Bioactive surfaces for antibody-antigen complex detection by Atomic Force Microscopy

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Abstract. Recently there has been a great develop of new antibodies immobilization procedures, that keep antibodies to retain their orientation and functionality after the binding to a solid support. This allows the formation of immune-complexes useful for the detection of biomarkers from biological samples. We have developed a new method of functionalization for solid substrates that involves an initial surface activation, then a functionalization by means of 3-aminopropyltriethoxysilane-followed by another functionalization step with a layer of very small peptides, which have a high affinity to the Antibody Fc portion, acting as antibody linkers. These antibody binding peptides can immobilize the antibodies with a proper configuration that allows an unambiguous detection of antibody-antigen complexes by means of atomic force microscopy (AFM). The AFM can act as a powerful label free detection technique, which allows to detect, in principle, single molecule interactions, with the only limitation to use substrate with low-roughness surfaces; in this case, the roughness can be interpreted as background noise in the AFM analysis. Moreover, our functionalization method can be used to obtain bioactive surfaces on a wide range of solid supports, making them capable to suitably immobilize the antibodies for the antigenic binding.
1. Introduction

Recently, there has been a growing development of antibody immobilization procedures, which are able to stabilize the antibodies in order to retain an optimal orientation and functionality after the binding to a solid support, improving the antibody avidity for the specific antigen thus providing a new generation of analytical assays with increased sensitivity and specificity toward biomarkers present in biological samples. In fact in the last decade the Enzyme-Linked ImmunoSorbent Assay (ELISA), the conventional analytical assay largely used to measure protein biomarkers from biological fluids, has been partially replaced with the protein microarray technique, which can detect simultaneously several analytes [1]. In these assays each spot of the array contains antibodies for a single antigen, i.e. the antigens are captured specifically by antibodies and revealed usually by a fluorochrome-based detection system [2]. Although microarrays can be applied to the study of several aspects of cell activity, they still have a limited sensitivity since they cannot detect and measure any analyte below a specific cut-off [3]. Therefore, the only possibility for detecting biomarkers at very low concentrations and/or present in extremely small samples (such as could be the case for some biopsies, laser capture microdissected samples, forensics microspecimens, cell lysates or supernatants from few cell cultures), is to develop new high sensitive techniques able to detect the target at the single molecule scale. Certainly a further miniaturization of analytical assays could offer several advantages in terms of higher array density, smaller volumes, reduced amount of analyte [4-7]. However current ultramicroarrays and nanoarrays system are still proof of concept prototypes in which the spot size range from few nanometers to several microns [8]. Moreover current conventional scanners used to detect fluorochrome-based recognition systems in microarrays are not capable to resolve nano-spots. All these considerations induce studies aimed at exploring the applicability of label-free techniques for single-molecule detection to analyze the cell activity in physiological and pathological conditions [9].

Our idea is to use the Atomic Force Microscopy (AFM) as label-free revealing system [10]. This approach can allow us to detect the formation of antigen-antibody immune complexes without the need of fluorochrome labeled probe, since the AFM is able to visualize single molecules only if the substrate roughness does not interfere with the measure. Therefore the substrate functionalization method plays a central role in the process of detection of the antigen-antibody complexes by AFM imaging.

There are several functionalization methods that allow antibodies to retain their orientation and/or function [11]. The most common approach consists in the use of antibody binding proteins (protein A and protein G) that specifically bind the Fc region of antibodies. Although this method is simple, cheap and does not require antibody modification, it is not suitable for AFM imaging because it produces substrates presenting high superficial roughness (1-5 nm) [12]. Others approaches are time and cost consuming as they use tagged recombinant antibodies [13-15]. Indeed, the functionalization of a suitable substrate for AFM imaging should create a mono-layer of molecules that allows antibodies to retain their orientation and functionality without enhances the substrate roughness.

The antibody height detected by AFM varies from 4 to 6.5 nm [16, 17] [4]. These sizes are lower than that reported by crystallographic analyses: 14.5 x 8.5 x 4 nm [18]. This discrepancy is mainly due to the antibody high flexibility [19, 20]. Therefore, in order to permit unambiguous identification of the antibody by AFM imaging the substrate roughness must be very low. In this paper a new functionalization procedure that use a smooth solid substrate (e.g. mica, glass and silicon) and a short peptide as antibody linkers, which has a high affinity to the antibody Fc portion, is described. This procedure allows the production of a bioactive surface with a very low roughness (below 1 nm) able to bind properly the antibody, thus keeping its bioactivity. The bioactive surface preparation involves an initial surface activation, then a functionalization by means of 3-aminopropyltriethoxysilane.
(APTES) followed by another functionalization step with a layer of the antibody binding peptide, which immobilize the antibodies with a proper configuration that allows an unambiguous detection of antibody-antigen complexes by means of atomic force microscopy (AFM).

2. Materials and Methods

2.1. Preparation of the antibody binding peptide monolayer

The antibody binding peptide could have 6-9 aminoacidic residues. We have manually synthesized three different peptides using the standard method of solid phase peptide synthesis which follows the 9-fluorenylmethoxycarbonyl (Fmoc) strategy with minor modifications.

The analysis of the crude powder was performed by liquid chromatography-electrospray mass-spectrometry (HPLC-ESI-MS) using an Agilent 1100 series LC/MSD ion trap instrument. The compound was purified by reverse phase high performance liquid chromatography (RP-HPLC) on a Shimadzu LC-9A preparative HPLC equipped with a Phenomenex C18 Luna column (21.20 x 250 nm). The molecular weight of the product was finally confirmed by electrospray ion trap mass spectrometry (ESI-TRAP-MS).

2.2. Preparation of the antibodies array for AFM and fluorescence investigation

The muscovite mica sheet was cut into (2 x 1) cm² pieces and cleaved in air. The cleaved mica was placed immediately into an oxygen plasma treatment chamber and then exposed for 2 min to oxygen RF plasma.

APTES-mica surface was obtained by placing the activated mica in a vacuum glass dessicator, which contained 50 µl of APTES 98% (Sigma-Aldrich) for 1h. The silanized substrate was then cured at 110°C for 1h.

To create a monolayer of antibody binding peptide on APTES-mica surface, 2 mg/ml peptide in DMF (Sigma-Aldrich) were treated with HATU and DIPEA (Sigma-Aldrich) to activate the carboxylic groups. The solution was then deposited on the APTES-mica substrate overnight, at room temperature, for the formation of the peptide bond between the amino groups of the APTES and the carboxylic groups of the peptide. The substrates were then washed in MilliQ water.

Five solutions with different concentrations (50 µg/ml, 1µg/ml, 10 ng/ml, 100 pg/ml, and 1 pg/ml) of anti-IL10 antibody (Biosource-Invitrogen, Carlsbad, CA) in PBS 30% Glicerol were spotted on the mica solid substrate by Piezoarray non-contact microarraying system (Perkin Elmer, Waltham, MA). The antibody array was incubated for 1 h at room temperature with a 1% mixture of aminoacids (Sigma-Aldrich) in PBS to block non-specific binding. The substrates were then washed in MilliQ water.

In order to test this procedure by means of fluorescence techniques, solutions of biotinylated recombinant human IL-10 (Peprotech, London, UK) in PBS were up to fifteen times more concentrated than the antibody solution, were deposited on the array and incubated for 1 h at room temperature, then washed with PBS. Streptavidin-Cy3 was then deposited on the array and incubated for 20 min in the dark and the fluorescence was detected by a standard microarray scanner system.

2.3. AFM imaging

The AFM analysis was performed using a Dimension 3100 Veeco AFM (Santa Barbara, CA) with a hybrid XYZ head, using Olympus OMCL-AC160 tips with nominal apical radius < 7 nm (Olympus corporate, Tokyo).
3. Results and Discussion

The idea to use AFM as detection technique induces some conditions on the characteristics of the substrate. In others words after each step of preparation the substrate must be smooth with very low roughness and must be able to bind antibodies with a proper orientation promoting the formation of the antibody-antigen complexes. Molecules such as protein A and G were not considered because of their elevated dimension. A review of the literature on this topic had shown that particular peptides could have antibody binding activity, so we focused our attention to some small peptides, which have the ability to recognize the Fc immunoglobulin portion [21]. Therefore, using peptides sequences with high affinity for the Fc region of anti-IL10 as model system, we have identified a class of peptides suitable for antibodies AFM investigation. Different peptides have been synthesized and tested. They are able to bind with high affinity the antibody Fc portion. In particular we have used the anti-IL10 monoclonal antibody as model. Indeed these peptides do not induce remarkable roughness on the substrate surface, reducing the AFM background signal, have high affinity for the antibody Fc portion and are able to link the antibody to the substrate with the proper orientation.

Figure 1: Tapping Mode AFM images of the four antibodies binding peptides investigated.
For this work we have analyzed four small peptides, which sequences are shown in table 1. They give us surfaces with very low roughness, even though with different morphology. In fact an AFM analysis of the surface obtained with these peptides shown a very different morphology, as it is possible to see in Figure 1. For instance, in figure 1c it is possible to identify globular structures, these structures disappear in the specimen a), but they are still present, although in less number, in the specimens b) and d). These structures very likely are due to the different value of the critical aggregation concentration (CAC) of these small peptides [22]. In spite of the morphological differences, the AFM-images show that all these peptides produced surfaces with very low roughness, as reported in table 1. In order to obtain the more suitable surface for our scope we have chosen the peptide that gives a surface with minor roughness. Therefore, five solutions with different concentrations of anti-IL10 antibody were deposited onto two solid supports of mica, previously coated with a layer of the peptide b). One support was used in order to verify the procedure by means of an immunofluorescence assay and the second one was used to perform AFM measurements.

Table 1. Peptides sequences and associated roughness. The roughness $R_q$ was evaluated by Nanoscope 6.14 (Digital Instrument/Veeco) AFM software.

| Peptides Investigated | Roughness $R_q$ (nm) |
|-----------------------|----------------------|
| a) COOH-G-C$_8$-V-W-G-R-W-H-NH-COCH$_3$ | 0.28 |
| b) COOH-G-C$_8$-L-L-V-T-R-P-NH-COCH$_3$ | 0.16 |
| c) COOH-G-C$_8$-L-L-T-R-P-NH-COCH$_3$ | 0.55 |
| d) COOH-G-C$_8$-L-L-T-R-P-A-M-V-NH-COCH$_3$ | 0.59 |

$R_q$ is the root mean square (RMS) average of height deviations taken from the mean image data plane.

The specimen used in order to perform the immunofluorescence assay was treated with a 1% mixture of aminoacids in PBS in order to block non-specific binding. Afterward was incubated for 1 h at room temperature with a solution of biotinylated recombinant human IL-10 in PBS up to fifteen times more concentrated than the most concentrated antibody solution. After a gentle washing with PBS it was treated with a solution of Streptavidin-Cy3 and incubated for 20 min in the dark. next the fluorescence was immediately measured.

![Figure 2: Fluorescence image of the immune assay experiment.](image)

As it is possible to see in Figure 2, the signal is very clear for the spots with higher antibodies concentration while the signal disappears for the spots with a lower antibodies concentration. In spite of the scanner resolution limit, this experiment confirms that the antibodies maintain their bioactivity after the binding with the peptide.

The second specimen was analyzed by means of AFM in order to measure the height and the width of the antibodies. We have measured an antibody average height of 4.2 ± 0.3 nm.
and an antibody average width of 34.0 ± 7.0 nm. These values, within the tip convolution effects, are in accordance with literature data [20, 23]. Further details on the possible configurations that the antibodies can assume on the surface can be find in [20]. In addition, an important step in the immunoassay setting is the blocking of the binding sites of the antibodies in order to avoid unspecific interaction. In conventional immunoassay this is performed by various blocking agents with high molecular weight, such as bovine serum albumin. However, the large dimension of these agents makes them not suitable for an AFM based detection system because their presence would interfere with the antibody-antigen complex detection. We overcame this problem using a mixture of low molecular weight aminoacids, which did not produce relevant variation of both antibody height and surface roughness. It is also important to notice that the best AFM images of the antibodies were obtained analyzing the regions corresponding to the spots with the antibodies concentration of 100 pg/ml, whereas was impossible to identify unambiguously the antibodies in the regions corresponding to the spot with higher concentration because of the high surface density of antibodies. In order to measure the heights of these structures, several AFM images were collected. The height of every single antibody was measured taking the higher point of the antibody cross-section. In Figure 3 it possible to see an example of antibodies AFM image and the histogram of the antibodies height.

![Figure 3](image-url)

**Figure 3**: A) Tapping Mode AFM image of antibodies, the red lines above the antibodies are the tracks along which the antibodies heights were measured. B) Antibody height histogram. The height of antibodies is 4.2 ± 0.3 nm.

The same regions were analyzed by AFM after that the antibodies arrays were incubated with IL10. The AFM analysis shows an increase of the average height of the antibodies. On the average this increasing is around 2 nm, more precisely the average height of these structures is 6.6 ± 0.3 nm. This increase of the height is due to the formation of the antibody-antigen complexes, in fact this value of the height is in agreement with the expected one. The AFM analysis of the antigen IL-10, performed on a sample prepared by solution casting of the antigen solution on a surface similar to that one used for the preparation of the antibody array, indicates that the antigen has an average height of 2.3 ± 0.1 nm, as it possible to see in Figure 4 where it is shown an example of antigen AFM-image and the histogram of its height.
Accordingly the difference between the height of the antibody and the height of the antibody-antigen complex fits very well the height of the antigen. This is more evident in Figure 5, where is shown an example of the difference between the cross-section of an antibody and an antibody-antigen complex.

![Figure 5](image)

Figure 5: A) Cross-section of antibody and antibody-antigen complex. B) Antibody-antigen complexes height histogram. The average height of antibody-antigen complexes is 6.6 ± 0.3 nm.

### 4. Conclusions

In conclusion our procedure of surfaces functionalization is a clear advance on current methods. In fact we developed a bioactive surface that results to be efficient in binding properly the antibodies, keeping their bioactivity, as confirmed by the immunofluorescence test. Although further studies are needed in order to set up an analytical procedure to use with biological samples, we have the proof of principle that it is possible to detect the formation of antibody-antigen complexes using the AFM as label-free technique. This result was achieved taking advantage of small peptides with a peculiar and well defined aminoacidic sequence, which confer them high affinity for antibody Fc portion. In addition we have obtained an ultra-flat surface that allows us to use the AFM as label-free detection. Major advances can be attained by the described procedure in comparison with the current
ones, if we consider that, thanks to the high resolution capability of the AFM can be possible to extend this analysis to the study of the interaction antigen-antibody in samples in which the analyte is present at extremely low concentration. Moreover, this procedure can be employed also to functionalize materials different of the mica, such as glass, silicon and some polymers as polydimethylsiloxane (PDMS).
References

1. Dufva, M., Christensen C.B., Diagnostic and analytical applications of protein microarrays. Expert. Rev. Proteomics 2005. 2(1): p. 41.
2. Pavlickova, P., Schneider E.M., Hug H., Advances in recombinant antibody microarrays. Clin. Chim. Acta 2004. 343(1-2): p. 17.
3. Borrebaeck, C.A., Wingren C., Design of high-density antibody microarrays for disease proteomics: key technological issues. J. Proteomics 2009. 72(6): p. 928.
4. Lee, K.B., Park S.J., Mirkin C.A., Smith J.C., Mrksich M., Protein nanoarrays generated by dip-pen nanolithography. Science, 2002. 295: p. 1702.
5. Nettikadan, S., Radke K., Johnson J., Xu J., Lynch M., Mosher C., Henderson E., Detection and quantification of protein biomarkers from fewer than 10 cells. Mol. Cell Proteomics, 2006. 5: p. 895.
6. Sekula, S., Fuchs J., Weg-Remers S., Nagel P., Schuppler S., Fragala J., Theilacker N., Franzreb M., Wingren C., Ellmark P., Multiplexed lipid dip-pen nanolithography on subcellular scales for the templating of functional proteins and cell culture. Small 2008. 4: p. 1785.
7. Zhang, G.J., Tanii T., Kanari Y., Ohdomari I., Production of nanopatterns by a combination of electron beam lithography and a self-assembled monolayer for an antibody nanoarray. J. Nanosci. Nanotechnol., 2007. 7: p. 410.
8. Wingren, C., Borrebaeck C.A., Progress in miniaturization of protein arrays--a step closer to high-density nanoarrays. Drug Discov. Today, 2007. 12: p. 813.
9. Ray, S., Mehta G., and Srivastava S., Label-free deection techniques for protein microarrays: Prospect, merits and challenges. Proteomics, 2010. 10: p. 731.
10. Menotta, M., Crinelli R., Carloni E., Bianchi M., Giacomini E., Valbusa U., Magnani M., Label-free quantification of activated NF-kappaB in biological samples by atomic force microscopy. Biosensors and Bioelectronics, 2010. 25(11).
11. Nakanishi, K., Sakiyama T; Kumada Y., Imamura K., and Imanaka H., Recent Advances in Controlled Immobilization of Proteins onto the Surface of the Solid Substrate and Its Possible Application to Proteomics. Current Proteomics, 2008. 5: p. 161.
12. Ohnishi, S., Murata M., Hato M., Correlation between surface morphology and surface forces of protein A adsorbed on mica. Biophys. J., 1998. 74(1): p. 455.
13. Steinhauer, C., Wingren C., Khan F., He M., Taussig M.J., Borrebaeck C.A., Improved affinity coupling for antibody microarrays: engineering of double-(His)6-tagged single framework recombinant antibody fragments. Proteomics, 2006. 6: p. 4227.
14. Torrance, L., Ziegler A., Pittman H., Paterson M., Toth R., Eggleston L., Oriented immobilisation of engineered single-chain antibodies to develop biosensors for virus detection. J. Virol. Methods, 2006. 134: p. 164.
15. Vallina-Garcia, R., del Mar Garcia-Suarez M., Fernandez-Abdul M.T., Mendez F.J., Costa-Garcia A., Oriented immobilisation of anti-pneumolysin Fab through a histidine tag for electrochemical immunosensors. Biosens Bioelectron, 2007. 23: p. 210.
16. Lee, J.M., Park H.K., Jung Y., Kim J.K., Jung S.O., Chung B.H., Direct immobilization of protein g variants with various numbers of cysteine residues on a gold surface. Anal. Chem., 2007. 79(7): p. 2680.
17. Lynch, M., Mosher C., Huff J., Nettikadan S., Johnson J., Henderson E., Functional protein nanoarrays for biomarker profiling. Proteomics, 2004. 4(6): p. 1695.
18. Silverton, E.W., Navia M.A., Davies D.R., Three-dimensional structure of an intact human immunoglobulin. Proc. Natl. Acad. Sci. U S A 1977. 74(11): p. 5140.
19. Martinez, N.F., Lozano J.R., Herruzzo E. T., Garcia F., Richter C., Sulzbach T. and Garcia R., Bimodal atomic force microscopy imaging of isolated antibodies in air and liquids. Nanotechnology, 2008. 19.
20. San Paulo, A., García R., High-resolution Imaging of Antibodies by Tappin-Mode Atomic Force Microscopy: Attractive and Repulsive Tip-Sample Interaction Regimes. Biophysical Journal, 2000. 78: p. 1599.
21. Fassina, G., Verdoliva A., Odierna M. R., Ruvo M., and Cassini G., Protein A Mimetic Peptide Ligand for Affinity Purificazione of Antibodies. Journal of Molecular Recognition, 1996. 9: p. 564.
22. Tiné, M.R., Alderighi M., Duce C., Ghezzi L., Solaro R., Effect of temperature on self-assembly of an ionic tetrapeptide. Journal of Thermal Analysis and Calorimetry, 2011. 103(1): p. 75-80.
23. Browning-Kelley, M.E., Wadu-Mesthrige K., Hari V., and Liu G. Y., Atomic Force Microscopic Study of Specific Antigen/Antibody Binding. Langmuir, 1997. 13: p. 343.