00

Chemiluminescence of a Functionalized Graphene Surface

© A.Yu. Plekhanov[1,2], M.V. Puzyk[3]¶, A.S. Usikov[4,5], A.D. Roenkov[6], A.A. Lebedev[5], S.P. Lebedev[7], S.A. Klotchenko[7], A.V. Vasin[7], Yu.N. Makarov[4]

[1] St. Petersburg Nuclear Physics Institute, National Research Center „Kurchatov Institute“, 188300 Gatchina, Russia
[2] Institute of Macromolecular Compounds, Russian Academy of Sciences, 199004 St. Petersburg, Russia
[3] Herzen State Pedagogical University of Russia, 191186 St. Petersburg, Russia
[4] Nitride Crystals Inc., 9702 Richmond, USA
[5] Ioffe Institute of RAS, 194021 St. Petersburg, Russia
[6] Nitride Crystals Group, 194156 St. Petersburg, Russia
[7] Peter the Great Saint-Petersburg Polytechnic University, 195251 St. Petersburg, Russia
¶ e-mail: puzyk@mail.ru

Received April 28, 2022
Revised June 3, 2022
Accepted June 16, 2022

To analyze the modification of the functionalized surface of graphene by protein molecules, a chemiluminescent enzyme-linked immunosorbent assay based method was proposed. Using the example of functionalized graphene (FG) purification, the possibilities of chemiluminescent control of the state of its surface are shown. Methods for purifying FG from protein molecules with the restoration of the ability to resorb protein molecules are discussed. It has been shown that the FG surface can be cleaned from sorbed proteins, and a biosensor can be designed again based on such purified graphene, including with a different specificity. Therefore, the graphene sensor can be used repeatedly.

Keywords: functionalized graphene, 1-pyrenemethylamine hydrochloride, cyclic voltammetry, chemiluminescence, monoclonal antibodies, biosensors

DOI: 10.21883/EOS.2022.09.54835.3628-22

Introduction

At present, the development and improvement of methods for obtaining and studying graphene, one of the widely studied carbon materials, is an important task. One atom-thick developed surface, chemical and mechanical resistance, high electrical and thermal conductivity, the ability to easily functionalize the surface with various molecules, good biocompatibility of graphene materials — all this attracts specialists from various fields of science and technology. The range of studies of graphene materials is very wide: supercapacitors [1,2], CO_2 [3] utilization systems, water purification [4], catalysis [5,6], nanocarriers for drug delivery [7], sensors [8–12].

The electrical conductivity of graphene changes significantly when other molecules are attached to its surface. The operation of graphene sensors is based on this principle. However, the graphene film itself is not a selectively sensitive sensor, since it easily sorbs various molecules on its surface. When creating a biosensor in order to increase the avidity and selectivity of graphene with respect to protein molecules, its surface is functionalized with active amino groups [13,14], on which protein molecules (antigens and antibodies) are then anchored. Antibodies attached to the surface of graphene can be detected, in particular, with a quantum dot-labeled secondary antibody using confocal fluorescence microscopy [15].

The attachment of biologically active molecules, such as antigens or antibodies, to the surface of graphene makes it possible to create biosensors capable of selectively detecting biological molecules in various media, which can be applied in medical diagnostics. Note that the antibody-antigen interaction is of fundamental importance for the functioning of the human immune system [16]. This reaction of specific binding of antigens to their corresponding antibodies leads to the formation of an immune complex. In other words, the antibody-antigen interaction is carried out according to the key-lock principle of three-dimensional spatial complementarity of the external electron clouds of the antibody and antigen molecules. *In vitro* these reactions form the basis of many immunological methods and are widely used in laboratory practice.

Our studies of graphene biosensors are based on the universal concept of controlled interaction of an antibody

with a specific antigen protein on the surface of graphene grown on SiC substrates. Only complementary antibodies and antigens take part in the interaction reaction, which ensures the selectivity and high sensitivity of the biosensor.

In our experiments on the sorption of fluorescein and monoclonal antibodies on functionalized graphene (FG), a change in electrical conductivity was recorded at a concentration of test molecules $1 \cdot (10^{-3} - 10^{4})$ ng/ml [17]. Separate experiments were carried out to determine group and other blood antigens [11,14,18]. We also studied sensors for detecting influenza viruses *A* and *B* at their concentration ~ 100 pg/ml for less than 1 min [19]. The studies performed have shown the need for repeated use of the same graphene sensors (chips) to optimize the modes of graphene surface treatment and detection.

In this paper, we describe the application of the chemiluminescent method for the detection of protein molecules on the FG surface and for monitoring the cleaning of the graphene surface after biochemical studies. Graphene films grown on SiC substrates were used as initial samples.

Experimental part

To create biosensors, we obtained graphene by sublimation of the surface of semi-insulating substrates SiC (0001) $\pm 0.25^{\circ}$ with the size of 11×11 mm [12]. The process of growing graphene was carried out in a graphite crucible inductively heated by a high-frequency generator in an argon atmosphere (720–750 Torr) at a temperature of $\sim 1750^{\circ}$C. The presence of a graphene monolayer on the SiC substrate was confirmed by Raman spectra [20]. The size of the graphene region in contact with the solution in the described experiments was about 5×5 mm.

The surface quality of the obtained graphene film was evaluated by cyclic voltammetry. Electrochemical studies were carried out on a potentiostat-galvanostat PI-50-*Pro*, (Elins, St. Petersburg, Russia) in a three-electrode cell in 96 wt.% ethanol solution (concentration of background electrolyte — 0.1 mol/l NH_4BF_4) at room temperature. The working electrode was a graphene/SiC chip, a reference electrode — a saturated silver chloride electrode, and an auxiliary electrode — a platinum plate. 96 wt.% ethanol containing 4 wt.% water was chosen as an electrolyte in order to observe the behavior of graphene material in the field of water oxidation and reduction during research.

All further operations, unless otherwise indicated, were performed at room temperature.

Three selected 5×5 mm graphene/SiC chips with good surface quality were functionalized with amino groups by a method based on sorption of pyrene derivatives from a solution [21]. Functionalization of the graphene/SiC chip was carried out by placing the chip in a 1 wt.% aqueous solution of 1-pyrenemethylamine hydrochloride for 20 min. Further the chip was washed with running tap water (1 min).

Monoclonal antibodies against influenza virus *A* nucleoprotein (4*H*1 clone produced by Smoroditsev Research Institute of Influenza of the Ministry of Health of the Russian Federation, St. Petersburg, hereinafter „primary antibodies“) were used as test protein molecules. Immobilization (sorption) of antibodies on graphene was carried out according to the following procedure. Graphene chips were kept in a solution of antibodies with a concentration in 250 μg/ml antibodies solution in phosphate-buffered saline (PBS) for 60 min, followed by rinsing with running tap water (0.5 min), then once in PBS (5 min). The excess sorption capacity of FG was exhausted by soaking in ultrapasteurized milk with a fat content in 1,5 wt% fat ultrapasteurized milk („Petmol“, St. Petersburg) (20 min) [22].

Further the graphene chips were kept in a solution of goat antibodies against mouse *G* immunoglobulins labeled with horseradish peroxidase labeled goat antibodies against mouse immunoglobulin G (hereinafter „labeled secondary antibodies“) (BioRad, 1 : 3000, 20 min) followed by rinsing with running tap water (30 s), then once — in PBS (5 min).

The peroxidase label on the thus prepared graphene surface was detected using a solution containing luminol, a chemiluminescence enhancer, and hydrogen peroxide (BioRad ECL). For that purpose, a 10 μl drop of that solution was applied to the graphene chip. Chemiluminescence was detected using Chemidoc device (BioRad). A $\sim 1 \times 1$ cm nitrocellulose membrane was used as a witness (chemiluminescent label).

Desorption of proteins from graphene (hereinafter, washing or purification of graphene) was carried out in three ways:

1) washing with a detergent, as which sodium dodecyl sulfate (1 wt.%) was used, dissolved in tris-glycine buffer (25 mmol/l tris — 250 mmol/l glycine, pH 8.3) at 50°C for 30 min;

2) washing with an aqueous solution of alkali: 1 mol/l NaOH;

3) enzymatic destruction of proteins. Subtilisin (Avizor proteolytic tablets for contact lenses at the manufacturer's recommended concentration of 1 tablet per 5 ml of PBS solution) was used as a proteolytic enzyme. Graphene chips with immobilized proteins were placed in the subtilisin solution for 15 min at room temperature and then for 15 min at 50°C.

After soaking in these solutions, the washed graphene chips were rinsed for 0.5 min in running tap water and kept in PBS for 5 min, followed by detection of residual peroxidase activity, as described above.

The washed graphene surface was tested for the ability to bind protein molecules. Labeled secondary antibodies were now used as a test molecule. Graphene chips were kept in a solution of labeled secondary antibodies (1 : 3000 in PBS) for 60 min, followed by rinsing with running tap water (0.5 min), then once in PBS (5 min). The peroxidase label on the thus prepared graphene surface was detected as described above.

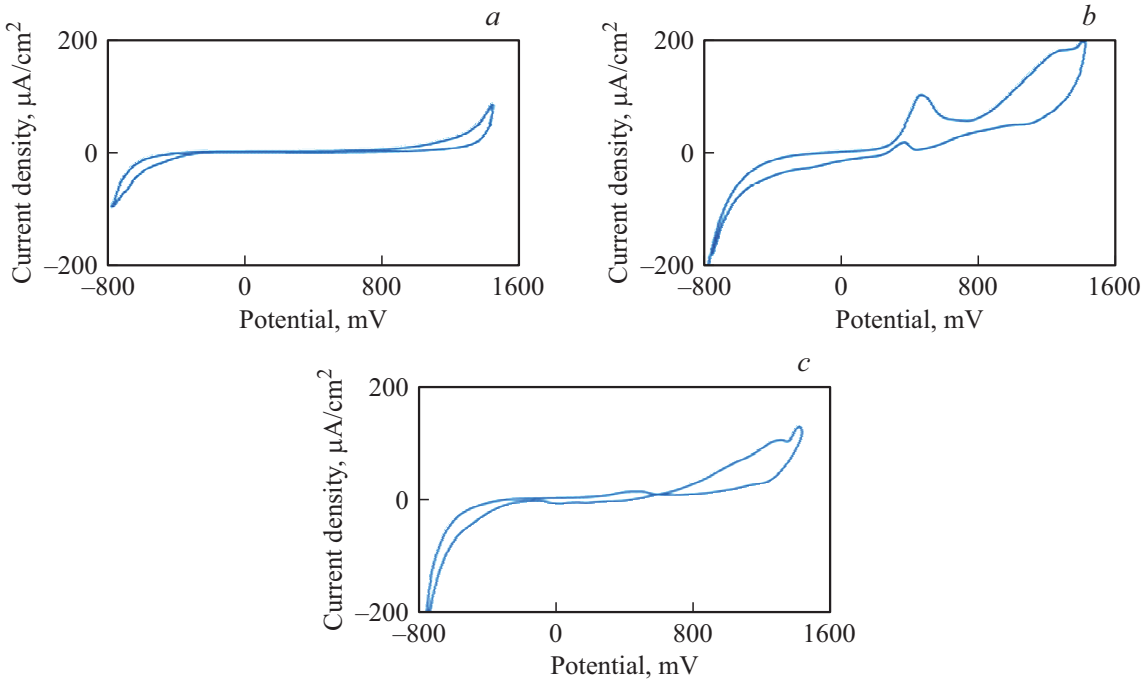

Figure 1. Cyclic voltammograms of graphene/SiC electrodes; potential sweep rate 50 mV/s. The potential values are given with respect to the silver chloride electrode. *a* — high-quality graphene *b, c* — low-quality graphene.

The spectrum and dynamics of luminol chemiluminescence of luminol were determined in an extemporaneously prepared solution containing 0.012 wt.% luminol, 0.005 wt.% p-coumaric acid, 5 wt.% dimethyl sulfoxide, and 0.003 wt.% H_2O_2 in 0.1 mol/l tris-HCl (pH 8.8). For determination of chemiluminescence spectrum in solution, 1 μl wt.% of horseradish peroxidase aqueous solution (Sigma) was added to 1 ml of the solution above immediately before the measurement. For determination of chemiluminescence spectrum on solid surface, 1 μl 1 wt.% peroxidase solution was applied to $\sim 1 \times 1$ cm nitrocellulose membrane, which was then covered with 50 μl of the above developing solution. The measurements were carried out using „Fluorat-02-Panorama“ spectrofluorimeter (GK Lumex, St. Petersburg).

Results and discussion

The nature of graphene is similar to grafite, i.e. consists of sp^2-hybridised carbon connected in a two-dimensional hexagonal lattice [23], and therefore should have a wide region of ideal polarizability. Figure 1 shows typical cyclic voltammograms (CVAG) of graphene/SiC chips in the supporting electrolyte for three samples. In the potential region less than -100 mV and more than $+1100$ mV, the processes of water reduction and oxidation are observed, respectively. A decrease in the width of the polarizability region and the presence of additional peaks in the CVAG in this potential region (Fig. 1, *b, c*) indicate shortcomings associated either with graphene itself or with the manufacture of graphene/SiC chips : low-quality graphene film in the chip. Therefore, graphene/SiC chips with a wide region of ideal polarization (~ 1200 mV) were selected for further experiments (Fig. 1, *a*).

The selected samples of graphene were then functionalized and incubated in test protein solution molecules. However, in our experiments, we encountered significant difficulties in trying to identify and characterize the functionalization of graphene and/or the sorption of protein molecules by electron microscopy (scanning electron microscope Tescan MIRA-3). To resolve this difficulty, we have developed a chemiluminescent enzyme-linked immunosorbent assay based method for detecting protein molecules on graphene surface

The spectrum of luminol chemiluminescencein solution is shown in Fig. 2, *a*. The spectrum of luminol chemiluminescence catalyzed by horseradish peroxidase immobilized on a solid substrate (1 μg of horseradish peroxidase applied to $\sim 1 \times 1$ cm nitrocellulose membrane in 1 μl of water, developed in 10 μl of BioRad ECL solution) is shown in Fig. 2, *b*. A certain hypsochromic shift of the chemiluminescence spectrum is noticeable in the case of the enzyme immobilized on nitrocellulose compared to the enzyme in solution, apparently due to the phenomenon of solvatochromism.

The combination of low concentration of peroxidase on the FG surface and low sensitivity of the spectrofluorimeter did not allow to record chemiluminescence spectra on the graphene surface. Therefore, FG chemiluminescence experiments were performed using a more sensitive Chemi-

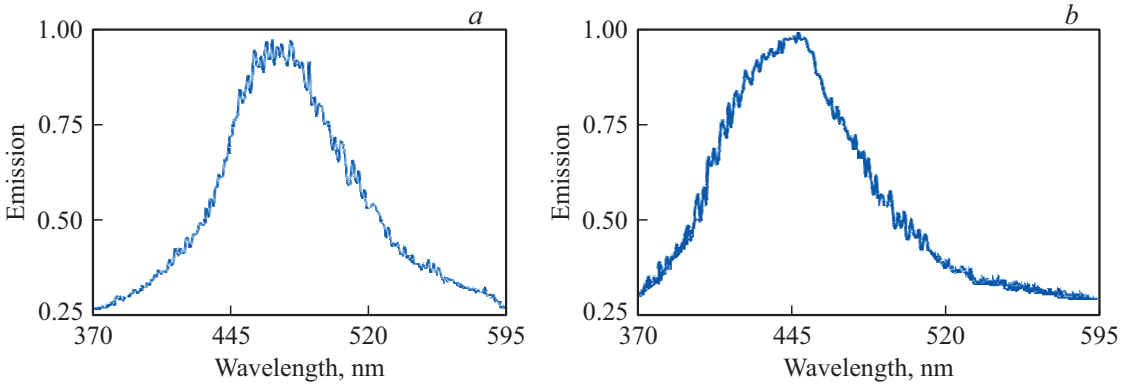

Figure 2. Chemiluminescence spectra of luminol in the solution (*a*) and on nitrocellulose (*b*).

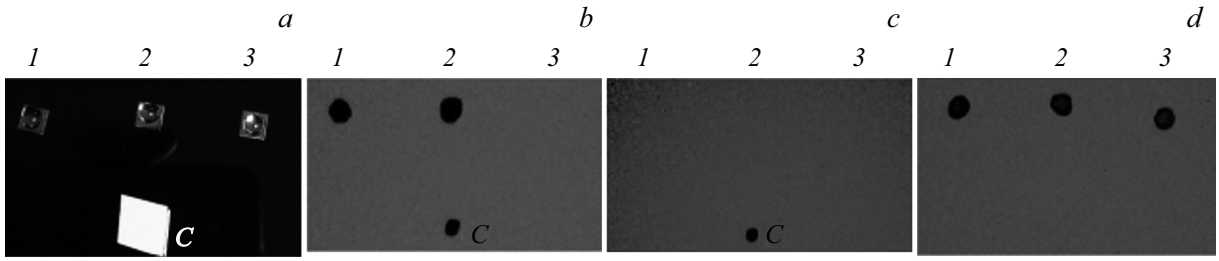

Figure 3. Photographs of FG surface chemiluminescence experiment. Numbers above *1, 2, 3* — graphene samples, below them is C — witness with 1 μl of labeled secondary antibodies on nitrocellulose membrane), in Fig. 3, *d* there was no witness. (*a*) Visible light photograph of graphene chips with droplets of a developing solution droplets and the witness. (*b*) Chemiluminescence. *1, 2* — samples are incubated in primary antibodies, then in milk, then in labeled secondary antibodies; *3* — the sample was incubated in milk, then in labeled secondary antibodies. (*c*) Chemiluminescence. *1* — washing with 1 wt.% SDS; *2* — washing with 1 mol/l NaOH; *3* — washing with subtilisin. (*d*) Chemiluminescence. After washing, all FG samples were incubated in labeled secondary antibodies.

Doc instrument (BioRad). The sensitivity of the latter also allowed the use of peroxidase at a much lower concentration. In particular, we used labeled secondary antibodies in a dilution typical for routine immunochemical studies. The results of experiments on chemiluminescence of graphene surface are shown in Fig. 3.

Fig. 3 shows that with modern methods of optical detection of chemiluminescence, enzyme-linked immunosorbent assay (ELISA) can be used as a method for analyzing the functionalization of graphene. Indeed, in our case, ELISA allows to recognize FG with primary antibodies (Fig. 3, *b*, 1, 2). With high specificity: in Fig. 3, *b*, chemiluminescence of the third graphene chip (3) is not registered (because primary antibodies on the sample of Fig. 3, *b*, 3 were not applied, and proteins adsorbed from milk make the FG surface inaccessible to labeled secondary antibodies).

The FG surface is an aromatic (hydrophobic) matrix containing amino groups in our case. These structures can interact with protein molecules in various ways. Thus, ionic interactions can occur between the amino groups of FG and the carboxyl groups of Glu and Asp in the structure of the protein; the same amino groups of FG can form hydrogen bonds with the hydroxyl groups Ser, Tre, Tyr in the structure of the protein molecule. In addition, the aromatic groups Phe, Tyr, Tri and the developed aliphatic groups Val, Leu, and Ile can enter in hydrophobic interactions with the aromatic surface of graphene.

Thus, three methods can be proposed for cleaning (washing) FG from protein molecules immobilized on it:

1) ionic detergent cleaning: 1 wt.% solution of sodium dodecyl sulfate (SDS), which destroys hydrophobic interactions;

2) alkali cleaning, in particular, 1 mol/l NaOH. In this case, the destruction of ionic bonds occurs; in addition, under strongly alkaline conditions, the solubility of proteins increases and their partial hydrolysis occurs;

3) enzymatic degradation of proteins by proteolytic enzymes such as subtilisin.

It can be seen in Fig. 3, *c* that, after washing by three methods, no chemiluminescence occurs on any of the graphene chips. Strictly speaking, Fig. 3, *c* only implies that peroxidase is damaged on chips 1 and 2. If graphene is truly free of protein molecules, it will restore its protein-binding activity.

Indeed, Fig. 3, *d* shows that after treatment with 1 wt.% SDS, 1 mol/l NaOH, and subtilisin, FG is again capable of sorbing secondary labeled antibodies, which can be seen by chemiluminescence. Note that the third FG sample (Fig. 3, *d*, 3) also restored its sorption capacity after cleaning

(compare the chemiluminescence in Fig. 3, *d* and Fig. 3 , *b*, 3), since milk proteins were washed out from it.

Conclusion

In this work, we observed the chemiluminescence on FG surface. We have shown that chemiluminescent enzyme-linked immunosorbent assay of the FG surface is a useful alternative to electron microscopy in biosensor design. In particular, the simplicity of enzyme-linked immunosorbent assay makes it easy to select the conditions for antibody binding to FG when creating biosensors. It should be noted that labeled secondary antibodies capable of catalyzing chemiluminescence have been used in biosensors [19] before, but only to enhance the electrical response.

We also proposed three methods for purifying FG from protein molecules with the restoration of the ability of FG to sorb protein molecules. This observation has a specific practical outcome, namely, it opens up the possibility of multiple use of graphene biosensors. Indeed, the working element of a used biosensor is a FG surface contaminated with proteins (antibodies and antigens, sometimes secondary antibodies and ballast proteins, such as serum albumin or milk proteins). As we have shown here, the FG surface can be washed away from these proteins. And on the basis of such purified graphene, a biosensor can be designed again, including with a different specificity. Considering the multi-stage production (including, for example, deposition of gold contacts) and the significant laboriousness of manufacturing graphene biosensors, the possibility of their repeated use contributes to the rapid optimization of graphene surface processing and detection modes.

Funding

The work was partially funded by the Ministry of Science and Higher Education of the Russian Federation under the Strategic Academic Leadership Program „Priority-2030" (Agreement 075-15-2021-1333 of 09/30/2021), as well as within the framework of the Russian Science Foundation project 22-12-00134 .

Conflict of interest

The authors declare that they have no conflict of interest.

References

[1] X. Fan, B.D. Phebus, L. Li, S. Chen. Sci. Adv. Mat., **7**, 1916 (2015). DOI: 10.1039/C000417K
[2] L.Q. Xu, Y.L. Liu, K.-G. Neoh, E.-T. Kang, G.D. Fu. Macromol. Rapid Commun., **32**, 684 (2011). DOI: 10.1002/marc.201000765
[3] M.-Q. Yang, Yi-J. Xu. Nanoscale Horizons, **1**, 185 (2016). DOI: 10.1039/C5NH00113G
[4] R. Sharma, P. Saini. Diamond and Carbon Composites and Nanocomposites, **2**, 21 (2016). DOI: 10.5772/63666
[5] N. Zhang, M.Q. Yang, S. Liu, Y. Sun, Y.J. Xu. Chem. Rev., **115**, 10307 (2015). DOI: 10.1021/acs.chemrev.5b00267
[6] M.Q. Yang, Y.J. Xu, Phys. Chem. Chem. Phys., **15**, 19102 (2013). DOI: 10.1039/c3cp53325e
[7] J. Liu, L. Cui, D. Losic. Acta Biomater., **9**, 9243 (2013). DOI: 10.1016/j.actbio.2013.08.016
[8] L.Q. Xu, Y.L. Liu, K.G. Neoh, E.T. Kang, G.D. Fu. Macromol. Rapid Commun., **32**, 684 (2011). DOI: 10.1002/marc.201000765
[9] A.D. Smith, K. Elgammal, F. Niklaus, A. Delin, A.C. Fischer, S. Vaziri, F. Forsberg, M. R.sander, H. Hugosson, L. Bergqvist, S. Schröder, S. Kataria, M. .stlinga, M.C. Lemme. Nanoscale, **7**, 19099 (2015). DOI: 10.1039/C5NR06038A
[10] A.A. Lebedev, V.Y. Davydov, C.N. Novikov, D.P. Litvin, Y.N. Makarov, V.B. Klimovich., M.P. Samoilovich. Technical Physics Letters, **42** (7), 729 (2016). DOI: 10.1134/S1063785016070233.
[11] S. Lebedev, A. Usikov, S. Novikov, E. Shabunina, N. Schmidt, I. Barash, A. Roenkov, A. Lebedev, Y. Makarov. Materials Science Forum, **924**, 909 (2018). DOI: 10.4028/www.scientific.net/MSF.924.909
[12] A.A. Lebedev, S.P. Lebedev, S.N. Novikov, V.Y. Davydov, A.N. Smirnov, D.P. Litvin, Y.N. Makarov, V.S. Levitskii. Technical Physics, **61** (3), 453 (2016). DOI: 10.1134/S1063784216030130.
[13] V. Georgakilas, M. Otyepka, A.B. Bourlinos, V. Chandra, N. Kim, K.C. Kemp, P. Hobza, R. Zboril, K.S. Kim. Chem. Rev., **112**, 6156 (2012). DOI: 10.1021/cr3000412
[14] A. Usikov, K. Borodkin, S. Novikov, A. Roenkov, A. Goryachkin, M. Puzyk, I. Barash, S. Lebedev, A. Zubov, Y. Makarov, A. Lebedev. Proc. Estonian Acad. Sci., **68**, 207 (2019). DOI: 10.3176/proc.2019.2.13
[15] Z. Tehrani, G. Burwell, M.A. Mohd Azmi, A. Castaing, R. Rickman, J. Almarashi, P. Dunstan, A. Miran Beigi, S. H. Doak, O.J. Guy. 2D Mater. **1**, 025004 (2014). DOI: 10.1088/2053-1583/1/2/025004
[16] R. D. Davies, E.A. Padlan, S. Sheriff. Annual Review of Biochemistry, **59**, 439 (1990). DOI: 10.1146/annurev.biochem.59.1.439
[17] A.S. Usikov, S.P. Lebedev, A.D. Royenkov, I.S. Barash, S.V.Novikov, M.V. Puzyhk A.V. Zubov, Yu..N.. Makarov, A.A. Lebedev. Pisma v ZhTF, **46** (10), 3 (2020) (in Russian). DOI: 10.21883/PJTF.2020.10.49421.18250
[18] A. Usikov, M. Puzyk, S. Novikov, I. Barash, O. Medvedev, A. Roenkov, A. Goryachkin, S.P. Lebedev, A. Zubov, Y. Makarov, A.A. Lebedev. Key Engineering Materials, **799**, 197 (2019). DOI: 10.4028/www.scientific.net/KEM.799.197
[19] A.A. Lebedev, S.Y. Davydov, I.A. Eliseyev, A.D. Roenkov, O. Avdeev, S.P. Lebedev, Y. Makarov, M. Puzyk, S. Klotchenko, A.S. Usikov. Materials, **14**, 590 (2021). DOI: 10.3390/ma14030590)
[20] V.Y. Davydov, D.Y. Usachov, S.P. Lebedev, A.N. Smirnov, V.S. Levitskii, I.A. Eliseyev, P.A. Alekseev, M.C. Dunaevskiy, O.Y. Vilkov, A.G. Rybkin, A.A. Lebedev. Semiconductors, **51** (8), 1072 (2017). DOI: 10.21883/FTP.2017.08.44800.8559.
[21] R.M. Torrente-Rodr.guez, H. Lukas, J. Tu, J. Min, Y. Yang, C. Xu, H.B. Rossiter, W. Gao. Matter., **3** (6), 1981 (2020). DOI: 10.1016/j.matt.2020.09.027
[22] A.Y. Plekhanov. Analytical Biochemistry, **239** (1), 110 (1996). DOI: 10.1006/abio.1996.0298
[23] F. Schedin, A.K. Geim, S.V. Morozov, E.W. Hill, P. Blake, M.I. Katsnelson, K.S. Novoselov. Nature Mater., **6**, 652 (2007). DOI: 10.1038/nmat1967