Formation of sensor array on the AFM chip surface by magnetron sputtering

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Abstract. Development of atomic force microscopy (AFM)-based nanotechnological approaches to highly sensitive detection of proteins is a perspective direction in biomedical research. These approaches use AFM chips to concentrate the target proteins from the test solution volume (buffer solution, diluted biological fluid) onto the chip surface for their subsequent registration on the chip surface by AFM. Atomic force microscope is a molecular detector that enables protein detection at ultra-low (subfemtomolar) concentrations in single-molecule counting mode. Due to extremely high sensitivity of AFM, its application for multiplexed protein detection is of great interest for use in proteomics and diagnostic applications. In this study, AFM chips containing an array of sensor areas have been fabricated. Magnetron sputtering of chromium and tungsten nanolayers has been used to form optically visible metallic marks on the AFM chip surface to provide necessary precision of AFM probe positioning against each sensor area for scanning. It has been demonstrated that the marks formed by magnetron sputtering of Cr and W are stable on the surface of the AFM chips during the following activation and intensive washing of this surface. The results obtained in our present study allow application of the developed chips for multiplexed protein analysis by AFM.

1. Introduction

Development of methods for the analysis of protein markers of human diseases at low and ultralow concentrations is a relevant area of research in modern biochemistry. In this regard, the use of nanotechnology-based approaches employing molecular detectors [1] including AFM is promising. Atomic force microscopy (AFM) enables to register single protein molecules and their complexes on the support (AFM chip) surface in counting mode [2-4], reaching subfemtomolar sensitivity of protein detetion [1, 3]. For protein analysis by AFM, preliminary concentrating of target protein molecules on the AFM chip surface at the expence of specific intermolecular interaction of target proteins with chip-immobilized probe molecules — so called biospecific fishing — is used [3]. The use of biospecific AFM-based fishing for highly sensitive analysis of viral hepatitis C [3] and HIV [5] oncomarkers is experimentally demonstrated.

Nevertheless, in the previous studies [3, 5, 6] one AFM chip was used to determine only one target protein, as only two sensor areas – an area with immobilized probe molecules against the target protein, and a control area were formed on its surface. The formation of an array containing multiple
sensor areas which can contain different types of immobilized probe molecules, will allow multiplexed analysis of protein biomarkers by AFM with single-molecule sensitivity. It should be noted that due to small scanned area of AFM (of the order of several square microns), precise positioning of the AFM probe against each sensor area should be provided to obtain correct data. In the present study, the use of an array of marks formed on the AFM chip surface by magnetron sputtering is proposed to provide visual orientation upon precise positioning of the AFM probe.

The choice of method applicable for the formation of an array of marks and sensor areas is conditioned by the surface quality requirements upon the single-molecule experiments using AFM. As a rule, the size of protein molecules is below 5 nm [7]. The surface roughness therefore should not exceed 1 nm. Hence, no particulates causing excessive roughness of the AFM chip surface must be produced during the surface marking. The marks thus formed must also be stable on the chip surface, i.e. must have good adhesion to this surface, and must not disintegrate during the further use of the chip.

Magnetron sputtering allows obtaining coatings of high purity with good adhesion to the substrate surface and low porosity even at small thicknesses. This method also enables to vary deposition rate in a broad range by changing sputtering gas pressure and power applied to the target [8, 9]. In the case of high (of the order of 10^-7 Torr) basic vacuum in the sputtering chamber, high quality coatings can be produced using not only heavy (Os, Pt) metals, but also the lighter ones (for instance, Cr). In the present study, DC magnetron sputtering deposition of chromium and tungsten nanolayers was used for the formation of an array of marks on the surface of AFM chips. After that, an array of sensor areas with immobilized probe molecules against hepatitis C protein biomarker (HCVcoreAg) was formed.

2. Methods

Rectangle (of 7×15 mm size) pieces of silanized muscovite mica (SPI, USA) treated as described in [7] were used for AFM chips fabrication. After metallic marks were formed on the chip surface, it was chemically activated to provide covalent attachment of probe molecules to the surface. Then, an array of sensor areas was formed on the activated surface by covalent immobilization of probe molecules from microdrops of their solutions dispensed on the surface. After that, the sensor areas were scanned using AFM to estimate the quality of the fabricated chips.

2.1. AFM chips marking

The chips were covered with copper shield with 16 round orifices. The diameter of each orifice was 450 μm, and the distance between the adjacent orifices was 1450 μm. The shield was fixed on the chip surface with a spring holder, and then the chip was placed into the sputtering chamber. Orion-3 magnetron sputtering system (AJA Inc., США) was used to form metallic marks (consisting of Cr and W nanolayers) on the AFM chip surface. The basic vacuum was 5.6×10^-7 Torr. The sputtering was carried out using argon plasma at sputtering gas pressure 3×10^-3 Torr. The distance between the metallic target and the specimen (AFM chip) was 15 cm. During sputtering, rotation of the substrate to be coated was provided with constant angle velocity 40 rpm. Sputtering was carried out in DC mode at constant power 70 W. In these conditions, the coating rate was 0.5 Å/s and 0.4 Å/s for Cr and W, respectively. At the first stage of coating process, Cr was deposited onto the substrate for 300 - 1200 s; At the second stage, W was deposited for 400 s. Both metallic targets were pre-sputtered for three minutes with the closed shutter prior to the deposition onto the substrate. It has to be noted that the marks formed on the AFM chip surface were insufficiently stable if Cr deposition time was less than 400 s, including the case of sputtering W directly on the chip surface without preliminary deposition of Cr (data not shown).

2.2. Formation of sensor areas

After the array of metallic marks was formed on the AFM chip surface, it was chemically activated using 3,3’-dithiobis(sulfosuccinimidy] propionate) (DTSSP) analogously to the procedure described in our previous studies [4, 5]. Briefly, 100 μL of 0.12 mM DTSSP solution in Dulbecco modified
phosphate buffered saline (PBSD, Pierce, USA; pH 7.4, 10 mM phosphate, 150 mM NaCl) containing 30\% v/v ethanol was dispensed onto the chip surface and incubated for 20 minutes in humid chamber, and then the chip was washed in EtOH : H$_2$O (1:1 v/v) at 650 rpm for 10 minutes and dried in nitrogen flow. Immediately after surface activation, the solutions of probe molecules — aptamers (Evrogen, Russia; 1 $\mu$M aqueous solution; nucleotide sequences are described in [10]) and antibodies (clone 1E5; Virogen, USA; 1 $\mu$M solution) against HCVcoreAg — were precisely dispensed onto the activated chip surface as an array of microdrops (of ~3 nL each) using Piezorray high accuracy system (Perkin Elmer Inc., USA). The microdrops were incubated on the activated AFM chip surface for 30 minutes in humid chamber, and after the incubation were intensively (650 rpm, 37°C) washed off with ultrapure water in a shaker for 45 minutes. In such a way, an array of sensor areas with immobilized probe molecules against HCVcoreAg was formed on the surface of AFM chips.

2.3. AFM scanning
NTEGRA atomic force microscope (NT-MDT, Zelenograd, Russia) was used to obtain AFM images of the chip surface. AFM scanning was performed in tapping mode in air using standard NSG10 AFM tips. The scan resolution was 256×256, and the scan size was 5×5 $\mu$m$^2$. The number of scans collected for each sensor area was no less than 5.

3. Results obtained
Figure 1 displays optical images of AFM chip with metallic marks before (figure 1 (a)) and during (figure 1 (b)) the incubation of probe molecules solutions on the chemically activated surface of sensor areas. The optical image of AFM chip after the chip washing after the incubation is also shown (figure 1 (c)). As seen from Figure 1, the marks formed by magnetron sputtering did not undergo any visible changes during the activation and subsequent washing of the chip surface.

![Figure 1](image1.png)

**Figure 1.** Optical images of AFM chip before (a), during (b, array fragment) and after (c, array fragment) the immobilization of probe molecules. Thick yellow arrows indicate the metallic marks, thin white arrows indicate microdrops (of ~3 nL each) of probe molecules solutions in sensor areas. The diameter of each mark is ~450 $\mu$m, the distance between the centers of the marks is ~1450 $\mu$m.

Figure 2 (a) displays typical AFM image of the surface of sensor area with immobilized aptamers against HCVcoreAg; the corresponding cross-section is also shown in figure 2 (B).
Figure 2. Typical AFM image of the sensor area surface with immobilized aptamers (a) and corresponding cross-section (b) indicated by a thin line in (a). AFM scan size $5 \times 5 \mu m^2$, Z scale 3 nm. The data were obtained with NTEGRA AFM (NT-MDT, Russia).

As seen from figure 2, no foreign objects with heights exceeding 1 nm is observed in the AFM image of the chip surface with immobilized aptamer probe molecules. The surface topography in the scanned sensor areas corresponds to that of silanized mica surface with immobilized aptamers [5]. This indicates that the process of AFM chip surface marking by magnetron sputtering does not cause any obstructive particulates to appear on the chip surface, and the metallic marks thus formed are sufficiently stable on the chip surface during the chip activation with further formation of sensor areas and washing. The fabricated (magnetron sputtering)-marked chips with an array of sensor areas are therefore suitable for further use in highly sensitive detection of HCV core Ag by AFM-based fishing.

4. Conclusions
Novel method of AFM chip surface marking has been developed. The results obtained allow application of the developed AFM-chips for multiplexed protein analysis. This is of interest for analytical proteomics and medical diagnostics.

5. Acknowledgements
The present study was performed in the framework of the Program for Basic Research of State Academies of Sciences for 2013-2020 and was granted with Russian Federation President scholarship for young scientists for 2016-2018 (project identifier SP-4280.2016.4).

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