Capabilities of Graphene Oxide Application as a Nanostructured Quencher at the Development of New Fluorescent Test Systems for DNA-Diagnostics

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ABSTRACT

Review is devoted to the description of approaches to using the graphene oxide as a nanostructured quencher for DNA diagnostics of SNPs. High importance of the field of DNA diagnostics for modern medicine, and also the need for its further development in the direction of the mass introduction of modern DNA diagnostics methods in clinical practice are substantiated in the introduction.

The review discusses the most widely used modern methods of DNA diagnosis of hereditary diseases at the present time. Advantages and disadvantages of modern fluorescent DNA diagnostic techniques (techniques based on real time PCR and PCR with end-point signal detection) as the most widely used in modern medical practice are discussed more precisely. There are unique physicochemical properties of graphene oxide with respect to DNA, allowing to create a new type of test systems based on graphene oxide and intended for DNA diagnostics of hereditary diseases observed. The details of several approaches to the establishment of such test systems based on the use of graphene oxide as a substitute of molecular quenchers were described. The scheme and operation of test systems, its component composition and key performance indicators was examined. Economic benefit of replacement of molecular quenchers by graphene oxide is substantiated.

This review outlines the shortcomings and limitations of existing approaches to the creation of test systems based on graphene oxide, examined the potential of their adaptation for use in laboratory DNA diagnostics.

Conclusion gives the withdrawal of the prospects and the need for fluorescent test systems based on graphene oxide intended for laboratory DNA diagnostics of hereditary diseases.

INTRODUCTION

Innovative methods of medical diagnosis based on cutting-edge developments and principles are important in the modern world. At the end of XX - beginning of XXI century one of these methods became the DNA diagnostics, which opening previously unachievable horizons in the field of predictive and preventive medicine [10]. DNA diagnostics is widely used for the detection of hereditary and multifactorial diseases, diagnostic of infections, determination of consanguinity, forensic practice and veterinary medicine. The volume of production of test systems for DNA diagnostics in Russia was estimated at 15.7 million units in 2010 [2]. Significant part of the ongoing medical genetic researches is currently focused on the diagnostics of monogenic diseases (which total number currently stands at more than 5,000 nosology [13]).

An important aspect of this developing field of medicine should be its mass character - the widespread introduction of DNA diagnostics to medical practice will significantly reduce the genetic load of the population, improve the gene pool, health and quality of life. To speed up this process, people must have modern and affordable test systems for DNA diagnostics which will provide fast, high quality and inexpensive tests.

Problems of modern DNA diagnostics methods

At the present time there are some basic principles for the molecular diagnosis of mutations [1]. There are classical methods based on PCR - RFLP with registration of results by electrophoresis; method of DNA sequencing according to Sanger; fluorescent techniques: Real-Time PCR and End-Point PCR; and methods based on biochips technology. But almost all of the standard methods of analysis used in the present time have both advantages and disadvantages that limit their potential of applications in the field of laboratory DNA diagnostics.

Currently, methods of fluorescent DNA diagnostic of polymorphisms have emerged as a popular tool in the practical medicine: PCR with the endpoint detection and the Real-time PCR [6] allowing a determination of amplicons quantity during PCR process. In the past five years fluorescent methods are successfully have been used in major diagnostic and research centers of developed countries due to the simplicity and rapidity of analysis performance, high reliability of the results, economizing of production space, reducing the number of staff and demand quantification of DNA / RNA. In the practice due to these advantages there is a gradual ousting of the classical PCR – RFLP. However, the best existing fluorescent test systems for DNA diagnostics of polymorphisms are based on the usage of complex circuits with different DNA probes (TaqMan, LightCycler, Scorpion, Molecular Beacons). The structure of these probes usually includes a fragment of the fluorophore and a fragment of fluorescence quencher molecule.

Fluorescence quencher provides the absence of fluorescent signal in the case if the probe is not embedded in the structure of the amplicons (the amplification have not occur), and if amplification is successful and the probes are embedded into amplicons during PCR, the accumulation of the fluorescent signal can be observed. Using of such structure provides a high reliability of the results and allows even quantitative DNA diagnostics with an assessment of the kinetics of the fluorescent signal accumulation in the case of real-time PCR.

However, such fluorescent test systems have one major drawback - the complex structure of DNA probes which leads to stability problems and increases the cost of the test system due to the complicated process of synthesis and purification of probes. It leads to high price/quality ratio for these test systems and hinders their wide introduction into practice of public health [16]. But at the moment, namely fluorescent methods are the most promising (in qualitative and

economic aspect) for introduction to clinical medicine. Therefore topical problem is a development of its potential - creation of such test systems based on improved or new principles of work that will simultaneously allow improvement of qualitative results and economic viability of DNA diagnostics.

The unique properties of graphene oxide for use in fluorescent test systems

At recent years scientists came to the discovery of new materials with unique properties, including properties of fluorescence quenchers [7]. In particular, researchers have found that grapheme oxide and its derivatives can be very effective quenchers for different organic fluorophores [11] and quantum dots [8]. Comparing to other quenchers, grapheme oxide showed the highest quenching effect of different fluorophores with low background and high signal-to-noise ratio [9,12]. This effect is caused by adsorption of fluorophores on the surface of graphene oxide and realization of FRET-effect in the form of excitation energy transfer from the fluorophore molecules to the surface of graphene oxide with its subsequent scattering.

A key feature of some derivatives of graphene (in particular - its oxide) is also its high affinity to the single-stranded DNA molecules, in contrast to double-stranded [15] (figure 1).

Due to these properties of graphene oxide, it is possible to develop various biosensor test systems on the basis of graphene oxide - better (in quality and economically) analogs of fluorescent systems for DNA diagnostics of mutations based on methods of real-time PCR and PCR with endpoint detection.

Approaches of development of fluorescent test systems based on graphene oxide for DNA diagnostic of SNPs

Last 2-3 years active study on the development principles of working of such test systems in the world has been made. To date, there were published different approaches for the diagnosis of point mutations in DNA, which may cause hereditary diseases and cancer; were formulated the general ideas and principles on prototyping biosensor test systems based on graphene oxide. Such several approaches developed over the last few years will be examined next:

One of the easiest approaches of development of such test was described in the study of S. He et al. [3]. Researchers applied the scheme, which uses a hybridization reaction of fluorescently labeled oligonucleotide probes (**P5-P7**) with the target molecule (**T7**) in a solution followed by addition of graphene oxide suspension into the solution (Fig. 2)

Hybridization of fully complementary single-stranded oligonucleotides forms a DNA duplex, which does not adsorb on the graphene oxide surface. It leads to presence of fluorescent signal as the characteristic of presence of hybridized probe in the final solution.

It was found that the difference in one nucleotide in the target molecules (**M1-M3)** does not lead to their complete hybridization with the probe **P1** (Fig. 3). It allows detecting any point mutations in DNA structure using fluorescent signal with the scheme described above.

This approach is the result of development of the earlier ideas laid down in 2009 [4]. However, unlike the old idea new approach allows the realization of multiplex analysis: different target oligonucleotides can be determined with a high signal/noise ratio using different probes labeled with fluorophores with different wavelengths of emission (**P5-P7**). In this case, each target oligonucleotide being added to a solution will only match the fluorophore that is associated with the fully complementary oligonucleotide probe.

Authors also found that the order of addition of graphene oxide and oligonucleotides can be any for this test system (Fig. 4 a, b). Even by adding a solution of the target molecule **T1** to a solution of graphene oxide with adsorbed probe molecules **P1** on its surface, the final solution later becomes to have fluorescent properties due to probe and target hybridization. It leads to form a poorly adsorbable DNA duplex and its subsequent desorption from the surface of graphene oxide.

The approach described above has been also improved in other work [14]. There was created new test system using covalent linking of fluorescently labeled oligonucleotide probes with the graphene oxide surface (Fig. 5 b). This approach, unlike the others (Fig. 5 a) allows to enhance the quality of DNA analysis results because of small probability of nonspecific offsetting substitution of probes which are adsorb on the surface of the graphene oxide in the process of analysis.

Researchers used the carbodiimide method of linking probes with graphene oxide surface. It affords to make the reusable test system for DNA diagnostics due to the possibility of "cleaning" of modificated grapheme oxide. It is important to note that in terms of reusable graphene oxide for DNA diagnostics, this work is so far unique.

Investigating the kinetics of fluorescence signal appearance in a solution of the modified graphene oxide after the addition of an oligonucleotide target (with and without the mutation), the authors were able to obtain the intensity ratio ~ 1.5 for the fully complementary and mutant target (Fig. 6). Limit of detection of complementary target for this test system was 1.5×10^{-10}M, which is a good indicator in the comparison of this approach with test systems with a "non-covalent" ways of oligonucleotides linking.

Other authors [5] developed the idea of using a fluorescent intercalation agent as SYBR Green (instead of fluorescent probes) and the suspension of graphene oxide for the detection of mutations in DNA. Scientists have found that by adding a mixture of oligonucleotides fully complementary to each other (probes and targets) with SYBR Green to a suspension of graphene

oxide is retain the fluorescence in the final solution, indicating the intercalation of a SYBR Green into a double helix structure of formed DNA duplex. But complete hybridization does not occur in the case of the presence of a mutation in the probe molecules. In that case fluorophore is adsorbed on the surface of the graphene oxide, which results to absence of fluorescence in the final solution (Fig. 7).

Experiments have shown that the registration of single nucleotide mutations using graphene oxide suspension attained significant signal / noise ratio (25-90) at the 10 nM concentration of the oligonucleotides. That provides high sensitivity for this method of analysis (Fig. 8).

Using the data obtained in the course of research experience, the authors have created a prototype of fluorescent microchip with the ability to distinguish the quantitative composition of the test mixture of oligonucleotides (Fig. 9).

Prospects and possibilities of adaptation of existing approaches for laboratory DNA diagnosis of hereditary diseases.

In general, over the last 3-4 years in the foreign literature there were described more than ten entirely different approaches to the development of fluorescent test systems based on graphene oxide for DNA diagnostics of mutations. In all works graphene oxide is used for two main purposes:

1) For logic recognition of single and double-stranded DNA molecules due to their different affinity with respect to graphene oxide

2) As a nanostructured quencher of fluorescent agents instead of molecular quenchers, which are prevalent in DNA probes in modern fluorescent test systems.

It should be noted that so far all of the above studies are generally aimed at demonstrating the potential use of graphene oxide in the field of creating DNA test systems. But graphene oxide have not yet found application in the field of laboratory DNA diagnostics of diseases. Published approaches need serious adaptation for use in DNA diagnostics laboratory because of two main reasons:

1) In all developed approaches presently used single-stranded oligonucleotides, while in the standard PCR - a key step in any procedure of DNA diagnostics - formed double-stranded DNA molecules (amplicons).

2) All developed test systems designed to operate with a sufficiently short oligonucleotides (15-25 b.), while the amplicons formed during the classic PCR cannot have length less than 50 bp.

The distinguishing characteristic of graphene oxide is the simplicity and low cost of its synthesis, as well as high stability colloidal suspensions. For comparison, on the basis of the data

about using graphene oxide in DNA diagnostics [3, 4, 5, 14], the mass of graphene oxide required for quenching 1 nM fluorophore is from 0.1 to 1 mg. In test systems with molecular quenchers (TaqMan, LightCycler) used are equivalent amounts of the fluorophore and quencher: for 1 nM fluorophore - 1 nM of quencher. The ratio of the average commercial prices of 1 mg of graphene oxide (eg from European brand «Graphenea») and 1 nM of quencher (eg Black Hole type from «Synthol", Russia) is about 1: 3. The share of the quenchers cost are usually 10-15% of the total cost of test system. Therefore, the use of graphene oxide as nanostructured quencher fluorescence can lead to creating a new class of fluorescent test systems with reduced cost and improved quality of DNA diagnosis in comparison with existing fluorescent test systems which are used expensive and low stable molecular quenchers.

CONCLUSIONS

Thus, despite of extensive research opportunities for application of graphene oxide in the field of creating test systems for DNA diagnostics, it have not been currently created such test systems intended for laboratory use. Ongoing researches of finding new approaches and adaptation of already existing approaches for the development of test systems based on graphene oxide and their implementation in clinical laboratory practice is an interesting challenge for researchers.

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