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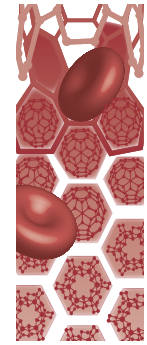

Surface modification of SOI-FET sensors for label-free and specific detection of short RNA analyte

Aim: A new type of surface modification of SOI-FET sensors with ultrathin sensor-probe transition layer and uncharged probes for highly specific detection of short RNA (saRNA) was suggested. **Materials & methods:** Carbonyldiimidazole (CDI) or glycidoxypropyltrimethoxysilane were used as precursors of sensor-probe interface layers, together with peptide nucleic acids and new NA analogues – phosphoryl guanidine oligo(2′-OMe)ribonucleotides (PGO) as probes for RNA hybridization. RNA sequences corresponding to mRNA *NELFA (NM_005663)* and microRNA-29a (cancer markers) were used as saRNA targets. Real-time saRNA detection by SOI-FET sensors and fluorescence analysis were applied. **Results:** Highly specific response with femtomolar sensitivity to saRNA was demonstrated for CDI-PGO-modified sensors fabricated by optical lithography. **Conclusion:** The proposed CDI–PGO protocol of modification of Si sensor surface is a promising procedure for biomedical applications.

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Keywords: short RNA analyte • SOI-FET biosensor • surface modification

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Regulatory RNAs, such as microRNAs (miRNAs) and long-noncoding RNAs (lncRNAs) play an important role in transcriptional and post-transcriptional regulation of gene expression. They are involved in a variety of vitally important processes, namely cell proliferation, senescence, migration and apoptosis, and therefore take part in progression of human diseases, including cancer [1]. Lung cancer is the leading cause of cancer-related mortality worldwide, accounting for ~1.5 million deaths annually [2,3]. Early diagnosis and treatment could sufficiently increase the survival rate of lung cancer patients with a potential for cure [4].

Circulating miRNAs, mRNAs and lncRNA were proposed as prospective molecular markers for cancer detection, and serve as natural RNA analytes [5,6]. Therefore, highly sensitive detection of individual miRNAs and fragments of mRNA and lncRNA in body fluids is conceptually a good approach for early diagnosis of cancer and assessment of disease progression. To this end, a major challenge is to develop tools with both high sensitivity and specificity for rapid quantitative detection of marker RNAs.

It is well known that Si nanowire (NW) field effect transistor (FET) biosensors are high-sensitivity analytical devices [7–17]. They transform the interaction of bio or chemical target particles with sensor surface into the change of their conductivity, and enable macromolecular detection in real-time mode. Such devices belong to the class of 'label-free' detectors. Compatibility of their manufacturing procedures with current commercial silicon-based complementary metal-oxide semiconductor technology provides a possible solution for the implementation of System-on-Chip Integrated Circuit fabrication with a huge potential for a large variety of biomedical applications. Nowadays, Si-NW FET biosensors are employed for detection

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of DNA [7–11], proteins [7,11–14] and miRNA [7,11,15–17], including cancer markers with extremely low (10^{-13}–10^{-16} M) concentration. The theoretical limit of detection for NW biosensor (experimentally achieved for viral particles [11]) is as low as one molecule per sensor element. As a comparison, the range of typical serum levels of circulating miRNAs is between 2×10^{-16} and 2×10^{-11} M [17]. The review [17] gives a overview of recent advances in the area of electrochemical detection of miRNAs, including the NW-FET sensors, with future prospects and challenges.

Surface modification, including immobilization of probes, is a key issue determining the selectivity and specificity of binding of target particles with sensors. The whole protocol includes four main steps: cleaning and activation of sensor surface; formation of interface layer between sensor and probes; attachment of probes and capture of analyte (targets) and signal processing (detection of biomolecular interactions). In addition, the surface passivation may be also used to reduce nonspecific binding.

As a general matter, activation and cleaning of Si-sensor surface is achieved by the use of strong oxidizers such as H_2SO_4/H_2O_2, NH_3/H_2O_2 or oxygen plasma and sonication, which result in the consistent surface abundance of Si–OH groups [8]. The formation of sensor/probes interface is generally achieved by the use of functional organosilanes. The latter can covalently interact with silanol residues on NW surface, and bear functional groups for the attachment of probes. For RNA detection, their hybridization with complementary DNA- or peptide nucleic acids (PNA)-probes is commonly used [8,11].

The key tasks for preparing the surface of the sensors are: formation of a well-ordered layer of highly specific probes and minimization of the total thickness of the interface- and probe-layers, as biomolecular recognition and detection events must occur within the Debye length from the sensor surface. Therefore, small molecules (e.g., carbonyldiimidazole [CDI] [18]) are more preferable as linker precursors than organosilanes. For comparison, the Debye length is about 10 nm in the 1 mM diluted salt solution, and only about 1 nm in the 100 mM physiological solution [10,11], while the silane monolayer thickness reaches 0.8–1.2 nm [13]. Besides, there are some problems with the ordering for organosilane monolayers deposited from solutions [8].

In physiological solutions RNAs and DNAs are negatively charged, and PNAs, which are the artificial synthetic oligomers, are neutral under the same conditions (Figure 1). Binding of saRNAs with neutral probes: allows hybridization in solutions with low ionic strength and reduces the electrostatic repulsion between probes or hybridized strands, hence increasing the probes/strands stability and minimizing the background response. From this point of view, neutral PNA-probes are more attractive than negative DNA ones [15]. Otherwise, the cost of synthesis, which is one of the essential factors for manufacturing of nanoscale biosensors, is significantly greater for PNA, as compared with nonmodified DNA or RNA oligomers. Moreover, PNA-probes can form both antiparallel and parallel complexes with complementary sequences [19,20], which can cause the unspecific response from NW sensor. The problem may be solved by the use of another type of uncharged nucleic acid analogues: phosphorodiamidate morpholino oligomers (PMO) [21] or recently developed oligomers of new type with internucleosidic phosphoryl guanidine groups (PGO) [22,23].

The aim of this study was the development of convenient approach to surface modification of Si-FET based sensors for highly specific detection of the saRNA, which should meet the following requirements: minimizing the thickness of the modifying layers on NW interface and the use of the low-cost uncharged probes.

To this purpose, we used the modification of surface by carbonyldiimidazole or, for comparison, the conventional silane modification by glycidoxypropyltrimethoxysilane to form the interface layers between sensor and probes. We investigated the different protocols of Si sensor surface modification and their effects on the sensor response to of different concentrations of saRNA and the specificity of saRNA binding to the modified sensor surface. The new type of nucleic acid analogues with internucleosidic phosphoryl guanidine groups or, PNAs were used as neutral probes. Chemical passivation by glycine was applied to minimize the nonspecific binding of saRNA to the sensor surface.

Two short RNAs analytes were chosen as molecular markers. The sequence of the first saRNA (saRNA1) corresponds to the fragment of mRNA *NELFA (WHSC2)*. This mRNA *NELFA (NM_005663)* encodes a member of the negative elongation factor (*NELF*) protein complex participating in the regulation of RNA polymerase II transcription elongation. *NELFA* factor was suggested as a target for specific immunotherapy for a large number of cancer patients [24]. In addition, the significant change of *NELFA* mRNA level in blood plasma was reported for lung cancer patients [25]. The second saRNA (saRNA2) was represented by clinically significant biomarker microRNA-29a (hsa-miR-29a-3p) which was chosen because its level is also significantly changed in blood plasma of lung cancer patients [26], and recently hsa-miR-29a was reported to be the strongest prognostic marker for overall survival of the lung cancer patients [24].

Silicon-on-insulator (SOI) FETs-sensors were employed in this study for real-time detection of saRNAs. We use the sensors in the shape of SOI-strips to

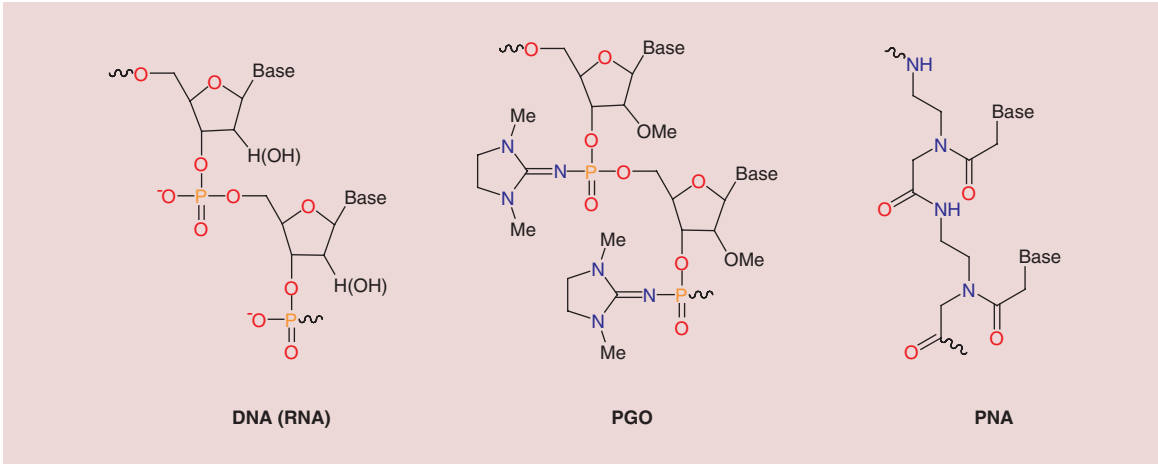


Figure 1. Structure of natural nucleic acids and their analogues.

increase the sensor square (the probability of saRNA binding to sensor surface) and to minimize the background response due to NW-related effects, such as the dependence of response on the target position at the NW-FET surface (near the source or drain, at the front or lateral site of NW, etc. [27]). Fluorescence assay was also applied to reveal the specific and nonspecific saRNA binding with different types of probes.

Materials & methods

Chemicals

3-Glycidoxypropyltrimethoxysilane (GOPS, 97%) was purchased from Sigma Aldrich (USA). 1,1′-CDI was purchased from Acros Organics (USA). Nucleotide sequences of target and capture probes are listed in Table 1.

Phosphoryl guanidine oligo(2′-OMe)ribonucleotide probes (PGO1 and PGO2) complementary to saRNA1 and saRNA2 were synthesized by NooGen LLC (Russia) and purified by HPLC. PNA oligomeric probes were synthesized in Laboratory of Bionanotechnology (ICBPM SB RAS, Russia). Molecular saRNA markers (saRNA1 and saRNA2) were synthesized in Laboratory of RNA Chemistry (ICBPM SB RAS, Russia). Capture PGO-probes were 3′-modified with an amine functionality. RNA targets were 3′-modified with fluorescein for hybridization monitoring. All probes were dissolved in H_2O and stored at -20°C until use.

Preparation of GOPS- or CDI-modified surface

GOPS- or CDI-modified surface was prepared according to [18]. The GOPS-functionalized surface forms a secondary amine moiety and CDI-functionalized surface forms an amide moiety upon reaction with aliphatic amine groups of capture probes. Both surfaces were treated with glycine solution in water (0.2 M) to inactivate the unreacted active groups.

Immobilization of capture probe on the silane- or CDI-modified surface

Figure 2 shows scheme of immobilization of capture probe on the sensor surface. For chips with SOI-FET sensors used for electrical measurements, 0.3 µl of capture probes (5×10^{-5} M) was dripped on a chip and was left up under wet conditions. After 12 h, sensors were sequentially rinsed twice with deionized water, 50% aqueous ethanol and finally with pure ethanol to remove noncovalently bound probes.

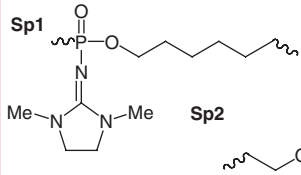
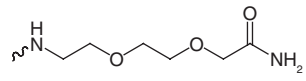
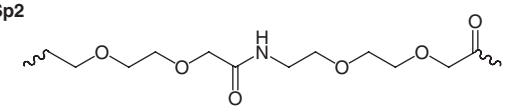
For biochips on a glass used for fluorescence analysis, 1 nl of capture probes (5×10^{-5} M) was immobilized using BioRad BioOdyssey Calligrapher (USA). Biochips with 360 nm spots were prepared by commonly used technique [28].

To prevent nonspecific binding of RNA, all chips were capped by 0.2 M glycine, 30 min, then rinsed sequentially twice with deionized water, 50% EtOH, 100% EtOH.

Hybridization of synthetic probes with saRNA was carried out at room temperature in 1 mM PB, pH 7.4. Fluorescence images of hybridized target probes were taken using Perkin Elmer ScanArray Express (Perkin Elmer, USA).

SOI-FET sensors & electrical measurements

We used n-channel SOI-FET sensors manufactured by a top-down fabrication process based on conventional photolithography. The fabrication process flow and the cross-sectional view of biosensor are schematically shown in Figure 3A & B, respectively. Main steps of manufacturing of sensors are described elsewhere [29]. Starting SOI wafers have 30 nm thickness of the top Si layer and 200 nm thickness of the buried oxide (BOX). Figure 3C & D show the optical images of the fabricated sensors on a chip, and the single SOI-FET sensor, respectively. Each chip contained 12 individual addressable sensors. The

Table 1. Sequences of oligonucleotides (5′→3′).			
1	saRNA1	r(GAGGCGAAGCGGAGAAGGAAGAC)-FAM	Target
2	saRNA2	r(CUAGCACCAUCUGAAAUCGGUUA)-FAM	
3	PGO1	2′-OMe-r(GUCUUCCUUCUCCGCUU)-Sp_1-NH_2	Capture probes
4	PGO2	2′-OMe-r(UAACCGAUUUCAGAUUU)-Sp_1-NH_2	
5	PNA1	H_2N-Sp_2-GTCTTCCTTCTC-Sp_3-C(O)NH_2	
6	PNA2	H_2N-Sp_2-TAACCGATTTCA-Sp_3-C(O)NH_2	
Sp1  Sp3  Sp2 			Spacers

length of sensor element was 10 µm, the width was 3 µm. After fabrication, surface of sensors covered by natural oxide was modified by CDI or GOPS interface layers, as described above. Part of sensors on the chips was functionalized by PGO1 and PGO2 or PNA1 and PNA2-probes (working sensors), the other part was without any probes (reference sensors) (Figure 3C). To provide the simultaneous detection of targets on the working and reference sensors, 1 µl miRNA-contained samples were dripped on the 3 mm^2 area of the chip with 12 sensors. The concentration of saRNA in the samples was varied in the range of 10^{-15}–10^{-5} M.

Electrical measurements were made using Multifunction DAQ NI USB-6363 (http://www.ni.com). During all measurements for saRNA detection, reference electrode placed into solution was grounded (Figure 3C), the constant low voltage V_{ds} = 0.15 V was applied between source and drain. SOI substrate was used as the back-gate for setting of the subthreshold mode, thus providing the highest signal response for back-gated SOI-FET sensors [29].

The response of sensors to saRNA was calculated as a relative change in current $\Delta I_{ds}/I_{ds}^{0}$, where ΔI_{ds} is the change of the current in the assay, and I_{ds}^{0} is the initial current of sensor (before introduction of saRNA in buffer solution). Specificity of saRNA attachment was defined as the ratio of the responses of working and reference sensors. Functionalization of sensors on the one chip by different probes (probe 1 and probe 2 which are fully complementary or noncomplementary to target saRNAs; Figure 3C) was also used to verify the selectivity of saRNA detection.

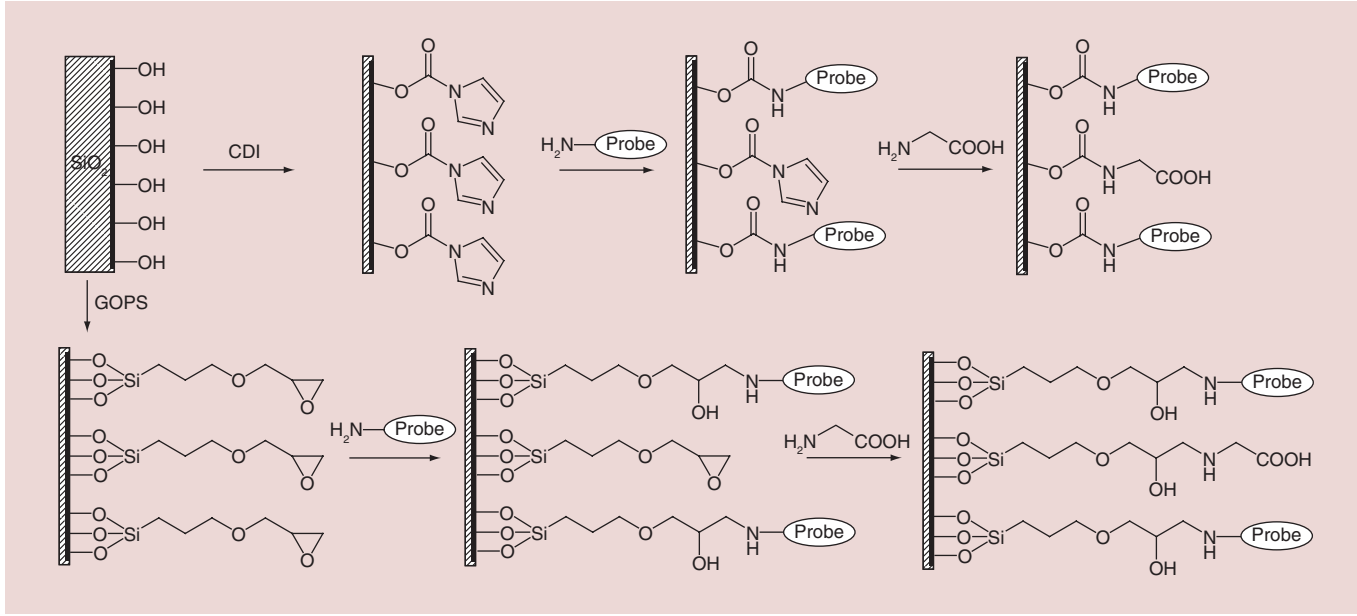


Figure 2. Scheme of immobilization of capture probe on the CDI- or silane-modified surface.

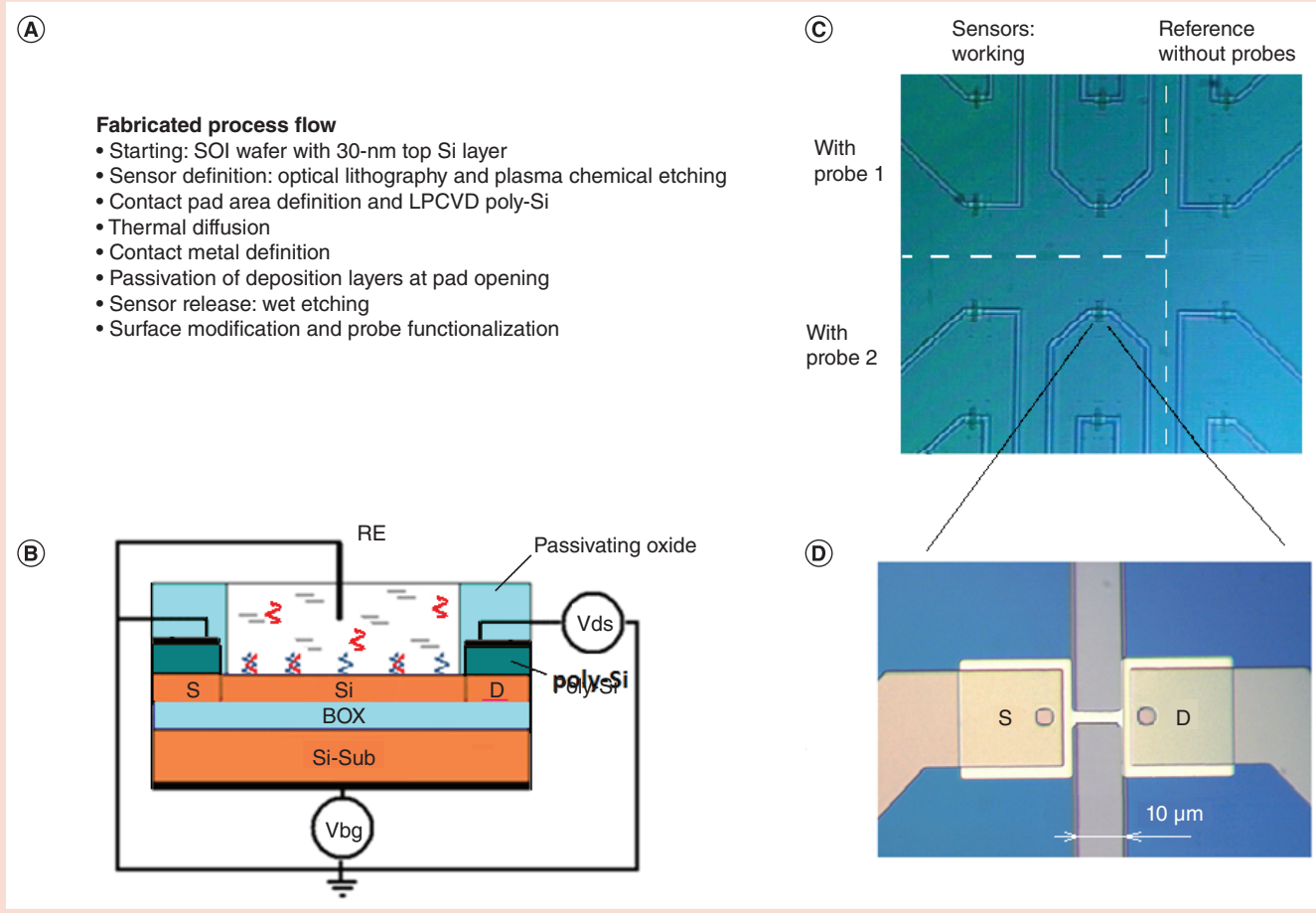


Figure 3. (A) Schematic description of the fabrication process flow of an SOI-FET biosensor. **(B)** A general scheme of biosensing measurement setup of sensors. The optical image of a fabricated chip with sensors. **(C)** Each chip consists of 12 individual addressable sensors with probes or without any probes on the surface. **(D)** The optical image of one of the sensors.

Results & discussion

Figure 4 shows the real-time detection of electronic signals (current) from the working CDI-PGO1 and GOPS-PGO1 sensors (see Table 1) and references to them (CDI- and GOPS-modified sensors) before and during binding with saRNA1 samples. The concentration of saRNA1 was varied in the range of 10^{-12}–10^{-5} M. Inset in Figure 4A shows the $I_{ds}(t)$ dependency for CDI-PGO1 sensors at saRNA1 concentration ranging from 10^{-15} M to 10^{-12} M and at 10^{-6} M concentration of saRNA2.

It was shown that the increase of the saRNA1 concentration leads to the decrease in sensor current. The changes in current are more obvious for working sensors. This confirms the specificity of saRNA1 binding with PGO1-probes, since under optimal conditions the hybridization of target saRNAs with the complementary probes increases the negative charge on the sensor surface and, correspondingly, increases the change of current, decreasing the electron conductance in the n-channel of SOI FETs. The inset shows that CDI-PGO1 sensor reacts on the femtomolar concentration of saRNA1 molecules. In contrast, sensor has a weak response even on micromolar concentration of saRNA2 molecules that are noncomplementary to PGO1-probes.

Figure 5 shows the response of sensors to saRNA1 molecules calculated on the basis of $I_{ds}(t)$-dependences given in Figure 4. The CDI-PGO1 sensor displayed a wide dynamic range up to 10^{-5} M and was able to detect of saRNA1 as low as 10^{-15} M (Figure 5, inset). Both CDI-PGO1- and GOPS-PGO1-sensors demonstrated 1 pM sensitivity to saRNA1 molecules with about 20% response. The response of the GOPS-PGO1-sensors in the range of saRNA1 concentration of 10^{-11}–10^{-5} M is more prominent than that for CDI-PGO1-sensors (compare curves 1 and 3; Figure 5). However, a significant response is also observed for the reference silane-modified sensors (curve 2; Figure 5). The same results were obtained for sensors with PNA1-probes.

Figure 6 shows the average values of responses and their standard deviation for the reference sensors. The data were obtained from three experiments (from mea-

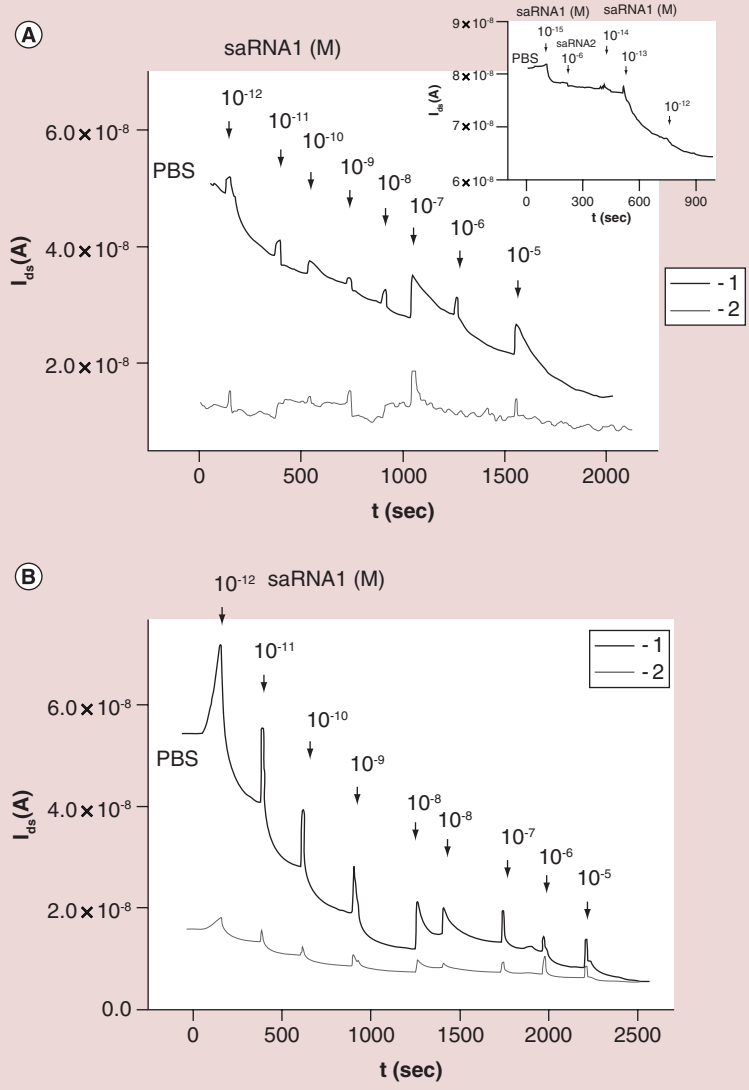


Figure 4. Current-versus-time data recorded for the simultaneous detection of saRNA1-containing samples on the sensors with (A) CDI- and (B) GOPS-modified surface functionalized by PGO1-probes – (1) and without any probes – (2). The concentration of saRNA1 was varied in the range of 10^{-12} to 10^{-5} M. The inset shows I_{ds}(t) dependence and response of CDI-PGO1 sensors for saRNA1 concentration ranging from 10^{-15} M to 10^{-12} M and for 10^{-6} M concentration of saRNA2.

surements on the three chips) for each concentration of saRNA1. As a comparison, the response of sensor with nonmodified surface is also shown in Figure 6. One can see that response of sensors with silane-modified surfaces varies in the range of (6–60)% at saRNA concentration ranging from 10^{-12} to 10^{-5} M. The response of sensors with nonmodified surface gave comparable values which were about 50% at the maximal saRNA concentration in solution. The CDI-modification of the sensor surface provides the lowest signal values which does not exceed 20% at the 10^{-5} M saRNA1 concentration.

Figure 7 shows the average values of response of sensors with the different types of the surface modification to saRNA1 and saRMA2-contained samples.

From the data shown in Figure 7, one can easily estimate that the ratio of the responses of working and reference sensors (i.e., the specificity of sensor) is about 9–10 for CDI-modified sensors (CDI1-PGO1 and CDI-PNA1) and 1–3 for silane-modified sensors (GOPS-PGO1 and GOPS-PNA1) in the concentration range of 10^{-12}–10^{-5} M. The data in Figure 7 also demonstrate the high selectivity of the saRNA binding. The response of sensors with the fully complemen-

tary complex probe1-saRNA1 (or probe2-saRNA2) is about order higher than that with noncomplementary probe1–saRNA2 (or probe2–saRNA1). In order to confirm this results, we used the fluorescence analysis after binding of different probes with the fully complementary- or noncomplementary-labeled saRNAs (Figure 8).

The glass wafer was employed as biochip platforms bearing capture probes corresponding to saRNA of type 1 or type 2 (see Table 1). Originally labeled unspecific oligonucleotides (oligo (dT)n) and pure water were also used for immobilization as controls. Then, on each wafer the saRNA1, fully complementary to the sample 1 and the saRNA2, fully complementary to sample 2, were added. It can be seen that in the first case only the spots corresponding to probe1/saRNA1 complex are detected (Figure 6A), while in the second case – the spots for probe2/saRNA2 (Figure 8B).

Thus, the fluorescence assay (Figure 8) together with results of the real-time saRNA detection on SOI-FET sensors (Figure 7) demonstrate the high selectivity and specificity for CDI-probes sensors. Additionally, the data presented in Figure 7 evidence that: the specificity of binding of saRNA1 molecules to sensor surface has little dependence on the type of probes (PGO1 or PNA1) (this result was *a priori* expected since both type of probes are neutral), and relative to the reference sensors, CDI-modified sensors have greater specificity (9–10) than sensors with silane modification of surface (1–3). This result can be explained as follows.

The specificity of saRNA binding is defined as the ratio of the responses of working and reference sensors. Results presented in Figures 5 & 6 show that the response of reference CDI-modified surface is smaller than that of silane-modified sensors. As we mentioned above, sensors were treated with glycine at the final stage of the surface preparation to suppress nonspecific binding of saRNA. As can be concluded from Figure 6, GOPS-glycine treatment does not suppress nonspecific binding of saRNA1 molecules (compare curves 1 and 3 in Figure 6). In contrast, CDI-glycine treatment successfully blocks unspecific binding sites, and leads to the formation of 'oligo-(saRNA)-phobic' surface. We expected that CDI-based protocol should result in hydrophilic interface, thus preventing unacceptable adsorption on the working area of NW-sensor.

Thus, the glycine treatment provides the suppression of the nonspecific saRNA binding and the decrease of background signal for CDI reference sensors. This results in the increase of sensor specificity.

To summarize, a new type of surface modification of SOI-FETsensors with ultrathin sensor-probe transition

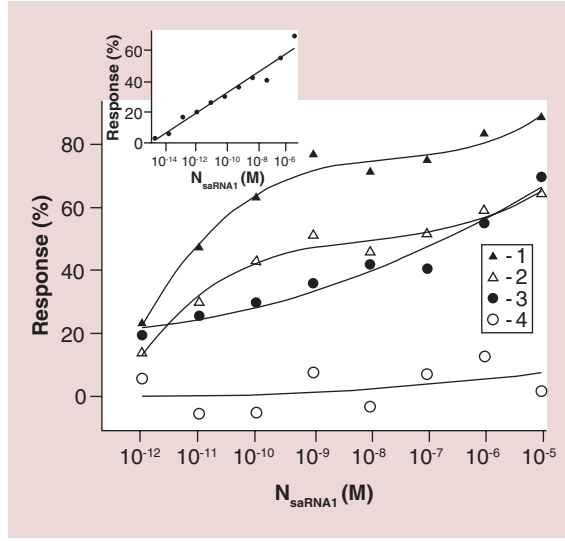


Figure 5. Responses for GOPS- (1, 2) and CDI- (3, 4) – modified sensors versus saRNA1 concentration ranging from 10^{-12} to 10^{-5} M: 1, 3 – sensors with PGO1-probes; 2, 4 – without any probes. The inset shows the response for CDI-PGO1 sensor to samples with the concentration of saRNA1 in the range of 10^{-15}–10^{-5} M.

layer and uncharged probes for highly specific detection of short RNA analyte was suggested in this study. We used carbonyldiimidazole molecules as the sensor-interface layer precursor and new NA analogues, PGO as immobilized probes [22]. A low cost of synthesis of neutral PGO-probes make them more attractive than PNA analogues. RNA sequences identical to biologically relevant miRNA lung cancer markers [24] were used in this work as target saRNAs.

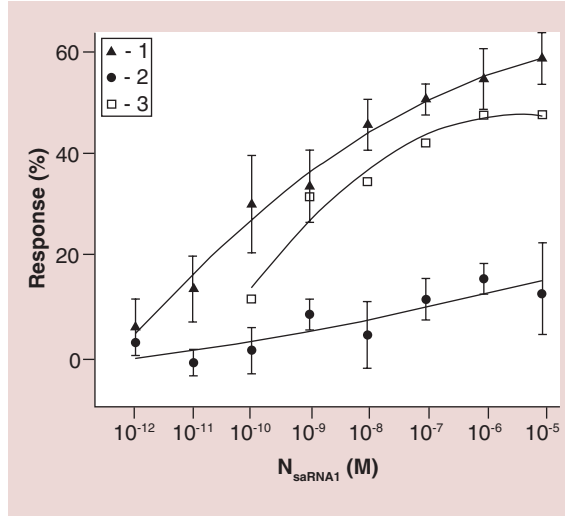


Figure 6. The average values of response for GOPS- (1) and CDI- (2) modified reference sensors at different saRNA1 concentrations. The error bars represent standard deviations of measurements obtained on three chips. Response of sensor with nonmodified surface (3) is shown for comparison.

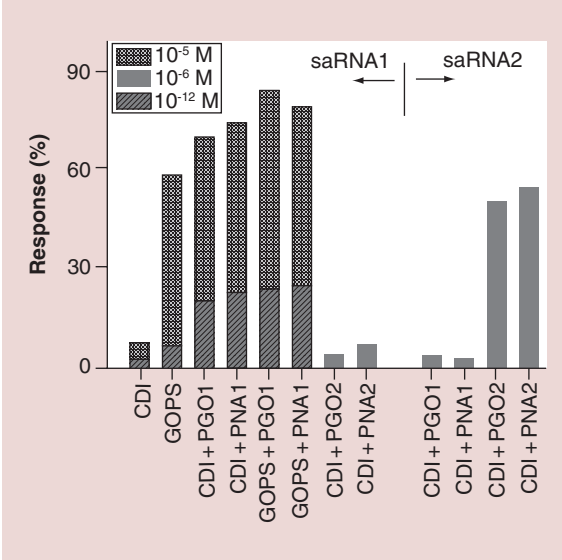


Figure 7. Response to saRNA1 and saRMA2-contained samples for different types of SOI-FET sensors.

The results of the study prove the feasibility of the proposed approaches. Indeed, the response of the CDI-PGO sensors varies between 4 and 20% for 10^{-15}–10^{-12} M concentration of saRNA (see Figures 5 & 7), that is, for the concentration range which is interesting for biological and medical diagnostics. Similar values of responses (2–30% in the same concentration range) are reported in [7,11,15] for detection of the various miRNA cancer markers on the NW sensors. However, in our study we used the sensors in the shape of 3 μm strings. Sensors were fabricated by optical lithography and standard complementary metal-oxide semiconductor-compatible process that significantly reduces the cost of devices manufacturing. It should be also noted that the modern biosensors are sensitive enough to detect miRNA at atto- and subattomolar concentrations [17,30–32]. However sophisticated signal amplification techniques (e.g., target recycling [30] or primer generation-mediated rolling circle amplification [31]), probe-miRNA hybtidization into solution followed by long-time (for 1 h) incubation of the reaction mixture with nanobiosensor [32], and the others are used to achieve such sensitivity. Importantly, these methods are also applicable for SOI-FET sensors, yet we used fast (for 200–300 s) real-time detection of saRNA in sample (see Figure 4). The 1 fM detection limit was achieved without employing any amplication techniques in this study.

We are sure that the proposed way of sensor surface modification is very promising for many biomedical applications. Neutral probes are more attractive than negative ones for the hybridization with negative targets under low-salt conditions [15]. Neutral PGO probes make it possible to perform the hybridization under low-salt conditions (and even in the absence of salt), which is undoubtedly obligatory for FET biosensors and would also provide the resistance of sensor oligonucleotide layer toward cellular enzymes during the analyses of biological samples.

Future perspective

Si-FET-based sensors represent a universal platform for highly sensitive label-free, real-time detection of various molecular markers and could be a possible solution for the implementation of System-on-Chip IC fabrication.

The results of this study are essential for the development of the systems for early diagnosis of cancer on

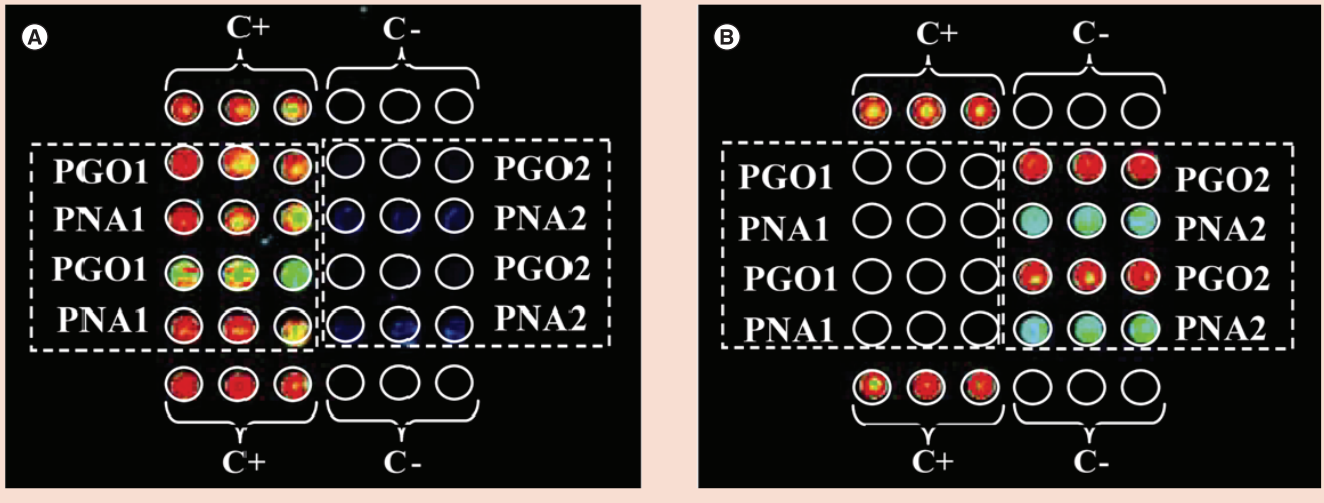


Figure 8. Fluorescence images of hybridized target probes. The results of the binding of fluorescein-labeled saRNA1 **(A)** and saRNA2 **(B)** to the biochips with. Control spots: C+: originally labeled unspecific oligonucleotides (oligo (dT)n) were immobilized; C-: pure water was used as a sample for immobilization.

the base Si-NW FETs providing the highly sensitive and specific detection of miRNA.

The next steps of investigation will be direct measurement of analytes in body fluids and the clinical trials. When the problem of the long-term storage stability will be overcome, and the biosensor technology will be more reliable, an array of such sensors could be incorporated into lab-on-a-chip devices. This should allow fast and low-cost screening of wide variety of miRNA biomarkers in the early diagnosis and assessment of disease progression, using small samples of patient material.

Financial & competing interests disclosure

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Executive summary

- In this report, we studied different protocols of the Si-surface modification for highly specific detection of the short RNA (saRNA), meeting the requirements of the minimization of thickness of the modifying layers and the use of low-cost neutral probes.
- We investigated the new type of nucleic acid analogues with internucleosidic phosphoryl guanidine groups in comparison with PNA as neutral probes for saRNA hybridization, carbonyldiimidazole with extremely short linker moiety in comparison with glycidoxypropyltrimethoxysilane as sensor-probe interface layers and the treatment by glycine for blocking the unspecific binding sites.
- Two short RNAs analytes (RNA sequences corresponding to mRNA *NELFA (NM_005663)* and microRNA-29a) were used as target saRNA cancer markers.
- A series of different concentrations of saRNA ranging from 10^{-15} M to 10^{-5} M were measured in real-time mode using SOI-FET sensors fabricated by optical litography to determine their response and specificity during saRNA detection.
- Real-time detection on SOI FET-sensors and fluorescence analysis after binding of specific and nonspecific saRNA with different probes were applied without employing any amplification techniques.
- The results evidence that CDI-PGO protocol of modification of the Si-FET sensor surface in combination with CDI-glycine treatment can be considered as promising procedure for different biomedical applications implying highly specific, label-free and low-cost femtomolar detection of saRNAs.

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