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

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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.

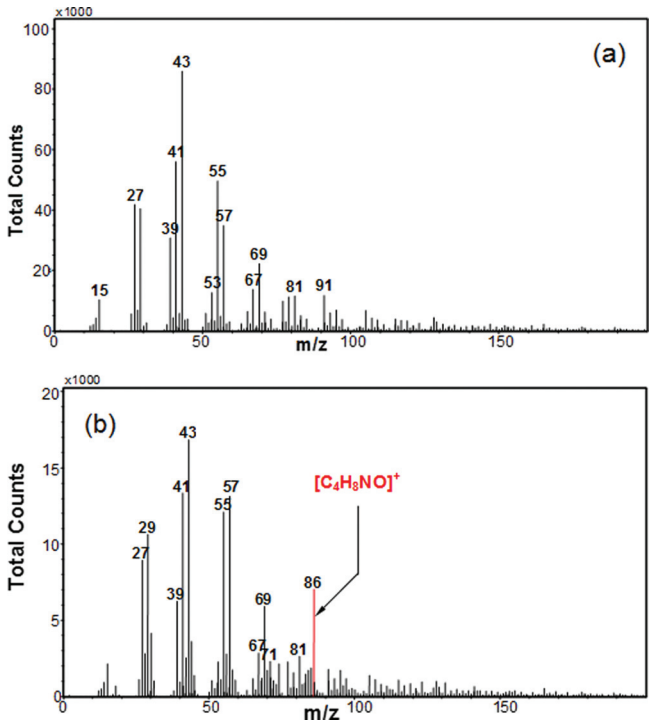

FIG. 2. Positive ion ToF-SIMS spectra recorded on (a) control ALD-pp sample and (b) caspofungin grafted sample.

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

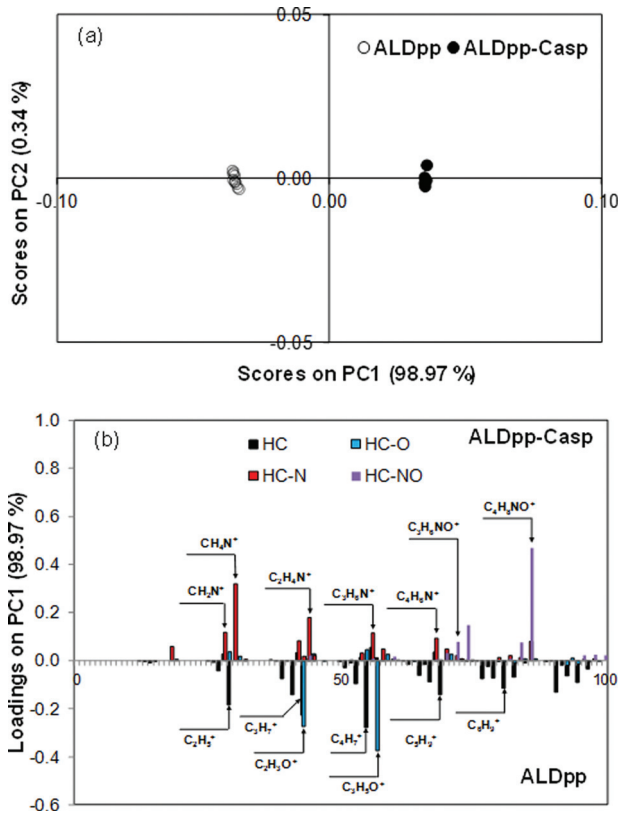

FIG. 3. PCA Scores plot on PC1 and PC2 (a), and loadings on PC1 (b), of positive ion mass spectra recorded on ALDpp and ALDpp-caspofungin (ALDpp-Casp) surfaces.

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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