A Surface Modifier for the Production of Selectively Activated Amino Surface Groups

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Abstract: The formation of self-assembled monolayers with the possibility of selective activation is an important goal of surface chemistry. In this work, a new surface modifier which creates amino surfaces based on aminopropylsilatrane (APS) with a protected amino group was obtained. The utilization of protected APS allows producing a self-assembled monolayer (SAM) and obtaining reactive surface amino groups at distinct times. Furthermore, a precise selective deprotection with a further modification of the activated amino groups could be performed without affecting the protected groups. To demonstrate the practical applicability of this modifier, a trinitrotoluene-sensitive sensor based on an ion-sensitive field-effect transistor (ISFET) was obtained.

1. Introduction

Organic linkers used for the covalent immobilization of biological and organic molecules, as well as nanoparticles have a wide range of roles in the modern natural sciences. The most common class of such surface modifiers is organosilanes [1]. The presence of alkoxy or halogen groups in these molecules allows them to easily interact with surface hydroxy groups, in particular, in silicon structures. This allows for the immobilization of various molecules in microelectronic devices [2].

Aminosilanes play a distinct role in organosilanes. The most commonly used aminosilane is (3-aminopropyl) triethoxysilane (APTES) [3]. It is used in various applications, for example, as the coupling agent for the modification of silica surfaces to increase adhesion in fiberglass–epoxy composites, as a polymer film on glass, for the production of temperature- and photo-responsive films, to immobilize proteins and cells, and to attach nanoparticles [4]. Despite such a wide distribution, APTES has drawbacks, e.g., it is very sensitive to moisture, which leads to its agglomeration and polymerization on a substrate during deposition and can cause multilayer deposition and irregular surface morphology [5]. γ-Aminopropylsilatrane (APS) is used to obtain a surface amino layer as an alternative to APTES [6]. Unlike APTES, APS is less reactive and extremely resistant to hydrolysis and polymerization at neutral pH [7]. However, neither APTES nor APS can withstand long-term storage at room conditions because they both contain a terminal amino group.

The amino surfaces resulting from treatment with APTES or APS are subject to degradation over time and can react with various reagents. Protective groups are used to solve these problems [8].

The presence of protective groups also allows for the selective deprotection of certain areas of the surface, which itself allows for further modifications. One method of deprotection is the electrochemical method [9]. The benzoquinone–hydroquinone system is often used to generate acid during electrochemical deprotection [10]. The acid released at the anode creates a local decrease of pH, thereby removing the protective groups near it. Cathode products react with H^+, preventing the removal of protective groups outside the anode region [11,12]. The compound 1,2-Diphenylhydrazine is also used to electrochemically decrease the pH to remove *tert*-butoxycarbonyl (Boc) protecting groups [13].

In this work, a new surface modifier was obtained based on an N-protected APS. *tert*-Butoxycarbonyl (Boc) was chosen as the protective group because of its capacity for deprotection in an acidic medium, as well as its availability and the ease of formation of the protection. The main reason for using this modifier is to separate in time the formation of thin films and the removal of the protective groups to obtain surface amino groups. This will allow the introduction of additional stages in the processing of the sample prior to the activation of the amino groups, as well as the selective removal of protective groups for subsequent functionalization. Such a modifier can be useful for the selective immobilization and fabrication of biosensors.

2. Materials and Methods

2.1. Materials

All chemicals received from commercial sources were used without further purification. Streptavidin–peroxidase conjugate and recombinant *Escherichia coli* nitroreductase K12 were obtained from Sigma Aldrich (St. Louis, MO, USA). Streptavidin–glucose oxidase conjugate was obtained from Fitzgerald Industries International (Acton, MA, USA). Solvents were purified and dried according to standard procedures. γAPS was synthesized according to published procedures [14].

For the synthesis of N-*tert*-butoxycarbonylaminopropylsilatrane (BocAPS), APS (0.4 g, 1.7 mmol), di-*tert*-butyl dicarbonate (Boc_2O, 1.2 mL, 5.1 mmol), and triethylamine (Et_3N, 1.4 mL, 10.2 mmol) were mixed in anhydrous dimethylformamide (DMF, 50 mL) and stirred at room temperature for 2 days. After this time, the solvent was evaporated. The residue was recrystallized from toluene, resulting in a colorless crystallin powder (0.56 g, 99% yield).

The p-type silicon boron-doped wafer (Ameks, Russia) was cleaned with Caro solution followed by chemical etching in an HF–HCl solution. The cleaned surface was treated by plasma oxidation followed by annealing at 300 °C for 30 min. The wafer was then cut into 4 mm × 4 mm or 7 mm × 20 mm slides.

n-Channel silicon-on-insulator (SOI) ion-sensitive field-effect transistors (ISFETs) were manufactured at the SMC Technological Center, Russia, according to the 1.2 μm complementary metal–oxide–semiconductor (CMOS) process. The sensitive surface of the ISFETs was a SiO_2 layer.

2.2. NMR and Crystallography Measurements

All NMR spectra were acquired at 25 °C on a Bruker AVANCE 400 spectrometer (Karlsruhe, Germany). Chemical shifts are reported in ppm, referenced to solvent signals (see Supplementary material Figures S1 and S2). BocAPS NMR [1]H ($CDCl_3$, 400 MHz), δ, ppm: 4.91 b.s. (1H, NH), 3.76 t (6H, J = 5.8 Hz, CH_2O), 3.09 m (2H, C$\underline{H}_2$NH), 2.80 t (6H, J = 5.8 Hz, CH_2N), 1.58 m (2H, $CH_2C\underline{H}_2CH_2$), 1.42 s (9H, $C(CH_3)_3$), 0.42 m (2H, CH_2Si). NMR [13]C ($CDCl_3$, 100 MHz), δ, ppm: 156.01 (C=O), 78.29 (CH_2NH), 57.64 (CH_2O), 50.99 (CH_2N), 43.46 ($CH_2\underline{C}H_2CH_2$), 28.45 (C($\underline{C}H_3)_3$), 24.88 ($\underline{C}(CH_3)_3$), 13.17 (CH_2Si).

For the interaction with glutaraldehyde, 6.6 mg (0.02 mmol) BocAPS or 4.6 mg (0.02 mmol) APS and 640 μL D_2O were mixed in an NMR tube. Then, 12 μL (25%, 0.03 mmol) glutaraldehyde was added. After 30 min, the ^{1}H NMR spectrum was recorded (Figure S3). No studies were carried out with (3-aminopropyl) triethoxysilane (APTES), because it was rapidly hydrolyzed under our experimental conditions.

The X-ray single-crystal data were collected at 290 K on a Enraf-Nonius CAD4 diffractometer using graphite monochromatized Cu–Kα radiation (λ = 1.54178 Å) in the ω-scan mode. Absorption corrections based on measurements of equivalent reflections were applied [15]. The structure was solved by direct methods and refined by the full-matrix least-squares method on F^2 with anisotropic thermal parameters for all nonhydrogen atoms in the Olex2 software package [16]. Hydrogen atoms were placed in calculated positions and refined using a riding model. The silatrane fragment was found to be rotationally disordered over two positions with occupancies of 0.62/0.38. The second component of the disorder was rotated about the Si–N bond by 38.4(3) with respect to the first component. Crystallographic data are presented in the Supporting Information (Table S1).

2.3. Monolayer Preparation and Modification

The slides were treated with UV-generated ozone in a BioForce UV/Ozone Pro-Cleaner ozone generator (Ghent, NY, USA) for 10 min to refresh the surface oxides. The slides were then submerged into a toluene solution of BocAPS (6.6 mg BocAPS in 15 mL toluene) and incubated for 3 h at 70 °C, prior to rinsing with dichloromethane and drying. The obtained films were then characterized by contact angle. The ISFET monolayers were prepared in a similar manner.

Water-wetting contact angle measurements were conducted at room temperature (20 °C) using an OCA 15EC DataPhysics Instrument (Filderstadt, Germany). Deionized water (electrical resistance = 18.2 MΩcm) was dropped onto each surface, and the contact angles were assessed using SCA 20 software (Version 4.3.19).

X-ray photoelectron spectroscopy (XPS) was performed with a Specs PHOIBOS 150 MCD (Berlin, Germany) instrument using monochromatic Mg Kα X-rays (1253.6 eV). The source power was 225 W. The pass energy was set at 40 and 10 eV for the survey regions and the narrow regions, respectively. Spectral calibration was determined by setting the main C_{1s} component at 286 eV.

Before removing the protective groups from the self-assembled monolayer (SAM) on the ISFET, epoxy structures of the well type were created on their surface according to the described procedure [17].

The general method of chemical removal of protective groups was as follows: The slides modified with BocAPS were placed in a solution of trifluoroacetic acid and dichloromethane (1:1) for 30 min at room temperature. Next, they were rinsed with dichloromethane and dried.

The general method of electrochemical removal of protective groups was as follows: Two silicon slides were connected to an Agilent B1500A semiconductor device parameter analyzer and immersed in an acetonitrile solution of benzoquinone (25 mM), hydroquinone (25 mM), and tetrabutylammonium hexafluorophosphate (25 mM). One of the slides was maintained at 0 V, making it the cathode, the other was maintained at a variable voltage (with the limit of 30 V) to establish a current of 200 µA between the slides, making this slide the anode. This setup was maintained for 40 min, then the slides were washed with acetonitrile and dried. In the case of the ISFET, a platinum reference electrode was used as the anode (V_g = 2.5 V), the sensitive surface of the ISFET was used as the cathode, and the drain and the source were connected to the source ground (V_d = V_s = 0 V).

The slides were incubated in a 5% glutaric dialdehyde solution in water for 1 h at room temperature. The slides were then thoroughly washed with phosphate-buffered saline (PBS, pH 7.4) and incubated overnight with 0.01 mg/mL of enzyme (glucose oxidase or nitroreductase) solution in PBS at 1 °C, then washed 5 times with PBS.

2.4. Measurement of Enzyme Activity

Glucose oxidase activity was measured using a microplate spectrofluorimeter (Infinite® M200, Tecan, Switzerland) and Bio-One 655001 microplates (Greiner Bio-One, Kremsmünster, Austria). The reaction was carried out in 0.1 M citrate–phosphate buffer, pH 5.5. The reaction mixture contained 3,3′,5,5′-tetramethylbenzidine (TMB, 0.3 mM), glucose (1.2 mM), and horseradish peroxidase (HRP, 6×10^{-8} M). The volume of the reaction mixture in the wells was adjusted to 100 µL. Enzyme-modified

slides were placed in the wells. Kinetic measurements were performed at room temperature for 4 h, and the increase in absorbance at 650 nm was recorded.

The measurement of nitroreductase activity using an ISFET was performed according to a method previously described [18], using a semiconductor device parameter analyzer (Agilent B1500A, Santa Clara, CA, USA) and a probe station (CascadeMicrotech PM5, Beaverton, OR, USA) operated by the Agilent VEE Pro software (Version A.04.20).

3. Results and Discussion

3.1. Synthesis and Characterization of BocAPS

The synthesis of BocAPS was carried out in two stages (Figure 1). In the first stage, APS was obtained according to the described procedure by condensing APTES with triethanolamine (TEA) in the presence of sodium as a catalyst [14]. In the second stage, the amine protective group was introduced by reacting it with Boc$_2$O. BocAPS, obtained with a yield of 99%, turned out to be a fairly stable compound. In contrast to most common surface modifiers with a terminal amino group (APS and APTES), BocAPS does not degrade when stored without special conditions (at room temperature without an inert gas) for several years (see Supplementary data Figure S4).
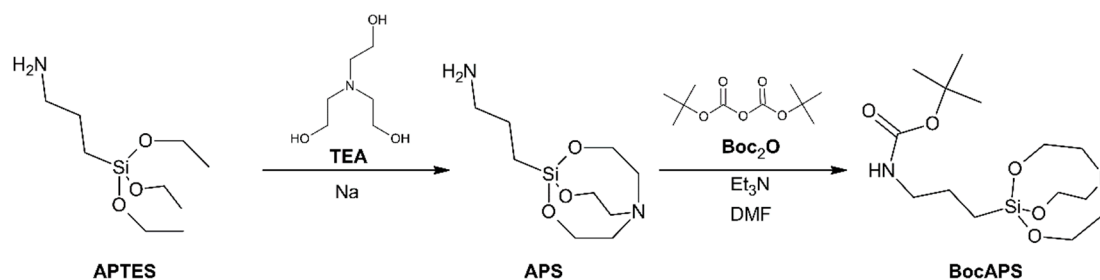

Figure 1. Synthesis of N-*tert*-butoxycarbonylaminopropylsilatrane (BocAPS).

The structure of the obtained BocAPS was confirmed by ^{1}H and ^{13}C NMR spectra, as well as X-ray structural analysis. X-ray-quality single crystals of BocAPS were grown by slow cooling a toluene solution of the substance (Deposition Number CCDC-1952855, Figure 2). The Si←N donating bond length is 2.154(3) Å, which is similar to those observed in other γ-organopropylsilatranes and in methylsilatrane [19]. The molecule has a moderate intramolecular N–H—O hydrogen bond between the amide fragment and the oxygen atom of the silatrane fragment (d(N—O) = 2.950(3) Å).
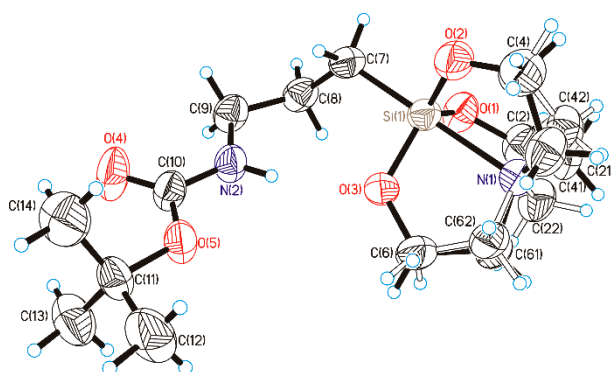

Figure 2. Molecular structure of BocAPS. Displacement ellipsoids are shown at the 50% probability level.

In order to check the protective properties of the Boc-group, NMR studies of the interaction of BocAPS with glutaraldehyde were carried out. Glutaraldehyde was chosen as a commonly used reagent for the further modification of surfaces with terminal amino groups [20]. When glutaraldehyde

was added to the BocAPS solution in D_2O, there was no noticeable change in the ^1H NMR spectra for 30–60 min: the signals of both BocAPS and glutaraldehyde were present in the spectrum without changing intensity over time (see Supplementary data Figure S3). In turn, unprotected APS reacted with glutaraldehyde under the same conditions within a few seconds. Thus, BocAPS can be used to achieve selective interaction with amino modifiers: Removing Boc groups activates amino groups, while the presence of Boc groups prevents their interaction with amino modifiers.

3.2. Formation and Modification of a BocAPS SAM

The formation of a BocAPS SAM on silicon slides bearing a surface silicon dioxide layer was investigated. The slide surface was initially treated with UV-generated ozone to remove any organic admixtures and regenerate free hydroxyl groups on the surface to provide maximum density of immobilized BocAPS molecules. BocAPS was attached to the surface by prolonged heating of the slides in a BocAPS–toluene solution. During the immobilization of BocAPS, the surface was covered with terminal *tert*-butyl groups (Figure 3d), which led to a change in its hydrophilicity. Thus, the completeness of the immobilization process could be estimated by measuring the wetting angle. The wetting angle of the bare slide was 5 ± 3° (after clearing with UV–ozone, Figure 3a) and after the modification of the slide by BocAPS, it increased to 75 ± 3° (Figure 3b). This confirmed the successful immobilization of BocAPS on the surface of SiO_2.
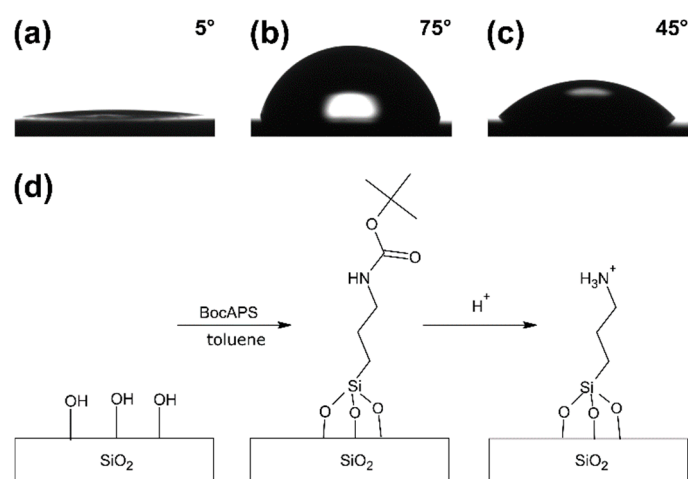
Figure 3. Water-drop profiles on the bare SiO_2 surface (after clearing with UV–ozone) (**a**), BocAPS-modified SiO_2 surface (**b**), and BocAPS-modified SiO_2 surface after removing the Boc groups (**c**). Scheme of self-assembled monolayer (SAM) formation and removal of the protective group (**d**).

It is known that the protective Boc group is removed in an acidic medium. Thus, for the chemical removal of Boc groups, the most frequently used method in the literature involves a solution of trifluoroacetic acid in methylene chloride [21]. It was shown that the optimal solution for deprotection was a 1:1 mixture of trifluoroacetic acid and methylene chloride applied for 30 min. In this case, the contact angle of the surface decreased to 45° (Figure 3c), which is close to the wetting contact angle value for the NH_3^+-group indicated in the literature [2].

For the electrochemical removal of protective groups, an anode–cathode system consisting of two silicon slides connected to an Agilent B1500A and immersed in an acetonitrile solution of benzoquinone, hydroquinone, and tetrabutylammonium hexafluorophosphate, was used. It is worth noting that, in contrast to works where the protective groups are removed by electrochemical generation of an acid in the anode area [22], in this case, the acidity of the medium was not enough to remove the Boc groups. The removal of the protective groups occurred at the cathode, similar to the cathodic removal of the benzyloxycarbonyl protecting group in the production of amine and CO_2 [23]. Variations in voltage, current, and time in the experiments showed that for 7 mm × 20 mm silicon slides, the treatment for

40 min with a voltage corresponding to a current of 200 μA between the electrodes (with a voltage limit of 30 V) was sufficient for the removal of the protective group (this result was reproducible; see Supplementary data Figure S5).

XPS studies were also used to analyze the attachment of BocAPS and the consequent surface modifications. The full survey spectrum (see Supplementary data Figure S6) showed characteristic N_{1s}, C_{1s}, and O_{1s} signals from immobilized BocAPS. A decrease in the N_{1s} signal was observed in the survey spectra of the slides which underwent both chemical and electrochemical deprotection. This was due to the hydrolysis of residues of triethanolamine, which were not completely removed at the stage of immobilization. The removal of the Boc group was confirmed by the dramatic changes in the high-resolution spectra of C_{1s} (Figure 4): the component at 290 eV decreased upon removal of the Boc group, which corresponds to the O–C=O fragment.
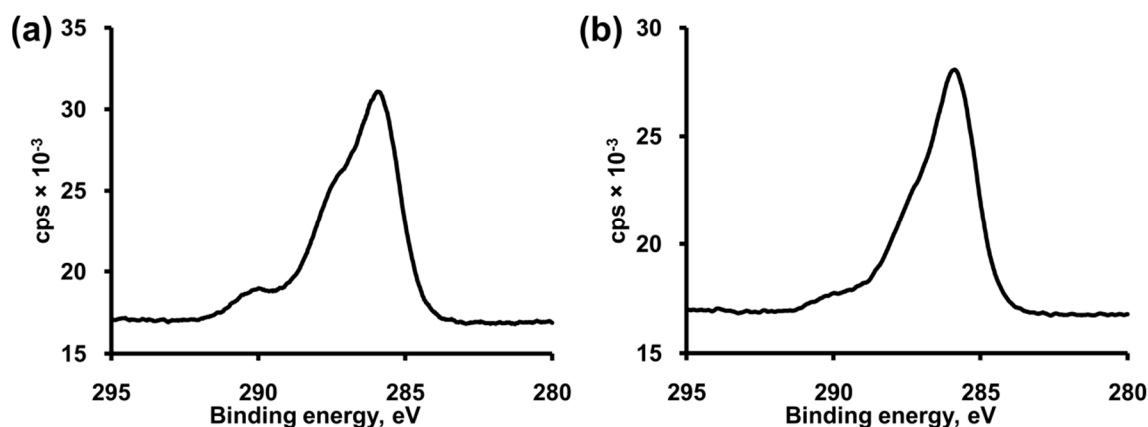
Figure 4. XPS spectra recorded over the C_{1s} region of the BocAPS-modified SiO_2 surface (**a**) and BocAPS-modified SiO_2 surface with removed Boc groups (**b**).

3.3. Biosensor Fabrication Based on BocAPS SAM

BocAPS surfaces could be used in biochemical experiments at the stage of biosensor production during biomolecule immobilization. Enzymes are often used as active components of a biosensor [24], thus they were chosen for further modifications.

An assessment of the suitability of the resulting amino surfaces for the immobilization of the enzyme glucose oxidase was carried out. Immobilization of the enzyme was also performed on slides where the Boc group had not been removed. In the case of electrochemical deprotection, in order to assess the selectivity of deprotection at the cathode, anode slides, where the protecting Boc group must remain, were also used in the studies with glucose oxidase. As a control, bare slides (without binding linkers) were used under the conditions of enzyme immobilization; thus, the contribution of possible physical sorption of the enzyme was evaluated. The enzyme activity on the samples in which the Boc group had been removed by trifluoroacetic acid turned out to be significantly greater than the enzyme activity on the samples in which the Boc group had not been removed; furthermore, it was slightly lower than the enzyme activity on the slides in which the amino layer was created using APTES (Figure 5a). This was probably due to the lower planting density of the amino groups when using BocAPS. With the electrochemical removal of the protective groups from the slides, a significantly greater enzyme activity was observed on the cathode samples (with the protective group removed) with respect to the anode samples (with the protective group intact) (Figure 5b). It is worth noting that the activity of the anode slides was comparable to the activity of the control slides, which indicates the preservation of protective groups outside the cathode area during electrochemical deprotection.
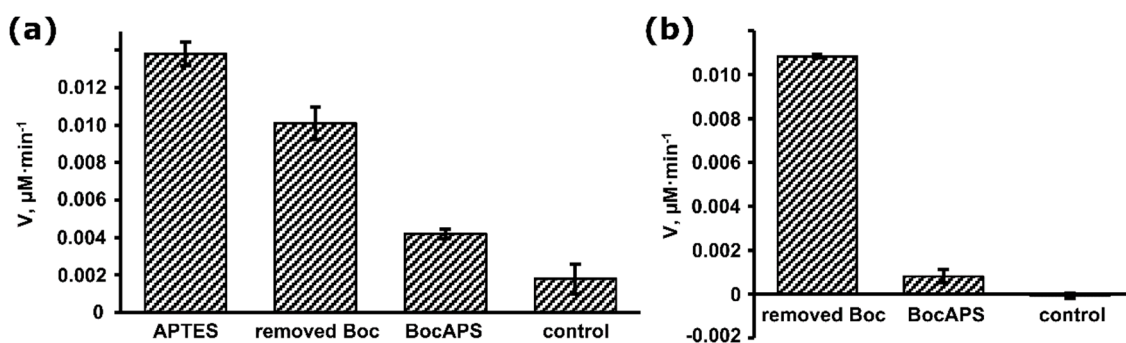
Figure 5. The activity of glucose oxidase immobilized on silicon slides using BocAPS. Chemical deprotection (**a**), electrochemical deprotection (**b**).

The fact that a silicon slide with a surface silicon dioxide layer was successfully used as a cathode for electrochemical deprotection indicates the suitability of such an approach for the removal of the Boc group from similar structures, such as ISFETs. This opens the possibility of the selective removal of the Boc group from the sensitive surfaces of an array of ISFETs for further surface modifications.

To demonstrate a practical application, BocAPS was used to create a trinitrotoluene-sensitive sensor based on an ISFET. Unlike surfaces with terminal amino groups, the modified BocAPS surface is stable over time (see Supplementary data Figure S7), and the additional technological stages required for biosensor fabrication can be carried out between BocAPS immobilization and the removal of the protection. After BocAPS was immobilized on the surface of the ISFET, a well-type epoxy structure was made on the top of the crystal (Figure 6a), which included such technological steps as applying a vaseline sacrificial layer, pouring on epoxy resin, and washing the vaseline sacrificial layer (using heating to 95 °C). Then, the Boc-group was removed electrochemically from the surface of the transistor, with the surface of the transistor acting as the cathode, and the platinum reference electrode as the anode. Next, the enzyme nitroreductase was immobilized using glutaraldehyde as a linker on the sensitive surface of the ISFET with the terminal amino groups. When the substrate (trinitrotoluene) was added to the well (at a total concentration of TNT 1×10^{-6} M), the surface potential increased compared to that in the control experiment (Figure 6b). The response of the ISFET to the enzymatic reaction showed the suitability of the obtained SAM as a linker for binding enzymes when creating sensors.
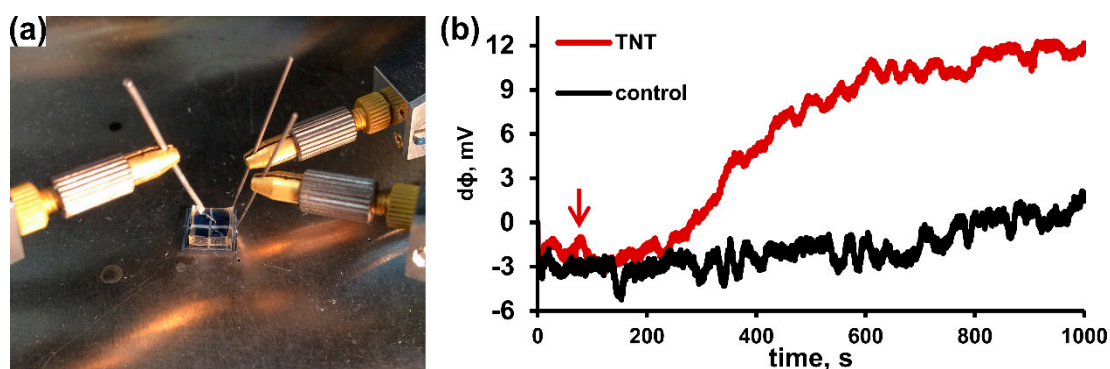
Figure 6. The ion-selective field effect transistor (ISFET) with a well-like structure during the experiments (**a**). Real-time signal of the ISFET modified with nitroreductase after the addition of trinitrotoluene (indicated by the red arrow) (**b**).

Further research will aim at creating multicomponent sensors on a single chip, which will be obtained by the sequential removal of protective groups and point modifications with various enzymes.

4. Conclusions

A new surface modifier based on aminopropylsilatrane with a protected amino group, N-*tert*-butoxycarbonylaminopropylsilatran (BocAPS), was obtained. Its synthesis is simple and can be carried out in any chemical laboratory. BocAPS has greater storage stability compared to APS and APTES, the two most common surface modifiers with a terminal amino group. The protecting group on the BocAPS-modified surface can be removed using chemical and electrochemical methods. The presence of a protective group allows the separation of the two processes of surface modification and creation of the surface amino layer and, thus, the introduction of additional technological steps; this was demonstrated in our example in which a TNT-sensitive biosensor was created. Selective removal of the protective groups on the BocAPS-modified surface can be used for further selective surface modifications with various reagents. This expands the use of BocAPS in technical design.

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