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НАНОЧАСТИЦЫ ОКСИДА ГРАФЕНА В РЕГУЛЯЦИИ ОКИСЛИТЕЛЬНОЙ АКТИВНОСТИ МОНОЦИТОВ ЧЕЛОВЕКА

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Резюме. Благодаря своим свойствам, материалы на основе графена обладают потенциалом к использованию в биомедицине, но из-за их цитотоксического и провоспалительного воздействия на клетки практическое применение препаратов на его основе затруднено. Известно, что различные поверхностные модификации (функционализация) наночастиц оксида графена (ОГ) полиэтиленгликолем (ПЭГ) – один из способов снижения негативных эффектов графена на живые клетки. Так как применение наночастиц подразумевает их взаимодействие с иммунной системой, которая участвует в защите организма и в регуляции его функций, изучение этого вопроса крайне важно. Моноциты – клетки врожденного иммунитета и первая линия защиты человеческого организма от микроорганизмов и других чужеродных объектов. Одна из реакций моноцитов на стимул любой природы – производство активных форм кислорода (АФК). Ранее опубликованные данные демонстрируют неполную картину влияния наночастиц модифицированного оксида графена на образование АФК моноцитами человека. Таким образом, целью нашего исследования явилось оценка влияния пегилированного оксида графена (ОГ-ПЭГ и ОГ-8армПЭГ) на продукцию АФК моночитами человека в тесте спонтанной и стимулированной люминол-зависимой хемилюминесценции (ЛЗХЛ).

В качестве объектов исследования использовались $CD14^+$-клетки, выделенные из мононуклеаров периферической крови здоровых доноров. Продукция АФК стимулировалась опсонизированным зимозаном (ОЗ), в качестве контроля выступал спонтанный вариант ЛЗХЛ. В работе использовались частицы оксида графена модифицированные линейным и разветвленным (армированным) ПЭГом (ОГ-ПЭГ и ОГ-8армПЭГ) размерами 100-200 нм («малые») и 1-5 мкм («большие»), с количеством покрывающего ПЭГ около 20%. Наночастицы применяли в концентрациях 5 и 25 мкг/мл.

Установлено, что на уровне спонтанной продукции АФК наночастицы малого размера в низкой концентрации (5 мкг/мл) и наночастицы большой размерности, покрытые разветвленным ПЭГом, в обеих концентрациях, оказывают достоверные подавляющие эффекты. На уровне стимулированной продукции АФК было обнаружено, что наночастицы графена малой размерности в концентрации 25 мкг/мл также подавляли продукцию АФК, как и частицы большой размерности, покрытые линейным ПЭГом, в той же концентрации. Таким образом, нами впервые установлено, что наночасти-

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цы оксида графена, функционализированные ПЭГом, способны ингибировать продукцию активных форм кислорода моноцитами человека, в связи с чем, можно говорить об антиоксидантной активности ОГ-ПЭГ.

Ключевые слова: оксид графена, функционализированные наночастицы, полиэтиленгликоль, люминол-зависимая хемилюминесценция, моноциты, активные формы кислорода

GRAPHENE OXIDE NANOPARTICLES IN THE REGULATION OF THE OXIDATIVE ACTIVITY OF HUMAN MONOCYTES

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Abstract. Graphene-based materials have an opportunity for use in biomedicine, thanks to their properties. Nevertheless, due to its cytotoxic effects, the use of graphene-based drugs is problematic. However, the surface modification of graphene oxide (GO) nanoparticles with a polyethyleneglycol (PEG) is one way to reduce the harmful effects of graphene on cells. Applying nanoparticles implies their interaction with the immune system, which protects the body. Monocytes are innate immunity cells and the first line of defence of the human organism from microorganisms and other alien objects. One of the monocytes' reactions to a stimulus of any nature is to produce reactive oxygen species (ROS). Published data shows an incomplete picture of modified graphene oxide nanoparticles' effects on ROS formation by human monocytes. Thus, it was essential to evaluate the pegylated graphene oxide (GO-PEG and GO-8armedPEG) effect on ROS production by human monocytes, assessed by the luminol-dependent chemiluminescence (LCL). The objects of the study were $CD14^+$-cells isolated from mononuclear cells of healthy donors. ROS production was stimulated by opsonized zymosan (OZ), spontaneous LCL was used as a control. PEG-modified (GO-PEG and GO-8armedPEG) GO nanoparticles with sizes of 100-200 nm ("small") and 1-5 μm ("big") with PEG covering ~ 20% were used at concentrations of 5 and 25 μg/ml.

The study showed that small size nanoparticles at a low concentration of 5 μg/ml and big nanoparticles coated with 8-armed PEG at both concentrations have a significant suppressive effect on spontaneous ROS production. In the stimulated LCL reaction variant, it was found that small nanoparticles (25 μg/ml) also have a suppressive effect on ROS production, such as big-sized particles coated with linear PEG at the same concentration. Thus, we have established for the first time that graphene oxide nanoparticles functionalized with PEG are capable of inhibiting the ROS production by human monocytes, and therefore, we can speak of the antioxidant activity of GO-PEG.

Keywords: graphene oxide, surface modification of nanoparticles, polyethyleneglycol, luminol-dependent chemiluminescence, monocytes, reactive oxygen species

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Introduction

Graphene is a lightweight two-dimensional material made from a monatomic carbon layer. Graphene and its modifications, particularly graphene oxide (GO), are currently being investigated for use in various biomedicine fields [9, 10]. However, the GO cytotoxicity and proinflammatory effects are the factors limiting the use of GO-based drugs and hinder the translation of scientific developments into practice. It is known that the surface modification (functionalization) of GO nanoparticles using the polymer-adsorbed particles is one of the ways to reduce the harmful graphene-related cell effects [2, 3]. In particular, polyethylene glycol (PEG) is the most common material used for functionalization of GO nanoparticles. In general, the use of graphene-based materials in medicine requires a thorough assessment of their biocompatibility and a detailed understanding of their interaction with immune cells.

Our work was focused on studying the modified GO preparations effect on the formation of free radicals and reactive oxygen species (ROS) by mono-

cytes, which play an important role in the body nonspecific immune defense. Scarce data on how GO nanoparticles modulate the functions of human monocytes are available. Nevertheless, it is known that GO nanoparticles could reduce the viability THP-1 monocytic line cells, whereas the GO functionalization via the amino groups (GO-NH_2) significantly reduced graphene cytotoxicity. Simultaneously, GO, and GO-NH_2 nanoparticles increased the level of proinflammatory intracellular cytokines (TNFα, MIP-1β, IL-6) in human monocytes. [11, 14]. In 2020, it was evidenced that GO, coated with $AgInS_2$ crystals, increased the level of TNFα and MIP-1β in human monocytes [5]. Thus, **our work aimed** to study PEG-modified GO nanoparticle effect on the oxidative activity of human monocytes, assessed by luminol-dependent chemiluminescence (LCL).

Materials and methods

The study was carried out in accordance with the 2000 Helsinki Declaration of the WMA and the protocol of the 1999 Council of Europe Convention on Human Rights and Biomedicine; permission from the Ethics Committee of the Institute of Ecology and Genetics of Microorganisms of the Ural Branch of the Russian Academy of Sciences (IRB00010009) dated of 30.08.2019 was obtained to use peripheral blood samples.

We used linear and armed polyethylene glycol (PEG and PEGarm, respectively)-based coated GO nanoparticles sized of 100-200 nm ("small", GO-S) and 1-5 μm ("big", GO-B) (Ossila Ltd, Great Britain). For functionalization procedures, we used monochloroacetic acid (99%), 1-Ethyl-3- (3-dimethyl aminopropyl) carbodiimide (EDC, 98%), N-hydroxysuccinimide (NHS, > 98%), 8-arm-polyethylene glycol- (tripentaerythritol core) -NH_2 (8armPEG-NH_2, Mw 10,000 g·mol^{1}), methoxy polyethylene glycol amine mPEG-NH_2 (MW 5 kDa), manufactured by Alfa Aesar, USA. All reagents were used without additional purification. GO modification with linear PEG and branched 8armPEG was carried out by using the covalent attachment of amino groups PEG-NH_2 and 8armPEG-NH_2 to the surface carboxyl groups of GO with the formation of an amide bond. For this, GO-B and GO-S solutions (2 mg/ml) were sonicated for 30 min at 25 W and 150 W, respectively. GO carboxylation was carried out in an alkaline medium (NaOH) in the presence of Cl-CH_2-COOH with ultrasonic treatment for 1 hour. The resulting GO-COOH solution was neutralized by repeated washing with deionized water. Next, NHS (10 mmol/L), EDC (4 mmol/L), and PEG-NH_2 (or 8armPEG-NH_2) (2 mg/ml) were added to the suspension of GO-COOH (pH 5.6) under ultrasonic treatment for 5 min., to complete the reaction of PEG covalent crosslinking, the solution was left at room temperature for 24 hrs. The resulting suspensions of GO-PEG or GO-8armPEG were purified by dialysis and triple washing by centrifugation with ethyl alcohol with final drying at 65 °C under vacuum.

Fourier-transform infra-red spectra of the initial and modified GO were obtained on an IFS 66/S Bruker spectrometer in the range 400-4000 cm^{-1}. Samples for analysis were prepared by pressing KBr tablets (2 mg sample to 299 mg KBr). The absorption spectra of the initial and modified GO solutions were determined on a UV 2600 double-beam spectrophotometer in the wavelength range of 200-900 nm. The size distribution of the initial and modified GO, as well as their zeta potential in aqueous solutions, was determined by dynamic light scattering on a ZetaPALS Brookhaven instrument. Thermogravimetric analysis of the initial and modified GOs was carried out on a TGA/DSC 1 Mettler-Toledo combined TG-DSC instrument at a heating rate of 10 K min^{-1} within the temperature range 30-900 °C in an inert atmosphere.

The studies of the pegylated GO samples showed that the chemical modification process was successful regardless of the size of the graphene particles, the aromatic structure of GO did not change, and the average size of pegylated GO nanoparticles decreased compared to the initial parameters declared by the manufacturer. The characteristics of the obtained particles are presented in Table 1.

Here we examined peripheral blood mononuclear cells (PBMC) obtained from apparently healthy donors (n = 7) isolated by density gradient centrifugation (1.077 g/cm^3) (Diacoll, Dia-M, Russia). The cells were then washed from serum three times by centrifugation at 350 g for 20 minutes using RPMI-1640 medium (Sigma, USA). PBMC-derived $CD14^+$-cells were isolated by the immunomagnetic separation

TABLE 1. CHARACTERISTICS OF GO-PEG NANOPARTICLES USED IN THE WORK

	OG-PEG-S	OG-PEGarm-S	OG-PEG-B	OG-PEGarm-B
Average effective diameter, nm	184±73	287±52	569±14	1376±48
Polydispersion index	0.25±0.02	0.23±0.02	0.21±0.02	0.30±0.01
Zeta-potential, mV	-31.70±1.70	-34.28±0.41	-39.98±1.17	-53.56±1.23
Coating degree, weight %	17.2±1.4	20.5±1.8	19.4±2.2	20.5±1.1

based on the MACS® technology (Miltenyi Biotec, Germany). The isolated cells were adjusted to a concentration of 10^6 cells per ml dissolved in Hanks's solution (Biolot, Russia).

The LCL reaction was carried out in the wells of a 96-well sterile plate (Nunc, Denmark) added with Hanks's solution (Biolot, Russia), graphene nanoparticles in various pharmacological concentrations (5 μg/ml; 25 μg/ml; control – spontaneous LCL), 10 μL of cell suspension and the luminol sodium salt (Sigma-Aldrich, USA) at a concentration of 20 μM [7]. In addition, pooled inactivated human serum was added to the samples to a final 10% concentration. Opsonized zymosan (OZ) acted as an inductor of oxygen burst added to a final concentration of 1.5 μg/ml.

The luminescence intensity was measured for 90 minutes with an interval of 3 minutes on a Synergy H1 hybrid reader (BioTek). The dynamic change in luminescence within 90 minutes at 37°C was assessed, and the integral indicator was calculated: the light sum (S), i.e., area under the chemiluminescence curve characterizing the total ROS synthesis in 90 min of the study and equal to the sum of all values of the luminescence intensity for each sample.

The percentage of live and dead cells was assessed by staining with propidium iodide (Thermo Fisher Scientific, USA) followed by analysis on flow cytometer (CytoFlexS, Beckman Coulter, USA). The viability of monocytes in the presence of nanoparticles averaged 96% of the total number of $CD14^+$-cells.

Statistical data processing was performed by using GraphPad Prism 8 software in Friedman's test and Dunn's posthoc test for multiple comparisons.

Results and discussion

One of the key functions of monocytes is the ROS generation, which can occur when cells interact with nanoparticles of almost any nature. Thus, cell stimulation is accompanied by the superoxide anion, hydrogen peroxide, hydroxyl radical, and chlorine active form generation, which have a potent bactericidal effect; and the LCL reaction characterizes the total ROS production by isolated monocytes.

The study showed that small size nanoparticles (GO-PEG-S and GO-PEGarm-S) at a low concentration of 5 μg/ml have a significant suppressive effect on spontaneous ROS production. Interestingly, a high concentration (25 μg/ml) of small nanoparticles (GO-PEG-S and GO-PEGarm-S) did not affect this parameter. It was shown that big nanoparticles also suppressed ROS production, while the effect was exerted only by particles coated with armed PEG (GO-PEGarm-B) at both concentrations (Table 2).

I case of the stimulated ROS production, small nanoparticles (GO-PEG-S and GO-PEGarm-S) were also found to exert a suppressive effect, which, however, was evident after using a high concentration of particles (25 μg/ml) only. Only nanoparticles coated with linear PEG at a high concentration (GO-PEG-B, 25 μg/ml) displayed a significant suppressive effect among the large-sized particles.

There are various possible ways of the GO nanoparticle effect on LCL. First, GO nanoparticles can directly interact with ROS and luminol, changing the intensity of LCL, and secondly, GO can affect the ROS production by monocytes. Since GO and GO-PEG reduced the LCL in blood monocytes, we assumed that it might be accounted for by an effect of

TABLE 2. GO-PEG EFFECT ON THE OF SPONTANEOUS AND ZYMOSAN-STIMULATED CHEMILUMINESCENCE LIGHT SUM (n = 7), Me ($Q_{0.25}$-$Q_{0.75}$)

	Control	GO-PEG-S (μg/ml)		GO-PEG-arm-S (μg/ml)		GO-PEG-B (μg/ml)		GO-PEG-arm-B (μg/ml)	
		5	25	5	25	5	25	5	25
Light sum	Spontaneous LCL (90 min incubation)								
	419 (417-521)	265 (254-307) p < 0,05*	312 (285-358)	259 (256-305) p < 0,05*	313 (300-359)	285 (263-324)	343 (333-404)	265 (244-311) p < 0,05*	271 (258-313) p < 0,05*
	OZ-stimulated (1.5μg/ml) LCL (90 min incubation)								
	4354 (3848-4812)	4026 (3757-4234)	2426 (2256-2653) p < 0,05*	4111 (3863-4498)	2538 (2249-2674) p < 0,05*	4190 (3903-4383)	2585 (2335-2871) p < 0,05*	3981 (3611-4220)	3133 (2782-3287)

Note. * P < 0.05, significant differences according to Friedman's test and Dunn's test for multiple comparisons compared to the control. Control, sample without GO-PEG (first column).

particle-driven quenched luminescence, which is not associated with its impact on cells. For this, we used a cell-free model of LCL, where hydrogen peroxide was used as a luminescence inductor. As a result, the effect of luminescence quenching by GO-PEG nanoparticles was not revealed [12].

Thus, it has been established that GO nanoparticles coated with linear and armed PEG have, first of all, suppressive effects on human monocyte ROS production. Interestingly enough, to enable such an effect, the concentration of small nanoparticles was primarily critical, and the type of pegylation was not of fundamental importance. However, for large nanoparticles the type of pegylation was of fundamental importance. Thus, at the level of spontaneous monocyte ROS production, only armed PEG-coated GO "were active". Simultaneously, in zymosan-stimulated samples, the suppressive effect was conferred if the particles were coated only with linear PEG. In general, it is quite interesting that the overall pattern of effects was suppressive, but the nuances depended on the size, concentration, and type of nanoparticle pegylation.

Thus, we have established for the first time that graphene oxide nanoparticles functionalized with PEG are capable of inhibiting the ROS production by human monocytes, and therefore, we suggest GO-PEG-related antioxidant activity.

Specific response of monocytes, macrophages, and neutrophils to a stimulus is coupled to increased ROS production called "oxygen burst", which is aimed at destroying foreign objects. However, the data on the antioxidant activity of GO are contradictory. In 2019 studies, it was shown that GO exhibits low antioxidant activity against H_2O_2 and OH*, while Qui et al. considered about high antioxidant activity of GO (10 μg/ml) to OH*, as well as its ability to neutralize the superoxide anion (at a concentration of about 80 μg / treated / untreated ml) [4, 8, 13]. Chemical modification of GO (doping with nitrogen, the introduction of various functional groups) significantly increases its antioxidant activity, in this regard, it can be assumed that treatment with PEG affects the neutralization of ROS [6]. However, in the study by Nilewski et al., pegylation did not significantly affect the antioxidant activity of GO to the superoxide anion [1]. At the same time, our study allowed to demonstrate that the type of pegylation is essential for conferring GO antioxidant effect.

In general, the data we obtained expand the knowledge about modified forms of GO nanoparticles in the context of their antioxidant activity, and demonstrate for the first time the data on the interaction between GO-PEG and monocytes affecting formation of reactive oxygen species therein.

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