Talanta 93 (2012) 139–146

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Macroporous methacrylate-based monoliths as platforms for DNA microarrays

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ARTICLE INFO

Article history:
Received 21 November 2011
Received in revised form 28 January 2012
Accepted 31 January 2012
Available online 7 February 2012

Keywords:
Macroporous monolithic materials
DNA microarrays
Aptamers

ABSTRACT

Macroporous monoliths with different surface functionalization (reactive groups) were utilized as platforms for DNA analysis in microarray format. The slides based on a copolymer glycidyl methacrylate-*co*-ethylene dimethacrylate (GMA-EDMA) have been chosen as well known and thoroughly studied standard. In particular, this material has been used at optimization of DNA microanalytical procedure. The concentration and pH of spotting solution, immobilization temperature and time, blocking agent and coupling reaction duration were selected as varied parameters. The efficiency of analysis performed on 3-D monolithic platforms was compared to that established for commercially available glass slides. As a practical example, a diagnostic test for detection of CFTR gene mutation was carried out. Additionally, the part of presented work was devoted to preparation of aptamer-based test-system that allowed successful and highly sensitive detection both of DNA and protein.

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1. Introduction

The microarrays have been successfully used in different practical fields, namely, molecular biology, medicine, biotechnology, pharmacology and ecology [1]. Comparatively to other conventional methods, these miniaturized devices allow simultaneous analysis of several hundreds or even thousands compounds. Thereby, the principle of biological test-systems for highly sensitive spot-analysis is based on a specific binding of target molecule (e.g. protein, DNA, virus) to its complementary partner (ligand) attached to a solid surface. The types of a substrate for microarrays can be categorized according to the dimensionality of the surface (2-D or 3-D), and whether the binding of a ligand to the surface is covalent or occurred by means of a nonspecific adsorption. Nowadays, the two-dimensional (2-D) platforms are based on glass slides typically treated with organosilanes of different functionality (epoxy, amino or aldehyde) [2,3], thin films of non-porous synthetic polymers [4] or metals [5,6]. However, the functional properties of biosubstances used as complementary ligands strongly depend on their conformation and structure. Therefore, sometimes a labile ligand may not be able to survive being immobilized at uncomfortable conditions on 2-D surfaces. Alternatively, the intraporous space of three-dimensional (3-D) solid supports represents more suitable surrounding for biomolecules to be immobilized, as well as such supports are characterized by much larger surface area that provides much higher ligand loading comparatively to 2-D matrices. Some prominent examples of 3-D systems include polyacrylamide gels [7,8], agarose [9], dextran gel [10], nylon [11] and porous nitrocellulose films [12].

Obviously the surface properties of the matrix affect directly such important aspects of analysis on microarrays as probe loading, spot morphology, background influence and the yield of target-ligand pair formation. Recently, the idea of fabrication of a new type of 3-D microarray based on rigid macroporous monolithic materials has been realized in our group [13–17]. The main advantages of these supports are their high mechanical and chemical stability along with the singularities of porous structure allowing efficient operating with different classes of substances. The crucial point of microarray manufacturing was an attachment of thin and fragile macroporous monolithic layer to a glass surface playing the role of inert support. Recently developed microarrays based on a copolymer glycidyl methacrylate-*co*-ethylene dimethacrylate

Abbreviations: GMA, glycidyl methacrylate; EDMA, ethylene dimethacrylate; HEMA, 2-hydroxyethyl methacrylate; CEMA, 2-cyanoethyl methacrylate; GDMA, glycerol dimethacrylate; 2-D and 3-D, two and three-dimensional, respectively; DMAP, 4-(dimethylamino)pyridine; CDI, 1,1-carbonyldiimidazole; HOBt, hydroxybenzotriazole; EDC, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; BSA, bovine serum albumin; SDS, sodium dodecyl sulphate; EA, ethanolamine; SA, succinic anhydride; PS, polystyrene; PMT, photomultiplier tube; SM, signal mean; BM, background mean; SNR, signal-to-noise ratio; CF, Cystic fibrosis; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator; PEI, polyethylenimine; PFEI-His, *Pseudomonas fluorescence* esterase I.

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doi:10.1016/j.talanta.2012.01.064

(GMA-EDMA) were used as a reference. This copolymer is well known as an efficient solid phase applied in the processes based on dynamic interphase mass transition [18–21]. Original epoxy groups in its chemical structure make possible a realization of one-step surface biofunctionalization with different classes of ligands. The succeeding tests of GMA-EDMA copolymer as a microarray platform for detection of virus-like particles and proteins promoted the development of several new macroporous matrixes differed by reactivity, hydrophobic–hydrophilic and porous properties [14–17]. We have previously reported that all materials obtained were characterized by excellent sensitivity for protein analysis [16].

The current work demonstrates the application of macroporous monoliths as platforms for DNA microarrays. Regarding to the properties of macroporous polymer supports, it was necessary to optimize probe printing procedure, as well as analytical conditions appropriate for DNA analysis. The optimization has been carried out using GMA-EDMA platforms as a model matrix. In addition, glycidyl methacrylate-*co*-glycerol dimethacrylate (GMA-GDMA), 2-cyanoethyl methacrylate-*co*-ethylene dimethacrylate (CEMA-EDMA), 2-cyanoethyl methacrylate-*co*-glycerol dimethacrylate (CEMA-GDMA) and 2-hydroxyethyl methacrylate-*co*-glycerol dimethacrylate (HEMA-GDMA) were tested as probable monolithic materials for DNA microarrays construction. The comparison of the efficiency of developed 3-D layers to the widely used for purposes discussed 2-D glass slides was performed. To demonstrate practical potential of developed microarrays, one example of diagnostic procedure is presented and discussed. As well, the first attempt to prepare an aptamer-based test-system is demonstrated in this paper.

2. Experimental

2.1. Materials

Bovine serum albumin (BSA), 4-(dimethylamino)pyridine (DMAP, 99% pure), 1,1-carbonyldiimidazole (CDI, 98% pure), Tween-20, sodium dodecyl sulphate (10% SDS), polyethylenimine (PEI), and ethanolamine were purchased from Sigma–Aldrich. The 25 mm × 75 mm × 1.2 mm glass slides used for lab-made microarray fabrication were obtained from Menzel. 2-D glass slides functionalized with reactive aldehyde groups were purchased from CEL Associates Inc. (CSS-100 Silyleted Slides). Amino-modified oligonucleotides (B2573RpoE: 5′-TCCGAGCGAGGGAAGCTATTGATAACAAAGTTCAACCGCTTATCAGGC -GT-3′ and B4142GroES: 5′-TCGACAATGAAGAAGTGTTGATCATGTCCGAAAGCGACATTCT GGCAATT-3′) were from Invitrogen. The probes for mucoviscidosis analysis (Y1: TCATCCTCCGGAAAATATT, Y2: CATCCTCTGGAAAATATTC and Y3: GGCTTGTCTTTTACCCTGC) were purchased from Beagle.

Anti-His-tag aptamer (6H7, 5′-GCT ATG GGT GGT CTG GTT GGG ATT GGC CCC GGG AGC TGG C-3′) was obtained from Biospring and 5′-modified with amino group directly at its synthesis. The following buffers were used: PBS 50 mM K_2HPO_4, 150 mM NaCl, pH 7.5; PBST–PBS containing 0.05% Tween 20, pH 7.5; 20× SSC – 3 M sodium chloride containing 0.3 M sodium citrate, pH 7.0. All buffer salts of analytical grade quality were purchased from Fluka and Sigma–Aldrich. The solutions were prepared using deionized water (ARIUM, Sartorius AG) and were filtered prior to use.

2.2. Instruments

200 pL oligonucleotide probes were spotted onto the surface of monoliths using a piezoelectric biochip arrayer Nano Plotter NP 2.1. The washing and surface blocking procedures were performed on the shaker Eppendorf. The hybridization procedure was carried out using Thermomixer Comfort Eppendorf in the special secure seal chambers (Sigma–Aldrich and Grace Biolabs). All slides were scanned at a photomultiplier tube (PMT) with variable gain. Scanning was performed using a GenePix 4000B (Axon Instruments). ImaGene 5 software (BioDiscovery, El Segundo, CA) was used for image analysis. Mean values of all replicates were applied for calculations. Relative signal intensity was calculated as a difference of mean signal (SM) and mean background signal (BM).

2.3. Methods: 2-D glass DNA microarray

For fabrication of DNA microarray based on aldehyde-bearing glass slides the protocol published elsewhere was used [22]. Briefly, 50 μM solution of oligonucleotide in 3× SSC was printed on the surface of glass slides in 10 replicates per one column. After spotting, the slide surface was baked at 80 °C for 1.5 h. Thereafter the arrays were stored overnight at room temperature. To remove unbound oligonucleotides after spotting and to deactivate the excessive aldehyde groups, the slides were washed with 0.2% SDS for 2 min, twice with ddH_2O for 1 min and once with a solution containing 1 g $NaBH_4$ dissolved in 300 ml PBS buffer plus 100 ml EtOH for 5 min. To carry out surface blocking, 1.0% BSA solution in 5× SSC buffer including 0.1% SDS was used. The slides were incubated at 42 °C for 45 min, washed 5× 1 min with ddH_2O then dried with CO_2.

2.4. Target preparation

Escherichia coli strain K12 MG1655 (Sigma–Aldrich) was used in this study to prepare cDNA to be applied for hybridization with oligonucleotides *B2573RpoE* and *B4142 GroES*. The cultivation was performed in shaking flasks with 100 mL of LB medium (10 g/L casein peptone, 10 g/L NaCl, 5 g/L yeast extract). The culture was grown overnight at 37 °C at optical density, OD_{600}, of 1.4. Afterwards the cells were shocked for 5 min at 50 °C and centrifuged at 3300 × *g* for 5 min (4 °C). Then the cell pellets were dried and stored at −80 °C until required. The isolation of RNA from *E. coli* was carried out using standard protocol [23]. In order to remove all DNA before RT (reverse transcriptase) reaction, the isolated RNA sample was treated with RNase-free DNAse I (Invitrogen) at 37 °C for 30 min [23]. The concentration of RNA was equal to 4 μg/μL and the value of ratio A_{260}/A_{280} was determined as 1.9 using spectrophotometer Nanodrop ND-1000 (NanoDrop Technologies). The targets for coupling were prepared from RNA as a template by incorporation of fluorescent-labelled Cy5-deoxyribonucleotides during first-strand cDNA synthesis. 3 μL dNTP's, 5 μL hexamer (Invitrogen) were added to 50 μg RNA sample dissolved in RNAse-free water to reach a final reaction volume of 32.5 μL. The mixture was incubated at 65 °C for 5 min and in ice for 1 min. After that 1.5 μL Cy5-dCTP (PerkinElmer), 10 μL 5× Buffer (Invitrogen), 3 μL DTT (Invitrogen) and 2 μL Superscript III (reverse Transcriptase, Invitrogen) were supplemented and the reaction was carried out at 42 °C for 2 h. RNA was removed by alkaline hydrolysis (10 μL 1 M NaOH, 10 min, 65 °C). After neutralization with 10 μL 1 M HCl, cDNA was purified with QIAquick PCR Purification Kit 250 (QIAGEN). The target was concentrated by vacuum centrifugation (Eppendorf), combined with 350 μL hybridization buffer (30% formamide solution in 4× SSPE and 2.5× Denahards solution, Sigma–Aldrich). The probe was denatured at 95 °C for 3 min, then additionally in ice for 2 min. After that 1/10 volume of 20 mg/mL Topblock (Fluka) was added. For each hybridization experiment, 130 μL volume of a target DNA solution was used. During the incubation, the slides were shaken at 650 rpm. The coupling reaction was allowed to proceed for 16 h at 42 °C. After hybridization step, the slides were washed using following washing buffers: 2× SSC including 0.1% SDS (pH 7.0) for 5 min, 1× SSC for 5 min and 0.5× SSC (pH 7.0) for 5 min. The

Table 1
Characteristics of polymer matrixes used for DNA microarrays.

Copolymer	Average pore size, nm	Specific surface area, $m^2\ g^{-1}$	Quantity of reactive groups, $mmol\ g^{-1}$
GMA-EDMA	1500	25	4.0
GMA-GDMA	900	26	4.0
CEMA-EDMA	1100	36	4.1
CEMA-GDMA	900	28	4.2
HEMA-GDMA	1300	27	6.0

The error of measurement of pore characteristics is 5–7%.

microarrays were dried with CO_2 and scanned using the 635 nm filter.

Signal-to-noise ratio (SNR) was calculated as:

$$SNR = \frac{(\text{SM-BM})}{\text{standard deviation of background}} \quad (1)$$

In this study, optimal PMT settings were established by variation of gain settings using intervals of 50. For data analysis, PMT settings were chosen according to found highest SNR values.

2.5. Fabrication of macroporous monolithic platforms

The procedure of fabrication of 3-D platforms includes the synthesis of polymer monolith inside the specially treated operative well on the glass surface. The procedure of operative well manufacturing was developed earlier [13,16]. The optimization of synthesis conditions taken place in this research was also performed as published elsewhere, e.g. for GMA-EDMA [24], GMA-GDMA [15], HEMA-GDMA [17], CEMA-EDMA and CEMA-GDMA [25]. The characteristics of macroporous monolithic layers used as platforms for DNA microarray are displayed in Table 1.

2.6. Optimization of printing procedure and analytical conditions using model DNA system

To optimize ligand immobilization procedure, some important parameters, such as the concentration of oligonucleotides, pH of printing buffer, influence of temperature and reaction time were investigated. For this purpose, GMA-EDMA as a standard matrix was used.

B2573RpoE was spotted onto a monolithic surface using two printing buffers, namely, 0.01 M sodium borate buffer, pH 9.4, and in 3× SSC, pH 7.0. Loading sample volume was equal to 200 pL in 10 replication in one column. The concentration of oligonucleotide solutions was varied from 10 to 50 μM.

The time of ligand immobilization included a bake at 80 °C and incubation at room temperature. The slides containing spotted probes of *B2573RpoE* were placed in thermostat at 80 °C. The bake time was varied from 1 to 3 h; the incubation at room temperature was ranged from 0.5 to 14 h.

After the immobilization process was stopped, the slides were washed with 0.2% SDS for 10 min and ddH_2O for 5 min. To choose the best pre-hybridization surface blocking agent, the solutions of 1% BSA in 6× SSC containing 0.1% SDS, 50 mM ethanolamine in 0.1 M Tris, pH 9.0, including 0.1% SDS or fresh solution of 0.55 g of succinic anhydride (SA) in 34 mL of DMSO and 2 mL $NaHCO_3$, pH 9.4, were tested. The surface blocking with BSA was performed for 45 min at 42 °C. In the cases of SA and ethanolamine, the slides were incubated for the same time but at room temperature. Finally, the microarrays were washed twice with ddH_2O for 10 min and dried with CO_2.

For coupling reaction the same target probe as in case of glass slides, but for a time varied from 2 to 14 h was used. After hybridization, the microarrays were consequentially washed with 2× SSC including 0.1% SDS (pH 7.0) for 15 min, 1× SSC for 10 min and 0.5× SSC (pH 7.0) for 10 min, then dried and scanned.

For further experiments the following printing and analytical conditions found to be optimal from the obtained results were applied: spotting ligand concentration was 50 μM in 3× SSC, pH 7.0; the immobilization time was 6 h thereof 2 h at 80 °C and 4 h at room temperature; the solution of 1% BSA in 6× SSC containing 0.1% SDS was used as blocking agent; the coupling reaction was carried out for 4 h.

To compare the efficiency of developed monolithic platforms of different functionality at optimal conditions, another gene, namely, *B4142GroEs* was immobilized on a surface of macroporous matrixes and hybridized with fluorescent target.

2.7. Diagnostics of mucoviscidosis

The 19-mer probes, namely, Y1, Y2 and Y3 were immobilized on a surface of monolithic platforms according to previously developed protocol. To perform the diagnostic test, the 218-mer PCR product containing mutation was generated from genomic DNA using following primers: Cy5-5-CACTCAGAACCCATCATAGGATACAATGAA-3 and 5-CTCCAGAGCTTCTGAAATTAATTGACCAC-3. The target, as well as PCR product without mutation, was purchased from D.O. Ott Research Institute of Obstetrics and Gynecology, Russian Academy of Medical Sciences. For realization of coupling reaction a volume of 20 μL Cy5-labeled target PCR product was mixed with 350 μL hybridization buffer and denatured. 130 μL of solution obtained was used for hybridization proceeded at 42 °C for 4 h. After the coupling was finished the microarrays were washed, dried and scanned.

2.8. Aptamer-based test-system

Aptamer 6H7 was activated with cyanuric chloride according to the protocol published by Ferguson et al. [26].

To incorporate a spacer into chemical structure of macroporous polymer matrixes, a polyethylenimine (PEI) solution was used. The monolithic platforms were immersed for 10 min in PBS, pH 7.5, and then incubated in 5% PEI in PBS for 4 h. Thereafter the slides were washed fourfold with PBS for 15 min and dried in CO_2.

The aptamers were spotted in PBS buffer with sample volume equal to 200 pL. The concentration of 6H7 was varied from 100 to 400 μM and for 6H7-Cy3 – from 10 to 200 μM. After printing, the slides were incubated at room temperature overnight in the dark.

To remove the excess of amino groups on the surface of macroporous matrixes, the microarrays were immersed in a fresh solution of SA in DMSO (the proportions were described above) for 30 min. Consequently, the slides were washed twice with ddH_2O for 5 min. The blocking procedure was carried out using a 1% BSA solution in a PBS buffer for 45 min at room temperature. Then microarrays were washed with PBS for 10 min. The denaturation of aptamers was performed by incubation in boiling ddH_2O (4 min). Afterwards, the slides were immersed into selected buffer (PBST) for 30 min in order to obtain the correct aptamer folding. For target binding, the microarrays were incubated with fluorescently labeled protein (900 μL, 3 $\mu g\ mL^{-1}$ Cy3-labeled PFEI-His – *Pseudomonas fluorescence* esterase I) in thermomixer at 20 °C and at 300 rpm for 4 h. *Pseudomonas fluorescence* esterase I (PFEI-His) encoding plasmid was kindly donated by Prof. Uwe Bornscheuer (University of Greifswald). Transformation of *E. coli* K12 was carried out utilizing the TSS method [27]. The preparation of fluorescent target was described earlier [28]. After coupling reaction the slides were washed three times with PBST for 5 min at room temperature.

Finally, the microarrays were dried and scanned using the 532 nm filter at varied gains.

3. Results and discussion

3.1. DNA analysis on monolithic surface: procedure optimization

The present work is devoted to the application of macroporous polymethacrylate materials directly synthesized in a shape of thin monolithic layers as a novel type of platforms for DNA microarrays. In fact, this research represents a part of our systematic investigations. The platforms based on GMA-EDMA copolymer were chosen as a checkpoint and used for optimization of analytical procedure. As a reference, the protocol for widely used glass slides was accepted [22]. To demonstrate the potential of developed platforms regarding their application for DNA arrays, a model bioaffinity system consisting of *B2573RpoE* oligonucleotide and cDNAs (from *E. coli*) conjugated with fluorescent label Cy5 was used.

It is well established that the results of solid-phase analysis based on affinity interactions between complementary biological molecules significantly depends on a quantity of immobilized ligand. Therefore, determination of optimal immobilization conditions was thoroughly studied. This part of investigation included the choice of a concentration and pH of oligonucleotide (ligand) solutions, as well as the reaction temperature and time. To establish an optimal quantity of immobilized oligonucleotide, the concentration of printing solutions was varied from 10 to 50 μM (Fig. 1A).

The results obtained demonstrate that the value of signal to noise ratio (SNR) for monolithic matrix was 3 times higher than that observed for 2-D glass analogues at 50 μM ligand concentration.

The reason seems to be obvious: contrary to 2-D slides, where the ligands are located only on a planar surface, in the case of developed macroporous materials, they will definitely penetrate inside 3D-structure of polymer matrix. Therefore, ligand immobilization capacity and, correspondingly, the quantity of formed complementary pairs will be significantly higher. Moreover, the result obtained confirms the absence of steric limitations at the formation of such pairs inside the intraporous space of solid matrix.

As a negative feature, the developed platforms based on macroporous polymer monoliths, similarly, for example, to well known nitrocellulose membranes also used for the same purposes, demonstrate higher self-noise in comparison with glass surface. In microarray experiments there are mainly two reasons for high background detecting signal: (1) fluorescence caused by non-specific binding of labeled, and correspondingly, detected molecules, and (2) fluorescence signal that originates from microarray base. Therefore, the autofluorescence, or self-noise, of the surface is an important parameter of applied for such purposes materials. The analysis of self-noise of monoliths obtained has shown that the values of mean and background signals depend both on photomultiplier tube (PMT) gain and the nature of material. For example, the autofluorescence of blank surfaces of aldehyde glass slide and GMA-EDMA material at constant PMT gain 700 was detected as 50 and 2150 AU, respectively. However, the mean signals detected after hybridization at equivalent conditions were equal to 2100 for glass slide and 17,650 for GMA-EDMA platform. Correspondingly, mean background signal was detected as 155 for 2-D and 2550 for 3-D microarrays. In general, two-dimensional glass surfaces exhibit lower self-noise comparatively to 3-D layers [12]. Though, the big difference between SM and self-noise of material makes this disadvantage appropriate for highly sensitive analysis.

The increase of surface concentration of a ligand leads to dramatic increasing of a signal, while the background fluorescence stays on the same detection level. As a result, the values of SNR are enlarged.

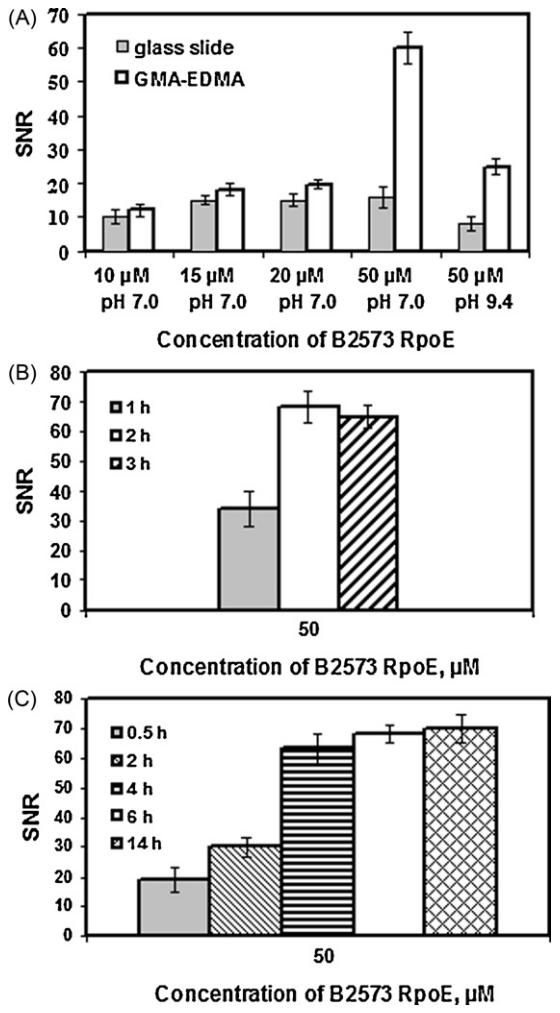

Fig. 1. Dependence of maximal signal to noise ratio (SNR) on immobilization conditions: (A) concentration and pH of oligonucleotide solution; (B) immobilization time at 80 °C; (C) incubation time at room temperature. *Conditions*: each concentration of printed oligonucleotide was replicated 10 times; PMT gains 550 and 700 were applied for GMA-EDMA platform and glass slide, respectively.

To choose appropriate pH, two buffer solutions were tested for spotting probe: 3× SSC, pH 7.0, and sodium borate buffer, pH 9.4. First buffer is widely used for printing of oligonucleotides [22], whereas the second one was used because of necessary alkaline conditions for the reaction between amino group of oligonucleotide (ligand) and epoxy group of polymethacrylate matrix.

Fig. 1A clearly confirms the advantage of the buffer with neutral pH value.

In the case of standard glass slides, the immobilization procedure includes two steps, namely, baking at 80 °C for 2 h and storage overnight at room temperature. High analytical sensitivity at minimal reaction time was considered as important element of optimized protocol. Fig. 1B confirms that chosen baking conditions (80 °C, 2 h) are appropriate for this step. The following incubation of monolithic microarrays at room temperature from 30 min up to 14 h (Fig. 1C) proved that 4 h seemed to be enough for stabilization of ligand conformation to reach high signal intensity.

In order to see the better contrast between detected spots and surface background, a blocking procedure followed by a target hybridization step has been carried out. One of the advantages of polymethacrylate monolithic materials consists of a unique

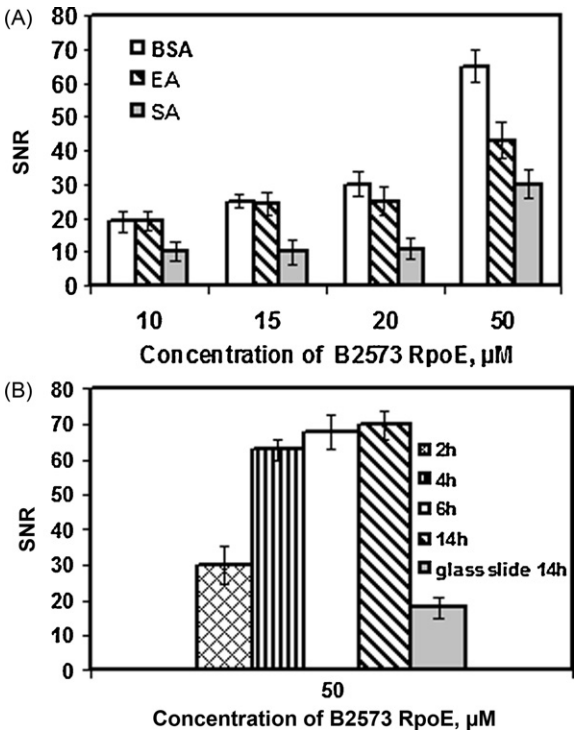

Fig. 2. Effect of analytical conditions on SNRs obtained: (A) variation of blocking agents: bovine serum albumin (BSA), ethanolamine (EA) and succinic anhydride (SA); (B) dependence of SNRs on hybridization time. *Conditions*: PMT gain was equal to 550, printing buffer – 3× SSC, pH 7.0; each solution of oligonucleotide was spotted in 10 replications; immobilization time – 2 h at 80 °C and 4 h at room temperature.

capability to be treated not only in aqueous solutions but also in water-organic and organic liquids without any changes of their porous morphology. The solutions of BSA, ethanolamine (EA) and succinic anhydride (SA) were tested as blocking agents. All three reagents are often used for pre-hybridization blockage in DNA and aptamer-based test-systems [22,28,29]. As it can be seen in Fig. 2A, the best result was reached for microarray blocked with BSA.

The optimal time of hybridization of fluorescently labeled target cDNAs with oligonucleotides immobilized on the surface of macroporous matrix was chosen in corresponding experimental series. According to referent protocol, the coupling reaction was carried out for 16 h at 42 °C [22]. The dependence of analytical results on hybridization time was investigated (Fig. 2B). From presented figure it is obviously that the magnitudes of SNR were strongly changed at increasing of time of hybridization from 2 to 4 h. After that the value of SNR changed insignificantly. The comparison of results obtained on hybridization time for developed 3-D platforms with aldehyde-bearing glass slides allowed conclusion that macroporous monolithic platforms demonstrates much faster hybridization with higher value of detected SNR.

3.2. The comparison of analytical efficiency of different 3-D microarrays

To investigate the influence of chemical nature of different macroporous monolithic matrixes on the results obtained at DNA analysis, all developed platforms were tested. The comparison of efficiency of discussed microdevices at optimal analytical conditions with commercial 2-D glass biochip was carried out using *B4142GroEs* gene immobilized on a surface of both types of slides and coupled to fluorescently labeled target. The values of maximal SNR obtained at optimal gain for each test-system were used as comparable criterion. All developed materials were characterized

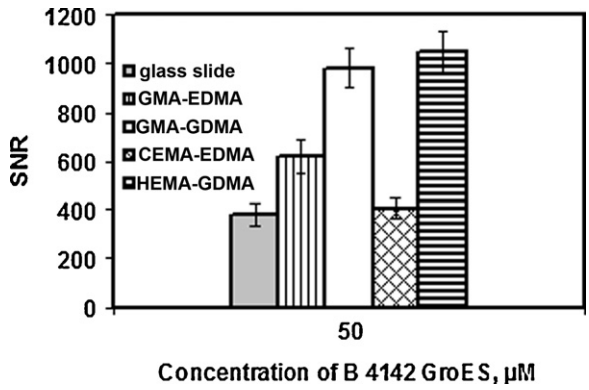

Fig. 3. Comparison of maximal SNRs obtained using different monoliths and aldehyde glass slide. *Conditions*: for GMA-EDMA, GMA-GDMA and CEMA-EDMA PMT gain was 350, for HEMA-GDMA and glass slide PMT gains were 400 and 600, respectively.

by high spot quality, as well as reproducibly high sensitivity. However, the best results were observed for hydrophilic GMA-GDMA and functionalized HEMA-GDMA matrixes (Fig. 3).

Both macroporous materials were synthesized using the solution of 1% PS in toluene and dodecanol as a porogenic mixture. Earlier these platforms obtained with PS/toluene/dodecanol porogenic system and characterized by the highest detection signals were found to be the best for protein microarrays [15,17].

Fig. 3 demonstrates the great privilege of coupling reaction for *B4142GroEs* oligonucleotide with target cDNAs comparatively to that observed for *B2573RpoE* gene (Fig. 1A) that expressed by 10-fold increasing of SNR. It is well known, if the bioaffinity interactions are strong, the requirements to operative support can be reduced. Thus, in the case of *B4142GroEs* gene, the difference of SNR values between glass slide and GMA-EDMA material results in a decrease (magnitudes of SNR are 400 and 630, respectively) in comparison to the first model system (magnitudes of SNR are 20 for 2-D slide and 65 for GMA-EDMA support). For CEMA-EDMA matrixes the result was approximately the same as for 2-D glass slides. However, as an advantage, the immobilization and hybridization processes on macroporous material still occurred substantially faster. Table 2 shows that the duration of ligand immobilization on a surface of macroporous platforms is 6 h versus 16 h used for glass slides. Thus, monolithic microarrays allow decreasing the immobilization and hybridization by 2.5 and 3 times, respectively.

To evaluate the reproducibility of results obtained, intra- and interfield coefficients of variation were determined [16]. The coefficients of variation were calculated as ratio of standard deviation to mean signal intensity (Table 2). The calculated data indicate that all developed monolithic platforms demonstrate sufficient values of these important characteristics.

The developed and described in presented paper macroporous polymer materials have definite advantages. First ones can be related to the simplicity of their synthesis (direct polymerization

Table 2
Times of immobilization and hybridization procedures, intrafield and interfiled coefficients of variation.

Type of microarray surface	Time of immobilization, h	Time of hybridization, h	Intrafield coefficient of variation, %	Interfield coefficient of variation, %
Glass slide	16	14	16	19
GMA-EDMA	6	4	6	10
GMA-GDMA	6	4	6	11
CEMA-EDMA	6	4	12	13
CEMA-GDMA	6	4	9	10
HEMA-GDMA	6	4	5	11

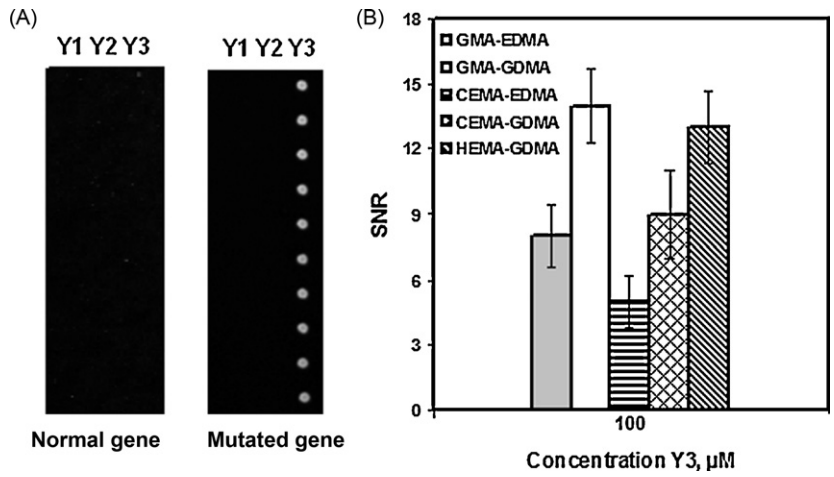

Fig. 4. Results of mucoviscidosis diagnostics performed on macroporous polymer platform: (A) images of fragment GMA-EDMA layers after coupling reaction with PCR products containing normal genes and its mutated derivative; (B) comparison of SNRs obtained for different macroporous matrixes. *Conditions*: printing buffer – 3× SSC, pH 7.0; each solution of oligonucleotides (Y1, Y2 and Y3) was spotted in 10 replications; immobilization time – 2 h at 80 °C and 4 h at room temperature; coupling time – 4 h, for GMA-EDMA, GMA-GDMA and CEMA-EDMA PMT gain was 550, for CEMA-GDMA and HEMA-GDMA PMT gains were 450 and 600, respectively.

time needs 20–30 min only), as well as to the possibility to vary in a wide range surface functionality used at further step of microarray preparation, namely, ligand covalent attachment at as mild as possible conditions. For comparison, to produce polyacrylamide-based microarray it is necessary to realize long enough procedure of incorporation of unsaturated bond into DNA molecule (for example, via acylation of amino groups), then to carry out the polymerization with participation of modified bioligand. Additionally, it was established that the time of nucleotides' hybridization on the surface of macroporous supports can be significantly reduced comparatively that published elsewhere [30]. Third, as it was mentioned above, the ligand immobilization in our case occurs via covalent binding of nucleotide amino groups with surface reactive groups. This step positively distinguishes suggested monoliths from functionalized polymer membranes, such as nylon or cellulose, where the immobilization most often is a result of physical adsorption. And finally, in known gel-like surfaces the porous structure seriously depends on gel swelling. In fact, the molecules move in these media similarly to the movement in highly viscous liquid with correspondingly diminished diffusivity forming local concentration gradients [31]. From this point of view, rigid macroporous structure of developed monoliths is stable with defined interphase border and provides non-limited fast mass transfer from non-viscous liquid into porous space (immobilization, hybridization) and back from pores to the liquid (washing procedures). As one example, the microarrays based on agarose layers needed more than twice higher time for oligonucleotide immobilization in comparison with all described monolithic platforms (overnight and approximately 6 h, respectively) [9].

3.3. Microarray-based detection of CFTR gene mutations

Mucoviscidosis, also known as Cystic fibrosis (CF), is a common recessive genetic disease which affects the entire body causing progressive disability and often early death. CF is caused by a mutation in the gene responsible for synthesis of a special protein, namely, Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). This gene represents a big nucleotide consequence including 250,000 pairs.

Currently, approximately 1500 mutations are identified in this gene, but usually 15–32 of them are screened with medical purposes. It is quite important that the individuals with risk of CF suffering can be diagnosed by genetic testing before birth or in early childhood.

In order to estimate the possibility of detection of mutation in CFTR gene, two kinds of PCR products were used, namely, the product containing the widespread mutation (del21 kb), as well as the same fragment of CFTR gene but without mentioned mutation. Regarding to CF diagnostics, only a hybridization of mutated gene fragment with specific oligonucleotide (Y3 ligand) leads to detection of difference between fluorescent signal and background noise. This result is an opposite of that obtained with normal DNA where the absence of fluorescent spots is observed (Fig. 4A).

The SNR values obtained using different macroporous platforms confirmed recently established with model test-system (see above) advantage of hydrophilic GMA-GDMA and HEMA-GDMA supports demonstrating the best analytical sensitivity (Fig. 4B). Moreover, it was proved that hydrophilic materials GMA-GDMA and CEMA-GDMA appeared to be also appropriate comparatively to more hydrophobic matrixes with the same reactive groups, e.g. standard GMA-EDMA and CEMA-EDMA copolymers.

3.4. Aptamer-based test-system for protein detection

Aptamers represent short oligonucleotides of single-stranded DNA or RNA selected in vitro to bind targets with high specificity. Nowadays, aptamers have demonstrated diverse applications in biosensors development, investigations of molecular interactions, as well as in protein microarrays fabrication [28]. Similarly to antibodies, aptamers can form three-dimensional structures which allow their binding to complementary proteins. Thus, to produce efficiently operating test-system, it is necessary to provide favorable conditions where immobilized aptamer could fold into its correct three-dimension conformation. For a prosperous reaction the specific properties of microarray's platform are very important.

For example, native nitrocellulose membranes often used in protein and DNA microarrays cannot be applied to aptamer-based test-systems because of mostly used adsorption principle of ligand immobilization that assumes physical oligonucleotide attachment to the surface due to hydrophobic and electrostatic interactions [31]. This type of immobilization undoubtedly leads to decreasing of aptamer efficiency at complementary pair formation related to steric limitations and non-defined conformation of a ligand. In contrast, macroporous monoliths have significant advantage which distinguishes them from other materials, namely, the developed

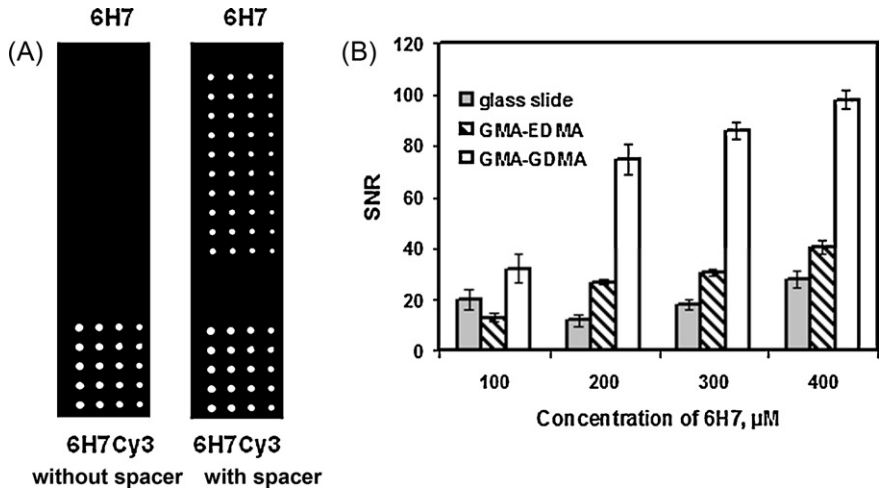

Fig. 5. Aptamer-based test-system: (A) images of monolithic surface after microanalysis performed without and with polymer spacer; (B) dependence of SNRs on 6H7 aptamer concentrations obtained on macroporous platforms and aldehyde glass slide. *Conditions*: spotting buffer – PBS, pH 7.5; each concentration of 6H7 aptamer was replicated 10 times and the solution of fluorescently labeled aptamer (6H7Cy3) was spotted in 5 replications; PMT gain 350 was used for GMA-EDMA and GMA-GDMA microarrays and 400 – for glass slide.

matrixes contain the reactive groups allowing covalent binding to different types of ligands.

It is well known that the introduction of a spacer into a surface structure results in decreasing of steric limitations at biospecific pair formation. To confirm or exclude such a need at aptamer-based microarray fabrication, two parallel experiments were performed under the same conditions. In first case, a spacer polyethylenimine (PEI) was covalently attached to the surface before aptamer immobilization, while in another one, no spacer was introduced. Two probes were printed onto the surfaces of macroporous platforms, namely, 6H7 aptamer and the same but fluorescently labeled ligand (6H7Cy3). Afterwards blocking and coupling steps were carried out. The results of detection (Fig. 5A) show that direct immobilization (without PEI spacer) allows attachment of aptamer that is confirmed by visible columns where fluorescently labeled 6H7Cy3 (left image in Fig. 5A) has been spotted.

At the same time, the absence of registered spots in the case of immobilization of non-labeled ligands indicates the inability of immobilized ligand to form a specific pair with fluorescently marked protein of interest. In contrast, the immobilization via long spacer led to detection of specific complex of both native 6H7 and labeled 6H7Cy3 aptamers (right image in Fig. 5A). The following Fig. 5B illustrates the dependences of SNR on aptamer 6H7 concentration plotted on three different platforms (glass slide, GMA-EDMA and GMA-GDMA). Obviously, the increase of aptamer concentration in spotting solution resulted in higher SNR values detected for monolithic materials.

This result coincided with tendency revealed in the experiments with model DNA system. Moreover, in both cases the hydrophilic GMA-GDMA monoliths demonstrated the best sensitivity.

4. Conclusions

In this work five macroporous polymer monolithic materials with different hydrophilic–hydrophobic properties and reactive groups were suggested as platforms for DNA microarray fabrication. The comparison of results obtained on the surface of macroporous platforms with those performed on standard glass slides clearly justified the advantage of developed devices. In particular, it was established that the time of ligand (oligonucleotide) immobilization, as well as the time of coupling (hybridization) reaction can be significantly reduced. All tested matrixes were characterized by high spot quality, as well as excellent and reproducible sensitivity. The coefficients of variation for developed test-systems were in the range 5–13%. Among all suggested materials, the best analytical potential was established for hydrophilic GMA-GDMA and HEMA-GDMA copolymers. As an practical example, the diagnostic test on Cystic fibrosis (CF) disease was developed. It was shown that the macroporous materials can be successfully applied to distinguish reliably positive (mutated) or negative (fragment of healthy DNA) samples of gene material. In addition, it was demonstrated that polymer macroporous matrixes can be used for aptamer-based test-system preparation.

The successful construction of DNA and aptamer-based microarrays using modern macroporous monolithic materials recently suggested as efficient 3-D platforms for protein microanalysis testifies the wide universality of these supports.

Acknowledgements

This work was supported by Russian Foundation of Basic Researches (grant RFBR #11-03-00829-a) and Russian Federal Program “Research and scientific-pedagogical personnel of innovative Russia in 2009-2013” (contract #14.740.11.0382). The authors are grateful to German Academic Exchange Service (DAAD) for providing the scholarship for PhD student E. Sinitsyna for five-month work at the Institute of Technical Chemistry, Leibniz University of Hannover (TCI LUH). We also thank Prof. Thomas Scheper for great organization help and valuable discussions.

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