PHYSICAL ELECTRONICS
A DIVISION OF ULVAC-PHI

TOF-SIMS Molecular Imaging of a Micropatterned Biological Ligand

Introduction

Micropatterning of biomolecules on surfaces has a number of applications, which include modulation of cell-substrate interactions in biomaterials and tissue engineering, the fabrication of multi-analyte biosensors, and genome arrays.[1,2] Micro contact printing methods are attractive for micropatterning of biomolecules, because of their simplicity and ease of use.

A new method of microcontact printing which enables biological ligands and proteins to be patterned onto polymer surfaces with a spatial resolution of at least 5 µm is called MAPS, microstamping on activated polymer surfaces. Although initial proof-of-principle of MAPS demonstrated its feasibility[3], detailed molecular characterization of each step is critical to further develop MAPS as a technologically useful micropatterning method, and to optimize end-use applications on the patterned polymer surface.

The analysis of organic polymer surfaces that are derivatized by a multi-step reaction scheme with organic reagents is a challenging problem, because of the following reasons: (1) the complex and heterogeneous chemistry and structure of polymer surfaces; (2) the alterations in surface chemistry at each stage of the functionalization procedure can be relatively subtle; (3) the minute amounts of material that are localized in a surface zone, extending less than 100 Å into the bulk; and (4) the subtle chemical differences between patterned regions and background that need to be discriminated with lateral resolution of a few microns.

Time-of-flight secondary ion mass spectrometry (TOF-SIMS) is a powerful technique that addresses many of these analytical needs.[4] TOF-SIMS can be used to interrogate each step of the polymer derivatization and micropatterning process. TOF-SIMS provides molecular chemical characterization of the top 10-30 Å of the sample. Its high mass resolution capabilities and its detection limits, on the order of 1 ppm, allow evaluation of subtle chemical differences of small quantities. Moreover, the imaging mode allows molecular mapping with submicron lateral resolution.

Experimental

Instrumentation

TOF-SIMS spectra and images were obtained using a PHI *TRIFT II* TOF-SIMS instrument. A mass-filtered ^{69}Ga liquid-metal primary ion gun was used with a DC current of approximately 600 pA. For spectral acquisition, the ion gun was operated at 15 keV with a pulse width of 12 ns. For imaging, the ion gun was operated at 25 keV with a pulse width of 30 ns. The data was acquired over a mass range of m/z 0-1500. The data was collected using an ion dose below the static SIMS limit of 10^{12} ions/cm^2. A low energy electron beam was used for charge compensation.

Sample preparation

The MAPS process is shown schematically in Figure 1. Poly(ethylene terephthalate) (PET) was derivatized in two steps to introduce carboxylic acid groups at the surface (see Figure 1A). The first step, the hydroxymethylation of the aromatic ring in PET, introduced a benzylic hydroxyl group within the PET repeat unit, which was then converted to a carboxyl group by reaction with bromoacetic acid. It is likely that a substantial fraction of the reactive groups arise from side reactions such as hydrolytic cleavage of the PET backbone, therefore, the scheme shown in Figure 1 is only approximate. After introduction of carboxylic acid groups on the surface of PET (the carboxyl derivatized PET surface is termed PET-COOH), the carboxylic acid groups were activated by reaction with pentafluorophenol (PFP) (see Figure 1B). The activated PET surface was then patterned with an amine-terminated biotin ligand by spatially-resolved reagent transfer using a PDMS stamp inked with an amine-terminated ligand (see Figure 1C). Unreacted esters were quenched by reaction with 2-aminoethoxyethanol (AEE). After printing the amine-terminated biotin on PET-COOH with a PDMS stamp, the substrate was incubated with Alexa™ 488 labeled streptavidin in HEPES buffered saline containing 0.1% (w/v) BSA and 0.02% (w/v) Tween 20 detergent for one hour (see Figure 1D).

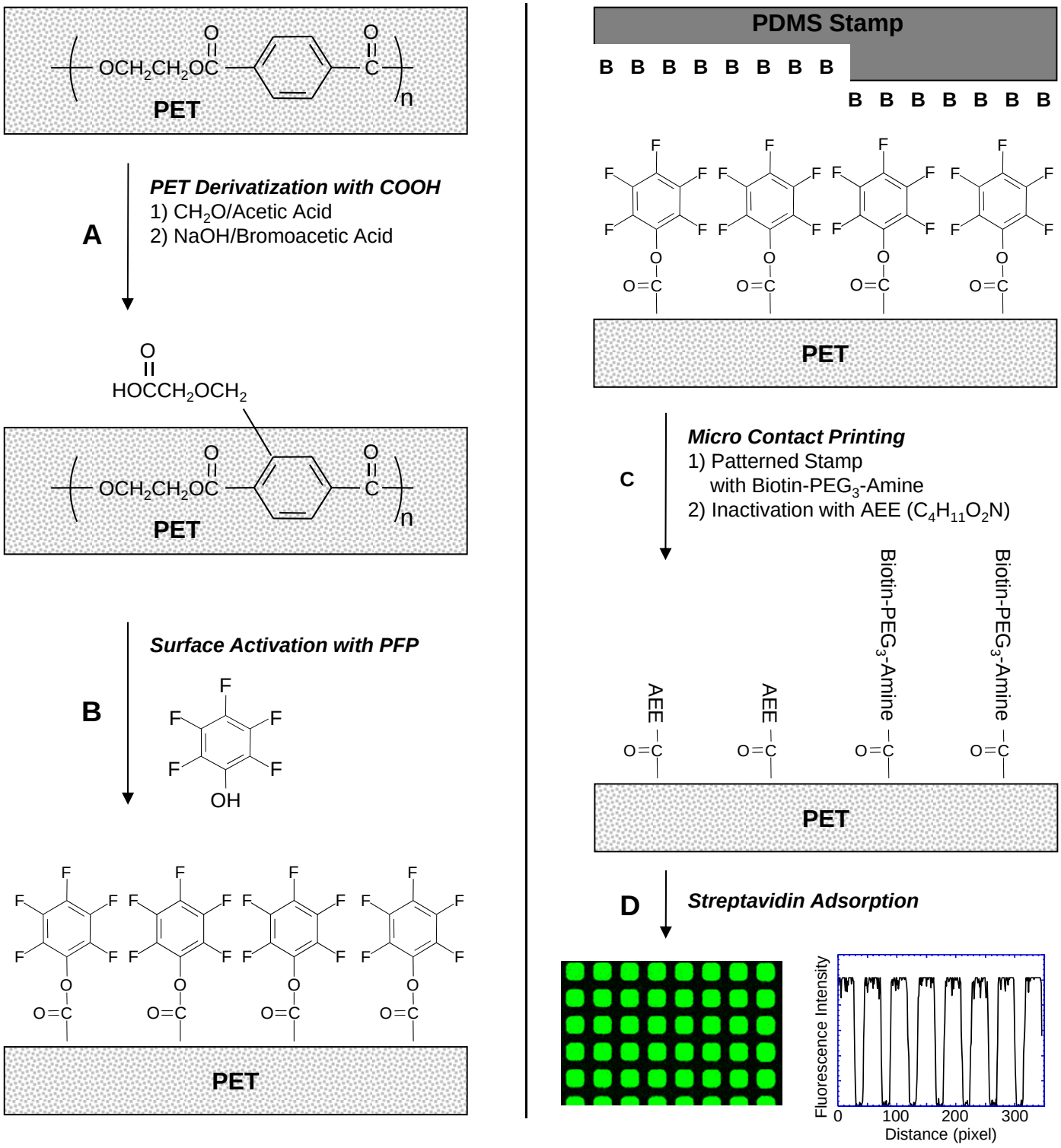


Figure 1. Schematic diagram of MAPS for micropatterning biotin onto PET, followed by adsorption of streptavidin.
A) Derivatization introduces carboxylic acid groups into PET
B) Carbolxylic acid groups are activated by reaction with pentafluorophenol (PFP)
C) An oxidized PDMS stamp inked with amine terminated biotin patterns biotin onto the sample, then AEE serves to quench the PFP molecules not derivatized with biotin
D) After streptavidin adsorption, the regions of streptavidin are shown by a Confocal image of Alexa 488 labeled streptavidin bound to amine-terminated biotin, and a line profile of the fluorescence intensity

Results

Chemical Characterization Through TOF-SIMS Spectroscopy

In order to monitor each step of the functionalization of PET by MAPS, it was necessary to first identify secondary ions that are unique to each step of the derivatization procedure. Control samples were prepared at each functionalization step and analyzed by TOF-SIMS. The positive ion spectrum of native PET shows characteristic peaks for PET at m/z 76, 104, 149, 193 ($M + H^+$) (M = the repeat unit of PET), 237, 341, 385 ($2M + H^+$), 429, and 577 ($3M+ H^+$) (Figure 2A). The positive ion spectrum of PET-COOH is qualitatively similar to that of PET (Figure 2B); the series of molecular cations, characteristic of PET are also observed in the TOF-SIMS spectrum of this sample. The peaks at m/z 45 ($C_2H_5O^+$) and m/z 65 ($C_5H_5^+$), however display increased intensity relative to unmodified PET. This increase in intensity was observed both in positive (Figure 2B) and negative ion mode (results not shown), suggesting an increased concentration of PET oligomers on the surface of the modified polymers.

PET-COOH substrates were reacted with biotin-amine by conformal contact of a flat PDMS stamp (inked with the reagent) with the surface or by reaction from solution. TOF-SIMS provided evidence for the reaction of biotin with the COOH moieties. Figure 2C shows the TOF-SIMS spectrum of biotin-amine reacted with PET-COOH in solution, where new peaks at m/z 44 ($C_2H_4N^+$), 58 ($C_3H_8N^+$), 26 (CN^-) and 42 (CNO^-) indicate the introduction of a nitrogen containing moiety. Molecular ions of low intensity at m/z 227 ($C_{10}H_{15}O_2N_2S^+$) and 270 ($C_{12}H_{20}O_2N_3S^+$) were also observed. These molecular ions represent the entire biotin molecule, and arise from the most surface oriented end of the biotin on PET-COOH.

After derivatizing the PET-COOH surfaces with biotin-amine using a patterned stamp, the unpatterned regions were quenched with the reagent 2-aminoethoxyethanol (AEE). Therefore it was necessary to analyze a control sample of AEE modified PET-COOH to identify the characteristic TOF-SIMS peaks for AEE. Compared to the reference spectra of PET-COOH, the peaks at m/z 44 ($C_2H_4N^+$), 58 ($C_3H_8N^+$), 26 (CN^-) and 42 (CNO^-) are of significance (Figure 2D). However, there are no distinct AEE ions compared to the biotin-amine. These peaks are also observed in the spectrum of PET-COOH functionalized with biotin-amine because both species contain nitrogen functionalities.

The important distinction between the spectra of PET-COOH derivatized with AEE and that with biotin-amine is the presence of the molecular biotin species (m/z 227^+ and 270^+) and the greater intensity of the 26^- and 42^- peaks in the biotin-amine spectra.

In the final step, the biotin-modified PET was incubated with Alexa 488 labeled streptavidin to demonstrate protein-ligand bonding. The TOF-SIMS spectrum of this sample exhibited unique peaks for streptavidin at m/z 70 ($C_4H_8N^+$) and 130 ($C_9H_8N^+$) in the positive ion mode (Figure 2E), and at m/z 46 (NO_2^-) and 62 (NO_3^-) in the negative ion mode.

Molecular Mapping Through TOF-SIMS Imaging

TOF-SIMS imaging allowed spatial mapping of biotin, patterned onto the activated PET, using a PDMS stamp with 40 µm square features. The square regions of biotin were best demonstrated by mapping the distribution of CN^- in the negative ion mode. The image clearly indicates the biotin ligand is spatially localized in the 40 µm square contact regions (Figure 3A). Note the CN^- map shows some signal in the regions between the biotin squares. Most likely this is due to the contribution of AEE, the reagent used to quench the regions lacking the biotin ligand. The AEE also gives a characteristic peak at m/z 26^-, but its relative significance is much lower. The peak intensity at m/z 227^+, the parent molecular peak of biotin, can be used to map the distribution of biotin unequivocally, however, its intensity is very low. In the TOF-SIMS technique the surface of the sample is destroyed; therefore, the biotin molecule most likely fragmented before significant signal-to-noise could be obtained in the imaging analysis. The image of m/z 227^+, nonetheless, demonstrates the biotin was contained in the 40 µm square regions (Figure 3B).

PFP was used to activate the entire PET-COOH surface before stamping with biotin. Figure 3C shows the image of PFP (the parent molecular ion $C_6F_5O^-$ at m/z 183) for the sample after patterning and after quenching with AEE. The PFP peak intensity corresponds strictly to the regions where biotin is absent. This suggests that the PFP hydrolysis is successful where biotin has bound to the PET-COOH but incomplete in the other regions.

Figure 3D shows the distribution of PET. A characteristic molecular fragment, m/z 104 ($C_7H_4O^+$), shows PET is most significant in the regions that lack biotin. The large biotin molecules mask the PET in the regions where biotin is present.

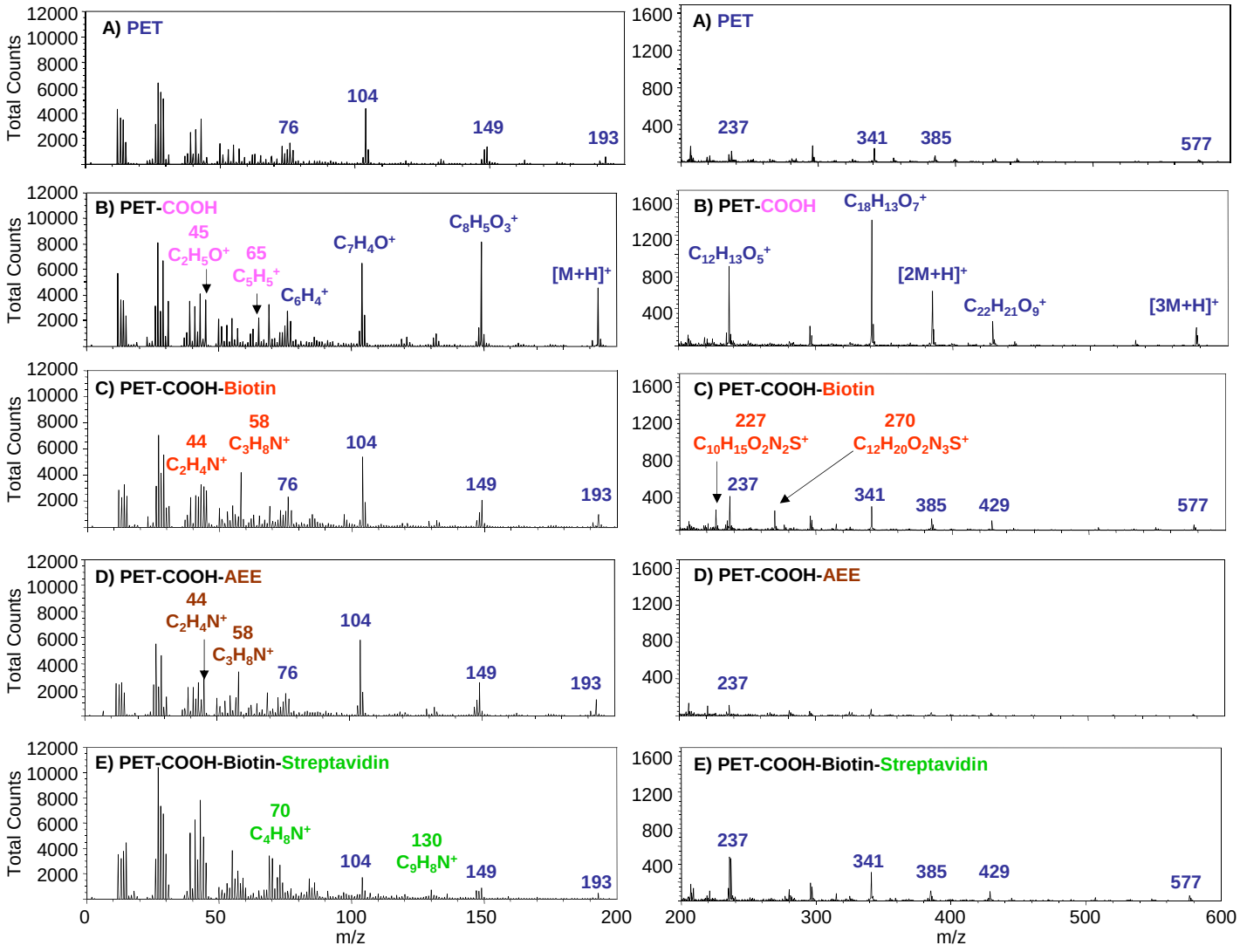


Figure 2. Positive mode TOF-SIMS spectra of PET and chemically derivatized PET. Each modification step is confirmed by the presence of unique molecular ions.

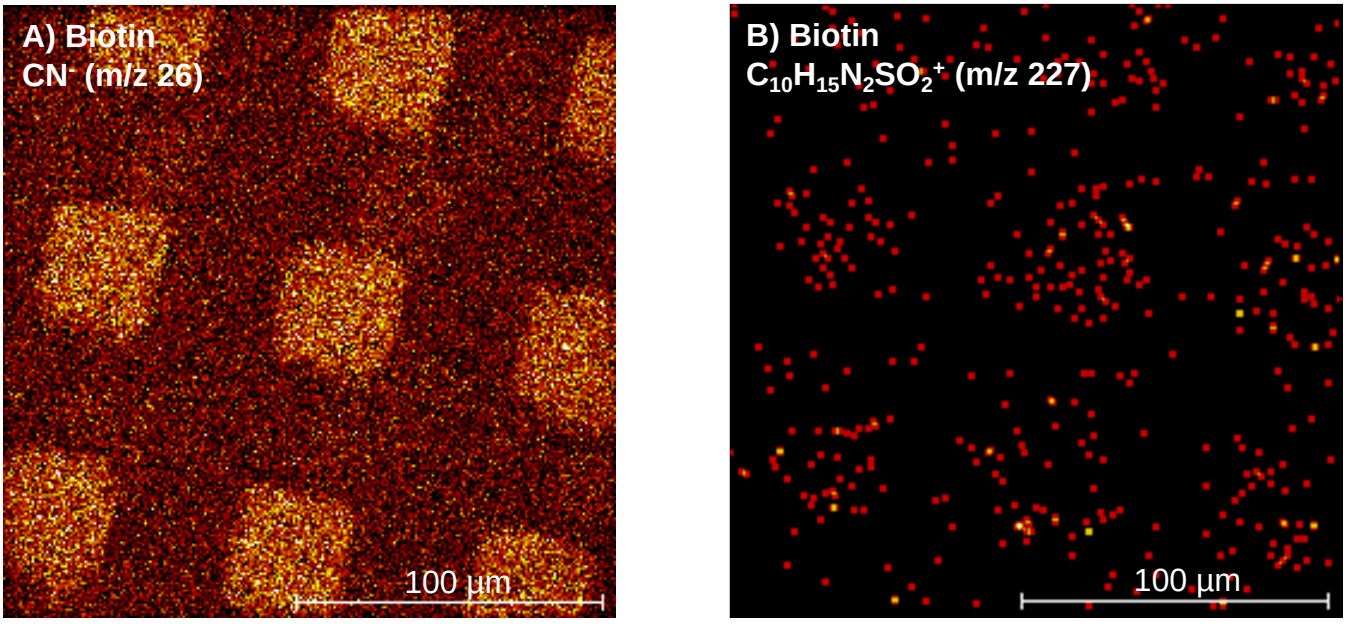


Figure 3. TOF-SIMS images obtained after patterning the PET with biotin.

In other regions, PFP and AEE are present, but these molecules are smaller and their coverage may be lower, resulting in increased PET peak intensities.

The patterned biotin samples were also analyzed by imaging TOF-SIMS after incubation with streptavidin. The 10 µm pattern in this example demonstrates the chemical modification steps are successful with very small features. The image of m/z 70^+, which is unique to streptavidin, shows the spatial localization of sterptavidin and reveals that streptavidin binds selectively to the 10 µm square patterned biotin regions (Figure 4B). In contrast, the image of m/z 104^+ for PET, shows higher intensity for regions of PET that were not in contact with the PDMS stamp (Figure 4A). The two images show a contrast inversion and demonstrate the successful patterning of PET with streptavidin.

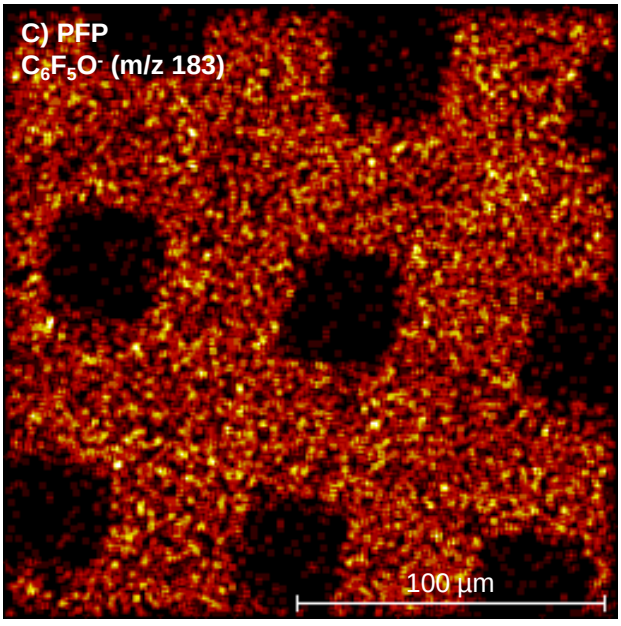
Streptavidin was incubated in the presence of Tween 20, a blocking agent composed of polyoxyethylene sorbitan monostearate. As a result, the TOF-SIMS data shows the presence of Tween 20 by significant peaks at m/z 227, 255, and 283 in the positive ion mode (Figure 4C), Note that the m/z 227 peak also is characteristic of biotin, but that no Tween 20 was present in the above example where biotin was imaged. The peaks represent the fatty acid series of myristic, palmitic, and stearic acids and are sidechains of the sorbitan molecule.

The TOF-SIMS image of this series of peaks shows the Tween 20 is preferentially located on the unstamped regions. This localization of Tween 20 helps explain the high 250:1 contrast observed for streptavidin in fluorescence microscopy. The high contrast of the fluorescence images can be attributed to the selective binding of streptavidin to the patterned biotin, as well as the preferential adsorption of the surfactant, Tween 20, to the background.

Conclusions

TOF-SIMS spectroscopy gave insight into the chemistry that resulted from chemical modification in the multi-step MAPS process. The modification steps could be confirmed by unambiguous identification of characteristic ions for expected molecular species. Since TOF-SIMS spectroscopy reveals all the species in a multi-component sample, it also served to identify contamination, uncompleted reactions, and unexpected chemistry at the surface.

The use of TOF-SIMS for molecular imaging clearly confirmed the spatial distribution of biological ligands on a polymer. It confirmed the micropatterning of biotin onto PET, and the preferential adsorption of streptavidin on the regions of biotin. The molecular ions of biotin and streptavidin were used to unambiguously map their lateral distribution.



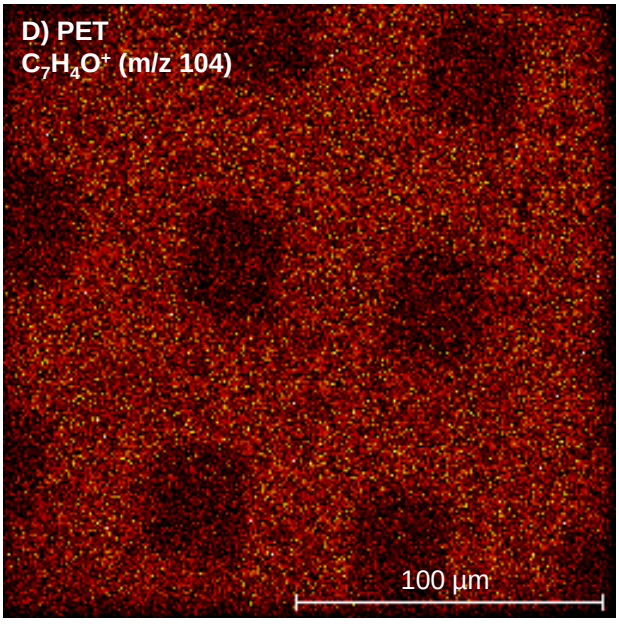


Figure 3 continued. TOF-SIMS images of a sample of biotin patterned onto activated PET using a 40 µm stamp.
A) The CN^- map confirms the presence of biotin created by the stamp
B) The map of the molecular biotin ion corresponds well with the CN^- biotin map
C) The map of PFP shows it is present in the "unstamped" regions
D) The map of the m/z 104 molecular ion of PET indicates PET is exposed in the "unstamped" regions
Note: Images A and C were acquired from the same area and images B and D were acquired from a different area

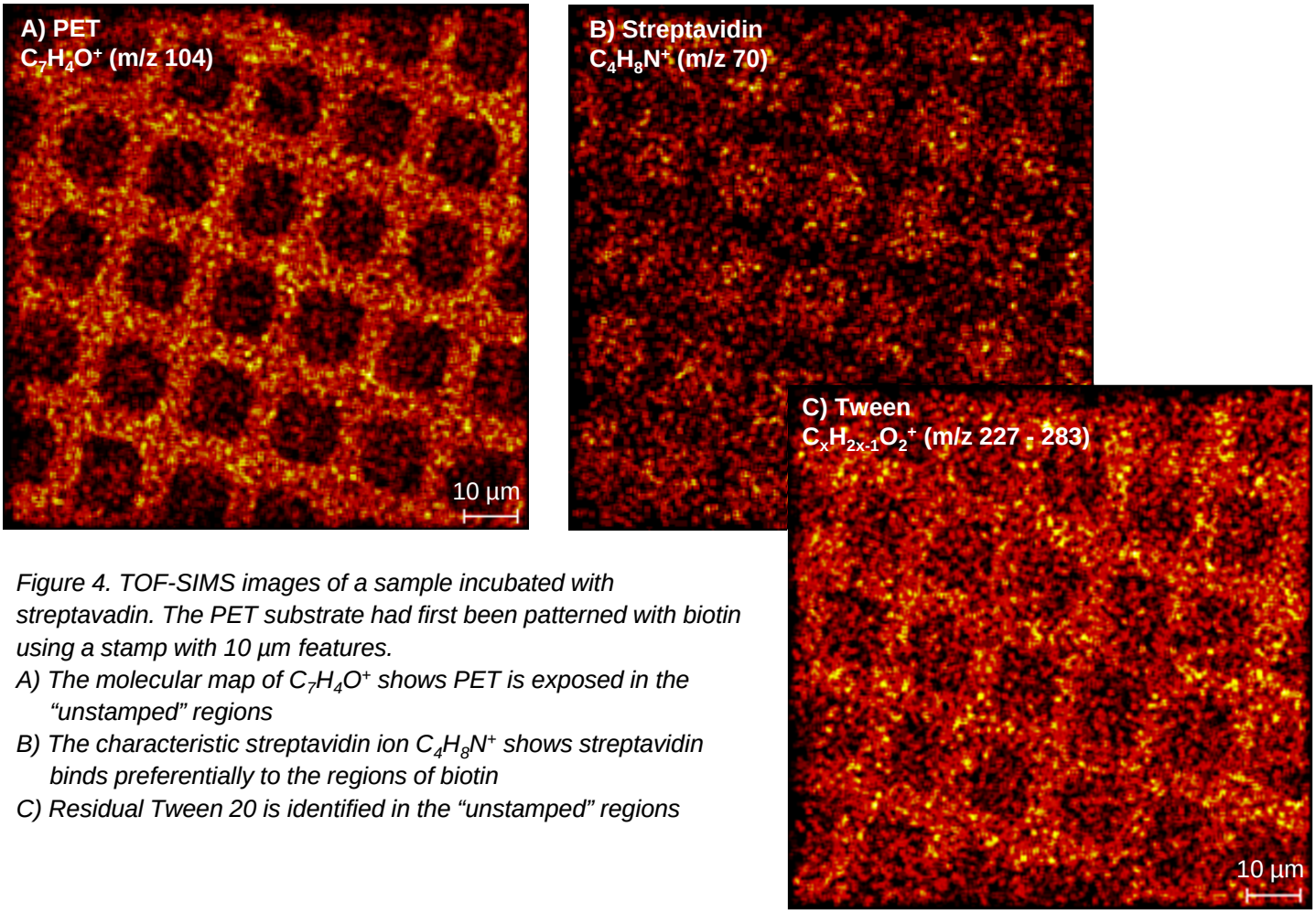


Figure 4. TOF-SIMS images of a sample incubated with streptavadin. The PET substrate had first been patterned with biotin using a stamp with 10 µm features.
A) The molecular map of $C_7H_4O^+$ shows PET is exposed in the "unstamped" regions
B) The characteristic streptavidin ion $C_4H_8N^+$ shows streptavidin binds preferentially to the regions of biotin
C) Residual Tween 20 is identified in the "unstamped" regions

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