Influence of the temperature conditions of the graphene oxide synthesis on graphene oxide – induced fluorescence quenching of ssDNA

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Abstract

Graphene oxide (GO) is the high-biocompatible, good aqueous dispersible and low-cost material. Fluorescence quenching and adsorption capacity of GO, different affinity of single-stranded and double-stranded DNA molecules to GO are used to design GO-based fluorescent sensors to detect complementary single stranded DNA. In this work, in the framework of the development of graphene oxide-based test systems for the diagnosis of point mutations in DNA, we study fluorescence quenching efficiency of GO. The graphene oxides were prepared by the modified Hummers method at different synthesis conditions and were characterized. During the study, it was found that the reaction temperature is the most dominant parameter to control GO properties. GO suspension synthesized at 75^0C of the reaction mixture showed the most high fluorescence quenching efficiency. Basing on XPS O1s, FT- IR spectra analysis, on data of the fluorescence emission spectra of dye-labeled DNA in the presence of various concentrations of GO it is found the effect of oxygen functional groups such as carboxyl, phenol, carbonyl, and epoxy on the efficiency of fluorescence quenching by GO. These results will be useful for in-depth studies of oligonucleotides and GO interaction and opens new opportunities for sensitive detection of biorecognition events. Copyright © 2017 VBRI Press.

Keywords: Graphene oxide nanoplates, fluorescence quenching, FssDNA, aqueous suspensions of GO, oxygen-containing functional groups.

Introduction

Graphene oxide (GO) is extensively studied due to its useful characteristics including large surface area, facile surface modification, high-biocompatibility, good aqueous dispersibility [**1-4**]. GO can be synthesized in large scales using inexpensive methods. It can act as a quencher of the fluorescence of organic dye [**5-8**]. The noncovalent binding of GO with dye-labeled single stranded DNA (ssDNA) and the quenching capacity has been widely used to design GO-based fluorescent sensors to detect complementary single stranded DNA [**6**, **9**, **10**]. The recognition binding with the complementary DNA molecule results in the dye-labeled DNA release and restoration of the fluorescence intensity due to the weak affinity of double-stranded DNA molecules to GO. Recently, sensitive, rapid, and cost-effective test system was developed on the base of the fluorescence of FAM-ssDNA quenching by aqueous GO suspension for the diagnosis of the point mutation in native DNA [**11**]. In the work [**12**] graphene oxides (GOs) were tested as a fluorescent quencher in the field of DNA-diagnostics. The various suspensions of GO nanoplates were prepared by changing the synthesis conditions: mixing time, temperature of the reaction mixture and amount of the oxidant. The main finding of this work is the fact, that the reaction temperature was found to be the most dominant parameter to control the GO properties. It was revealed that the T= 75^0C of reaction temperature produced the GO nanoplates with a maximum specific level of fluorescent quenching (16.39 nmol/mg). However, this study is not possible to make firm conclusions about the nature of the influence of the GO synthesis conditions on the efficiency of fluorescence quenching, and this trend is for further study. In this work, we carried out more complete study and show that not only the degree of oxidation of GO nanoplates in aqueous suspensions, but also an abundance ratio of oxygen-containing functional groups leads to different quenching efficiency of the fluorescence of FssDNA. Here it is established as in the previous work that the GO suspension synthesized at the temperature 75^0C is the most efficient fluorescent quencher. GO nanoplates produced at this temperature could be an effective substitution of molecular fluorescent quenchers in test-systems for DNA-diagnosis.

Experimental

Sample preparation

The powder of highly oriented pyrolytic graphite (HOPG, Sigma Aldrich) was the source material for aqueous GO suspensions preparation. GO was synthesized according to Hummer's method with some modification. An amount of the oxidizing agent was varied. Time of stirring of the reaction mixture was from 2 to 3 weeks at temperature T=25^{0}C and 75^{0}C. Graphite powder (0.1 g) and sodium nitrate (0.05 g) were put into solution of concentrated H_2SO_4 (7 mL), then successively little portions of potassium permanganate $KMnO_4$ (0.2 or 0.3g) were added gradually under stirring. The mixtures were then diluted with 5% hydrogen peroxide solution until the manifestation of the brilliant yellow colouring. The brilliant yellow mixture containing GO was washed with 300 ml deionized (DI) water until neutral pH of the filtrate in Buchner funnel with ashless filter 70 mm in diameter. Here at, a dark brown jelly- like mass of GO is formed. The jelly-like mass of GO was removed from filter to beaker, diluted by 50 ml DI water and then exfoliated to generate GO nanoplates by ultra-sonic waves. After ultrasonication the obtained GO-suspension was centrifugated at 14500 rev/min for 5 minutes to remove particles of graphite oxide. The dark brown GO-suspension was dialyzed to remove residual metal ions and acids. Dialysis was carried out in Cellu·Sep Cellulose Tubular Membranes (MWCO 12-14 kDa). Samples of suspensions of GO nanoplates were prepared with some variations of above mentioned method. Experimental solutions with volumes of 200 µl were prepared by serial mixing of DI distilled water, phosphate buffer PBS2X (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 и 2 mM KH_2PO_4, pH= 7.4), aqueous suspension of GO nanoplates and the dye-labeled ssDNA with the consentration 10^{-8} M. The phosphate buffer solution of ions of metals is added to supply an effective adsorbtion of the ssDNA on GO nanoplates and sustain pH to level up oscillations of fluorescence intensities of different samples. In the experiment oligonucleotide sequences are as follows: 6-carboxyfluorescein (FAM) -labeled ssDNA sequence is 5`-FAM –CCTCAAGATTTCGAGA TGGC-3`. Fluorescent oligonucleotide for DNA fluorescent quenching analysis (95 % purity according to HPLC) was purchased from Evrogen CJSC.

Characterization technique

Concentrations of GO in aqueous suspensions of GO were determined by the weight method. The element composition in the GO films was carried out by the energy-dispersive X-ray microanalysis using microanalysis system (Nano Analisys, OINA, Oxford Instruments) attached with a scanning electron microscope (SEM, Jeol 7800 F). The lateral size distribution of GO nanoplates was studied using their optical and SEM images. The thickness of GO nanoplates was determined by atomic force microscope (AFM, Solver Next NT-MDT, Russia). Raman spectra of GO were measured with Raman microscope (Integra Spectra, NT-MDT, Russia). The oxygen functional groups to be part of GO were investigated over FTIR and XPS spectra. FTIR spectra of GO films were obtained in a spectrometer Varian 7000 FTIR. XPS spectra were recorded on a spectrometer SPECSPHOIBOS 100 MCD (SPECS Surface Nano Analysis GmbH, Germany) using monochromatic AlKα radiation (hν = 1486.6 эВ). The spectrometer is calibrated with respect to peaks of $Au4f_{7/2}$ (energy of binding– 84.0 эВ), $Ag3d_{5/2}$ (368.3 эВ) и $Cu2p_{3/2}$ (932.7 эВ) [**13**]. As an internal standard was used the peak C1s (284.5 эВ). Emission fluorescence spectra of the mixture of oligonucleotides and OG measured on a fluorometer Filter Max F5 and luminescence spectrometer Perkin Elmer LS50B. UV–visible absorption spectra of GO were obtained using P330 nanofotometer (IMPLEN). Fluorescence of GO nanoplates in water was studied by the use of steady state spectroscopic techniques. The emission spectrum of FAM was recorded by the fluorometer Filter Max F5 at the wavelength λ=535 nm. The pulse excitation of the carboxyfluorescein (FAM) fluorescence with 400 ms duration at the 485-nm wavelength is used.

Results and discussion

Raman spectra for samples of GO films obtained from aqueous suspensions of GO in the frequency range of 200-3400 cm^{-1} at room temperature presented in **Fig. 1.** The Raman spectrum shows two characteristics for GO bands: D at ~ 1350 cm^{-1} and G at ~ 1600 cm^{-1}. The band G is due to the stretching motion of sp^2 pairs of C and band D due to the vibration of carbon atoms with dangling bonds [**14, 15**]. In contrast, the Raman spectrum of graphite showed very weak peaks assigned to the D. The difference in the Raman spectra of GO and graphite may indicate the formation of some sp^3 –carbon atoms in GO, which was consistent with earlier reports.

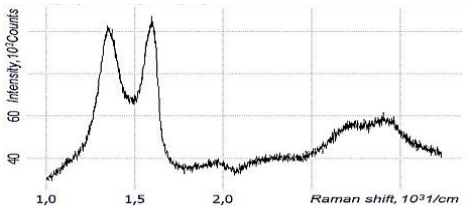

Fig. 1. Raman spectrum of GO, synthesized at T=75^{0}C.

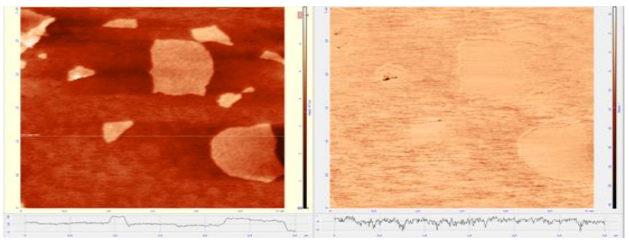

Fig.2. AFM images of GO nanoplates with 3 mg/l concentration on mica (left - topography and cross-section profile of sheets, right - phase contrast mode).

GO nanoplates on mica and SiO_2/Si substrates were obtained from GO aqueous suspensions drops with [GO]=1 mg/l . GO nanoplates were imaged by AFM. As

shown in **Fig. 2**, the thickness of GO nanoplates were being about 1.2-2 nm. This confirms that they are mono- and bilayer nanoplates and they exist in solutions primarily as exfoliated single sheets. This also occurs due to oxidation of the sheets leading to a net negative charge on them.

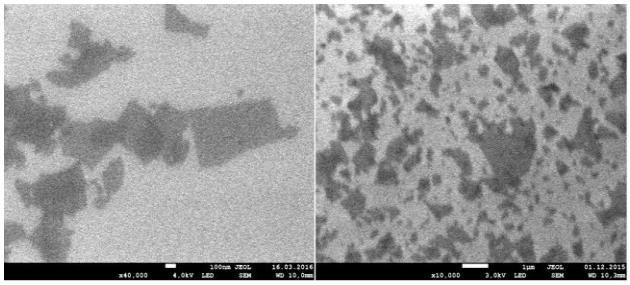

Fig 3. SEM images of GO nanoplates with different oxygen content (left, O- 26,8 %), and (right, O-41, 6 %). at different magnification.

SEM images of nanoplates (**Fig. 3**) were used to determine the lateral size distributions of GO nanoplates on (**Fig. 4**). GO nanoplates thickness averaged 1.2nm and lateral dimensions - 0,5-1μm. GO synthesized upon reaction mixture heating at the temperature T=75^{0}C (T-GO) is found to be more finely dispersed and lateral size of T-GO nanoplates varies from 0.2 to 0.6 μm. In this case, the nanoplates content with lateral dimensions of ~ 0.2 μm prevails (**Fig. 4**).

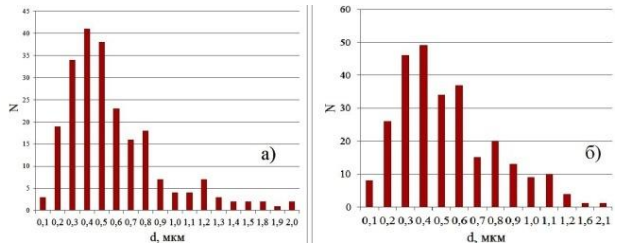

Fig. 4. GO nanoplates lateral sizes histogram: a) T-GO (O-40,6 %), b) GO (O-41, 6 %).

In the work [**12**] it was found that the GO nanoplates with a maximum specific level of fluorescent quenching (16.39 nmol/mg) are produced at the temperature T= 75^{0}C of a reaction mixture. However, the results of this study not gave conclusions about the nature of the influence of the GO synthesis conditions on the degree of fluorescence quenching. It is known that the conditions of GO suspensions preparation influence their physico-chemical properties [**16**]. Therefore, we performed the study of contents of oxygen functional groups (hydroxyl, carboxyl, epoxy) and oxygen in GO samples synthesized at different oxidant, reaction temperature, time of stirring of mixture. We prepared GO films from aqueous GO suspensions on silicon substrates and in transparent vials. The element composition of the five μm-thick GO films on the silicon substrates (Figure 5) were determine on SEM using energy dispersive analysis (EDS). SEM using energy dispersive analysis (EDS).

EDS data are following: O- 45 % for the base sample GO-1(t-3 weeks, m=0.3g, T=25^{0}C), O-41,6 % for the sample GO- 2 (t-2 weeks, m=0.3g, T=25^{0}C, 300 ml), O- 26,8 % for the sample GO-3 (t-3 weeks, m=0.2g, T=25^{0}C, 300 ml), O- 40,6 % for the sample GO -4 (t-3 weeks, m=0.3g, T=75^{0}C, 300 ml), O-40,3 % for the sample GO-5 (t -2 weeks, m=0.3g, T=25^{0}C, 300 ml, heating at 75^{0}C for hour), where t is mixing time, T is the temperature of the reaction mixture, 300 ml is the volume of washing water, m is the amount of oxidant.

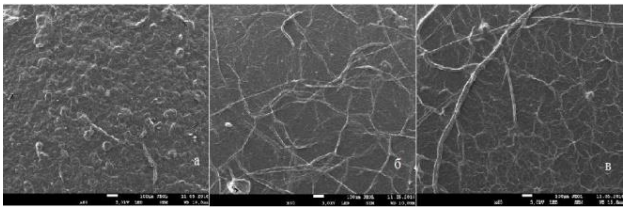

Fig. 5. SEM images of GO μm-thick films a) O-26,8 %, б) O-40,3 % and в) O-41,7 %.

The FT-IR spectra of GO gave the characteristic vibrations of GO, as those reported in the work [**17**] and further provided the information of the successful synthesis of GO. IR spectra were recorded in the range of 550-4000 cm^{-1} with a resolution of 8 cm^{-1}.

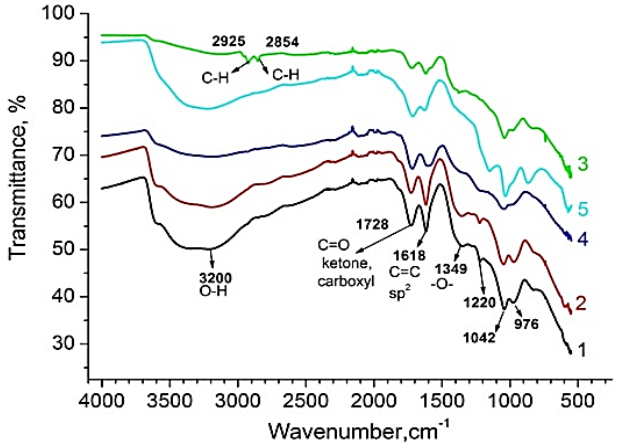

Fig. 6. IR spectra of various GO films.

GO showed FTIR peaks (**Fig.6**) at 1728 cm^{-1} (ketone and/or C=O stretching vibrations in carboxylic group COOH ~1700-1900 cm^{-1}), 1618 cm^{-1} (sp^2 -hybridized C=C in-plane stretching vibrations, ~1550-1650 cm^{-1}), 1349 cm^{-1} (epoxy C-O-C, ~1350 cm^{-1} and 800-900 cm^{-1}) [**18**], 1220 cm^{-1} (β-region, C=O contribution, ether, anhydride and epoxy, 1100-1280 cm^{-1}), 1042 cm^{-1} and 976 cm^{-1} (α-region, hydroxyl, C=O contribution, carboxyl and epoxy, 900-1100 cm^{-1}). The maximum amount of adsorbed and intercalated water at ~ 3200 cm^{-1} (vibrational modes C-OH of hydroxyl COOH and H_2O, 3000-3750 cm^{-1}), is observed for the base sample 1 (**Fig. 6**, curve 1), the smallest amount – for the sample 3, obtained with a reduced amount of oxidant (**Fig. 6**, curve 3). The shift of O-H stretching peak in GO film to lower wave number as compared to 3428 cm^{-1} is probably due to destification of GO gel. The view of the IR-spectrum of GO sample 4 differs from the other spectra by very weak peak at 1349 and 1220 cm^{-1} corresponding C-O-C

epoxide and β-region (C=O contribution, ether, anhydride and epoxy).

The degree of oxidation of GO nanoplates in aqueous suspensions influences on their absorption efficiency. UV-vis absorption spectra (200−600 nm) GO are shown in **Fig. 7(a)** for samples of GO synthesized at T=25^{0}C with a weight concentration of [OG] = 50mg/l. The two spectral features at ca. 230 and 300 nm correspond to the $\pi\rightarrow\pi*$ transitions of aromatic C−C bonds and the $n\rightarrow\pi*$ transitions of C=O bonds, respectively [**15**]. With increasing of the degree of oxidation of GO nanoplates the UV-vis absorption efficiency increases.

Plotted curves of XPS O 1s spectra of GO are shown in **Fig.7**.: the peak with the binding energy E_b (C1s) ~284.5 eV corresponding C-C bonds of sp^2 orbitals (blue line), the peak E_b (C1s) ~ 286.8 eV - C-OH, epoxy, ether groups, aldehyde and ketone groups, and C with N, C with S (red line), the peak E_b (C1s) ~288.2 eV - carboxyl group (magenta line) [**15,13**].

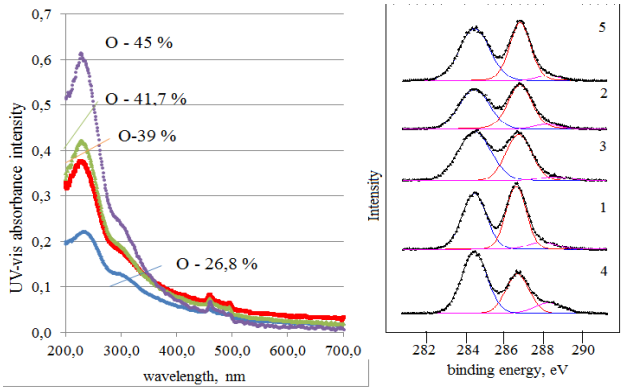

Fig. 7. UV-vis absorption curves of nanoplates of GO (a) and fitted results of XPS O1s spectra of GO samples (b).

XPS O 1s spectra in **Fig.7 (b).** were used to calculate content of each oxygen functional group. For this atomic ratios [**19**] with respect to main element- carbon with the binding energy 284.5 eV were calculated according to the equation,

$$X/C = \left(I_X/\left(Sc_X \times TF_X\right)\right)/\left(I_C/\left(Sc_C \times TF_C\right)\right),$$

where, X/C – an atomic ratio of atoms X to carbons atoms; I_X and I_C – intensities of choosed XPS lines; Sc_i – Scofield coefficients, TF_i –values of transmission function for corresponding lines. Deconvolutions of spectra were performed in the program XPS Peak 4.1c using Gauss-Lorenz functions. The phone substraction was carried out according to the Shirley method. Atomic percentages from dissected spectra of XPS O 1s for the GO are listed in **Table 1**.

XPS O1s spectra of GO show that the atomic ratio C (286.7 eV)/C(284.5 eV)= 0.6 for T-GO and 0.94 for GO-1 at ~ equal value of C(288.2 eV)/C(284.5 eV) =0.18, 0.19 for T-GO and GO-1, where GO-1 is the base GO (**Table 1**). We can conclude that the content of sp^2 carbons maximal for T-GO. Comparison of FT-IR and XPS spectra shows minimal content of epoxy group and maximal content of carboxyl group for T-GO (T = 75^{0}C). Such a distinction of T-GO results to some consequences, which exhibit in FssDNA fluorescence quenching.

Study of GO –induced FssDNA fluorescence quenching was carried out at different temperatures, concentrations and degrees of oxidation of GO.

Table 1. Atomic percentages from dissected spectra of XPS O 1s for the GO.

Atomic ratio	Sample 1	2	3	4	5
C(286.7eV)/C(284.5eV)	0.94	0.96	0.72	0.60	0.79
C(288.2eV)/C(284.5eV)	0.19	0.12	0.07	0.18	0.07
S/C (284. eV)	0.059	0.035	0.034	0.035	0.032
N(sum.)/C(284.5eV)	0.13	0.061	0.051	0.085	0.045

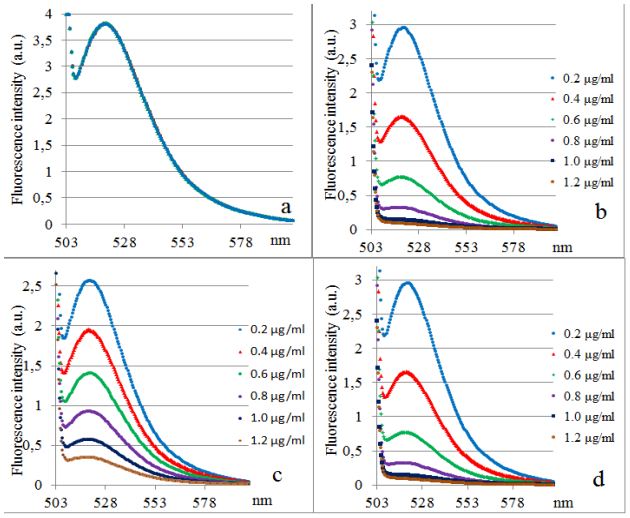

Fig. 8. The fluorescence quenching of F-ssDNA(10 nM)+PBS+H_2O systems by various concentrations of GO a) control sample - without GO (PBS+F-ssDNA+H_2O), after addition of GO ([GO]=0.2, 0.4, 0.6, 0.8, 1, 1.2 μg/ml): b) T-GO (O-40,6 %), c) base GO-1 (O-45 %) d) GO-2 (O-41,6%).

As shown in **Fig. 8**., the fluorescence emission intensity of FssDNA gradually decreases with [GO] (concentration of GO) increasing. As [GO] of T-GO reaches 1.0 μg/mL (**Fig. 8.b**), the fluorescence nearly vanishes. As-synthesized GO-1 quenchs 87% of FssDNA (length of DNA is about 7 nm) fluorescence at [GO] =1.0 μg/mL. At the same time, GO-2 quenches 43% of FssDNA fluorescence at [GO] =1.0 μg/mL. There is no correlation with the degree of oxidation of OG and the fluorescence quenching efficiency. This indicates that the composition of functional oxygen groups T-GO leads to FssDNA fluorescence quenching enhancement. The difference in kinetics of T-GO and GO – induced fluorescence quenching of FssDNA is observed due to different content of oxygen functional groups (**Fig. 9**).

The experimental results show that the mechanism of fluorescence quenching depends on temperature conditions of GO synthesis. At T=28^{o}C the Stern-Volmer plot is a downward curvature for the short incubation time of FssDNA in T-GO and it is linear for the long incubation time. A complex fluorescence quenching mechanism occurs for GO synthesized without heating at the same temperature.

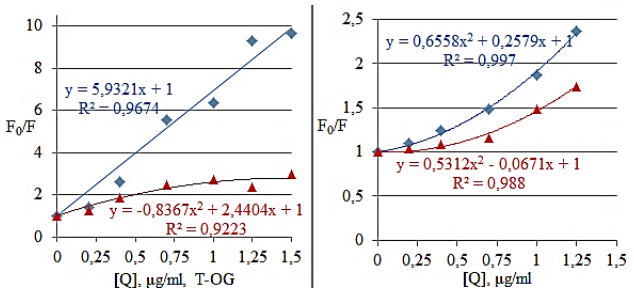

Fig. 9. FssDNA fluorescence quenching mechanism depends on temperature conditions of GO synthesis The Stern-Volmer plots: a) T-GO (O- 40,5%) at T = 28°C and incubation time of FssDNA with GO t=144 s (red) and at t = 1248 s (blue); b) GO (O-45%) at a temperature T = 28°C.

Conclusion

To develop the high efficient fluorescent quencher, it is important to consider the issues related to its structure and properties of GO, which are dependent on the method of synthesis. The graphene oxides were prepared by the modified Hummers method at different synthesis conditions (stirring time for the reaction of mixture, temperature of the reaction mixture under stirring, the amount of oxidant in the reaction mixture-potassium permanganate, and the volume of deionized water for washing of GO on the filter) and characterized. During the study, it was found that the reaction temperature is the most dominant parameter to control the GO properties. Fluorescence emission spectra of aqueous suspensions of GO nanoplates and buffer are stable in time and depend on the degree of oxidation. The greater the percentage of oxygen content of GO nanoplates in aqueous suspensions, the greater the intensity of the fluorescence emission and absorption at the same concentration [GO]. However, the efficiency of fluorescence quenching of FssDNA depends on the amount of different oxygen- functional groups of nanoplates. Heating of the reaction mixture at the mixing step at the temperature T=75^{0}C leads to a greater content of carboxyl groups and smaller content of epoxy groups according data of FT-IR and XPS spectra. Quantitative analysis of XPS O1s spectra of GO show that the atomic ratio C (286.7 eV)/C(284.5 eV) = 0.6 for T-GO (75^{0}C) and 0.94 for the base sample of GO-1 (25^{o}C). T-GO has a higher quenching efficiency, thus reaction temperature of 75^0 C produced the GO nanoplates with a maximum level of fluorescent quenching. The difference in quantitative content of oxygen functional groups is confirmed by exhibition of different fluorescence quenching mechanism. These results will be useful for in-depth studies of oligonucleotides and GO interaction and opens new opportunities for sensitive detection of biorecognition events.

Acknowledgements

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