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Cellular Adhesion to Carbon Nanotubes-anchored Oligonucleotide: Electro-chemical Detection

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In the study a new biosensor contained an oligonucleotide-carbon nanotubes-stearic acid monocrystalline clusters in its sensitive coating has been developed. The electrochemical characteristics of sensors with different coatings distinguishing by content of oligonucleotide and cerium ions have been measured. It was found that the presence of Ce in the coating leads to decrease of sensor capacity changes at measurements in mediums of different content. The oligonucleotides anchored in sensor coating attenuate cell non-antisense adhesion to sensor surface.

Keywords: Oligonucleotides, Multi-walled carbon nanotubes, Electrochemical biosensor, Impedance spectroscopy

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1. INTRODUCTION

Impedance analysis of biological objects is the promising method for the development of biomedical diagnostic test systems [1]. Impedance monitoring of cell monolayer grown or adhered to nanobiosensors is a cheap, practical and non-invasive method of quantitative estimation of cell proliferation, cell-cell communication and cellular functional activity at the influence of different chemical compounds, as well as for the evaluation of their cytotoxic effects [2-5].

Nucleic acids offer some advantages, which include combinatorial encoding of interactions, easiness of synthesis and modification, as well as minimal interaction with cell membrane molecules. DNA programmed adhesion is also strong, rapidly formed and is reversible upon addition of DNase [6-7]. DNA-based attachment strategy is widely developed for the generation of artificial tissues and the incorporation of living cells into device settings.

The cells with membrane ss-DNA- or ss-oligonucleotides linking to the complimentary ss-DNA- or ss-oligonucleotide modified surface is used to direct the physical interactions between cells and substrates independent of the adhesion machinery. Cells fixed on a surface interact weakly with the substrate and cell-cell communication is insignificant. Currently, there are no developed electrochemical techniques to detect functional changes that occur in cells fixed near the surface of a sensor at their stimulation or destruction. Registration of such weak signals demands a high sensitivity of a sensor. With this in mind, the improvement of the method for express electrochemical measurement of cellular parameters is of great interest.

The purpose of the study is to develop the electrochemical impedance sensor that contains in a sensing coating the oligonucleotide sequence, anchored on modified carbon nanotubes (CNT) and to evaluate non-antisense effect of anchored CNT-oligonucleotides complexes on C6 rat glioma cells.

2. MATERIALS AND METHODS

In this work a system of electrode pairs with interdigital structure has been used. Each pair located on a glassceramics substrate represents itself an "open type" electrical capacity.

Dielectric coating of sensor aluminum electrodes represents a layer of porous anodic aluminum oxide with pores of 10 nm diameter on which five metal-contained Langmuir-Blodgett (LB) monolayers of thiophene derivatives oligomer 3-hexadecyl-2,5-di(thiophen-2-yl)-1H-pyrrole (HDTP) [8] and two LB-monolayers of multi-walled carbon nanotubes (MCNTs) were sequentially deposited. MCNT with diameters ranging from 2.5 to 20 nm and a length of 2.5 μm were covalently functionalized with carboxyl groups and non-covalently modified with stearic acid molecules [9]. The oligonucleotide ON1 (structure of the sequence: 5'-GCCATATACTCTCCTTGGTGACA-3') was synthesized at OOO Primetex (Minsk).

To obtain LB-monolayers with oligonucleotide-MCNT complexes, alcoholic solution of oligonucleotide-MCNT complexes were mixed with a solution of stearic acid in hexane. The resulting mixture was homogenized to form reverse micelles of stearic acid with oligonucleotide-MCNT complexes inside it [10].

HDTP LB-films formed on the subphase containing iron ($Fe(NO_3)_3$) and/or Ce ($Ce_2(SO_4)_3$), concentration ratio of [Ce] to [Fe] in subphase was equial to 0 or 0.01.

LB-sublayers of MCNT or MCNT-oligonucleotide complexes have been formed on subphases with or without Ce.

Measurements were performed in a cuvette with a buffer medium. Earle's balanced salt solution consisted

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of 0,12 M NaCl, 5,4 mM KCl, 0,9 mM $NaHPO_4 \cdot 2H_2O$, 0,8 mM $MgSO_4 \cdot 7H_2O$, 2,5 mM $CaCl_2$, 5,6 mM glucose, 26,2 mM $NaHCO_3$ ("Analys X", Belarus) was used.

All other reagents were of analytical grade. All aqueous solutions were made with bidistilled deionized water.

C6 rat glioma cells obtained from culture collection of Institute of Epidemiology and Microbiology (Minsk) were grown on a sensor surface in a Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum and $1 \cdot 10^{-4}$ g/ml gentamycin at 37 °C in a humidified 5 % CO_2 atmosphere.

Registration of the sensor characteristics was carried out by impedance spectroscopy. Interdigital sensors were connected into the pulse-adjustable RC-autooscillator. Electrochemical measurements were carried out in auto-oscillate regime at a frequency of quasi-resonance [11].

Microdiffraction patterns and electron microscopic images were obtained by means of transmission electron microscope JEM-100CX (JEOL, Japan) (TEM) at an accelerating voltage of 100 kV. Structural analysis have been performed on the objects previously deposited on a copper grid with a formvar polymer coating.

Visualization of the cellular monolayer was carried out using a confocal micro-Raman spectrometer Nanofinder HE («LOTIS-TII», Tokyo, Japan).

3. RESULTS AND DISCUSSION

3.1 Oligonucleotide-MCNT LB-complex structure

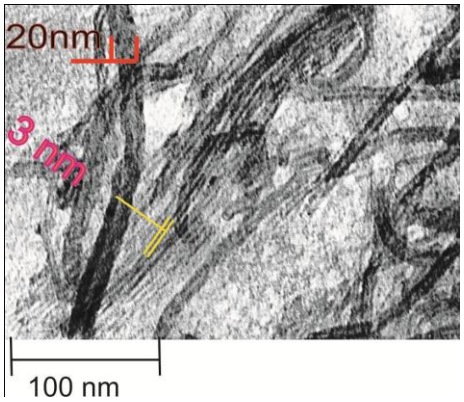
Carbon nanotubes form highly ordered LB-CNT-clusters in the LB-monolayers. According to the TEM images shown in Fig. 1a, a diameter of MCNTs in LB-cluster equals to 3 nm. Fig. 1b demonstrates that the oligonucleotide-MCNTs LB-complexes represent themself MCNTs covered close-packed layer of oligonucleotide molecules, nucleotide layer thickness is of the order of 2 to 10 nm.

Highly oriented MCNTs arrays are formed in the epitaxial growth on an iron-containing crystalline LB-films of HDTP [10]. It has been shown that the diffraction patterns of LB-nanotube clusters on the HDTP LB-films are characterized by Kikuchi lines [10] being characteristic for mono-crystallites with size of 0.5 – 1 μm.

The oligonucleotide molecules located on the MCNT in LB-film are in a highly ordered state. In the zero-node of the reciprocal lattice a diffraction pattern (Laue pattern) of the LB-monolayers consisting of oligonucleotide-MCNT-stearic acid mixture represents itself a three reflexes with the class of symmetry C2. This indicates that the non-covalently functionalized by stearic acid MCNT-oligonucleotide complexes placed on HDTP LB-films are also in the monocrystal form. The oligonucleotide crystal interplanar spacing d_p in the direction being orthogonal to the surface of MCNT is equal to 0.438 nm.

3.2 Electrochemical impedance spectroscopy

The sensors have been tested for 6 different types of coatings: 1) iron-containing HDTP LB-film and two LB-MCNT-monolayer without Ce (coating 1a) with oligonucleotide (coating 1b), 2) Fe-and Ce-containing HDTP LB-film and two LB-MCNT-monolayer without Ce (coating 2a) with oligonucleotide (the coating 2b), 3) Fe-containing HDTP LB-film and the two Ce-containing LB-MCNT-monolayer (coating 3a) with oligonucleotide (coating 3b).



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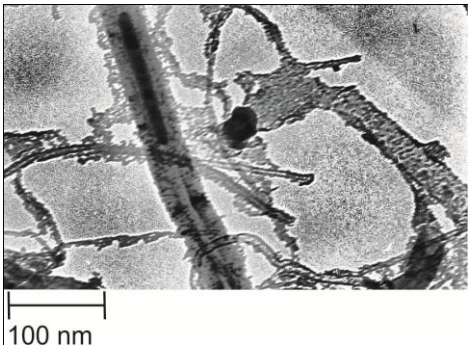


b

Fig. 1 – TEM image of two-monolayer MCNTs-stearic acid mixture LB-films formed on $Ce_2(SO_4)_3$ salt aqueous solution (a) and deposited on three HDTP LB-monolayers formed on $Fe(NO_3)_3$ salt aqueous solution with pH finished by hydrochloric acid;
(b) TEM image of two-monolayer MCNTs-oligonucleotide complexes-stearic acid mixture LB-film deposited on five-monolayer iron-containing HDTP LB-film

In bidistilled water, the dielectric properties of LB-MCNT-films and oligonucleotide-MCNT complexes in LB-films appear as a change of capacity of the double-charged Helmholtz layer which is formed at the interphase boundary between insulating barrier layer of anodic aluminium oxide and water. At low frequency range 200 – 400 kHz a sharp decrease of capacity of the sensor placed into electrolyte has been registered, which indicates the presence of a significant ionic component of the double layer capacity. At the higher frequency range (600 – 1300 kHz), the capacity of sensor varies slightly due to the large distribution of relaxation times of ions (H^+, OH^-) in the pores and small dipole relaxation times. The measurements of electrical capacity of the sensor with deposited LB-MCNT-films showed that a change of capacity of the sensors with and without oligonucleotide has opposite sign with respect to sensor without MCNTs. The capacity change for sensors with LB-MCNT-cluster coating noncontaining oligonucleotide equals to –0.8 pF in the frequency range 500 – 900 kHz, whereas for sensors with LB-MCNT-oligonucleotide-clusters, the capacity change equals to +(0.8 – 1.2) pF.

Let us estimate the sensor capacity change when the ion concentration in the medium varies. According to the Maxwell-Garnett law the capacity of sensor upon its immersion in a conducting medium increases with the increase of ion concentration in the medium. The minimal sensor capacity is observed for 0.15 M NaCl. If the sensor were immersed in Earle's medium (pH = 7.4) that does not contain $CaCl_2$, then its capacity increased by 65 ± 35 pF, 180 ± 20 pF and 90 ± 10 pF, that in the percentage ratio was 6 ± 4 %, 22 ± 5 % and 10,7 ± 0,9 % of the capacity of sensor in the Earle's medium without $CaCl_2$ for coatings type 2b, 1b and 3b, respectively. In Earle's medium with 2.5 mM $CaCl_2$ a further increase of capacity by 33 ± 10 pF (3,3 ± 1,1 %) was observed, regardless of a coating type.

For coatings that does not contain oligonucleotide, the capacity of sensor, which is in the Earle's medium, is higher by 235 ± 95 pF (21 ± 4 %) than in 0.15 M NaCl. For coating 1a, the capacity of sensor, which is in the Earle's medium, is higher by 66 ± 11 pF (6,0 ± 0,5 %) than in Earle's medium without $CaCl_2$. The corresponding recordings for sensors with coating 2a and 3a were 117 ± 27 pF (10,7 ± 0,5 %) and 80 ± 21 pF (8,2 ± 0,5 %).

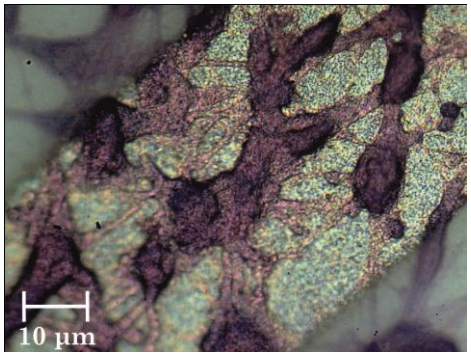
3.3 Morphology of cells in monolayer

By means of light microscopy the study of morphological characteristics of cells in a monolayer formed on the sensors on the fourth day of cell growth in culture has been performed. It is found that the number of cells on the coating 2b is less than a number of cells on the coatings 1b and 3b. As Fig.2 shows, morphology of cells depends on the coating type. On the coating 1b the population of cells is heterogeneous: there are both star-shaped cells with a high degree of flatness and having a large number of cell-cell contacts and cells being rounded and slightly flattened. On the coating 3b cells are strongly flattened and mainly elongated. Each cell has no more than three cell-cell contacts at the ends of highly elongated threadlike pseudopodia. Cells on the coating 2b are rounded, with a small area of contact to the surface, processes are short, cell-cell contacts are few.

3.4 Dielectric spectroscopy of cellular monolayer

Previously it has been shown that the formation of the cellular monolayer on the sensor surface with coatings 1a – 3a containing no oligonucleotide is accompanied by arising of a Helmholtz double layer electric field shielding. The shielding becomes apparent as sensor capacity decreasing on 1 – 3 days of cell growth and depends on monolayer density and a degree of intracellular communication [12]. It has been found that for sensors with coatings 1b – 3b containing oligonucleotide, the sensor capacity was increasing during the time of cell growth. It is stipulated by a Coulomb repulsion between negatively charged phosphate groups of oligonucleotide and negatively charged hyaluronic acid residues in cellular glycocalyx, obstructing the non-antisense cell adhesion to oligonucleotide-contained coatings.

As Fig. 3 shows, for coating 1b the sensor capacity decrement in regard to capacity of sensor without cells varies insignificantly on the second and third day of cellular growth as compared with the first day of growth.



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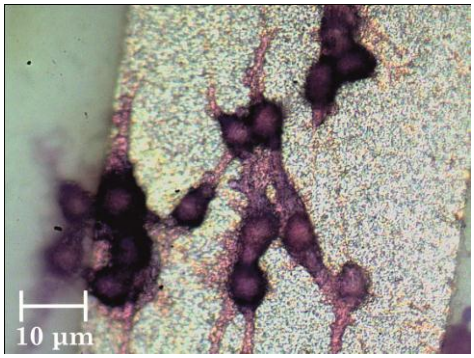

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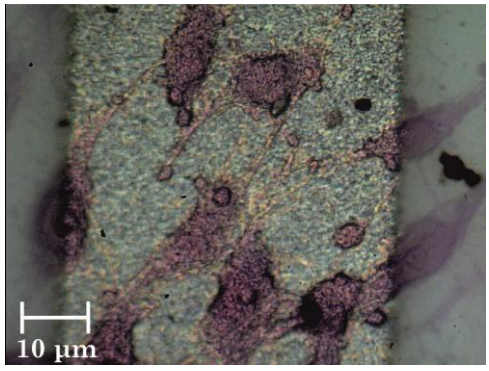

c

Fig. 2 – Light microscopy images of cells grown on coating 1b (a), coating 2b (b) and coating 3b (c)

The compensation of two processes is observed, namely the capacity increase due to the rise of the number of cells and the capacity decrease due to the shielding effect, that is enhanced at establishing of cell-cell contacts. For the coating 2b the capacity increase on the first day of cellular growth is less by 200 pF, as compared with coatings 1b and 3b. This indicates that the number of cells adhered to the coating 2b is smaller than number of cells adhered to the coatings 1b and 3b. For the coatings 2b and 3b the rise of the sensor capacity on the 2nd and 3rd day of cell growth demonstrates a low rate of intracellular contacts formation. These data agree with the data of cell morphology.

The experimental results obtained in the work are evidence of the following. First, the positively charged ions Ce^{4+} in the HDTP LB-film of coating 2b hydrolyzed oligonucleotides [13]. This is accompanied by breaking of π-π stacking interaction in oligonucleotide monocrystal following formation of double helices carrying a large negative charge. The negatively charged double-stranded oli-

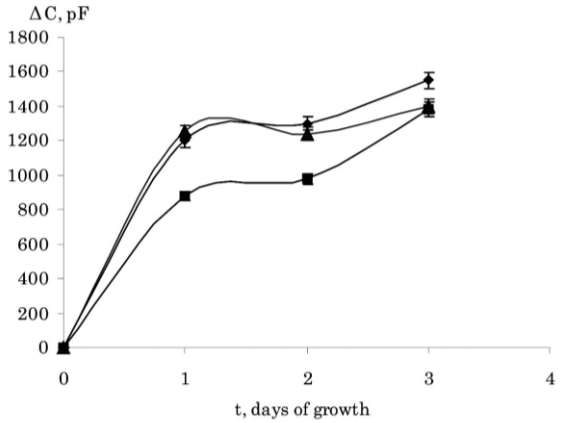

Fig. 3 – Dependency of sensor capacity decrement on cell growth time for sensor capacity with cellular monolayer. The coatings of sensor used were coating 1b (triangle), coating 2b (square), coating 3b (rhomb)

gonucleotide prevents non-specific adhesion of negatively charged cells due to strong Coulomb repulsion.

Second, oligonucleotide hydrolysis is absent for Ce^{3+}-containing MCNT-oligonucleotide clusters of coating 3b. Therefore, π-π stacking interaction in the oligonucleotide monocrystal leads to the transfer of electrical charge along the π-π coupling chain which shields the Coulomb field of the negatively charged phosphate groups.

The capacitive sensor characteristics testify a physiological state of cells in different medium. The dielectric properties of the cellular monolayer depend on the transmembrane potential of cells and are determined by the state of ion channels. It was revealed that the capacity of sensors with cellular monolayer depends on the composition of the measuring medium. It is shown that the capacity of sensor with cellular monolayer increases as follows: DMEM <Earle's medium (pH = 7.4) <Earle's medium (pH = 7.4) without 2.5 mM $CaCl_2$ <0.15 M NaCl. The capacity increases by 150 ± 62 pF (4,3 ± 1,1 %) for coatings 2b and 3b, and by 300 ± 50 pF (9 ± 2 %) for coating 1a when cellular monolayer is transfered from Earle's medium to Earle's medium without $CaCl_2$. Capacity of sensors with cellular monolayer formed on the surfaces that do not contain oligonucleotide, increases by 14 ± 4 % for coatings 1a and 2a at changing the Earle's medium on the Earle's medium without $CaCl_2$.

4. CONCLUSIONS

Thus, a nanobiosensor having an oligonucleotide-MCNT clusters layer in its sensitive coating has been developed. The sensor has a high-ordered monocrystalline structure of LB-MCNT-clusters and a close-packed oligonucleotide layer. Such type of oligonucleotide deposition is promising for high-sensitive detection of antisense oligonucleotide-bonded biological objects by means of electrochemical analysis due to the distance to the object must be about pair nucleotides size.

The oligonucleotide anchored in sensor coating attenuates cell non-antisense adhesion to sensor surface resulting in capacity increasing during cell growth. The presence of Ce in the sensor coating leads to prevent non-specific cellular adhesion.

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REFERENCES

1. T. Henning, M. Brischwein, W. Baumann R. Ehret, I. Freund, R. Kammerer, M. Lehmann, A. Schwinde, B. Wolf , *Anti-Cancer Drugs* **12**, 21 (2001).
2. L. Ceriotti, J. Ponti, P. Colpo, E. Sabbioni, F. Rossi, *Biosens. Bioelectron.* **22**, 3057 (2007).
3. J.H. Yeon, J.K. Park, *Anal. Biochem.* **341**, 308 (2005).
4. C. Xiao, J. Luong, *Biotechnol. Prog.* **19**, 1000 (2003).
5. R. Ehret, W. Baumann, M. Brischwein, A. Schwinde, B. Wolf, *Med. Biol. Eng. Comput.* **36**, 365 (1998).
6. N.S. Selden, M.E. Todhunter, N.Y. Jee, J.S. Lui, K.E. Broaders, Z.J. Gartner, *J. Am. Chem. Soc.* **134**, 765 (2012).
7. S.C. Hsiao, B.J. Shum, H. Onoe, E.S. Douglas, Z.J. Gartner, R.A. Mathies, C.R. Bertozzi, M.B. Francis, *Langmuir* **25(12)**, 6985 (2009).
8. A.V. Kel'in, O.G. Kulinkovich, *Folia Pharm. Univ. Carol.* **18**, 96 (1995).
9. H.V. Grushevskaya, V.V. Hrushevsky, N.G. Krylova, T.A. Kulahava, I.V. Lipnevich, T.I. Orekhovskaya, G.N. Semenkova, B.G. Shulitsky, *J. Nonlin. Phen. Com. Sys.* **13**, 396 (2010).
10. A. S. Egorov, H. V. Krylova, I. V. Lipnevich, B. G. Shulitsky, L. V. Baran, S. V. Gusakova, and M. I. Govorov, *J. Nonlin.Phen. in Complex Sys.* **15** No2, 121- (2012).
11. I.I. Abramov, V.V. Hrushevsky, G.G. Krylov, H.V. Krylova, I.V. Lipnevich, T.I. Orekhovskaya, *Peterburg journal of electonics (in Russian)* **4(73)**, 59 (2012).
12. N.G. Krylova, H.V. Grushevskaya, I.V. Lipnevich, T.A. Kulahava, , T.I. Orekhovskaya, B.G. Shulitsky, *Materials of international conference "Molecular, membrane and cellular foundations of biosystems function"* (June 19-21, 2012, Minsk, Belarus) **2**, 244 (2012).
13. S.J. Franklin, *Curr. Opin. Chem. Biol.* **5**, 201 (2001).