Article

Novel Structures of Functionalized Graphene Oxide with Hydrazide: Characterization and Bioevaluation of Antimicrobial and Cytocompatibility Features

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Citation: Zarafu, I.; Limban, C.; Radulescu, C.; Dulama, I.D.; Nuta, D.C.; Chirita, C.; Chifiriuc, M.C.; Badiceanu, C.D.; Popa, M.; Bleotu, C.; et al. Novel Structures of Functionalized Graphene Oxide with Hydrazide: Characterization and Bioevaluation of Antimicrobial and Cytocompatibility Features. *Coatings* **2022**, *12*, 45. <https://doi.org/10.3390/coatings12010045>

Received: 28 October 2021

Accepted: 28 December 2021

Published: 31 December 2021

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Abstract: Graphite was oxidized to graphene oxide and activated by thionyl chloride, for further covalently linking three hydrazides with potential biological activity. The obtained materials were characterized by scanning electron microscopy with energy dispersive spectroscopy, Fourier-transform infrared and Raman spectroscopies. The presence of various functional groups specific to graphene oxide (GO) functionalized with different hydrazides was confirmed by spectral data. The ratio between D- and G-bands, observed in Raman spectra, allowed for an evaluation of the disorder degree and the mean crystallite size of the samples. The micrographs highlighted that the samples lead to the occurrence of disorders, probably caused by the sp^3 carbons, the formation of oxygen-containing functional groups in the basal planes, and by various structural defects. The new graphene oxide–hydrazide derivatives were tested for their antimicrobial and cytotoxicity activity. Their antimicrobial activity against planktonic and biofilm-embedded cells was inferior to that of free hydrazides, except for GO-3 against planktonic *Escherichia coli* and GO-2 against *Pseudomonas aeruginosa* biofilm, demonstrating that further optimization is needed to be able to exploit the huge potential of GO for developing potent antimicrobials.

Keywords: hydrazide; graphene oxide; functionalization; antimicrobial activity; cytotoxicity activity

1. Introduction

The rising prevalence of multi-drug resistant microbial infections has become a serious health care problem. For this reason, the search for new antimicrobial drugs will remain a

challenging task for medicinal chemistry. In the search for materials with improved biological activity, one of the major options is the association, through different physicochemical processes, of well-known antibacterial agents with various inert materials, like silica [1], cellulose [2], nanoparticles (including graphene) [3], gold nanoparticles, [4] and so on. In this paper, we report the synthesis, characterization, and antimicrobial activity of obtaining novel graphene oxide–hydrazide derivatives, combining the same multiple-molecule pharmacologically active compounds with previously demonstrated antimicrobial potential. Graphene oxide is a 2D material that can be easily obtained in large amounts, has a low toxicity, and, according to previous studies, might exhibit an inherent antibacterial effect. Graphene oxide (under the name of graphitic oxide) has been known since 1958 [5], but only later was graphene (the reduced form of graphene oxide) fully characterized and its astonishing properties were discovered [6]. Graphene oxide is an insoluble material of nanometric size harboring a large number of hydrophilic, oxygen-containing groups, like carboxyl, keto, hydroxyl, and epoxy, allowing for easy functionalization by different means, including covalent binding. Recent studies show that graphene oxide-based materials, due to their high porosity and surface area, may facilitate the microbial adherence and further development of microbial biofilms [7,8]. This could have positive consequences in both the biotechnological and bioremediation fields [9–12], or a negative impact for the biomedical field, taking into account that the biofilm cells can be up to 1000–4000 times more resistant than their planktonic counterparts.

Hydrazides are a well-known class of biologically active organic compounds, in which at least one hydrogen atom of hydrazine is replaced by an acyl group, the most well known being the tuberculostatic agent isoniazid (isonicotinic acid hydrazide, INH; pyridine-4-carbohydrazide) [13–19]. The hydrazide chain seems to be an attractive pharmacophore in medicinal chemistry, especially for the development of novel antimicrobials [20]. It has been shown that the presence of certain structural fragments in hydrazide molecules as substituents influences the biological and pharmacokinetic properties of these compounds. For example, the hydrazides bearing benzimidazole and thiophene nuclei in their structure exhibited large-spectrum antimicrobial activities against methicillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, and *Eberthella typhosa* [21]. The presence of a long-chain alkyl group in the case of *N'*2,*N'*5-bis((1-decyl-1*H*-benzoimidazol-2-yl)methyl)-3,4-dimethylthiophene-2,5-dicarbohydrazid increased anti-*Staphylococcus aureus* activity, probably by increasing the lipophilicity, which makes it easier to penetrate the bacterial cell membrane.

The sulfonamide group is present in the structure of compounds with antimicrobial, as well as other biological properties, such as diuretics [22,23], anticonvulsants [24], or carbonic anhydrase inhibitors [25]. The 4-(benzensulfonamido)benzoic acid hydrazide was used as an intermediate compound in the synthesis of pharmacologically active *N*-[4-[(phenylcarbamoyl)amino]phenyl]benzenesulfonamide derivatives [26]. The 2-furancarboxylic acid hydrazide was the key intermediate for the synthesis of a new Pd complex with antibacterial activity [27].

Quinazolin-4-ones are the building blocks of many natural and synthetic products that have a wide range of biological activities. The stability of the quinazolin-4-one nucleus allows the introduction of several bioactive moieties in different positions to synthesize new potential therapeutic agents [28]. The 2-(2-methyl-4-oxoquinazolin-3(4*H*)-yl)-acetohydrazide exhibited bioactivity and low genotoxicity in the *Triticum* assay [29].

This study reports the synthesis of new architectures of graphene oxide–hydrazide derivatives and the evaluation of their influence on the growth of free planktonic microorganisms and biofilm-embedded cells. Although these new compounds are derivatives of known graphene oxide structures, the evaluation of their antimicrobial features against *E. coli*, *P. aeruginosa*, *S. aureus*, and *Enterococcus faecalis* planktonic cells and biofilms has not been reported before.

2. Materials and Methods

All chemicals and reagents for synthesis and analyses were purchased from commercial suppliers (MilliporeSigma, Burlington, MA, USA and Chimopar, Bucharest, Romania, respectively) and were of the highest available purity.

2.1. Synthesis of Graphene Oxide

Graphene oxide was synthesized by the well-known Hummers method, with slight modification [5,30–33]. This method was chosen because it is the fastest and most scalable, and provides good quality products. The preparation technique involves oxidation of graphite powder with strong oxidant potassium permanganate (KMnO_4) in concentrated sulfuric acid (H_2SO_4), producing a dark-yellow-to-brown powder with high yields. The degree of oxidation is quite high, providing a lot of carboxyl groups required in the next step. Thus, under external ice-cooling, 5 g of graphite and 2.5 g of sodium nitrate (NaNO_3) were carefully added to 125 mL of cold sulfuric acid, and the mixture was vigorously stirred. After few minutes, a total of 15 g of potassium permanganate was added in small portions, in about 30 min. The mixture was stirred continuously for another hour without cooling, and then 250 mL of cold water was added slowly.

During water addition, the temperature increased to about 90 °C and the mixture was kept at this temperature for another 15 min. Another 500 mL of water was added and the mixture was left to cool to room temperature, then hydrogen peroxide (H_2O_2) was added until the disappearance of excess potassium permanganate. The next day, the supernatant was removed, and the slurry was washed several times with diluted hydrochloric acid, followed by methanol (CH_3OH). Finally, the solid was dried under vacuum at 60 °C.

2.2. Functionalization of Graphene Oxide with Hydrazides

Knowing the importance of hydrazide in therapeutics, as well as other pharmacophores (e.g., sulfonamide group, furan moiety, or quinazoline-4-one nucleus) [34], in this study new compounds, such as 2-furancarboxylic acid hydrazide (H1), 4-(benzensulfonamido) benzoic acid hydrazide (H2), and 2-(2-methyl-4-oxoquinazolin-3(4H)-yl)-acetohydrazide (H3) (Figure 1) were synthesized. These compounds were previously used as intermediates for the synthesis of new compounds with antimicrobial potential. In addition, these compounds chains were grafted on graphene oxide (GO) to obtain various functionalized graphene oxide-based hydrazides (Figure 2), intended for biomedical applications with promising performance as anti-biofilm agents.

Figure 1. Hydrazides used for the synthesis of new functionalized graphene oxide: GO-1 graphene oxide functionalized with H1 (i.e., 2-furancarboxylic acid hydrazide); GO-2 graphene oxide functionalized with H2 (i.e., 4-(benzensulfonamido)benzoic acid hydrazide); GO-3 graphene oxide functionalized with H3 (i.e., 2-(2-methyl-4-oxoquinazolin-3(4H)-yl)-acetohydrazide).

On the other hand, it has been used in the two-step synthesis method to covalently link the organic compounds to graphene oxide, as previously reported [31]. In the first step, the activation of carboxyl groups is made by thionyl chloride, and the material reacts further with the hydrazides (Figure 2).

Figure 2. Synthesis of functionalized graphene oxide (* is marks the linkage position) with hydrazides.

An amount of 0.5 g of dried graphene oxide was suspended into 30 mL dichloroethane (DCM), to which a few drops (about 0.1 mL) of dimethyl-formamide (DMF) and 3 mL of thionyl chloride were added. The mixture was refluxed for 1–2 h, filtered off on a G3-sized sintered glass filter, the solid mass was washed with a large amount of solvent (i.e., DCM). The obtained solid was further suspended into 30 mL DCM, to which 0.25 g of hydrazone and 2.5 mL of triethylamine were added. The mixture was left overnight, and then the collected solid was washed with DCM and methanol, centrifuged, and further dried in an open atmosphere, yielding about 0.5 g of black solid in all cases. For agitation, a classical magnetic stirrer was employed using dry solvents in the reaction with thionyl chloride; no inert atmosphere is required (compulsory) in such reactions.

2.3. Characterization of Functional Materials

2.3.1. FE-SEM-EDS

Scanning electron microscopy with energy dispersive spectroscopy (SEM-EDS) results were obtained using a SU-70 microscope (Hitachi, Ibaraki, Japan) coupled with an UltraDry detector (Thermo Fisher Scientific, Waltham, MA, USA). The SU-70 microscope operates under high-vacuum conditions, and due to the field-emission (FE) it reveals the uniform dispersion of electrons on sample surfaces, allowing it to obtain high-resolution images. The SEM images were obtained under a 5–10 kV accelerating voltage and a 16–21 mm working distance range, meanwhile the EDS data (EDS spectra and elemental composition) were obtained under a 15 kV accelerating voltage using the Phi-Rho-Z correction method available in NSS software (Version 3.0, Thermo Fisher Scientific, Waltham, MA, USA).

2.3.2. ATR-FTIR Spectroscopy

Fourier-transform infrared spectroscopy (FTIR) was performed using a Vertex80 spectrometer (Bruker Optik GmbH, Billerica, MA, USA) equipped with a diamond attenuated total reflection (ATR) crystal accessory. All spectra were recorded in transmittance mode (32 scans/sample) in the range of $4000\text{--}400\text{ cm}^{-1}$, with 0.2 cm^{-1} spectral resolution and 0.1% T accuracy. The samples were analyzed without any preparation and the data were collected and processed by Opus software (Version 7.0, Bruker Optik GmbH, Billerica, MA, USA).

2.3.3. Raman Spectroscopy

Raman spectra were recorded with a Xantus-2™ Raman analyzer (Rigaku, Boston, MA, USA) equipped with two laser sources (785 and 1064 nm) and two detectors (i.e., TE-cooled CCD and InGaAs). For this study, the following parameters were used: 1064 nm excitation source, 50 mW laser power, 500 ms integration time, and 5 scans/spectra. The spectral range was 200–2000 cm^{-1} , with 15–18 cm^{-1} spectral resolution. Xantus-2™ reduces intrinsic fluorescence issues and offers an extensive range of analysis capabilities.

2.4. Antimicrobial Activity Assay

The antimicrobial activity of the new compounds was assessed on Gram-positive (*S. aureus* ATCC 25923, *E. faecalis* ATCC 29212) and Gram-negative (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) bacterial reference strains from the American Type Culture Collection (ATCC), using stock solutions of 10 mg/mL prepared in dimethyl sulfoxide (DMSO). All antimicrobial activity assessments were performed in a Mueller–Hinton broth medium, a rich culture medium recommended by international guidelines (CLSI, EUCAST, etc.) to be used for antimicrobial susceptibility testing assays.

The efficiency of the tested compounds was assessed against the free floating planktonic cells by using the broth microdilution method, allowing the establishment of the minimum inhibitory concentration (MIC), as well as against biofilm-embedded cells through the violet crystal microtiter method, allowing for the establishment of the minimal biofilm eradication concentration (MBEC) [35]. For this purpose, serial two-fold dilutions varying from 5 to 0.009 mg/mL were performed in 96-well microplates to which the bacterial suspension in a final density of 10^5 CFU/mL (colony forming units) was added, and then, the plates were incubated at 37 °C for 24 h. The positive growth control was represented by the bacterial culture developed in the absence of the tested substance, while the negative growth control was represented by the sterile broth. After incubation, a volume of 5 μL of each well was spread on agar plates and incubated at 37 °C for 24 h to determine the MIC and MBC (minimal bactericidal concentration) values. After sampling the wells for determining the MIC/MBC values, the microplates were emptied and gently washed to remove the non-adherent bacteria, and then the biofilm formed on the plastic well was fixed by methanol, stained by violet crystal, and then resuspended by acetic acid 33%. The optic density of the colored suspension was read at 490 nm, the MBEC corresponding to the lowest concentration inhibiting the biofilm development in comparison with the positive growth control [36].

2.5. Biocompatibility Assays

2.5.1. Human Cellular Viability Assay

In order to determine the number of viable cells that actively proliferate, the CellTiter 96® AQueous One Solution Cell Proliferation Assay was used. The epithelial cells HaCaT and HeLa (ATCC®CRM-CCL-2) were seeded at a concentration of 1×10^4 cells/well, in a 96-wells plate in 1:1 Dulbecco's modified essential medium (DMEM) and Ham's F-12 Medium (DMEM:F12, Life Technologies, Scotland, United Kingdom) supplemented with 10% fetal bovine serum (ThermoFisher Scientific, Waltham, MA, USA). The treatments were performed using binary dilutions of GO-1,2,3, between 1 mg/mL and 31 $\mu\text{g/mL}$. After 24 h, the GO-1,2,3 effects were evaluated by adding 20 μL of CellTiter Reagent for 2 h at 37 °C, and thereafter reading absorbance at 490 nm with TriStar² S LB942 Multimode Reader (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The IC50 was calculated with a Quest Graph™ IC50 Calculator [37].

2.5.2. Fluorescein Diacetate (FDA)-Propidium Iodide (PI) Cell Staining for Microscopic Examination of Treated Cells

One hundred thousand cells were added to each well of a 24-wells plate and treated with GO-1,2,3 at concentrations ranging between 1 mg/mL and 31 µg/mL. After 24 h, the culture medium was removed, and the cells were stained for 5 min in the dark by addition of staining solution containing 10 µg/mL of FDA plus 5 µg of PI. FDA-PI-stained cells were examined with an Observer D1 Zeiss microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) using a band pass 488 nm exciter filter.

2.5.3. Assessment of the Compounds' Influence on the Cell Cycle Analysis

For cell cycle analysis, the cells were also treated with GO-1,2,3 at concentrations between 1 mg/mL and 31 µg/mL, for 24, 48, or 72 h. At specified times, treated and untreated cells were harvested with trypsin, fixed in cold ethanol (70%), and stained with 100 µg/mL propidium iodide [38]. For events acquisition, a Beckman Coulter flow cytometer (Beckman Coulter, Nyon, Switzerland) was used, and analyses were performed using the FlowJo software 7.2.5. (Ashland, CA, USA).

3. Results and Discussion

The SEM investigation of the morphology of the graphene oxide (GO) functionalized with hydrazides reveals that all synthesized samples have different structures according to the types of hydrazides grafted onto the GO (Figure 3).

As shown in Figure 3, discrepancies in morphology are probably related to the sample preparation technique, as well as to the hydrazide used in the functionalization of graphene oxides. Figure 3b–d show the SEM images of the GO-1 samples with lamellar structure characteristic to graphene [39]. The lamellar structure is the result of the alignment and self-assembly of molecules (due to intermolecular forces) in an organized way to form various nanostructures. It can be observed that the lamellar structure of GO-1 (Figure 3b–d) presents thinner layers than the initial graphene oxide, as well as the expected smooth surfaces with wrinkles and folded regions. These observations could be accounted for by the sp^3 carbons, the formation of oxygen-containing functional groups in the basal planes, and to various GO structural defects [40]. The reduction of oxygen-containing functional groups located in the basal plane of the sp^2 carbon hybridization allowed the lamellas of the GO to be held together via weak van der Waals forces [22]. Figure 3e–g show SEM images of the GO-2 characterized by flake structures with graphite particles are in the platelet-like crystalline form of carbon [41]. As shown in Figure 3h–j, GO-3 is characterized by straight rods (~5 µm length) with a square cross-section [42]. The SEM micrographs highlight that the GO functionalized with hydrazides leads to the formation of disordered nanostructures [43,44].

The elemental composition of the studied samples was determined by energy dispersive spectroscopy (EDS) and data are presented in Table 1. These results revealed a high content of carbon (76.05 wt.%–76.69 wt.%), oxygen (11.82 wt.%–14.97 wt.%), and nitrogen (5.99 wt.%–8.00 wt.%) in all studied samples. For GO-1 and GO-2 samples, an important content of sulfur (1.59 wt.%–2.25 wt.%) was determined. Other minor constituents of all analyzed samples were identified, i.e., aluminum (0.04 wt.%–0.13 wt.%), silicon (0.05 wt.%–0.21 wt.%), chlorine (0.28 wt.%–1.10 wt.%), and potassium (0.17 wt.%–1.82 wt.%). Just the GO-2 sample has a small content of sodium (0.06 wt.%), magnesium (0.04 wt.%), and calcium (0.31 wt.%).

The SEM images highlighted the modification of graphene oxide structures: the initial layers (Figure 3a) were transformed into thin sheets (Figure 3b–d), flakes (Figure 3e–g), or needles (Figure 3h–j), depending on the used hydrazide. Additionally, nitrogen incorporation in the GO structures is demonstrated by the reduction of oxygen (Table 1).

To confirm the insertion of hydrazide derivatives into graphene oxide and the absence of interactions between both hydrazides and GO, Raman scattering with an excitation wavelength of 1064 nm and FTIR spectroscopies were investigated. Both analytical techniques

represent versatile nondestructive and high-resolution tools to identify and characterize the chemical structure and properties of current graphene-based materials with a high scientific interest for different applications. FTIR data of new graphene oxidehydrazides derivatives (Figures 1 and 2) are shown in Figure 4 and Table 2.

Figure 3. SEM photomicrographs of: (a) initial graphene oxide with compact thick layers ($\times 3$ k); (b) GO-1 lamellar structure ($\times 10$ k); (c) GO-1 lamellar structure ($\times 35$ k); (d) GO-1 lamellar structure ($\times 130$ k); (e) GO-2 flakes structure ($\times 8$ k); (f) GO-2 flakes structure ($\times 15$ k); (g) GO-2 flakes structure ($\times 15$ k); (h) GO-3 acicular structure ($\times 2.5$ k); (i) GO-3 acicular structure ($\times 6$ k); (j) GO-3 acicular structure ($\times 10$ k).

Table 1. The elemental composition determined by FE-SEM-EDS in the studied samples, expressed in wt. % $\pm$ S.D.%, normalized to 100 wt. %.

Element \ Sample	GO	GO-1	GO-2	GO-3
C	62.84 $\pm$ 0.29	76.69 $\pm$ 0.33	76.05 $\pm$ 0.29	76.36 $\pm$ 0.38
N	nd *	7.87 $\pm$ 0.08	5.99 $\pm$ 0.08	8.00 $\pm$ 0.18
O	33.42 $\pm$ 0.22	11.82 $\pm$ 0.18	13.08 $\pm$ 0.22	14.97 $\pm$ 0.35
Na	nd *	nd *	0.06 $\pm$ 0.01	nd *
Mg	nd *	nd *	0.04 $\pm$ 0.01	nd *
Al	0.14 $\pm$ 0.01	0.05 $\pm$ 0.01	0.13 $\pm$ 0.01	0.04 $\pm$ 0.01
Si	nd *	0.05 $\pm$ 0.01	0.21 $\pm$ 0.01	0.15 $\pm$ 0.01
S	2.15 $\pm$ 0.04	2.25 $\pm$ 0.01	1.59 $\pm$ 0.01	nd *
Cl	0.82 $\pm$ 0.05	1.10 $\pm$ 0.01	0.72 $\pm$ 0.01	0.28 $\pm$ 0.01
K	0.63 $\pm$ 0.03	0.17 $\pm$ 0.01	1.82 $\pm$ 0.02	0.20 $\pm$ 0.02
Ca	nd *	nd *	0.31 $\pm$ 0.02	nd *

nd *—undetermined element or with concentration < LOD (0.01 wt.%).

It is well known that infrared is the most common vibrational spectroscopy for assessing the molecular motion and fingerprinting of investigated structures, based on the inelastic scattering of a monochromatic excitation source using an energy range from 400 to 4000 cm^{-1} [45–47]. The presence of different functional groups in graphene oxide, as well as GO–hydrazide derivatives, is revealed in medium intensity peaks at 3404 (GO), 3412 (GO-1), 3413 (GO-2), and 3410 (GO-3) cm^{-1} for O–H, and strong peaks at 1630 (GO), 1633 (GO-1), 1632 (GO-2), and 1635 (GO-3) for C=C stretching/bending vibrations modes, and for C=O with the molecular bond δ (scissoring vibration) from amide (Table 2). The peak around the value of 3404 cm^{-1} is attributed to the O–H stretching vibration mode of –COOH and C–OH functional groups. In addition, GO, GO-1, GO-2, and GO-3 show medium intensity peaks at 1720, 1724, 1727, and 1731 cm^{-1} , which are assigned to the C=O stretching vibration mode [48].

Figure 4. Overlap of FTIR spectra for graphene oxide functionalized with hydrazides: GO-1, graphene oxide-2-furanecarboxylic acid hydrazide; GO-2, graphene oxide-4-(benzensulfonamido)benzoic acid hydrazide; GO-3, graphene oxide-2-(2-methyl-4-oxoquinazolin-3(4*H*)-yl)-acetohydrazide.

Table 2. The tentative assignment of significant FTIR spectra peaks.

Wavenumber (cm ⁻¹) & Relative Intensity *				Vibrational Assignment
GO	GO-1	GO-2	GO-3	
3680 w	3687 w	3690 w	3689 w	O–H stretching
3641 w	3646 w	3639 w	3641 w	O–H stretching
3620 w	3627 w	3622 w	3629 w	O–H stretching
3408 m	3412 m	3413 m	3410 m	–OH stretch in carboxylic; C–OH group (GO)
–	3316 s	3318 s	3327 s	N–H proton stretching vibration from amide
–	3293 s	3289 m	3302 s	N–H proton stretching vibration
–	–	3229 m	–	N–H stretching vibration of sulfonamide
3169 m	3161 m	3170 m	3162 m	C–H stretching of hydrocarbon
–	3107 m	–	3051 w	C–H stretching from furan cycle; C–H stretching of hydrocarbon
2976 m	2978 w	2980 m	2971 w	C–H stretching vibration
2350 w	2355 m	2354 w	2354 w	C=O stretching vibration
–	2206 w	–	–	C–N, N–H stretching hydrazide
–	2108 w	–	–	C=C stretching vibration from furan cycle
–	1989 w	–	–	C=C stretching vibration from furan cycle
–	1962 w	–	–	C=C stretching vibration from furan cycle
1720 m	1724 m	1727 m	1731 m	C=O stretching vibrations in carboxylic (GO)
–	–	1682 m	1682 m	C=C aromatic ring; N–C=O stretching on quinazolin-4-one
1630 s	1633 s	1632 s	1635 s	C=C aromatic stretch (GO); C=O with molecular bond δ from amide
1616 m	1614 m	1616 m	1615 m	C=C stretching vibration from aromatic ring (GO)
1562 w	1545 w	1554 w	1570 w	C=C stretching vibration (GO)
–	1538 w	1533 w	1539 w	C–N, N–H stretching vibration from hydrazide group
–	1516 w	1517 m	1523 w	C–N, N–H stretching vibration from hydrazide group
–	1470 w	1471 m	1473 m	C–N of stretching vibration from hydrazide group
–	1455 w	1446 m	1444 m	C–N stretching vibration from hydrazide group
1404 m	1405 m	1408 m	1409 m	C–O–H stretching vibration (GO)
1388 m	1390 m	1389 m	1389 m	C–OH stretching vibration (GO)
1347 w	1334 w	1331 w	1347 w	C–OH stretching vibration from GO; S=O stretching of –SO ₂ –NH– group (sulfonamide)
–	1319 m	1322 m	1326 m	C–N stretching vibration from hydrazide group
1238 m	1240 m	1239 m	1236 m	C–O–C stretching vibration (GO)
1221 m	1227 m	1224 m	1229 m	C–OH stretching vibration (GO)
–	–	1180 s	–	S=O stretch from –SO ₂ –NH– group (sulfonamide)
–	1187 m	–	1145 m	C=O–C stretch of furan cycle; N–C=O on cycle
1085 m	1082 m	1085 m	1086 m	C–O–C stretching vibration (GO)
–	–	903 w	–	S–N stretching vibration from –SO ₂ –N
–	802 w	–	–	C–H stretching vibration from furan cycle
–	787 w	–	–	C–H stretching vibration from furan cycle
–	752 w	–	–	C–H stretching vibration from furan cycle
698 w	706 w	716 w	697 w	C–H stretching vibration (GO)

* w = weak, m = medium, s = strong.

Regarding FTIR spectra, the medium/weak signals are observed for the synthesized compounds in the range from 1600 to 1800 cm⁻¹, excepting strong peaks around 1630–1635 cm⁻¹ which can be assigned to a C=C aromatic stretch (GO), as well as what can be attributed to C=O with the molecular bond δ (scissoring vibration) from amide. On the other hand, the strong signals (peaks) for GO-1, GO2, and GO-3 around 3316–3327 cm⁻¹ can be assigned to amide groups, mainly from the H–N stretch vibration. It is well known that vibrational spectroscopy is a tool widely used in the study of molecular structures and surfaces, but usually it is used in terms of qualitative analysis.

The medium intensity peaks around 1404 and 1238 cm⁻¹ (Table 2) are attributed to functional groups such as C–O–H and C–O–C, respectively [30,49]. Furthermore, the normalized FTIR data of GO, GO-1, GO-2, and GO-3 revealed peaks with medium intensities around 1616, 1390, 1221, and 1085 cm⁻¹ for the C=C aromatic stretch, C–O–C ether, the C–OH stretch in acids, and C–O–C epoxy groups, respectively [48,50]. The intensities of peaks for oxygen-containing hydrazide functional groups were observed in the range of

1444–1539 cm^{-1} (Table 2); for C-N and the N-H stretching vibration of the hydrazide group (more than one band varied from medium to weak intensities in FTIR data, but there were prominent peaks in the Raman spectra, as shown by Table 3 and Figure 4).

Table 3. The tentative assignment of significant Raman spectra peaks.

Wavenumber (cm^{-1}) & Relative Intensity *				Vibrational Assignment
GO	GO-1	GO-2	GO-3	
–	–	1680 w	1680 w	C=C aromatic stretch; N-C=O stretching vibration from quinazolin-4-one
1622 w	1623 w	1628 w	1631 w	C=C aromatic stretch (GO)
1573 s	1579 s	1558 s	1571 s	C=C stretching vibration mode of sp^2 hybridized carbon atoms (GO)
–	1533 m	1538 m	1542 m	C-N, N-H stretching vibration from hydrazide group
–	1516 m	1517 m	1523 m	C-N, N-H stretching vibration from hydrazide group
–	1471 m	1473 m	1479 m	C-N of stretching vibration from hydrazide group
–	1452 w	1457 m	1459 m	C-N stretching vibration from hydrazide group
1424 w	1417 w	1428 w	1419 w	C-O-H stretching vibration (GO)
1348 s	1355 s	1346 s	1351 s	C-OH stretching vibration from GO
1230 w	1223 w	1227 w	1216 w	C-O-C stretching vibration (GO)
1220 w	1217 w	1223 w	1229 m	C-OH stretching vibration (GO)
–	–	1165 m	–	S=O stretch from $-\text{SO}_2\text{-NH-}$ group (sulfonamide)
–	1181 m	–	1128 w	C=O-C stretching from furan cycle; N-C=O on cycle
1080 m	1089 m	1091 m	1090 m	C-O-C stretching vibration (GO)
–	–	910 m	–	S-N stretching vibrations from $-\text{SO}_2\text{-N}$
–	801 m	–	–	C-H stretching vibration from furan cycle
–	780 m	–	–	C-H stretching vibration from furan cycle
–	751 m	–	–	C-H stretching vibration from furan cycle
690 m	704 m	721 m	695 m	C-H stretching vibration (GO)
–	–	691 m	–	S=O stretching from $-\text{SO}_2$ group
–	659 m	–	–	C-H stretching vibration from furan cycle
–	–	635 m	–	S=O stretching vibration from $-\text{SO}_2$ group
–	584 m	584 m	585 m	C=O stretching vibration from amide group

* w = weak, m = medium, s = strong.

The same assignment of hydrazide functional groups can be made for strong intensity peaks in the range of 3289–3327 cm^{-1} for N-H proton stretching, and a medium signal around 1320 cm^{-1} for the C-N stretching vibration mode, respectively (Table 2). A new peak with a medium intensity was observed at 1682 cm^{-1} for both GO-2 and GO-3, and was attributed to C=C stretching on the aromatic ring and the N-C=O stretching vibration mode from quinazolin-4-ones' nucleus, respectively. On the other hand, the weak intensity peaks observed in FTIR data (Table 2) for all compounds (i.e., GO, GO-1, GO-2, and GO-3) around 1560 cm^{-1} , with complementary very-strong peaks in Raman spectra (Figure 5 and Table 3), are attributed to the C=C stretching vibration mode of sp^2 hybridized carbon atoms from graphene oxide structures. The medium intensity peaks, observed in FTIR spectra of GO, GO-1, GO-2, and GO-3 compounds, at 3169, 3161, 3170, and 3162 cm^{-1} , respectively, were assigned to the C-H bonds of aromatic hydrocarbons. The sp^2 hybridization of the carbon from C-H group affects the position of the absorption, which means the stiffer bonds vibrate at higher frequencies such as 3000–3200 cm^{-1} . The furan cycle in GO-1 is revealed by the medium intensity peaks at 3107 and 1187 cm^{-1} for the C=O-C stretching vibration mode correlated with the weak intensity peaks in the range of 667–802 cm^{-1} , but with prominent peaks (more than one band with medium intensity) in the Raman spectrum (Table 3). However, in the FTIR spectrum of GO-2, we observed medium intensity peaks at 3229 cm^{-1} and a strong signal at 1180 cm^{-1} attributed to the S=O stretching vibration mode of the $-\text{SO}_2\text{-NH-}$ group (sulfonamide). On the other hand, for the GO-2 compound, two new peaks at 903 and 694 cm^{-1} were observed, with weak intensities in the FTIR spectrum (Table 2), but with medium intensities in Raman (Table 3). By the introduction the quinazolin-4-one hydrazide derivatives on GO, it can be concluded that the FTIR data of GO-3 are more completed by various peaks with different intensities (i.e., strong to weak)

at 3162 and 3051 cm^{-1} from the C–H stretch of hydrocarbon, 1682 and 1145 cm^{-1} from the C=N stretching vibration mode of the 4-substituted quinazolin ring, and 1326 cm^{-1} from the C–N stretching of the quinazolin cycle [51]. The minimum intensities of peaks from the FTIR data of all synthesized compounds, including GO, in the range of 3620 to 3690 cm^{-1} (Table 2), are attributed to the O–H stretching vibration mode from graphene oxide.

Figure 5. Overlap of Raman spectra for graphene oxide functionalized with hydrazides: GO-1, graphene oxide-2-furanecarboxylic acid hydrazide; GO-2, graphene oxide-4-(benzensulfonamido)benzoic acid hydrazide; GO-3, graphene oxide-2-(2-methyl-4-oxoquinazolin-3(4*H*)-yl)-acetohydrazide.

The Raman spectra of new functionalized graphene oxides (GO-1, GO-2, and GO-3) show few prominent features regardless of the final structure. In this respect, in Figure 5, the positions, the line shapes, and intensities of peaks recorded in the Raman spectra reveal important information regarding the structures and properties of synthesized compounds [52].

In Figure 3, the overlap of the Raman spectra of the new structures' graphene oxide-hydrazides derivatives (i.e., GO-1, GO-2, and GO-3), reveal the spectral regions D and G specific to GO. In addition, the D peak is important to characterize, firstly, the graphene oxide, and then, the functionalized graphene oxides. The G-band is characteristic of sp^2 hybridized carbon–carbon bonds in graphene oxide and in Raman spectra (Figure 5 and Table 3), which is observed as strong intensity peaks at 1573 , 1579 , 1558 , and 1571 cm^{-1} for GO, GO-1, GO-2, and GO-3, respectively [53,54].

The ratio between the D-band and G-band, observed in Raman spectra, in terms of peak area (i.e., I_D/I_G), allowed for the measurement of the defects which occurred in GO nanostructures after functionalization [55]. For the analyzed samples, the I_D/I_G ratios have recorded the following values: 4.93 (GO-1), 4.50 (GO-2), and 3.17 (GO-3). Taking into account these values, it can be assumed that the number of the defects in the GO functionalized with hydrazine is very low, but according to the data reported in the scientific literature, the G-band peak is influenced by the D-band ($\sim 1600\text{ cm}^{-1}$) [56].

The Raman spectroscopy can be also used to determine the mean crystallite size of the synthesized GO samples (L_{GO}) using the following equation [48]:

$$L_{GO}(\text{nm}) = \frac{2.4 \times 10^{-10} \lambda^4}{I_D/I_G}$$

where λ represents the wavelength of the LASER source. The obtained results for the functionalized GO samples were: 62.392 nm (GO-1), 68.354 nm (GO-2), and 97.033 nm (GO-3), respectively.

In addition, the strong intensity peaks for GO, GO-1, GO-2, and GO-3 compounds at 1348, 1355, 1346, and 1351 cm^{-1} , revealed the presence of sp^3 hybridized carbon atoms in GO and functionalized GO, known as D-band [57]. Obviously, the weak intensity peaks observed in FTIR data (Table 2) in the range from 1080 to 584 cm^{-1} are more remarkable in Raman spectra as one or more bands for the various values of identified peaks (Table 3).

The antimicrobial activity was assessed on susceptible, reference ATCC strains which are used for antibiogram quality control and have known antibiotic susceptibility profiles. All antibiotics recommended by CLSI (Clinical Laboratory Standard Institute) to be tested on the four strains have MIC values in the range of 0.0016 and 0.152 $\mu\text{g/mL}$, much lower than the MIC recorded for the tested graphenes and hydrazides. Regarding the antimicrobial activity against the planktonic bacterial cells, from the four tested strains, a better antimicrobial activity was recorded against the Gram-negative strains, *E. coli* and *P. aeruginosa* (Figure 6).

Figure 6. The MIC values of the tested compounds (presented values were calculated as the average of three replicates). The results for antibiotics are not represented, as the MIC values were much lower, i.e., in the range of 0.016 and 2.5 $\mu\text{g/mL}$.

The graphenes functionalized with the three hydrazides, 2-furancarboxylic acid hydrazide (H1) in case of GO-1, 4-(benzensulfonamido)benzoic acid hydrazide (H2) for GO-2, and 2-(2-methyl-4-oxoquinazolin-3(4*H*)-yl)-acetohydrazide (H3) for GO-3, respectively, exhibited a low inhibitory effect, inferior to that of free hydrazides (H1, H2, and H3) and in the majority of the cases, even to that of the unfunctionalized GO, as revealed by the higher MIC values, ranging from 1.04 to 2.5 mg/mL. The only case in which a slightly improved antimicrobial activity was observed for the graphene oxide–hydrazide complex (as compared to the free hydrazide (H3)) was GO-3 against the *E. coli* strain. However, the MIC values for both free hydrazide H3 and the corresponding functionalized graphene GO-3 were higher than those recorded for the unfunctionalized graphene GO.

Regarding the antibiofilm activity, the free hydrazides (H1, H2, and H3) exhibited a similar antibiofilm activity with the unfunctionalized GO against all four tested strains. However, the graphene functionalized with hydrazides (GO-1,2,3) exhibited lower inhibitory activity than that of the free hydrazides with one exception, i.e., the GO-2 that exhibited a slightly improved antibiofilm activity against *P. aeruginosa*, a fearful, opportunistic, and nosocomial pathogen, included in the list of ESKAPE bacteria, as compared to the corresponding free hydrazide H2 and the unfunctionalized GO (Figure 7). It is well accepted that biofilm cells can be up to 1000–4000 times more resistant than their planktonic counterparts. Despite this, currently, in the medical field, there is no standardized method to assess the biofilm cell's susceptibility, with the selection of antibiotics based on the MIC results evidently leading in many cases to antibiotic treatment failures [58].

Figure 7. The MBEC values of the tested compounds (presented values were calculated as the average of three replicates). The results for antibiotics are not represented as the MIC values were much lower, i.e., in the range of 0.064 and 5 µg/mL.

Although the fact that GO did not improve the antimicrobial activity of hydrazides could be represented as a weakness of this study, by taking into account that both GO and hydrazide derivatives are intensively studied to synthesize potent antimicrobials, we strongly believe that the results of this type of research should be published to avoid unproductive leads and the waste of resources.

The cytotoxicity of the obtained functionalized graphenes was evaluated in vitro, using the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay that quantifies the production of formazan proportionally with the number of living cells. Unfortunately, the range of the tested concentration could not overlap on the range used in the antimicrobial assays, due to the cytotoxic effects recorded at the active antimicrobial concentrations. Therefore, the highest tested concentration was 1 mg/mL. The results are presented as percent of viable cells reported to untreated cells. Using the colorimetric method, it was

observed that after 24 h, the concentrations over 250 $\mu\text{g/mL}$ for HeLa cells and over 125 $\mu\text{g/mL}$ for HaCaT cells had a concentration-dependent toxic effect (Figure 8).

Figure 8. Effect of GO treatment on viability of HeLa cells and HaCaT cells. The results are presented as percent of treated/affected cells reported to untreated cells.

Taking into account the general criteria and the conditions regarding a cytotoxic activity [59], the newly synthesized GOs are fit into the weakly-cytotoxic group (IC_{50} between 201 and 500 $\mu\text{g/mL}$) (Table 4).

Table 4. The IC_{50} values ($\mu\text{g/mL}$) of the GO-1,2,3.

Cell Line	HeLa			HaCaT		
Functionalized graphene oxide	GO-1	GO-2	GO-3	GO-1	GO-2	GO-3
IC_{50} ($\mu\text{g/mL}$)	417.69	448.47	569.62	201.43	181.13	186.45

The comparative analysis of the two cell lines reveals a higher cytotoxic effect on HaCaT cells (Figure 8), as also revealed by the lower IC_{50} for this cell line in comparison with HeLa cells.

These results were confirmed through FDA-PI cell staining. FDA measures cell-membrane integrity and the enzymatic activity of the cells. FDA is an esterase substrate that is hydrolyzed to the AM ester that yields fluorescein. If FDA is a membrane-permeant dye, PI is a membrane-impermeant one, entering into the cells only when the membranes are affected. In this fluorescence assay, FDA stains viable cells in green while PI stains dead cells in red. At high concentrations, cell mortality has been increased and only a few cells from those that adhered to the surface of the flask were alive and stained green by the FDA (Figure 9).

Cell cycle analysis has shown that these substances are not affecting the cellular cycle of HeLa cells, as shown in Figure 10.

In contrast, HaCaT cell cycle is affected by the GO treatment, as revealed by the occurrence of a sub-G0 peak (marked by a red arrow in Figure 11, generally associated with cellular death).

Figure 9. Fluorescence microscopy images of HaCaT and HeLa cells exposed to different concentrations of GO-1, 2, and 3 and stained by FDA and PI (200×).

Figure 10. Overlaid histograms of GO treatment in HeLa cells.

Overall, taken together, the results of the cytotoxicity and antimicrobial assays show that the active antimicrobial concentrations are generally higher than the cytotoxic ones, suggesting that more optimization studies are required in order to tailor these compounds for potential clinical applications. Additionally, they could be potentially used for developing novel disinfectants or antiseptics that could be applied on inert surfaces at higher concentrations.

Figure 11. The effects induced by the treatment with functionalized graphenes on the HaCaT cell cycle progression (analysis at 48 h).

4. Conclusions

In this research, graphene oxide–hydrazide derivatives were successfully synthesized through a slightly modified Hummer’s method and characterized using different spectroscopy (FTIR and Raman) and microscopy (SEM-EDS) techniques. The various functional groups specific of GO, as well as of functionalized GO-1, GO-2, and GO-3, were proved by FTIR and Raman spectra analyses. Taking into account the obtained values for the ratio between the D-band and G-band (i.e., 4.93 for GO-1, 4.50 for GO-2, and 3.17 for GO-3), it can be assumed that the number of defects in the GO functionalized with hydrazine is very low, but according to the data reported in the scientific literature, the G-band peak is influenced by the D-band ($\sim 1600\text{ cm}^{-1}$). From the Raman spectroscopy data, it can be concluded that the crystallite size calculated for the functionalized GO depends on the excitation wavelength. In addition, the SEM micrographs highlight that the GO functionalized with hydrazides leads to the formation of disordered nanostructures. This phenomenon could be accounted for by the existence of sp^3 carbons and the formation of oxygen-containing functional groups in the basal planes and by various GO structural defects. The in vitro antimicrobial activity evaluation suggests that the functionalization of the graphene oxide with the three hydrazides does generally not improve their antimicrobial features against

planktonic and biofilm cells, in comparison with the unfunctionalized graphene oxide and free hydrazides. The cytotoxic concentrations are below the antimicrobial active ones; therefore, they cannot be used for in vivo applications, unless structure optimization is made. However, further bioevaluation studies could reveal other potential applications of the functionalized graphene oxides.

Author Contributions: Conceptualization, I.Z., C.R. and P.I.; methodology, I.Z., C.R., I.D.D., P.I., C.L., M.C.C., D.C.N., M.P. and C.B.; software, I.A.B. and S.G.S.; validation, I.Z. and C.R.; investigation, I.D.D., I.A.B., R.M.S., C.R., S.G.S., P.I., C.L., D.C.N., M.C.C., C.D.B., M.P., C.C., C.B., L.D.D. and I.Z.; resources, C.R. and C.L.; data curation, C.R., C.L. and I.Z.; writing—original draft preparation, I.Z., P.I., C.R., C.B., C.L., M.C.C. and I.D.D.; writing—review and editing, I.Z., C.R. and I.D.D.; visualization, I.Z., P.I. and C.R.; supervision, I.Z.; project administration, C.R.; funding acquisition, C.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Competitiveness Operational Programme (COP) A1.1.4. ID: P_37_798 MyeloAL-EDiaProT, MyeloAL Project [SMIS 106774, 149/26 October 2016] and by the project CNFIS-FDI-2021-0075-ProResearch: Quality, Performance, Excellence—concepts for a stimulating and competitive environment in research (2021).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data sharing is not applicable to this article.

Conflicts of Interest: The authors declare no conflict of interest.

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