Marek Jasieniak, Bryan R. Coad and Hans J. Griessera
ToF-SIMS multivariate analysis of surface-grafted small bioactive molecules
Biointerphases, 2015; 10(4):04A310-1-04A310-11

Originally published at: http://dx.doi.org/10.1116/1.4937464

ToF-SIMS multivariate analysis of surface-grafted small bioactive molecules

Marek Jasieniak, Bryan R. Coad, and Hans J. Griesser

Citation: Biointerphases **10**, 04A310 (2015); doi: 10.1116/1.4937464
View online: https://doi.org/10.1116/1.4937464
View Table of Contents: http://avs.scitation.org/toc/bip/10/4
Published by the American Vacuum Society

ToF-SIMS multivariate analysis of surface-grafted small bioactive molecules

Marek Jasieniak, Bryan R. Coad, and Hans J. Griesser[a)]
Mawson Institute, University of South Australia, Mawson Lakes, South Australia 5095, Australia

(Received 16 October 2015; accepted 25 November 2015; published 15 December 2015)

In the development of bioactive coatings on biomaterials, it is essential to characterize the successful fabrication and the uniformity of intended coatings by sensitive surface analytical techniques, so as to ensure reliable interpretation of observed biointerfacial responses. This can, however, be challenging when small bioactive molecules are grafted onto biomaterials surfaces at sub- and near-monolayer densities. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) provides the required sensitivity, but ion signals from small grafted molecules may still be dominated by fragment ions from the underlying polymer. In such cases, multivariate analysis provides valuable enhancement of spectral data, as illustrated here by examples comprising the surface grafting of bioactive serrulatane molecules, the peptide GRGDSP, the oligonucleotide 15-thymidine, and the antifungal compound Amphotericin B. The authors also show how ToF-SIMS plus principal component analysis can distinguish between covalent grafting and physisorption of the antibiotics caspofungin and micafungin. © *2015 American Vacuum Society*. [http://dx.doi.org/10.1116/1.4937464]

I. INTRODUCTION

Detailed surface characterization is essential for reliable rational interpretation of observed biological responses, be they protein adsorption, cell attachment and spreading, or bacterial biofilm formation.[1] In the encounter of a synthetic material with a biological environment, it is the chemical composition and properties (such as surface roughness, phase separation, etc.) of the *surface* of the material that determine the interfacial forces affecting approaching proteins and other biomolecules, as well as cells and tissue, thus governing the ensuing biological responses. Yet, many reports in the biomaterials and biodiagnostics literature contain insufficient, inadequate, or at times manifestly incorrect, surface analytical data, casting doubt on interpretations of biological responses.

Part of the reasons for this deficiency may be the availability and expense of surface analysis instrumentation, and the technical challenges in expert surface characterization. Some of the most valuable surface analysis techniques require expensive instruments and in-depth expertise. A good illustration is time-of-flight secondary ion mass spectrometry (ToF-SIMS); this method can provide unrivalled details of chemical information for surface layers. There are several excellent reviews describing the application of ToF-SIMS to biomaterial surfaces.[2–5] Skilled operation of ToF-SIMS instruments is, however, an art that requires thorough training, and experience is even more important for skilled interpretation of ToF-SIMS spectral data, particularly when employing multivariate analysis techniques rather than a simple study of individual peaks in mass spectra.

Yet, ToF-SIMS can provide essential data that clarify the surface chemical composition of biomaterials with unparalleled sensitivity and molecular structural details. The purpose of this article is to illustrate this with some examples of biomaterials surface characterization in which ToF-SIMS provided data that were not accessible by other methods, thereby demonstrating the power of ToF-SIMS for the characterization of grafted bioactive layers and thus its utility in elucidating rational understanding of biointerfacial interactions.

ToF-SIMS has proved to be of great value for detecting and probing proteins on biomaterials surfaces.[6–11] Yet, where ToF-SIMS really provides unique capability in biomaterials surface science is for the analysis of grafted layers of small (molecular weight of a few hundred Daltons) bioactive molecules, such as oligopeptides, oligonucleotides, antibacterial drugs, and the like. For such small molecules, XPS analysis can struggle with detection limits for elements such as N, S, and P, and in addition, some molecules of interest may not contain a unique element. Surface plasmon resonance has a high sensitivity but cannot provide chemical information and thus is incapable of verifying grafting of the intended compounds by the intended chemistry, versus adsorption of the compound or adventitious contaminants such as organosilicones, hydrocarbons, and fatty acids. With its extremely high sensitivity and chemical information content, ToF-SIMS is the method of choice for detecting grafted layers of small bioactive molecules.

II. ToF-SIMS METHODOLOGY

Briefly, in ToF-SIMS, ions from a pulsed "primary" source gun impinge on a surface, which leads to bond scissions and thus the creation and ejection of "secondary" fragment ions, as well as neutral molecular fragments, from the analyte surface. Secondary ions are extracted electrostatically into a mass spectrometer and their mass-to-charge ratios, m/z, are determined by measuring the time it takes for the ejected ions to arrive at the detector (time of flight); in this way, a mass spectrum is acquired. The analysis mode

[a)]Author to whom correspondence should be addressed; electronic mail: hans.griesser@unisa.edu.au

can be selected for positive ions and for negative ions. For the analysis of sputtered neutrals, postionization can be applied using a pulsed laser; the ejected neutrals then become accessible for mass analysis, and since their number is much greater than that of the ions, a significant increase in sensitivity is achieved,[12] although this is often not necessary as the mass spectra usually contain sufficient intensity when analyzing ions ejected from grafted organic layers.

When analyzing "soft" samples such as grafted organic molecular layers on polymeric biomaterials, ToF-SIMS must be used in the so-called static mode in order to avoid significant surface damage due to excessive ion impact density. By its nature, SIMS is destructive as it ablates the analyte surface. Not all molecular structures may sputter equally; hence, as the analysis proceeds, the composition of the analyte surface and the observed mass spectra may change. By using a primary ion beam of sufficiently low current and higher mass (Au or C60); however, it is possible to limit beam damage and derive data from a virtually intact surface, yet acquire sufficient numbers of secondary ions. In this "static" mode, the sample surface is not significantly altered during the analysis. In practice, meeting the requirements of the static regime means that less than 0.1% of the surface groups should be struck by the primary ion beam during the time of measurement.[13]

Its combination of high surface sensitivity (ca. 1 nm information depth[14]), information content (numerous peaks), extremely low detection limits for many molecular structures, molecular specificity, and submicrometer lateral resolution for imaging the distribution of the ejection of selected ions from the analyte surface provides surface information unavailable by other techniques. ToF-SIMS can be applied to the characterization of biomaterials prior to biological exposure and then again after contact with biological media. In particular, it is well-suited to the study of the adsorption of biomolecules onto materials surfaces since, for example, adsorbed proteins can be detected at amounts as low as 0.1 ng cm^{-2}.[7,8]

The main analytical limitation of ToF-SIMS originates from difficulties in quantifying the concentrations of surface species due to matrix effects. However, for samples in which a specific surface chemical group is in the same chemical environment, the secondary ion emission intensity is proportional to the surface density of that group.[15] Accordingly, for chemically similar materials, it is possible to perform semiquantitative analysis. More powerful, however, is a combination of ToF-SIMS and XPS, with the latter method used for quantification if its lesser chemical information content allows this.

The primary ion gun markedly affects the quality of ToF-SIMS spectra for grafted organic layers. While early work used a Ga liquid metal ion gun, this monoatomic source delivers marginal information for soft organic surfaces, since it produces a low yield of higher mass secondary molecular ions, which contain much more structural information than the lower mass ions. Sensitivity and yield decrease dramatically with increasing molecular weight of the fragment ions and with the complexity of the analyte,[16] meaning that to generate higher mass signals of sufficient intensity, long acquisition times may be required, beyond the static limit, leading to substantial surface damage and unrepresentative spectra. Ga and Cs ions penetrate into the material, depositing most of their energy at considerable depths, with only a small portion of the energy available at the surface.[17] The development of ion gun sources for polyatomic cluster ions such as SF_5, Au_n, Bi_n, C_n, and Ar cluster sources has been a major instrumental development for molecular ToF-SIMS surface analysis[18,19] as they much increase the relative yield of higher mass secondary ions.

However, the extremely high sensitivity of ToF-SIMS exacerbates problems arising from possible surface contamination. The spectra of secondary ions originating from contaminants superimpose on the spectrum of the analyte of interest,[20] and this can lead to ambiguity of interpretation, particularly with hydrocarbon contaminants. The most common contaminant tends to be poly(dimethylsiloxane); polymer additives have also been detected.[21,22] Contaminated samples should be discarded. For grafted bioactive layers, sodium and potassium ions can be present, and they can affect the accuracy of analysis, but as they are water-soluble, they can usually be eliminated by washing with ultrapure deionized water.

III. PRINCIPAL COMPONENT ANALYSIS

In some cases, the aim might just be to look for some diagnostic peaks that confirm the presence of grafted bioactive molecules and also check for absence of surface-adsorbed contaminants. In other analyses, however, interpretation may not be as straightforward, for example, when relatively low surface densities of grafted molecules give rise to peaks that are not immediately evident among the peaks arising from fragment ions from the substrate. Not surprisingly, for samples comprising grafted small molecules, the contribution of ions from the substrate is much more pronounced than for grafted layers of larger molecules. With the complexities inherent in ToF-SIMS spectra, it can be challenging to compare, digest, and interpret spectra that can contain hundreds of peaks. A comparison of relative intensities can quickly become time-consuming. Among the various multivariate analysis methods available, principal component analysis (PCA) is the most popular for working up interpretations of ToF-SIMS spectra.

Detailed explanations of PCA and how best to use it have been given in the literature.[23,24] Briefly, PCA determines the greatest directions of variance within a data set such as spectra from related samples or from different spots on one sample. This is performed through the singular value decomposition of the variance–covariance matrix from the data set, which produces characteristic eigenvalues and eigenvectors of the matrix. The result of this process is a set of new uncorrelated variables called principal components (PCs), which are linear combinations of the original variables (ToF-SIMS peak intensities). The output from PCA consists of the scores, the loadings, and the residuals. Scores describe the relationship (spread) between the samples as evidenced by the new PC axes. Mathematically, the scores are the

projection of the sample data onto a PC axis. Loadings describe how the original variables relate to the PC axes. The number of principal components necessary for detailed analysis can vary; for simplicity, we have selected examples, below, for which the first principal component, PC1, is dominant and sufficient for our purposes. In other cases, however, the analyst may need to scrutinize further PCs until the residual matrix contains the remaining variance not described in the scores and loadings, and represents random noise in the data. The reader interested in in-depth PCA is referred to specialist treatises.[23,24]

The scores and loadings must be used together when interpreting the data set. The loadings plot is used to determine the main variables (peak intensities) that are responsible for the separation between the samples. For straightforward cases of PCA where PC1 dominates, as in the examples below, peaks with positive loadings are more intense in samples with positive scores on the same PC axis, and peaks with negative loadings are more intense in the spectra of samples with negative scores. When higher PCs need to be considered, however, interpretation becomes more complex, and the reader is referred to Refs. 23 and 24.

PCA can reduce large data sets into more manageable sets, help find trends within data sets that are not readily apparent, find differences between samples when the chemistry is similar (for example, proteins/peptides), identify peaks of interest for unknowns, show surface contaminants, eliminate user bias, and simplify interpretation of complex data sets. It is, however, essential to be aware of potential bias, such as in peak selection.[23,24] Also, statistical significance must be assured. Random noise in peak intensities as well as (hopefully small) variations in chemistry across the surface of a particular analyte mean that spectra should be recorded from multiple separate spots on each sample; in this way, one can assure that when comparing two samples, for example, before and after grafting of a bioactive molecule, one probes for significant differences in chemistry rather than spot-to-spot variability. In our laboratory, we typically record spectra from 8 to 10 independent spots for each sample, across a sample area of at least 10×10 mm. Sample topography (surface roughness) can also affect ToF-SIMS spectra; in all the examples below, this was avoided by using silicon wafer substrates for depositing coatings.

Interpretations should be examined closely to make sure the results are sensible and consistent. The PCA scores and loadings plots must be scrutinized and checked against the raw data, and if any discrepancies occur, it may be necessary to review the assumptions, such as peak selections, made during the preprocessing steps. Data preprocessing can require some trial and error, but can soon be learnt by using a systematic approach to the trends in the analysis method when dealing with ToF-SIMS data.

IV. EXAMPLES

The applicability, information content, and limitations of ToF-SIMS are best illustrated by specific examples. We will discuss several examples; in all cases, the small bioactive molecules are grafted covalently not directly onto the solid bulk substrate but onto a plasma polymer interlayer. The use of such an interlayer is convenient, since an optimized coating-plus-grafting method can be readily transferred onto a wide range of substrate materials and biomedical devices, with constant chemistry and no need for reoptimization. We use 10–20 nm thick plasma polymer interlayers from *n*-heptylamine,[25] propionaldehyde (aka propanal),[26] or allyl glycidyl ether (AGE)[26,27] for their ease of deposition and their amine, aldehyde, and epoxide, respectively, surface groups that are suitable for immobilizing a wide range of bioactive molecules under aqueous conditions. This wide use also means that we have a detailed historical database on the spectral features of these plasma polymer interlayers to serve as control data sets for assessing surface chemistries before and after grafting.

The first example relates to the covalent grafting of antibacterial compounds of the diterpene class of serrulatanes.[28] These compounds are found in Australian desert plants that were used by Aboriginal people for medicinal recipes. Amphiphilic serrulatanes have been shown to be active against a number of Gram-positive bacteria;[29,30] hence, there was interest in exploring whether they could be used as grafted antibacterial layers. Grafting was performed in three steps: first, a propanal plasma polymer was deposited onto polymeric substrates and Si wafers, then polyallylamine was grafted onto its surface aldehyde groups, and finally, the carboxylic acid group of 8-hydroxyserrulat-14-ene-19-oic acid[29] was reacted with surface amine groups by carbodiimide chemistry (Fig. 1).

The presumed grafting was rather challenging to verify; FTIR lacked sensitivity, and in XPS, there was no unique element available for detection. XPS C1s spectra[28] (not shown) showed a small decrease in the C–O and C=O components in favor of the C–C and O–C=O components, and a low intensity shake-up satellite, in agreement with expectations, but these small changes were unsuitable for guiding optimization of grafting conditions. In ToF-SIMS spectra (Fig. 2), on the other hand, successful grafting was immediately evident. Aromatic ring structures and unsaturated structural elements often produce relatively high fragment ion yields, and in this case also, there were clearly distinguishable peaks from the serrulatane molecule [Fig. 2(c)] and its isoprene "tail" (m/z 69). Remarkably, even the entire parent molecule (PA) could be detected, albeit with a mass consistent with its carboxyl group having been converted into an amide group, as expected in carbodiimide-mediated amide formation.

In this case, the ToF-SIMS spectra recorded before and after grafting of the antibacterial serrulatane molecules differ so markedly that there is no need for multivariate analysis, thanks to the relatively high intensity often seen with aromatic ions in positive ToF-SIMS. The new peaks with significant intensity are all readily assignable to fragment ions from the serrulatane molecule. There is no evidence of any detectable adventitious surface contamination (due to

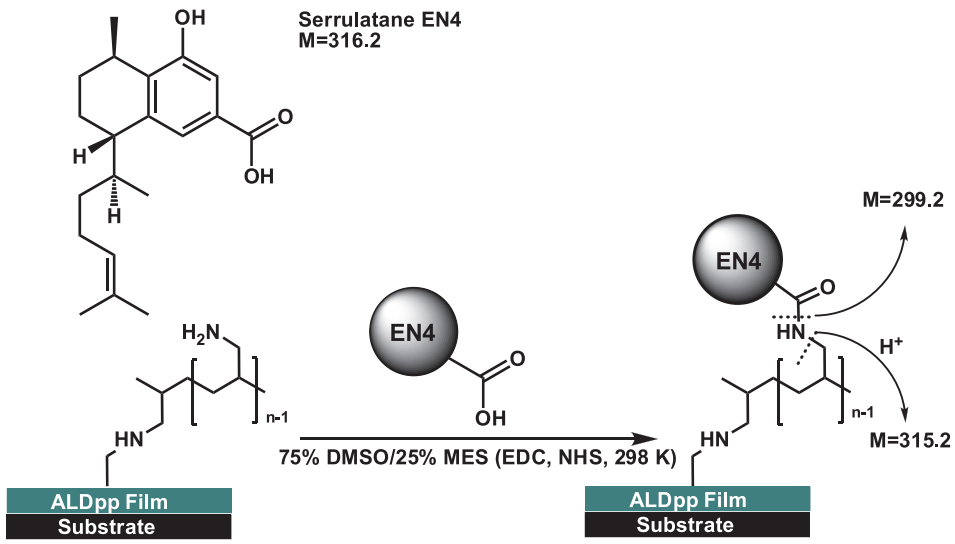

FIG. 1. Schematic diagram of grafting the carboxy-serrulatane EN4 onto a polyallylamine layer that had been grafted onto an aldehyde plasma polymer film via reductive amination. Also shown are the fragmentation pathways releasing two observed ToF-SIMS peaks.

stringent clean lab conditions), and hence, the altered biological responses (bacterial resistance and 3T3 cell attachment[28]) can be confidently assigned to the presence of grafted serrulatane and interpreted in terms of interfacial interactions with its chemical structural elements and properties.

The next example concerns the grafting of small synthetic oligopeptides that replicate an active region of a larger protein. This may be more attractive than grafting an entire large protein for reasons of cost and sterilization. There is substantial literature on grafting small oligopeptides onto reactive biomaterials surfaces, albeit not always with adequate surface characterization. The most popular motif has been the amino acid sequence RGD, which is found in several adhesive glycoproteins that bind to cell-wall integrins and thereby promote cell attachment when an RGD-containing oligopeptide is grafted onto biomaterials surfaces.

Whereas XPS is well suited to detecting and quantifying grafted protein layers mainly via their N signal (if the substrate does not contain N), for small oligopeptides the amount of N is so small that XPS detection is close to the sensitivity limit even if the oligopeptide is grafted to close to monolayer density; yet, biological activity may result from coverage well below monolayer density. Again, XPS is not well suited to assess the efficiency of different grafting conditions. ToF-SIMS, on the other hand, can detect low amounts of surface-grafted oligopeptides, as shown by the example of the grafting of the oligopeptide GRGDSP. While strictly speaking ToF-SIMS is not a quantitative analysis method due to matrix effects, for chemically closely related samples the relative intensity of ions can be compared semi-quantitatively, and hence, we consider it feasible to assess grafting density versus grafting conditions by ToF-SIMS, as long as the substrate is the same. Polypropylene was surface-functionalized with epoxy groups by the plasma polymerization of allyl glycidyl ether,[27] and the GRGDSP oligopeptide was grafted by covalent reaction in buffered aqueous solution (Fig. 3). The process of grafting was monitored by ToF-SIMS.

The positive ion ToF-SIMS spectra before and after grafting (Fig. 4) looked superficially rather similar. After grafting, the peaks below m/z 50 are difficult to assign as these small fragment ions could be ejected both from the plasma polymer surface and from the peptide. The clearest changes are an increase in the relative intensity of the signal at m/z 69 and a decrease in the signal with m/z 57. Unlike for the example above, here there were no clearly discernible peaks from aromatic structures, and instead of visual inspection of differences in peak height, in this case, PCA is a much preferable way of ensuring reliable interpretation.

The PCA scores plot [Fig. 5(a)] showed clear differences between spectra from the plasma-coated layer and the subsequent GRGDSP grafting. We note that the individual data sets, while containing small spectral differences between different spots on the same sample, cluster as two well-separated groups, thus allowing meaningful comparison of chemical changes upon grafting, with statistical significance. We also note that the first principal component, PC1, dominates (>90%) and the two sets of spectra separate well along the PC1 axis, whereas they do not separate along the PC2 axis. Accordingly, it suffices to undertake interpretation by considering the loadings of peaks on PC1 only.

Differences could, however, also arise from the presence of surface contaminants on one or both of the samples; it was important to check that spectral differences could be attributed to chemically meaningful peaks. For clarity, the PC1 loadings plot was separated into three plots, showing peaks of different chemistries: hydrocarbon, N-containing peaks, and O-containing peaks. Figure 5(d) shows that all the N-containing peaks had negative loadings, consistent with the scores plot showing a negative score on PC1 for the

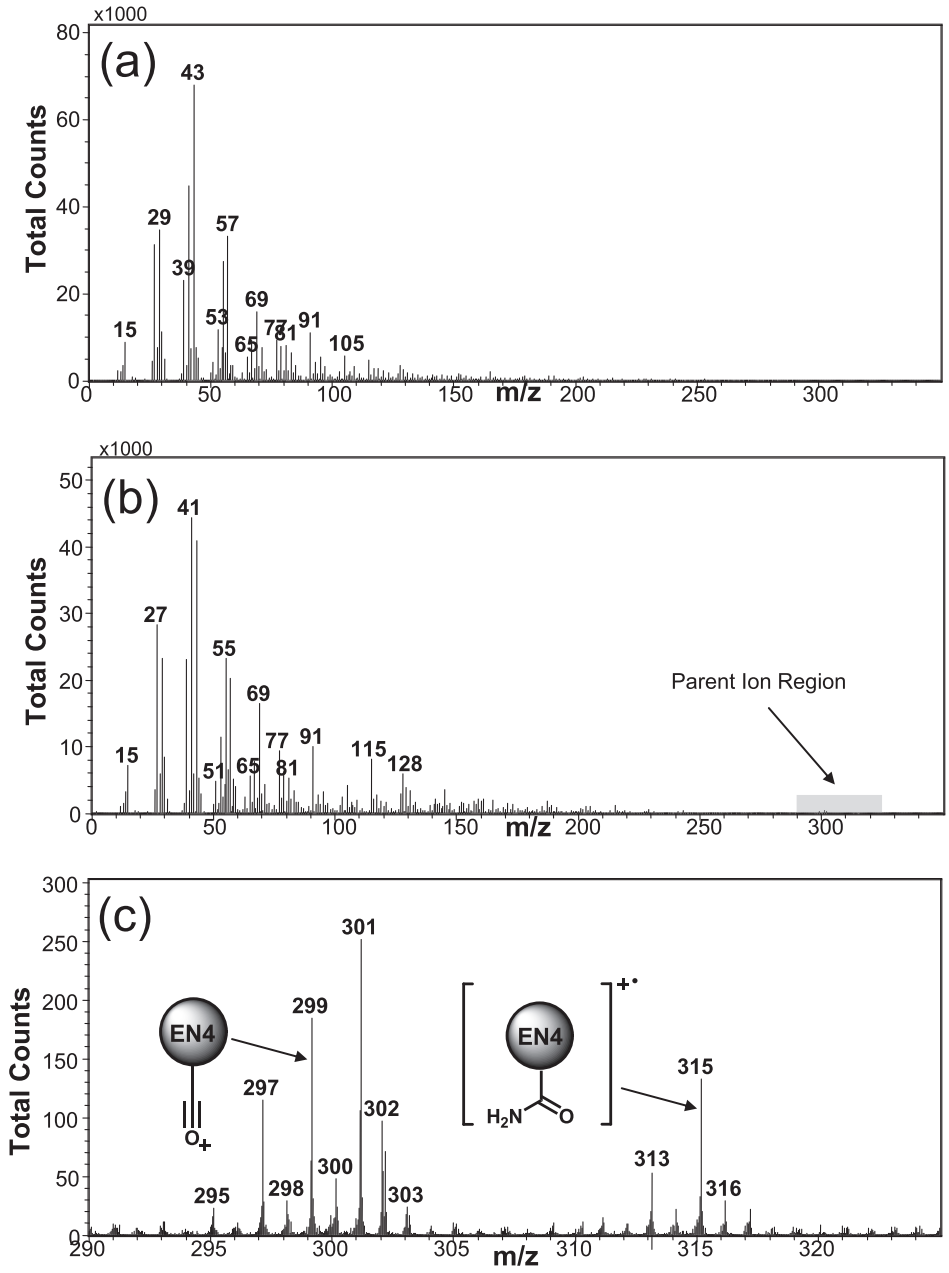

FIG. 2. ToF-SIMS spectra (positive ions) recorded on (a) polyallylamine grafted onto aldehyde plasma polymer; (b) serrulatane grafted onto polyallylamine; and (c) expanded parent ion region. From Ref. 28.

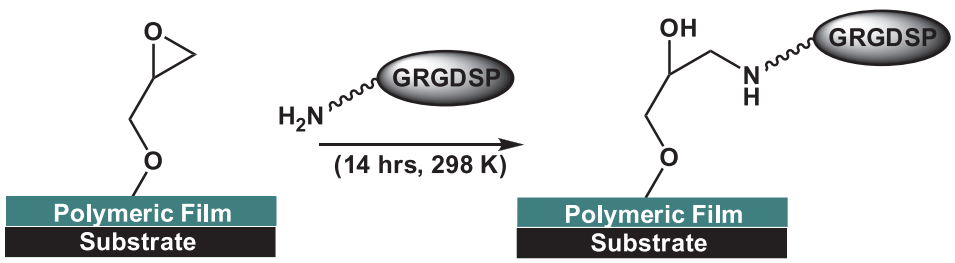

FIG. 3. Schematic diagram of grafting the oligopeptide GRGDSP onto polypropylene modified with a thin allyl glycidyl ether plasma polymer layer.

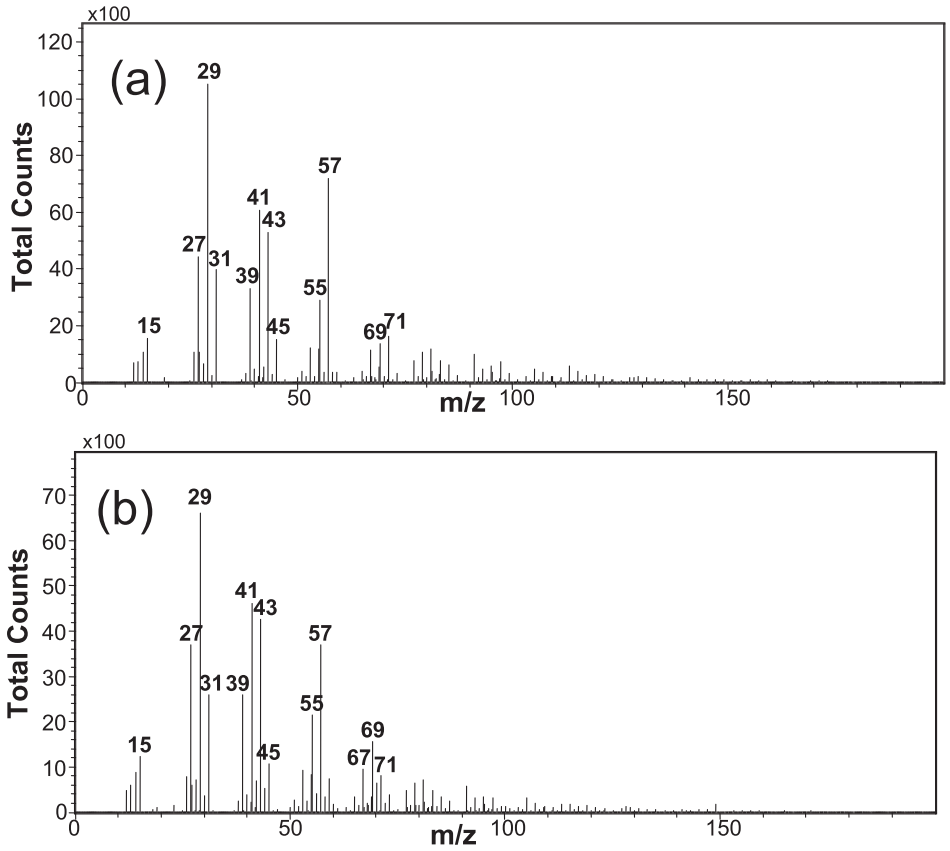

FIG. 4. ToF-SIMS spectra (positive ions) recorded on (a) an allyl glycidyl ether plasma polymer surface and (b) the same plus grafting GRGDSP.

GRGDSP-grafted sample. Thus, these peaks can be assigned to immonium ions and smaller N-containing fragments from the grafted oligopeptides. In contrast, the three O-containing peaks with the highest loading values all loaded positively [Fig. 5(c)], meaning reduced intensity after grafting, consistent with their origin from the AGE plasma polymer which became partially covered by the grafted oligopeptide molecules. The hydrocarbon peaks [Fig. 5(b)] are less instructive as they can originate both from the plasma polymer and the oligopeptide, but the peak m/z 29 ($C_2H_5^+$) is a strong feature of the AGE plasma polymer surface. Its molecular fragmentation origin is not entirely clear.

It is often instructive to highlight the peaks with high loadings as they provide the most reliable spectral information. For example, the high loading peak at 70 amu reflects the presence of a $C_4H_8N^+$ fragment, which is the fingerprint immonium ion originating from the amino acid proline, which forms part of GRGDSP.

While ToF-SIMS has been used to study the orientation of proteins on surfaces,[4,31] for our samples of small grafted peptides, there was no evidence that we could discern that would speak to the question of their orientation on the surface.

Another instructive example concerns the grafting of oligonucleotides, functionalized for reaction by the addition of a terminal amine group, onto a propanal plasma polymer interlayer.[32] For the grafting of 15-thymidine-amine, for example, examination of the positive ion spectrum [Fig. 6(a)] shows some characteristic peaks assignable to the oligonucleotide. PCA can again bring out the spectral differences with better clarity, but in this case, the *negative* ion spectrum [Fig. 6(b)] is much more immediately instructive and does not need PCA to verify grafting, as it clearly shows two dominant peaks that can be assigned to fragments originating from the phosphate groups of the oligonucleotides, such as PO_2^- at m/z 63 and PO_3^- at m/z 79 (which are absent in the corresponding spectrum from the plasma polymer, not shown). The ready production of such negatively charged ions from phosphate groups is chemically intuitive. Often ToF-SIMS spectra are acquired only in the positive ion mode because many surfaces do not produce informative negative ion spectra and only relatively low intensities of negative ion signals, but this example shows that in some cases, the negative fragment ions can yield valuable information, and that intuition and experience can serve to plan effective analysis strategies.

A further example of a challenging surface analysis problem arose when studying the covalent surface grafting of the antifungal compound amphotericin B onto a propanal plasma polymer interlayer.[33] This compound has rather limited solubility in phosphate buffered aqueous solution and only one amine group within its molecular weight of 924 g/mol, and thus, it was not surprising that by XPS there was a very low intensity (0.6%) N1s signal after grafting.

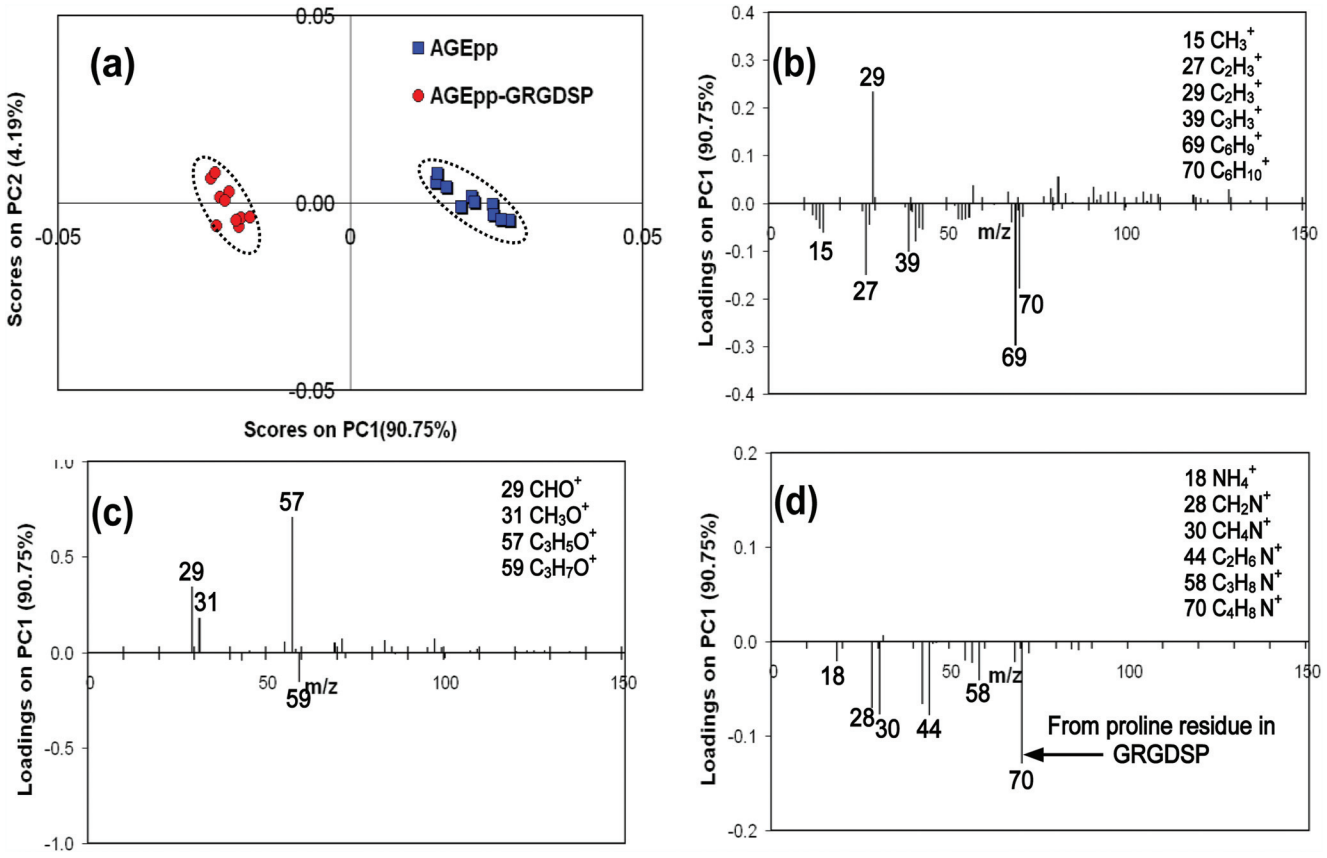

FIG. 5. PCA scores and loadings plots comparing samples before and after grafting of GRGDSP onto AGE plasma polymer; (a) scores plot for PC1 and PC2; (b) PC1 loadings for hydrocarbon ions; (c) PC1 loadings for O-containing ions; (d) PC1 loadings for N-containing ions.

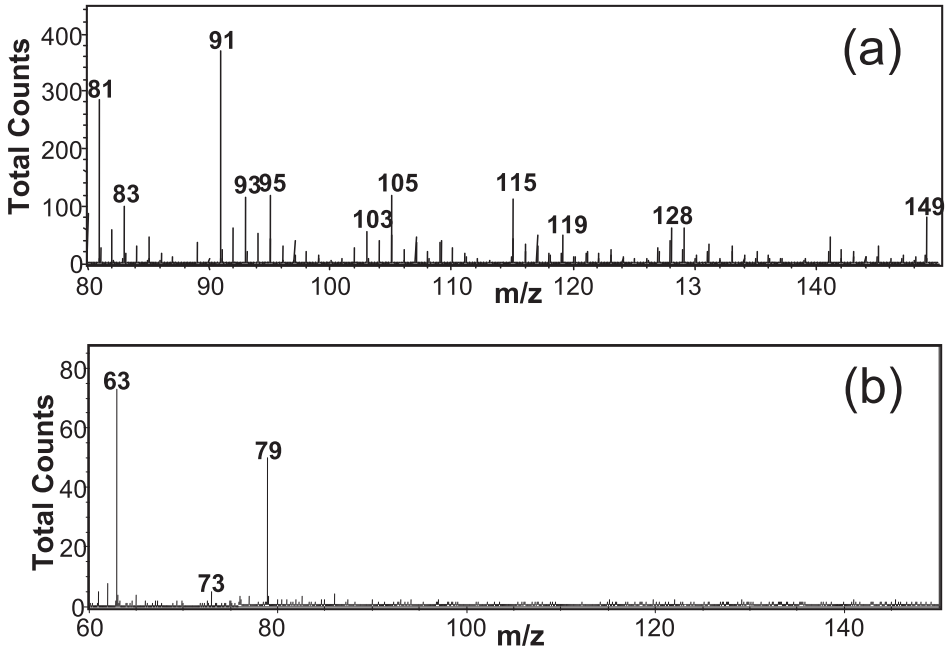

FIG. 6. ToF-SIMS spectra recorded on 15-thymidine grafted onto propanal plasma polymer: (a) positive ion spectrum and (b) negative ion spectrum. From Ref. 32.

ToF-SIMS spectra before and after grafting (not shown) appeared indistinguishable by visual inspection, akin to the situation in Fig. 4. PCA, however, brought out the differences quite clearly with the scores plot [Fig. 7(a)] demonstrating distinctly different surface compositions and the loadings plot on PC1 [Fig. 7(b)], showing that nitrogen-containing fragment ions and C_xH_yO fragment ions were more intense for the amphotericin-grafted sample, whereas the plasma polymer sample was characterized by more intense hydrocarbon fragment ions. This is clearly consistent with expectations. No silicones were detected, and on the basis of this, PCA verification of amphotericin immobilization one can infer that the biological effects (prevention of fungal biofilm formation)[33] are indeed assignable to the presence of a surface-grafted layer (at probably submonolayer density) of this antifungal compound.

The final example illustrates the utility of ToF-SIMS in probing for specific chemical immobilization of bioactive compounds through covalent bonds as differentiated from nonspecific surface attachment through adsorption (physisorption). Echinocandins comprise a drug class of small molecular weight lipopeptides with an excellent antifungal activity. Caspofungin (Merck and Co., Inc.) and micafungin (Astellas Pharma) are structurally related; yet, a key difference is the presence of two primary amine functionalities in the former, whereas the latter does not contain amine groups

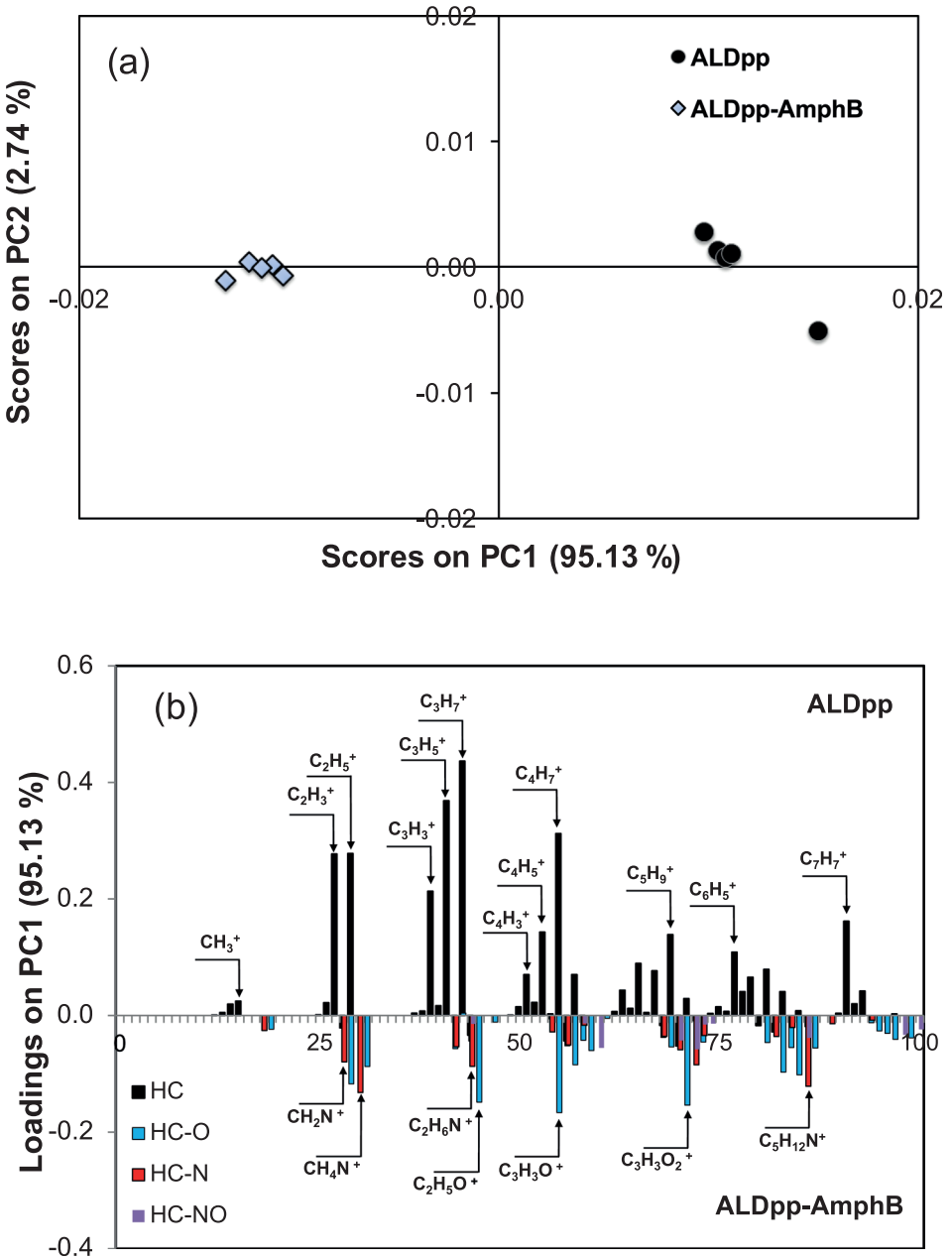

FIG. 7. PCA of positive ToF-SIMS spectra recorded on propanal plasma polymer and Amphotericin B grafted onto propanal plasma polymer; (a) scores plot, (b) loadings on PC1. From Ref. 33.

(Fig. 8). One would expect that only caspofungin can chemically react to form imine bonds with surface aldehyde groups (again on a propanal plasma polymer interlayer), followed by reductive amination to form stable amine bonds. However, both compounds might also adsorb onto surfaces through noncovalent interfacial forces, and such physisorption might be superimposed on apparent covalent immobilization. Washing protocols can be used to attempt disruption of interfacial physisorption forces, but they are not always conclusive. The particular challenge for surface analysis is to determine whether or not low amounts of physisorbed compound remain after washing, since physisorption could confuse interpretation of bioassays via release of highly bioactive compounds that would affect fungal cells in solution even at low concentrations, and interfere with the desired testing of interfacial interactions between fungal cells and covalently grafted antifungal compounds. As micafungin is incapable of forming a covalent bond with the propanal plasma polymer layer, whereas caspofungin should do so, they form a suitable pair for analysis of grafting versus physisorption. The compounds are otherwise structurally sufficiently similar so that it seems reasonable to assume that (noncovalent) interfacial binding should be of similar strength.

The sensitivity of ToF-SIMS allowed us to confidently address this issue. On the Caspofungin surface, the PCA scores and loadings on positive mass spectra (Fig. 9) provided clear evidence that Caspofungin remained on the surface after binding and extended washing. For example, the dominant $C_4H_8NO^+$ fragment can be assigned to a specific part of the caspofungin molecular structure (Fig. 8); there is no evidence of contaminants. It would be of much interest if ToF-SIMS analysis could directly identify and verify the intended covalent bond, as had been possible in the above case of an amide bond when immobilizing a serrulatane (Fig. 2), but in the case of caspofungin, the spectral evidence is less clear cut. One might take the fragment ion $C_4H_9^+$ to represent evidence of the intended interfacial imine bond, as this ion is less likely to originate from the peptide part of caspofungin, but isotope labeling studies would be needed to seek direct evidence.

For the micafungin surface versus the aldehyde control sample, in contrast, PCA evaluation of the data sets (eight separate analysis spots per sample) led to a scores plot (Fig. 10) that showed such overlap that the two sample surfaces cannot be distinguished by ToF-SIMS. Hence, we conclude that no significant amount of physisorbed micafungin was present and thus that the washing protocol for removing physisorbed compound was effective. This accords well with the conclusion that after such washing there was no further removal of caspofungin. The use of these two structurally related compounds (one covalently binding, one nonbinding) and results from surface analysis allowed us to confirm grafting of caspofungin to surfaces through a specific covalent

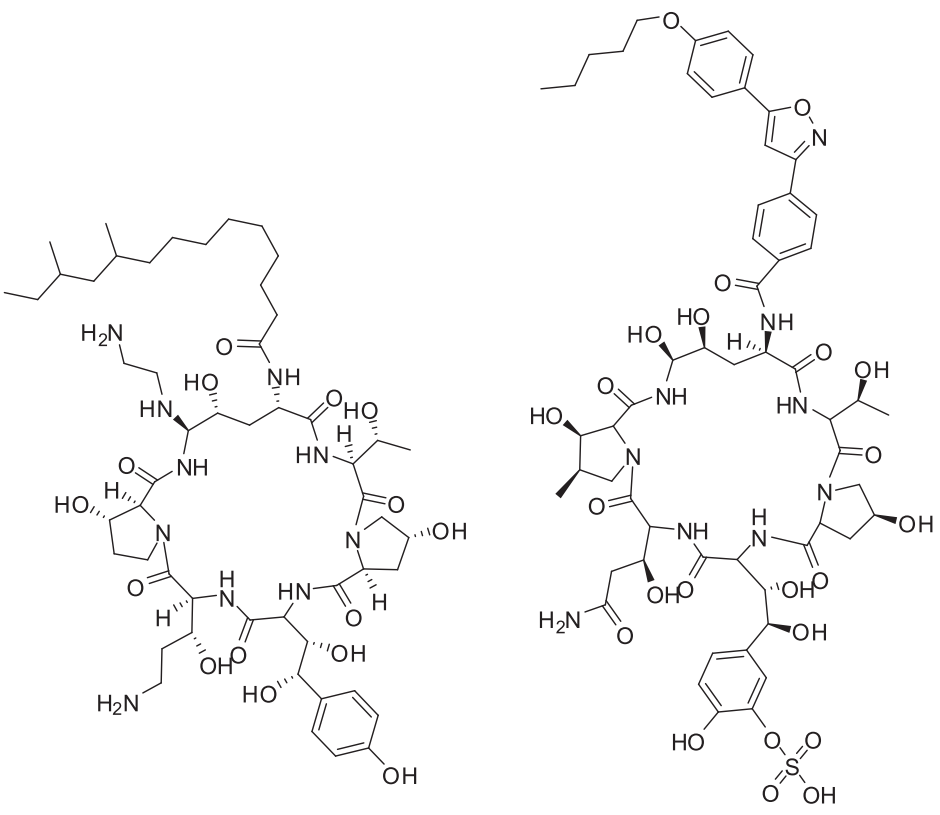

FIG. 8. Chemical structures of two members of the echinocandin class of antifungal drugs: caspofungin ($C_{52}H_{88}N_{10}O_{15}$) and micafungin ($C_{56}H_{71}N_9O_{23}S$).

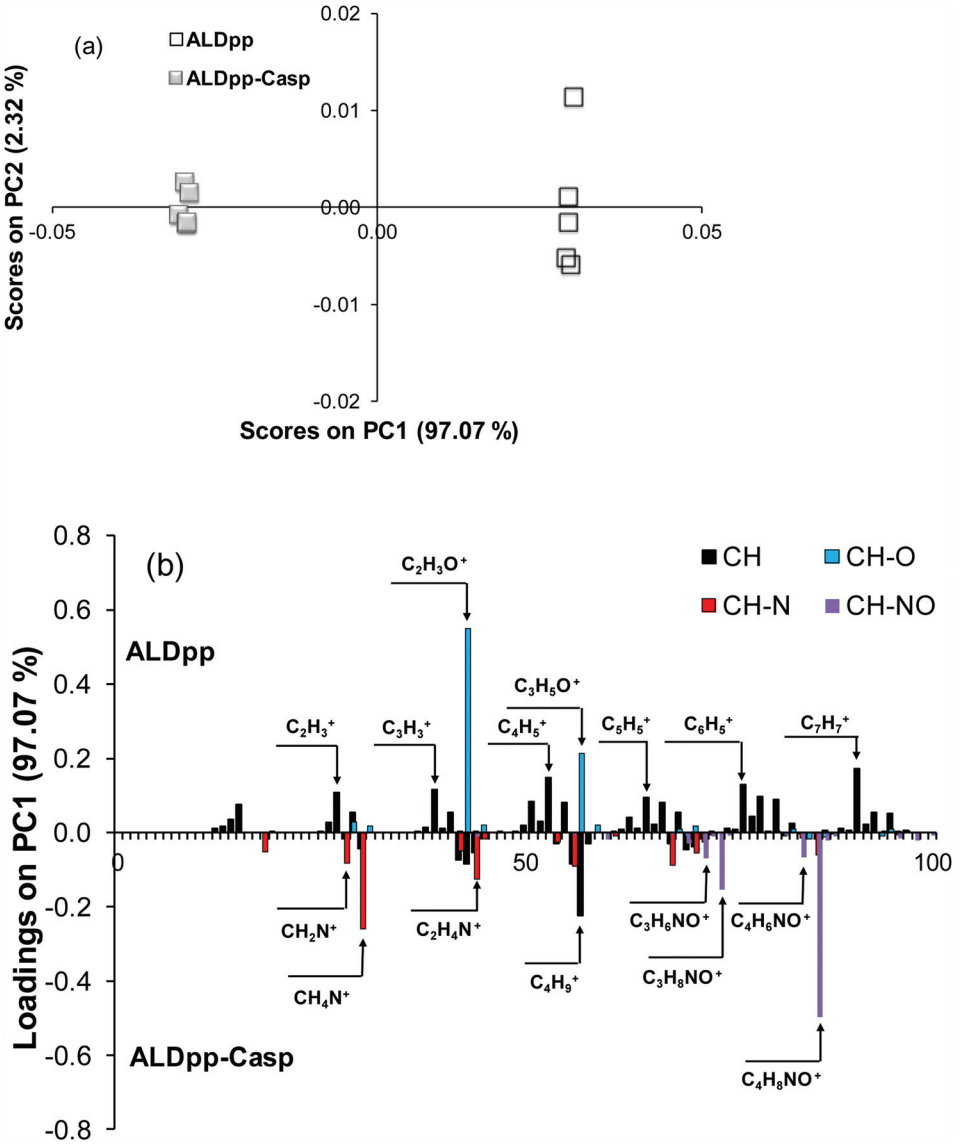

FIG. 9. Scores plot on PC1 and PC2, and loadings on PC1 of positive mass spectra for aldehyde plasma polymer before and after immobilization of caspofungin.

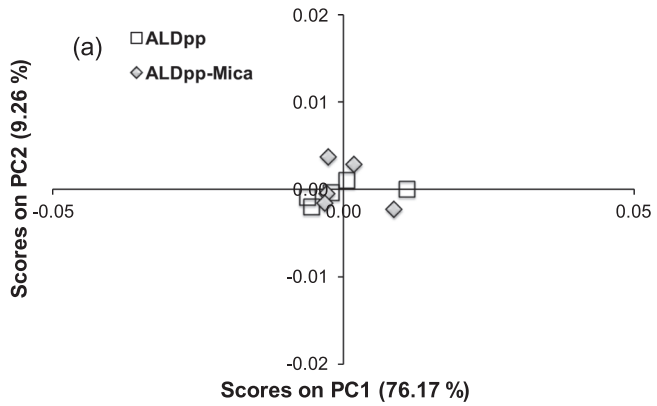

FIG. 10. Scores plots on PC1 and PC2 of positive mass spectra for aldehyde plasma polymer before and after attempted immobilization of micafungin.

chemical linkage while providing strong evidence against concurrent physisorption through nonspecific interfacial forces. Thus, results from bioassays can be interpreted with confidence as arising only from covalently grafted molecules, rather than having to include confounding possible additional effects from physisorbed molecules diffusing into solution.

V. CONCLUSIONS

ToF-SIMS is ideally suited, particularly when used in combination with PCA, for the surface characterization of samples bearing covalently grafted layers of relatively low molecular weight bioactive molecules. Typically, in such cases, XPS provides only a very small signal with poor signal-to-noise ratios, whereas detection is unambiguous via ToF-SIMS analysis. While usually positive ions are collected, in some cases, the negative ion spectrum can be

informative, for example, when a grafted molecule contains groups such as phosphate that readily liberate negative ions. Characteristic fragment ions can also directly attest to the occurrence of the intended immobilization reaction; for example, an amide-bearing fragment ion attested that a carboxylated serrulatane had indeed been grafted via amide bond formation. Often the small molecular masses of the grafted molecules lead to ToF-SIMS spectra being dominated by fragment ions from the polymer substrate; while visual inspection can reveal peaks assignable to fragment ions from the grafted molecules, much more reliable and detailed information is furnished by PCA evaluation. ToF-SIMS with PCA can also provide insights into covalent grafting versus physisorption, with the latter often not being considered in grafting studies.

ACKNOWLEDGMENTS

The authors thank Phuong-Cac Nguyen and Stefani Griesser for fabricating samples. This work was supported in part by the Australian Government via NHMRC Project Grant No. 1066647.

[1]D. G. Castner and B. D. Ratner, Surf. Sci. **500**, 28 (2002).
[2]D. Lèonard and H. J. Mathieu, J. Anal. Chem. **365**, 3 (1999).
[3]H. J. Mathieu, Y. Chevolot, L. Ruiz-Taylor, and D. Leonard, Adv. Polym. Sci. **162**, 1 (2003).
[4]A. M. Belu, D. G. Graham, and D. G. Castner, Biomaterials **24**, 3635 (2003).
[5]R. Michel and D. G. Castner, Surf. Interface Anal. **38**, 1386 (2006).
[6]D. S. Mantus, B. D. Ratner, B. A. Carlson, and J. F. Moulder, Anal. Chem. **65**, 1431 (1993).
[7]M. S. Wagner, S. L. McArthur, M. Shen, T. A. Horbett, and D. G. Castner, J. Biomater. Sci., Polym. Ed. **13**, 407 (2002).
[8]P. Kingshott, S. McArthur, H. Thissen, D. G. Castner, and H. J. Griesser, Biomaterials **23**, 4775 (2002).
[9]M. S. Wagner and D. G. Castner, Langmuir **17**, 4649 (2001).
[10]N. Xia, C. J. May, S. L. McArthur, and D. G. Castner, Langmuir **18**, 4090 (2002).
[11]M. S. Wagner, T. A. Horbett, and D. G. Castner, Biomaterials **24**, 1897 (2003).
[12]"Tof-SIMS 5," http://www.ion-tof.com/technique-timeofflight-IONTOF-TOF-SIMS-TIME-OF-FLIGHT-SURFACE-ANALYSIS.htm.
[13]D. Briggs, *Surface Analysis of Polymers by XPS and Static SIMS* (Cambridge University, Cambridge, 1998).
[14]J. C. Vickerman and D. Briggs, *TOF-SIMS Surface Analysis by Mass Spectrometry*, 1st ed. (IM/Surface Spectra Limited, UK, 2001), Chap. 5.
[15]A. Benninghoven, Surf. Sci. **299/300**, 246 (1994).
[16]C. W. Diehnelt, M. J. Van Stiponk, and E. A. Schweikert, Int. J. Mass Spectrom. **207**, 111 (2001).
[17]Z. Postawa, B. Czerwinski, N. Winograd, and B. J. Garrison, J. Phys. Chem. B **109**, 11973 (2005).
[18]E. A. Jones, J. S. Fletcher, C. E. Thompson, D. A. Jackson, N. P. Lockyer, and J. C. Vickerman, Appl. Surf. Sci. **252**, 6844 (2006).
[19]E. A. Jones, N. P. Lockyer, and J. C. Vickerman, Int. J. Mass Spectrom. **260**, 146 (2007).
[20]U. Oran, E. Űnveren, T. Wirth, and W. E. S. Unger, Appl. Surf. Sci. **227**, 318 (2004).
[21]N. Medard, C. Poleunis, X. Vanden-Eynde, and P. Bertrand, Surf. Interface Anal. **34**, 565 (2002).
[22]D. Briggs, *Comprehensive Polymer Science, Polymer Characterization*, edited by C. Booth and C. Pricev (Pergamon, Oxford, 1989), Vol. 1, pp. 543–599.
[23]J. E. Jackson, *A Users's Guide to Principal Components* (Wiley, New York, 1991).
[24]M. Jasieniak, D. Graham, P. Kingshott, L. Gamble, and H. J. Griesser, *Surface Analysis of Biomaterials, Handbook of Surface and Interface Analysis*, edited by J. P. Riviere and S. Myhra, 2nd ed. (CRC, Boca Raton, 2009), pp. 529–564.
[25]H. J. Griesser and R. C. Chatelier, J. Appl. Polym. Sci. Appl. Polym. Symp. **46**, 361 (1990).
[26]B. R. Coad, M. Jasieniak, S. S. Griesser, and H. J. Griesser, Surf. Coat. Technol. **233**, 169 (2013).
[27]B. Thierry, M. Jasieniak, L. C. P. M. de Smet, K. Vasilev, and H. J. Griesser, Langmuir **24**, 10187 (2008).
[28]H. Ys, "Antibacterial coatings for biomedical devices by covalent grafting of serrulatane diterpenes," Ph.D. thesis (University of South Australia, 2011).
[29]C. P. Ndi, S. J. Semple, H. J. Griesser, S. M. Pyke, and M. D. Barton, J. Nat. Prod. **70**, 1439 (2007).
[30]O. Anakok, C. P. Ndi, M. Barton, H. J. Griesser, and S. Semple, J. Appl. Microbiol. **112**, 197 (2012).
[31]C. D. Tidwell, D. G. Castner, S. L. Golledge, B. D. Ratner, K. Meyer, B. Hagenhoff, and A. Benninghoven, Surf. Interface Anal. **31**, 724 (2001).
[32]P. C. T. Nguyen, "Oligonucleotide immobilisation via a patterned propanal plasma polymer coating: A potential surface chemistry for DNA microarray fabrication," Ph.D. thesis (University of South Australia, 2006).
[33]S. S. Griesser, "Antimicrobial coatings for biomedical and wound healing devices," Ph.D. thesis (University of South Australia, 2015).