Reduced graphene oxide based nanobiocomposite as basis for flexible biosensors

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Abstract. Flexible selective electrochemical biosensor based on reduced graphene oxide film and short oligonucleotides (aptamers) was developed. Laser scribing was applied for graphene reduction due to controllable reduction rate and simple devices patterning. Optimal parameters of film reduction were determined for effective aptamers coupling, by varying laser output power. Mild reduced graphene oxide, as was expected, revealed better reactivity for aptamers coupling. Resistive response to biosensors exposure to thrombin and albumin proteins was measured. As a result we developed bionanocomposite that can be used in a new generation of available low-cost biosensors.

1. Introduction

Personal healthcare is one of the powerful and prospective trends in device development. There are lots of different fitness trackers, smartwatches and even sensors in smartphones that gives a possibility to monitor such parameters as pulse, step number, stress level etc. However, up to nowadays one can't monitor personal health on different illnesses excluding cardiovascular disease or diabetes due to the lack of the appropriate sensors. One of the ways to solve problem of health monitoring is to create new sensor with the sensitivity to different illnesses. Main goal of this work is to show possibility of coupling biosensitive agent – aptamers to the reduced graphene oxide to form one of the main part of biosensor – transducer. Carbon nanomaterials, in particular, graphene [1] and its derivatives [2], and carbon nanotubes [3], meet well with requirements of flexibility, acceptable conductivity and presence of reactive functional groups [4] with which different biomaterials can be coupled.

Modified or reduced graphene oxide [1] is one the most promising material for transducer formation due to the presence of different functional groups including carboxyl, epoxy, amino etc. [5]. On the other hand, reduced graphene oxide have large surface area, flexibility, relatively good conductivity and possibility of reduction ratio management.

Two main mechanisms of graphene oxide reduction: chemical [6] and thermal [7] can be implemented by different techniques, with laser reduction as the most perspective for nanodevice fabrication. During the last decades, there appeared some other reduction methods including UV...
treatment in vacuum [8] or laser treatment [9], that based on photochemical [10] or thermal [11] reduction mechanism. In case of femtosecond laser pulses it can be realized both thermal and photochemical reduction mechanisms, the last one allows to attach or remove various functional groups [12]. Laser induced reduction contrary to thermal or chemical reduction have such advantages as possibility of local and easy patterning of graphene oxide and possibility of providing different photochemical reactions in case of femtosecond lasers.

In our work we used pulsed microsecond laser to locally pattern graphene oxide film with manageable reduction ratio and further aptamer coupling to form nanobiocomposite that works as transducer. Aptamers are ideal sensitive substrates for immobilization. In addition to high sensitivity and affinity, aptamers have such advantages over their nearest competitors - antibodies: higher stability, much easier and cheaper synthesis [13 - 15].

2. Materials and methods
Electronic part of a biosensor consists of flexible substrate (175 µm thick poly(ethylene terephthalate)) and GO/rGO film with about 1 µm thickness. Substrates with 20 x 20 mm dimensions were precleaned with 2-propanol and water. GO suspension (4,7 mg/ml, LLC “MIP Graphene”, Yakutsk, Russia) [16] was diluted to 1,5 mg/ml and deposited by drop-casting method. Films were dried for 24 h after deposition under normal conditions.

Local laser treatment was carried out by the own-designed system with 445 nm solid-state laser facility with motorized table [17]. Output laser power was varied in 10-400 mW range by pulse width modulation (PWM) at 30 MHz frequency. Pulses width ranged from 1 to 20 µs. Treatment time in a spot (~1,5·10³ µm²) was constant 30 ms.

For biocomposite formation, we used the amine-modified derivative of the thrombin binding aptamer AmTBA (5’-GGTTGGTGTGGTTGG-3’) with amino group at the 5’-end. The amino-derivative aptamer AmTBA of 50 µkM coupled with carboxylic groups on the surface of the rGO by standard conjugation method using EDC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide, Sigma-Aldrich, USA) in 100mM MES buffer solution, pH = 6.0 (2-(N-morpholino)ethanesulfonic acid, Sigma-Aldrich, USA) with 25% ethanol addition, at room temperature in an inert gas atmosphere within 1 hour, after that the solution was collected, centrifuged and analysed by HPLC.

We performed Raman spectroscopy for characterization of reduction depth by estimating relative intensities of specific GO and rGO Raman peaks. We got Raman spectra with Ntegra Spectra (NT-MDT Ltd, Moscow, Russia).

Structures resistance before aptamer coupling and sensor response were measured by IPS-16 (Practtic NC Ltd., Russian Federation) sensor parameter measurement system that allows performing long-term measurements up to 48 hours long. We also used the self-made platform with the OMRON XF2J connector that allows high stability of electric contact and thus resistance and response measurement.

3. Results and discussion

3.1. Graphene oxide reduction and biosensor design
In our work we processed graphene oxide film by laser irradiation with different fluence parameters to find out optimal reduction ratio. We also performed aptamer coupling to structures with low, medium and high fluence (25, 35 and 42 J/cm²) and investigated approximate amount of functional groups (table 1). We also found a reduction limit at about ~ 50 J/cm² when the film resistance starts to increases, which indicates the onset of rGO ablation and oxidation by atmosphere oxygen. When fluence excess about 60 J/cm² substrate degradation begins thus such fluence value considered as unacceptable for sensors fabrication.

Investigation of aptamer coupling showed that low fluence (25 J/cm²) is better for aptamer coupling due to presence of a larger number of functional groups that is discussed further.

Based on the obtained data we choose low fluence value to perform GO film reduction and made a set of biosensor structures with 13 × 5 mm dimensions and Π-shaped pattern of reduced graphene oxide
area (figure 1a). After formation of set of biosensor structures we conducted a reaction of aptamer coupling to reduced graphene oxide.

We performed measurement of sensor structures resistance stability for 120 minutes. Statistics of resistance values of different structures is of figure 1b. We can notice that 19 of 30 (i.e. 63%) of sensor structures have resistance not more than 200 kOhm. On the other hand, resistance of some structures can achieve more than 5 MOhm that we attribute to superposition of such factors as local film roughness, nonuniformity of laser movement and local non-flatness of the substrate. Thus, we can predict about 60% yield of sensor structures ready for aptamer coupling.

![Figure 1](image)

Figure 1. (a) 3D model of a biosensor based on reduced graphene oxide, (b) histogram of the resistances of the obtained sensors (photo in the insert image).

3.2. Biocomposite formation
To confirm the presence of functional groups and the possibility of aptamer coupling we used well-studied thrombin binding aptamer with amino-modification (Am-TBA) [18]. TBA recognizes the thrombin with a very high affinity using its TT-loops, and the core structure of the G-quadruplex stabilizes exact loops arrangement [19]. The functional amino-group of Am-TBA reacted with carboxylic groups of GO film preactivated by carbodiimide (EDC) giving conjugation product.

This approach is an extremely widespread practice for bioconjugation: such reaction proceeds in quantitative yield. However, when we applied this method for the amino-modified aptamer derivative conjugation to the film of reduced graphene oxide, we observed the hydrophobic effect of reduced graphene oxide surface appear to play an important role. We showed that the addition of 25% v/v ethanol to the reaction mixture improves the coupling reaction. The method allows visualizing reactive carboxylic groups only. However, the number of active carboxyl groups determines the number of conjugated sensing molecules on the surface of the reduced graphene oxide and therefore it determines the effectiveness of the biosensor.

In practice, a biosensor is often produced as a construct, one of the elements of which is the conductive layer containing sensing molecules for binding the target. Realization the reaction of covalent attaching an aptamer to the whole construct differs from attaching an aptamer to a substrate that is not packaged.

In particular, for whole constructs, it is difficult to carry out the conjugation reaction in solution, since some parts of the biosensor constructs may be damaged during prolonged contact with aqueous solutions of buffers. The conjugation reaction in the case of whole biosensors should be carried out locally. Therefore, it is the reason to increase the concentration of the EDC activator to increase the efficiency of the conjugation reaction. The reaction of the aptamer conjugation with reduced graphene oxide within constructs demands more mixing to conduct the reaction in an inert gas atmosphere. An important factor that ensures high conjugation efficiency is the mixing regime of the reaction.

Based on amount of reacted amino-modified aptamer AmTBA we calculated that the specific amount of carboxyl groups reaches 29 pmole per 1 mm2, of maximum fluence 42 J/cm2, 32 pmole per 1 mm2, of fluence 35 J/cm2 and 43 pmole per 1 mm2 for the film of minimum fluence 25 J/cm2 (Table 1).
Table 1. Results of aptamer conjugation.

| Laser fluence, J/cm² | The percentage change in the concentration of AmTBA, % | Quantitative change in the quantity of AmTBA, nmol | The specific amount of reactive carboxyl groups per unit area, 10¹²/nm² |
|----------------------|------------------------------------------------------|-----------------------------------------------|---------------------------------------------------------------|
| 25                   | 32                                                   | 3.2                                           | 43                                                            |
| 35                   | 29                                                   | 2.9                                           | 32                                                            |
| 42                   | 26                                                   | 2.6                                           | 29                                                            |

Thus, we observe a trend on increasing of functional groups present with decreasing of laser fluence and identified that laser treatment with fluence near 25 J/cm² is suitable for aptamer coupling and, so, for the whole sensor.

3.3. Structure characterization

We made series of sensor structures with coupled aptamers. Typical Raman spectra from these samples before and after aptamer coupling presented on figure 2. In these spectra, we can observe D band (~1350 cm⁻¹), G band (~1600 cm⁻¹) and 2D band (~2700 cm⁻¹). It is known that the D band arises due to A₁g symmetry and originates from the zone boundary phonons. The D band is usually attributed to various defects in the graphite lattice. The G band on the other hand is first-order scattering, related to E₂g symmetry and corresponds to the Brillouin zone of crystalline sp² lattices in graphite [20].

Higher ID/IG ratio in the reduced graphene oxide indicates larger defect density [21, 22]. It has been reported that the 2D peak profile is sharp for pristine graphene, whereas low and wide 2D peak intensity as compared to D and G peaks indicates higher disorder rate in the GO structure [23].

From figure 2 and abovementioned information on the nature of Raman bands we can make next observations and deduction. One can see that there are 2 main features in spectra after coupling: change in ID/IG ratio (0.98-0.99 before to 0.86 - 1.13 after aptamer coupling) and decrease of 2D peak intensity.

As we assume these effects could be corresponded to increase of defect number due to presence of aptamer-rGO conjugates with the trend and values ID/IG ratio before and after aptamer coupling very similar to the effects obtained in [24, 25]. Authors of [25] corresponded this effect to the coupling of polyethylenimine (PEI) to the graphene oxide. In our case, such comparison is legal due to low reduction rate of graphene oxide that thus contains different functional groups.

Figure 2. Raman spectra of reduced graphene oxide surface before and after aptamer coupling (S7 sensor structure).
3.4. Response measurements

We performed exposition of a sensitive area with thrombin (target) and albumin (reference) proteins to get response from sensor. Sensors with coupled thrombin binding aptamer were exposed to thrombin (figure 3a) and albumin (figure 3b) proteins. We observed the significant difference between sensor signals exposed to different proteins. Sensors also showed different signs of change in resistance, the response to albumin was about 0.9%, whereas to thrombin it was ten times higher, 8.5%.

![Figure 3](image)

**Figure 3.** Sensor response on (a) thrombin, (b) albumin exposition.

We attributed such a difference in the signals to the specificity of the aptamer to the target protein. The decrease in resistance of the reduced graphene oxide film modified by an aptamer can be explained by the p-type conductivity of this material in normal conditions due to the presence of carboxyl groups on its surface [11]. The low change in resistance of the sensor exposed to albumin can be explained by a nonspecific reaction of the protein with the surface of the reduced graphene oxide. We also showed that the lower detectable concentration is 10 μM for this sensor design.

4. Conclusion

In this work we showed possibility of local manageable reduction of graphene oxide and pattern graphene oxide film to prepare biosensor drafts. We made series of sensor structure with about 60% yield of structures with relatively low resistance less than 200 kOhms. We also showed possibility of covalent coupling of aptamers to the reduced graphene oxide that is one of main challenges in development of a new generation of electrochemical biosensors.

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