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Comparative Analysis of the Interaction of Cytochrome c with Supported Lipid Films and DNA Aptamers Using QCM-D Method †

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Abstract: Cytochrome c (cyt c) is important indicator of the cell apoptosis and can be therefore used for diagnosis of cancer. We performed comparative analysis of cyt c detection at surface of lipid films or monolayer of 11-mercaptoundecanoic acid (MUA) with immobilized specific or nonspecific DNA aptamers. The quartz crystal microbalance with dissipation monitoring (QCM-D) in a multi-harmonic mode has been used to study the interaction of cyt c with various surfaces. For this purpose, the changes of resonant frequency, Δf, and dissipation, ΔD, were determined. We have shown that the strongest interaction of cyt c has been observed with sensor based on specific DNA aptamers that was accompanied by decrease of frequency and increase of dissipation. The limit of detection (LOD) for this aptasensor has been established as 2.89 ± 0.12 nM. The interaction of cyt c with supported lipid films also resulted in decrease of resonant frequency, but its significant changes occurred only in μM concentration range of cyt c. Changes in dissipation were much lower in comparison with aptamer-based surfaces, which suggest weaker contribution of cyt c adsorption to the viscosity.

Keywords: cytochrome c; biosensor; lipid films; DNA aptamers; QCM-D

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1. Introduction.

Cytochrome c (cyt c) belongs to the most essential proteins in living organisms. This relatively small hemoprotein (molecular weight of 12 kDa, diameter 5 nm) has a significant role in electron transport in mitochondria. It is positively charged and revealed redox properties [1], which is often used in electrochemical studies [2]. Cyt c plays also important role in cell apoptosis, which is accompanied by release of cyt c from mitochondrial membrane to the cytoplasm [3,4]. Therefore, detection of cyt c can serve as suitable tool for evaluation of the effectivity of chemotherapy. Monitoring of cell apoptosis during chemotherapy can prevent not desirable damage of the healthy tissues [5,6].

Standard methods of cyt c detection such as flow cytometry, enzyme linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC) are commonly used for cyt c detection [7]. However, these methods are time consuming, require expensive instruments and well-trained personnel. Current trends for cyt c detection are focused on application of biosensors based on DNA aptamers as receptors [8]. Aptamers are relatively short single-stranded RNA or DNA (typically around 30–80 bases) that are selected by combinatorial chemistry known as SELEX (Systematic Evolution of Ligands by EXponential enrichment) [9,10]. Using this method, the DNA aptamers specific to cyt c were developed. However, because cyt c is a positively charged, interaction with the negatively charged DNA aptamers can be also non-specific due to electrostatic binding. DNA aptamers, as an alternative to more expensive and less stable antibodies [7], were

used for development of several biosensors for cyt c detection [11,12]. Recent research reports sub nanomolar limit of detection (LOD) of cyt c for electrochemical and acoustic biosensors [13].

Analysis of the mechanisms of these interactions requires certain model studies. Cyt c can be immobilized on a surface of the supported lipid films bearing negatively charge, or covalently immobilized at the monolayers of 11-mercaptoundecanoic acid (MUA) as it has been reported in our recent work [14]. Using quartz crystal microbalance with dissipation monitoring (QCM-D) we have shown that cyt c is released from the surface of lipid films following interaction with DNA aptamers. As a result, the resonant frequency increased. However, when the cyt c has been covalently attached to the surface of MUA, the frequency decreased which is evidence of the formation of aptamer binding layer at the surface. But there is still not clear how to eliminate specific and not specific binding of cyt to the negatively charged DNA aptamers and other molecules or structures such as lipid films. So far such a comparative analysis of cyt c binding has not been reported. In this work we therefore extended our studies recently published in [14] on the analysis of the changes of the resonant frequency, Δf, and dissipation, ΔD, following adsorption of cyt c at the MUA layers modified by specific or nonspecific aptamers as well as with supported lipid films and those with adsorbed model protein- bovine serum albumin.

2. Materials and Methods

2.1. Chemicals

The experiments have been performed using phosphate buffered saline (PBS) composed of 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4. We also used phosphate buffer (PB) (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) contained 2 mM $MgCl_2$. MiliQ water with a resistance of 18 MΩ.cm prepared by Purelab Classic UV (Elga, High Wycombe, UK) has been used for preparation of all aqueous solutions. The standard chemicals such as ethanol, NaCl, HNO_3, NH_3, H_2O_2, $MgCl_2$, and $CaCl_2$ were purchased from Slavus (Bratislava, Slovakia). The supported lipid membranes were prepared by liposome fusion from 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (Avanti Polar Lipids Inc., Birmingham, AL, USA) and 1,2-Dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DMPG) (Sigma Aldrich, Darmstadt, Germany). 1-dodecanethiol (DDT), 11-mercapto-1-undecanoic acid (MUA), bovine serum albumin (BSA), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) were from Sigma Aldrich (Darmstadt, Germany). DNA aptamers sensitive to cyt c were purchased from Biosearch Technologies (Risskov, Denmark). The aptamers were modified by amino group at 5′ end (NH_2-Apt-cytc) and have following sequence [15]: 5′-NH_2-TTTTTTTTTTATCGATAAGCTTCCAGAGCCGTGTCTGGGGCCGACCGGCGCATTGGGTACGTTGTTGCCGTAGAATTCCTGCAGCC-3′. 10-mer thymidine spacer at 5′ end has been included to improve conformational flexibility. We also used DNA aptamer that doesn′t specifically bind to the cyt c with following sequence: 5′-NH_2-CTGAATTGGATCTCTCTTCTTGAGCGATCTCCACA-3′. The aptamer was purchased from Generi Biotech Ltd. (Hradec Králové, Czech Republic).

2.2. Preparation of Aptamer-Based Sensing Surfaces, Supported Lipid Films and QCM-D Measurements

The lipid films and other layers were prepared on a surface of 8 MHz AT-cut quartz crystal of the working area 0.2 cm^2 consisted of thin gold layer (Total Frequency Control Ltd., Storrington, UK). Before lipid application, crystal has been incubated for 16 h at the room temperature in 2 mM 1-dodecanthiol (DDT) in ethanol. The modified crystal was placed in an acryl flow cell (JKU Linz, Austria) connected to the syringe pump (Genie Plus, Kent Scientific, Torrington, Connecticut, USA). The frequency, Δf, and dissipation, ΔD, changes were measured by computer-controlled vector analyzer Sark 110 (Seeed, Shenzhen, China). Lipid films were prepared by fusion of the liposomes. Small

unilamellar liposomes (diameter approximately 20 nm) were prepared by sonication of the lipid solution in a PB. For this purpose, 8 mg mixture of phospholipids (equimolar ratio of DMPC: DMPG) was dissolved in small volume of chloroform in a glass round shaped flask. Chloroform was then evaporated by gentle stream of nitrogen and constant circular motion of flask caused deposition of lipid film on its wall. Aliquot containing PB was then added and incubated for the 30 minutes. Incubation was followed by the 20-minute ultrasonication at the room temperature in the sonicator (Bandelin Sonorex RK31, Berlin, Germany) [15]. Final liposome solution with a concentration of 0.5 mg/mL was then applied by a flow on the surface of DDT-modified gold layer of quartz crystal. MUA layer on a quartz crystal surface was prepared by chemisorption from 2 mM MUA during 16 hours incubation. The crystals were then washed with MiliQ water and incubated for 35 minutes in 20 mM EDC and 50 mM NHS mixture to activate carboxylic group of MUA. The aptamer layers were formed by addition of 1 μM aptamers to the surface of MUA activated by NHS/EDC. This resulted in covalent immobilization of the aptamers.

Cyt c has been added in the flow mode (flow rate 50 μL/min) to the surface of lipid films or aptamer layers at various concentrations. Changes of resonant frequency, Δf, caused by adsorption of cyt c were evaluated by Sauerbrey equation [17]

$$\Delta f = \frac{-2nf_0^2}{\sqrt{\rho_q \mu_q}} \frac{\Delta m}{A} \quad (1)$$

where n represents harmonic number, f_0 is fundamental resonance frequency, Δm is mass change, A is working area of crystal (0.2 cm^2), μ_q is shear modulus of elasticity (2.947×10^{11} g·cm^{-1}·s^{-2}), and ρ_q is density of the crystal (2.648 g·cm^{-3}).

3. Results and Discussion

In the first series of experiments, we studied the changes of the frequency, Δf, and dissipation, ΔD, following addition of cyt c to the layers formed by NH_2-aptamers covalently immobilized at MUA layer. Remaining amino-reactive MUA sites were blocked by BSA. The kinetics of the frequency and dissipation changes following addition of BSA and cyt c for 3rd to 9th harmonics are shown on the Figure 1. The decrease of the resonant frequency and increase of dissipation can be seen.

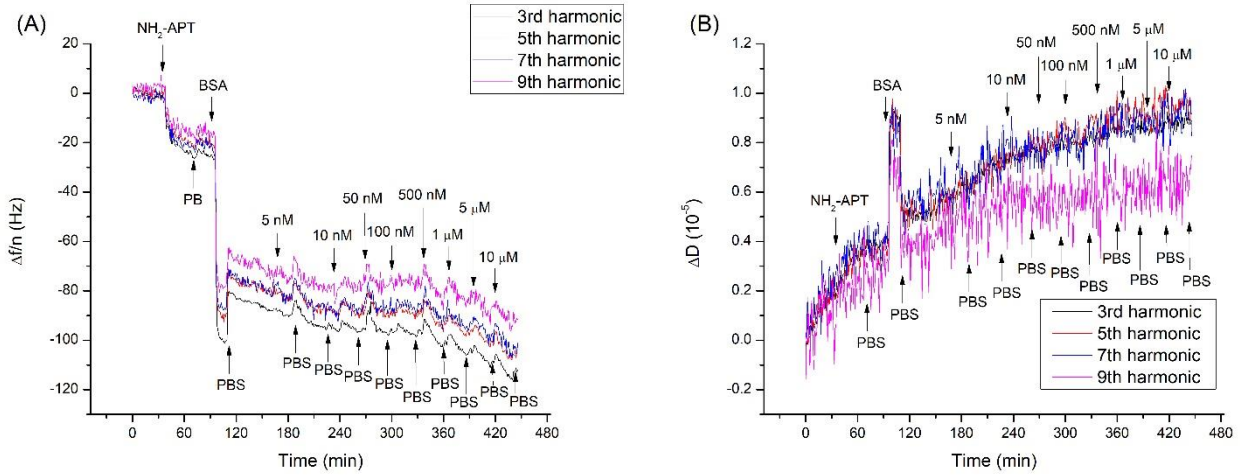

Figure 1. The kinetics of the 3rd to 9th harmonic frequencies, $\Delta f/n$, normalized by harmonic number (A) and dissipation ΔD (B) following addition of specific DNA aptamers (NH_2-APT), BSA and cyt c in a concentration range 5 nM–10 μM on MUA layer chemisorbed on the thin gold layer of piezo-crystal. The moments of addition of aptamers, BSA, cyt c, and PBS wash are shown by arrows.

We also performed similar experiments on supported lipid membranes. In the experiments we used PB contained 2 mM $MgCl_2$. The kinetics of the changes for normalized frequency and dissipation following addition of various concentrations of cyt c are shown on Figure 2.

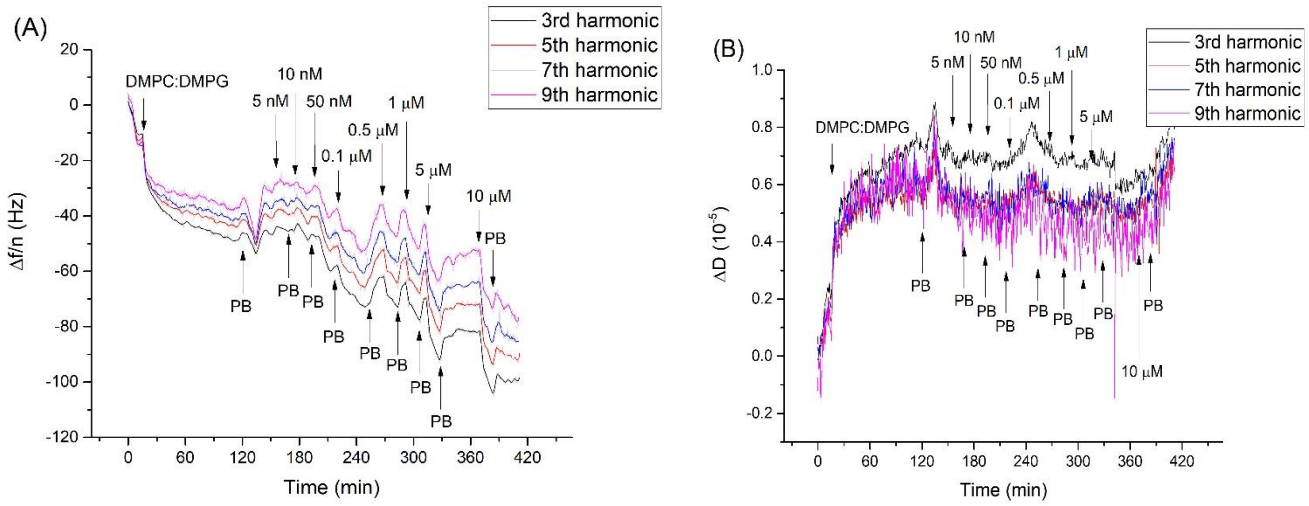

Figure 2. The kinetics of the 3rd to 9th harmonic frequencies, $\Delta f/n$, normalized by harmonic number (**A**) and dissipation ΔD (**B**) following addition of DMPC:DMPG liposomes (0.5 mg/ml), cyt c in a concentration range 5 nM–10 μM on chemisorbed layer of DDT on the gold surface of the piezocrystal. The moments of addition of liposomes, cyt c and PB wash are shown by arrows.

As it can be seen, the substantial changes of the acoustic values occurred following addition of cyt c in μM concentrations. We also tested interaction of cyt c with the surface formed by nonspecific DNA aptamers and BSA immobilized at MUA monolayer. Specific and nonspecific interactions of cyt c with the aptamers resulted in similar changes of the acoustics parameters. Much lower changes in frequency and dissipation were observed for interaction of cyt c with BSA covalently immobilized at MUA monolayer. The plot of the changes of frequency (A) and dissipation (B) vs. concentration of cyt c for various surfaces studied are shown on Figure 3. The obtained results suggest that there is the increased adsorption of cyt c on the surface formed by specific DNA aptamers. Cyt c interacted also with surfaces formed by nonspecific aptamers or BSA, but the frequency changes were lower. Estimated limit of detection (LOD) for cyt c by specific aptamers was 2.89 ± 0.12 nM. It is higher than those reported by Poturnayova et al. [18] (0.50 ± 0.05 nM). However, in this article the sensor has been formed by biotinylated aptamers attached to the neutravidin layer. It can be also seen that with increased concentration of cyt c the dissipation substantially increased for aptamer-based sensors, while practically no changes of this value occurred for lipid layers as well as for BSA. In later case, however, sharp increase of dissipation occurred at rather high cyt c concentration (10 μM). Substantial changes of dissipation for interaction of cyt c with aptamer layers can be due to influence of the cyt c on conformation and flexibility of aptamers. At the same time, cyt c at the surface of lipid films forms probably tightly packed rigid protein layer that practically does not contribute to the surface viscosity.

Using Sauerbrey equation, we also compared the surface density of cyt c at different surfaces and at the maximally applied cyt c concentrations, 10 μM. Results of calculations are presented in the Table 1. From frequency changes, we firstly calculated surface densityand then number of molecules that were adsorbed on the surface. As it can be seen the surface density of cyt c at specific aptamers has been 3.7 times higher than the surface density of these aptamers. Considering that the specific aptamer should have probably one binding site for cyt c, the larger number of adsorbed cyt c molecules can be also due

to non-specific interactions between positively charged cyt c and negatively charged aptamers. It can be also seen that the surface density of cyt c is only slightly higher for specific aptamer in comparison with non-specific one. This is additional confirmation of existence non-specific interactions between cyt c and aptamers. The adsorption of cyt c at the lipid membrane was most extensive and resulted in the cyt c surface density of (18.73 ± 2.88) × 10^{12} molecules.cm^{-2}. Considering that changes of dissipation for lipid films was rather small, one can assume that Sauerbrey equation can be more correctly used in comparison with the sensing layers based on aptamers. In later case viscosity contribution on the frequency changes can cause overestimated frequency decrease. This means that the real surface density of cyt c at such surface can be lower in comparison with those showed in Table 1.

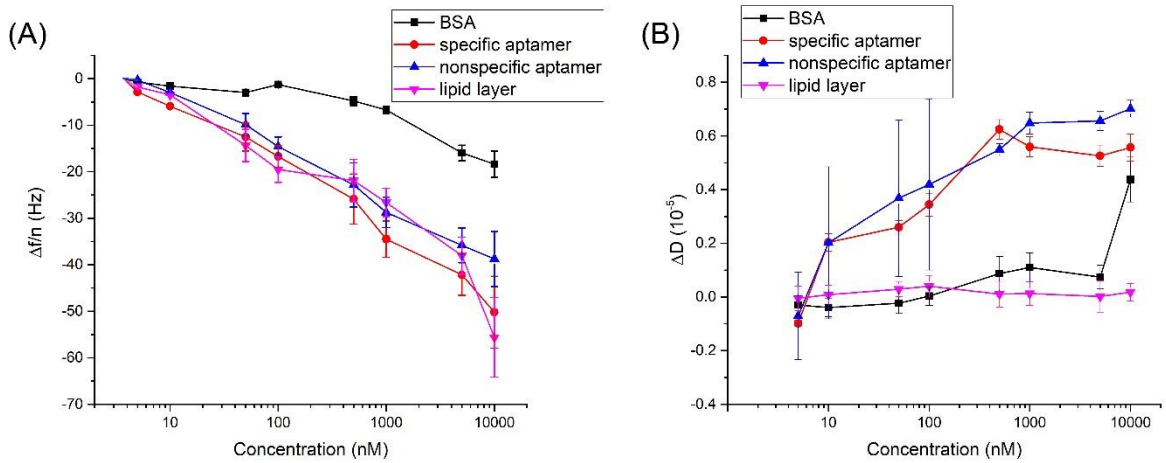

Figure 3. The plot of the changes of resonant frequency Δf/n (A) and dissipation ΔD (B) vs. cyt c concentration for surfaces based on physically adsorbed BSA on a gold surface (black), specific aptamer (red), nonspecific aptamer (blue) and on mixed lipid layer (purple). Measurements were performed in PB buffer. The results are mean±S.D. obtained at least for 3 independent measurements in each system.

Table 1. The frequency changes, Δ*f*, mass density, Δ*m*/*A*, and surface concentrations of molecules, ***σ***, calculated by Sauerbrey equation for the systems studied. For calculation we used molecular weight of BSA (66 kDa), specific aptamer (26.165 kDa) nonspecific aptamer (10.647 kDa) and cyt c (12.327 kDa). The surface density of the molecules has been calculated as mass density x (N_A/Mw), where N_A is Avogadro's number (6.02205 × 10^{23} mol^{-1} and Mw is molecular weight.

Adsorbed molecule	**Δ*f* (Hz)**	**Δ*m*/*A* (μg.cm^{-2})**	***σ*** **(10^{12}.cm^{-2})**
BSA (on MUA)	−47.67 ± 2.4	0.33 ± 0.02	3.00±0.15
Specific apt. (on MUA)	−28.75 ± 4.14	0.20 ± 0.03	4.57± 0.66
Non-specific apt. (on MUA)	−10.15 ± 4.40	0.07 ± 0. 03	3.96 ± 1.72
Cyt c (on BSA)	−18.38 ± 2.80	0.13 ± 0.19	6.20 ± 0.94
Cyt c (on specific apt.)	−50.21 ± 7.70	0.35 ± 0.05	16.92 ± 2.59
Cyt c (on non-specific apt.)	−38.75 ± 5.96	0.27 ± 0.41	13.06 ± 2.01
Cyt c (on lipid film)	−55.58 ± 8.55	0.38 ± 0.59	18.73 ± 2.88

4. Conclusion

In this study we demonstrated that cyt c interacts with both specific and non-specific DNA aptamers covalently attached to MUA layers. The surface density was, however, slightly higher for specific aptamers. The fact that cyt c interacts rather strongly with non-specific aptamers is challenging. This phenomenon requires further analysis. The mixed

DMPC/DMPG monolayers also revealed high adsorption of cyt c. This effect can be used for further study of detection of cyt c at lipid surface for example by nanowires modified by DNA aptamers.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Geng, R.; Zhao, G.; Liu, M.; Li, M. A sandwich structured SiO_2/cytochrome c/SiO_2 on a boron-doped diamond film electrode as an electrochemical nitrite biosensor. *Biomaterials* **2008**, *29*, 2794–2901.
2. Lee, T.; Kim, S.-U.; Lee, J.-H.; Min, J.; Choi, J.-W. Fabrication of nano scaled protein monolayer consisting of cytochrome c on self-assembled 11-MUA layer for bioelectronic device. *J. Nanosci. Nanotechnol*. **2009**, 9, 7136–7140.
3. Martinou, J.C.; Desagher, S.; Antonsson, B. Cytochrome c release from mitochondria: All or nothing. *Nat. Cell Biol.* **2000**, 2, 41–43.
4. Goldstein, J.C.; Waterhouse, N.J.; Juin, P.; Evan, G.I.; Green, D.R. The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nat. Cell Biol.* **2000**, 2, 156–162.
5. Eleftheriadis, T.; Pissas, G.; Liakopoulos, V.; Stefanidis, I. Cytochrome c as a potentially clinical useful marker of mitochondrial and cellular damage. *Front. Immunol.* **2016**, 7, 1–5.
6. Yadav, S.; Sawarni, N.; Kumari, P.; Sharma, M. Advancement in analytical techniques fabricated for the quantitation of cytochrome c. *Process Biochem.* **2022**, 122, 315–330.
7. Manickam, P.; Kaushik, A.; Karunakaran, C.; Bhansali, S. Recent advances in cytochrome c biosensing technologies. *Biosens. Bioelectron.* **2017**, 87, 654–668.
8. Subjakova, V.; Oravczova, V.; Hianik, T. Polymer nanoparticles and nanomotors modified by DNA/RNA aptamers and antibodies in targeted therapy of cancer. *Polymers* **2021**, 13, 341.
9. Zhou, W.; Huang, P.-J.J.; Ding, J.; Liu, J. Aptamer-based biosensors for biomedical diagnostics. *Analyst* **2014**, 139, 2627–2640.
10. He, F.; Wen, N.; Xiao, D.; Yan, J.; Xiong, H.; Cai, S.; Liu, Z.; Liu, Y. Aptamer-based targeted drug delivery systems: Current potential and challenges. *Curr. Med. Chem.* **2020**, 27, 2189–2219.
11. Jalalvand, A.R.; Akbari, V.; Bahramikia, S. Two- and multi-way analyses of cardiolipin-cytochrome c interactions and exploiting second-order advantage for bio-sensing of cytochrome c. *Sensing Bio-Sens. Res.* **2022**, 38, 100518.
12. Stepanova, V.B.; Shurpik, D.N.; Evtugyn, V.G.; Stoikov, I.I.; Evtugyn, G.A.; Osin, Y.N.; Hianik, T. Label-free electrochemical aptasensor for cytochrome c detection using pillar[5]arene bearing neutral red. *Sens. Actuators B Chem.* **2016**, 225, 57–65.
13. Poturnayova, A.; Leitner, M.; Snejdarkova, M.; Hinterdorfer, P.; Hianik, T.; Ebner, A. Molecular addressability of lipid membrane embedded calixarenes towards cytochrome c. *J. Nanomed. Nanotechnol.* **2014**, 5, 202.
14. Tatarko, M.; Spagnolo, S.; Csiba, M.; Šubjaková, V.; Hianik, T. Analysis of the interaction between DNA aptamers and cytochrome c on the surface of lipid films and on the MUA monolayer: A QCM-D study. *Biosensors.* **2023**, 13, 251.
15. Chinnapen, D.J.; Sen, D. Hemin-stimulated docking of cytochrome c to a hemin-DNA aptamer complex. *Biochemistry* **2002**, 41, 5202–5212.
16. Mirsky, V.M.; Muss, M.; Krause, C.; Wolfbeis, O.S. Capacitive approach to determine phospholipase A2 activity toward artificial and natural substrates. *Anal. Chem.* **1998**, 70, 3674–3678.
17. Sauerbrey, G. Verwendung von schwingquarzen zur wägung dünner schichten und zur mikrowägung. *Z. Phys.* **1959,** 155, 206–222.
18. Poturnayova A.; Castillo G.; Subjakova V.; Tatarko M.; Snejdarkova M.; Hianik T. Optimization of cytochrome c detection by acoustic and electrochemical methods based on aptamer sensors. *Sens. Actuators B Chem.* **2017**, 238, 817–827.