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Mendeleev Commun., 2023, **33**, 790–792

Mendeleev Communications

Non-covalent conjugate based on graphene oxide and a cytotoxic agent containing 1,3,5-triazine derivative and (5-phenyl-2*H*-tetrazol-2-yl)acetoxy moiety: synthesis, characterization, properties and cytotoxic activity

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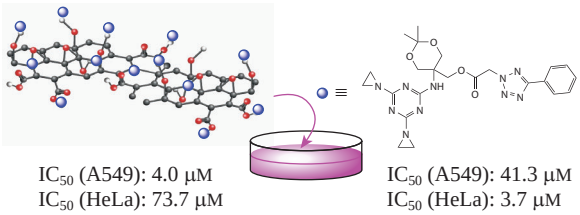
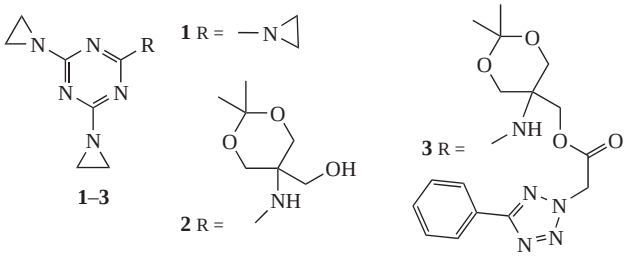
DOI: 10.1016/j.mencom.2023.10.017

The synthesis, identification and evaluation of the biological activity of a new non-covalent conjugate based on graphene oxide and (5-{[4,6-di(aziridin-1-yl)-1,3,5-triazin-2-yl]-amino}-2,2-dimethyl-1,3-dioxan-5-yl)methyl (5-phenyl-2*H*-tetrazol-2-yl)acetate were carried out. It was shown that the synthesized nanoconjugate is hemocompatible over the entire studied concentration range (10–100 μM) and demonstrates antiradical activity under irradiation. In addition, the conjugate exhibits cytotoxicity against A549 and HeLa cell lines with IC_{50} values of 4.0 and 73.7 μM, respectively.



Keywords: graphene oxide, 1,3,5-triazine, tetrazole, hemolysis, cytotoxicity.

One of the ways to increase the specificity and biocompatibility of cytostatic agents is to obtain conjugates of drugs with carbon nanoforms.[1–3] To solve this problem, various nanocarriers can be used, such as fullerenes, nanodiamonds, graphene and its forms, carbon nanotubes, as well as polymer structures, liposomal forms, *etc*.[1–6] Graphene and its oxidized form, graphene oxide (GO), have become promising materials in the field of nanomedicine due to their chemically reactive and developed surface, which can undergo covalent and non-covalent functionalization to ensure the immobilization of drugs.[2,7–9] A large number of studies have been published on the synthesis of GO-based conjugates with various cytostatic drugs.[8,10–13] It has been shown that some 2,4,6-substituted 1,3,5-triazine derivatives, such as tretamine **1**, (5-{[4,6-di(aziridin-1-yl)-1,3,5-triazin-2-yl]amino}-2,2-dimethyl-1,3-dioxan-5-yl)methanol **2** and (5-{[4,6-di(aziridin-1-yl)-1,3,5-triazin-2-yl]amino}-2,2-dimethyl-1,3-dioxan-5-yl)-methyl (5-phenyl-2*H*-tetrazol-2-yl)acetate **3**, exhibit an antitumor effect due to DNA alkylation.[14–19]



An *in vitro* study showed an antitumor effect of compound **3** against A549, PA-1, T98G and SK-HEP-1 cell lines.[20] To reduce or minimize some of the side effects observed during therapy with compound **3**,[21] it may be promising to conjugate it to a carrier. In this work, nanoform GO-**3** based on GO and compound **3** was synthesized. Conjugate GO-**3** was characterized by elemental analysis, XRD, XPS and solid-state ^{13}C NMR spectroscopy. The morphology of nanoparticles was determined using scanning electron microscopy (SEM). The thermal stability of the samples was studied by thermogravimetric analysis.

The synthesis of conjugate GO-**3** was carried out according to previously published methods.[†,12,22,23] In the ^{13}C NMR spectrum, the signal at 98 ppm refers to the quaternary carbon atom in the dioxane fragment of compound **3**, and the signal at 130 ppm is associated with the C=C structural fragment of the graphene plane. Signals at 163 and 29 ppm are caused by the carbon atoms of the tetrazole and aziridine rings in compound **3**, respectively, while the resonance at 19 ppm corresponds to the methyl groups of the dioxane fragment of compound **3** (see Figures S1–S3).

[†] *Synthesis of* GO-**3**. GO powder (0.5 g) was dispersed in an alkaline (pH 9) medium (40 ml) by sonication for 25 min. Then, an aqueous solution of compound **3** (0.5 g, 0.98 mmol) was added to the resulting mixture. For compound **3**, all characteristics are consistent with the observed data (Figures S1–S6, see Online Supplementary Materials). The reaction mixture was kept in an ultrasonic bath for 80 min. The precipitate of conjugate GO-**3** was separated from the reaction mixture, washed with methylene chloride and then with deionized water. The resulting GO-**3** was dried at 38 °C for 12 h and characterized by elemental analysis. Found (%): C, 59.7; O, 25.4; N, 15.1.

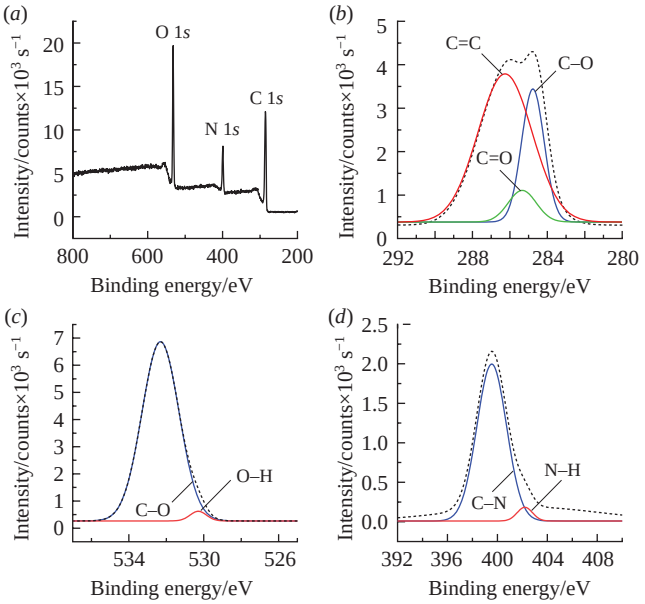


Figure 1 (*a*) XPS spectrum of GO-**3** and deconvolution of the (*b*) C 1*s*, (*c*) O 1*s* and (*d*) N 1*s* peaks.

According to the elemental analysis of GO-**3**, the loading of compound **3** is 1.07×10^{-3} mol per 1 g of GO or 54.8 wt%. In addition, to confirm the loading of compound **3**, the drug loading efficiency was also calculated using equation S1 (see Online Supplementary Materials). The obtained value of 55 wt% is consistent with the result of elemental analysis.

The XPS spectrum of GO-**3** contains C 1*s* (285 eV), O 1*s* (531 eV) and N 1*s* (399 eV) peaks [Figure 1(*a*)]. Figure 1(*b*) shows the result of deconvolution of the C 1*s* peak into four peaks corresponding to binding energies of 286 eV (C=C groups of aromatic domains on the graphene surface), 285 eV (C=O groups of the graphene framework) and 284 eV (C–O groups). Deconvolution of the oxygen 1*s* peak [Figure 1(*c*)] revealed a signal at 532 eV, attributed to the C–O fragment in the composition of epoxy, carboxyl and hydroxyl groups on the GO surface, and a signal at 530 eV related to the O–H fragment of the carboxyl and hydroxyl groups of GO.[24] Figure 1(*d*) demonstrates the result of deconvolution of the N 1*s* peak, which consists of peaks at 402 eV (the C–N fragment of the aziridine group of compound **3**) and at 399 eV (the N–H fragment of compound **3**).

The X-ray diffraction pattern of nanoconjugate GO-**3** is a superposition of individual patterns of GO and compound **3** and contains peaks at 2θ 10.825°, corresponding to the GO 001 plane, and peaks at 2θ 7.6°, 9.2°, 18.2°, 22.3° and 28.0°, which agrees with the values obtained for compound **3**.

The Raman spectrum contains the D, G and 2D bands. If in the case of single-layer graphene the I_{2D}/I_G ratio is 2, then for conjugate GO-**3** this ratio turned out to be equal to 1.85. Thus, the synthesized nanoconjugate has a predominantly single-layer structure.[25] The I_D/I_G ratio makes it possible to estimate the degree of functionalization of the GO surface (Figure 2). Analysis of the Raman spectrum in Figure 2 shows the presence of D and G bands at 1344 and 1562 cm^{-1}, respectively, and the I_D/I_G ratio is 0.84. These data are consistent with the previously obtained values for GO enriched in oxygen-containing functional groups.[26] This fact, in turn, confirms that functionalization has occurred.

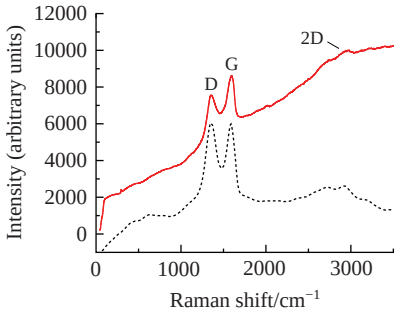


Figure 2 Raman spectra of GO-**3** (solid line) and GO (dashed line).

As was demonstrated by DLS measurements, in an aqueous dispersion GO-**3** forms associates with an average size of 90–100 nm, and their size does not depend on the concentration. At the same time, the average values of ζ-potentials for such dispersions range from −42 to −40 mV, which may indicate the stability of GO-**3** in aqueous dispersion.

The SEM image of GO-**3** (Figure S7) shows the distribution of molecules of compound **3** on the surface of GO. It can also be seen that GO flakes are individual domains with lateral sizes from several hundreds of nanometers to several microns and a thickness of several nanometers. The results obtained confirm the functionalization of GO.

To assess the hemocompatibility of GO-**3**, its effect on spontaneous hemolysis was studied. The effect of compound **3** and GO-**3** on hemolysis was determined by measuring the released hemoglobin. It was found that compound **3** and GO-**3** (Figure 3), when incubated for 1 and 3 h, caused insignificant hemolysis in the studied concentration range, while the intensity of hemolysis depended on the dose and time. It is generally accepted that nanosystems are hemocompatible if the hemolysis rate does not exceed 5%.[27]

In addition, compared with the control, GO-**3** inhibited hemolysis induced by Radachlorin, which was manifested in an increase in the time of hemolysis of 50% of erythrocytes. Based on the data obtained, it can be concluded that GO-**3** exhibits antioxidant activity, which is dose-dependent.‡

The cytotoxic activity of compound **3** and GO-**3** against A549 and HeLa tumor cell lines was studied *in vitro* using the MTT assay. Analysis of the obtained data on the cytotoxicity of compound **3** and GO-**3** shows a dose-dependent decrease in the survival of A549 and HeLa cells (Figure 4). The cytotoxic activity (IC_{50} values) of compound **3** and conjugate GO-**3** was 41.3 and 4.0 μM against A549 cells and 3.7 and 73.7 μM against HeLa cells, respectively.

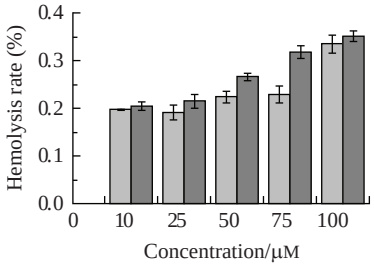


Figure 3 Concentration dependence of spontaneous hemolysis rate calculated as $[(A_{test} - A_{control})/A_{100}] \times 100\%$ in the presence of GO-**3** after 1 h (light gray) and 3 h (dark gray).

‡ The study of photoinduced hemolysis in the presence of GO-**3** was carried out according to a known procedure.[28] Erythrocytes were obtained from citrated blood by centrifugation at 1500 rpm for 10 min, followed by three washings with saline. The cells were then stabilized at 4 °C for at least 24 h in Alsever's reagent, consisting of sodium chloride (0.42%), citric acid (0.055%), sodium citrate (0.8%) and D-glucose (2.05%). Before use, erythrocytes were washed three times from Alsever's reagent with physiological saline, and a standard cell suspension was prepared in PBS (pH 7.4). The optical density of the standard suspension after eightfold dilution with PBS was 0.560 ± 0.020 at 800 nm. The measurements were carried out on an SF-2000 spectrophotometer (Russia) in a cuvette with an optical path length of 1 cm. The cytolytic activity of GO-**3** was recorded by registering a decrease in the optical density of the cell suspension at 800 nm at five-second intervals until complete hemolysis.

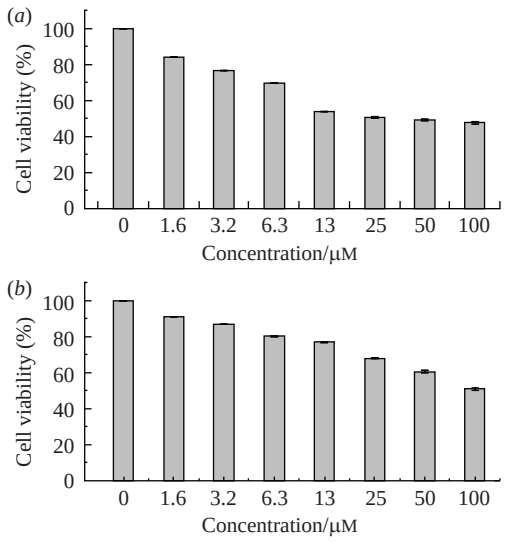


Figure 4 Effect of GO-**3** on (*a*) A549 and (*b*) HeLa cells lines.

Thus, a non-covalent conjugate of GO and (5-{[4,6-di(aziridin-1-yl)-1,3,5-triazin-2-yl]amino}-2,2-dimethyl-1,3-dioxan-5-yl)-methyl (5-phenyl-2*H*-tetrazol-2-yl)acetate **3** was synthesized and characterized. The loading of compound **3** is 55 wt%. The resulting nanomaterial is hemocompatible over the entire range of concentrations (10–100 μM) and exhibits cytotoxicity against A549 and HeLa cell lines. Comparison of cytotoxic activity values with previously published results for a non-covalent conjugate based on GO and unsubstituted 1,3,5-triazine (1,3,5-triazine loading 62 wt%) showed comparable cytotoxic activity against A549 and HeLa cell lines.[22]

This work was supported by the Ministry of Health of the Russian Federation within the framework of the project 'Creation and evaluation of the antitumor activity of conjugates of unannelated 1,3,5-triazinyl-tetrazoles with targeted delivery molecules to targets in the tumor cell microenvironment'. Research was performed using the equipment of the Center for Optical and Laser Materials Research, the Research Center for X-ray Diffraction Studies, the Magnetic Resonance Research Center, the Center for Physical Methods of Surface Investigation, the Thermogravimetric and Calorimetric Research Center, the Computer Center of SPbU and the Center for Chemical Analysis and Materials Research of the Research Park of Saint Petersburg State University.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2023.10.017.

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Received: 8th June 2023; Com. 23/7187