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Mechanisms Related to Inhibition of Fungal Biofilm Formation on Medical Device Coated with Poly(Methylmethacrylate-*co*-Dimethylacrylamide)

Fernanda Perasoli [1‡], Luan Silva [1‡], Bruna Figueiredo [1], Juliana Bastos [1], Simone Carneiro [1], Jéssica Sampaio [1], Vânia Araújo [2], Felipe Rafael Beato [3], Fernando Araújo [2], Ana Paula Barboza [3], Luiz Fernando Teixeira [1], Maurice Gallagher [4], Mark Bradley [5], Seshasailam Venkateswaran [5], Orlando dos Santos [1,*]

[1] Laboratory of Fitotechnology, Department of Pharmacy, Federal University of Ouro Preto, Ouro Preto, Minas Gerais, 35400-000, Brazil
[2] Nano Lab, Department of Metallurgical Engineering and Materials, Federal University of Ouro Preto, Ouro Preto, Minas Gerais, 35400-000, Brazil
[3] Laboratory of Microscopy, Department of Physics, Federal University of Ouro Preto, Ouro Preto, Minas Gerais, 35400-000, Brazil
[4] School of Biological Sciences, University of Edinburgh, King's Buildings, David Brewster Road, Edinburgh, EH9 3FJ, UK
[5] School of Chemistry, University of Edinburgh, King's Buildings, David Brewster Road, Edinburgh, EH9 3FJ, UK
* Correspondence: orlando@ufop.edu.br (O.S.);
‡ Equal contribution

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Abstract: *Candida albicans (C. albicans)* are the most common cause of urinary fungal infections. *C. albicans* biofilms are of increasing clinical importance due to their resistance to antifungal therapy. Since the use of medical devices causes most hospital infections, polymeric coatings that reduce microorganisms adhesion and biofilm formation are considered an attractive strategy. In this work, the ability and possible mechanisms of poly(methylmethacrylate-*co*-dimethylacrylamide) (PMMDMA) to inhibit *C. albicans* biofilms on medical devices have been studied. Scanning electron microscopy was used to evaluate fungal adhesion at various pH conditions, while the surface roughness of the coated and uncoated catheters was analyzed by atomic force microscopy. The surface charge was assessed, and the contact angle was determined to evaluate the surface hydrophobicity. PMMDMA coated catheters showed reduced binding of *C. albicans* at all pH values studied and presented a hydrophilic contact angle of $\Theta = 71°$. Negative zeta potential values of PMMDMA enhanced the reduction in *C. albicans* binding. AFM images demonstrated a smoother and homogeneous surface of PMMDMA-coated catheters. Coating with PMMDMA provided a smoother, more hydrophilic, and negative-charged surface, contributing to a substantial reduction of *C. albicans* binding.

Keywords: poly(methylmethacrylate-co-dimethylacrylamide); *Candida albicans*; coatings; biofilm inhibition; adhesion.

1. Introduction

Hospital-acquired infections, i.e., infections acquired by patients during hospital care [1], are a global public health issue. About 8% of hospitalized patients develop a hospital-acquired infection, 20% are caused by multidrug-resistant organisms [2]. They are described

as infections appearing during or after the care process that was not present or incubating at the time of the patient's admission to a hospital or other healthcare facility [3].

50% of the patients in intensive care units have infections, [2] and infections associated with biofilms are particularly difficult to treat. Urinary tract infections are the most common Hospital-acquired infections, and 80% of them are associated with medical devices, such as catheters [4, 5].

Biofilm formation starts with the attachment of microorganisms (typically bacteria or fungi) [2] to the surface influenced by acting Van der Waals forces and is affected by factors like charge, roughness, or hydrophobicity of the surface [6]. Upon attachment, the microorganisms multiply, adhere, and produce an extracellular matrix composed mostly of polysaccharides, nucleic acids, and proteins to form so-called microcolonies [7, 8]. Also, biofilms begin to form. In the fourth stage, more and more biofilms are formed, and three-dimensional structures containing shrouded cells are developed within several groups connected. In the last stage, the development of the biofilm structure results in the cell dispersion stage so that the cells are released from the biofilm, attach to new substrates and form new biofilms [9]. Biofilms offer a physical barrier of benefit to these microorganisms. They are linked to antimicrobial resistance, both due to limited diffusion of antimicrobials through this matrix and the low metabolic activity of the microorganism, thus, protecting the microbes from the drugs [10].

Fungal infections are the second most common hospital-associated urinary tract infections. Among them, infections of *Candida albicans* are the most common [11] and account for ~15% of all sepsis cases [12]. Candidiasis is the most common, multifactorial, and opportunistic yeast infection [13], and candidemia can arise with subsequently systemic invasive infections of tissues and organs [14]. In addition, *Candida* biofilms have huge clinical relevance because of their greater resistance to antifungal therapy and host immune defenses [15], where the resistance to antibiotics from fungal biofilms can be 1000 times higher than that of free microorganisms [16]. Also, Candida is an opportunistic pathogen, commensal and normally found in the oral cavity and found more frequently in younger children because the immune system in children is still in the development stage [17]. Hence, there is an urgent need to design medical devices that resist the formation of fungal biofilms. One of the strategies to achieve this is to use a biofilm-resisting polymer coating. Such coatings could either be designed to kill the microbes on contact or minimize the binding of the microbes due to their surface properties. Here, the latter strategy was explored.

Biofilms formed by *C. albicans* can be considered to happen over three developmental stages: the basal layer, containing founder cells that allow the adhesion of the developing biofilm to the substrate; the middle layer, which consists of hyphae and pseudohyphae; and the uppermost biofilm layer consisting of a thicker, porous layer of hyphae which produce abundant extracellular material [18-20].

The initial binding of the microorganism plays a key role in biofilm formation [21] in that reduced binding would inhibit the subsequent ability of the microbes to establish colonies and hence, a mature biofilm. This initial adhesion of the microbes to polymeric surfaces is a complex interplay between (a) the surface characteristics of the microorganism, (b) the nature of the medium components, and (c) physicochemical properties of polymer-coated surface such as roughness, hydrophobicity, and superficial charge [22]. Thus a smooth polymer surface could reduce the binding of microbes and hence the formation of biofilms. In contrast, the

charge carried by the polymer surface might promote or inhibit biofilms, depending on the surface charge of the microbes.

To overcome infections related to medical devices, medical devices are fabricated with materials that present intrinsic antimicrobial characteristics, highlighting the importance of catheters. This can be achieved by applying surface grafting with functionalized groups and surface coating by means of antimicrobial compounds [22, 23].

Here, we aim to use a simple, low-cost polymer that can be readily synthesized and scalable for commercial use. In this context, poly(methyl methacrylate-*co*-dimethyl acrylamide) (PMMDMA) (identified via high-throughput polymer microarray screening) has been shown to inhibit bacterial attachment onto catheter surfaces [10, 21]. Despite these encouraging results, the efficacy of PMMDMA against *C. albicans* had not been investigated, and here we study the ability of this simple but highly effective polymer to reduce *C. albicans* binding and biofilm formation as a means to fight fungal Hospital-acquired infections.

PMMDMA was synthesized, characterized, and coated onto urinary catheters and analyzed for surface roughness by atomic force microscopy (AFM) and superficial hydrophobicity determined using contact angle measurements. The inhibition potential of PMMDMA-coated catheters against *C. albicans* adhesion was assessed by scanning electron microscopy (SEM), and the superficial charge of *C. albicans* and the polymer surface was evaluated.

2. Materials and Methods

2.1. Materials.

PMMDMA was synthesized using methyl methacrylate (MMA) (Sigma Aldrich®), N,N-dimethylacrylamide (DMA) (Sigma Aldrich®), 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Tokyo Chemical Industry®), acetone (Impex®) and 365 nm illumination (Empalux®). Solvents for polymer characterization were stabilized tetrahydrofuran UV/HPLC (THF) (Vetec®) and chloroform-D (Sigma Aldrich®). Sabouraud broth was obtained with 5% meat peptone (microMED®), 5% casein peptone (microMED®), and 20% D-glucose (Neon®). Urinary catheter number 14 (Foley probe) was obtained from Solidor®.

2.2. Methods.

2.2.1. Polymer synthesis and characterization.

Monomers MMA (36 mmol) and DMA (4 mmol) (in proportion 9:1), the chain initiator 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone 0.100 mmol, and acetone (4 mL) were added to a glass vessel, and polymerization carried out for 3 h inside a UV light reactor (composed of 3 lamps Empalux®, 25W - 365 nm). The polymer was precipitated by dropwise addition into hexane, collected by filtration, solubilized in acetone, and precipitated again in hexane. This purification process was performed 3 times, and the polymer was dried in vacuo at 40 °C until complete removal of the solvent.

PMMDMA (Mn 48,000 g/mol, Mw 113,000 g/mol, PDI 2.3, and 75% yield) was characterized by gel permeation chromatography (GPC), infrared spectroscopy (FTIR), and nuclear magnetic resonance (^{1}H NMR). GPC was conducted on an Agilent GPC instrument, fitted with a PL gel 5 µm Mini MIX-D column (250 × 4,6 mm), with THF as eluent (flow rate 0.25 ml min^{-1}), pre-calibrated using polystyrene standards. IR analysis was conducted using an

ABB Bomem MB 3000 spectrometer with an ATR (attenuated total reflectance) device. ^{1}H NMR analysis was conducted using an Avance III HD NMR SPECT 400 One Bay spectrometer (Bruker®).

2.2.2. Coating of the urinary catheter.

Silicon latex urinary catheters were cut into cylindrical pieces approximately 4 mm long (measured using a digital caliper, Mitutoyo®) and coated by dipping into a 10% PMMDMA solution in acetone for 2 minutes. The pieces were dried for 30 minutes at 25 ºC. The process was repeated for the other side of the piece and dried overnight [22].

2.2.3. Analysis of fungal adhesion.

Uncoated and PMMDMA-coated catheter pieces were sterilized by UV light (20 min) and placed in 24-well culture plates. A suspension of *C. albicans* (ATCC 14408) was prepared in 0.9% saline to give a McFarland scale of 0.5 turbidities (1×10^8 CFU/mL). This fungal suspension was diluted in Sabouraud broths at pH 5, 7, or 9 to obtain a final concentration of 1×10^6 CFU/mL. 1 mL of these suspensions at specific pH was added to each well in triplicates and incubated with the catheters (coated and uncoated), incubated (37 °C, with shaking at 100 rpm for 48 hours). Following this, the catheter pieces were carefully washed with PBS (2 × 2 mL) to remove non-adherent microorganisms, transferred to wells of another culture plate containing 1 mL of 10% formaldehyde solution (30 minutes), washed with PBS (1 × 2 mL) and dried overnight at 25 °C. Coated and uncoated catheters were gold-coated in a metallizer (Quorum Tech® 150RES) for 90 seconds under vacuum (1×10^{-1} mbar) and analyzed by a field emission SEM (VEGA3 Tescan® 15 kV) [24].

2.2.4. Determination of PMMDMA and *C. albicans* surface charge.

The surface charge (zeta potential) of PMMDMA and *C. albicans* was determined in 0.9% saline at different pH (5, 7, and 9) using the Nano ZS Zetasizer (Malvern Instruments®). Through the use of a high-performance disperser at 15,000 rpm (IKA® T25 digital ULTRA-TURRAX®), fine particles of the dispersed polymer were obtained in saline solutions. Fungal suspensions were obtained by diluting C. albicans in saline solutions at different pH's to obtain 1×10^8 UFC/mL. Analysis was performed in triplicate using 1 mL of each sample.

2.2.5. Evaluation of superficial hydrophobicity of urinary catheter and PMMDMA.

Superficial hydrophobicity was determined by contact angle measurements. A piece of the urinary catheter was opened and glued to a flat glass surface in order to flatten the sample. To evaluate PMMDMA, a 10% solution of the polymer in acetone was used to coat a glass slide and dried overnight. A drop (10 µL) of deionized water was deposited onto each of the surfaces from a distance of 0.5 cm. Images of each of the evaluated samples were obtained, and the images were processed using *ImageJ*®.

2.2.6. Analysis of roughness of PMMDMA and urinary catheter material.

The roughness of the uncoated and PMMDMA coated urinary catheter pieces were analyzed by AFM (XE-7 SPM, Park Instruments®), using an intermittent contact mode and Si cantilevers, with resonant frequencies of 330 kHz and a spring constant of 42 Nm−1. All AFM

images were processed (profiling and 3D rendering) using the software package Gwyddion [25].

3. Results and Discussion

3.1. Polymer characterization.

FTIR spectrum (Figure 1) of the polymer showed that the sp^3 or sp^2 type C-H stretch around 3000 cm^{-1}, a band close to 1500 cm^{-1} equivalent to the folding of CH_3 or CH_2, a C-N or C-O stretch band close to 1140 cm^{-1} and a typical ester C=O stretch band around 1720 cm^{-1}. Typical amide CO-N stretch near 1640 cm^{-1} indicated that the N,N-dimethylacrylamide monomer was inserted into the chain [26].

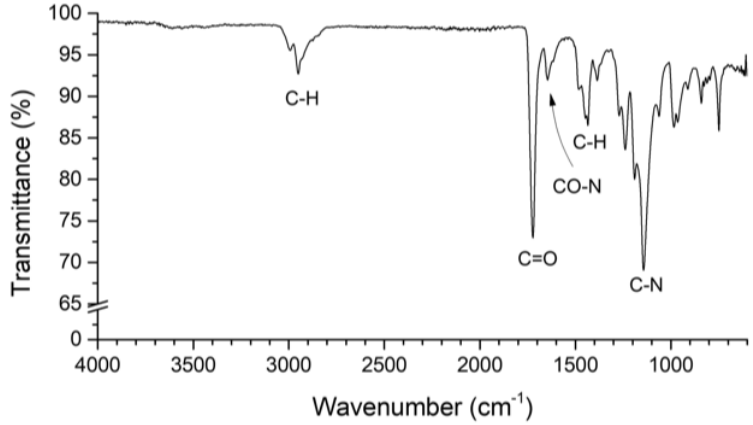

Figure 1. FTIR spectrum of PMMDMA.

^{1}H NMR analysis (Figure 2) gave chemical shifts consistent with the polymer, specifically at 3.6 ppm (3H, f) assigned to the methoxy protons and δ = 1.8 ppm (2H, c) attributed to the methylene group for the incorporated MMA unit [27, 28]. The polymer also showed signals related to the DMA unit, including at 2.8-3.1 (6H, e) corresponding to the methyl groups [29, 30]. The ratio of protons (f) and (e) integration values was approximately 27:6, indicating the presence of both MMA and DMA units in the polymer at a ratio of 9:1.

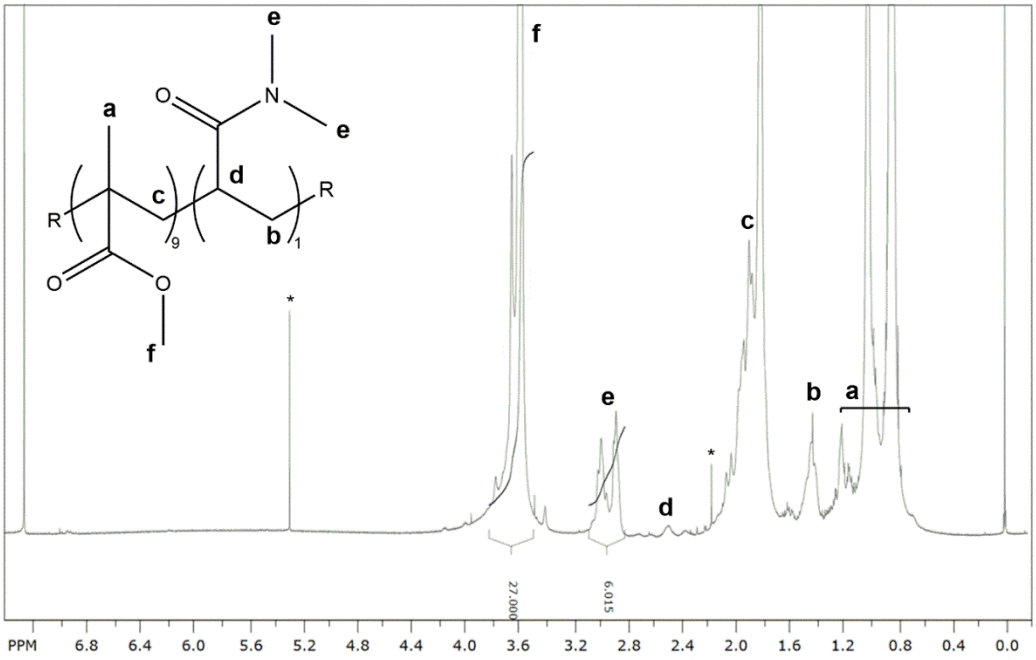

Figure 2. ^{1}H NMR spectrum of PMMDMA. The peaks marked with an asterisk (*) are impurities.

3.2. Analysis of C. albicans adhesion.

Previous studies have shown that the mature *C. albicans* biofilm has complex three-dimensional architecture and spatial heterogeneity, composed of a dense network of yeasts, hyphae, and pseudo-hyphae covered by an exopolymeric matrix [31, 32] with extracellular polymeric material on the surfaces of some of these morphologies [33]. In other work, using confocal microscopy, biofilms formed by *C. albicans* on silicone elastomer strips made from catheters were highly structured and composed of various cell types (budding round yeast cells, oval cells, pseudohyphae, and elongated hyphae cells) encased in the extracellular matrix [18]. Importantly is that chemical and structural differences in the surfaces are key factors in biofilm formation [34].

We made similar observations – thus, although the morphology of the cells varied with the pH of the medium, (i) acidic medium (Figure 3 (a) and (b)) showed rounded cells (yeast), hyphae, and extracellular material; (ii) the neutral medium (Figure 3 (e) and (f)) showed, hyphae covered by abundant extracellular material while (iii) basic medium (Figure 3 (i) and (j)) showed the presence of yeasts, hyphae, pseudohyphae, and extracellular material. In comparison, the PMMDMA-coated catheters showed a much-reduced binding of *C. albicans* at all three pH values studied. We also observed that the pH of the media influenced cell adhesion, with higher cell adhesion under acidic conditions (Figure 3 (c) and (d)). In contrast, few cells were attached under neutral (Figure 3 (g) and (h)) and basic conditions (Figure 3 (k) and (l)).

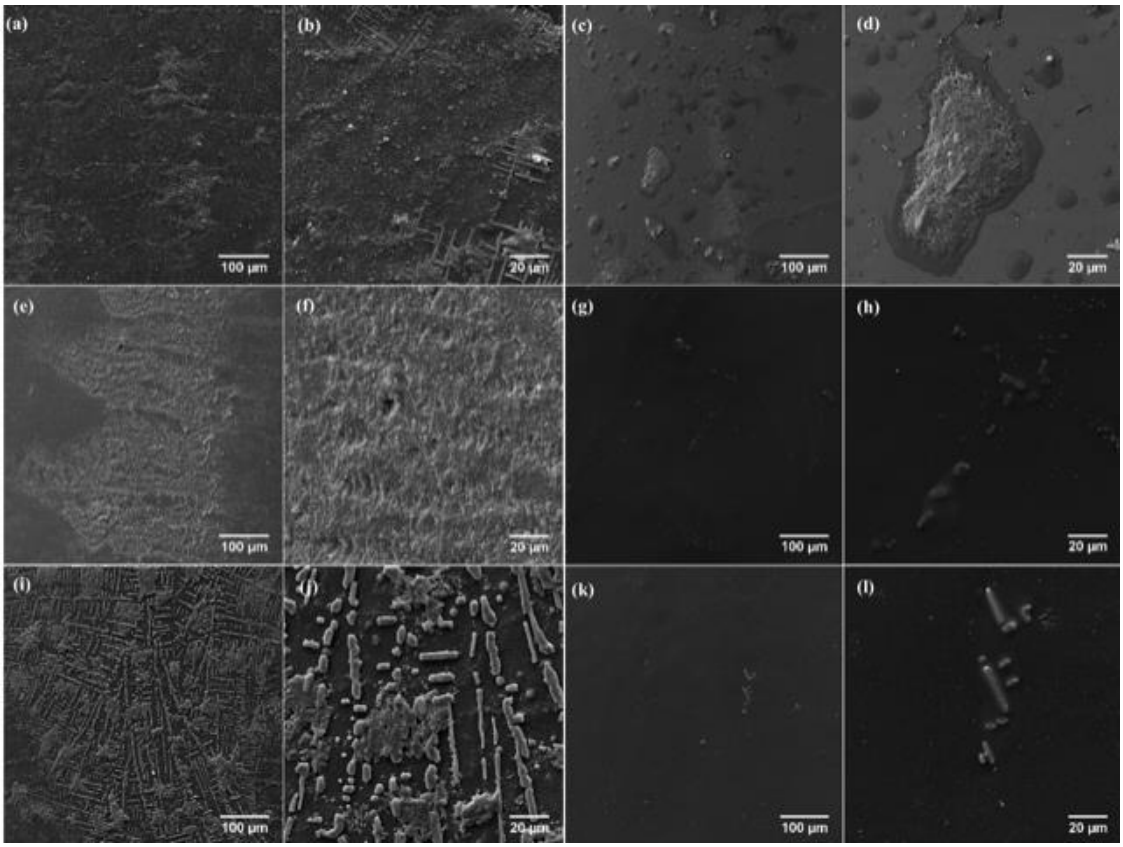

Figure 3. SEM images of uncoated and coated catheters with PMMDMA after incubation with *C. albicans* for 48h. ((**a**) and (**b**)) Uncoated catheter incubated in culture medium pH 5. ((**c**) and (**d**)) Coated catheter incubated in culture medium pH 5. ((**e**) and (**f**)) Uncoated catheter incubated in culture medium pH 7. ((**g**) and (**h**)) Coated catheter incubated in culture medium pH 7. ((**i**) and (**j**)) Uncoated catheter incubated in culture medium pH 9. ((**k**) and (**l**)) Coated catheter incubated in culture medium pH 9. Scale bar = 100 and 20 µm. Magnification from 500x and 2,000x, respectively.

A previous study demonstrated the effectiveness of coating surfaces with copolymer 2-methacrylyloxyethyl phosphorylcholine (MPC) in reducing the *C. albicans* binding when compared to uncoated surfaces. The authors attributed this reduction to the MPC's super hydrophilicity [35]. Other studies have also shown that modifying surfaces through polymeric coatings is considered a promising strategy to combat the formation of *C. albicans* biofilm, corroborating our study [36-39].

3.3. Determination of surface charge and hydrophobicity surface.

The surface properties of microorganisms and catheter materials, as well as environmental factors, may influence the adherence of *Candida* species [40, 41]. During the adhesion process, microorganisms must firmly adhere to the material surface through physicochemical interactions [42, 43].

In the process of initial microbial adhesion to solid surfaces, both repulsive hydrophobic (London-Van der Waals) and/or electrostatic (stronger) interactions are important [44, 45].

According to previous studies, microorganisms attach more easily to hydrophobic surfaces than hydrophilic ones [46, 47]. It has also been shown that in the adhesion of fungi to solid surfaces, both electrostatic and hydrophobic interactions are involved and depend on the physicochemical properties of the solid surfaces and the yeast [48].

Microbial cells usually have a negative surface charge that can be attributed to the cell wall and membrane components such as teichoic acids, charged lipopolysaccharides, and phospholipids [49]. *C. albicans* likewise have a formal negative surface charge. When interacting with negatively charged polymeric surfaces, this provides an electrostatic repulsion environment, making it difficult for the microorganism to adhere to these surfaces [46, 50]. It has been previously reported by *in vitro* experiments that some carboxylated PMMA derivatives inhibit *C. albicans* adhesion by means of the presence of a negatively charged surface[51, 52].

A widely used method for assessing surface wettability is determining the contact angle, which measures the angle between a water droplet and the solid surface substrate [53]. The measurements of contact angles (Figure 4) for uncoated and PMMDMA coated urinary catheters were $\Theta = 92°$ and $\Theta = 71°$, respectively, showing that the urinary catheter material was hydrophobic and PMMDMA made the surface more hydrophilic, a possible explanation for the low adherence of *C. albicans* to the catheter coated with PMMDMA.

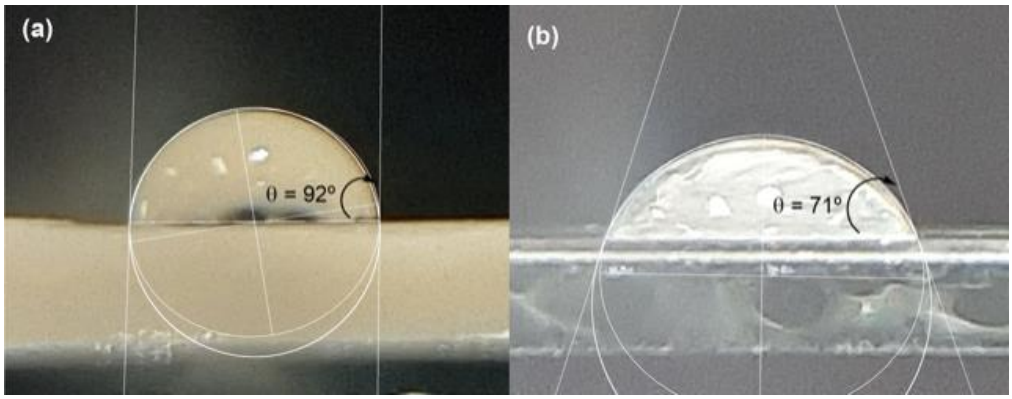

Figure 4. Determination of contact angle. Liquid drops in contact with a solid surface. The contact angle between a drop of deionized water (10 µL) and a solid surface of the urinary catheter (**a**) or PMMDMA coated glass slide (**b**). The images of the catheter and PMMDMA surface were processed with the *ImageJ*® software using the contact angle plugin.

We observed that the zeta potential (surface charge) values of the PMMDMA and *C. albicans* varied with the pH of the medium (Table 1). While the fungus had a negative surface charge, ranging from -3.58 ± 0.48 (pH 5) to -3.83 ± 0.05 mV (pH 7), PMMDMA had a negative surface charge in both neutral and basic media (-5.09 ± 0.32 and -9.66 ± 0.67 mV, respectively) and a positive surface charge (+8.00 ± 0.16) in acidic pH. This explains the higher adhesion of *C. albicans* in an acidic medium, with the stronger positive charge of the polymer enabling initial binding of the microorganism with a negative surface charge, while neutral and basic pH reduce binding due to the negative charge of the polymer under these conditions.

Table 1. Potential Zeta values of PMMDMA and *C. albicans* diluted in saline 0.9% (w/v) at pH 5, 7, and 9.

Samples	**Zeta potential (mV) ± SD**
PMMDMA pH 5	+8.00 ± 0.16
PMMDMA pH 7	-5.09 ± 0.32
PMMDMA pH 9	-9.66 ± 0.67
C. albicans pH 5	-3.58 ± 0.48
C. albicans pH 7	-3.83 ± 0.05
C. albicans pH 9	-3.59 ± 0.50

SD: Standard deviation.

The zeta potential of poly(methyl methacrylate) (PMMA) surface remains negative over a wide range of ionic strength, pH, temperature, and the nature of aqueous electrolyte co- and counterions [54]. Therefore the amide function may influence the zeta potential of PMMDMA at different pH. A similar result was found in a study with pH-sensitive copolymeric micelles. A decrease in pH of the medium from 7.4 to 5 promoted an increase in the zeta potential of the particles from -2.33 mV to +4.07 mV. The authors suggested that an increased positive charge occurs from the protonation of tertiary amides along the polymeric chains, which are organized on the micellar surface, even in a weakly acidic medium [55]. In another study, it was reported that protonation of tertiary amides at pH 5 increased the zeta potential of functionalized graphenes to +40 mV [56].

We could not determine the zeta potential of the catheter material, as it was impossible to crush the catheters into small particles. Also, milling and solubilization (and re-precipitation) did not produce reproducible values of its zeta potential.

3.4. Roughness analysis.

The surface of a biomaterial (roughness) is a relevant property for *Candida* adhesion since the polymeric surfaces' irregularities usually promote Candida attachment and biofilm accumulation [43, 46, 57, 58].

Previous studies showed that biofilm formation in latex urinary catheters was approximately 1.5 times greater than the amount of biofilm formed on a silicone-based urinary catheter. This was because the surface of the latex catheter was more porous (rough), which facilitated the binding of microorganisms [34]. Compared to highly polished smooth surfaces, a greater adherence of C. albicans to rougher surfaces has also been demonstrated. Microorganism retention occurs readily on rough surfaces as they provide a large area for attachment and a more protective environment [59].

AFM images in our study showed similar results (Figure 5). The morphology of the surface showed cracks in the urinary catheter sample (Figure 5 (a)) and a more homogeneous surface on the PMMDMA-coated catheter samples (Figure 5 (b)). Roughness measurements indicated considerable differences. While Rq was 92.5 nm for catheter samples, the Rq values

for the PMMDMA coated catheter samples were 3.2 nm (Rq is the root-mean-squared roughness, and the values are given for the standard deviation of the height value in the selected region).

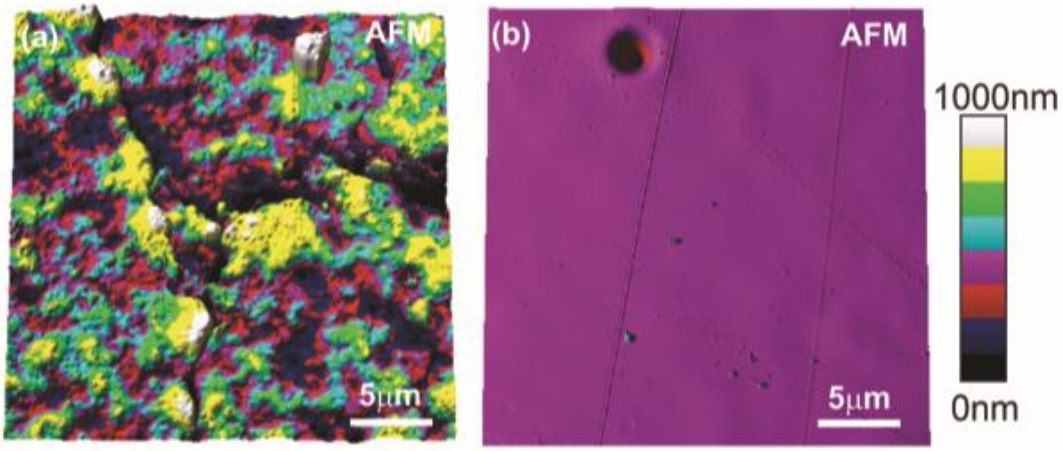

Figure 5. AFM - Topography image of catheter sample. (**a**) uncoated and (**b**) coated with PMMDMA polymer.

4. Conclusions

This study demonstrates the development of a promising strategy to combat hospital-acquired infections and *C. albicans* associated-biofilms on medical devices using a polymer, PMMDMA. The PMMDMA polymer was successfully synthesized and characterized and then used to coat the surface of urinary catheters. We demonstrated that the reduced binding of *C. albicans* to PMMDMA polymer is related to its physicochemical surface characteristics (negatively charged, hydrophilic, and smooth/uniform surface) offered by the polymer at physiological pH. Thus, PMMDMA polymer is a promising alternative for coating medical devices in combating hospital-acquired infection and reducing morbidity and mortality rates.

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Conflicts of Interest

The authors report no conflict of interest.

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