

REVIEW

Why 3-D? Gel-based microarrays in proteomics

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Gel-based microarrays (biochips) consisting of nanoliter and sub-nanoliter gel drops on hydrophobic substrate are a versatile technology platform for immobilization of proteins and other biopolymers. Biochips provide a highly hydrophilic environment, which stabilizes immobilized molecules and facilitates their interactions with analytes. The probes are immobilized simultaneously with gel polymerization, evenly distributed throughout individual elements, and are easily accessible because of large pores. Each element is an isolated nanotube. Applications of biochips in the studies of protein interactions with other proteins, nucleic acids, and glycans are described. In particular, biochips are compatible with MALDI-MS. Biochip-based assay of prostate-specific antigen became the first protein microarray approved for clinical use by a national regulatory agency. In this review, 3-D immobilization is compared with mainstream technologies based on surface immobilization.

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1 Introduction

A search in Medline shows that as much as 20% of publications on protein microarrays in the last 5 years are reviews. This indicates that the field is still in its early phase with no clearly dominating technology. Because of the abundance of all-embracing reviews of this topic, we will discuss primarily immobilization of proteins on 3-D supports, focusing on gel-based technology of biochips developed in the laboratory of the late Andrei Mirzabekov. In 2001, a new generation of gel drop biochips was developed by his group; the manufacture and applications of these biochips, including immobilization of proteins, were

described in detail elsewhere [1–3]. We will summarize our work with gel drop biochips and review pertinent papers that appeared after the publication of our previous review in 2001 [4].

Technological advances have affected all aspects of protein array technologies, from manufacturing to interpretation of results and practical applications. Most importantly, protein microarrays are now being used in high-throughput studies of protein interactions with other proteins, nucleic acids, carbohydrates, and small molecules serving crucial biological functions. The diversity of chemical properties of proteins makes their immobilization in microarray format a much more challenging task than the immobilization of nucleic acids, especially when it is necessary to preserve biological activity. Proteins tend to denature at the interface between water and solid support; besides, some proteins can be attached to solid surfaces in a certain orientation that could mask their active centers, or they aggregate at a high concentration in the process of immobilization.

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Abbreviations: PAAG, polyacrylamide gel; POCT, point-of-care testing; PSA, prostate-specific antigen; OS, oligosaccharides; SAW, surface acoustic wave; SPR, surface plasmon resonance

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The choice of substrate for protein immobilization depends on many factors: stability of the probe, the nature of the target (analyte), the required density and sensitivity of the arrays, methods of registration, cost, *etc.* Some of the most popular technologies were compared and later reviewed by Angenendt *et al.* [5, 6]. These authors outlined three main categories of protein microarrays based on the principles of immobilization and spacial distribution of molecules.

An overwhelming majority of commercially available products employ 2-D surfaces chemically modified or coated for optimal performance in combination with fluorescent labeling of the analyte-detecting reagent.

Another rapidly developing category of methods is based on the immobilization of protein molecules within molecular structures organized at a nanoscale level [7]. This category encompasses a broad range of true nanotechnologies, from inclusion of proteins into complex dendromers attached to a substrate, to immobilization of protein molecules on patterned surfaces in controlled orientation. In many cases, these approaches employ direct detection of binding using complex physical methods of registration [8]. Despite the extremely high sensitivity of these methods, they require special expertise, sophisticated and expensive equipment, and, by and large, have not yet entered the mainstream of practical applications.

The third category of immobilization media, according to Angenendt *et al.* [6], is “3-D slides”, which includes four types of immobilization media: slides coated with polyacrylamide gel (PAAG), or modified PAAG; agarose slides; NC-coated slides (for instance, FAST slides <http://www.whatman.com/>); and proprietary SuperProtein slides coated with hydrophobic polymer (Telechem International, Inc., <http://arrayit.com/>).

Despite its porous structure, which significantly increases the binding capacity of the FAST slides, NC coating does not provide the true 3-D hydrophilic environment similar to solution, characteristic of PAAG and agarose gels (see below); in fact, the manufacturer describes FAST slides as having a “three-dimensional surface.” The exact chemistry of the SuperProtein coating is not disclosed, and no publications with this keyword could be found in Medline.

We will focus on gel-based technologies, in which proteins are immobilized in highly hydrophilic 3-D media with high water content and in which conditions are similar to solutions [9]. In addition, we will describe gel-based arrays with immobilized nucleic acids and glycans, which complement protein arrays in studies of protein interactions with corresponding compounds.

2 Main types of 3-D chips

There are several reasons for the need of a 3-D hydrophilic environment for protein immobilization. One of the most important reasons was eloquently formulated by Shuguang Zhang [10]: “Wet or let die,” and further: “Proteins are like fish in that they need water to survive—without it they lose

vitality and become unable to carry out their functions”. Development of microarray technologies able to provide such an environment is an essential field of proteome research.

2.1 Gel-based biochips

Immobilization of proteins in a small array of elements consisting of pre-formed polyacrylamide gel (biochip) was first reported by Gushchin *et al.* in 1997 [11] and further developed by Arenkov *et al.* [12], as reviewed by us earlier [4]. The technology of gel drop biochips constituted a major refinement of this approach [2, 3].

The original rectangular gel elements, which required complicated molding devices, were replaced by hemispherical drops easily applied on a variety of supports using automated arraying equipment with pin dispensers.

New gel chemistry—the substitution of methacrylamide for acrylamide—resulted in stronger, heat-resistant, and more porous gel. Polymethacrylamide gel elements sustain 30–40 standard cycles of PCR without any noticeable damage. As for the size of the pores, antibodies with molecular mass 150 kDa penetrate readily into gel elements by passive diffusion (see Section 5).

Proteins are immobilized in the gel using photoinduced copolymerization. In this reaction, the proteins are modified by incorporation of a non-saturated group, which then participates in the radical polymerization of gel-forming monomers. As the result of copolymerization, the protein forms a strong covalent bond with the growing polymer network of the hydrogel.

We developed two approaches to the aforementioned modification of protein with a non-saturated group. In the first approach, the non-saturated group is incorporated into protein by its modification prior to polymerization with *N*-hydroxysuccinimide ester of 6-(*N*-methacryloyl)-aminohexanoic acid or any other activated ester of non-saturated carbonic acid. In the second, simpler approach, the reactions of protein modification and gel polymerization are carried out simultaneously, with the protein being modified at its NH₂- and SH-groups by bifunctional gel-forming monomers in the course of polymerization [2, 3]. Since all chemical reactions—polymerization, modification of probe molecules, and their cross-linking to the gel—occur simultaneously, the immobilized molecules are distributed evenly throughout each element, as demonstrated by confocal microscopy [3, 13] (see Section 6). The efficiency of immobilization depends almost exclusively on the nature of protein within a broad range of the extent of its modification by methacrylic groups and varies from 40 to 75%.

The 3-D gel elements (hemispherical drops) are placed on a hydrophobic surface of the chip at a fixed distance. The size of the drops is determined by the diameter of the robotic pin and varies from 50 to 500 μm in diameter (Fig. 1). The size and the pitch of the elements in the array, as well as the amount of immobilized material, depend on the specific

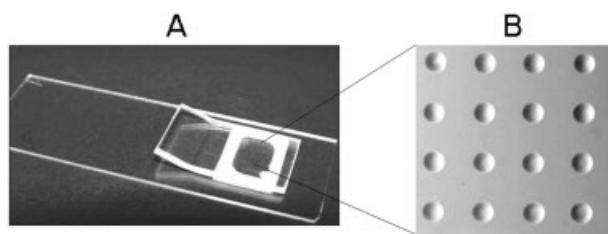


Figure 1. Biochip manufactured for commercial application. (A) Glass slide with the array of semispherical gel drops. The array is covered with a transparent plastic reaction chamber with two outlets for the addition of reaction mixture. (B) Array of gel elements 0.1 nL in volume and 100 μm in diameter in bright-field microscope.

goal and design of the experiment. In a typical case, 100 fmol of protein can be immobilized in a gel element 200 μm in diameter; in these conditions the average distance between immobilized molecules exceeds 100 nm, preventing their undesirable interactions. This results in a broad linear range of measurements of the concentrations of interacting molecules.

Reproducibility of microarrays is an important issue, as noted, for instance, in a recent review [14], especially for some coated slides. In particular, commercial gel-coated slides demonstrate very high level of variability [15] (also, see Section 2.2). In this respect, the gel drop technology allows one to increase substantially the reproducibility of the results since this technology makes it possible to select microchips with the intra-array variation of drop dimensions not exceeding 5%, and inter-array variation about 10% during the manufacturing step [2].

2.2 Agarose-coated slides

The use of slides coated with NaIO_4 -activated agarose for microarrays was first reported by Afanassiev *et al.* [16], who demonstrated their applicability for hybridization of nucleic acids and protein immobilization. However, agarose-coated slides have been used mostly for immobilization of oligonucleotides and hybridization. Only recently, another study was published on further optimization of the immobilization of antibodies on agarose-coated slides [17]. The authors demonstrated that agarose slides offer substantial advantage as compared with commercial Hydrogel-coated slides. Signals produced by sandwich immunoassay were linear within the whole range of concentrations of immobilized antibodies, indicating a high capacity of the agarose layer. Notably, the intra- and inter-array signal variations were very low, under 2 and 9%, respectively, contrary to Hydrogel-coated slides (see below). In addition, agarose-coated slides have low background, are easy and inexpensive to prepare, and are compatible with arraying and recording equipment. The applicability of agarose-coated slides is limited by their fragility and incomplete separation of probes. Besides, the

authors did not test their shelf life beyond 2 months, which does not exclude their potential for longer storage.

2.3 Comparison of various 3-D technologies

Gillaume *et al.* [18] performed a systematic comparison of eight commercially available protein arraying technologies, including Hydrogel™ slides, similar slides Nexterion™ manufactured by Schott AG (<http://www.us.schott.com/english/index.html>), and several 2-D technologies. Slides coated with an even layer of PAAG-based Hydrogel™ were developed by Packard Biosciences and are currently distributed by Perkin-Elmer, which acquired Packard in 2001.

Hydrogel-coated slides demonstrated better overall performance, including high LOD, S/N, and sensitivity. However, the intraslide variability was 20–40% and interslide variability 60–100%, far above the level of variability of gel drop biochips and certainly unacceptable for any mass applications.

The main drawbacks of the Hydrogel-coated slides and other similar substrates are defined by the application of probes on the surface of the gel and the continuous nature of the coating. The minimal spot size for all tested media [18] was about 200 μm , thus limiting the potential density of spots in the arrays. The probes penetrate in the coating layer by passive diffusion, their penetration depends on the size of the molecules, and the resulting distribution within the spots is uneven.

Another comparison of different substrates for protein arrays together with a large compilation of published data was made by Kusnezow *et al.* [19]. The data obtained by the authors were inconsistent. Even the relative performance of different types of substrates depended on the nature of the antibody–antigen pair. In some cases the signals on Hydrogel-coated gels were 100 times lower than on simple homemade epoxysilane glass. The authors wrote: “. . . Interpretation of experimental microarray results can be quite fortuitous. Moreover, changing any experimental condition, such as incubation chamber and time, spot size and so on, it is impossible to predict the consequences on assay performance.”

Because of the practical importance of this issue, we recently investigated the sensitivity of gel-based and surface microchips in the sandwich-type immunoassay commonly used for the assessment of antigen concentration, as well as direct immunoassay [20]. We used the detection of prostate-specific antigen (PSA) as the model.

The maximal density of antibodies immobilized on 2-D surface is limited by their size and is $3\text{--}4 \times 10^4$ molecules *per* μm^2 . Theoretically, the maximal density of gel-immobilized antibodies projected onto the basis of the gel element is $5 \times 10^6\text{--}5 \times 10^7$ molecules *per* μm^2 . In fact, the maximal working density in the gel is significantly lower because of kinetic considerations, as demonstrated by Zubtsov *et al.* [20], and is approximately an order of magnitude higher than in 2-D arrays. The signals at saturation on 3-D arrays were up to eight times higher in sandwich-type assays and up to 13 times higher in direct assays than on 2-D arrays (Fig. 2) [20]. Most

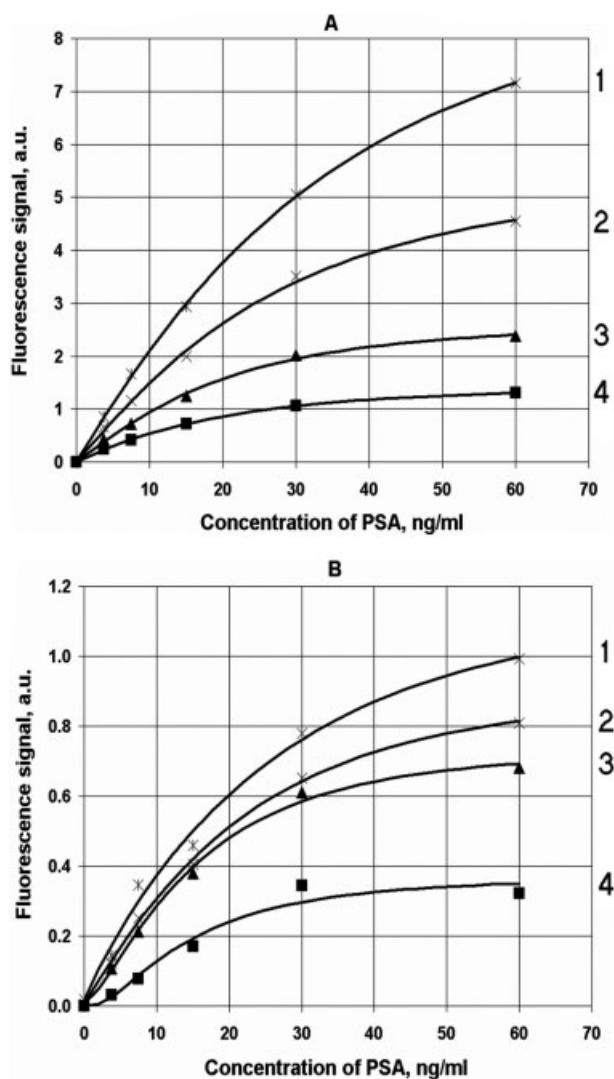


Figure 2. Calibration curves for sandwich-type immunoassay of PSA obtained with gel-based (A) and surface (B) protein microchips at different concentrations of antibodies in spotting solution: 0.8 mg/mL (1); 0.4 mg/mL (2); 0.2 mg/mL (3); and 0.1 mg/mL (4).

interesting, in accordance with theoretical predictions conveyed in the paper, the kinetics of binding of the analyte to immobilized antibodies was identical for 2-D and 3-D chips.

3 Design of assays

Assays on protein microarrays in proteomic research and clinical applications can be divided into several main categories (see, for instance, [14]).

The goal of specific assays is the identification and sometimes quantification of certain proteins. This category can be further subdivided into (i) forward-phase assay, in

which the capture proteins (in most cases, antibodies) are immobilized on the chip, so that different analytes in the target can be screened simultaneously, and (ii) reverse-phase (RP) assay, in which complex protein mixtures are immobilized on the chip and screened by applying specific probes.

Functional assays on protein microarrays analyze protein interactions with other molecules; these experiments can be complemented by immobilizing the protein-binding molecules (nucleic acids, oligosaccharides, small ligands) and testing their interactions with proteins in solution.

In the course of the development of gel drop biochips, Rubina *et al.* [1, 2] successfully applied all these types of assays. In particular, antibodies were immobilized in the gel elements and coupled with corresponding proteins with proper controls; in other experiments, antigens (proteins) were placed on the array and stained with antibodies using both direct and sandwich immunoassays. In all cases association constants for the antigen-antibody complexes formed in the gel were very close to those determined by the manufacturers of antibodies in solution. This was demonstrated for the complex of insulin with anti-insulin antibody [21] and diphtheria toxin and immobilized antibodies against the toxin [13]. In the latter work, Rubina *et al.* [13] examined whether random modification of antibodies followed by covalent immobilization affected their performance. Antibodies against ricin were immobilized in gel pads by two different methods: chemical modification with simultaneous copolymerization or biotinylation with subsequent binding with gel-immobilized avidin. The LOD achieved using both methods (0.1 ng/mL) was very similar, despite completely different strategies used for both immobilization and development.

In their initial work on immobilization of proteins using copolymerization, Rubina *et al.* [2] also studied the activity of immobilized HRP depending on the conditions of immobilization. The enzyme retained 70% of its specific activity in optimal conditions. This suggests a modest impact of chemical treatment on protein structure.

The experiments with barnase, extracellular ribonucleoside-3'-transferase with strong preference for G-containing sequences, proved that chip-immobilized protein maintained both enzymatic activity and substrate specificity. Ribog-G-containing oligonucleotide was completely hydrolyzed by gel-immobilized barnase at room temperature in less than 1 min, whereas the hydrolysis of ribo-U-containing nucleotide took several hours [22].

The stability of immobilized proteins was tested in the original paper [2] by comparing the activity of immobilized HRP immediately after manufacturing and after storage for 6 months; the enzyme fully retained its initial activity. Similar data were obtained for gel-immobilized antibodies stored for 6 months [13]. Finally, biochips for clinical measurements of PSA show no signs of degradation of the immobilized antigen over 12 months of storage as illustrated in Fig. 3 [23].

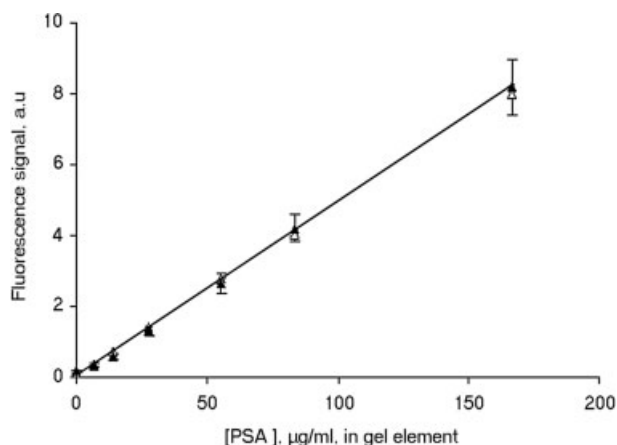


Figure 3. Stability of immobilized proteins during storage. The signals from the biochip-immobilized PSA after the interaction with fluorescently-labelled antibodies were obtained immediately after manufacture (open triangles) and after 1 year of storage at 2–8°C (filled triangles). Each point is a mean value of ten measurements.

The possibility of more detailed chemical analysis of gel-immobilized protein was shown by tryptic hydrolysis of gel-immobilized lysozyme with subsequent analysis of the resulting peptides by MALDI-TOF (see Section 8).

4 Recording of data

Measurements of fluorescence or chemiluminescence of individual elements on gel drop biochips are performed using equipment, algorithm, and software developed at the Engelhardt Institute of Molecular Biology (EIMB) in Moscow [24, 25].

The software allows one to calculate fluorescence intensity from each gel element after subtracting the background level. To control the variations in quantitative assays, each protein concentration on a chip is represented by four identical gel elements (see, for example, Fig. 6), and the results are calculated as median values of signals from four elements.

The threshold of statistically valid measurements was universally chosen at a threefold background level. The sensitivity of the research reader developed in the laboratory reaches 0.3 amol *per* gel element with the dynamic range of 4000:1 [24].

Due to the large capacity of gel elements, gel drop technology enables one to register the binding (or dissociation) of the molecules inside the gel in real time using relatively simple technology. The only other method that offers such a possibility is surface plasmon resonance (SPR) (for review, see [26]), which has been used lately in multiplex experiments with protein arrays. One advantage of SPR is that it uses direct registration, *i.e.*, it does not need any fluorescent

labeling. However, SPR requires constant flow of the analyte solution along the surface of the array. This excludes some of the possibilities offered by gel chips, for instance, dependence of binding on temperature in quasi-equilibrium conditions (melting). Studies of protein interactions using SPR require immobilization of proteins on a 2-D surface of thin gold film. To prevent the denaturation of proteins on the gold surface, it has to be coated with a hydrophilic monolayer.

5 Protein diffusion in gel drop elements

In both 2-D and 3-D arrays, the interactions with immobilized molecules may occur only after the diffusion of their counterparts from surrounding solution. Although there is no universally accepted model of diffusion in PAAG, it has been estimated that the diffusion coefficients for proteins in 5% PAAG at 2.7% cross-linking are about 1/3 of the corresponding values in water, and this slows down the interactions within the gel pads [27]. The diffusion coefficient in high-porosity gels obtained by copolymerization of gel-forming derivatives of methacrylamide and N-substituted sugars and described in our latest work [28] should certainly be higher.

Still, there are ways to further accelerate the diffusion. Arenkov *et al.* [12] used microelectrophoresis. Zubtsov *et al.* [21] forced constant flow of solution over the biochip using peristaltic pump. This procedure accelerated the interaction of antigen (insulin) with the corresponding immobilized antibody about fivefold (Fig. 4). The real-time data allowed the authors to estimate key parameters of the antigen–antibody interaction. The association constant $(4.5 \pm 0.4) \times 10^7 \text{ M}^{-1}$ was in good agreement with the value determined by an unrelated method of SPR, and the calculated diffusion coefficient for insulin (molecular mass 5.7 kDa) in the gel was $5 \times 10^7 \text{ cm}^2/\text{s}$, in line with existing estimates for diffusion of proteins in gels with the concentration of PAAG below 5% and low level of cross-linking, especially taking into account potential formation of insulin oligomers. For instance, the diffusion coefficient in 5% PAAG for BSA (67 kDa) is $6 \times 10^7 \text{ cm}^2/\text{s}$ and for RNase (13.7 kDa) $11 \times 10^7 \text{ cm}^2/\text{s}$ (see [27]).

We compared our method of mixing using a peristaltic pump with surface acoustic wave (SAW) microagitation [29]. The SAW device (SAWstation with the Extension Module) is available commercially (<http://www.advalytix.com>). We found that the formation of binary and tertiary immune complexes accelerated to the same extent regardless of the method of mixing.

In addition, in accordance with theoretical predictions, both the rate of signal saturation and the intensity of signal at saturation showed linear dependence on the concentration of antigen in solution within a broad range of concentrations. The mixing procedure was also successfully used in the study of gel chips with immobilized glycans (see Section 10).

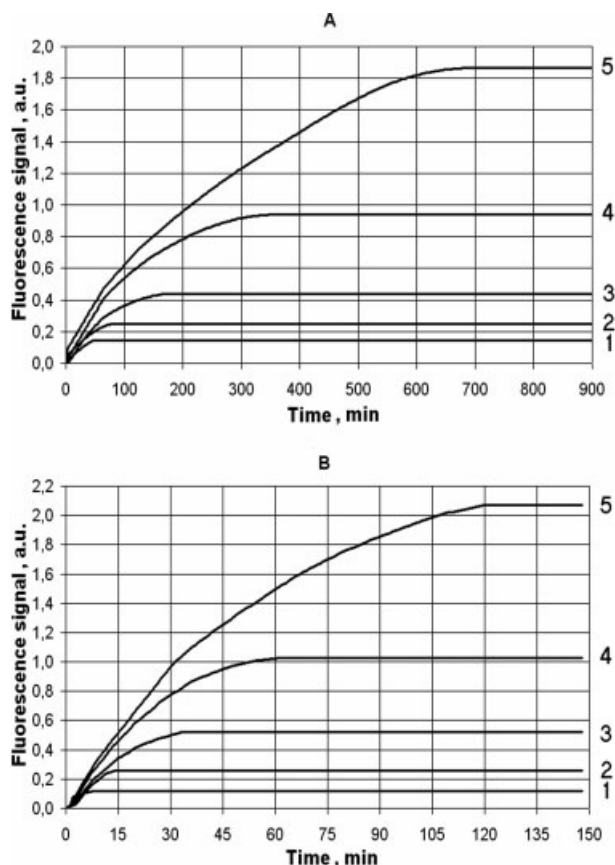


Figure 4. Kinetic curves for (A) diffusion-reaction kinetics and (B) kinetics forced by peristaltic pump. Interaction of Cy3-labeled insulin with antibodies to insulin immobilized into gel elements. Concentration of insulin in solution was 100 ng/mL, concentration of antibodies in the gel pads was 40 (1), 80 (2), 150 (3), 300 (4), and 600 (5) $\mu\text{g/mL}$ of gel. Note the difference in time scale between panels A and B.

6 Application of biochips with immobilized proteins for clinical diagnostics

It is widely accepted that one of the major practical goals of proteomics is the search for new biomarkers of human malignancies for early and noninvasive diagnostics [30]. In addition, some of them are currently used to monitor the course of treatment. In the future, two other important applications of tumor markers are considered: mass screening for preclinical malignancies and the development of surrogate (or biological) markers for clinical studies.

Although a number of individual tumor markers have been approved by the FDA for diagnostics and treatment monitoring (PSA, alpha-fetoprotein, CA-125, carcinoembryogenic antigen, CA 19-9, CA 15-3, nuclear matrix protein 22, and, with certain reservations, chorionic gonadotropin),

all of them can be used only in combination with other extensive clinical data and not used by themselves either for diagnostics or for early screening. Radical improvement of the statistical validity of protein tumor markers will require simultaneous measurement of subsets of proteins identified through large-scale statistical analysis, *i.e.*, multiplex format. In a recent review of the current status of this approach, the author wrote: "...Multiplexed protein measurement has the capacity to identify surrogates that will be integrated into clinical indices, treatment algorithms, and, ultimately, into dynamic disease models that permit real-time, data-driven patient management" [31].

In just one example of multiplex protein analysis in current development, Mor *et al.* [32] compared 169 proteins in sera from healthy women and patients with ovarian cancer. They identified four proteins, which, when used in multiplex format, permitted the authors to achieve a sensitivity of 95%, positive predictive value of 95%, specificity of 95%, and negative predictive value of 94%. The authors used both microarray and conventional ELISA platforms and found complete concordance between the two.

Simple, inexpensive, and reliable tools are necessary for regular screening of the population for the early diagnosis of cancer. At EIMB, Moscow, Dementieva *et al.* [33] developed such a tool on the basis of gel drop biochips. The original biochip carried mAbs to six tumors markers; later this set was supplemented with two more markers. The markers, their clinical relevance, the sensitivity of the biochip elements, and comparison with existing commercial test-systems are listed in Table 1. The diagnostic cut-off values indicated in the table were chosen rather arbitrarily, since the statistical significance of these markers and their exact values remain controversial. The main advantage of such a diagnostic chip for several tumor markers is the possibility to use it for large-scale statistical analysis.

Fluorescent confocal microscopy demonstrated that complete penetration into gel elements of the chip is achieved even for a large protein like CA-125, whose molecular mass is at least 200 kDa (Fig. 5).

While the sensitivity of detection of tumor markers using gel biochip is comparable to existing commercial system, the required volume of the sample is much smaller, making the biochips especially convenient for multiple runs of the same samples, for testing of newborns and infants, as well as research applications in the studies of small animals. Taking into account the possibility of further miniaturization of gel biochips—gel elements 50 μm in diameter can be manufactured—these biochips should be considered prototypes for future multiplex analysis of larger sets of tumor markers.

6.1 PSA biochip

Measurement of PSA level is considered the most reliable early diagnostic tumor marker detectable as an antigen in serum. Despite its pitfalls—relatively high level of false-

Table 1. Diagnostic biochips for detection of tumor-associated protein markers in serum

Tumor marker (antigen)	Tumor	Normal concentrations	Diagnostic margin	Sensitivity using biochips	Sensitivity of commercial tests
α -Fetoprotein	Liver	<10 ng/mL	12.5 ng/mL	0.4 ng/mL	0.5–2 ng/mL
PSA _{tot}	Prostate	<4 ng/mL	10 ng/mL	0.3 ng/mL	0.01–1 ng/mL
PSA _{free}	Prostate	<1 ng/mL Free/Total >0.25	Free/Total <0.1	0.2 ng/mL	0.02–0.1 ng/mL
Carcino-embryogenic antigen (CEA)	Colon, rectum	<5 ng/mL (non-smoker) <10 ng/mL (smoker)	20 ng/mL	0.15 ng/mL	0.25–2 ng/mL
CA 125	Ovaries, uterus	<30 IU/mL	40 IU/mL	15 IU/mL	5 IU/mL
CA 19-9	Pancreas, GI	<30 IU/mL	40 IU/mL	2.0 IU/mL	3 IU/mL
CA 15-3	Breast, ovaries	<22 IU/mL	30 IU/mL	4.0 IU/mL	3 IU/mL
Chorionic Gonadotropin (hCG)	Choriocarcinoma, testicular tumors, bladder, prostate, kidney	<5 IU/mL	30 IU/mL	3.0 IU/mL	3 IU/mL
NSE (neuron-specific enolase)	Lung, neuroblastoma, pancreas	<12.5 ng/mL	20–50 ng/mL	0.5 ng/mL	0.5 ng/mL

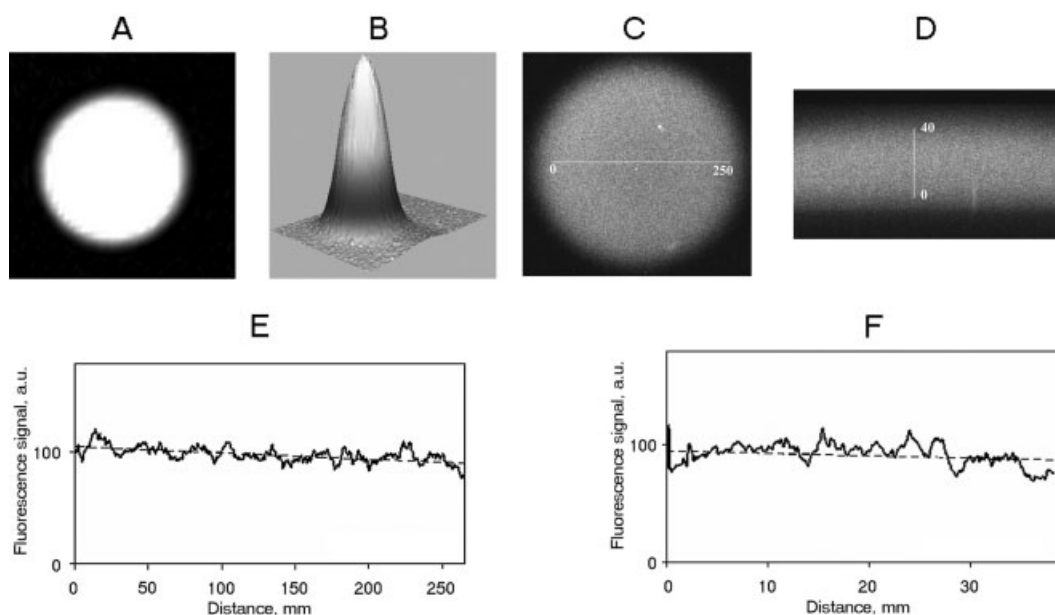


Figure 5. Distribution of fluorescent label within microchip gel elements. 2-D (A) and 3-D (B) fluorescent images of gel elements containing immobilized mAb against CA-125 (100 μ g/mL) after interaction with CA-125 (100 ng/mL) and development with Cy5-labeled secondary antibodies against CA-125 (10 μ g/mL). Confocal microscope fluorescence images of the same gel element in horizontal section 5 μ m from the glass surface (C) and vertical section through the center of the drop (D). Distribution of fluorescence intensity along the horizontal (E) and vertical (F) sections of the drop is also shown.

positive results and lack of PSA increase in some cancer patients —PSA measurement will remain a valuable cancer screening tool in the foreseeable future [34].

PSA exists in circulation in two forms: free (PSA_{free}) and bound with α -1-antichymotrypsin. Malignant condition is characterized by an elevated level of total PSA (PSA_{tot}) and low proportion of free (non-bound) protein (PSA_{free}/PSA_{tot}) [35]. PSA is the most specific among serum markers: simultaneous measurement of total (PSA_{tot}) and of the PSA_{free}/

PSA_{tot} ratio provides a statistically valid rationale for diagnostics. In addition, it is extremely helpful during the follow-up observation after the treatment of prostate tumors.

Although commercial FDA- and EMEA-approved tests are widely available, they have some common drawbacks [36]. The tests are performed in large batches in dedicated laboratories using complex equipment. Therefore, samples must be transported to large central facilities, which add to the cost of tests and administration. Most tests require a

relatively large volume of samples (sometimes up to 1 mL), and the turnaround time is rather long. For these reasons, a point-of-care testing (POCT) system would be highly desirable. Detection and measurement of PSA is a popular model for the demonstration of novel microarray and biosensor technologies [36].

Kononova *et al.* [23] describe a gel biochip for sandwich immunodetection and measurement of total and free PSA concentration in serum, which incorporated several major technological innovations.

The gel used for PSA biochips contains copolymerized N-substituted aminosaccharides. These highly hydrophilic moieties stabilize immobilized proteins, allow synthesis of low-percentage gel with a low level of cross-linking, and increase the water-retaining capacity of the gel and its porosity. A similar galactoside polyacrylate hydrogel was first used for immobilization of proteins on coated slides by Goldman *et al.* [37]. Among other tests, Kononova *et al.* compared calibration curves and test results on PSA biochips after 12 months of storage and found no changes in these parameters (see Fig. 3).

The chip consists of a set of elements with immobilized mAbs to total and free PSA. In addition, it contains a set of elements with different amounts of immobilized PSA itself, which are used to obtain an internal calibration curve on the same chip. Each concentration is represented in quadruplicate to increase the statistical validity of the data. Gel elements are 0.1 nL in volume and 80 μm in diameter. When a mixture of tested serum and developing anti-PSA fluorescently labeled antibodies is added to the chip, the antibodies bind in the sandwich mode with PSA from solution and with unlabelled anti-PSA antibodies located in the gel elements used for measurement, and also in direct mode with the immobilized antigen PSA (Fig. 6).

The concentration of both PSA_{tot} and PSA_{free} in samples is determined using dedicated software “ImaGelAssay” (EIMB). This program calculates integrated fluorescent signals from all individual gel elements and plots a calibration curve using signals from the elements containing immobilized antigen. Based on this internal calibration curve, the program then calculates the concentration of the two forms of PSA and their ratio in the sample. The intensity of fluorescence is calculated as a median value of four elements.

The reproducibility of the analysis was tested on 100 biochips. The dependence of fluorescence intensity on the concentration of PSA in solution—external calibration curve—was constructed using specially prepared calibration solutions containing known (diagnostically meaningful) concentrations of PSA up to 60 ng/mL for PSA_{tot} and up to 20 ng/mL for PSA_{free} . Each point on the curve in Fig. 7 corresponds to measurements performed on ten biochips, and the CV for each concentration of the antigen was 7–10%.

The sensitivity of the assay was defined as the lowest reliably detectable PSA concentration, *i.e.*, the concentration which produced fluorescence signal exceeding that of the control probe by at least two SDs (the so-called analytical

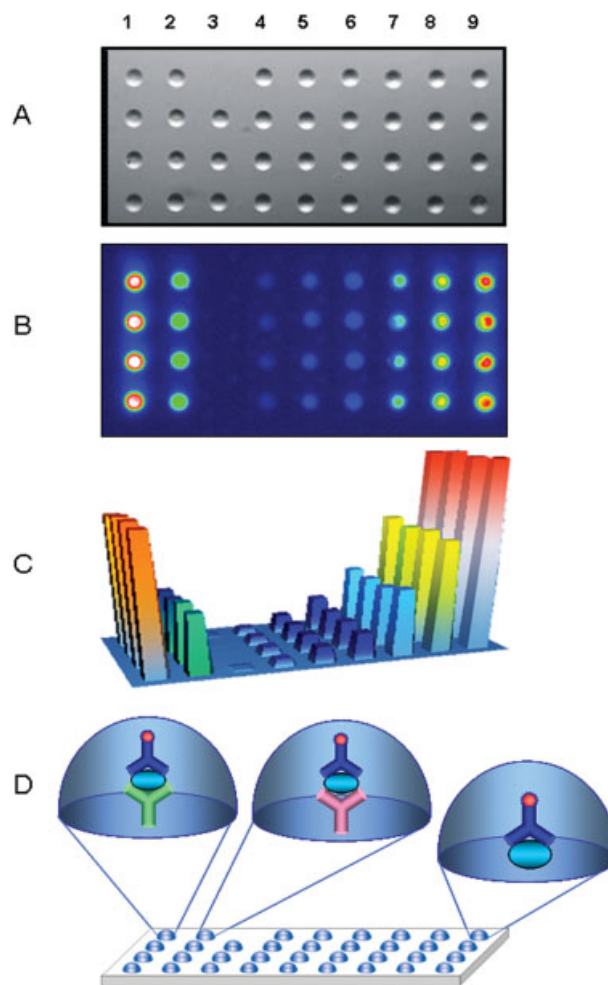


Figure 6. Quantitative detection of PSA_{tot} and PSA_{free} using gel-based microchips. (A) Diagnostic biochip in transmitted light. Columns 1 and 2 contain 200 $\mu\text{g/mL}$ of immobilized mAb to PSA_{tot} and PSA_{free} , respectively; column 3 contains three empty gel elements (controls); columns 4–9 contain immobilized PSA (7, 14, 25, 55, 80, and 160 $\mu\text{g/mL}$ PSA, respectively). (B) and (C) 2-D and 3-D computer emulations of the fluorescence image (B) or fluorescence intensities (C) of biochip after the assay. For final calculations, the median intensity of four identical elements was used. (D) Scheme of the reactions on the biochip. As the result of single-step sandwich immunoanalysis, two reactions take place simultaneously. In the analytical gel elements (left), triple complexes are formed between immobilized mAb and antigen and developing mAb in solution. Immobilized and developing antibodies recognize different epitopes of the antigen. In gel elements used for internal calibration (right), binary complexes are formed between immobilized antigen and developing antibody.

sensitivity [38]). The signal of the control probe without PSA was measured at least ten times. Thus, measured sensitivity was 0.3 ng/mL for PSA_{tot} and 0.2 ng/mL for PSA_{free} (Fig. 7). The functional sensitivity defined as the lowest concentration at which CV does not exceed 20% is very close to analytical sensitivity.

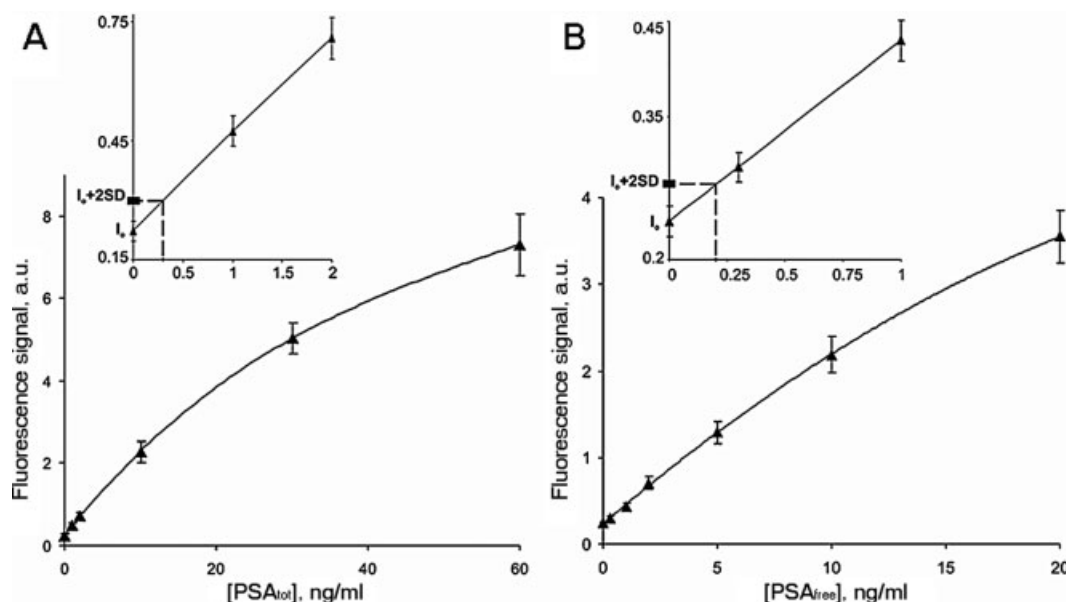


Figure 7. Sandwich immunoassay of PSA on biochips. (A) and (B) external calibration curve. Fluorescence intensity I_{fluor} of gel elements containing immobilized antibodies against PSA_{tot} (A) and PSA_{free} (B) is plotted against the concentration of the corresponding forms of PSA in solution. Each point represents measurements on ten biochips. SD is shown. CV in all cases does not exceed 10%. The insets show enlarged fragments of the same curves at low concentration of PSA used to determine the sensitivity of the tests. I_0 – fluorescence of the control probes, $I_0 + 2SD$ is the value that determined the sensitivity of the measurement.

As required by Russian regulations, a series of 60 samples of serum from healthy controls and prostate cancer patients was analyzed at three medical centers in Moscow specializing in oncological diseases using ELISA (EIA) diagnostic kits manufactured by CanAg (<http://www.canag.se/>; CanAg Diagnostics has been recently acquired by Fujirebio Diagnostics, Inc.) and using biochips with an internal calibration curve as described here. The results of this comparison are illustrated in Fig. 8. The correlation coefficient was 0.988 for PSA_{tot} and 0.987 for PSA_{free}.

Every batch of about 500 biochips undergoes scrupulous quality control procedures. First, the biochips are inspected in passing light and computer software rejects the chips with intra-array variations in the diameter of gel elements exceeding 5%. Then the whole batch of biochips is inspected for inter-chip variations of the size of gel elements: the inter-chip CV must not exceed 8%.

The biochips in the PSA detection kit are completely ready for the assay. Biochips are already treated with blocking compounds to minimize non-specific binding and are covered with a reaction chamber. The required volume of samples is 50 μ L and the reaction is performed for 17 h (overnight in a typical clinical laboratory).

In September 2006 Whatman PLC announced the release of Combichip™ for clinical diagnostics of the human autoimmune diseases collagenosis and vasculitis (see www.whatman.com and press releases therein). One important advantage of Combichip™ is its multiplex design, allowing identification of several autoimmune antibodies on a

single array. Combichip™ received CE certification, indicating that the device meets the safety, health, and environmental requirements of the EU, without any assessment of the quality or clinical relevance of the device.

Thus, the PSA detection kit is the first immune biochip-based kit available commercially and approved for clinical applications by national regulatory agency, the Federal Agency of Supervision of Health Care and Social Development of the Russian Federation, based on a multicenter clinical trial.

The design of the PSA chip is based on the principle “one patient—one chip.” The estimated cost of a single PSA chip is within the range of the cost of testing *per* sample using traditional ELISA.

A unique feature of the PSA biochip is the incorporation in the array of internal calibration elements containing standard amounts of immobilized antigen (PSA). Because of these calibration elements, there is no need to construct the calibration curve for every assay and no need to use special calibrators.

Besides the immediate clinical importance of the PSA biochip, we consider it the testing model for the development of multiplex gel-based arrays. Multiplexing is one of the major advantages of microarrays as compared with traditional ELISA diagnostics, while being comparable to advanced ELISA tests in terms of cost and sensitivity.

At the present time, the laboratory of biological microchips at EIMB, Moscow, has passed the medical tests for the next biochip-based immunological kit: a set of reagents (test

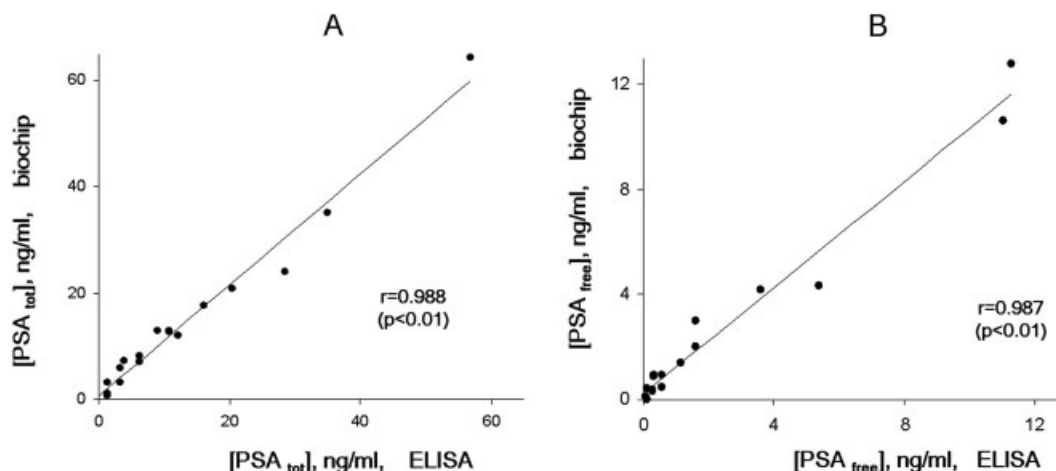


Figure 8. Comparison of the quantification of PSA_{tot} (A) and PSA_{free} (B) using biochips with internal calibration curve versus sandwich immunoassay using ELISA (EIA) kits manufactured by CanAg (Göteborg, Sweden).

system) for simultaneous quantitative detection of six markers of oncological diseases in human sera (total and free forms of PSA, α -fetoprotein, carcinoembryonic antigen, chorionic gonadotropin, and neuron-specific enolase). The principle of the assay is similar to that described here for the PSA determination kit.

6.2 Detection of biotoxins

Many bacterial, plant, and animal toxins are extremely toxic for humans, with lethal doses for some of them being just a few nanograms. While existing immunoassays are sensitive and convenient for rapid detection of biotoxins, they do not allow carrying out simultaneous tests for the presence of several toxins. Development of fast, sensitive, and versatile methods for the detection of biological toxins is of great importance because of the threat of bioterrorist attacks. Besides, biotoxin assays are used in the food and medical industries and in environmental monitoring.

In 2005, Rubina *et al.* [13] described a gel-based biochip for the detection of six protein toxins (ricin, viscumin, staphylococcal enterotoxin B, tetanus toxin, diphtheria toxin, and lethal factor of anthrax) using immobilized antibodies. Besides its practical importance, this work served as another test for essential features of the gel drop biochips.

First, the porosity of the methacrylamide-based gel was sufficient for complete penetration of molecules up to 150 kDa and their even distribution within the gel as demonstrated by confocal microscopy. Further, large complexes of antigens with antibodies, including triple complexes resulting from sandwich immunoassays with primary antibodies immobilized in the gel, were also evenly distributed in the gel elements.

Second, measurements on biochips enabled the researchers to determine the association constants for ricin and diphtheria toxins and corresponding antibodies. The

constants turned out to be close to those calculated in solution (of the order of 10^8 – 10^9 M⁻¹). This confirms that gel-immobilized antibodies fully preserve their antigen recognition ability and that moderate modification of the antigens with Cy3 fluorescent dye does not affect their binding.

Third, the highest sensitivity was consistently achieved using sandwich assays with direct fluorescent labeling of antibodies. Because of the high concentration of the gel-immobilized antibodies, the dynamic range of measured concentrations of the toxins in some cases exceeded three orders of magnitude, which cannot be achieved using either ELISA or 2-D array technology.

Fourth, and most important, the robust technology platform suitable for field applications made it possible to identify all six investigated toxins using a single chip.

Since the problem of biotoxin detection is extremely important, several scientific groups are developing biochips for multiplex assay of biotoxins. In particular, Rucker *et al.* [39] described at approximately the same time an assay of six biotoxins using 2-D protein arrays. Both direct and competition assays were carried out using antibody arrays on epoxy-functionalized slides, reaching the LOD within the 14–700 ng/mL range.

Since potential biological warfare includes not only toxins, but also viruses and bacteria, several groups are working on microarrays that could detect more than one type of agents. Thus, microarrays have been described for simultaneous detection of two toxins, five pathogenic viruses, and five bacteria [40].

One of the recent developments in toxin detection is the development of nanoscale microarrays of individual elements placed on a laser-patterned chip surface [41]. It is not yet clear which of the numerous emerging nanoscale technologies, if any, will be simple and robust enough for rapid and reliable deployment in field conditions.

7 Sensitivity of microarrays

All assays, in particular immunoassays, can be divided into two categories: ambient analyte assays and mass-sensing assays [42, 43]. In ambient analyte assay, the number of captured molecules is a negligible fraction of the total amount in the sample, and the assay measures the concentration of the target in the sample, regardless of its volume. In mass-sensing assays, the device (for instance, a well in an ELISA plate) captures virtually all target molecules from the sample, so the result is expressed as the amount of the target compound. As shown by Ekins [42], the former approach has a potentially greater sensitivity than the latter, and the smaller the elements of the array, the higher the sensitivity.

This theoretical prediction holds true up to a point, because of the limitations imposed by the rate of diffusion and its ultimate stochastic nature. Namely, as suggested by Sheehan and Whitman [44], “sensor size and shape profoundly affect the total analyte flux. <...> Without directed transport of biomolecules, individual nanoscale sensors will be limited to picomolar-order sensitivity for practical time scales.” Moreover, calculations show that the enhancement of diffusion by forced fluid flow is not effective for very small sensors. For instance, forced flow along the surface of a spot 100 μm in diameter can increase diffusion 30-fold, while for a 1- μm spot the potential increase is only threefold [44].

Both Ekins [42] and Sheehan and Whitman [44] emphasized the importance of the improvement of the S/N as the most promising way to increase the sensitivity of microarrays. In particular, Ekins illustrated his point by an interesting (though counterintuitive) diagram (Fig. 3.11 in [42]), showing that the lower the occupancy fraction of a microarray element, the higher the S/N. Since low occupancy requires very high sensitivity of the registration, the noise begins to be generated by the equipment. In other words, there seems to be an optimal level of occupancy as well as of the size of the elements and density of capture molecules (in the case of immunoassays, antibodies).

Simple calculations based on the formula from the Ekins paper (Fig. 3.6 in [42]) indicate that the gel drop technology is an ambient analyte assay, *i.e.*, its results and sensitivity do not depend on the volume of samples when they are larger than 1–2 μL . This notion is fully supported by our observations. The most important factor in the sensitivity of the assays and S/N is the choice of specific antigen/antibody combinations and the quality of antibody preparations.

Another factor is, of course, the sensitivity of the equipment. The dedicated homemade research microscope used in our laboratory and described by Barsky *et al.* [24] scans a gel element into a 16×16 -pixel image with a pixel size of $6 \times 6 \mu\text{m}$. Its highest sensitivity is about 20 molecules *per* μm^2 . Although this sensitivity is sufficient for most of our applications, commercially available instruments with higher sensitivity could further increase the sensitivity of gel drop arrays.

8 Combining protein biochip technology with MALDI-TOF MS

MS is one of the most specific and sensitive method of protein analysis [45]. In the last 10 years, new MS techniques based on m/z analysis, ionic fragmentation, and a broad range of enzymatic and chemical treatments became the gold standard of proteomics.

The gel elements of our arrays are similar in chemical composition to the gel used in 2-D PAGE-MS, a standard method in proteomics [46]. At the same time, they are comparable to biological tissues subjected to fixation procedures, which are investigated using a MALDI imaging approach [47]. Hence, experimental approaches to MALDI-MS analysis of the gel-based arrays draw analogies to these related methods. In particular, prior to laser treatment and spectroscopy, it is necessary to destroy the complex between the immobilized probes and the targets formed within the gel and extract the target to the surface of the gel element. Then the target compound should be mixed with excess of MALDI matrix and the mixture should be dried, while avoiding cross-contamination throughout the procedure.

Since direct analysis of antibodies and other high-molecular-weight proteins by MALDI-MS poses technical difficulties, it might be necessary to cleave such targets into smaller fragments. Direct MALDI-MS of target molecules is most promising for the analysis of the binding of low-molecular-weight ligands with immobilized substrates, as in high-throughput studies in drug design and combinatorial chemistry.

MALDI-TOF MS was first used for direct analysis of hybridization on gel-based biochips in 2000 [48]. Although in experiments involving MALDI-TOF it is preferable to place the elements of the array on an electrically conductive silicon or metal-coated substrate, it is not obligatory. For protein analysis using MALDI-TOF, Rubina *et al.* [2] manufactured an array of gel elements 0.4–1 mm in diameter with 3 mm pitch on hydrophobic glass to avoid cross-contamination. Bound proteins, as well as products of an enzymatic reaction, were extracted from the gel and successfully identified using MALDI-TOF MS. In another model experiment, an immobilized protein was digested with trypsin, the products extracted, subjected to MALDI-TOF, and the original protein identified from the molecular masses of the products using the PeptIdent program.

As with other applications of protein arrays, the detection of protein–protein interactions requires the preservation of the native state of both counterparts and sufficient yield of bound material. Gavin *et al.* [49] compared a 2-D array manufactured on a commercial support (SuperAldehyde, Telechem International), a commercial 3-D support (Hydrogel-coated slides, PerkinElmer), and a homemade polymethacrylate-based gel-based array as platforms for further identification of interacting proteins by MALDI-MS. The authors used as a model the interaction of gel-bound trypsin

with its inhibitor. The 2-D immobilization did not result in binding of the amount of protein sufficient for further analysis, while 3-D slides yielded enough for MALDI-MS.

9 Biochips with complete combinatorial set of oligonucleotides (generic chips)

The introduction of microarrays to study the abundance of individual mRNAs resulted in the accumulation of an enormous body of data on gene expression. To understand the regulatory mechanisms responsible for gene regulation, a number of high-throughput microarray-based techniques were devised (for review, see [50]). These techniques screen large sets of sequence-specific proteins or corresponding DNA sequences.

In principle, there are two ways to create DNA arrays: to synthesize oligonucleotides on the surface of a substrate *in situ*, or to immobilize pre-synthesized oligonucleotides. The former technology yields high-density arrays with up to 10^5 – 10^6 features, but the length of oligonucleotides is limited and their quality difficult to control. The latter approach allows purification of the oligonucleotides to homogeneity and checking of their quality before immobilization, but it is laborious, expensive, and provides arrays with fewer features [51].

As noted by Bulyk [50], screening of immobilized DNAs using specific DNA-binding proteins often requires prior knowledge of their consensus recognition sequences. An alternative to this requirement is a microarray containing a full combinatorial set of DNA sequences of a given length. Such an array carrying all possible hexamers flanked by one degenerated nucleotide on each side (a total of 4096 elements) was used by Krylov *et al.*, Zasedateleva *et al.*, and Chechetkin *et al.* in the studies of several DNA-binding proteins (the so-called generic chip; see [52–54]). These biochips were manufactured by immobilizing pre-synthesized, purified oligonucleotides in 3-D gel elements.

One obvious limitation of this approach is the short length of interacting oligonucleotide, which is nevertheless sufficient to investigate the binding specificity of some proteins. For instance, detailed quasi-equilibrium curves of “melting” of protein complexes with ssDNA on generic chip enabled us to deduce consensus sequences for the binding of bacterial HU-protein [52] and mammalian p50 protein [53] with ssDNA. A massive set of experimental data characterizing the sequence-specificity of p50 protein–oligonucleotide binding is presented in Fig. 9. It illustrates the binding affinity of this protein to G-rich ss oligodeoxyribonucleotides.

After converting the ssDNA of the generic chip into dsDNA by annealing with the set of octamers with complete combinatorial turnover of the central hexanucleotide and degenerated flanking nucleotides, data could be extended to include the melting curves of DNA duplexes in complexes with the protein and temperature-induced dissociation of the bound protein, depending on which component has been fluorescently labeled prior to the binding.

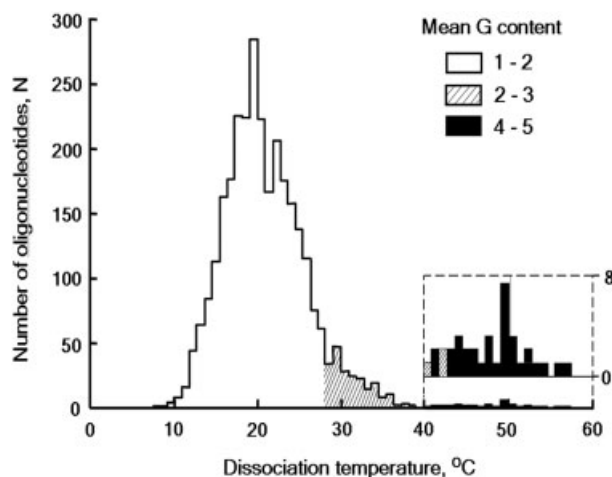


Figure 9. Application of generic microchip carrying the complete set of oligonucleotide hexamers immobilized into 4096 gel elements (see text) to the investigation of sequence-specific interactions between p50 protein and ssDNA. Distribution of the number of p50-oligonucleotide complexes is plotted against their dissociation temperatures.

Despite the short length of the DNA set immobilized on the chip, the ranking of affinities of the complete set of hexamers at least in some cases provided sufficient information to reconstruct a longer sequence consisting of primitive recognition domains (for discussion, see [53]). This reasoning might be especially valid in the case of oligomeric DNA-binding proteins, whose subunits might recognize partial recognition sequences with relatively low affinity [54]. Thus, generic chips, as well as gel-based chips with longer segments of immobilized DNA, may be particularly useful for studies of low affinity binding.

Real-time recording of dissociation and measurement of affinities enabled us to deduce consensus-binding sequences for p50 protein and lambda-Cro V55C repressor, which correlated well with previously known specificities [53, 54].

10 Binding of proteins with glycan biochips

The entire set of cellular glycans, or its glycome, is chemically the most diverse class of biopolymers. Most of these glycans interact specifically with proteins, and these interactions often play central roles in cell functions. To study these interactions is even more challenging than to study interactions of proteins with nucleic acids because of the chemical complexity of both partners.

As in the case of DNA-protein interactions, significant advances in the studies of glycan–protein interactions are expected from the use of microarray technologies [55]. Although in principle glycans can be immobilized using regular 96-well plates, microarrays offer many more possibil-

ities. First and foremost, the glycan consumption *per* element of a 2-D microarray may be three orders of magnitude and on 3-D array two orders of magnitude lower than on regular plates [56]. It makes it feasible and affordable to arrange microarrays from many more individual glycans. This, in turn, enables one to screen proteins for their interaction with a much more diverse sets of glycans, which could result in unexpected findings.

Immobilization of glycans can complement the studies of their interactions with immobilized proteins. However, the affinity of proteins towards carbohydrates is usually much weaker than towards proteins and nucleic acids, and requirements for immobilization of carbohydrates are much more stringent. The choice of substrate when the carbohydrates are immobilized by physical adsorption and of linker structure in the case of chemical immobilization must ensure as low background protein binding as possible. Generally, covalent immobilization is preferable, since it provides more structurally homogeneous immobilization, regardless of the chemical nature of the carbohydrate [55].

Thus, 3-D immobilization is the method of choice for immobilization of glycans. Because of their high capacity, gel elements provide strong signals *per* square unit with low consumption of material, and they are best suited for the studies of low-affinity interactions because of low background and the possibility of real-time measurements within a broad range of concentrations. The relatively low number of features on gel chips as compared with 2-D arrays is not as important as in the case of nucleotide sequences or massive sets of proteins, because the variety of glycans used in most studies is limited to several hundred compounds at most.

With this in view, Dyukova *et al.* [57] developed gel drop biochips with immobilized glycans. The amounts of immobilized oligosaccharides were 0.4–1.7 pmol *per* gel element 1 nL in volume. Quantitative assays of fluorescently labeled antibodies and lectins demonstrated specific interaction with chip-immobilized oligosaccharides. Antibodies against blood group antigens were successfully detected using biochips with immobilized trisaccharides A and B, and the procedure was equally efficient with both purified antibodies and biological fluids—serum and hybridoma cultural fluid. Association constants of ricin with carbohydrates were measured and appeared to be in good agreement with the existing data.

In a recent work, Shilova *et al.* [58] proposed an integrated approach to labeling, separation, characterization, and immobilization of oligosaccharides (OS). The protocol begins with modification of a mixture of OS by a fluorescent bifunctional reagent. The mixture is then separated by HPLC, homogeneous compounds characterized and quantified by MS, and the remaining active group used to immobilize the OS in gel elements of the chip. The fluorescent label is the most convenient way to monitor all these steps.

11 Future development and applications of hydrogel-based microarrays

Immobilization of proteins in gel-based arrays is a proven technological platform and a viable alternative to flat arrays for many applications. Gel drop biochips became the first protein arrays approved in Russia for clinical applications.

The 3-D biochips may be advantageous for immobilization of sensitive proteins that tend to lose their functional activity. Contrary to flat surfaces, immobilization of proteins in hydrogels stabilizes them [59].

Gel drop technology is extremely robust and perhaps the most promising microarray technology for field applications, particularly in combination with an inexpensive portable imaging device [24].

Another major advantage of gel immobilization is the possibility to obtain detailed real-time curves of binding and/or dissociation. This allows the recording of association/dissociation processes in quasi-equilibrium conditions and the study of weak interactions, which might escape detection using 2-D arrays and single-wash approaches.

Virtually any ELISA test can be converted into gel chip format, and several ELISA tests can be combined on a single biochip for multiplex analysis. The low cost of disposable gel chips and recording equipment allows their mass manufacturing and application.

It has to be stressed that the only chemical feature necessary for immobilization is a primary amino group. Obviously, such groups are present in any protein or peptide, be it the universal N-terminal amino group or lysine. Introduction of primary amino groups into nucleic acids, glycans, and other biological molecules is a routine procedure, which makes the gel drop immobilization technology especially flexible and universal. This chemical versatility allows several classes of biological molecules (proteins, DNA, oligosaccharides) to be immobilized on the same chips and used to test a broad range of analytes in single samples.

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The authors have declared no conflict of interest.

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