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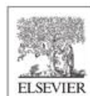

Volume 252, No. 19, 30 July 2006 ISSN 0169-4332

applied surface science

A journal devoted to applied physics and chemistry of surfaces and interfaces

Proceedings of the Fifteenth International Conference on Secondary Ion Mass Spectrometry, SIMS XV

The University of Manchester, UK
September 12–16, 2005

SIMS XV
MANCHESTER 2005

Edited by:
John C. Vickerman, Ian S. Gilmore,
Mark G. Dowsett, and Alex Henderson

SIMS Conference Series Founding Editor: Alfred Benninghoven

Volume 252, No. 19, pp. 6403–7326
30 July 2006
Available online at www.sciencedirect.com
ScienceDirect
http://www.elsevier.com/locate/apsusc

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Applied Surface Science 252 (2006) 6742–6745

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Mass spectrometric characterization of DNA microarrays as a function of primary ion species

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Received 12 September 2005; accepted 15 February 2006
Available online 9 May 2006

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Abstract

Recent studies have shown TOF-SIMS to be an appropriate method for the detailed examination of the immobilization process of PNA and its ability to hybridize to unlabeled complementary DNA fragments. Unlabeled single-stranded DNA was hybridized to Si wafer biosensor chips containing both complementary and non-complementary immobilized PNA sequences. The hybridization of complementary DNA could readily be identified by detecting phosphate-containing molecules from the DNA backbone. An unambiguous discrimination was achieved between complementary and non-complementary sequences.

In order to optimize detection parameters, different primary ions were applied, including monoatomic ions (Bi^{+}) as well as cluster ions (Bi_2^{+}, Bi_3^{+}, Bi_4^{+}, Bi_3^{++}, Bi_5^{++}), and secondary ion yield behavior and formation efficiencies were studied. It was found that cluster primary ions resulted in a significantly increased yield of DNA-correlated fragments, enabling higher signal intensities and better secondary ion efficiencies.

TOF-SIMS is undoubtedly a highly useful technique for identifying hybridized DNA on PNA biosensor chips. It is suitable for studying the complexity of the immobilization and hybridization processes and may provide a rapid method for DNA diagnostics. With the absence of the labeling procedure and the simultaneous increase of the phosphate signal as a result of increasing DNA sequence length, this technique comes to be especially useful for the direct analysis of genomic DNA.
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Keywords: DNA; PNA; TOF-SIMS; Biosensor; Microarray; Cluster ions

1. Introduction

In recent years, nucleic acid chip technology has been a subject of growing interest for clinical diagnostics as well as for DNA and cDNA sequencing, partial sequencing of clones, single nucleotide polymorphism (SNP) studies, and for identification of expressed genes. Nucleic acid chips are based on sequencing by hybridization, where unknown DNA fragments are hybridized to complementary nucleic acid sequences which are immobilized in an array format on a solid surface. The main variables in this process are the attachment of the probing nucleic acid sequences to a solid surface (immobilization), the hybridization conditions, and the detection of hybridized DNA sequences.

Currently, various techniques are used to detect hybridized DNA or RNA. Most of them use PCR for amplification, and labeling procedures such as fluorescent or radioactive tags for detection.

A recent advance combines peptide nucleic acid (PNA) microarray chips [1–6] as probes for hybridization of DNA sequences with time-of-flight secondary ion mass spectrometry (TOF-SIMS). PNA is a synthesized DNA analog in which both the phosphate and the deoxyribose of the DNA backbone are replaced by a polypeptide. Nevertheless, PNA retains the ability to hybridize with complementary DNA or RNA sequences, thus allowing PNA chips to be used in the same way as DNA chips. Combined with TOF-SIMS, a technique that can identify the presence of phosphates in a molecular surface layer with high sensitivity in the attomol range, amplification-free and label-free DNA diagnostics should enable detection of complementary hybridization of unmodified genomic DNA.

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doi:10.1016/j.apsusc.2006.02.281

To reduce the quantity of PNA/DNA needed for preparation, the bombardment conditions have to be optimized for higher sensitivity and efficiency. Therefore, the influence of different primary ion species on measurements of hybridized surfaces was investigated.

2. Terms and definitions

The following definitions have proven to be useful for a quantitative comparison of different primary ion species when analyzing molecular surfaces.

For a secondary ion species X_i^q, the yield $Y(X_i^q)$ is defined as the number of secondary ions $N(X_i^q)$ that can be detected per incident primary ion N_{PI}:

$$Y(X_i^q) = N(X_i^q)/N_{PI}$$

It is a measure of the intensity of secondary ions that can be achieved under static SIMS conditions by primary ion bombardment of the surface.

The disappearance cross-section $\sigma(X_i^q)$ corresponds to the surface area damaged by an incident primary ion and can be derived by monitoring the exponential decay of a species' secondary ion intensity $N(X_i^q)$ under primary ion bombardment according to

$$N(X_i^q) = N_0(X_i^q)\exp\{-\sigma(X_i^q)vt\}$$

with vt being the applied primary ion dose density. The area damaged by an incident primary ion is the surface area from which no further ion X_i^q can be detected due to fragmentation processes or other changes of the initial surface coverage.

The secondary ion formation efficiency is obtained by dividing the secondary ion yield by the disappearance cross-section:

$$E(X_i^q) = Y(X_i^q)/\sigma(X_i^q)$$

It indicates how much information can be derived per unit surface area and is therefore a measure for the effectiveness of the analytical process.

3. Experimental

All experiments were carried out using a reflectron-based TOF-SIMS instrument (TOF-SIMS IV) equipped with a liquid metal ion gun using bismuth cluster primary ions (Bi^+, Bi_2^+, Bi_3^+, Bi_4^+, Bi_3^{++}, Bi_5^{++}). The acceleration voltage was varied

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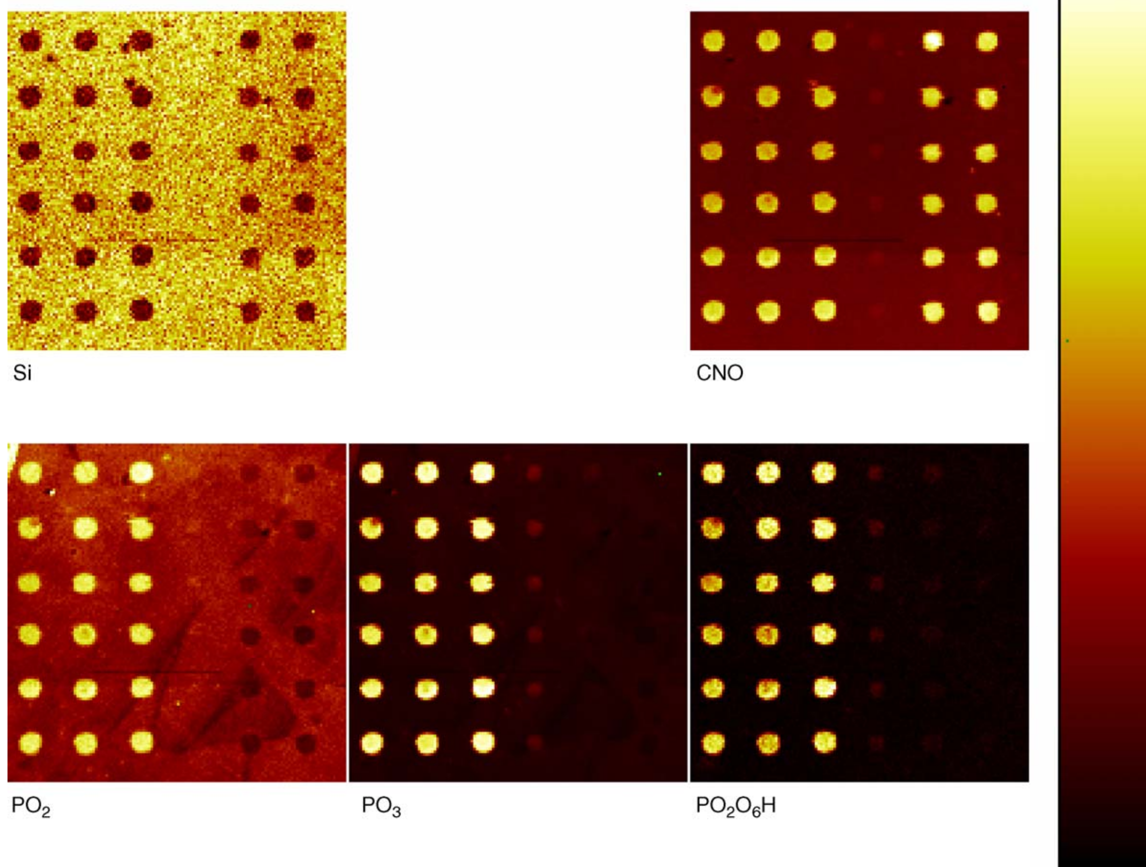

Fig. 1. Negative TOF-SIMS images (area 1500 μm × 1500 μm) of a Si wafer PNA/DNA biosensor chip acquired with 25 keV Bi_3^+.

between 10 and 25 kV resulting in effective ion energies between 10 and 25 keV for singly charged and 20–50 keV for doubly charged ion species.

Silicon wafers with a thin oxide layer (~15 nm) were used as substrate materials for PNA biosensors. They were prepared following a previous published procedure [5]. The PNA sequences spotted on the aminosilane-activated surface were 13mer oligonucleotides with the following sequences: Cxxttctccctctctc, a PNA sequence containing only cytosine and thymine (TC-PNA) and Cxxttgaatcgctcga, a mixed PNA sequence containing all four base sequences (Mix-PNA) with C being cystein, an amino acid which bonds to the surface and xx being an AEEA linker connecting the bonding molecule to the base sequence. The typical amount of PNA used per spot droplet was around 1 nl of a 160 μM solution, resulting in about 160 fmol per droplet. Note that of this amount only a small part of the molecules actually bind to the surface, with the rest washed from it in subsequent cleaning steps. Thus, the amount of PNA bound to the surface is lower by an order of magnitude or more. Typical resulting spot sizes for PNA on the chips used were about 100 μm in diameter. The remaining surface of the biosensor was deactivated with mercaptopropionic acid, which prevents hybridization of DNA by associating and non-specifically binding to the activated surface in the final step, where hybridization was done with DNA oligonucleotides complementary to one of the PNA sequences.

4. Results and discussion

Recent experiments have shown that TOF-SIMS mass spectra of negatively charged secondary ions can be used to identify DNA and PNA fragments and thus to characterize PNA/DNA biosensor surfaces and verify the hybridization process [2].

Fig. 1 shows negative secondary ion images obtained from a hybridized test PNA biosensor chip. In this experiment, two different PNA sequences were immobilized. TC-PNA was spotted in the first three columns followed by a column of neutral buffer, and Mix-PNA in the fifth and sixth column. Hybridization was carried out with a DNA sequence only complementary to the TC-PNA sequence.

In the first row, Fig. 1 displays images of Si^- as a substrate signal and CNO^- as a typical oligonucleotide fragment. Generally, these signals can be used for identifying the spot position on the chip. The second row contains images of phosphate signals PO_2^-, PO_3^-, and $P_2O_6H^-$. The data show that only for spots with the PNA sequence which is complementary to the hybridized DNA sequence (the first three columns), significant phosphate signals are observed. PO_3^- is the most intense phosphate signal with a signal-to-background ratio of more than one order of magnitude, and therefore is used for further chip characterization.

The results obtained from this biosensor chip show that TOF-SIMS can easily identify spotted areas on a biosensor chip. Unlabeled hybridized DNAs can be unambiguously and efficiently distinguished from immobilized PNAs by detecting the negative phosphate signals such as PO_3^- or other phosphate-containing DNA fragments.

In order to evaluate the best parameters for detecting hybridized unlabeled DNA on PNA biosensor surfaces, the secondary ion emission behavior of the phosphate signal PO_3^-, including secondary ion yields, disappearance cross-sections, and secondary ion formation efficiencies has been examined as a function of energy and various Bi primary ion species. For each primary ion setting, mean values were calculated from up to ten measurements. The error margin is around 10% of the mean value which is approximately the symbol size in the displayed graphs.

The left and center graphs in Fig. 2 display the variation of yields and disappearance cross-sections as a function of

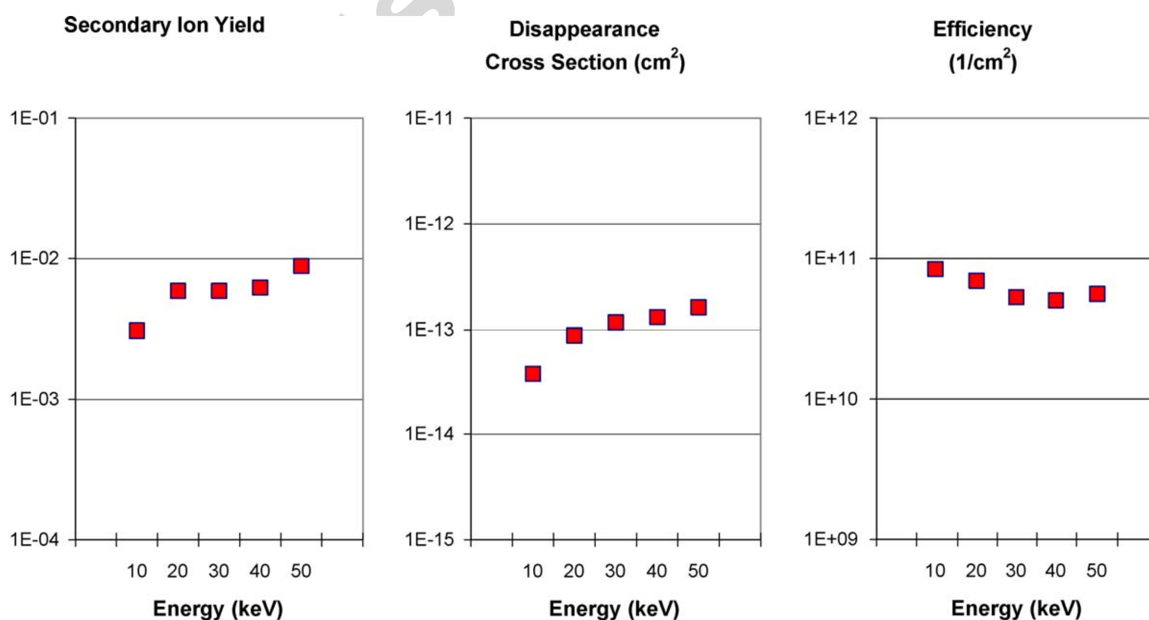

Fig. 2. Secondary ion yield, disappearance cross-section and efficiency as a function of the ion energy for Bi_3 primary ions.

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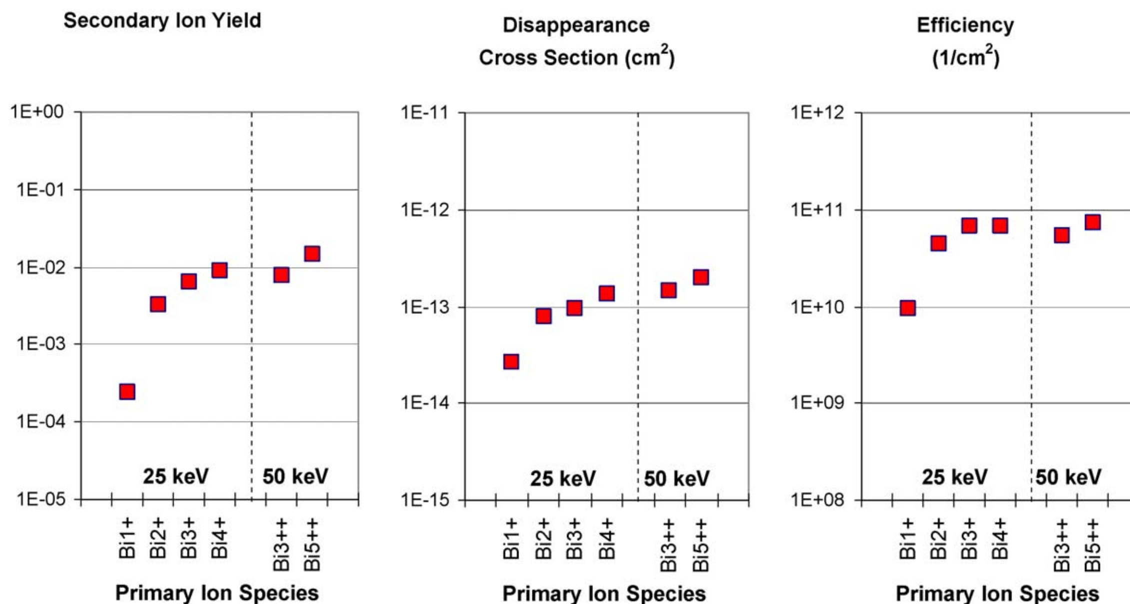

Fig. 3. Secondary ion yield, disappearance cross-section and efficiency as a function of the primary ion species for the Bi cluster ion gun operated at 25 kV acceleration voltage. Note that the doubly charged ions Bi_3^{++} and Bi_5^{++} effectively have twice the energy of singly charged ions.

different Bi_3 primary ion energies. In general, there is only a slight increase of the values with rising energy. The right graph in Fig. 2 shows the variation of the secondary ion formation efficiency. It shows no significant variation with ion energy, and even a slight decrease at higher energies was observed. The results suggest that the efficiency for the Bi_3 primary ion is near saturation over the entire energy range.

Comparing measurements with the same acceleration voltage, the change of primary ion species from Bi^+ to Bi_4^+, Bi_3^{++} and Bi_5^{++}, generally resulted in increasing yields and disappearance cross-sections. Although doubly charged species such as Bi_3^{++} effectively have twice the ion energy of singly charged ions, the influence of the ion energy itself is low as already discussed for Fig. 2.

As an example, the results for different Bi primary ion using an acceleration voltage of 25 kV will be discussed. The left and center graphs in Fig. 3 display the variation of yields and disappearance cross-sections as a function of Bi ion species. In general, the highest increase for the secondary ion yield (approximately an order of magnitude) is observed by changing from monoatomic Bi^+ to polyatomic Bi_2^+ primary ions. The disappearance cross-section increases with increasing cluster ion size.

The rightmost graph in Fig. 3 shows the variation of the secondary ion formation efficiency. The change from monoatomic to polyatomic primary ions leads to an increase of about an order of magnitude, whereas there is no significant difference between Bi_3^+, Bi_4^+, Bi_3^{++} and Bi_5^{++}, taking into account the error margin. The data indicate that there is an efficient saturation using cluster primary ions, with no further increase for higher clusters.

5. Conclusion

The data show that TOF-SIMS is a powerful technique for unambiguous identification of unlabeled DNA hybridized to PNA microarray chips, via detection of phosphate or phosphate-containing molecules resulting from DNA fragmentation. Utilizing unlabeled DNA has several advantages, including not needing any labeling or amplification procedure and the possibility of direct analysis of hybridized genomic DNA. In particular, the increase in the number of phosphates with increasing sequence length and the higher efficiency in analysis using polyatomic primary ions such as Bi clusters are advantageous for sequence analysis of genomic DNA even at low concentrations. In future PNA/DNA biosensor analysis, it should be possible to take advantage of a simplified low-energy cluster LMIG supplying doubly charged Bi_3^{++} primary ions, thus providing optimal bombardment conditions.

Acknowledgement

This work was supported by the BMBF under contract number 13N8507.

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