AFM-FISHING TECHNOLOGY FOR PROTEIN DETECTION IN SOLUTIONS

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The review considers the possibility of using atomic force microscopy (AFM) as a basic method for protein detection in solutions with low protein concentrations. The demand for new bioanalytical approaches is determined by the problem of insufficient sensitivity of systems used in routine practice for protein detection. Special attention is paid to demonstration of the use in bioanalysis of a combination of AFM and fishing methods as an approach of concentrating biomolecules from a large volume of the analyzed solution on a small surface area.

Key words: atomic force microscopy; biospecific fishing; protein detection

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INTRODUCTION

In the 1950s, Academician V.N. Orekhovich suggested in his report that the blood could contain hundreds of different high-molecular nitrogen-containing compounds [1]. According to [2], all proteins mentioned in this report, at the current stage of medical development, serve as markers of diseases used to determine the functional status of the liver, cardiovascular system damage, kidney dysfunction, blood coagulation system, autoimmune pathologies, etc. However, the search for new disease markers still remains an urgent task of biomedical research. But the modest number of tests that have found practical application contrasts sharply with the tens of thousands of potential biomarkers published over the past two decades. One of the reasons for this situation is the limited sensitivity of analytical methods in molecular profile analysis [3, 4].

In genomics, the problem is not so acute due to the fact that a single nucleic acid molecule can be multiplied by the polymerase chain reaction (PCR). To date, the genome has been successfully decoded, but the hopes of scientists and doctors for the medical significance of the data obtained have not been justified. Progress has been made in the diagnostics of rare diseases, due to the invention of PCR more than 30 years ago rather than development of postgenomic technologies [2]. One of the reasons, as noted above, is the insufficient sensitivity of analytical methods. Enzyme immunoassays work up to the level of 10^{-10} M, allowing high-copy proteins to be detected in biological samples. "Analytical methods work up to the level of 10^{-14} M; beyond that, terra incognita begins" [2]. "A concentration of 10^{-12} M is the threshold beyond which it is advisable to express the content of molecules not in concentration units, but in "number of molecules" — as a result of an "inventory" of the molecular composition of the sample" [5]. For the purpose of "inventorying" proteins, molecular detectors are used, based on sensor elements whose sizes are comparable to the sizes of biomolecules, i.e. nanosensors or molecular detectors [6]. The development of detection methods using molecular detectors is implemented within the framework of the development of technologies related to nanomedicine: nanoanalytical genomics and proteomics in the creation of nanodiagnostics. The use of molecular detectors is an opportunity to increase the sensitivity of protein detection methods in deep low- and ultra-low-copy areas [7].

The main measurement methods used in biology may detect a signal from only an insignificant part of protein molecules. In this case, the "part" is an ensemble of molecules, which does not take into account the "individuality" of each molecule. What sensitivity of the analytical method is necessary to identify single precursors associated with molecular pathology before clinical manifestations [2]? In a small tumor (less than 1 mm^3 [8]), which is not visible with modern imaging methods (magnetic resonance imaging), there are several million transformed cells, but it is not clear whether biomarkers are present in the blood. Hori and Gambhir [8] performed calculations based on a mathematical model describing the dynamic kinetics of biomarkers in plasma relative to tumor growth, starting from a single cancer cell. The calculations showed that for a tumor with a diameter of about 1 mm, a single biomarker would have a concentration in the blood of about 10^{-15} M. Thus, it is difficult to detect markers of the pathological process in blood plasma due to their low content (about 1 molecule of the marker in 1 μl of blood).

According to Rissin et al. [9], for the diagnosis of oncological and viral diseases, the detection limit (DL) of diagnostic methods should also be below 10^{-15} M. However, the sensitivity of most currently used standard high-throughput proteomics methods, such as mass spectrometric (MS) methods using

one-dimensional or two-dimensional electrophoresis, liquid chromatography, is characterized by DL ≤ 10^{-12}–10^{-14} M [10, 11].

It is necessary to increase the concentration sensitivity of analytical systems by several orders of magnitude in order to detect a wide variety of low abundant functional proteins, which can potentially be disease markers [3, 12, 13].

Where to look for low-copy proteins? In proteomic studies, blood serum is of considerable interest for the search for disease biomarkers. Protein changes in response to pathological processes in the body make serum an attractive sample for clinical studies [14]. Despite these advantages, serum proteome analysis is a very complex task due to the wide dynamic range of concentrations of proteins. Detection of target analytes at low concentrations is also difficult due to the presence of highly abundant proteins in blood serum, high levels of salts and other interfering compounds [3, 15, 16].

Limited volume of sample material (serum or plasma) is the second reason for the need to increase the concentration sensitivity of bioanalytical methods. Calculations have shown that the sample volume should be about 1 μl to identify a protein at a concentration of 10^{-8} M in serum using MS methods. To identify a protein at a concentration of 10^{-15} M, it should be about 10 l, which is obviously impossible in reality [3].

1. METHODS OF HIGHLY SENSITIVE DETECTION OF PROTEINS IN SOLUTIONS

Considering the methods used to solve biological problems, one should take into consideration that the task of studying the properties of individual biomacromolecules and the task of detecting these molecules in solution lie in different planes. Methods of highly sensitive detection of proteins in solutions should take into account the following factors: the possibility of detecting proteins in solutions at concentrations below 10^{-12} M; the possibility of detecting proteins in complex biological fluids (blood serum and plasma); "technologicity" of the method for serum proteome analysis, including the complexity of the sample preparation procedure, since the difference in sample preparation, including the need to introduce labels, influences the reproducibility of the proteome analysis results.

The Serdyuk's textbook [17] states that "we learn about the existence of macromolecules only through methods by which they can be observed ... There is no single method that provides all the necessary information about macromolecules and their interactions." The capabilities of some biophysical methods are shown in Figure 1. As can be seen from Figure 1, despite the very high resolution of NMR and crystallography methods (0.1–1 nm), a sufficiently large amount of biological material (10^{9}–10^{15} averaged biomolecules) is required to conduct research.

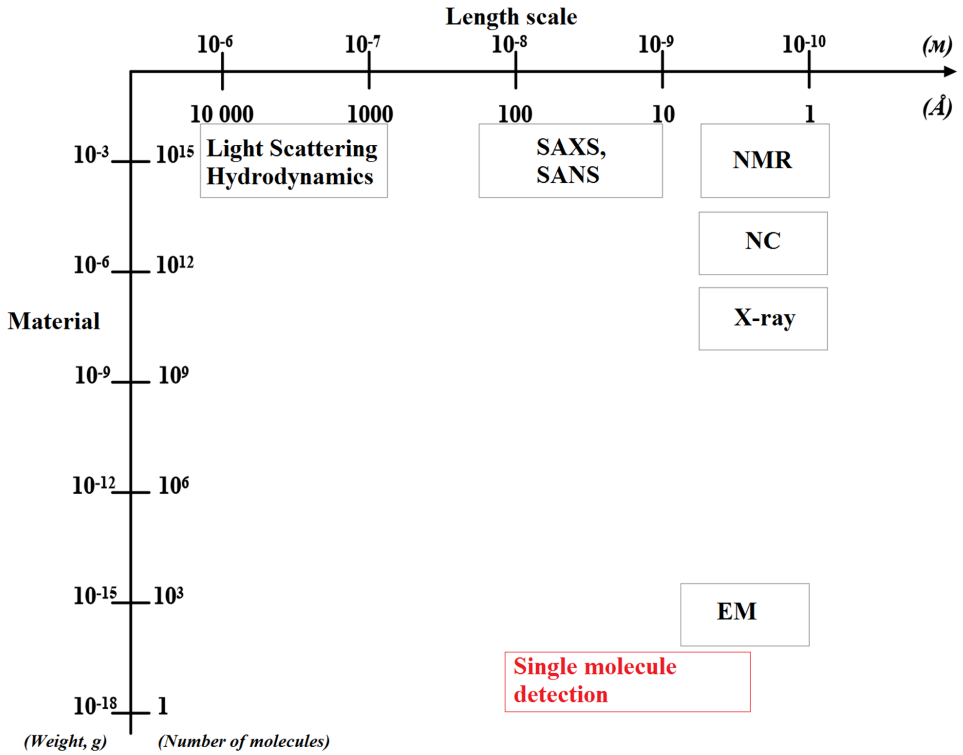


Figure 1. Characteristics of some biophysical methods in the study of biomolecules: the achievable spatial resolution ("Length scale") and the required sample mass for the application ("Material"). Abbreviations: g – grams; SAXS and SANS – small-angle X-ray and neutron scattering, respectively; NMR – nuclear magnetic resonance in solution; NC – neutron crystallography; EM – electron microscopy. The number of molecules is calculated based on the assumption that the molecular mass of the material is about 10^{5} g/mol. Adapted from [17].

The methods indicated in Figure 1 can be used to study the properties of individual biomacromolecules, but not to detect them.

Pleshakova et al. [18] have summarized the methods that basically record a signal from an ensemble of molecules; using these methods proteins in solutions at ultra-low concentrations (below 10^{-16} M) have been experimentally detected. The situation has not changed fundamentally since the publication of the review in 2015. In the paper by Efimov et al. [19], two methods were compared using the example of detecting the coronavirus nucleocapsid (N) protein by the DR LPG (double resonance long-period grating) method and SPR (surface plasmon resonance). The advantage of the DR LPG method was shown, since it was used to detect a concentration of 13×10^{-15} M, while using the SPR method a concentration of 126×10^{-15} M of the N protein was detected. Ahmadivand et al. [20] used an improved SPR method known as a plasmonic metasensor. Antibodies to the coronavirus spike protein were used as molecular probes, and bovine serum albumin (BSA) was used as a control protein. The signal was amplified using colloidal gold particles conjugated with the antibodies. The authors reported that this method could detect proteins at a level of 4.2×10^{-15} M. Bodily et al. [21] also detected synthetic analogues of beta-amyloid (Aβ), tau (τ), and α-synuclein (αS) proteins involved in the development of neurodegenerative diseases. The authors used DNA aptamers Aβ7-92-1H1 (Aβ), F5R1 (αS), and IT2 (τ 441), specific to the studied proteins, as molecular probes. Using the GFET biosensor, the above-mentioned proteins were detected at femtomolar concentration levels: 10×10^{-15} M Aβ, $(1–10)\times10^{-12}$ M τ, and $(10–100)\times10^{-15}$ M αS [21].

Below we consider in more detail the possibilities of using atomic force microscopy (AFM) as a basic method for detecting proteins in solutions with low concentrations.

2. COMBINATION OF AFM AND FISHING METHODS FOR DETECTING BIOMACROMOLECULES

AFM has certain advantages over other methods for studying nanoscale objects: the method is non-destructive, has a high resolution; it does not require complex sample preparation and can be used in both air and liquid phases thus providing information on a wide range of physical properties of the sample. At the same time, using this method it is not possible to analyze the chemical composition of the studied sample and the AFM-based analysis is time consuming. In addition there is an important precondition for the AFM application: the studied object must be fixed on the surface [22].

AFM allows measuring the height and volume of a protein globule immobilized on the surface of a substrate [23, 24]. Determining these parameters allows recording the event of formation of protein complexes on the surface. This ability to distinguish between a protein in a free state or as a part of the complex on the surface of a substrate justifies the use of AFM as a basic method in a bioanalytical system designed to detect proteins in solution. However, as mentioned above, the use of AFM requires protein fixation (immobilization) of the studied object on the surface. In the case of protein detection in solution this is realized by using the fishing procedure: concentrating target proteins on a small area of the surface from a large volume of the analyzed solution. The process of detecting proteins using a combination of AFM and fishing (AFM-fishing) consists of two stages [23]: (1) biomolecule concentrating from a large volume of biological fluid on a small surface; (2) signal recording from the molecules on the surface using AFM (registration of parameters and counting of single molecules or complexes).

2.1. AFM Chip Format

The surface (its properties and geometry) is an important factor for AFM-fishing. In the terminology of AFM analysis, a specially prepared atomically smooth substrate is called an “AFM chip”. Developing an AFM chip designed to detect proteins in solutions, it is necessary to take into account the prospects for using AFM chips for multiplex analysis. The most capacious term defining the essence of a biochip designed for both proteomic problems and medical diagnostics is the English term “microarrays”, i.e. an organized array of molecules on a substrate platform [25]. The word “chip” in this context means a “piece”, “fragment” of a plate made of glass, silicon, plastic. Traditionally, chip diagnostics is based on the registration of ligand-receptor interactions. One of the interacting partners, a ligand or receptor (generalized name “molecular biological probe”), is immobilized on a small, but strictly defined (specified) surface of the chip, a sensor zone, which is called a spot [25]. Each spot contains one type of molecular probe on the surface. In AFM analysis, an array of spots can also be organized on the surface; in this case the spot surface is modified with a cross-linker, a reagent aimed at immobilizing biomolecules due to the formation of a covalent bond between the cross-linker and biomolecule groups.

An example of bioanalysis using AFM-based chips can be a system implemented by the BioForce (USA) for rapid, sensitive and non-invasive detection of viruses [26]. The developed platform consists of a silicon chip with applied ultramicrofields of antibodies (20 fields per chip) and an AFM registration system.

Dubrovin et al. [27] demonstrated the use of oligonucleotide microarrays for gene diagnostics. The AFM has been used to characterize the morphology

of individual molecules on the surface of the carrier (a microarray designed to detect nucleic acids). An approach based on AFM surface imaging is proposed to evaluate the results of hybridization of nucleic acids labeled with gold nanoparticles on silicon microarrays.

Protein chips are less common than DNA chips, but they represent an actively developing technology that promises to become a powerful tool, including determination of biomarkers of various diseases [28, 29].

In addition to the registration system, one of the factors affecting the efficiency of protein detection using chip technologies is the choice of substrate (underlay) [28]. Choosing the optimal substrate, it is necessary to evaluate such parameters as the size and morphology of the sensor zones (spots), the efficiency of binding to antibodies (or other ligand molecules), background signal, sensitivity threshold, and reproducibility of results between different chips. Thus, the substrate should have a high binding capacity, the ability to retain the activity of ligand molecules, and a high signal-to-noise ratio. It should be taken into account that the optimal conditions may vary for each specific chip and the tasks for which it is designed.

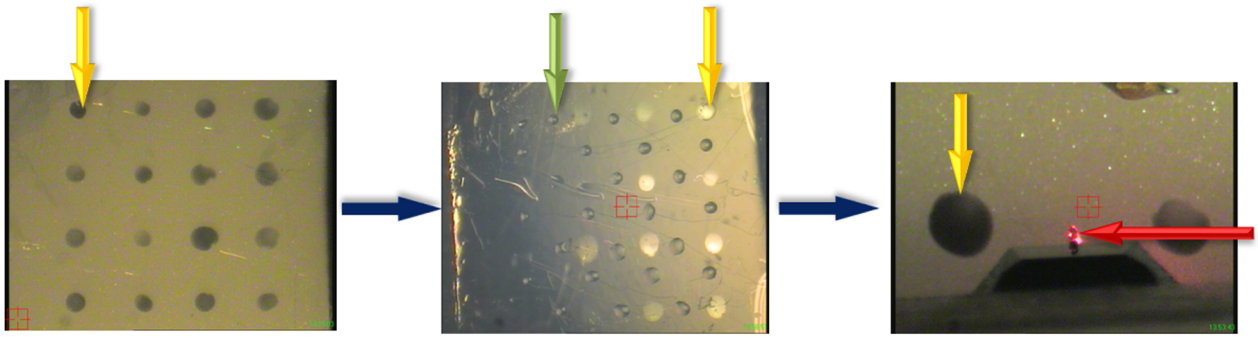
To ensure visual orientation and precise positioning of the cantilever over each spot (Figure 2 (III)) for subsequent AFM imaging, our group developed a surface marking method based on the formation of optically visible metal labels on the AFM chip using a magnetron sputtering device [30]. An example of the surface of the AFM chip with applied labels is shown in Figure 2 (I).

2.2. Size of the Sensor Zone on the AFM chip surface and the Calculated Estimate of the Analytic Sensitivity

The calculated justification of the fishing parameters and the theoretical estimate of the sensitivity were carried out based on the following initial data. Table 1 shows the maximum number of biomolecules that can be caught from a solution with different volumes and concentrations, designated as N_V, i.e. this is the maximum number of molecules in the solution, calculated as:

$$N_V = N_A \times C_V \times V \quad (1),$$

where $N_A = 6.02 \times 10^{23}$ is Avogadro's number, $mole^{-1}$; C_V is the volume concentration of protein mol/l (M); V is the volume of the sample, l.



I II III

Figure 2. Formation of a sensor zone array. (**I**) AFM chip with metal marks (yellow arrows); (**II**) Droplets of solutions (green arrow) are applied to the chip surface by using a pipetting robot between the labels, which form an array of molecules on the substrate; (**III**) To scan the surface after the washing and incubation procedure, the cantilever (red arrow) is positioned over a specified zone (spot) with control by the photo image.

Table 1. Number of biomolecules in solution with different volume and concentration (N_V)

Solution concentration (M)	Solution volume			
	1 µl	1 ml	100 ml	500 ml
10^{-10}	60 200 000	60 200 000 000	6 020 000 000 000	30 100 000 000 000
10^{-11}	6 020 000	6 020 000 000	602 000 000 000	3 010 000 000 000
10^{-12}	602 000	602 000 000	60 200 000 000	301 000 000 000
10^{-13}	60 200	60 200 000	6 020 000 000	30 100 000 000
10^{-14}	6 020	6 020 000	602 000 000	3 010 000 000
10^{-15}	602	602 000	60 200 000	301 000 000
10^{-16}	60	60 200	6 020 000	30 100 000
10^{-17}	6	6 020	602 000	3 010 000
10^{-18}		602	60 200	301 000
10^{-19}		60	6 020	30 100
10^{-20}		6	602	3 010

Another factor that must be taken into consideration for assessing the measurement parameters and fishing parameters is the scanning speed and the frame size of a single scan. Figure 3 shows an example of a chip surface scanning scheme.

The size of the scanning area, a single frame, which is an image of the surface topography (S_{fr}), — is usually no more than 25 μm². With such area and a number of scanning lines of 256, the scanning step is 20 nm; this allows registering a protein with linear dimensions of ~5 nm typical for a conventionally averaged macromolecule. The total scanning area is S_{scan} = n×S_{fr}, where n is the number of frames obtained during measurements. In the example in Figure 2, for the sensor zone in the callouts n = 20, and S_{scan} = 500 μm². To normalize the obtained AFM analysis results and ensure the possibility of comparing the results obtained in one case or another, all data are recalculated for the same scanning area (for example, 400 μm²).

Our research group uses AFM chips with two main sizes of the S_{area} sensor zone were used. For a "small" sensor zone (S_{area} = 17663 μm² or ~0.02 mm²) formed using a pipetting robot, and a "large" one formed by applying the solution using a laboratory pipette (S_{area} = 1766250 μm² or ~1.8 mm²), the scanning time of the entire zone can be estimated. Table 2 shows the calculation parameters and the obtained values, as well as the scanning time of an area of 400 μm², selected as the area for normalizing the results.

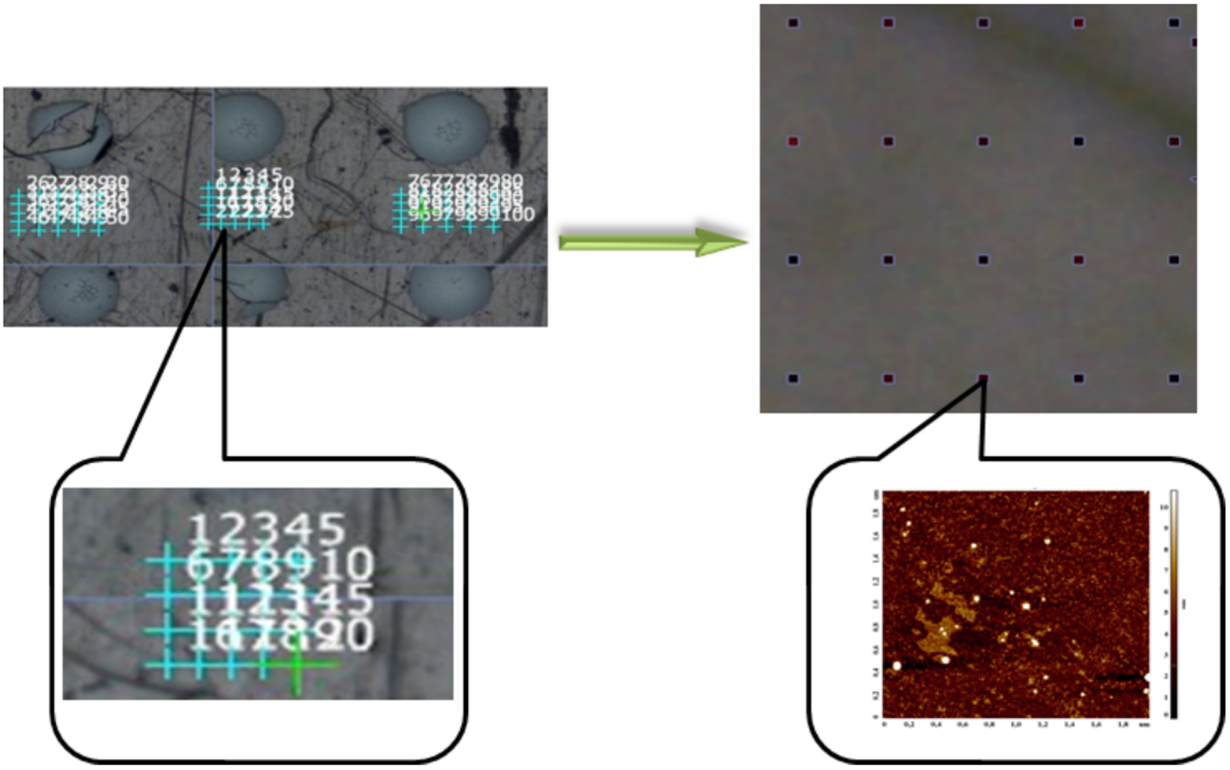


Figure 3. Example of the scheme for selecting scanning points on the AFM chip surface. The left panel is the assignment of scanning points on the video image of the chip surface with labels (an example is given for three sensor zones). The right panel is the scanning result – a set of frames at the specified scanning points. In the callouts, an enlarged image of a surface area with an array of points within one sensor zone (left panel) is shown; a separate frame in the array of obtained scanning data (right panel).

Table 2. Initial data and results of calculation of the total scanning time of the sensor area on the AFM-chip surface

	S_{area}	Frame number on the area S_{area}	Scanning parameters				Scanning time	
			S_{fr}, μm²	Scanning frequency, Hz	Number of points per frame	Scanning step, nm	Single frame	Whole area of the sensor zone
Standard AFM	400 μm²	16	25	1	256	20	4.3 min	1 h
	0.02 mm²	707						50 h
	1.8 mm²	70650						5024 h
Medium-speed AFM	400 μm²	16		5			0.9 min	0.2 h
	0.02 mm²	707						10 h
	1.8 mm²	70650						1005 h

As can be seen from Table 2, using standard AFM devices that allow scanning at a speed of 1 Hz (one line per second) it is possible to visualize 400 μm^2 in 1 hour, but if it is necessary to scan the entire area of the sensor zone, the time increases dramatically. In case of using medium-speed AFM and increasing the scanning speed by only 5 times, it is possible to obtain information about the surface of the "small zone" in 10 hours. Using standard AFM, S_{scan} = 400 μm^2 (designated S_{400}) is reasonably suitable for normalizing the obtained results. The time given in Table 2 is the calculated scanning value without taking into account positioning, approaching and retracting the probe to the surface, etc. Thus, the actual measurement time increases by 2–3 times. To evaluate the experimental results, it is necessary to take into account that S_{400} represents ~2.3% of the area of the "small" sensor zone or ~0.02% of the "large" zone.

The problem of long measurement times on AFM can be solved by increasing the scanning speed (as shown in Table 2), as well as by automating the process. Currently, using the Russian AFM Titanium device (NT-MDT), it is possible to obtain up to 700–800 frames from the surface of one sample within two days. Scanning can be performed at specified points, as shown in Figure 3, thus allowing a researcher to obtain a sample of frames reflecting the surface state of various areas of the sensor zone.

Table 3 shows the estimated number of biomolecules ($N_{400,theor}$), which can be recorded on a surface area of 400 μm^2 in the case of fishing of all the molecules from the volume and their uniform distribution over the entire area of the sensor zone.

The data are given for the types of sensor zones described above in the section "AFM chip format". $N_{400,theor}$, which can be used as approximate values of the detection limit $DL_{400,theor}$, are highlighted in color in Table 3. The limits were chosen based on the noise level (500 objects/400 μm^2) — the number of non-specific objects with a height of more than 1 nm present on the surface of silanized mica [31]. It should be noted that this assessment was made without taking into account the linear dimensions of biomolecules.

The calculation data presented in Table 3 have shown that the minimum volume from which it is advisable to fish a protein in the case of using AFM-fishing is 1 ml. It is also obvious that the minimum value of $DL_{400,theor}$, ~10^3 can be achieved with a minimum area of the sensor zone and a maximum volume of the analyzed solution: S_{area} = 0.02 mm^2 and 100–500 ml.

3. TYPES OF AFM-FISHING

The variety of types of AFM chips, differing in surface properties and geometry of sensor zones, is due to various research problems that can be solved using the AFM-fishing technology (Table 4).

The "Non-specific fishing" group can be used to solve methodological issues. In the "Non-specific irreversible (chemical)" fishing scheme (Table 4, line 1), a silanized mica substrate activated by a cross-linker is used. In the case of using the chemical AFM-fishing method [31], analyte molecules are fished from the solution not on ligand molecules, but directly on the surface of the chemically activated zone, which occupies a small part of the surface of the AFM chip (sensor zone). Non-specific irreversible fishing of protein molecules located in the analyzed solution occurs due to the use of a chemically activated zone. During incubation in a protein solution, a chemical bond is formed on the surface of the chip between the active groups on the surface and the groups of the fished biomolecule. The purpose of using a cross-linker instead of ligand molecules is to exclude the process of dissociation of the ligand-target complex. The formation of a chemical bond allows irreversible fixation of biomolecules on the surface.

In the "Non-specific reversible" fishing scheme (Table 4, line 2), highly oriented pyrolytic graphite (HOPG) is used as an AFM chip. Graphite is a conductive material, whose surface has hydrophobic properties. The purpose of using an HOPG AFM chip is to use electrical forces as a factor influencing the effectiveness of fishing [32].

In the case of using chips with immobilized ligand molecules (aptamers or antibodies), the target biomolecules are fished due to the biospecific ligand-target interaction. This type of fishing can be designated as "Biospecific". The ligand-target interaction is reversible, but the dissociation of the ligand-target complex can be avoided by forming a chemical bond between the groups of the probe molecule and the target molecule. In this case, the ligand-target binding becomes irreversible. In the case of using the traditional scheme of biospecific interaction between the ligand immobilized on the chip surface, fishing is designated as "Biospecific reversible fishing" (Table 4, line 3). In the case of reversible binding, the number of complexes formed on the surface is determined by the dissociation constant K_d. In irreversible fishing, the complexes formed on the surface are additionally covalently cross-linked; this converts the reversible complex formation reaction into an irreversible one and increases the concentration sensitivity of protein complex detection by removing the K_d limit. To achieve the goal of "irreversibility" of biomolecule binding in the complex, a procedure for modifying immobilized ligand molecules (e.g. antibodies) using cross-linkers can be used, as described in [3], or aptamers containing an active group capable of forming a covalent bond with the target protein molecule can be used, by analogy with SOMAmers [33, 34]. In this case, the ligand-target complexes are formed on the chip surface during incubation due to biospecific interaction. This is accompanied by the chemical bond formation between the groups of biomolecules

Table 3. Estimation of the expected number of biomolecules that can be registered in an area of 400 μm² after fishing from solutions with different volumes and concentrations ($N_{400,theor}$ assuming a uniform distribution of all biomolecules over the surface of on the sensor zone S_{area}

Sensor zone area	Solution concentration (M)	Solution volume			
		1 μl	1 ml	100 ml	500 ml
S_{area} = 17663 μm² ≈0,02 mm² («small»)	10^{-10}	1 363 340	1 363 340 410	136 334 041 047	681 670 205 237
	10^{-11}	136 334	136 334 041	13 633 404 105	68 167 020 524
	10^{-12}	13 633	13 633 404	1 363 340 410	6 816 702 052
	10^{-13}	1 363	1 363 340	136 334 041	681 670 205
	10^{-14}	136	136 334	13 633 404	68 167 021
	10^{-15}	14	13 633	1 363 340	6 816 702
	10^{-16}	1	1 363	136 334	681 670
	10^{-17}		136	13 633	68 167
	10^{-18}		14	1 363	6 817
	10^{-19}		1	136	682
	10^{-20}			14	68
S_{area} = 158963 μm² ≈0,16 mm²	10^{-10}	151 482	151 482 268	136 334 041 047	681 670 205 237
	10^{-11}	15 148	15 148 227	13 633 404 105	68 167 020 524
	10^{-12}	1 515	1 514 823	1 363 340 410	6 816 702 052
	10^{-13}	151	151 482	136 334 041	681 670 205
	10^{-14}	15	15 148	13 633 404	68 167 021
	10^{-15}	2	1 515	1 363 340	6 816 702
	10^{-16}		151	136 334	681 670
	10^{-17}		15	13 633	68 167
	10^{-18}		2	1 363	6 817
	10^{-19}			136	682
	10^{-20}			14	68
S_{area} = 1766250 μm² ≈1,8 mm² («large»)	10^{-10}	13 633	13 633 404	1 363 340 410	6 816 702 052
	10^{-11}	1 363	1 363 340	136 334 041	681 670 205
	10^{-12}	136	136 334	13 633 404	68 167 021
	10^{-13}	14	13 633	1 363 340	6 816 702
	10^{-14}	1	1 363	136 334	681 670
	10^{-15}		136	13 633	68 167
	10^{-16}		14	1 363	6 817
	10^{-17}		1	136	682
	10^{-18}			14	68
	10^{-19}			1	7
	10^{-20}				1

Cells with $N_{400,theor}$ values, which can be used as approximate values of the $DL_{400,theor}$ detection limit of proteins in AFM fishing are highlighted in color.

in the complex via a cross-linker. In this case, fishing can be designated as "Biospecific irreversible fishing" (Table 4, line 4).

The possibility of using the "Biospecific fishing" group determines the potential for practical application of AFM-fishing technology for detecting proteins in solutions, since biospecific interaction determines the specificity of the analysis in routine medical diagnostics and proteomics.

During realization of the above fishing schemes, one of the following techniques can be used: incubation in the volume or in the flow of the analyzed protein solution. During incubation "in the volume", the AFM chip is completely immersed in a test tube, incubation is carried out and then the chip is transferred to another test tube containing a washing solution. After incubation and washing, the AFM chip is dried and then transferred for surface scanning.

Table 4. Various types of AFM-fishing

Group	Scheme no.	Designation	AFM-chip	Factor determining fixation of target biomolecules on the surface
Non-specific	1	Non-specific irreversible (chemical)	silanized mica with cross-linker-activated surface	chemical (covalent) bond between groups on the chip surface and target groups, formed by cross-linker
	2	Non-specific reversible	highly oriented pyrolytic graphite (POPG) plate	physical sorption of target biomolecules on the chip surface
Biospecific	33	Biospecific reversible	silanized mica with immobilized ligand molecules	biospecific ligand-target interaction
	44	Biospecific irreversible	silanized mica with immobilized ligand molecules, ligand molecules modified with cross-linked	biospecific ligand-target interaction and chemical bonding between ligand and target groups formed by cross-linker

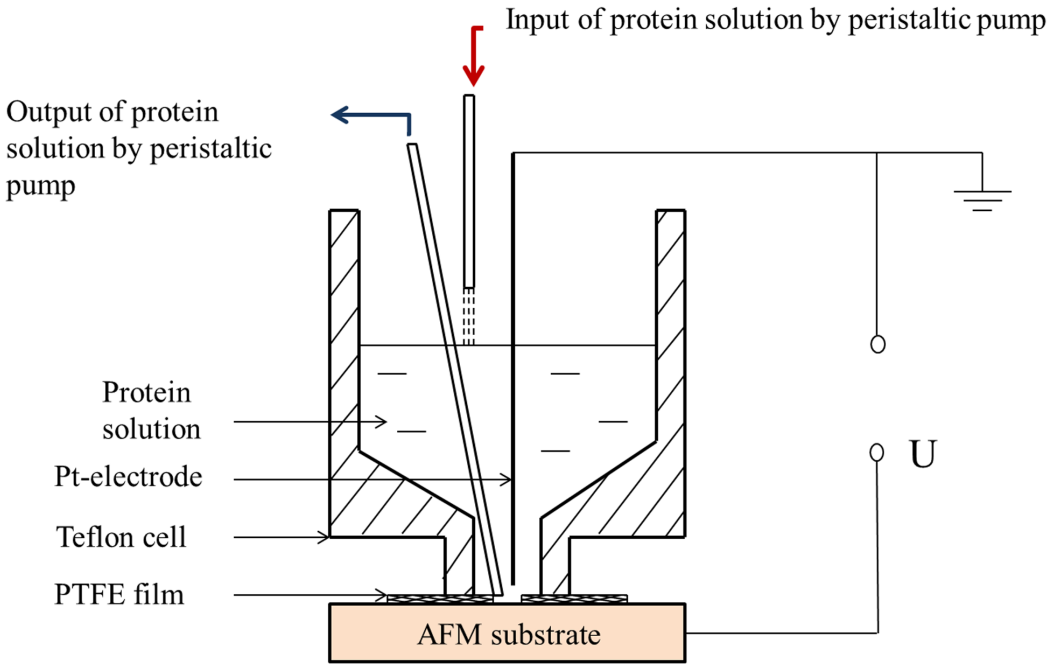


Figure 4. Schematic diagram of the device for fishing in the flow mode, "Non-specific irreversible fishing".

During "flow incubation", a flow system is assembled [35]; it includes a peristaltic pump (or automatic pipette) for feeding the analyzed flow and an incubation cell (Fig. 4).

In this case, the AFM chip "serves" as the bottom of the cell, into which the analyzed protein solution is fed using a peristaltic pump or an automatic pipette. The surface of the AFM chip is coated with a polytetrafluoroethylene (PTFE) film with a hole. Then, a fluoroplastic cell is placed on the surface of the chip. In the fishing process, the analyzed solution is fed into the cell using a peristaltic pump (or automatic pipette). Liquid is taken from the cell using a peristaltic pump through a pumping tube. The rate of feed and withdrawal of the solution is selected so that the cell always remains filled. After passing all the analyzed solution, the surface is washed, also using a flow system. The AFM chip is then removed from the system, dried and sent for scanning. The advantage of this method of sample delivery is the ability to use a large sample volume. The "flow incubation" method is described in detail in [35].

4. EXAMPLES OF USING AFM-FISHING TO DETECT PROTEINS

Ivanov et al. [36] have demonstrated the principal possibility of detecting the horseradish peroxidase (HRP) protein by means of irreversible fishing. Such fishing parameters as incubation time, volume and concentration of the analyzed solution had a significant impact on the efficiency of detecting the studied HRP proteins. A decrease in the concentration of the protein in the analyzed solution resulted in the decrease in the number of molecules fished during the same incubation time. The amount of protein caught during chemical fishing is directly proportional

to the volume of the analyzed solution: with an increase in the volume, the amount of caught protein increased. An increase in the volume of the analyzed 10^{-17} M solution made it possible to reliably register the protein (using the approach of preliminary 10-fold concentration of the protein). It was also experimentally shown that with a decrease in the concentration of the analyzed solution, it was important to increase the scanning area surface and decrease the area of the sensor zone to increase the reliability of the data obtained.

Ivanov et al. [35] demonstrated an example of using non-specific reversible AFM-fishing to detect proteins in solutions at low concentrations. Fishing of two proteins, human serum albumin (HSA) and cytochrome b_5, was performed on the surface of the conductive material HOPG. Fishing was performed in two modes of administration of solution: (1) rapid administration of the solution by an automatic pipette (injector administration); (2) slow administration of the solution by using a peristaltic pump. Fishing with injector administration was performed in the presence or absence of an applied external voltage to the HOPG surface. The volume of the analyzed solution (100 ml) was the same in all cases. The experimental series showed that effective protein fishing occurred under conditions of rapid injector administration of the protein solution. Two proteins, HSA and cytochrome b_5, were detected in solutions with a minimum concentration of 10^{-17} M. When the solution was slowly injected by a peristaltic pump, no fishing occurred and the protein was not adsorbed onto the HOPG chip surface. As shown by Ivanov et al. [35], one of the determining factors for efficient protein delivery during fishing onto the HOPG chip surface was the injection method of the analyte solution administration into the measuring cuvette. With this method of administration of the solution, an additional electric charge arises in the system, which promotes rapid sorption of molecules on the sensor surface. The presence of an additional charge leads to efficient protein fishing even without an applied electric field. In the injection mode, applying an external positive voltage does not affect the amount of sorbed proteins, while negative voltage application leads to a decrease in the fishing efficiency.

Biospecific AFM-fishing was applied to detect the serological marker, hepatitis C core antigen (HCVcoreAg), in a buffer solution by using antibodies against the hepatitis C core antigen (anti-HCVcoreAg) as a molecular probe [37]. After incubation of the AFM chip functionalized with anti-HCVcoreAg molecules in the analyzed solution containing HCVcoreAg, the size of the objects on the surface increased due to the formation of the antigen-antibody complex. It was shown that the height of anti-HCVcoreAg was in the range of 1–1.5 nm, the height of HCVcoreAg was in the range of 1.5–2 nm, and the height of their complexes was in the range of 3–7 nm. As expected the heights of the molecular probes and target proteins were less than the height of the antibody-antigen complex. The results of the work [16] showed that using reversible biospecific fishing it was possible to detect the protein in 1 ml of solution at a minimal concentration of 10^{-11} M. HCVcoreAg was concentrated on the surface due to reversible antigen/antibody binding. In the case of switching to the “irreversible” binding mode [16] due to additional cross-linking of the antibody/antigen complex by means of the photocross-linker, the protein was registered in 1 ml of solution at a minimal concentration of 10^{-15} M. Converting the reversible antigen/antibody interaction in the complex to irreversible one increased the sensitivity of the analysis by 4 orders of magnitude. Modification of immobilized anti-HCVcoreAg with a photocross-linker does not impair their affinity. Thus, during incubation in the analyzed solution, antibodies of the same class were present on the surface of the control zone and they were also modified with a photocross-linker (anti-HBsAg). However, the target anti-HCVcoreAg/HCVcoreAg complexes were formed on the surface of the working area during irreversible fishing and were subsequently registered using AFM.

Biospecific fishing can be used to detect proteins in a biological sample. This method was used to detect hepatitis C and hepatitis B viral particles in serum [38–40]. In [40], after incubation of a chip functionalized with antibodies in serum containing the hepatitis C virus, new objects with a height in the range of 10–35 nm were visualized using AFM, while such objects were not detected in the control virus-negative serum samples. Typical objects in the control experiment had a height below 5 nm. A similar registration scheme was used to detect hepatitis B viruses. In this case, objects with a height in the range of 10–40 nm were observed after incubation of an AFM chip with immobilized antibodies to the hepatitis B virus antigen (HBsAg) in serum. MS analysis confirmed the selectivity of the proposed analytical system for the detection of hepatitis C virus marker proteins using antibody-functionalized AFM chips [41, 42].

Biospecific fishing was applied to detect the human immunodeficiency virus (HIV-1) envelope glycoprotein gp120, used as a biomarker in diagnostics [43]. In this case, aptamers, amino-modified biotinylated DNA oligonucleotides (M_w ≈23 kDa) containing t-amino groups as a terminal insert to facilitate both aptamer immobilization on the chip surface and its interaction with the target protein, were immobilized on the surface as molecular probes. The use of aptamers increased the contrast of the AFM image compared to the approach based on the use of antibodies [43–45]. The contrast value determined on the basis of experimental data was 0.8 in the case of using aptamers; this value was 2 times higher than the similar value

obtained in the case of using immobilized antibodies ($IC_{gp120/ab}$ ~0.4). Such increase in contrast is expected and follows from the fact that the ratio of the molecular masses of gp120 and aptamer is higher than the ratio of the molecular masses of gp120 and antibodies to it, and therefore the heights of the images.

Using increased contrast of the AFM image of aptamer/protein complexes it is possible to achieve higher sensitivity of the analysis [38, 46, 47]. Pleshakova et al. [38] have shown that AFM chips with immobilized aptamers can be used to detect HCVcoreAg in a buffer solution and in the presence of a protein matrix at a concentration of 10^{-12} M; the antigen can be detected as part of the conjugate. In the case of AFM chips with immobilized aptamers, two criteria can be used to evaluate the fishing results: qualitative and quantitative. Pleshakova et al. [46] have shown that the protein marker of viral hepatitis C HCVcoreAg can be detected in solutions with concentrations from 10^{-10} M to 10^{-13} M. In this case, an AFM chip with immobilized aptamers that differ in sequence but contain the same number of bases can be used as an affinity reagent. For one of the aptamers, the possibility of analysis in the above-mentioned concentration range was demonstrated. It should be noted that modified aptamers against HCVcoreAg, developed for therapeutic purposes, were used as molecular probes on the surface. The achieved sensitivity of the analysis is two orders of magnitude better than in the case of using antibodies immobilized on the surface of the AFM chip.

The possibility of detecting the marker protein HCVcoreAg in human blood serum by using AFM chips with immobilized aptamers against HCVcoreAg was shown in [47]. Satisfactory agreement between the AFM analysis data and the ELISA data was noted: the agreement was 100% and 80% in the case of analysis of positive and negative sera, respectively. It was not possible to correctly establish the specificity and sensitivity of the AFM analysis due to the small sample size. All the used types of aptamers can be used as molecular probes, but the use of a matrix of sensor zones containing different types of aptamers is especially promising. Such array can contain aptamers against other marker proteins of viral hepatitis C. In this case, multiplexity of the analysis will be ensured, which will certainly increase the reliability of the data obtained.

The high sensitivity of the analytical system based on AFM-fishing is due to the effective concentration of the protein and the high sensitivity of the recording system at the level of individual protein molecules.

In [3], a concentration factor (F) was introduced to assess the efficiency of protein concentration during irreversible fishing. Under certain conditions, F can reach a value of about 10^8. This means that in the case of irreversible fishing from 1 ml of an analyte solution with a concentration of 10^{-17} M, the concentration of molecules will lead to an increase in the volume concentration of 10^{-17} M to a near-surface concentration of 10^{-9} M, which will significantly facilitate detection of proteins.

The sensitivity of the detector has a significant impact on the sensitivity of the analytical system designed to detect proteins [3, 16]:

$$DL = \frac{N_{AB}}{N_A V} \quad (2),$$

where NAB is the number of protein molecules or their complexes on the chip surface that the detector can register. For example, if N_{AB} ~10^8 protein molecules (Mr ~50 kDa), then DL ~10^{-12} M. In the limiting case, when the detector sensitivity increases to N_{AB} ~1, with an analyte solution volume of V = 1 l, the DL value will be:

$$DL = \frac{1}{N_A V} \approx 10^{-24} \text{ M} \quad (3),$$

which is the reverse Avogadro's number. High detection sensitivity can be achieved by using an AFM probe with a size comparable to the size of a biological macromolecule (1–10 nm).

From the above equations (2) and (3) it follows that:

(1) the fishing sensitivity can be increased by increasing the volume of the analyte solution both due to an increase in the total number of molecules available for catching and due to an increase in F;

(2) the fishing sensitivity increases with a decrease in the chip surface area S.

In order to decrease the surface area S and increase the factor F, it is reasonable to use a small sensor zone. The efficiency of this method has been demonstrated in this work and in other works, for example [48, 49], which show the possibility of reducing DL for proteins to 10^{-19}–10^{-20} M using an activated zone with an area of S<0.1 μm^2. However, on the other hand, the use of a small sensory zone limits the capacity of the chip.

To estimate the theoretically possible capacity based on experimental data, one can use the value $N_{400,ab}$ ≈14500, the number of antibodies on an area of 400 μm^2 under the immobilization condition in the monolayer form. Thus, if we assume that the target biomolecules correspond to the linear dimensions of the antibody (~5 nm), then the maximum number of biomolecules on the surface of the entire sensory zone S_{area} = 1.8 mm^2 (1766250 μm^2) would be:

$$(N_{400,ab} \times S_{area} / S_{400}) \approx 6.4 \times 10^7 \quad (4).$$

Therefore, theoretically, all biomolecules can be fished and placed on the surface of the sensor zone, for example, from 1 ml of 10^{-13} M solution (Table 5). But if it is necessary to concentrate a larger number of biomolecules, it is necessary to increase the area of the sensor zone. The maximum

Table 5. Experimental values of DL (DL_{exp}) obtained using various types of AFM-fishing

Type of fishing	Ligand	Target protein	Fishing conditions			Estimated number of biomolecules fished on the chip, N_{area}	DL_{exp}, *M*
			Solution volume, V	Sensor zone area, S_{area}	Fishing time		
Biospecific reversible	Antibody	HCVcoreAg	1 ml	1.8 mm^2	60 min	—	10^{-11}
			50 ml			—	10^{-11}
	Aptamer (A15)		1 ml			4.4×10^5	10^{-13}
Biospecific irreversible	Modified antibodies	HCVcoreAg	1 ml	1.8 mm^2	60 min	—	10^{-15}
			50 ml			—	10^{-16}
Non-specific reversible	—	Cytochrome b_5	100 ml	0.07 mm^2	90 min	2.0×10^5	10^{-17}
Non-specific irreversible (chemical)	— (chemically activated surface)	Horseradish peroxidase	1 ml	0.03 mm^2	180 min	3.7×10^4	10^{-15}
			500 ml (lyoph.)	1.8 mm^2	—	1.3×10^5	10^{-17}

possible S_{area} corresponds to the area of the entire chip (~1 cm^2 or 100 mm^2), in the case of using an AFM chip based on mica. In this case, a maximum of $\sim3.5\times10^9$ biomolecules can be fished.

CONCLUSIONS

The fundamental possibility of registering a signal from single biomolecules determines the use of AFM as the basis for a new bioanalytical system for protein detection. However, implementation of such system requires combination of AFM with fishing methods, allowing the concentration of target molecules on the surface due to non-specific or biospecific interaction. Currently, the parameters of target biomolecules on the surface used for analysis are their height and number. However, the development of new generations of devices, such as BioScope Resolve™ BioAFM [50], which simultaneously register several signals via independent channels, defines the prospects for using AFM as the basis for a new nanotechnological platform that will expand the range of registered parameters for biomacromolecules. The implementation of serial high-speed devices will make it possible to use AFM in medical diagnostics, as well as to widely introduce the AFM fishing method for detecting medically significant proteins. The combination of high-speed scanning and a multi-probe device [51] will be promising for the use in proteomic screening. AFM has great potential in combination with other research approaches. AFM and MS analysis methods are combined for visualization and further identification of proteins and protein-protein complexes. AFM-fishing allows detecting proteins with low content and characterizing their properties. Such combinations are especially useful for solving problems of proteomics and medical diagnostics.

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any research involving humans or the use of animals as objects.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ТЕХНОЛОГИЯ АСМ-ФИШИНГА ДЛЯ ОБНАРУЖЕНИЯ БЕЛКОВ В РАСТВОРАХ

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Рассмотрена возможность использования атомно-силовой микроскопии (АСМ) как базового метода для обнаружения белков в растворах с низкими концентрациями. Востребованность новых биоаналитических подходов обусловлена проблемой недостаточной чувствительности систем, используемых в рутинной практике для детекции белков. Приведены примеры использования в биоанализе комбинации методов АСМ и фишинга — способа концентрирования биомолекул из большого объёма анализируемого раствора на небольшом участке поверхности.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: атомно-силовая микроскопия; биоспецифический фишинг; детекция белков

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