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Localization and Quantitative Analysis of Antigen−Antibody Binding on 2D Substrate Using Imaging NanoSIMS

Stéphanie Dauphas,[†] Thomas Delhaye,[‡] Olivier Lavastre,[†,‡] Anne Corlu,[§] Christiane Guguen-Guillouzo,[§] Soraya Ababou-Girard,[||] and Florence Geneste*[,†]

Laboratoire des Sciences Chimiques de Rennes, Equipe catalyse et organométalliques, Université de Rennes 1, UMR-CNRS 6226, Campus de Beaulieu, 35042 Rennes cedex, France, Plateforme d'imagerie analytique ONIS (GIS Europia), Université de Rennes 1, Campus de Beaulieu, 35042 Rennes cedex, France, INSERM U522, IFR 140, Université de Rennes 1, Campus de Villejean, 35042 Rennes cedex, France, and UMR 6627, Laboratoire de Physique des Atomes, Lasers, Molécules et Surfaces, Université de Rennes 1, Campus de Beaulieu, 35042 Rennes cedex, France

Iron containing-antigen bound specifically to antibody immobilized on a surface is analyzed by nanoscale secondary ion mass spectrometry (NanoSIMS). This technique is well adapted compared with X-ray photoelectron spectroscopy and energy dispersive spectroscopy, which do not allow the detection of iron. The obtained Fe^+ map gives a good representation of the antigen repartition on the surface. NanoSIMS analysis of competition experiments performed with albumin and iron-free antigen are in good accordance with results obtained by a classical fluorescence microscopy approach. These results underline the interest of imaging NanoSIMS as a label-free method, allowing the localization and quantitative analysis of antigen−antibody binding with better spatial resolution than imaging ellipsometry and SPR.

Antibody-based bioassays have been developed for various applications, including clinical diagnostics, environmental control, and food analysis.[1,2] Immunosensors are based on the selective binding of antigens to immobilize antibodies by molecular recognition. Then, a transducer converts the recognition event into a measurable signal. Imaging the protein surface binding events is essential for protein microarrays and comprehension of biological systems. Numerous techniques have been employed with inherent limitations for each of them. Labeling techniques such as fluorescence are usually applied.[3] However, the labeling process has to be well controlled otherwise it can introduce potential errors, as it can change the antigen binding properties. Label-free methods such as imaging ellipsometry[4] and surface plasmon resonance (SPR)[5–7] have also been used to detect protein adsorption. However, these methods present also some disadvantages such as limited lateral resolutions (5 μm for imaging ellipsometry[8] and 1 μm for SPR[9]) and the difficulty of estimating the refraction index necessary to obtain mass coverage of proteins on surface, especially for a mixture of adsorbed proteins.[10,11] Moreover, SPR analysis also lacks the sensitivity to detect the interaction of proteins with low molecular weight ligands. Subnanometer resolution can be reached with atomic force microscopy (AFM). This technique gives information about surface morphology but not about its composition. In addition, the immobilization of the antigen or the antibody on the tip, which is necessary to identify specific types of proteins in compositionally complex samples, is a difficult step.[12–14]

Nanoscale secondary ion mass spectrometry (NanoSIMS) is an imaging technique, allowing the detection of trace elements and isotopes.[15] It has found applications within such diverse fields as the analysis of presolar materials from meteorites,[16,17] material science,[18,19] and geology and mineralogy.[20–22] The interesting possibilities offered by the last generation of NanoSIMS 50 have also opened the way to new applications in life sciences: (i) a spatial resolution down to 50 nm if Cs^+ primary ions are used for negative ion spectrometry and

* To whom correspondence should be addressed. E-mail: Florence.Geneste@univ-rennes1.fr.

[†] Laboratoire des Sciences Chimiques de Rennes, Equipe catalyse et organométalliques.

[‡] Plateforme d'imagerie analytique ONIS (GIS Europia).

[§] INSERM U522, IFR 140.

[||] UMR 6627, Laboratoire de Physique des Atomes, Lasers, Molécules et Surfaces.

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200 nm (routinely 400 nm) with the oxygen source for positive ion spectrometry (Fe^+, Ca^+, Cu^+); (ii) the ability to detect simultaneously five different ions; (iii) a high mass resolution ($M/\Delta M$ > 5000) at high transmission (70%).[23] For example, imaging of unlabeled cells and tissue has been performed with the analysis of nitrogen atoms using $^{12}C^{14}N^-$ secondary ion.[24] The labeling of molecules with stable or radioactive isotopes also allowed the semiquantitative analysis of these isotopes (for example, $^{13}C/^{12}C$ or $^{12}C^{15}N/^{12}C^{14}N$ ratios) within subcellular compartments. Another example of biological application is the analysis of membranes to obtain quantitative information on the composition of isotopically labeled supported lipid bilayers, which is the basic assembly common to all biological membranes.[25,26]

The performances of NanoSIMS 50 led us to investigate the ability of this technique to image antigen−antibody recognition. We have previously reported a method to achieve the covalent attachment of antibodies onto carbon surfaces.[27] A classical fluorescent microscopy approach was used to confirm that the specific binding of antigen to antibody still occurred, when antibodies were immobilized according to our protocol. In this work, we report that NanoSIMS analyses allows quantitative imaging of unlabeled antigens bound specifically to antibodies with better spatial resolution than imaging ellipsometry and SPR. Moreover, the comparison with other more classical methods for surface analysis such as X-ray photoelectron spectroscopy (XPS) and energy dispersive spectroscopy (EDS) underlined the interest of secondary ion mass spectrometry techniques, which allows the detection of elements inside the sample without any depth limitations.

EXPERIMENTAL SECTION

Materials. Holo- and apotransferrin and human serum albumin (HSA) were purchased from Sigma, and delipidated fraction V bovine serum albumin (BSA) was from Roche. Texas Red human apotransferrin was from Molecular Probes (Eugene, OR).

Phosphate-buffered saline (PBS) was prepared at pH 7.4 from 1.5 mM potassium phosphate monobasic, 8.6 mM sodium phosphate dibasic dodecahydrate, 137 mM sodium chloride, and 2.7 mM potassium chloride in deionized water.

Surface Functionalization. The functionalization of graphite disks leading to *N*-hydroxy-succinimide-activated graphite disks has been published elsewhere.[27] These surfaces were allowed to react either with holotransferrin antigen (1 g L^{-1}) or with antitransferrin antibody (2 g L^{-1}) in pH 7.4 PBS for 3 h at room temperature and then washed using three baths of 20 mL of pH 7.4 PBS for 10 min each, before analyses.

Instrumentation. XPS analyses were performed under a base pressure of 1×10^{-9} mbar using a VSW HA100 spectrometer. The analyzer was operated with constant pass energy of 22 eV. X-ray source used Mg Kα excitation radiation at 1283.6 eV. The spectrometer binding energy scale was initially calibrated against the Au $4f_{7/2}$(84.0 eV) level. C 1s level (284.4 eV) on clean graphite disk served as a reference for all spectra.

The SEM micrographs were obtained with a Jeol JSM-6301F (9 kV) microscope, and EDS analyses were performed on a Jeol JSM-6400.

NanoSIMS analyses were performed on a NanoSIMS 50 standard ion microprobe (Cameca). Samples were cut and directly introduced into the instrument. An oxygen primary ionic source was used for the measurement of $^{56}Fe^+$. A 16-keV O-beam (~30 pA) was focused and raster-scanned on the surface sample. Sputtered positive secondary ions were extracted for mass analysis and collected in an electron multiplier. A mass resolving power of ~5000 ($M/\Delta M$) enabled to remove potential mass interferences. All $^{56}Fe^+$ images (50 μm × 50 μm) of the same set were recorded under the same primary ion current and with the same parameters (diaphragms, slits, time/pixel). The spatial resolution for all images was 0.4 μm.

Preparation of samples for NanoSIMS imaging of antigen−antibody recognition were done as follows. Graphite surfaces modified with antitransferrin antibodies (see above) were saturated by a solution of BSA (10 g L^{-1}) in PBS for 1 h at room temperature to avoid nonspecific binding of antigens. Then, they were placed in a BSA solution containing 10 or 100 μg mL^{-1} holotransferrin and supplemented or not with HSA (10 mg mL^{-1}) or human apotransferrin (10 mg mL^{-1}). The samples were washed three times in a solution of BSA (10 g L^{-1}) in PBS before analysis. Quantification of NanoSIMS intensity was performed by imaging analysis using Image J.

Fluorescence analyses have been described in detail previously.[27]

RESULTS AND DISCUSSION

Preliminary Study. Since antigens and antibodies are both mainly composed of C, N, O, and S atoms, it is difficult to discriminate them by their atomic composition. However, many proteins contain prosthetic groups, which may be organic (vitamin, sugar, or lipid) or inorganic (metal ions). The presence of these groups in the protein can be a good means to discriminate antigens from antibodies if the prosthetic groups contain atoms other than C, N, O, and S. It is the case for the holotransferrin used as antigen. The polypeptide chain is folded into two structurally homologous lobes (N- and C-lobes), joined by a short stretch of α helix and with each lobe binding a single ferric iron in a deep cleft between two domains (Scheme 1).[28]

Since antitransferrin antibodies do not contain iron, it can be possible to distinguish holotransferrin from antibodies by the analysis of iron. With this aim in mind, we covalently immobilized

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Scheme 1. Holotransferrin Antigen (An) Covalently Attached to the Surface (A) or Bound Specifically to the Immobilized Anti-Transferrin Antibody (Ab) (b). Texas Red Labeled Apotransferrin (An) Bound Specifically to the Immobilized Anti-Transferrin Antibody (Ab) (c)

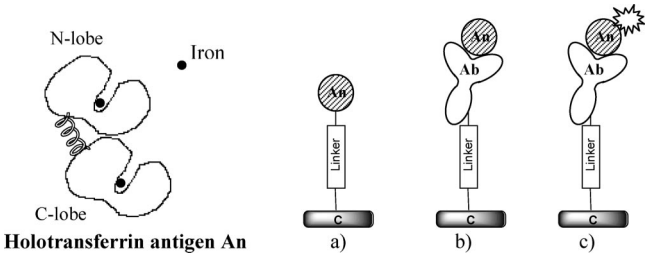

Table 1. XPS Analyses of Modified Graphite Surface

nitrogen and sulfur/carbon ratio	fresh graphite	antitransferrin modified graphite	transferrin modified graphite
% N_{1s} 400−401 eV	2−3[27]	13[27]	15
% $S_{2p1/2 + 3/2}$ 164 eV	0[27]	0.5[27]	0.4

the antigen, holotransferrin, on a graphite surface (Scheme 1a), according to a previously reported procedure (Scheme 1).[27] XPS, EDS, and NanoSIMS were employed to analyze the atomic composition of the modified surface. XPS analyses of the sample (Table 1) showed a large increase of N/C percents compared with a fresh graphite surface and the appearance of a peak of S 2p, with contents similar to what is found in antitransferrin antibodies immobilized by the same procedure.[27]

However, ferric irons present in holotransferrin antigens were not detected. A possible explanation is that XPS analysis has a sampling depth of only a few atomic layers and an element sensitivity of 0.1−1% of surface monolayer.[29] Fe^{3+} ions are not detected since they are located inside the protein at a depth of ~2 nm,[28] with a very low concentration compared to C, under the sensitivity of the technique. Since EDS analyses can determine the elemental composition of a sample with a depth of 0.5 μm, we also tried to detect ferric irons by this method. However, the presence of Fe and even S atoms was not observed by this technique. The low depth resolution (0.5−3 μm) of EDS led to a dilution of the signal, preventing the detection of low concentrated elements.

The holotransferrin modified surfaces were then analyzed by NanoSIMS. The good depth resolution of NanoSIMS (10 nm) and the possibility to analyze elements inside a sample without strong limitations of depth allowed this time the detection of iron. The Fe^+ map is given in Figure 1A.

The spatial localization of iron (spatial resolution 0.4 μm) was observed on the surface, with some iron-enriched spots ~2-μm diameter dispersed in a uniform distribution of iron and some dark regions (without iron). This result encouraged us to map the distribution of antigens specifically bound to the antibodies on the surface through iron analysis. For this purpose, antitransferrin antibodies were immobilized onto carbon surfaces,[27] (Scheme 1b) and the nonspecific binding sites of antibody modified surfaces were saturated by a solution of delipidated fraction V BSA. At this stage, NanoSIMS analysis (Figure 1B) revealed the presence of iron, probably coming from the buffer solution. Then, samples were prepared by incubation of 10 or 100 $\mu g \cdot L^{-1}$ holotransferrin in BSA solution with the coated surface. The samples were washed 3 times in BSA solutions and analyzed by NanoSims. The obtained Fe^+ maps revealed the presence of iron unevenly distributed over the surface (Figure 1C and D). The concentration of iron on the surface seemed visually to be higher in Figure 1 D than in Figure 1 C. This result is in agreement with the concentration of antigens used during incubation to prepare the two different samples.

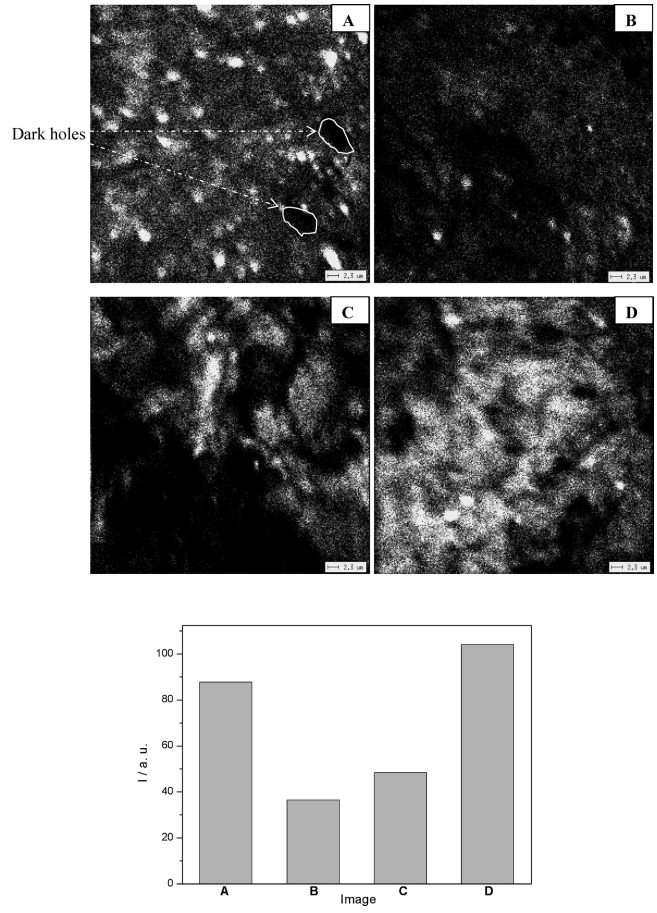

Figure 1. Fe^+ maps and NanoSIMS quantitative analysis of holotransferrin antigens attached to the surface (A) and antibody-modified surfaces saturated by a solution of BSA (B) and after incubation in a solution of 10 (C) and 100 $\mu g\ L^{-1}$ (D) of holotransferrin antigens. At least, two regions of each sample were imaged to check the repeatability of the quantification.

To understand the iron repartition observed on the different samples of Figure 1, an image of the same size of the polished graphite disk was done by Scanning Electronic Microscopy (SEM) (Figure 2).

The graphite surface exhibited a rough structure with holes of different sizes. The dark regions observed in Fe^+ map of Figure 1A, C, and D probably reflected the presence of such holes on the surface. This phenomenon can be explained by a lower collection efficiency of the secondary ions coming from the holes or by a less efficient coating of the surface inside the holes owning to the difficulty of penetration of the protein solutions.

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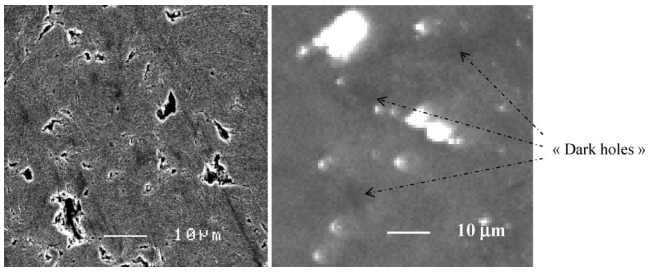

Figure 2. SEM micrograph of a polished carbon surface (left) and florescence image of an antibody-modified surface after incubation in a solution of 100 μg L^{-1} Texas Red labeled apotransferrin (right). The images are presented with the same scale as Figure 1 for purpose of comparison to NanoSIMS maps.

The highly concentrated regions (spot) observed in Figure 1 A, C, and D can be attributed to aggregated proteins. For comparison, an image obtained by fluorescence microscopy (the same size as in Figure 1) of the saturated modified surface after incubation with 100 μg L^{-1} apotransferrin labeled with Texas Red in BSA solution (Scheme 1c) is given in Figure 2. The fluorescent image is fuzzy at this size because its resolution is lower than these obtained by NanoSIMS. A heterogeneous fluorescent film can be observed with more fluorescent regions, also attributed to aggregated proteins. Some darker regions were also detected, owning to the presence of holes on the graphite surface.

As it is usually done to quantify fluorescence images, images of Figure 1 were quantified as mean gray per pixel per unit of surface. The histograms confirmed visual perception showing high concentrations of antigens in Figure 1A and D and low concentrations in Figure 1B and C. At least, two regions of each sample were imaged to check the repeatability of the quantification.

Competition experiments. To confirm that Fe^+ maps obtained by NanoSIMS analyses described above really reflected the presence of antigen bound specifically to the antibody on the surface, we performed antigen competition experiments, as it is usually done in Elisa tests. First, antitransferrin antibodies were immobilized onto the surface and the nonspecific binding sites were saturated by a solution of delipidated fraction V BSA (Figure 3A).

Then, a reference sample was prepared by incubation of 100 μg L^{-1} holotransferrin in BSA solution with the coated surface (Figure 3B). Fixation of the antigen induced an increase of the signal intensity measured by NanoSIMS (Figure 3A/B). Competition experiments were then performed using HSA (Figure 3C) and apotransferrin (iron-free transferrin) (Figure 3D). When holotransferrin was incubated with the surface in presence of a 100-fold higher concentration of HSA, no decrease in the fluorescence intensity was evidenced (Figure 3B/C). In contrast, when the incubation of holotransferrin was performed with a 100-fold higher concentration of apotransferrin, a drastic decrease in signal intensity was detected (Figure 3B/D). Good reproducibility has been obtained with two different samples in each series and on several regions of the same sample. Similar competition experiments performed using a fluorescence microscopy approach are given in Figure 4 for comparison.[27]

Both results are in accordance showing the efficiency of NanoSIMS as a quantitative imaging method for the detection of specifically bound antigens: the intensity of the signal does not decrease in presence of non-competing proteins but decreases in the presence of antigens, which must be recognized by the antitransferrin antibodies.

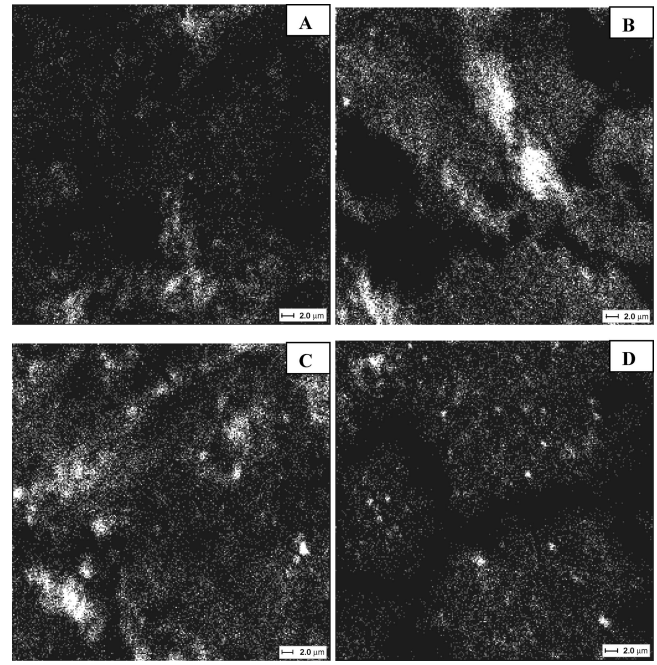

Figure 3. Fe^+ maps of the antibodies' modified surfaces (A) in the presence of holotransferrin antigens alone (B) or in competition with human albumin (C) and apotransferrin (without iron) (D).

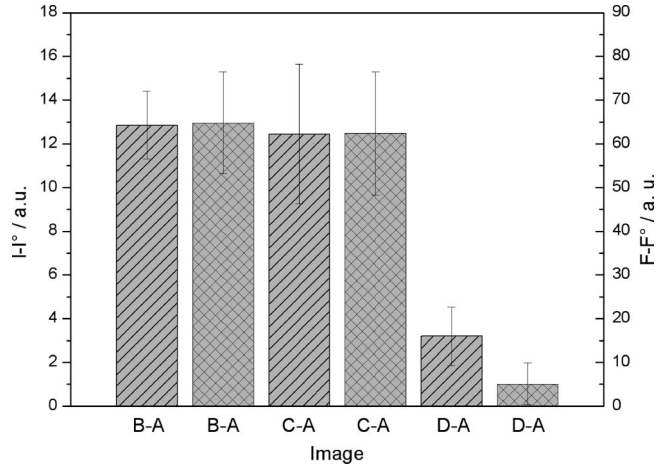

Figure 4. NanoSIMS (hatched area) and fluorescent (crossed area) quantitative analyses of the antibodies' modified surfaces in the presence of holotransferrin antigens, alone (B-A) or in competition with human albumin (C-A) and apotransferrin antigens (D-A). I (or F) is the iron intensity obtained in the presence of holotransferrin antigens and I° (or F°) is the signal in absence of antigen. Reproducibility is based on two different samples for NanoSIMS analysis.

CONCLUSIONS

We demonstrated the applicability of NanoSIMS as a label-free method to allow both the localization and quantitative analysis of antigen−antibody binding on surfaces. It provides an additional method for quantitative imaging of antigen−antibody recognition and presents several advantages: (i) compared with fluorescence,

it can avoid complicated preparation procedures to produce labeled antigens and the possible modification of antigen properties due to the introduction of a fluorophore; (ii) it can image the repartition of antigens on the surface with a spatial resolution of 0.4 μm (5 μm for imaging ellipsometry[4] and 1 μm for SPR[5–7] in the literature); (iii) it is more simple than AFM, which requires the chemical modification of tips.

Since the feasibility has been demonstrated with holotransferrin, the method should be applicable to other iron-binding proteins such as heme or nonheme iron proteins, lactoferrin, ferritin, and cytochrome *c*. The extension to other metal-binding proteins, for example, copper or calcium, should also be possible since these elements have a sensitivity close to iron (or higher for calcium) in NanoSIMS.[28]

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