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Antifungal coatings by caspofungin immobilization onto biomaterials surfaces via a plasma polymer interlayer

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Not only bacteria but also fungal pathogens, particularly *Candida* species, can lead to biofilm infections on biomedical devices. By covalent grafting of the antifungal drug caspofungin, which targets the fungal cell wall, onto solid biomaterials, a surface layer can be created that might be able to provide long-term protection against fungal biofilm formation. Plasma polymerization of propionaldehyde (propanal) was used to deposit a thin (~20 nm) interfacial bonding layer bearing aldehyde surface groups that can react with amine groups of caspofungin to form covalent interfacial bonds for immobilization. Surface analyses by x-ray photoelectron spectroscopy and time-of-flight secondary ion mass spectrometry confirmed the intended grafting and uniformity of the coatings, and durability upon extended washing. Testing for fungal cell attachment and ensuing biofilm formation showed that caspofungin retained activity when covalently bound onto surfaces, disrupting colonizing *Candida* cells. Mammalian cytotoxicity studies using human primary fibroblasts indicated that the caspofungin-grafted surfaces were selective in eliminating fungal cells while allowing attachment and spreading of mammalian cells. These *in vitro* data suggest promise for use as antifungal coatings, for example, on catheters, and the use of a plasma polymer interlayer enables facile transfer of the coating method onto a wide variety of biomaterials and biomedical devices. © *2015 American Vacuum Society*. [http://dx.doi.org/10.1116/1.4933108]

I. INTRODUCTION

The clinical problems arising from bacterial colonization of biomedical devices and ensuing infections have long been well documented and have given rise to a considerable body of research on antibacterial coatings.[1] More recently, it has also become evident that fungal pathogens can also form infectious biofilms, either by themselves or in coexistence with bacteria.[2–5] Fungal infections can be life-threatening for immunocompromized patients and, in some cases, may have higher mortality rates than bacterial infections.[6] Invasive biomedical devices such as catheters, lines, drips, and implants are a major risk factor for fungal infections, as they are associated with nonsterile sites providing environments for colonization by opportunistic and pathogenic bacteria and fungi to form biofilms.[7] The most common hospital acquired infections are urinary tract infections, ventilator associated pneumonia, and bloodstream infections,[8] all of which can lead to septic shock and death, as well as to the formation of biofilms on medical devices; once formed, biofilms are much more difficult to eradicate. The impact of fungal *Candida* species in infections has been underappreciated although they cause over 400 000 life-threatening infections globally per year and are the third most common cause of catheter-associated bloodstream infections, with a mortality reaching 45%.[9,10]

The most frequently encountered pathogen in this genus, *Candida albicans*, is a fungal organism that normally colonizes mucosal tissues, forming part of the microflora in an opportunistic manner. Its population is normally controlled by the mucosal microenvironment, the bacterial flora, and through a healthy innate immune system.[11] It can colonize humans from birth and persist as a lifelong commensal in the gastrointestinal tract. It is dimorphic in that it can undergo a reversible transition between yeast and hyphae growth forms; invasive infection usually occurs in the hyphal form. When fungal cells infect the blood stream (referred to as candidemia), and when hyphae spread to internal organs, it can become a deep-seated, invasive infection with high mortality rates.

Evidently, *C. albicans* can also spread to and colonize the surfaces of biomedical devices to form sessile biofilms. Their treatment can be challenging and seriously affects patients already weakened by disease or trauma. Thus, there is a need for surface treatments or thin coatings that can be applied onto existing medical devices so as to make the surface unsuitable for attachment and biofilm formation by *Candida* species. Such antifungal surfaces must also meet criteria such as compatibility with human cells and tissue; antiseptic coatings can stop microbial attachment, but they are cytotoxic.[12] Given the biological similarities in fungal and human cells (both eukaryotic), selective antifungal strategies are required. Approaches fall into two classes: release or covalent surface grafting of antifungal compounds.[13] Release approaches are inherently of limited duration of effectiveness, and as drug molecules diffuse away or move with secretions, they may not reach the minimum inhibitory concentration.[14] Accordingly, we prefer the approach of equipping biomaterials with a covalently grafted layer of antifungal molecules, which might provide long-lasting

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protection against colonization and prevents accumulation of released drug molecules in organs. However, this approach would seem unsuitable for drugs that have intracellular targets, even with the use of spacer linkers, and we thus focus on the grafting of drugs that have known targets in the cell envelope. Even then, a drug might become inactive when grafted covalently onto solid biomaterials surfaces due to steric hindrance or conformational effects. In addition, one must be careful to consider and eliminate effects arising from physisorbed molecules diffusing into the medium and killing fungal cells in solution during testing, before concluding that a grafted layer indeed exhibits activity.

For this study, we have selected the antifungal compound caspofungin (Fig. 1), a member of the echinocandin class of lipopeptides. Its fatty acid side chain is thought to intercalate with the phospholipid bilayer of the cell membrane, and caspofungin then acts by noncompetitive inhibition of the synthetic cell wall enzyme complex β-1,3-D-glucan synthase. This prevents the synthesis of β-1,3 glucan, an essential component of the fungal cell wall, and causes osmotic lysis of the fungal cells. Effects are both fungistatic and fungicidal.[15,16]

Most biomaterials and medical devices, however, do not possess chemical groups on their surfaces capable of covalent interfacial immobilization of caspofungin. Accordingly, we first apply a thin interfacial bonding layer, being a plasma polymer deposited from the process vapor propionaldehyde (propanal); this polymeric interlayer has been found to be useful for immobilizing a variety of bioactive molecules bearing primary amine groups.[17,18] Distinct advantages of this approach also comprise that plasma polymers adhere well to most substrate materials, exhibit excellent uniformity and process reproducibility, and the coating technology can readily be transferred to a wide range of potential products. Here, we report the method of immobilization of caspofungin and data showing effectiveness of this coating against colonization by *C. albicans* as well as cytocompatibility with fibroblast cells.

II. EXPERIMENT

A. Samples

Caspofungin (Sigma-Aldrich) was covalently immobilized onto intermediate coatings approximately 18–20 nm thick, as

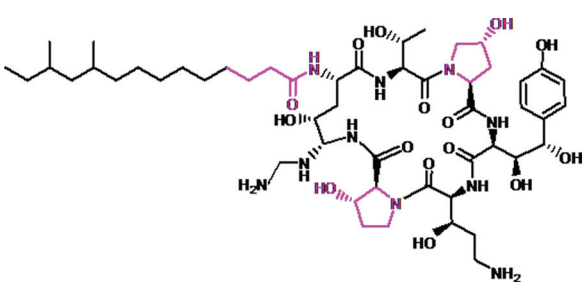

FIG. 1. Chemical structure of caspofungin; MW 1093.31 $C_{52}H_{88}N_{10}O_{15}$. Highlighted fragments with m/z = 86 (shown in pink) were useful for confirming the surface attachment (see main text for details).

determined by ellipsometry, prepared by the plasma polymerization (pp) of propionaldehyde (ALD, 99%, Aldrich) using a plasma deposition system described previously.[19] The initial reaction between primary amine groups of caspofungin and aldehyde groups of the ALD-pp leads to interfacial imine bonds, which were reduced to stable amine bonds using $NaBH_3CN$; this has been documented previously to lead to stable covalent attachment.[18] Substrates used included silicon wafers (Micro Materials and Research Consumables, Australia), Thermanox cover slips (ProSciTech, Australia), and IBIDI 15 μ-slide 12-well uncoated microscopy chambers (Cat. no. 81201, IBIDI, Australia). All work was undertaken in a clean biohazard cupboard, except plasma polymerization. Caspofungin was prepared as a 0.1 mg/ml solution in phosphate-buffered saline. For grafting, 100 μl of this solution was added per sample to cover the surface, and 100 μl of 1 mg/ml $NaBH_3CN$ added after 1–2 h contact. Samples were incubated overnight at 18 °C. Before fungal testing, samples were either soaked and rinsed a minimum of 12 times over 2 h each, with Milli-Q water, or soaked for 2 weeks and rinsed before testing. The final rinse was collected and tested against fungi to prove that no antifungal compounds were eluting and prevention of fungal attachment was indeed from a covalently attached caspofungin surface layer only.

B. Surface analysis

Chemical compositions of coatings and their uniformity across different positions on the surface were analyzed by x-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (ToF-SIMS). XPS was performed with a Kratos AXIS Ultra DLD spectrometer, using monochromatic AlKα radiation ($h\nu = 1486.7$ eV) and a magnetically confined charge compensation system. Spectra were recorded using an acceleration voltage of 15 keV at a power of 225 W. Survey spectra were collected with a pass energy of 160 eV and an analysis area of 300×700 μm. High-resolution spectra were obtained using a 20 eV pass energy and an analysis area of $\sim 300 \times 700$ μm. Data analysis was performed with CASAXPS software (Casa Software, Ltd.). All binding energies were referenced by charge correction to the "neutral" C 1s peak at 285.0 eV. Core level envelopes were curve-fitted with the minimum number of mixed Gaussian–Lorentzian component profiles. The Gaussian–Lorentzian mixing ratio (typically 30% Lorentzian and 70% Gaussian functions); the full width at half maximum, and the positions and intensities of peaks were left unconstrained to result in a best fit but outcomes were also checked by constrained fitting.

ToF-SIMS measurements were performed with a PHI TRIFT V nanoTOF instrument (PHI Electronics, Ltd., USA). A 30 keV, pulsed primary Au+ ion beam was used to sputter and ionize species from each sample surface. The instrument's dual beam charge neutralization system using a combination of low energy argon ions (up to 10 eV) and electrons (up to 25 eV) was employed to provide excellent charge neutralization performance. Positive mass axis

calibration was done with the ions CH_3^+, $C_2H_5^+$, and $C_3H_7^+$. Spectra were acquired in the bunched mode for 60 s from an area of $100 \times 100\,\mu$m. The corresponding total primary ion dose was $<1 \times 10^{12}$ ions cm^{-2}, and thus met the static SIMS regime.[20] A mass resolution m/Δm of >7000 at nominal m/z = 27 amu ($C_2H_3^+$) was typically achieved.

Some samples were further characterized by collecting multiple positive ion mass spectra from at least eight distinct areas that did not overlap on a given sample surface, and working them up by principal component analysis (PCA). All recognizable, clear (i.e., unobscured by overlap) fragment ions from 2 to 175 amu were used in PCA calculations. Peaks were normalized to the total intensity of all selected peaks and the multiple spectra processed using previously reported procedures and checks,[21] using PLS_TOOLBOX version 3.0 (Eigenvector Research, Inc., Manson, WA) along with MATLAB software v. 6.5 (MathWorks Inc., Natick, MA).

C. Fungal testing

C. albicans (ATCC 90028) was plated on SAB + ANTI (Oxoid) medium from −80 °C frozen stock and incubated overnight at 37 °C, then one colony was picked from the plate and spread on fresh medium and incubated at 35 °C overnight as a stock plate. The other colonies were collected and frozen for future experiments. One colony was selected from the stock plate and grown in 10 ml YPD Broth (20 g glucose, 20 g peptone, 10 g yeast in 500 ml RO water, Oxoid)[22] overnight at 35 °C. The following day this was measured for optical density and made up to 0.5 McFarland using a Vitek colorimeter, then diluted 1/100 to give approximately 1×10^6 cfu/ml. Depending on the sample sizes, *Candida* solutions were added at 100 or 300 μl (thus providing fungal numbers of $\sim 1 \times 10^5$ or 3×10^5) and incubated for 3 days at 35 °C, followed by rinsing twice to remove loosely attached and yeast-form fungi. Surfaces were analyzed using LIVE/DEAD BacLight Bacterial Viability assays kits (Invitrogen), which is the same as the fungus live/dead imaging kit, and also using the FUN1 kit (Invitrogen).

D. Mammalian cell toxicity studies

Primary human fibroblasts were harvested from fresh skin and maintained according to published protocols using Dulbecco's Modified Eagle's Medium and 10% fetal bovine serum.[23] Thermanox cover slips without a coating (as a control) and with coatings were placed in sterile 24-well culture plates (NUNC, Invitrogen) and prewarmed in a cell culture oven. Cell solutions were made up to 10 000 cells/ml in medium/serum and added till 1 ml to each well or 100 μl to IBIDI wells. Well plates were placed into a culture oven and left for 48 h. Cover slip samples were rinsed to remove nonadherent cells and placed on a glass slide and covered with a cover slip for immediate microscopic analysis. Cells in IBIDI wells were stained using 4′,6-diamidino-2-phenylindole nucleus stain (Invitrogen) used as per instructions.

III. RESULTS

Prior to biological testing, it was necessary to confirm the presence and uniform coverage of surface-grafted caspofungin molecules by surface analytical techniques, so that reliable interpretation of observed biological responses can be assured, free from possible artefacts such as adventitious surface contaminants and coating nonuniformity.

A. XPS analysis

XPS is well suited to detect the presence of surface-grafted caspofungin, since this compound contains amine and amide groups, whereas the substrates used and the ALD-pp interlayer do not contain N. The atomic percentage of N could in principle also be used to estimate the surface coverage of caspofungin via an overlayer model. Table I shows representative XPS data with the elemental surface composition for the ALD-pp control coating and after grafting of caspofungin. The composition of the ALD-pp surface was similar to previous work.[24] After exposure to caspofungin and reducing agent and extensive washing, the observed composition of the caspofungin surface showed the presence of N and a higher percentage of O compared to the control surface, which accords with expectations based on the chemical structure of the compound. The relative abundance of N is lower than the theoretical percentage for pure caspofungin, which indicates that the grafted layer is thinner than 10 nm. This is entirely reasonable for a (vacuum-dried) monolayer of a molecule of this size. The observed %N suggests that the surface coverage is likely to be close to monolayer, though detailed overlayer calculations are not warranted given that we cannot ascertain the orientation/conformation of grafted caspofungin.

High-resolution XPS N 1s spectra (not shown) recorded with the caspofungin-grafted surface show both amine N (at 399.3 eV) and amide N (400.0 eV), in accord with expectations based on the molecular structure of caspofungin.

XPS analysis was repeated after soaking caspofungin-grafted samples in water for 2 weeks; elemental compositions (not shown) were identical within experimental uncertainty (estimated at ±5% for N at this atomic ratio), thus confirming that the initial rinsing had removed any physisorbed caspofungin that might have been present initially. Hence, these data suggest that the extensively rinsed samples subjected to biological tests contained only covalently grafted caspofungin with no measurable amounts that could desorb and affect fungal cells in solution.

TABLE I. Compositional analysis by XPS.

	Composition (at. %)		
Sample	O	N	C
ALDpp	12.5	0	87.5
ALDpp-caspofungin	13.2	2.6	84.3

B. ToF-SIMS analysis

While XPS analysis provides quantitative data on elemental compositions, it has limited chemical information content, and it is desirable to complement XPS data with a chemically more informative analysis technique, so as to check the expected surface chemistry. ToF-SIMS is well suited due to its ability to reveal molecular fragments and hence confirm specific molecular structures, though it is not a quantitative method. Figure 2 shows positive ion ToF-SIMS spectra recorded on ALD-pp and ALD-pp + caspofungin samples. While in the lower mass region the peaks were rather similar, corresponding to fragment ions that can be ejected both from the ALD-pp and from caspofungin, there are signals from higher mass fragment ions that are more informative. The most pronounced of these was a signal at m/z = 86. A high resolution, narrow-range scan (not shown), coupled with this instrument's resolution of Δm/m of 1/11 000, established its identity as $C_4H_8NO^+$, a fragment ion that can readily be assigned to originating from at least three parts of the molecular structure of caspofungin, as shown with pink highlighting in Fig. 1. Such signals are useful in providing confidence that the analyte surface is indeed the intended grafted caspofungin layer, as opposed to possible adventitious contaminants.

ToF-SIMS can be used to probe for uniformity of coating coverage by recording spectra on multiple independent areas of a sample and performing PCA.[21] This is important in work on antimicrobial coatings because coating defects could give rise to localized attachment of microbes followed by nucleation of biofilm clumps. Eight sets of spectra each were collected from distinct areas of two samples and subjected to PCA analysis. The clear separation of the two data clusters along the PC1 axis [Fig. 3(a)] is confirmation of clearly distinct surface chemical compositions, and the tight clustering of the individual data points within the two sets indicates a high degree of uniformity of the surface compositions of both samples.

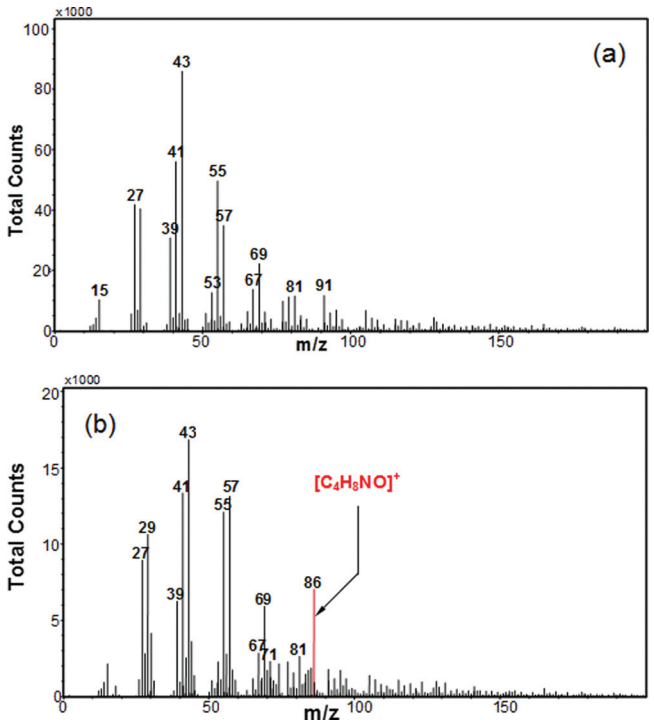

FIG. 2. Positive ion ToF-SIMS spectra recorded on (a) control ALD-pp sample and (b) caspofungin grafted sample.

The loadings plot of positive ions [Fig. 3(b)] was examined to determine which ion signals provided the most significant contributions to compositional differences between the two samples. With a positive score on PC1 for the caspofungin-grafted sample, the fragment ions that load positively are those that are relatively more intense in the spectra from the grafted surface. The most pronounced differences are observed for ions that contain N, for example, $C_3H_6NO^+$ and $C_4H_8NO^+$, which is consistent with the putative presence of caspofungin. We also note that several $C_xH_yO^+$ ions load negatively, consistent with expectation that their intensity would be greater from the ALD-pp surface.

Thus, the ToF-SIMS data indicate effective and uniform grafting of caspofungin onto the ALD-pp surface. The peaks identified in the loadings plot can all be assigned to fragments within the molecular structure of caspofungin; there are no peaks, suggesting the presence of surface contamination.

C. Fungal colonization

Prior to assessing ALD-pp and ALD-pp + caspofungin coatings for colonization by *C. albicans*, the viability of assessment of colonization of surfaces via live/dead staining was ascertained using a standard tissue culture polystyrene

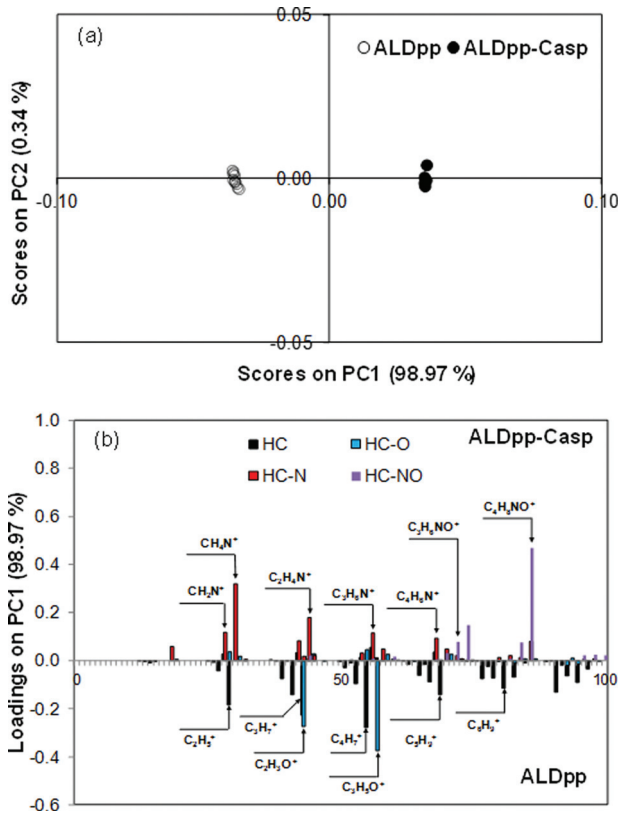

FIG. 3. PCA Scores plot on PC1 and PC2 (a), and loadings on PC1 (b), of positive ion mass spectra recorded on ALDpp and ALDpp-caspofungin (ALDpp-Casp) surfaces.

(TCPS) surface. After 48 h, visual assessment showed partial surface coverage by a fungal biofilm. For metabolically active yeast cells, the dye kit stains cells green, as shown in Fig. 4(a). On the other hand, cells that are dead or in the process of dying are indicated by an intermediate orange color. This was shown when the cells were exposed to Virkon for 10 min [Fig. 4(b)]. Virkon is composed of oxone, sodium dodecylbenzenesulfonate, and sulfamic acid and is a broad spectrum antiseptic solution.

On the aldehyde plasma polymer surface also, *C. albicans* managed to attach and form colonies, though the rate of formation of biofilm was reduced compared with the TCPS surface (Fig. 5).

In contrast, over the same time period of 48 h, caspofungin-grafted samples displayed excellent prevention of biofilm formation (Fig. 6). While some fungal cells managed to attach (to an extent resistant to removal by gentle rinsing), they then were killed, as evident by the staining, and the killing must have been sufficiently fast to prevent any initiation of biofilm formation. Higher resolution also shows shapes indicative of cells with damaged membranes, and there were no intact yeast cells or hyphae found on caspofungin-grafted surfaces. It should be noted that there is some green color

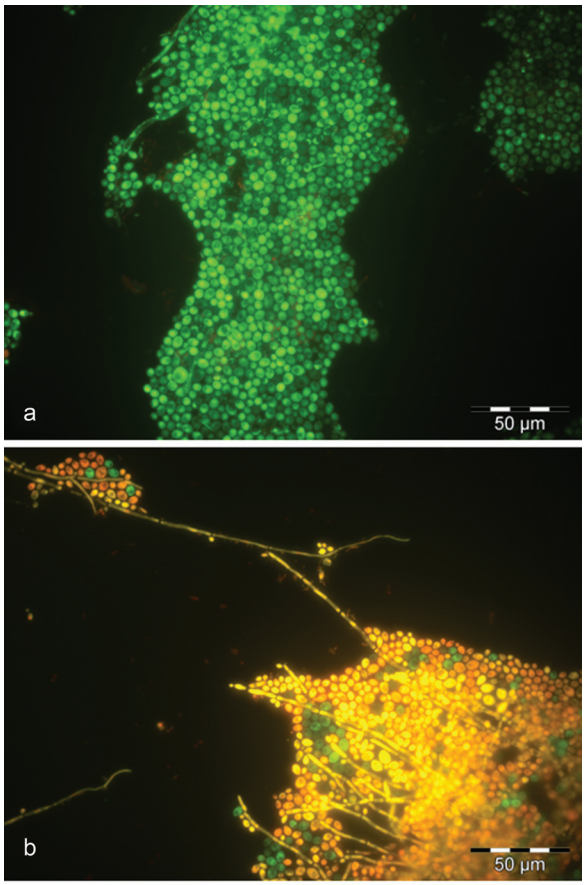

FIG. 4. Live/dead staining of *C. albicans* colonies grown for 48 h on TCPS: (a) positive control with mostly live fungal cells (stained green), and (b) after treatment with Virkon antiseptic for 10 min (dead/dying cells stained yellow–orange; some live cells are also visible). Rounded yeast cells are evident with also some elongated hyphal structures.

[Fig. 6(a)], but not in the shape of live cells; rather, there is some absorption of the green dye into the ALD-pp coating. This diffuse background color is easily distinguished by visual observation but unfortunately interferes with measurement of optical absorbance; accordingly, we prefer to present these images rather than absorbance data with problematic background correction. For improved visualization of dead fungal cells against the diffuse green background, a filter eliminating the green fluorescence can be utilized [Fig. 6(b)]. Figure 6(c) shows an enlarged image of dead fungal cells that are misshapen and appear to be leaking. In the 100× image [Fig. 6(d)], again only dead fungi were observed, with apparent leaking and broken hyphae. This appearance is consistent with substantial damage to the cell wall and membrane, as one would expect when caspofungin inhibits the synthesis of a crucial cell wall component.

While XPS data indicated that caspofungin was stably attached onto the ALD-pp interlayer, biological testing was also performed after extended (2 weeks) soaking in Milli-Q water. As for fresh (but well-rinsed) samples, there was a clear difference in performance between the ALD-pp and the ALD-pp + caspofungin samples (Fig. 7). As for fresh (but well-rinsed) samples, there was a clear difference in performance between the ALD-pp and the ALD-pp + caspofungin samples (Fig. 7), but for both types of surfaces the performance did not change upon such extended washing. Caspofungin has a high solubility in water and hence any physisorbed molecules should be removed after 2 weeks of soaking. Thus, we conclude that caspofungin was covalently grafted and thus able to maintain activity after extended contact with water, amounting to a long-lasting antifungal coating. While the aldehyde control surface showed multiple yeast cells and some pseudohyphae [Fig. 7(a)], the caspofungin surface treatment showed only a few attached cells [Fig. 7(b)], which may be a consequence of some small coating defects due to the unavoidable presence of some dust particles during coating. Those few cells, however, seemed unable to spread and form colonies, presumably due to the inhospitable caspofungin surface around them. Combined with the observation of no antifungal activity of the soaking solution, we can exclude a possible contribution by leaching caspofungin. Thus the data indicate that the ALD-pp + caspofungin coating acts by a surface-contact-killing mechanism and not one that requires release of the compound. These experiments demonstrate a good qualitative overview of the fungal cell killing and prevention of biofilm formation on surfaces and provide a basis for future investigations. Subsequent studies will quantify the antifungal surface effect by comparing the number of colony-forming units that remain viable or are killed after exposure to control and caspofungin-treated surfaces, respectively. Such quantitative studies will be important for statistically evaluating the data.

D. Cytotoxicity assessment

Aldehyde plasma polymer "control" surfaces and caspofungin-grafted surfaces were examined for possible toxicity to primary human fibroblasts, harvested from human

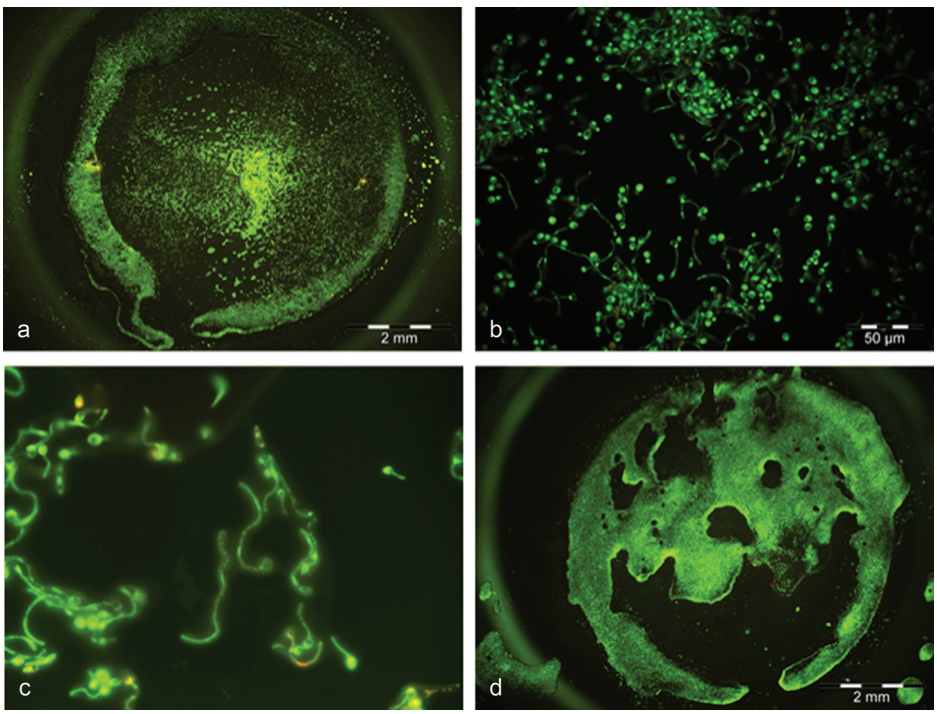

FIG. 5. *C. albicans* biofilm formation on ALD-pp after 48 h: (a) entire sample inside well (24-well plate), (b) 60× image, (c) 100× image, and (d) on TCPS surface, entire sample imaged, showing more extensive but patchy biofilm formation.

skin. Figures 8(a) and 8(b) show microscopy images of cultured cells on these two surfaces.

On both types of samples, fibroblast cells were able to attach and grow to similar extent, and there were no observable differences in cell morphology examined up to 3 days of growth. Figure 9 shows cell counting data showing no significant difference in the number of cells observed in the microscopy images.

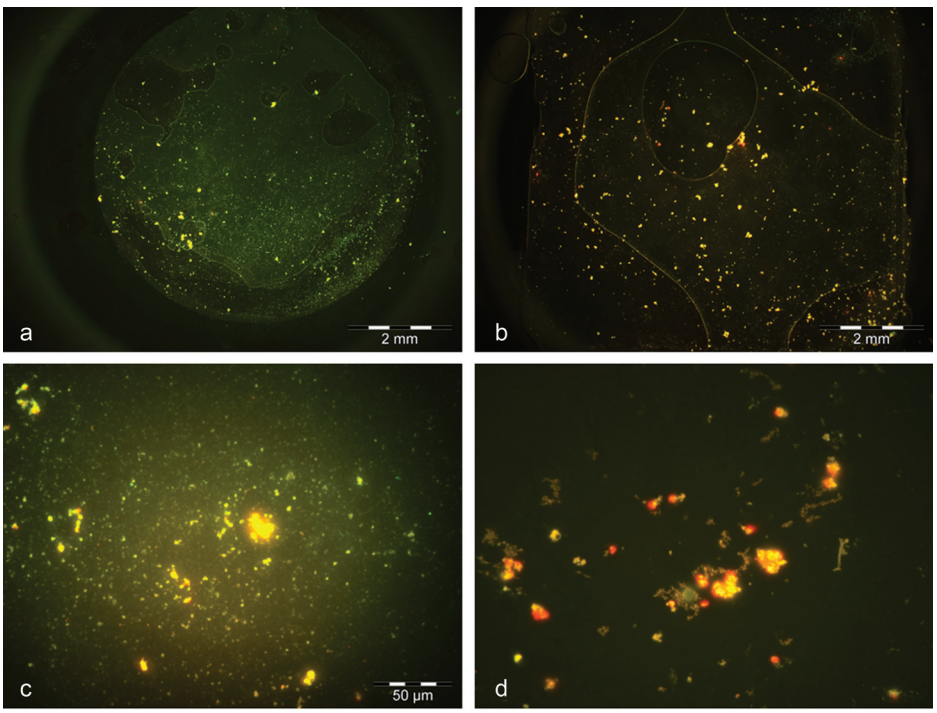

FIG. 6. *C. albicans* on caspofungin-grafted surfaces after 48 h incubation: (a) full view of sample; (b) same with filtering of green background, (c) 60× image showing dead yeast cells, and (d) 100× image showing changes in yeast and hyphal morphology.

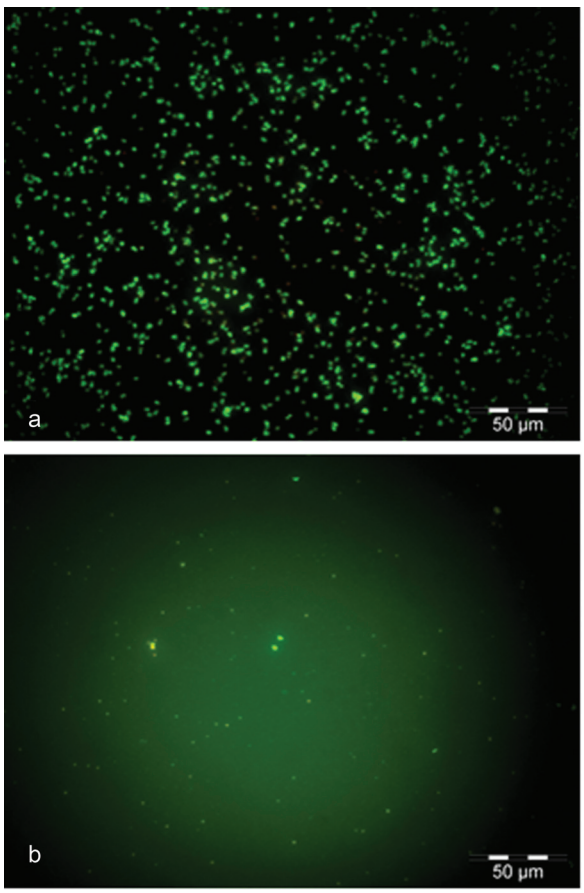

FIG. 7. *C. albicans* on (a) aldehyde plasma polymer surface, and (b) caspofungin-grafted surface, both soaked for 2 weeks prior to testing.

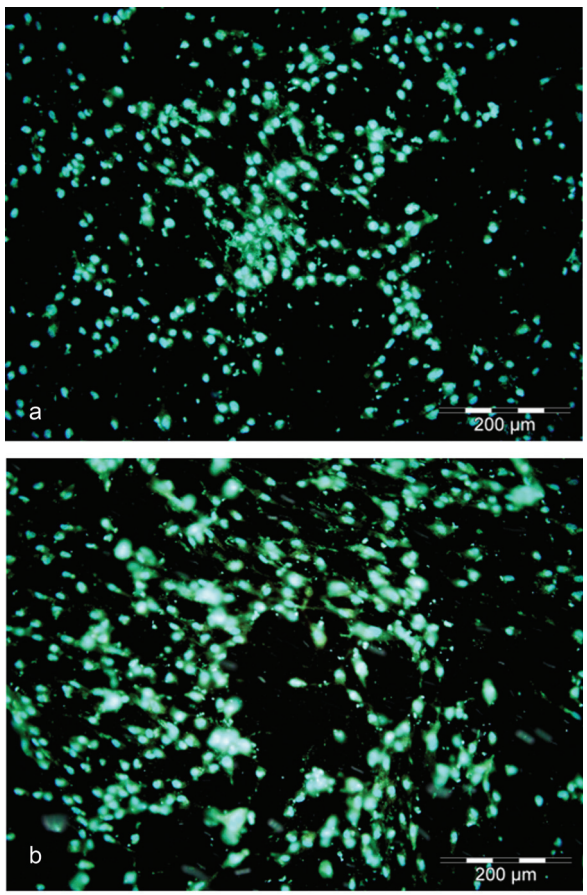

FIG. 8. Attachment and growth for 3 d of primary fibroblasts on (a) ALD-pp surface, and (b) on caspofungin-grafted surface.

IV. DISCUSSION

Surface analysis data indicate that covalent grafting of caspofungin onto the aldehyde plasma polymer interlayer leads to a good grafting yield and uniform surface coverage, and the biological tests show selective changes in activity upon grafting of this antifungal compound. The aldehyde plasma polymer by itself supports the attachment and growth of *C. albicans* and primary human fibroblasts, to an extent similar to such colonization observed on a range of other polymeric surfaces. Low molecular weight aldehyde compounds leaching from the aldehyde plasma polymer could be toxic to both fungal and mammalian cells, but do not seem to be present in any significant quantities. Besides, samples are routinely rinsed. Upon covalent immobilization of caspofungin, however, the biological performance changes markedly; while the colonization of the coating by fibroblasts is unchanged, biofilm formation by *C. albicans* is inhibited completely. Microscopy images show that some *C. albicans* cells manage to attach but are then killed before they can initiate biofilm formation. Testing after extended soaking in water suggests that the observed effects are not attributable to physisorbed, leaching drug molecules. Hence, we conclude that our data support the hypothesis that irreversibly surface-immobilized caspofungin retains antifungal activity.

Based on traditional solution studies, the putative antifungal mechanism of caspofungin is to disrupt β-1,3-D-glucan synthase, an important enzyme in the production of the protective cell wall. The *C. albicans* strain chosen for this work was determined to be susceptible to caspofungin with a solution minimum inhibitory concentration (MIC) in the range of 0.125 mg/l in accordance with the observation of others.[25] When attaching onto solid surfaces, *C. albicans* undergoes

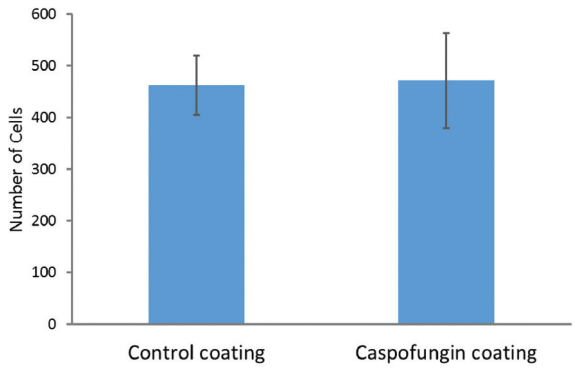

FIG. 9. Primary human fibroblast cells per view area; three independent replicates each. Control surfaces were coated with aldehyde plasma polymer. Caspofungin surfaces were prepared by binding 1.0 mg/ml caspofungin onto aldehyde plasma polymer surfaces as described in the methods.

morphology changes including budding, and transitioning to hyphae. Therefore, it is difficult to compare MIC values to an expected surface density of immobilized caspofungin molecules because of the different ways that yeasts would be exposed to either freely circulating or immobilized caspofungin. Additionally, hyphal development is associated with the virulence of this organism and would also seem important in the first stages of biofilm formation on surfaces. One possible mechanism by which surface-attached caspofungin could cause a fungicidal effect is that the antifungal agent is able to disrupt the integrity of the cell wall during these morphological changes. When attempting to either replicate or spread on the surface through hyphal formation, the increased reliance on cell wall remodeling and therefore, the production, reorganization and dismemberment of glucans in the cell wall requires upregulation of these enzymatic pathways. Therefore, we would expect an increased reliance on β-1,3-D-glucan synthase in the construction of new cell wall elements particularly at the cell/biomaterial interface—a process which is met by inhibition by the surface-attached drug.

It should be noted that there has been discussion of other mechanisms by which caspofungin may act, including altering the cell morphology,[26–28] lowering the cell wall mechanical strength, and increasing the sensitivity to osmotic pressure, which could eventually lead to cell lysis and death.[29,30] Studying the relevance of these postulated mechanisms in the context of surface-bound echinocandins would need to be established through further experimentation. However, the cytotoxicity data shown above indicate that only fungal cells and not human fibroblasts (which do not contain glucan-based cell walls) are susceptible to caspofungin. This lends support to the hypothesis that the inhibition of β-1,3-D-glucan synthase by the contacting caspofungin surface coating is important in the selective elimination of fungal cell colonization. Further investigation will be needed to establish mechanistic details of fungal responses upon contact with solid surfaces both with and without attached drug molecules and in the presence of protein. A useful methodology would seem to be the use of continuous microscopy live cell imaging, which may pick up the kinetics of dimorphic changes that may be affected by the presence of coatings. Additionally, studies investigating phenotypic changes could be undertaken to advance the understanding of the relevance of morphological changes.

Even in the absence of detailed molecular mechanistic understanding, however, it is clear that covalently grafted caspofungin coatings offer promise for combatting fungal infections on biomedical devices, given that *C. albicans* plays a prominent role in device-related infections in human medicine.[31,32] Clinical evidence shows that fungal infections are far more difficult to diagnose, and the importance and severity of fungal infections has only recently started to be recognized.[10] Because of the marked differences in cell walls between bacteria and fungi, it is not appropriate to expect that antibacterial surfaces will also be antifungal; while graft coatings with antiseptics, such as quaternary ammonium compounds, kill both bacteria and fungi, they also damage human cell membranes.[12]

Thus, our conjecture is that to combat fungal infections and mixed bacterial/fungal infections, it is essential to utilize a selective antifungal compound, and for mixed infections it may well be that two-component coatings, one antibacterial and one antifungal, are required. While some antibacterial coatings act by disturbing the stability of the bacterial cell wall, fungi have a different cell wall composition and a much thicker cell wall, with a thickness of around 100 nm (Ref. 33) as compared with bacteria where it is only nearly half as thick.[34] When attempting to combat fungal colonization of biomedical devices, one must also consider that both fungal pathogens and mammalian cells are eukaryotic. This consideration raises concerns whether a prospective coating will be selective enough to pass cytotoxicity testing. Accordingly, it would seem preferable to construct antifungal coatings by using compounds, such as caspofungin, that employ a specific biomolecular pathway unique to fungi (i.e., glucan pathway), rather than mechanistically acting nonspecifically through physicochemical disturbance via electrostatic or surfactant effects. There is, of course, the important question whether steric hindrance or conformational changes upon surface immobilization might inhibit the desired biological activity, but our results show that at least in some cases a surface-grafted drug may still be able to interact with a specific biomolecular target, in this case a cell wall enzyme. Of particular interest may be compounds that target a biological process that is upregulated upon attachment of microbes to solid surfaces, as in this case where caspofungin inhibits the synthesis of an essential cell wall component that would be in demand as the cell adapts to the surface. This increased susceptibility may also enhance selectivity. There would seem to be considerable scope in defining such approaches based on molecular microbiology and biomaterials surface science, leading to rationally designed novel antimicrobial coatings.

V. SUMMARY AND CONCLUSIONS

Caspofungin can be covalently grafted onto biomaterials surfaces via a propanal plasma polymer interlayer, by reaction between amine groups on caspofungin with surface aldehyde groups followed by reductive amination. Its presence has been verified by the surface analytical methods XPS and ToF-SIMS. While *C. albicans* attached and grew on the propanal plasma polymer surface, after immobilization of caspofungin, the surface was able to inhibit the formation of *Candida* biofilm. Some fungal cells managed to attach but then were killed and thus unable to progress biofilm formation. In contrast, the presence of caspofungin did not alter the attachment and spreading of human primary fibroblast cells, which showed good colonization and normal morphology both on the plasma polymer surface by itself and after the grafting of caspofungin. This immobilization strategy enables the application of antifungal coatings onto a wide variety of biomaterials and biomedical devices.

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