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Graphene Nanosystems as Supports in siRNA Delivery

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The nature of graphene-based nanosystems, as well as the possibility to synthesize them at low cost, have made them in the last few years, an interesting proposition in tumour therapy as a drug delivery system. Here, a reduced form of graphene oxide (RGO) has been synthesized and conjugated with a specific antibody for active targeting against tumour cells (RGOY). Furthermore, its bi-dimensional nature permits also π- π stacking interactions with planar molecules like siRNA (RGOY-siRNA). All these nano-complexes were characterized by DLS and DSC analysis, TEM and Raman spectroscopy and their biocompatibility was demonstrated by viability assay on cell line ECV 304. The system is able to be internalized from cells, as demonstrated by uptake studies with confocal microscopy, performed using its red fluorescence variant (RGOY-AF). A high reduction of the stacking interactions between the graphene sheets was obtained by conjugating RGOY particles to polyvinylpyrrolidone (PVP) (RGOY-PVP). The addition of PVP did not alter the biocompatibility of the system, but limited the formation of aggregates due to the stacking interaction between the graphene sheets: the complexes appeared more dispersed and able to enter into the cells after only few minutes, and in higher amounts with respect to the complex without PVP. All obtained data indicate graphene nanosystems very good candidates as delivery system thanks to their specific properties that permit to link to both antibody and siRNA without any degradation effect.

1. Introduction

Cancer is one of the leading causes of death in the world. Classical pharmacological treatments (parenteral, oral, cutaneous or topic) for cancer chemotherapy can induce side effects because they are unable to discern the specific tumour target, acting both on normal and tumor cells. Furthermore, the drug dilution in the bodily fluids limits their absorption in the target tissue, so that it is necessary to administer substantial doses to have a high local concentration. Over the past several decades, the development of engineered nanosystems for targeted drug delivery have received great attention thanks to their possibility to overcome the limitations of classical cancer chemotherapy including poor solubility, targeting incapability, nonspecific action and, consequently, systemic toxicity (Ranganathan et al., 2012; Adamo et al., 2016). Indeed, nanosystems can be functionalized in order to recognize a specific tissue and to release the conjugated therapeutic agent through a controlled mechanism (Mauro et al., 2015; Mauro et al., 2016). In this contest, graphene based nanosystems and their chemically oxidized derivatives (GO) had generated great interest thanks to their specific structure and properties that make them protagonist for different applications including diagnosis, biosensing (Akhavan et al., 2012, Suvarnaphaet and Pechprasarn, 2017), antibacterial and antiviral development (Gurunathan et al.,2012) and cancer therapy. Reduced graphene oxide (RGO) is produced by treating GO under reducing conditions and it consists of two-dimensional nanosheets of hexagonally arranged sp^2-hybridized carbon atoms with a high surface-area to volume ratio (Fiorica et al., 2017; You et al., 2015). In the basal surfaces

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there are also hydroxyl (-OH) functional groups, uncharged but polar and the perimeter is characterized by the presence of carboxyl acid groups that confer pH dependent negative surface charge and colloidal stability.
Therefore, this particular structure can provide an ideal surface for the adsorption of molecules with aromatic rings via π–π interactions, increasing the drug loading and the following drug release capacity (Dorniani et al., 2016, Thapa et al., 2016). For example, small oligonucleotides like small interference RNAs (siRNAs) are perfect candidates, able to bind graphene based nanosystems by stacking interactions. They are very unstable molecules that can be subjected to degradation by ribonuclease enzymes in body fluids, therefore the association with RGO can protect them and their large surface can permit the adsorption of large amount of siRNA respect to the other nanosystems with a sphere or rod shape (Adamo et al., 2016).
On the other hand, the carboxyl acid groups can permit the installation of covalent bonds with molecules like antibodies used for a specific targeting activity: they can recognize a specific ligand expressed exclusively by target cells. The possibility to associate hydrophilic and hydrophobic regions supports both their solubility in aqueous environments and their interaction with lipids in cell membranes that could facilitate cellular internalization.
Here, reduced graphene oxide (RGO) nanosystems were synthesized and chemically conjugated with a specific antibody for the tumour targeting. Their capability to link planar molecules were also tested by adding siRNA against a transcription factor involved in tumour progression and metastasis. All the samples were physically and chemically characterized and tested for their biocompatibility and ability to be internalized by tumour cells. RGO nanosystems are stable in distilled water, but they can interact with the ions of the body fluids, which causes aggregation. To limit this problem, polyvinyl pyrrolidone (PVP) was conjugated to RGO and the biological evaluations were carried out. Indeed, PVP is a dispersant and stabilizing agent, nontoxic, nonionic and biocompatible polymer (Dispensa et al.,2012; Adamo et al., 2014).

2. Experimental

2.1 Nanosystems synthesis

RGOY was synthesized by a simple coupling reaction through the carboxyl acid function of RGO at the perimeter and the amino residues of the antibody selected. In particular, an EDC (1-Ethyl-(3-dimethylaminopropyl) carbodiimide)- NHS (N-hydroxysulfosuccinimid) protocol was adopted (Grimaldi et al., 2014). 50 mg of RGO were treated with 6.1 mg of EDC (Sigma Aldrich) and 5.5 mg of NHS (Sigma Aldrich) and the pH was adjusted to 6.4. Therefore, 0.75 ng (3.33 nmol) of antibody was added at once. The product was purified by dialysis against RNAsi free water, using 100 kDa cutoff dialysis tubes and so retrieved as a green powder (yield 95 %).
On the other hand, part of RGOY nanosystems was used for the conjugation with siRNA. 2 μg of RGOY was added to different amount of siRNA (RGOY/siRNA; 1:1, 1:2, 1:5, 1:10, 1:20) and incubated for 3 hours in static mode. Thus, samples were freeze-dried to facilitate the stacking interactions, re-suspended in RNAse free water and then subjected to sonication cycles in a 35 kHz Ultrasonic Bath (3 x 2 minutes). The best conjugation grade was obtained with the dilution 1:10.
Polyvinylpyrrolidone (PVP) was synthetized by passive adsorption with RGOY nanosystems to form RGOY-PVP nanocomplexes. RGOY was incubated at room temperature with an excess of PVP and immediately dialyzed against RNAsi free water, using 50 kDa cutoff dialysis tubes for 48 hours.
The nanosystems fluorescent variant was obtained using again the EDC-NHS protocol by conjugating RGOY or RGOY-PVP nanosystems with the red fluorescence probe Alexa Fluor 647.

2.2 Physico-chemical characterization

All the nanosystem variants were characterized through Dynamic Light Scattering (DLS), Transmission Electron Microscopy (TEM), Differential Scanning Calorimetry (DSC) and Raman Spectroscopy. For the DLS analysis, samples were dispersed in distilled water at the concentration of 10 μg/mL and measured by photo-correlation spectroscopy (Malvern Zetasizer Nano-ZS, UK). The measurements were done in triplicate. The morphology of RGO was observed using TEM: samples were dispersed in distilled water (20mg/mL) and placed on copper grids with films. After 1 min of absorption, excess liquid was blotted off with filter paper. The dried specimens were examined by using a JEOL JEM200CX microscope. The electron beam was accelerated with a voltage of 1000 V. TEM images were captured using a Gatan, Inc.,ORIUS SC200CCD Camera. DSC measurements were conducted using a Mettler Toledo DSC 823 instrument (Schwerzenbach, Switzerland). Approximately, 1-3 mg samples were accurately weighed in standard aluminum pans. An empty pan was used as a reference. A scan rate of 10° C/min was employed to heat the samples from 25 to 350 ° C. Analysis was performed under a nitrogen purge (60 mL/min). Calorimetric parameters were analysed using STARe Software.

Raman spectra were recorded with a LabRam Raman spectrometer (Horiba Jobin Yvon,Ltd), fitted with a Peltier-cooled CCD camera for detection and an Olympus BX40 microscope.

2.3 *In vitro* biocompatibility

Vein endothelial (ECV-304) cells were seeded in 96-well plates at the density of 1 x 10^4 cells/well and maintained in a humidified atmosphere of 5 % CO_2 at 37 ° C. In particular, they were growth in M199 medium (Sigma) containing 10 % (v/v) FBS (Euroclone, Celbar), 100 units per mL penicillin G, 100 µg mL^{-1} streptomycin (Euroclone, Celbar) and 2 mM L-glutamine (Euroclone, Celbar) (complete medium). After 24 h, cells were treated with different amount of RGOY (0.25, 0.5, 1, 2.5, 5, 10, 15, 20, 25, 30 and 50 µg/ mL) or RGOY-PVP (0.25, 0.5, 1, 2.5, 5, 10, 15, 20, 25, 30 and 50 µg/ mL) nanosystems for 24 h, 48 h and 72 h. Cells treated with Doxorubicin (DOXO) were used as positive control, while untreated cells like negative control. At the end of each time, cells were incubated for 2 h at 37 ° C with Cell Counting Kit-8 (CCK-8) diluted 1:10 in complete medium: water-soluble tetrazolium salt (WST-8) is reduced by mitochondrial dehydrogenases of the living cells into soluble formazan dye that, consequently, is directly proportional to the number of living cells. The absorbance was determined at 490 nm wavelength by the DU-730 Life Science spectrophotometer (Beckman Coulter). The viability was expressed as percent values related to the untreated cells (100 % of viability). Each experiment was done in triplicate.

2.4 Cellular uptake studies

ECV-304 cells were seeded at a density of 5 x 10^3 cells/well on 12-well plates containing sterile coverslips and grown for 24 h at 37 ° C. After that, cells were incubated with 50 µM of RGOY for 15', 30', 1 h and 2 h or with 5 µM of RGOY-PVP for 15', 30', 1 h, 2 h, 4 h and 6 h. At the end of each time, the samples were washed with complete phosphate buffer saline (PBS) and fixed with 3.7 wt % formaldehyde for 5 minutes. After other washings with PBS, nuclei were labelled with DAPI (1: 10000 in water) for 15minutes and the actin cytoskeleton with phalloidin-FITC (1:500) per 15'. The samples were detected by confocal microscopy (FLUOVIEW FV10i-LIV, Olympus).

3. Results and discussion

3.1 Antibody conjugated graphene nanosystems

In order to investigate the RGO nanosystem function as vector for drug delivery, it was analyzed for its capability to link biological molecules like antibodies. In this contest, a generic antibody was conjugated to the carboxyl acid groups of RGO to form the RGOY nanocomplex. The bidimensional nature of RGOY was evident in TEM images in which it was clear the structure like lamellar sheets (Figure 1a).

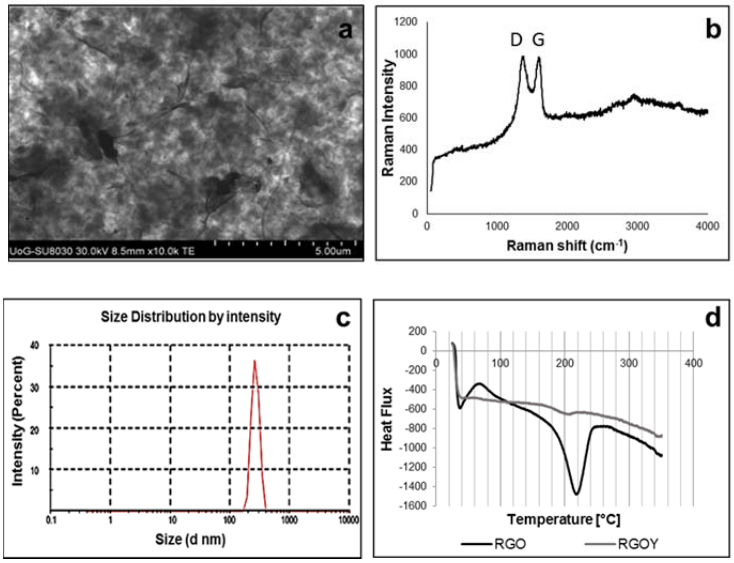

Figure 1. Characterization of RGOY nanosystems by TEM (a), Raman Spectroscopy (b), DLS (c) and DSC (d) analysis.

The intensity of electrons was attenuated by RGOY platelets of different thickness. Depending on the concentration of the sample, it is possible to individuate the dark areas indicating the stacking interaction of

different layers of RGOY, and the transparency areas relative to the thinner sheets of a few layers of reduced graphene oxide. Furthermore, Raman spectroscopy identified the graphene characteristic inner atomic structure with the two peaks D e G relative to sp^2 and sp^3 carbon stretching modes (Figure 1b). The size distribution, investigated by DLS analysis, showed that the RGOY nanocomplex presented a size of 263.6 nm and the population was homogeneous (Figure 1c). In particular, RGOY nanosystems presented a lower size respect to RGO nanocomplexes (368.9 nm) (data not shown), probably because of the presence of the antibody linked to each complex that limits the stacking interactions, reducing the aggregation formation as is evident by the tight peak. On the other hand, the antibody conjugation was confirmed by DLS analysis in which was evident a difference in the melting point peaks between the nanocomplex with or without the antibody (RGO:204 ° C, RGOY: 215 ° C) (Figure 1d).

3.2 Biological evaluation

One of the most important characteristics of nanosystems, when used as vectors for drug delivery, is their biocompatibility in cellular systems; therefore, their toxicity profile was investigated by CCK-8 assay on ECV-304 cells incubated with different amount of RGOY for 24 h, 48 h and 72 h. As shown in Figure 2a, after 24 h of incubation, cell viability was around 100 % (respect to untreated cells) at the lowest RGOY concentration (0.25 μg/mL) and it was maintained for higher concentrations until 50 μg/mL. The same trend was evident also after 48 and 72 h of RGOY incubation (Figure 2b and 2c), suggesting that the nanocomplex was biocompatible. Furthermore, their capability to be internalized from cells was investigated using a red fluorescence variant by adding Alexa Fluor 647 probe (RGOY-AF). Therefore, ECV-304 cells were incubated with RGOY-AF at different times and then stained with DAPI for nuclei (blue) and FITC-phalloidin for actin cytoskeleton (green). Confocal microscopy images (Figure 2) showed that, after 15 minutes of incubation, the red fluorescence of RGOY-AF was still diffused in the media and was partially present on the cells surface (a'''). After 1 h (c'''), the RGOY-AF red signal was higher and it started to appear also in the cytoplasmic compartment. The red signal increased with the duration of the treatment 2 h (d'''), suggesting that the particles were internalized in a time- dependent way.

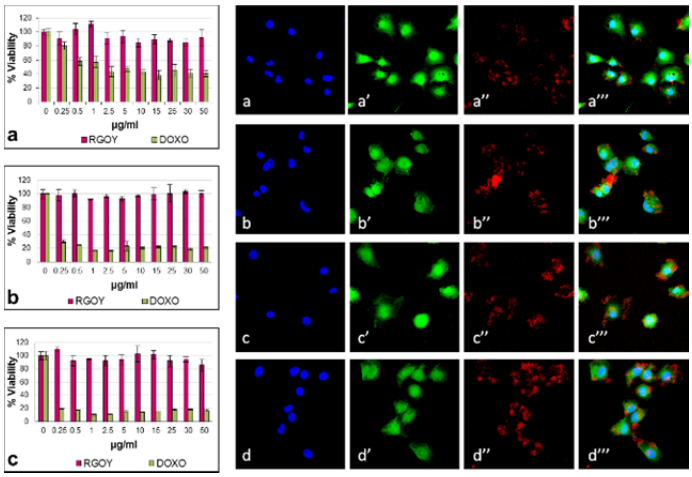

Figure 2. Biological evaluation of RGOY: on the left: biocompatibility of RGOY systems on ECV-304 cells after 24 h (a), 48 h (b) and 72 h (c) of treatment; on the right: localization studies of RGOY-AF on ECV-304 after 15' (a-a'''), 30' (b-b'''), 1 h (c-c''') and 2 h (d-d''') of incubation. Blue: nuclei, green: Phalloidin red: RGOY-AF. Magnificence 60 X.

3.3 siRNA conjugated graphene nanosystems

The planar structure of reduced graphene oxide offers a very large surface available for stacking interactions with many molecules. For example, the conjugation with planar bases of small oligonuotides like a siRNA is very simple to realize and permits to link a higher amount of them (RGOY-siRNA). The conjugation was confirmed by DSC analysis in which was evident that RGO and RGOY-siRNA samples were characterized by different melting points: 215 ° C and 196.2 ° C respectively (Figure 3a). Stunningly, compared with the RGOY size (263.6 nm), it was evident that the presence of siRNA reduced the size of the complexes (149.7 nm), probably because of its negative charge. Indeed, it was presumed that the repulsive force between the negative siRNAs limited the stacking interactions of the RGOY sheets, resulting in a lower size of the

aggregates (Figure 3b). On the other hand, Raman spectrum reflected the classical graphene spectrum (Figure 3c).

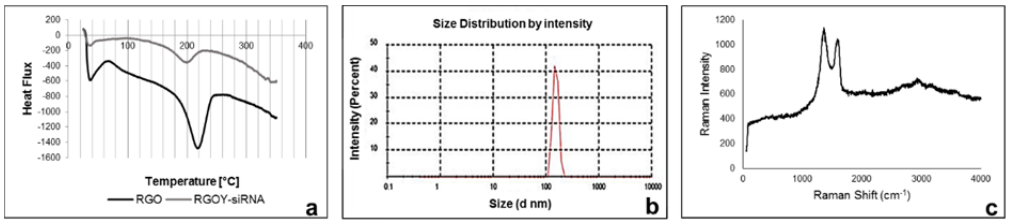

Figure 3. Characterization of RGOY-siRNA nanocomplex by DLS (a) and DSC (b) analysis and Raman Spectroscopy (c).

3.4 PVP- graphene nanocomplexes

Even if the presence of siRNAs conjugated can limit the formation of aggregates due to the stacking interaction between the graphene sheets, a more dispersed complex was created by adding polyvinylpyrrolidone (PVP) (RGOY-PVP). Also in this case, its biocompatibility was tested on ECV-304 cells by CCK-8 test. As reported in figure 4, cell viability was about 100 % for all the samples treated with different nanosystem concentrations (from 0.25 to 50 μg/mL) for 24 h (Figure 4a), 48 h (Figure 4b) and 72 h (Figure 4b), suggesting that also this nanosystem was biocompatible.
The internalization studies were conducted through confocal microscopy and revealed a time-dependent uptake (Figure 4). In particular, after 15 minutes (a'''), the RGOY-PVP-AF was still dispersed around the cells and partially localized in correspondence to the plasma membrane. It started to go inside the cells and situates in the cytoplasm after only 30 minutes or more (1 hour of treatment), when the red fluorescence inside the cells was higher. The signal increased after 2 hours of treatment, when the particles appeared localized in the perinuclear area (d''-d'''). For longer incubation times, the red mark was even brighter, and it was especially localized around the nucleus compartment.

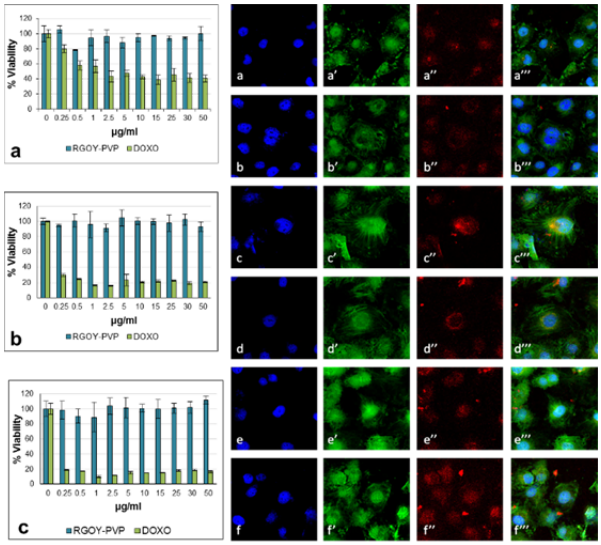

Figure 4. . Biological evaluation of RGOY-PVP: on the left: biocompatibility of RGOY-PVP systems on ECV-304 cells after 24 h (a), 48 h (b) and 72 h (c) of treatment; on the right: localization studies of RGOY-PVP-AF on ECV-304 after 15' (a-a'''), 30' (b-b'''), 1 h (c-c'''), 2 h (d-d'''), 4 h (e-e''') and 6 h (f-f''') of incubation. Blue: nuclei, green: Phalloidin red: RGOY-PVP-AF. Magnificence 100 X.

4. Conclusions

Graphene is a single-atom thick, two-dimensional sheet of hexagonally arranged carbon atoms isolated from its three-dimensional parent material, graphite. The reduced form of graphene oxide (RGO) consists of single-atom-thick carbon sheets with carboxyl acid groups on the periphery, where they provided pH dependent

negative surface charge and colloidal stability. The basal surface contains hydroxyl (-OH) functional groups, which are uncharged but polar. The basal planes also includes unmodified graphenic domains that are hydrophobic and capable of π-π interactions relevant to adsorption of dye molecules or some drugs. In this contest, the bi-dimensional and planar nature offers a large surface area for steaking interactions with a specific antitumoral siRNA. On the other hand, an antibody with targeting activity was conjugated to carboxyl acid groups by covalent linkage. The characterizing assays had shown the RGO nanosystems ability to link this kind of biological molecules with different interactions. Furthermore, it was biocompatible and can be internalized by cells *in vitro*, and these properties were not altered by adding PVP in order to reduce their aggregation. All these characteristics make reduced graphene oxide as a perfect candidate like drug delivery system, suggesting an innovative therapeutic approach for antitumour therapy.

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