MATERIALS
ENGINEERING

Graphene/SiC dies for electrochemical blood-type sensing

Alexander Usikov[a,b*], Konstantin Borodkin[c], Sergey Novikov[d], Alexander Roenkov[c], Andrei Goryachkin[c],
Mikhail Puzyk[e], Iosif Barash[c], Sergey Lebedev[f], Alexander Zubov[b],
Yuri Makarov[a], and Alexander Lebedev[f]

[a] Nitride Crystals Inc., 9702 Gayton Road, Suite 320, Richmond, VA 23238, USA
[b] ITMO University, Kronverkskiy pr. 49, 197101 St. Petersburg, Russia
[c] Nitride Crystals Group Ltd., Engel'sa pr. 27, 194156 St. Petersburg, Russia
[d] Aalto University, Micronova, Tietotie 3, FI-02150, Espoo, Finland
[e] Herzen University, Nab. r. Moyki 48, 194186 St. Petersburg, Russia
[f] Ioffe Institute, Politekhnicheskaya 26, 194021 St. Petersburg, Russia

Abstract. We discuss graphene-on-SiC dies for blood-type sensing. For the sensor application, chemical species to be detected
adsorb on the graphene surface and act as electron donors or acceptors resulting in resistance changes of the graphene channel. In
this work, graphene films were formed on 4H-SiC substrates by thermal decomposition of the (0001) silicon surface in Ar
ambient at a high temperature of 1800–2000 $^{\circ}$C. The graphene functionalization was performed by the covalent bonding of a
nitrophenyl group ($C_6H_5NO_2$) followed by its reduction to a phenylamine group ($C_6H_5NH_2$) by using a cyclic voltammetry
process. There was a clear and prompt response (current change) of the antibody-coated graphene/SiC dies when the blood
antigen matched the antibody. No response occurred when the antibody on the graphene surface mismatched the blood antigen.
The experiments demonstrated that a functionalized graphene-on-SiC die has capability in blood sensing, opening a way to
manufacture biosensors for detecting blood types and for other applications.

Key words: graphene film deposition, SiC sublimation, graphene functionalizing, graphene-based biosensor.

1. INTRODUCTION

The unique physicochemical properties of graphene,
associated with the presence of a highly mobile electron
shell in it, which provides high electrical conductivity,
theoretically indicate that any molecule capable of
acting as an electron donor or acceptor can, upon
contact with the graphene surface, lead to a change in
electrical resistance [1]. This property has been used in
graphene-based sensor for detecting molecules of certain
gases [2], for example, nitrogen oxide [3].

However, as-fabricated graphene is not selectively
sensitive, it attaches various biomolecules and can serve
as a biosensor only after special treatment. To provide
sensing ability, graphene functionalization is usually
accomplished by using various covalent and noncovalent
approaches [4,5]. Graphene functionalization modifies
the surface chemistry of graphene and creates covalent
bonds on its surface to attach a specialized immune
protein, an antibody. For the biosensor application, the
antibody has to be dispensed on functionalized graphene.
During the detection process, the protein molecules
chemically attach to the antibodies on the graphene
surface.

The antigen–antibody (AG–AB) interaction is fundamental for the functioning of the human immune system. These reactions are the basis of many immunological processes and are widely used in laboratory practice. For a number of diseases, the detection of specific antigens and antibodies is the main diagnostic tool [6].

The AG–AB reactions are promising for the mass use in the format of immunochromatographic analysis, which can be carried out in non-laboratory conditions, do not require equipment and highly qualified personnel, and allow getting the result of the examination in 10–15 min [7–10].

In this work, we investigated the possibility of using the AG–AB reaction with respect to graphene films grown on semi-insulating SiC substrates to create an electrochemical analyser (biosensor) in which acceptors or electron donors are protein antigens and antibodies that interact with each other.

A scheme of the biosensor operation adapted for this work is shown in Fig. 1. The AG–AB reaction causes an instantaneous change in the graphene channel resistance (current flow), which can be recorded by modern electronic equipment. The graphene response (resistivity change) takes place only if the antibody and the antigen are a matched pair. This feature provides selectivity of the graphene-based biosensors. The working capacity of the functionalized graphene dies is tested to determine blood type.

Currently, 36 human blood group system genes have been identified. Most blood group antigens are glycoproteins and their specificity is mostly determined either by the oligosaccharide (e.g. ABO) or amino acid sequence (e.g. MN, Kell, Duffy, Kidd, Diego) [11]. The blood group affiliation is due to specific groups of carbohydrates, proteins, and glycoproteins that are located on the erythrocyte membrane. Among them, the most important blood groups applied in medicine are the system ABO and Rh factor.

Here we report for the first time on the possibility of using a functionalized graphene chip to determine the blood group using the ABO system based on the AG–AB reaction. Graphene biosensors are promising for the further determination of blood groups also in other systems. The graphene-based sensor for blood type sensing can provide quick analysis on a large scale and operate in a simple manner.

2. EXPERIMENTAL

2.1. Fabrication of graphene chips

Graphene films were formed on 4H-SiC substrates by thermal decomposition in argon ambient at a high temperature of 1800–2000 ^{o}C [12]. The method allows getting high-quality graphene films on arbitrarily large areas, which is important in processing graphene chips (dies) for sensing applications. Laser photolithography was used to pattern the graphene/SiC die area. Reactive ion etching in Ar and oxygen plasma was used to remove the graphene layer from the uncoated areas. Metallization of Ti/Au (5 nm/50 nm) was used as ohmic contacts. After the processing, a 1.5 mm × 1.0 mm graphene/SiC die was mounted on a ceramic holder and bonded with Au wires. Figure 1 shows the typical view of the graphene/SiC die on the holder. Current-carrying parts of the holder, contact pads of the graphene/SiC die, and connection wires, which can be in contact with electrolyte liquid solutions and biological media, were covered with a chemical resistant lacquer to protect them from electrochemical corrosion. Then the chips were treated in Ar gas at an elevated temperature of 100 °C for one hour to remove residual moisture adsorbed on the
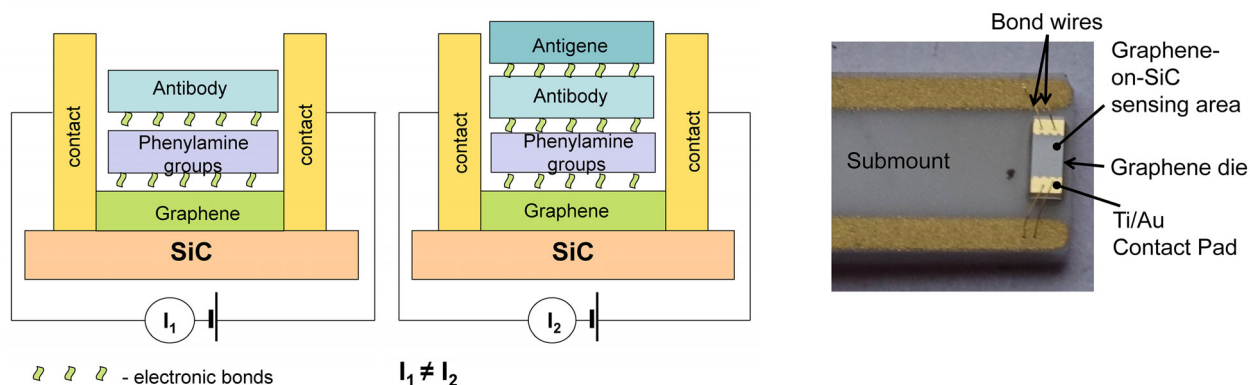

Fig. 1. Scheme of the biosensor operation (on the left, $I_1 \neq I_2$, ⌀ ⌀ ⌀ – covalent bonds) and the graphene sensor assembly image (on the right). Die size is 1.5 mm × 0.7 mm. Graphene area for sensing is 1 mm × 0.7 mm.

graphene from the environmental air. After the annealing, the chips were stored in argon ambient.

2.2. Graphene surface functionalization

In this work, we performed graphene functionalization by the covalent bonding of the nitrophenyl group ($C_6H_5NO_2$) followed by its reduction to the phenylamine group ($C_6H_5NH_2$) by using a two-step cyclic voltammetry (CV) process. All CV experiments were performed with an Elins P-20X potentiostat/galvanostat (Electrochemical Instruments, Russia) in a conventional three-electrode cell with an Ag/Ag^+ (or $Ag/AgCl$) reference electrode, a platinum wire counter electrode, and a graphene/SiC die as the working electrode. The three-electrode cell had a hermetic lid allowing the electrolyte and the space above it to be purged by dry Ar to remove traces of the moisture from the cell and the electrolyte.

At the first step, the nitrophenyl groups were attached to the graphene surface. For this, a graphene die assembled on a holder was immersed for 1–2 min into a non-aqueous electrolyte based on a mixture of 2 μM 4-nitrobenzenediazonium tetrafluoroborate (4NDT) and 0.1 M tetrabutylammonium tetrafluoroborate (TBATF) in acetonitrile (CH_3CN).

The covalent binding (attachment) of the nitrophenyl groups was controlled by monitoring the current when cycling the potential at the working electrode (graphene chip) from +200 mV to –600 mV and back at a scan rate of 20 mV s^{-1}. Figure 2a shows typical cyclic voltammograms of the attachment process of the nitrophenyl groups. The decrease of the current at each subsequent cycle indicates the completion of the surface attachment reaction. The first cycle shows a large reduction peak, related to the irreversible attachment of the nitrophenyl radical to the graphene electrode surface [5]. The 2nd

and 3rd cycles show that the attachment reaction was nearly complete and the surface was saturated with nitrophenyl groups attached to the surface. After three cycles, the current diminution was about an order of magnitude. Then the graphene chips were rinsed in CH_3CN and dried in Ar.

In the second CV process the graphene/SiC die was immersed in a 0.1 M KCl water/ethanol (9 : 1) solution to reduce the nitrophenyl groups to the phenylamine groups on the graphene die surface. Figure 2b shows typical cyclic voltammograms of the reduction process. We used a silver–silver chloride electrode (Ag/AgCl) as the standard electrode in this case. The reduction process was also monitored by decreasing the current while cycling the potential at the working electrode (on graphene) from +200 mV to –1000 mV and back. The current became stable and several times lower after 3 cycles. This means that almost all nitrophenyl groups became phenylamine groups on the graphene surface and the functionalizing process was completed. Then, the graphene dies were rinsed in deionized water and dried in Ar. The functionalized graphene chip was kept in a glovebox in Ar ambient before utilizing it in the blood sensing experiments.

2.3. Blood sensing

It is known that the antigen, a structure on the surface of a red blood cell, determines the blood group [6]. To detect the blood group, a reaction between the antigen and the antibody can be used. The functionalized graphene surface promotes the antibody adaption and the use of graphene as a biosensor. Note that the adapted antibody can only react to and bind one specific antigen.

For the antibody immobilization, graphene/SiC dies were kept for 3 h in a mix of an antibody in a buffered
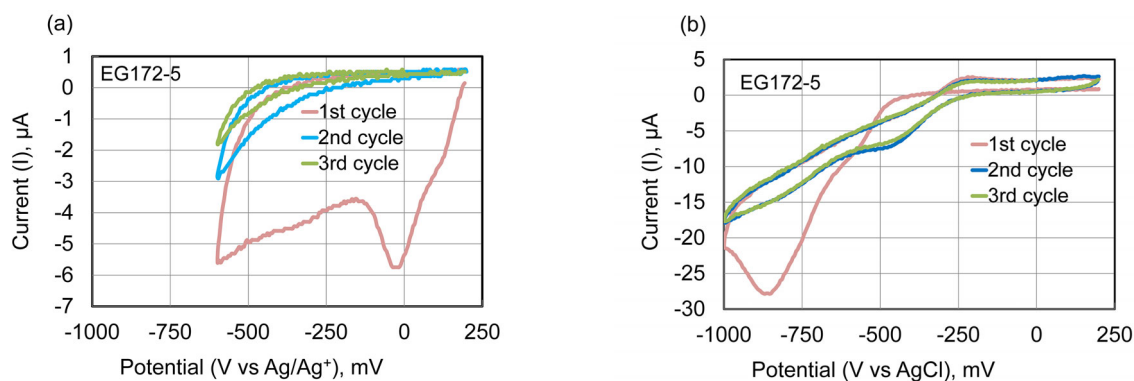

Fig. 2. Cyclic voltammograms (3 cycles) of binding nitrophenyl to the graphene/SiC die (a) and reduction of nitrophenyl to phenylamine (b). A water-free electrolyte was applied for the binding process (a) and Ag wire was the reference electrode. A water-based electrolyte was applied for the reduction process (b) and Ag wire in a water-based KCl solution was used as the reference electrode.

borate solution followed by washing in a pure buffered borate solution and water. Various antibodies of the ABO blood group systems and the Rh factor (anti-A, anti-B, anti-D) and phenotype systems (anti-E and anti-c of phenotypes E and c) were used for the blood analysis.

A die with a functionalized graphene surface coated with an antibody was placed in a physiological solution (0.9 wt% NaCl). Then a stabilized voltage of 50 mV was applied, and the whole blood was dropped on the graphene die sequentially (after 30–60 s and 90–120 s), first with a volume of 10 µL, then with a volume of 500 µL, after which the change in the current was recorded. The whole blood was dropped on the graphene die coated with the antibody. The attachment of the blood molecules (antigen) to the adapted (matched) antibody situated on the graphene surface changed the total resistance of the graphene film.

3. RESULTS AND DISCUSSION

At the detection process, the reaction combining the antigen with its complementary antibody results in a change in the resistance of the graphene channel, which can be promptly detected by the current passing through the graphene/SiC die. In the event that the AG–AB reaction does not occur, there is almost no change in the resistance (that is, a change in the magnitude of the flow current). This is what we observed in our experiments on the reaction of the functionalized graphene chip in contact with blood. Figures 3 and 4 show the response of the antibody-coated graphene/SiC dies after contact with different blood types and different blood phenotypes.

The response (r) is the relative change of the graphene/SiC die resistance $r = (R - R_0)/R_0$, where R_0 is the initial resistance and R is the resistance after blood has been applied. When 10 µL of whole blood was admixed, there was almost no response of any graphene dies. This blood volume was too small. When 500 µL of whole blood was admixed, a faint response of the graphene die was observed in the case the adapted antibody on the die matched the antigen on red blood cells in the whole blood. The response appeared step-wise after whole blood drops were placed on the die (Fig. 3a–c).

Below is a description of the response of graphene dies (biosensors) illustrated in Fig. 3 upon contact with the whole blood.

The response of the antibody-coated (anti-A) die EG168-13 after contact with A(II)Rh(+) blood is given in Fig. 3a. The whole blood A(II)Rh(+) has antigens A on the red blood cells [13]. The antigen A matches the
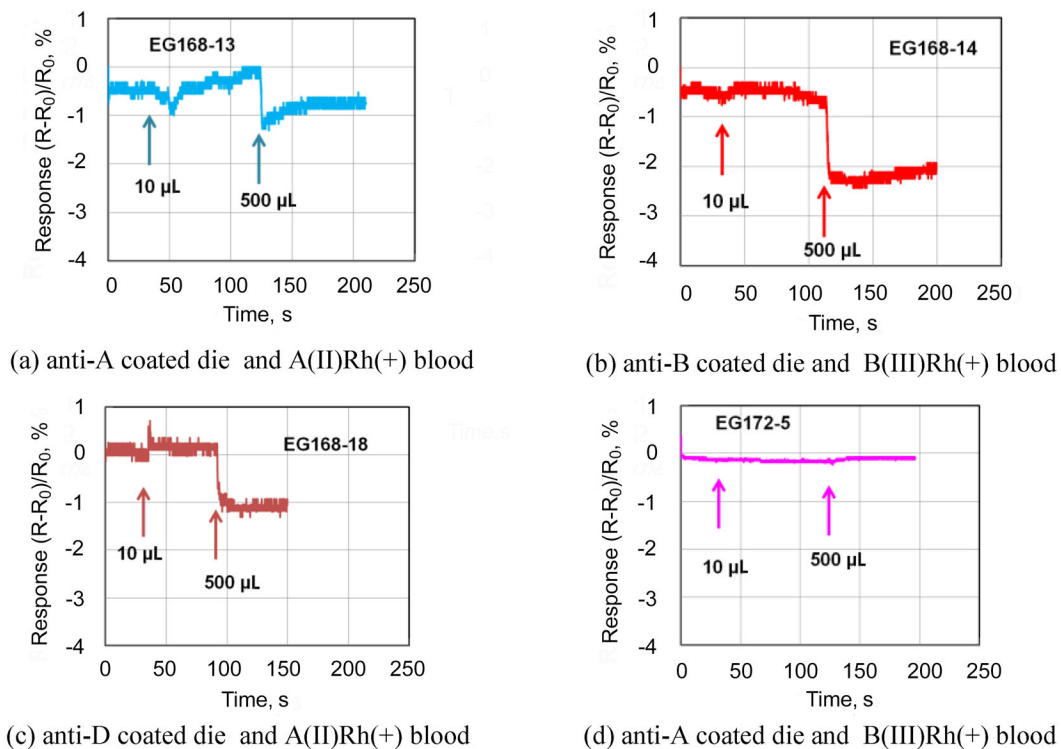

(a) anti-A coated die and A(II)Rh(+) blood

(b) anti-B coated die and B(III)Rh(+) blood

(c) anti-D coated die and A(II)Rh(+) blood

(d) anti-A coated die and B(III)Rh(+) blood

Fig. 3. Blood type sensing by graphene/SiC dies. The response of the antibody-coated graphene/SiC dies after contact with different blood types. The arrows indicate the moments when 10-µL and 500-µL drops of the whole blood were placed on the die.

anti-A situated on the graphene die, and the AG–AB interaction must occur (combination of an antigen with an antibody), which was proven by the step-like behaviour of the response in Fig. 3a.

The response of the antibody-coated (anti-B) die EG168-14 after contact with B(III)Rh(+) blood is illustrated in Fig. 3b. The whole blood B(III)Rh(+) has antigens B on the red blood cells [13]. The antigen B matches the anti-B situated on the graphene die and the AG–AB reaction must occur, which was correctly manifested by the step-like behaviour of the response in Fig. 3b.

The response of the antibody-coated (anti-D) die EG168-18 after contact with A(II)Rh(+) blood is shown in Fig. 3c. The whole blood A(II)Rh(+) is known to have antigens D on the red blood cells [13]. The antigen D matches the anti-D situated on the graphene die, and the AG–AB reaction must occur, which was correctly manifested by the step-like behaviour of the response in Fig. 3c.

The response of the antibody-coated (anti-A) die EG172-5 after contact with B(III)Rh(+) blood is illustrated in Fig. 3d. The whole blood B(III)Rh(+) has antigens B on the red blood cells [13]. The antigen B does not match the anti-A situated on the graphene die and no AG–AB reaction can be expected, which is correctly manifested by the horizontal line in Fig. 3d.

The experiments demonstrated that the functionalized graphene dies coated with antibodies are blood sensitive and can potentially be used to identify promptly (within 1–2 min or less) types of the blood. There was a fairly clear step-like response of the antibody-coated graphene/SiC dies when the blood antigen matched the antibody. There was no response if the antibody on the graphene surface mismatched the blood antigen.

Note that the initial resistances, R_0, of all chips in the experiments were within 0.9–13.3 kΩ. Such resistances had no noticeable effect on the reaction of the chips. However, more experiments need to be made to find the optimal resistance value of the original graphene chip.

ABO is the most important blood group system in transfusion medicine and transplantation immunology. The ABO blood groups differ in the presence or absence of antigens on the red blood cells and antibodies in plasma. Accurate determination of the ABO status is critical to reduce the occurrence of transfusion reactions. However, besides AB0 typing, there are other genetic differences between blood groups (blood phenotypes) of importance in transfusion medicine as well as in the emerging field of organ transplantation. The phenotype of any blood group refers to which antigens are detectable on red blood cells.

Another set of experiments was made to study the response of graphene die upon mixing 500 μL of whole blood with rare blood phenotypes. The results of blood phenotype sensing are illustrated in Fig. 4. Figure 4a shows the response of the antibody-coated (anti-E) die EG168-22 after contact with B(III)Rh(−) phenotype DCcEe blood having antigens E on the red blood cells [13]. The antigen E matches the anti-E situated on the graphene die, and the AG–AB interaction must occur. This is confirmed by the step-like behaviour of the response in Fig. 4a.

Figure 4b shows the response of the antibody-coated (anti-c) die EG168-23 after contact with two different blood phenotypes: A(II)Rh(+) phenotype DCCee blood and O(I)Rh(−) phenotype ddccee blood at times t_1 and t_2. No antigens in A(II)Rh(+) phenotype DCCee blood matched anti-c, and thus no response of the graphene chip at t_1 was expected. Still, an unexpected weak response can be observed at t_1 in Fig. 4b. It seems that free amine groups on the graphene surface were still not saturated with the primary antibody (anti-c in this case), which gave the unexpected response. To prevent this,
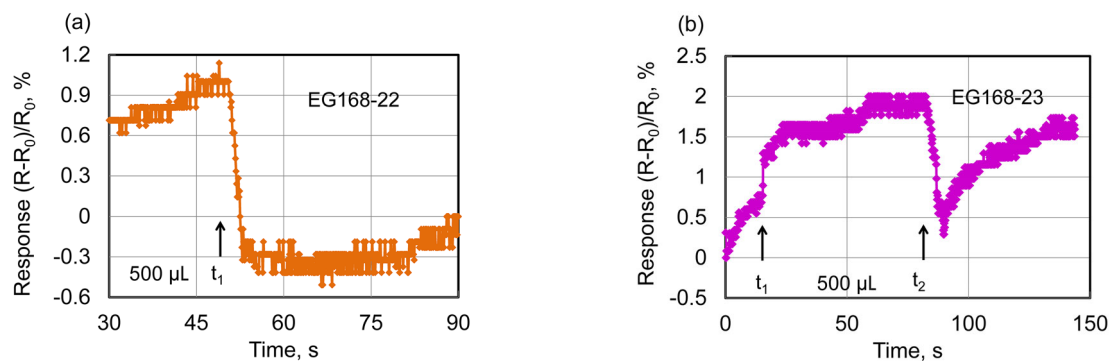

Fig. 4. Blood phenotype sensing by graphene/SiC dies. The response of the antibody-coated graphene/SiC dies after dropping 500 μL of blood of different phenotypes at t_1 and t_2 (shown by arrows): (a) anti-E coated die and B(III)Rh(−) phenotype DCcEe blood at t_1; (b) anti-c coated die and A(II)Rh(+) phenotype DCCee blood at t_1 and O(I)Rh(−) phenotype ddccee blood at t_2.

special passivation of the free amine group after the antibody immobilization is necessary [5].

Incomplete coverage of the graphene surface by the phenylamine group after the reduction process (incomplete reduction process) may also result in a false response or a weak correct response. Optimization of the electrolyte and use of a catalyst can be used to improve the conversion of NO_2 to NH_2 groups [14]. However, 40% NH_2 coverage was reported to be sufficient for successful bio-attachment [5].

In addition, to avoid a false (unexpected) response of the graphene/SiC dies, additional experiments should be performed with blood plasma to verify also the influence of specific blood molecules such as erythrocytes, leukocytes, and thrombocytes on the graphene/SiC die response. These molecules have different dimensions and may interfere with the AG–AB interaction on the graphene surface. Specifics of the functionalization process (electrolyte type concentration, number of the CV process cycles) and the graphene/SiC die layout should also be optimized.

The response at t_2 in Fig. 4b was observed after the contact of the antibody-coated (anti-c) die (EG168-23) with O(I)Rh(–) phenotype ddccee blood, which has antigens E on the red blood cells [13]. The antigen c matches the anti-c situated on the graphene die, and the AG–AB reaction is more noticeable than that at t_1. The response at t_2 looks peak-like rather than step-like, which is probably due to the fact that amine groups were not saturated by the anti-s amine group on the graphene surface as mentioned above.

4. CONCLUSIONS

Graphene dies formed on a SiC substrate were functionalized by binding nitrophenyl followed by its reduction to phenylamine and tested for blood sensing. The experiments demonstrated blood sensitivity of the functionalized graphene dies with applied antibody and can potentially be used to identify promptly (within 1–2 min or less) blood types and phenotypes.

There was a clear and prompt response (current changing) of the antibody-coated graphene/SiC dies when the blood antigen matched the antibody. No response occurred if the antibody on the graphene surface mismatched the blood antigen. The experiments demonstrated that a functionalized graphene-on-SiC die is capable of blood sensing, which opens a way for fabricating biosensors for detecting blood types as well as for other applications.

Based on the results obtained, benefits of the graphene/SiC dies for blood sensing can be formulated as follows:

- there is no visual control of the blood, which is mandatory in the case of other methods;
- this approach allows performing blood typing by a person with no special skills;
- the result can be obtained in less than 1 min;
- simultaneous determination (on one graphene/SiC die) of several variants of blood groups (ABO, Rh-factor, Kell, etc.);
- large possibilities for identifying other important substances in the body that are detected using an antigen–antibody reaction, such as cancer markers.

However, the issue of an unexpected response of the graphene dies when no matched antigens to be attracted by the adapted antibody on the die are present in the blood needs to be eliminated.

ACKNOWLEDGEMENT

REFERENCES

1. Liu, Y., Dong, X., and Chen, P. Biological and chemical sensors based on graphene materials. *Chem. Soc. Rev.*, 2012, **41**, 2283–2307.
2. Schedin, F., Geim, A. K., Morozov, S. V., Hill, E. W., Blake, P., Katsnelson, M. I., and Novoselov, K. S. Detection of individual gas molecules adsorbed on graphene. *Nat. Mater.*, 2007, **6**, 652–655.
3. Novikov, S., Lebedeva, N., Satrapinski, A., Walden, J., Davydov, V., and Lebedev, A. Graphene based sensor for environmental monitoring of NO_2. *Sensor. Actuat. B-Chem.*, 2016, **236**, 1054–1060.
4. Georgakilas, V., Otyepka, M., Bourlinos, A. B., Chandra, V., Kim, N., Kemp, K. C., et al. Functionalization of graphene: covalent and non-covalent approaches, derivatives and applications. *Chem. Rev.*, 2012, **112**, 6156–6214.
5. Tehrani, Z., Burwell, G., Mohd Azmi, M. A., Castaing, A., Rickman, R., Almarashi, J., et al. Generic epitaxial graphene biosensors for ultrasensitive detection of cancer risk biomarker. *2D Mater.*, 2014, **1**, 025004.
6. Ryan, K. J. and Ray, C. J. G. (eds). *Sherris Medical Microbiology: An Introduction to Infectious Diseases.* McGraw-Hill, New York, 2004.
7. Chareonsirisuthigul, T., Khositnithikul, R., Intaramat, A., Inkomlue, R., Sriwanichrak, K., Piromsontikorn, S., et al. Performance comparison of immunodiffusion, enzyme-linked immunosorbent assay, immunochromatography and hemagglutination for serodiagnosis of human pythiosis. *Diagn. Micr. Infec. Dis.*, 2013, **76**, 42–45.
8. Birhanu, H., Rogé, S., Simon, T., Baelmans, R., Gebrehiwot, T., Goddeeris, B. M., and Büscher, P. Surra Sero K-SeT, a new immunochromatographic test

for serodiagnosis of *Trypanosoma evansi* infection in domestic animals. *Vet. Parasitol.*, 2015, **211**, 3, 153–157.

9. Grimaldi, G. Jr., Teva, A., Ferreira, A. L., dos Santos, C. B., Pinto, I. d.-S., de-Azevedo, C. T., and Falqueto, A. Evaluation of a novel chromatographic immunoassay based on Dual-Path Platform technology (DPP® CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. *Trans. R. Soc. Trop. Med. Hyg.*, 2012, **106**, 1, 54–59.

10. Saghrouni, F., Gaïed-Meksi, S., Fathallah, A., Amri, F., Ach, H., Guizani, I., and Saïd, M. B. Immuno-chromatographic rK39 strip test in the serodiagnosis of visceral leishmaniasis in Tunisia. *Trans. R. Soc. Trop. Med. Hyg.*, 2009, **103**, 1273–1278.

11. ISBT. Red Cell Immunogenetics and Blood Group Terminology. http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology (accessed 2019-04-15).

12. Davydov, V. Yu., Usachov, D., Lebedev, S., Smirnov, A. N., Levitskii, V., Eliseyev, I., et al. Study of the crystal and electronic structure of graphene films grown on 6*H*-SiC (0001). *Semiconductors*, 2017, **51**, 1072–1080.

13. Reid, M. E. and Lomas-Francis, C. *The Blood Group Antigen Facts Book*. Second ed. Academic Press, New York, 2003.

14. Yu, S. S. C., Tan, E. S. Q., Jane, R. T., and Downard, A. J. An electrochemical and XPS study of reduction of nitrophenyl films covalently grafted to planar carbon surfaces. *Langmuir*, 2007, **23,** 11074–11082.

Grafeen-ränikarbiidandurid veregrupi tuvastamiseks

Alexander Usikov, Konstantin Borodkin, Sergey Novikov, Alexander Roenkov, Andrei Goryachkin, Mikhail Puzyk, Iosif Barash, Sergey Lebedev, Alexander Zubov, Yuri Makarov ja Alexander Lebedev

Vaatluse all on grafeen-ränikarbiidandurid veregrupi tuvastamiseks. Sensorite kasutamisel tuvastatavad keemilised ühendid adsorbeeruvad grafeeni pinnal ja toimivad elektronide doonorite või aktseptoritena, mis väljendub grafeeni kanalite takistuse muutustes. Käesolevas töös saadi grafeenikiled 4H-SiC alustel räni (0001) pinna termilise lagundamise teel argoonis kõrgel temperatuuril (1800–2000 °C). Grafeeni funktsionaliseerimine viidi läbi nitrofenüülgrupi ($C_6H_5NO_2$) kovalentse sidumise ja selle järgneva taandamisega fenüülamiingrupiks ($C_6H_5NH_2$), kasutades tsüklilist voltameetrilist protsessi. Saadi selge ja kiire signaal (voolu muutus) antikehadega kaetud grafeen-ränikarbiidanduritelt vere antigeeni antikehale vastavuse korral. Signaal puudus, kui vere antikeha grafeenpinnal ei vastanud vere antigeenile. Katsed näitasid, et funktsionaliseeritud grafeen-ränikarbiidanduritel on potentsiaali vere analüüsimiseks, mis avab võimaluse vere tüübi määramiseks mõeldud biosensorite loomiseks jm rakendusteks.