REVIEW

Nanotechnologies in proteomics

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Progress in proteomic researches is largely determined by development and implementation of new methods for the revelation and identification of proteins in biological material in a wide concentration range (from 10^{-3} M to single molecules). The most perspective approaches to address this problem involve (i) nanotechnological physicochemical procedures for the separation of multicomponent protein mixtures; among these of particular interest are biospecific nanotechnological procedures for selection of proteins from multicomponent protein mixtures with their subsequent concentration on solid support; (ii) identification and counting of single molecules by use of molecular detectors. The prototypes of biospecific nanotechnological procedures, based on the capture of ligand biomolecules by biomolecules of immobilized ligate and the concentration of the captured ligands on appropriate surfaces, are well known; these are affinity chromatography, magnetic biobeads technology, different biosensor methods, *etc*. Here, we review the most promising nanotechnological approaches for selection of proteins and kinetic characterization of their complexes based on these biospecific methods with subsequent MS/MS identification of proteins and protein complexes. Two major groups of methods for the analysis and identification of individual molecules and their complexes by use of molecular detectors will be reviewed: scanning probe microscopy (SPM) (including atomic-force microscopy) and cryo-massdetector technology.

Keywords:
Atomic-force microscopy / CD-ROM reader biosensor / Nanobiotechnology / Nanotechnology / Optical biosensors

1 Introduction

The major problem of the present-day proteomics lies in the lack of a reaction similar to PCR; hence, the impossibility of multiplying various protein molecules, which in turn makes it impossible to enhance the concentrations of assayed biological material [1]. Thus, there arises a methodological barrier in proteomics: protein molecules occurring with concentrations below 10^{-15} M cannot be identified in biological material. Of note, the protein identification method based on a combination of 2-DE or chromatography with MS has a sensitivity of 10^{-9}–10^{-12} M [2]. The sensitivity of immunoenzyme methods (ELISA, RIA) is 10^{-12}–10^{-15} M [2–4]. However, it is the concentration range starting from 10^{-15} M and under that is most interesting from the biomedical viewpoint. Thus, for those tissue proteins, whose concentrations upon their leakage into plasma are lowered manifold, the required limit of diagnostic sensitivity lies in subfemtomolar concentrations.

Another problem arising upon proteome analysis is that the dynamic range of protein concentrations in biological materials, especially in plasma, lies in a very broad interval – from milli- to zeptomolar concentrations [2]. Clearly, high-

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Abbreviations: Ad, adrenodoxin; **AdR**, adrenodoxin reductase; **AFM**, atomic-force microscopy; **BIA**, biomolecular interaction; **GFP**, green fluorescent protein; **HB**, hepatitis B; **MCP**, microchannel plate; **OB**, optical biosensor; **Pd**, putidaredoxin; **PdR**, putidaredoxin reductase; **RM**, resonant mirror; **SNOM**, scanning near-field optical microscopy; **SPR**, surface plasmon resonance; **STJ**, superconducting tunnel junction; **STM**, scanning tunnelling microscopy

abundance proteins, hampering the analysis of low-abundance proteins, have to be removed – effectively and without loss – which is no easy task. Attempts of investigators to descend to subfemtomolar concentration range at the cost of large amounts of biological material have thus far been unsuccessful [5]. Indeed, it is very difficult to secure high concentration sensitivity of low-abundance proteins in the presence of other proteins with much higher concentrations in the assayed sample, if the dynamic concentration range is about 10^{15}.

Latest nanotechnological achievements allow such problems to be effectively solved. That is why nanotechnologies enable to measure, visualize and manipulate objects at a nanometre scale [6]. The requirements for the sample volume are therewith lowered by several orders while the concentration sensitivity may reach a single-molecule level.

Two approaches for application of nanotechnology to present-day proteomics may be singled out: The first approach involves creation of nanosystems for the separation of proteins by exploiting their physicochemical properties – with the aim of subsequent identification and characterization of these proteins. To such systems may be assigned nanoelectrophoretic systems [7], nanocolumns for LC [8] and magnetic biobeads facilitating micropurification and recovery of bound material [9–11]. Special emphasis will be placed on those procedures which enable to separate and concurrently concentrate proteins from complex mixtures – at the cost of their biospecific capture by biomolecules immobilized onto solid matrices of appropriate nanosystems. Based on such an approach, proteomic arrays for the separation of multiprotein systems are currently being developed that allow the investigator to do without 2-DE, CE and chromatography. The traditional method of affinity chromatography – based on biospecific interactions between macromolecules – is in fact a prototype of nanosystems, exploiting the same interactions, specifically the modification of the adsorbent's surface by biomolecules [12]. In recent years, the exploitation of the biospecific capture effects led to creation of new interesting nanotechnological systems: an original and simple biosensor laboratory on the basis of CD-ROM to PCs allowing for protein capture from multicomponent mixtures; optical biosensors (OBs) with convenient real-time monitoring of the protein capture process [13] and others.

The second approach involves (instead of using the concentration detectors whose sensitivity is not higher than 10^{-15} M) the application of molecular detectors, counting individual molecules and their complexes. These are scanning atomic force microscopes [14], optical microscopes [15], cryomassdetectors [16] and others.

In practice, the combination of both nanoapproaches is possible; in particular, a very popular 'OB fishing' technique may be combined with mass detection – with the aim of selective capture of proteins from a multicomponent mixture and their subsequent identification [17]. Such a combination makes it possible to spend minimal amounts of material while reducing the analysis time to several minutes.

The general direction in which these two approaches in proteomics are being developed is creation of highly efficient multichannel systems; in particular, these systems are finding an increasing application (i) in proteomic microassays for the screening of low-molecular drugs [18] and (ii) in (not micro but nano) atomic-force microscopy (AFM)-based proteomic arrays for viral disease detection; the usage of such arrays has recently been reported [19].

Considered below are the potentialities of nanotechnologies and typical examples of their application in proteomic researches.

2 Nanotechnologies for separation of multicomponent protein mixtures

Nanotechnologies may be considered as the embodiment of the industrial revolution of the 21st century. They offer a potential ability to carry out massive parallel analyses of proteins in nanoamounts – in prospect, even at a single-molecule level. To carry out such analyses, appropriate separation methods are to be used, the most important of which will be considered in Section 2.1.

2.1 Nanoelectrophoresis, nanochromatography and magnetic biobeads technology

Traditionally, for the separation of multicomponent protein mixtures 1-D and, especially, 2-DE are used [20]. 2-DE method involves the separation of proteins based on their p*I*s and molecular weights [21]. In application to proteomics, both technologies enable to carry out the protein mapping of a wide variety of biological sources (tissue, blood plasma *etc.* [1, 22]) without the need for any prior knowledge. When combined with MS, they allow for the identification of separated proteins [23]. However, 2-DE is a slow-operating, non-automated and labour-consuming procedure. The results from 2-DE can be impaired by comigrating spots and complicated by solubility problems arising upon IEF, by difficulties in pattern reproducibility and the limited dynamic range for protein staining [24].

An alternative procedure to 2-DE separation of proteins is high-resolution chromatography [12]. Compared to 2-DE, high-resolution chromatography is more convenient for analysis: it is automatable and is capable of removing high-abundance proteins from a mixture for the subsequent revelation of low-abundance proteins – which are then subjected to 2-DE procedure [25]. For direct identification of proteins in complex mixtures, a chromatographic column is coupled to mass spectrometer. Especially popular among chromatographic methods is RP chromatography (RP-HPLC): being used in a combination with MS, it allows for the identification of proteins in an online mode; that is, after the liquid-phase chromatography the sample is directly delivered to mass spectrometer. Proteins are not generically amenable to high-quality, robust separation by RP-HPLC [1]. All proteins

tend to denature under RP conditions (because of low pHs and high organic solvent concentrations) making their quantitative elution rather difficult; therefore, the direct identification of the majority of intact proteins (especially of those with masses over 10 kDa) is severely hampered by a low accuracy of mass spectrometric analysis [26]. The analytic accuracy was improved by digesting the proteins to peptides with a protease, typically trypsin. The resulting peptides behave in a more predictable manner than do the intact proteins and can be readily separated by RP-HPLC. To date, nanoLC-MS approaches have been effectively used to characterize protein complexes and to specifically examine protein–protein interactions. For instance, the approach based on the original crosslinking procedure and on the usage of nanoLS with Q-TOF MS/MS was used for identification of numerous proteins that copurified with a constitutively active form of M-Ras (M-Ras^{071Q}) [27]. Among these proteins, authors identified the RasGap-related protein IOGAP1 to be a novel interaction partner of M-Ras^{071Q}.

The common disadvantage of chromatographic systems is that they necessitate painstaking selection of conditions for each particular protein type; this does not allow the investigator to obtain a qualitative separation spectrum for all the proteins of a given mixture under the same conditions. To attain qualitative separation of proteins from their mixture, a combination of several chromatographic procedures (the so-called multidimensional chromatography) is used, which however leads to further complications in the whole procedure.

Application of nanotechnologies in electrophoretic and chromatographic separation procedures makes it possible (i) to use nanovolumes and/or nanoflows thereby lowering the volume of the required material by several orders and (ii) to essentially reduce the assay time. A recently published paper [7] has reported on the nanoelectrophoretic separation of a multicomponent mixture involving more than 20 proteins of 10–100 kDa within 15 s; that is, a gain in time was more than two orders compared to traditional 2-DE. In addition, such an approach enables to reveal proteins that were not detected as distinct spots by 2-DE analysis. Usage of nanochromatographic columns in a combination with the nanoelectrospray ion source of MS (LC-MS) allows for the identification of nanoamounts of proteins, reduces the solvent flow rate to a few nanolitre *per* minute and consequently ensures an over 100-fold increase in sensitivity compared to common chromatography – at the cost of the system's minimization [28]. The concentration limit – upon application of electrophoresis or chromatography in a combination with MS – is determined by the electrophoretic or chromatographic stages since the sensitivity of mass-spectrometric analysis may reach the attomolar levels – as was demonstrated in experiments with bradykinin and cytochrome C [29].

While considering the present-day modifications of the chromatography method for the separation of multicomponent mixtures, special attention will be paid to such technologies as SELDI [30–33] and biomagnetic beads [11, 33–35, http://www.bioclon.com]; in these two technologies, the hydrophobic or charged surfaces of magnetic beads or SELDI chips, in combination with mass spectrometric analysis, can be employed for protein revelation or protein and peptide profiling in serum [11, 30, 32, 34]. Combination in the SELDI-MS technique of adsorption of biological molecules (proteins, peptides) on analytical surfaces chemically modified to mimic interactions (cation/anion exchangers) with precise analysis of their molecular mass offers a more advanced approach to the study of complex biological specimens compared with conventional mass-spectrometric methods. The disadvantage of this method (compared to the combination of chromatographic separation with mass spectrometric detection) is its inability to identify, with high resolution, numerous fractions of separate protein components from complex mixtures. Nevertheless, the method enables to obtain protein patterns on the basis of the anionic, cationic, metal-binding and hydrophobic surfaces. The ability of the method to register protein profiles has been put to use for the development of the so-called 'proteomic pattern diagnostics' based on the association of protein patterns with certain diseases. The fact is that in cancer diseases the protein pattern in biological material undergoes essential changes compared to the protein pattern characteristic for the group of healthy individuals. Therefore, the monitoring of protein pattern changes may form the basis for an early disease diagnosis. Thus, it is unnecessary to perform costly procedures for identification of proteins that appear or disappear in serum, tissues and other biological materials and that are characteristic for particular diseases; it is only necessary to register changes occurring in the mass spectra of the serum's protein pattern by use of SELDI-MS, which makes the establishing of the diagnosis less expensive. In Table 1, the examples of SELDI-MS application for the revelation of markers of ovarian, prostate and breast cancers are given.

One problem that arises upon SELDI-MS application is the technical limitations of the SELDI-MS platform, in terms of both sensitivity and reproducibility; the other is the fundamental problem of identifying the parent proteins responsible for each peak, using the current instrumentation. The second concern involves fundamental issues of experimental design and appropriate controls: do the patterns reflect cancer-specific phenomena, or epiphenomena related to general inflammatory responses or metabolic disturbances? Are the results comparable across labs? One of the studies especially

Table 1. Examples of application of SELDI to the revelation of disease markers

Disease	References
Ovarian cancer	[36–38]
Prostate cancer	[39–41]
Breast cancer	[42]

devoted to the problem of deviation of protein pattern data with regard to prostate cancer is the study by Diamandis [43] – which discusses the deviation in marker MS peaks typical for this disease and obtained by various authors [39–41]. Analysis of protein pattern data obtained by various authors reveals many discrepancies. Therefore, the question 'Can the technology detect known biomarkers as an internal validation?' is very actual. The answers will undoubtedly be forthcoming as more investigators apply these tools to similar problems [44].

Magnetic beads from silica with surface derivatized with common RP ligands (nonspecific affinity) effectively bind proteins or peptides from serum [11]. In [11] the authors described the automated technology platform for the simultaneous measurement of serum peptides that is simple, scalable and generates reproducible patterns. Peptides were captured and concentrated using RP batch processing in a magnetic particle-based format, automated on a liquid handling robot and followed by a MALDI TOF mass-spectrometric readout. This combined approach allows for a detection of 400 polypeptides (in 0.8–15 kDa range) in a single droplet (approximately 50 μL) of serum, and almost 2000 unique peptides in larger sample sets, which can then be analysed using common microarray data analysis software. A pilot study indicated that sera from brain tumour patients can be distinguished from controls based on a pattern of 274 peptide masses. The biobeads' size may be made less than micron (1 μm–50 nm) ([11], http://www.gentaur.com), thereby reaching the conventional upper borderline of nanotechnologies or the conventional lower borderline of microtechnologies.

There is yet another convenient procedure for isolating proteins without their preliminary purification from multicomponent mixtures (such as blood serum, urine or tissue homogenates) which makes use of biospecific intermolecular interactions of proteins on the biochip surface or on the protein array surface. Apart from the ability to perform protein's fishing, this procedure has an additional advantage of simultaneous concentration of fished-out proteins.

2.2 Employment of intermolecular interactions for the capture and concentration (*i.e.* 'fishing') of proteins and their complexes by biochips

By fishing it is meant a selective capture of a protein from a multicomponent mixture and its concentration with the aid of the protein partner immobilized onto the biochip surface [45].

The biospecific fishing is the most efficient method for selection of particular proteins from the mixture; it takes advantage of the individual properties of interacting partners, such as antibody/antigen, enzyme/substrate analogues, nucleic acid/binding protein, hormone/receptor *etc.* Such an approach, common for affinity chromatography, is used for the development of modern technologies like magnetic biospecific biobeads and SELDI with the ProteinChip system (http://www.ciphergen.com [31, 37, 46]). For the preparation of magnetic biospecific biobeads, their surface is modified by biomolecules ([33, 34, 47, 48], http://www.bioclon.com, http://www.biocompare.com). Then magnet suspension is mixed with a multicomponent mixture; during this procedure the biospecific adsorption of partner proteins onto the biobeads' surface (*e.g.* the adsorption of antigens onto immobilized antibodies) takes place, just as in the case of affinity chromatography. Then the magnetic particles are pulled by a magnet and the captured proteins are analysed. In SELDI with the ProteinChip system, the chip surface modified by specific biopartners is employed from which the captured proteins are recovered and delivered to mass spectrometer for identification. In the SELDI with the ProteinChip, the biochip is an eight-channel array, which is very convenient for the separation and concurrent analysis of protein mixtures. In the study by [49] the mass spectrometric profiling of amiloid β peptide variants was carried out; for this purpose the active ProteinChip array with anti-Aβ polyclonal antibody (antiNTA4) was employed to perform the biospecific capture and purification of multiple immunoreactive Aβ fragments from cerebrospinal fluid and cultured cell media. In [50] three new amyloid peptide markers of Alzheimer disease were identified by use of SELDI method with affinity matrix for selective detection of peptides from cerebrospinal fluid of patients and for subsequent MS-TOF identification of these peptides.

The examples of various applications of SELDI method with affinity matrix are given in Table 2.

Application of the 'biospecific-binding' principle in nanodevices provides additional possibilities. Thus, the apparent advantage of a very popular OB system over the above-described techniques is direct real-time monitoring of the capture of a given protein by its biopartner in the nanolayer, followed by real-time monitoring of its concentration increase; this feature of the method enables to directly calculate the amount of captured protein. The OBs – in particular, the flowing surface plasmon resonance (SPR) and the stationary resonant mirror (RM) biosensors – are commonly

Table 2. Examples of application of SELDI with an affinity matrix to the revelation of disease markers

Disease	References
Vascular endothelial growth factor (VEGF) from resected human bronchial carcinoma tissue	[51]
Profiling of amiloid β peptide variants	[49]
Identification of new peptide markers of Alzheimer disease	[50]
Revelation of transthyretin TTR in plasma and urine (might be of diagnostic interest associated with different diseases, such as amyloidosis *etc.*)	[52]

adopted. These two OB systems enable (i) to specifically bind proteins on the biochip surface (the surface size, a few μm^2 to cm^2; its thickness, a monomolecular layer) and thus to concentrate those proteins occurring in solution in small concentrations; (ii) to register in real time the intermolecular interaction reactions; (iii) to calculate the kinetic rate constants and affinities for the complexes formed on the biochip surface; and (iv) to identify biospecific protein complexes by use of MS [53].

2.2.1 OBs

Of all biosensor types, particular attention should be paid to noncontact OBs that have found an ever-widening application in biology and medicine [54–69]. They are used in the real-time analyses of protein – protein interactions including the interactions between antigen/antibody, oligonucleotide/oligonucleotide, protein/lipid, hormone/receptor and, also, drug/receptor – with the latter interactions being widely used for drug design and drug testing. OB techniques with resonant nanostructures–SPR [70] and RM [71] – take advantage, under total internal reflection conditions, of the evanescent wavelength as a probing element that registers refractive index changes occurring in the medium upon complex formation in the reaction zone limited to several hundred nanometres from the sensor surface. OB techniques enable to register complex formation of macromolecules with high concentration sensitivity (up to 10^{-12} M) and with time resolution of about a few seconds, providing the most convenient tools for studying macromolecules' complex formation in real time, for measuring association/dissociation rate constants as well as for determination of thermodynamic parameters of protein–protein complex formation. Of note, the labelling of proteins is not required with application of OB technique, which is very important for solution of proteomic problems.

The most commonly used commercial OBs are the four-channel SPR devices of the BIAcore type (Biosensor, Sweden) and the two-channel RM-biosensors of the IAsys+ type (Affinity Sensor, UK). Used as an SPR-based sensor chip in BIAcore biosensors is a glass prism coated with a thin gold film. IAsys chip is a nanolayer waveguide coupled with a glass prism (Scheme of IAsys+ is presented in Fig. 1). Ligate, immobilized onto the biochip surface, is complementarily bound to its partner, *e.g.* protein. Increasing the surface concentration of a protein due to the ligand/ligate complex formation increases the refractive index in the sensitive layer adjacent to the sensor surface, which in turn leads to the change of the resonant angle position of the probing light [71]. Such biosensors are capable of determining the surface concentration of the ligand bound to the sensor surface as a function of time with a very high sensitivity (up to 10^{-11} to 10^{-12} M). Lately, the trend has been towards creation of multichannel SPR biosensors, for example a 400-channel SPR biosensor (Applied Biosystems 8500 Affinity Chip Analyser), that is capable of registering in parallel up to

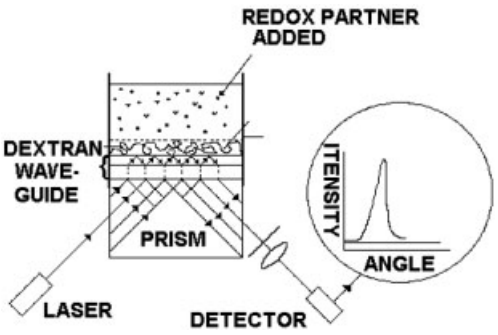

Figure 1. Scheme of IAsys OB. Ligate, covalently immobilized onto the biochip waveguide/prism surface, is complementarily bound to its partner from solution. Increase of the surface concentration of a protein caused by the ligand/ligate complex formation brings about the increase of the refractive index in the sensitive layer nearing the sensor surface, which in turn leads to the change of the resonant angle position of the probing laser.

400 complex formation reactions in real time. Recently reports have appeared about creation of colorimetric resonant reflection biosensors that do not require any labels and allow for the monitoring of protein/protein and protein/low-molecular ligand interactions in real time based on measuring the shift in the wavelength of light, reflected from the diffraction grating surface of the chip – with adsorption of biomolecules on the chip surface [72]. The system, possessing the SPR-comparable sensitivity, is able to markedly enhance the area of the analytic surface. Thus, the system may be used for the preparation of arrays capable of performing parallel analyses in a great number of channels at once. Of note, the preparation of this system is much cheaper owing to a much simpler measurement scheme than the scheme used for SPR- or RM-biosensors. Multichannel SRU BIND systems, based on the principle of colorimetric resonant reflection and having 384 channels, are presently being developed by SRU Biosystem [72].

2.2.1.1 Biomolecular analysis (BIA) of complex formation by use of OB and its application in protein system studies

The formation of the protein/protein complex may be described as

$$A + B \underset{k_{off}}{\overset{k_{on}}{\leftrightarrow}} AB \quad (1)$$

where k_{off} and k_{on} are the rate constants for the dissociation and association reactions, respectively. Their ratio, *i.e.* the equilibrium constant for the association (K_{eq}) or dissociation (K_d) processes is usually written as

$$[A][B]/[AB] = K_d = 1/K_{eq} = k_{off}/k_{on} \quad (2)$$

The complex association/dissociation reactions may be followed in real time while their k_{on} and k_{off} are calculated from the experimental curves describing the appro-

priate reaction processes (Eq. (1)); the ratio between these constants K_d is obtained from the corresponding Eq. (2).

This approach was taken to obtain kinetic constants for complex formation and decay and to measure complex lifetime in various protein systems [54–63, 73, 74].

By measuring the temperature dependence $K_d(T) = k_{off}(T)/k_{on}(T)$, the major thermodynamic parameters of the complex formation reaction, *i.e.* Gibbs free energy (ΔG), enthalpy change (ΔH) and entropy change (ΔS), may be calculated: For this purpose the following system of equations is used:

$$\Delta G = -RT \ln K_{eq} \quad (3)$$

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

where R is the gas constant.

Combination of this approach with the mass spectrometric analysis of complexes formed on the OB surface enables not only to identify partner proteins and determine complex composition but also to elucidate the mechanism of complex formation and decay. All this information cannot be obtained by using other approaches, commonly applicable in proteomics, such as the two-hybrid system, 2-DE and chromatographic methods. The mere revelation of a complex – without measuring its tightness, specificity and functional activity – is apparently insufficient for its characterization and estimation of its productivity/nonproductivity. This fact, which is well known in enzymology, has found additional confirmation in our studies [59–60]. It was also found that OBs are capable of monitoring in real time the processes of complex formation and decay not only in binary but also in multicomponent complexes. Elucidation of the mechanism of function of multiprotein enzyme systems requires much effort – as was demonstrated, for instance, for cytochrome P450 monooxygenase systems [62]. As is known, the multiprotein cytochrome P450-containing monooxygenase systems may be divided by their organization into three major types. The first, bacterial type is represented by water-soluble proteins putidaredoxin reductase (PdR), putidaredoxin (Pd) and cytochrome P450cam (P450cam). The intermediary type – the mitochondrial hydroxylase system of adrenal cortex – involves the water-soluble proteins adrenodoxin reductase (AdR), adrenodoxin (Ad) and the membrane protein cytochrome P450scc (P450scc). The third, membrane-bound type – the microsomal P450 2B4 containing monooxygenase system – involves three membrane proteins: cytochrome P450 reductase (Fp), cytochrome P450 2B4 (2B4) and cytochrome b5 (b5). The interprotein electron transfer from the reductase to P450 occurs in these systems either through Pd or Ad (as in the case of P450cam or P450scc) or directly (as in the case of 2B4) [75]. It was found, with the aid of OB, that the P450-containing systems of the above-mentioned three types (the microsomal, mitochondrial and bacterial ones) can form not only the binary complexes: PdR/Pd and Pd/P450cam (the P450cam system), AdR/Ad and Ad/P450scc (the P450scc system) or Fp/2B4 and b5/2B4 (the P4502B4-system) but also the PdR/Pd/P450cam, AdR/AdP450scc and Fp/2B4/b5 ternary complexes [59, 60, 62]. Importantly, not the binary but ternary complexes proved to be functionally active in the P450cam and P450scc systems. Binary complexes were, as a rule, nonproductive [59, 60].

Several examples of application of the biosensor fishing technique for selective capture of partner molecules out of a multicomponent mixture are presented below. By use of this technique, we have managed, following isatin immobilization, to fish out its protein partners from mitochondrial lysates [64]. It is well known that the most important problem of practical clinical medicine is the one of maximally fast and precise diagnostics. It is on correct diagnosis that an effective treatment of patients and even their life is largely dependent. The biosensor fishing technique appears to be one of the most perspective diagnostic tools. Based on this technique, the biosensoric system of real-time registration of social diseases' markers (*e.g.* the hepatitis B (HB) marker) was created [13]. Importantly, this system does not require special labelling of molecules. The HB marker in this system, HbsAg, was isolated from patient serum by use of a biochip. Employed as a biochip was the biosensor support with immobilized antibodies (antiHBs). The association and dissociation rate constants measured in this system for antiHBs had the following values: $k_{on} = (6.3 \pm 1.5) \times 10^3 M^{-1} \cdot c^{-1}$, $k_{off} = (0.20 \pm 0.16) \times 10^{-3}$/c and $K_d = (3.2 \pm 2.6) \times 10^{-8}$/M. The HBsAg/antiHBs complexes readily dissociated in 10 mM HCl, and thus the antiHBs-containing biochips were reusable. Upon testing serum samples for the presence of HBsAg, the serum was loaded onto the OB biochip wherein the formation of complexes in the HBsAg-containing serum was registered by refractive index changes. For the registration of complex dissociation, the serum was removed from the biochip and the standard phosphate buffer/Tween 20 (PBS/T) was added. Figure 2 presents typical curves of the biochip in response to the addition of serums no. 1 and no. 2. One can see that the HBsAg/antiHBs complexes were registered in serum no. 1 but not in serum no. 2. As was confirmed by ELISA, serum no. 1 contained HBsAg while serum no. 2 did not contain it. The advantage of the biosensor system as regards HBsAg revelation in sera lies in rapid (within 5–8 min) detection of the antigen and the repeated usage of the biochip. The same approach was taken for the revelation of antibodies to synthetic peptides, for modelling the antigenic determinants of hepatitis A virus [67] and herpes virus [68], as well as for the revelation of oncological markers [69] and for monitoring of patients with Goodpasture's diseases [76].

2.2.1.2 Revelation and identification of protein complexes by a combination of BIA with MS

The combination of BIA analysis (by use of the biosensor fishing technique) with MS (BIA/MS) increases the OB potential at the cost of subsequent mass-spectrometric identification of protein–protein complexes and their interaction sites [17, 18, 77].

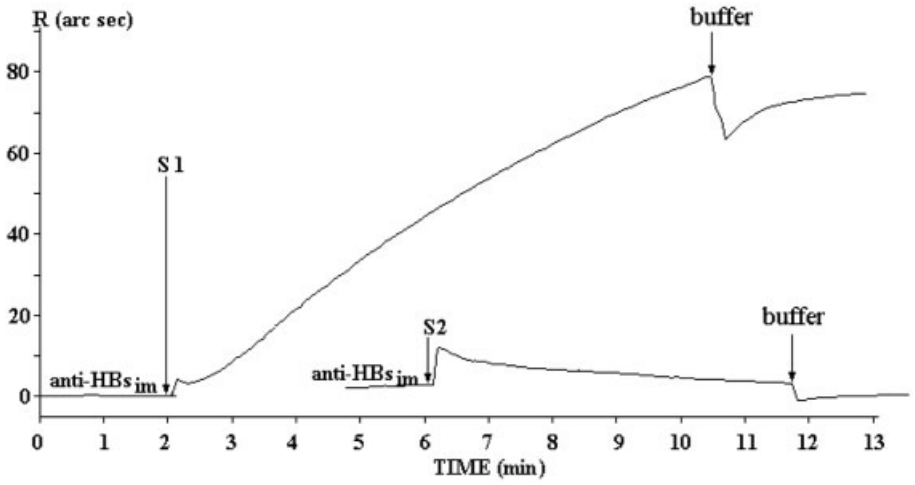

Figure 2. Detection of HBsAg in an IAsys aminosilane biochip. AntiHBs is immobilized onto the biochip bottom. Serum no. 1 (S1) contains HBsAg while serum no. 2 (S2) does not contain HBsAg. Ordinate axis is the biosensor response (in arcseconds) which is proportional to the amount of bound HBsAg. Incubation mixture contained 54 μL PBS/T buffer, pH 7.4. T = 25°C. Arrows indicate addition of 6 μL serums (S1 and S2) and replacement of the serum solution by PBS/T buffer.

Thus at the first step of the procedure the OB operates as selective and concentrating element, when the captured protein is concentrated on the sensitive layer of the device. On the other hand, subsequent mass-spectrometric analysis proved to be an efficient technique for identification of captured proteins and their complexes. Mass spectrometer enables to identify proteins and protein complexes being formed directly on the highly sensitive biosensor support. There are two ways of sample ionization in mass spectrometer after OB analysis: (i) ionization by the MALDI method, when sample is delivered to ion source of mass spectrometer – either directly onto the OB surface, as was demonstrated in [78] for identification of myotoxin a (which was selected from solution of whole venom from the prairie rattlesnake by antimyotoxin *a* Ig covalently bound with the biosensor chip surface) or indirectly, *i.e.* in the form of eluate from the OB biochip, as was demonstrated for myoglobin selected from solution by antimyoglobin also bound covalently with the biosensor chip surface [79]; and (ii) ionization by the electrospray method (ESI) that enables, for instance, to select calmodulin from rat brain extract and obtain its MS spectrum [80]. In the coupling scheme (i), according to which OB is combined with MALDI mass spectrometer, a peptide or protein recovered from biosensor is cocrystallized with an absorbing matrix such as CHCA; the mixture thus obtained is heated with a laser pulse of appropriate wavelength, after which analyte protein or peptide ions are formed that can be delivered to a suitable mass analyser [79]. As a pulsed ionization technique, MALDI is commonly combined with a TOF technique (MALDI-TOF). For a high-speed analysis the coupling scheme (ii) is applied. It presupposes the elution of proteins and their complexes from the biosensor support and their subsequent delivery to mass spectrometer with ESI in an online mode; ESI may work under atmospheric pressure and, hence, is applicable for transfer of sample solution from OB [80]. ESI is commonly combined with quadruples (ESI-Q) or with an IT (ESI-trap) mass analysers.

By coupling OB with mass spectrometer with only one component of ion analyser (*e.g.* TOF) it is possible to measure ion mass and register captured molecules on the sensor chip on the subpicomole level. However, this method often fails to characterize proteins for three reasons: (i) as a rule, with delivery of an intact protein to mass spectrometer the accuracy of determination of ion mass is insufficient for correct identification of this protein [26]; the mass accuracy frequently drops to 0.1% for a protein over 30 kDa, (ii) large proteins are often heterogeneous and hence possess no single molecular weight; (iii) proteins are often modified post-translationally (through glycosylation, phosphorylation, acetylation *etc.*) [80]. To obviate this problem, proteins and their complexes, prior to being delivered to mass spectrometer, are trypsin-treated and only then the identification of proteins and their complexes is carried out based on PMF. In certain cases PMF provides insufficient information for protein identification. MS/MS fragment ion analysis of selected peptides may then be used for improved protein identification. Application of a successive combination of two analytic procedures (MS/MS) (*e.g.* TOF/TOF, Q-TOF) enables not only to measure ion mass but also to perform in a collision chamber the fragmentation of an ion isolated in the first analyser with subsequent analysis of appropriate amino acid fragments in the second TOF for protein identification – as is exemplified by the analysis of calmodulin, a Ca^{2+}-binding protein in brain (100% sequence coverage) [80].

To improve the efficiency of identification of proteins and their complexes with the aid of tandem MS/MS, the electrospray and/or MALDI ionization techniques are commonly employed together with various combinations of analysers, with the advantages of each analyser being employed to the maximal degree. Thus, the merits of TOF are the best mass range (up to tens of millions of Da), fast action and high ion throughput, while its demerits are the requirement for high vacuum during analysis and limited selection of single mass. The merits of ITs are their ability to perform a multistep mass

analysis (MS/MS) and their high sensitivity. The merits of single quadruples are their high reproducibility, small sizes and cost-effectiveness while their demerits are lower mass range (the mass limit is about 10 kDa) and lesser efficiency of fragments' selection compared to TOF; usage of QTRAP (linear quadruple trap) as a Q-analyser enables to significantly increase the population of trapped ions, which – in contrast to toroidal IT – leads to the broadening of a dynamic range and the improvement of sensitivity. These advantages are gained owing to the trapping of ions within quadruple at the cost of blocking potentials at the inlet and outlet. The merits of Fourier transform ICR are high sensitivity, best resolution compared to other mass spectrometers, nondestructive ion detection, repeated measuring of ions and suitability for impulse ionization methods. The following combinations of analysers are commonly used: ESI-Q-TOF, ESI-ion-trap-TOF, MALDI-Q-TOF, MALDI-TOF-TOF *etc.* [81]. The combination of ESI-Q-TOF (*i.e.* usage of Q or IT as a first analyser) enables to use the merits of Q or IT (*i.e.* to deliver the sample in liquid phase to the analyser, to ion trap the necessary ions by the first analyser) and then to use the merit of TOF for performing an analysis of polypeptide fragments in the TOF component in a wide mass range. An example of ESI-Q-TOF operation is its usage for performing fast and precise characterization of a complex ganglioside mixture from human cerebellar sequencing [82]. The overcoming of disadvantages of Q or TRAP (a lesser efficiency of fragments' selection) is possible when the MALDI-LIFT-TOF/TOF scheme is used. Upon application of this scheme, the additional knot LIFT raises the potential energy of the ions after TOF1 analyser; second accelerator stage is realized in TOF2. This approach provides high performance of automated MS and MS/MS analyses and high sensitivity – as was demonstrated for protein from lysate of human endothelial cell line [83].

Further application of OBs in proteomics is connected with evaluation of schemes for OB sensitivity improvement and creation of reliable devices for coupling biosensors to mass spectrometers.

2.2.2 CD-ROM and other biosensors

In recent years new nanotechnological approaches for protein interaction studies without labelling have been developed. In this section we describe one of these approaches based on the biospecific registration of complex formation between macromolecules using a CD-ROM to a PC [84].

The CD is read by measuring the change in reflection of a polarized infrared laser. As the laser beam travels through a CD, the binary system is generated by modulating the reflection of light according to its reflection from land or from lowered pits within an internal layer. When the beam hits a pit, the reflected light is destructively interfered by the incoming beam. The player reads 1 when the beam hits land or 0 when the beam hits a pit (light is switched off by destructive interference). Standard writing onto a CD presupposes the availability of information allowing the CD-ROM to reveal and correct, by use of a special program, the numerous errors emerging upon contamination or damage of the disk surface. The CD-ROM performance makes it possible to estimate the state of the CD surface at any one of its points. The CD-ROM biosensor method is based on the analysis of errors made upon reading the information from a modified CD. A standard CD-ROM reader to a PC with biochip disks serves as an analyser. Biochip is a CD with a biomolecular monolayer immobilized onto it. Such a biolayer interferes with the optical transmission of the CD polycarbonate layer; as a result, first-level errors emerge upon reading by laser of digital data from the internal CD layer. With depositing onto the biochip of the sample containing partner biomolecules to the immobilized biolayer, the molecular complexes are formed, which leads to the increase in the number of reading errors due to the OD changes occurring on the CD surface. The principle of complex revelation is based on registering these second-level errors. By comparing the distribution of second-level errors caused by complex formation with the distribution of first-level errors on the CD bearing an immobilized ligate protein, the number of complexes formed may be calculated. By using CD-ROM biosensor, successful registration of streptavidin/biotin complexes ($K_d < 10^{-10}$ M) and low-affinity Con A/α-mannoside complexes ($K_d = 10^{-4}$ M) was demonstrated [84].

Thus, the biospecific fishing procedure based on standard reading of CD-ROM enables to reveal specific biomolecules in the biological sample by means of estimation of the increase in the number of errors that emerge upon formation of biomolecular complexes with biolayers immobilized on the CD surface. Although the use of this new and perspective technology makes possible formation of a rather cheap laboratory on a common CD-ROM-like disk, the technology has not as yet received wide acceptance.

Apart from the OBs that exploit biospecific interactions, some other devices operating on the same principle were designed. Among these there are potentiometric and amperometric biosensors capable of registering charge distribution changes [85] and the electric current generated by the redox reaction [86]; acoustic devices whose work is based on exploiting the effect of changes in resonators' acoustic properties upon enlargement of the product mass on the resonator surface [87, 88]; calorimetric devices capable of registering heat evolved during the reaction progress [89, 90] and some others. Importantly, with the miniaturization of biosensor's sensitive elements on the basis of a microfabricated cantilever – as is the case with calorimetric biosensors – the theoretical sensitivity may reach several attoJoules, *i.e.* the level of 10–100 molecules [91, 92]. In microbalance biosensors, a theoretical minimum detectable mass of 10^{-15} g (*i.e.* of about 1000 molecules) is proposed [93]. Miniaturization of such biosensor elements enables to use them as arrays.

It is to be noted that fabrication of biospecific protein arrays is finding an ever-widening application in proteomics and diagnostics – although these rapidly evolving technologies are still at the incipient stage of development [94]. Use of

protein arrays, *i.e.* the so-called 'Second-Generation Proteomics', presupposes separation of protein mixtures without application of other separation procedures such as 2-DE, CE and chromatography [95].

2.2.3 Protein arrays for separation of multiprotein mixtures

Technology of creation of proteomic arrays allows for uniting the multitude of biospecific biochips (about 1000 channels) in one construct. This abruptly enhances the efficiency of biochip usage for multiplex analysis of proteins in serum, plasma, cell lysates and tissue extracts – at the cost of automation of parallel measurement processes in many channels at once. The need for such an approach (*e.g.* in diagnostics) is determined by the obvious fact: although single biomarkers are often used as disease indicators, they cannot compete with the multianalyte profile or with the proteomic pattern – as was indeed demonstrated for patients with prostate and ovarian cancers [37, 96]. Proteomic arrays, on the contrary, make it possible to detect expression of a large number of specific proteins in normal and malignant tissues, *i.e.* to detect the protein expression profile [94]. Wiese *et al.* [3] have shown that with the aid of an array analysis the sensitivity of 0.05 pM for interleukin-6 detection is much higher than that obtained by a single classic ELISA. An essential limitation for ELISA is cross-reactivity of secondary antibodies with immobilized antibodies or other captured proteins in a multicomponent mixture. Lately, new specific capturing agents, photoaptamers, have been put to use. Photoaptamers bind proteins with two dimensions of specificity [94]. First, the protein is captured *via* a high-affinity noncovalent interaction, with a K_d in the nM to pM range, and, second, the captured protein is photocrosslinked to the photoaptamer if the protein is bound tightly and in the proper orientation to provide for a photocrosslinking site. Owing to such properties of photoaptamers, they are beginning to be used in fabrication of photoaptamer arrays: these latter do not require secondary antibodies, thereby enabling to avoid the significant limitation of the ELISA method [94].

The key problem in creation of proteomic arrays is their miniaturization: this feature is essential for lowering the quantity of analysed material. For the time being, the preparation of proteomic arrays is carried out by the use of high-precision contact printing or ink-jet technology, using a sample of subnanolitre volume and spot size of about 100 μm [94]. Such proteomic arrays allow for the monitoring of enzymatic reactions with the amount of enzyme in the spot of about 100–1000 molecules [97]. Gyros reported on creation of the CD-microlaboratory based on a microfluidic protein array (using different affinity binders) and a combination of this CD-microlaboratory with Bruker MALDI-MS and MS/MS (http://www.gyros.com). At the same time, creation of AFM nanoarrays with the spot size of less than 100 nm has recently been reported [19]. Proteins on the surface of micro- and nanoarrays may be immobilized by standard chemical procedure.

3 Molecular detectors

3.1 Need for the application of molecular detectors in proteomics

The information obtained with the aid of nanotechnologies is unique: it enables to register and characterize the properties of molecules both at the level of their assemblies in nanostructures [98, 99] and at a single-molecule level [100, 101]. Analysis of properties of single molecules provides new information compared to data from the average number of numerous molecule copies [102]. Single molecules are local reporters of the microenvironment. This is very important for studying heterogeneous systems in which various individual copies of a protein occur in various states of folding, or in various configurations or else at different stages of enzymatic activity [103]. Lately, the keen interest just in the 'nanoworld' of single atoms and molecules has been expressed. There are several devices that may register single molecules and examine their properties, *i.e.* function as molecular detectors. Use of waveguides with a decay wavelength (zero-mode waveguides) made it possible to analyse single macromolecules in volumes of up to 10^{-21} L [104]. By using such waveguide technique, it became possible to register the DNA-polymerase activity. Detection of single molecules based on the registration of ion current in a single transmembrane channel was also reported [105]. Original real-time detection of single viruses with high selectivity by using nanowire field effect transistors was demonstrated on the nanowire arrays modified with antibodies for influenza A [106]. It is apparent that for studying higher eukaryotes new technologies must be developed, suitable for top – down analysis of the real functional units of cell, namely whole proteins, which is essential for proteomics [107]. For physiologically relevant studies of higher eukaryotes, there is an urgent need to extend technology to 10 zmol protein amounts, *i.e.* these technologies must allow one to count single molecules, which opens new perspectives in the development of quantitative proteomics. From this viewpoint, the scanning microscopy technologies that make possible the imaging of single biomolecules in nanometre domains in their original location and position upon the biomolecule's adsorption on a surface are of particular interest. Among these technologies are scanning near-field optical microscopy (SNOM) [108], AFM [14] and scanning tunnelling microscopy (STM) [101]. Figure 3 presents the schemes for the probing of single molecules' surfaces based on these technologies. In SNOM optical fibre probe is coupled to a laser light, which excites fluorophores of proteins. SNOM probe does not touch molecules; therefore this microscopy type is noninvasive and does not perturb the sample. In contrast, in the AFM and STM techniques, probe exerts influence on sample. At the same time, these two technologies present interest from the viewpoint of high spatial resolving capacity of biological molecules – in the order of 1 nm and less.

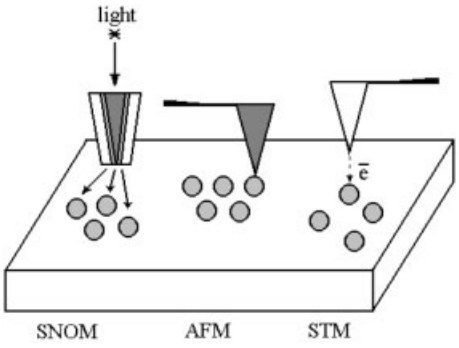

Figure 3. Scheme of molecule registration of SNOM, AFM and STM. Probes of microscopes are scanned over the surface of a single molecule. In SNOM, optical fibre probe is coupled to laser light that excites fluorophores of molecule. In the AFM the interaction force between probe and molecule is monitored. In the STM the tunnel current between the conducting (as a rule, metallic) probe tip and the surface of the sample is monitored.

The appearance, in recent years, of increasingly numerous studies dealing with the analysis of single molecules and based on the tunnelling and, especially, on the AFM techniques [109–123] is explainable. Indeed, the AFM offers a unique ability to easily detect and visualize proteins and their complexes in near-native conditions while not requiring protein labelling (in contrast to the earlier mentioned optical microscopy techniques) [114]. Clearly, the combination of OB technique with MS and AFM enables to reveal, identify and exhaustively characterize both the single molecules and their complexes. In view of this, we considered it necessary to describe in detail the potentialities, with regard to proteomics, of the AFM while only touching briefly on its closely related technique – the STM.

3.2 AFM and perspectives for its application in proteomics

The nanotechnological method of SPM includes a wide variety of microscopy techniques, all of which take advantage of local interactions of microprobes with surfaces and are aimed at examining the structure of studied objects at a nanometric resolution and, also, at elucidating their properties (mechanical, electrical and electronic). Among these techniques are the STM and the AFM of biomolecules. The STM and AFM may be classed with the 'scanning near-field microscopy' approach, and both are finding an increasing application in structural studies of biological systems with high nanometric or subnanometric resolution (such as proteins and their complexes, nucleic acids, biomembranes and cells) [110, 111] in near-native conditions. The principle of operation of the two techniques is the same: the probe tip of the device scans the area of the decay length of some nearly located field across the sample surface. In both devices the atomically smooth surfaces are used as supports. The STM measures the tunnel current between the conducting (as a rule, metallic) probe tip and the surface of the sample [101]. The STM images of membranes and proteins with a lateral resolution of about 1 nm and higher were obtained [110]. The inherent limitation of the STM is that it cannot be effectively used as an indirect tool for the revelation and examination of nonconductive objects – especially in proteomic researches. The advantage of the AFM over the STM is that the AFM may be applied to investigate not only conductive but also nonconductive bioobjects – to which proteins and their complexes are assigned. The AFM scheme is presented in Fig. 4. The scanning AFM [14] registers the force of interaction between the probe tip of the cantilever and the surface of the sample immobilized onto the atomically smooth support. As supports, highly oriented pyrolitic graphite or mica is commonly used. As a rule, the adhesion of proteins to their surfaces is not very high. To enhance the immobilization efficiency, the support surfaces should be modified. The commonly used measurement scheme is based on the monitoring of the cantilever's deviation upon scanning the biomolecular surface with the probe tip of the microscope. The deviation is registered with the aid of an optical system including a position-sensitive photodiode. This detection system is able to simultaneously register the vertical and the lateral cantilever deviation. To obtain the force characteristics of interaction between biomolecules and examine biomolecular topography, the constant force mode is usually employed, *i.e.* upon the scanning of sample with probe, the constant sample/probe interaction force is maintained owing to the feedback loop. To lower the forces that may deform and shift the sample on the support, the tapping mode of measurement is employed, with the cantilever's oscillation being close to the resonance frequency. When probe interacts with molecular surface, the oscillation amplitude is changed. These changes reflect the macromolecule topography. Such an approach enables to improve the quality of molecules' images [111]. With native proteins, the method ensures the vertical resolution of ~0.1 nm and the lateral resolution of up to 0.5 nm [112, 113]. The lateral resolution is less precise because of the tip-broadening effect.

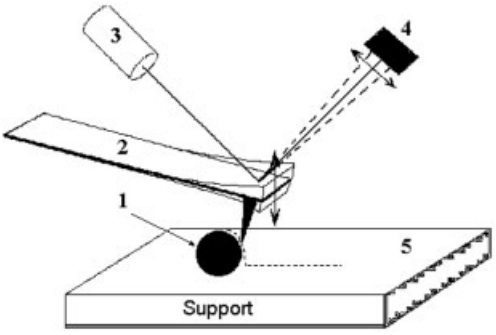

Figure 4. Scheme of AFM. Protein molecule (1) is located on the atomically smooth support (5). Image of protein molecule is obtained by means of its scanning with the cantilever (2) tip. Interaction between tip and molecule resulting in the cantilever's bend is registered by the reflected laser beam (3) on the photodiode area (4).

AFM application makes it possible to visualize a broad spectrum of water-soluble proteins and their complexes, such as immunoglobulins, ferritin and their complexes [114]; phosphorylase, phosphorylkinase and their complexes [115]; member proteins of a monooxygenase cytochrome P450cam system [116] and others. Interesting results were obtained in our studies of the complicated water-soluble cytochrome P450cam monoxygenase system involving three proteins – cytochrome P450cam, Pd and PdR [116]. Demonstrated was the ability of the AFM to reveal binary and ternary complexes of these partner proteins in multicomponent systems and to distinguish, in these systems, the binary from the ternary complexes – the latter finding being especially important for proteomic researches. It is essential that the AFM technique may be used to visualize membrane proteins in near-native condition. Thus in the study of [117], the image of the membrane protein P4502B4, incorporated into the phospholipid bilayer, was taken. In [118] the imaging of the extracellular surface of the protein porin OmpF with a vertical resolution of 0.1 nm and a lateral resolution of up to 0.6 nm was reported. We have undertaken the task to study the microsomal membrane-bound cytochrome P452B4 monoxygenase system involving three membrane proteins: cytochrome P4502B4, cytochrome P450 reductase and cytochrome b5, in the presence of the detergent Emulgen 913; as a result, the binary and ternary complexes of partner proteins were revealed based on the increase of imaged objects' heights upon complex formation [119].

The unique ability of the AFM technique to recognize individual proteins and their complexes may be used in important area of medical diagnostics such as immunoanalysis. The analytical procedure involved, at the first step, covalent immobilization onto the support of one protein molecule, *e.g.* the antibody or antigen molecule. Then this support was incubated in the biological fluid. With one of the partner proteins being immobilized, the formation of the antigen/antibody complex was registered. Based on the analysis of a series of such antigen/antibody pairs, it was shown that the heights of the complexes formed exceed those of individual protein molecules. For instance, the heights of individual HSA, antiHSA and their complex differ essentially, amounting to 0.6, 1.9 and 3.1 nm, respectively [120].

In our study, the images of hepatatis B diagnostic markers (antibodies) to HBsAg, HbsAg and HBsAg/antiHBsAg complexes were taken. It was found that the heights of the complexes formed (6–10 nm) exceed the heights of the isolated antigen (4 nm) and antibody (2 nm) molecules (Fig. 5).

Thus, convincing evidence has been provided to date that it is possible to register formation of antigen/antibody immunocomplexes by their increased heights, which opens up the opportunity of using AFM in immunoanalysis for the diagnostics of social diseases accompanied by markers' appearance – such as cancer, myocardium infarction and other disease states.

Described below are the potentialities of AFM application in measuring intermolecular forces. For this purpose the ligand was coupled to the AFM probe while the complementary molecule was coupled to the support. Then the studied surfaces were brought into contact while at the next step they were brought apart; in the course of this procedure, the investigator registers the dependence of the cantilever's deviation on the distance between probe tip and immobilized molecule. In such a way the interaction forces were measured between the pairs: biotin/avidin, antigen/antibody, as well as between the complementary strands of DNA, carbohydrates and lectin/carbohydrate [121]. As an illustration, the ranges of interaction forces between molecules will be presented. For biotin/avidin the range of these forces lies within 0.3–3 nN [121]. For antigen/antibody the interaction forces are much lower, amounting, for instance, to 0.244 nN with HSA and antiHSA [102]; and they are even lower (96 pN) for Con A and oligoglucose saccharides [123].

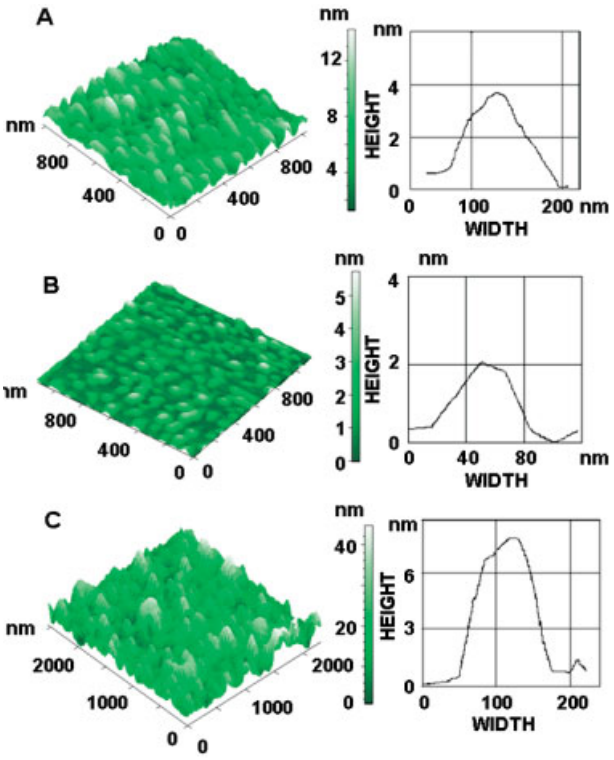

Figure 5. AFM images and cross-sections for HBsAg (A), antiHBs (B) and for antiHBs/HBsAg complexes (C) on mica on air. AFM was a SOLVER P47H device (NT-MDT). Tapping mode. Experimental conditions: 1 μM (2 μL) HBsAg in PBS/T buffer (A), 1 μM (2 μL) antiHBs in PBS/T buffer (B) and the (1 μM/1 μM) antiHBs/HBsAg mixture in PBS/T (mixture volume, 2 μL) (C), pH 7.4, were placed onto the AFM array, incubated for 2 min and rinsed in distilled water; t = 25°C. Image area was 1 × 1 μm.

3.3 Perspectives of development of AFM techniques in proteomics

Widening the AFM scope in proteomics is predicated on nanotechnology development. Further, AFM refining will help remove the presently existing limitations of this tech-

nique. One essential limitation of the AFM is slow data acquisition. For example, the commercial device manufactured by Solver P47H (Russia) allows for the revelation and analysis of proteins and their complexes on the $20 \times 20\ \mu m^2$ area for about 20 min. This time may be substantially reduced at the cost of application of probe arrays instead of one-channel probes. Minne *et al.* [124] reports on the development of an array containing 50 cantilevers; its application allowed these authors to enhance the throughput capability of the device by two orders. In the study of [125] the fabrication of 144-channel arrays is described. Another way to reduce the operation time is to apply high-speed mechanisms for the observation of the surface. Both of these approaches are now being effectively developed.

Along with the concentration detectors which are based on the microarray approach, the AFM-based molecular detectors are beginning to employ a similar, but more refined, nanoarray approach. Presently, the development of diagnostic systems having an antibody array (ViriChip™) and aimed at atomic-force detection of complex formation between antibodies and viruses has been initiated, as reported in (http://www.bioforcenano.com). These protein arrays are commonly fabricated by use of atomic-force manipulators, operating with such amounts of substance which allow the spot size to be lowered to 100 nm and below [19]. Such a system enables to selectively capture partner proteins (whole viruses) from a biological mixture onto the affinity matrix (*e.g.* onto antibodies covalently bound with surfaces) and to perform the AFM measurement of molecular complex sizes at a single-molecule level. By the use of this technology it became possible to capture and visualize 11 enteroviruses, the herpes simplex virus, vaccinia virus, canine parvovirus, BA phage, T4 phage and fd phage. The system is able to diagnose HIV and HCV and to carry out the assessment of noncultivable viruses (caliciviruses). In addition, such virichips may be employed as a single-state purification in PCR and RT-PCR (http://www.bioforcenano.com).

3.4 Cryomassdetectors: the present and the future

The application of nanotechnologies for cryodetectors' creation may lead to the breakthrough in mass spectrometric techniques. The efficiency of direct registration of molecules in a mass spectrometer equipped with a traditional ionizing detector decreases rapidly for massive proteins (with $W > 10$ kDa) due to the low transfer of energy of massive macromolecules to ionizing detectors. Of course, some improvement in this field is envisaged which can be attained at the cost of the increased energy of flight of heavy macromolecules–owing, for instance, to their additional acceleration in the electric field. However, the increased tension arising upon acceleration generates various technical problems which require untrivial solution.

Recently, cryomassdetectors have been developed [126, 127], in which the superconducting tunnel junction (STJ) – a sensitive element cooled to 0.1 K and consisting of superconducting films separated by a nanometric oxide layers – enables to register and identify single-protein molecules and their complexes in the mass range of up to 1 MDa without trypsinolysis. This is achieved owing to the cryodetector's construction: the device is, in fact, a calorimeter with a very low thermal heat capacity, enabling to register the arrival of a molecule by the transformation of its kinetic energy into the heat energy. Comparison of the MALDI-STJ cryodetector with the microchannel plate (MCP) detector–as used in the common MALDI-TOF (Applied Biosystems Voyager DE-STP) – shows that in the mass range 10–20 kDa detection with MCP offered better resolution, while the detection limits of the two devices are comparable. For a very important mass range (20–100 kDa and more) the resolution with MALDI-STJ is comparable with MCP resolution while the LOD with MCP is improved (more than ten-fold for IGG, with MW ~149 kDa). Therefore, both ion detection methods are complementary for investigation of a multicomponent protein mixture over a broad mass range [128]. The possibility of registering heavy protein masses makes the application of cryodetectors in proteomics very attractive. The advantages of cryodetector application for identification of proteins such as IgG, cytochrome C and BSA have been demonstrated in [16, 128]. Cryodetectors may be combined with OBs (serving as selective elements), which makes possible a performance of a single-molecule proteomic analysis based on direct identification of molecules fished out onto the OB from a multicomponent biomixture.

3.5 Potentialities of confocal and near-field microscopies

Confocal microscopy came into being as an extension of fluorescence microscopy. The presently existing device is a scanning microscope with a laser excitation source; it enables to perform scanning not only in the XY plane but also in the vertical direction. Principal resolution of commercial microscopes is determined by the diffraction limit and makes up $\sim\lambda/2$ (*i.e.* 200 nm). With application of special registration schemes, such as two-photon excitation, the resolution becomes even better (up to 100 nm) [129]. Using such devices, one may obtain the images of single molecules–as was demonstrated on the green fluorescent protein (GFP) of *Aequora victoria* [130].

In recent years, the possibility of studying nanoobjects with the aid of SNOM has arisen [108] – along with far-field microscopy [131]. Wide-field microscopy with total internal reflection enabled, for instance, to observe individual ATP turnovers by a single myosin molecule [132] and, also, to observe diffusion of partly immobilized molecules into the lipid membrane at video rate. In outline, the SNOM technique is based on the following principle. The part of light beam propagates along the metal-coated nanofibre probe and radiates through a small aperture with a diameter of much less than λ (Fig. 6). The radiation passing through aperture (about 50 nm) is exponentially evanescens from tip. This

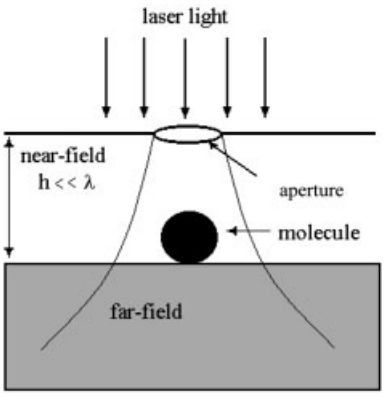

Figure 6. Scheme of molecular probing by light beam in SNOM Part of light beam propagates along the metal-coated nanofibre probe and radiates through a small aperture with a diameter of much less than λ. Radiation passing through an aperture is exponentially evanescens from tip. This probe scans the sample at the height $h \ll \lambda$ (near-field). At the distance within probe and object less than λ, the size of the probe's light spot on the sample is less than the diffraction limit.

probe scans the sample at the height $h \ll \lambda$ (near-field). If the distance within probe and object is made less than λ, the size of the probe's light spot on the sample becomes less than the diffraction limit, as was demonstrated by [133], and resolution in near-field appears to be much better than with the characteristic height $h \gg \lambda$ (far-field). Practically, SNOM resolution reaches 50 nm [134]. Theoretically, this limit may be decreased to less than 1 nm. Betzig and Chichester [135] were among the first pioneers to investigate biological tissues using SNOM. SNOM potential for the revelation of single molecules was demonstrated on the (S65T) mutant of the GFP [136]. GFP is considered by these authors as an individual marker for applications in molecular biology for detailed understanding of its photophysical and photodynamic properties. The authors investigated individual S65T mutants of GFP on a glass surface and embedded in a water-pore gel. In [137] the imaging of laminin and BSA with AFM and SNOM was carried out on a patterned glass surface in both dry and hydrated environment. Some authors [138], based on the study of fluorophore-labelled proteins, stress the perspectiveness of SNOM application in proteomics for the examination of a specific behaviour of one protein in response to the behaviour of the other protein.

The capability to generate patterned surfaces with biomolecules such as proteins for biosensor technology [139] is very important. In protein arrays, the spots contain a range of proteins, and/or visualization of these proteins in their original location and position with SNOM in nanometre resolution may play a critical role in the understanding of their interaction with other molecules.

As follows from the above, the 'molecular detection' approach brings out proteomic researches onto a new qualitative level of single-molecule proteomics – which enables to characterize physicochemical properties of proteins with regard to any one molecule.

4 Conclusions

This review is concerned with the use of nanotechnological approaches for the solution of a major problem of proteomic researches–the revelation and identification of proteins in a wide concentration range, from 10^{-3} M down to single molecules. Two approaches to the application of nanotechnologies in proteomic researches are highlighted: (i) nanotechnological physicochemical and biospecific procedures for the separation of multicomponent protein mixtures as well as for selection, concentration and identification of proteins from multicomponent protein mixtures; (ii) counting of single molecules by use of various molecular detectors.

Special attention is paid to the biospecific 'biosensor fishing' method that proved optimal for selection, concentration on a solid matrix and identification of proteins by MS/MS.

Yet another approach for the separation of protein mixtures may be taken – based on the usage of biospecific arrays. The review outlines the prospects for the application of both proteomic and aptameric arrays in proteomics and diagnostics. In describing the biospecific methods of protein revelation and concentration, a special emphasis is placed on the CD-ROM method as the most simple, cost-effective and convenient one. Application of such an approach will enable to integrate a simple laboratory on a common CD-ROM-like disk.

The sensitivity value of the biofishing method in combination with AFM detection may be theoretically estimated. Let us presume that proteins, *e.g.* antibodies, are covalently immobilized onto a 20×20 μm^2 array spot (as is known, the spot size may range from a few nanometres to a few square centimetres). In order for the proteins to be separated from one another, the density of their packing on this area must not exceed 40 000 molecules *per* 20×20 μm^2 – with a protein's molecular mass of about 50 000 kDa. It may well be suggested that on incubating the spot in 1 mL sample of blood serum, containing an antigen with a concentration of 10^{-15} M, about 40 complex molecules will be formed, with the dissociation constant of the complex $K_d = 10^{-12}$ M. The reversibility of the reaction, as determined by the dissociation constant, would limit the number of the complexes formed on the support surface. It is expedient to use a photoaptamer as an immobilized ligate when the complex formation reaction becomes irreversible. Photoaptamers enable to estimate specificity in two dimensions without requiring a secondary binding agent and, thus, reduce the challenges associated with antibodies detection. The process of fishing by use of photoaptamers involves two procedures: reversible binding of photoaptamers with proteins and covalent linking of tight binding partners. The two-dimension specificity decreases cross-reactivity. Upon formation of irreversible complexes in above-mentioned conditions, the number of captured protein molecules is increased from 40 to 40 000, *i.e.* to saturating amounts. With the antigen concentration in

the sample = 10^{-18} M, one may reveal up to 600 irreversibly bound protein molecules. By increasing the linear size of the spot by an order and hence the immobilization area by two orders, it is possible to considerably enhance the concentration sensitivity and thus to reveal up to 600 molecules in the sample at a protein concentration of 10^{-20} M. If single-molecule registration systems (*i.e.* molecular detectors) are employed, it is not difficult to register such molecule numbers. As molecular detectors, the biosensors with fluorescent detection, atomic force microscopes and some other devices may be used. Thus, the fishing method used in a combination with such detectors enables to lower the concentration sensitivity to 10^{-21} M. There is reason to believe that the photoaptamer-based fishing method in combination with molecular detectors will find a wide application in proteomics.

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