast biofilms by SEM. The results showed here reinforce that combining HPF or PF with FS methods improves the structural preservation of *Candida* biofilms formed *in vitro*, allowing the visualisation of both cells and ECM in the biofilms, and revealing novel aspects of the arrangement of these biofilm components.

Previous reports have shown that ESEM is one of the best microscopy tools to observe microbial biofilms since it allows imaging of fully hydrated samples (Little *et al.*, 1991). We used ESEM to confirm that *C. albicans* biofilms formed on CVCs were mature after 24 h, containing a clear and robust ECM component (Figs. 1A and B) and, thus, validate our *in vitro* biofilm-formation assay. Even though ESEM imaging has undeniable importance for biofilm detection, this imaging method is not appropriate for structural characterisation due to its limited resolution. ESEM allows only quick sample observation as the biological samples are unprotected by metal coating and are excessively damaged by the electron beam during the observation.

In contrast, samples subjected to cryofixation followed by FS can be coated with metals, reducing electron beam damaged and increasing the resolution of the final image. When PF/FS was used, two or three layers of ECM cover could be well distinguished in the biofilms structure (Fig. 4). This technique provided a better preservation of the biofilm surface, revealing a ‘blanket-like’ ECM layer covering *C. albicans* biofilms cells, similarly to that previously described for *A. fumigatus* plate colonies fixed using cryotechniques and observed by SEM (Beauvais *et al.*, 2007a). Interestingly, a very similar structure of a ‘blanket-like’ layer covering a network of ECM filaments that interconnect cells has been described for *Pseudomonas fluorescens* biofilms formed *in vitro* (Baum *et al.*, 2009) and *Hemophilus influenza* (Webster *et al.*, 2004) biofilms, after HPF/FS. The ‘blanket-like’ layer appears to be a continuous film covering the whole colony and may be responsible for isolating the internal biofilm structure from external threats. The internal fibrillar layer is less dense and may allow the traffic of water and nutrients between cells. Finally, the tightly adhered ECM layer around each cell reinforces that the ECM acts as an additional barrier for drug-penetration, contributing to increase biofilm drug resistance (Nett *et al.*, 2007, 2010a, b; Vediyappan *et al.*, 2010). The presence of these structurally different layers of ECM highlights the complexity of this component and of the biofilm itself.

Fixation by HPF/FS showed numerous fractured-cells of *C. albicans* biofilms, this could possibly happen during the sample release from the aluminium planchettes used for HPF/FS. These fractures exposed the intracellular content of biofilm cells, and also their periplasmatic space, revealing small spherical structures between the membrane and the cell wall, possibly representing fungal extracellular vesicles previously described by other authors (Rodrigues *et al.*, 2014). However, specific immuno-labelling is needed to confirm this event.

HPF/FS is the best method described in literature to preserve the internal structure of thick samples (up to 0.6 mm) for visualisation by electron microscopy (Dahl & Staehelin, 1989). Our results show that HPF/FS also preserves the network of ECM fibers in *C. albicans* biofilms, allowing a clear observation...
of the cells that compose the complex biofilm structure and the ECM network by SEM. It is important to clarify that, even though a better preservation of the ECM may be obtained using cryofixation, a small residue of ECM could still be detected in the supernatant of the freeze substitution liquid (Fig. 3C). Noteworthy, the FS step seems to be extracting mostly glucose (90–95%), which can also result from the cell wall, as well as from disruption of cells that may happen during the freezing process. Arabinose, an aldopentose, has been described as one of the major carbohydrates of the ECM of Candida biofilms (Zarnowski et al., 2014). Our analysis focused only on ECM hexoses, still, interesting enough, a high pentose pick was observed in the sugar spectrum of all samples obtained after room-temperature processing, but was absent in the FS supernatant (data not shown). Togther our data confirms that ECM extraction can be dramatically reduced by cryofixation of Candida albicans biofilm samples, yielding a more reliable analysis of this cell community structure. One can also use cryo-SEM that allows imaging of frozen samples directly, eliminating the FS step (Alhede et al., 2012; Wu et al., 2014) and further improving the ECM preservation. Unfortunately, this option was not available for our analysis and for this reason this comparison is not possible in this work. Because the in vivo environment is highly aqueous, every imaging technique that requires a drying step will impose some degree of artefact. Here we demonstrated that the combination of PF or HPF with FS improves biofilm preservation in comparison to RT techniques.

The improvement in SEM sample processing techniques and their application in biofilm studies might help us to elucidate the role played by ECM components on the biofilm organisation, its participation in the drug-resistant behavior of biofilm cells, as well as the effect of drugs on ECM production and how it impacts the overall fungal biofilm structure.

Conclusions

In summary, our work give proof of the idea that the ECM from C. albicans biofilms is extracted during room-temperature sample processing for SEM. Importantly, we demonstrate that cryofixation techniques allowed to a better preservation of ECM, showing three different layers tightly adhered to the C. albicans biofilms cells and also interconnecting the cells inside the biofilm structure. Although the cryotechniques used in this work have been applied to study bacterial biofilms with an enhancement of ECM preservation, this is the first study using yeast biofilms. Thus, we showed here that cryofixation represent the more adequate procedure to study the entire fungal biofilm structure (including ECM and cells), increasing the screening potential of in vitro biofilm models to address anti-biofilm antifungal development and function.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contribution

TV participated in all aspects of the reported laboratory studies, data analysis and manuscript writing. BBF participated in all microscopy experiments and data analysis, GRCs and EBB participated in GC-MS experiments and data analysis. MMLC, KI, WS and SR participated in study design and manuscript writing. All authors have read and approved the final manuscript.

References


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Supporting Information
Additional Supporting information may be found in the online version of this article at the publisher’s website:

Fig. S1. Gas chromatography analysis of supernatants from scanning electron microscopy sample different processing steps.

Fig. S2. Mass spectrometry (MS) analysis of gas chromatography (GC) peaks from supernatants of scanning electron microscopy sample processing steps.