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Graphene quantum dots for cancer targeted drug delivery

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ABSTRACT

A biocompatible and cell traceable drug delivery system Graphene Quantum Dots (GQD) based, for the targeted delivery of the DNA intercalating drug doxorubicin (DOX) to cancer cells, is here reported. Highly dispersible and water soluble GQD, synthesized by acidic oxidation and exfoliation of multi-walled carbon nanotubes (MWCNT), were covalently linked to the tumor targeting module biotin (BTN), able to efficiently recognize biotin receptors over-expressed on cancer cells and loaded with DOX. Biological test performed on A549 cells reported a very low toxicity of the synthesized carrier (GQD and GQD-BTN). In GQD-BTN-DOX treated cancer cells, the cytotoxicity was strongly dependent from cell uptake which was greater and delayed after treatment with GQD-BTN-DOX system with respect to what observed for cells treated with the same system lacking of the targeting module BTN (GQD-DOX) or with the free drug alone. A delayed nuclear internalization of the drug is reported, due to the drug detachment from the nanosystem, triggered by the acidic environment of cancer cells.

1. Introduction

Nanoparticle based Drug Delivery Systems (DDS) have shown unprecedented opportunity in cancer treatments for improving drug loading, targeting and efficacy (Liu et al., 2016). Innovative nanotechnology platforms, such as polymeric nanoparticles, liposomes, dendrimers, nanoshells, carbon nanotubes, superparamagnetic and nucleic acid based nanoparticles, have been employed to the targeted delivery of biologically active cargoes into living systems, showing improved safety profile and enhanced antitumor efficacy when compared with the free drugs (Nazir et al., 2014; Radenkovica et al., 2016). Among the different classes of nanomaterials the carbon allotropes, fullerene, carbon nanotubes and graphene have been widely used in biomedical field as biosensors, imaging probes, and DDS (Marchesan and Prato, 2013; Iannazzo et al., 2013, 2015a). Recently, graphene and graphene oxide (GO) aroused significant interest in biomedical research because of their single atomic-layered structure and chemical properties (Zhang et al., 2012; Iannazzo et al., 2015b). Functionalized graphene and GO have shown to improve the drugs solubility, to extend their half-life, and to reduce their side effects (Zhao et al., 2014).

Graphene quantum dots (GQD), fragments limited in size of a single-layer two-dimensional graphene, are considered as the next generation of carbon-based nanomaterials with enormous potential in biomedical field (Shen et al., 2012). Recent studies on this class of nanomaterials report that GQD are less toxic and more hydrophobic with respect to graphene and endowed of stable strong fluorescence (Zhu et al., 2013). Their intrinsic fluorescence have attracted increasing attention in anticancer therapy since it may allow the efficient tracking of human cells *in vitro* (Chen et al., 2013). Analogously to graphene and GO, the presence of more active groups on the GQD surface allows their multimodal conjugation, making them ideal carriers for the simultaneous treatment and tracking of cancer cells. Moreover, due to their unique structural properties, GQD have shown to increase chemotherapy efficacy of anticancer drugs that are suboptimal due to the drug resistance (Wang et al., 2013). Recent works demonstrated that GQD can efficiently accelerate the nuclear accumulation of drugs, such as doxorubicin or cisplatin and also

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enhance markedly the DNA cleavage activity and cytotoxicity of these drugs. These outstanding biological properties, highlight the superiority of GQD over the modified graphene or GO and many other nanoparticle based delivery systems (Sui et al., 2016).

However, the design of effective DDS to treat cancer should include a tumor targeting ligand unit that could specifically recognize cancer receptors on the cell surface and induce receptor-mediated endocytosis, in order to minimize systemic toxicity and undesirable side effects, typically associated with conventional chemotherapy. Targeting ligands such as arginine-glycine-aspartic acid (RGD), folic acid, biotin and antibodies have been inserted to carbon based DDS and have shown to improve therapeutic responses both *in vitro* and *in vivo* (Chen et al., 2008).

Based on these considerations, the aim of this study was to design a new biocompatible and cell traceable DDS, able to release the therapeutic agent to cancer cells in a selective manner. To achieve our goal we have used as nanocarrier, highly dispersible and water soluble GQD, synthesized by acidic oxidation and exfoliation of purified pristine multi-walled carbon nanotubes (MWCNT) where the presence of numerous defects in graphene layers can greatly contribute to afford GQD with a great oxidation extent and water solubility (Lavin et al., 2002).

GQD were covalently linked to the tumor targeting module biotin (vitamin K or vitamin B7), able to efficiently recognize biotin receptors over-expressed on cancer cellsby means of a strategically designed cleavable linker which can be specifically activated inside cells (Russel-Jones et al., 2004). The therapeutic agent used in this study, doxorubicin (DOX), was loaded to the GQD surface, taking advantage of the excellent absorption properties of carbon nanomaterials, by π-π interaction; moreover its inherent fluorescence allowed the drug release to be tracked (Fig. 1).

2. Experimental

2.1. Materials

Solvents and reagents were obtained from commercial suppliers and used without further purification. MWCNTs were produced by catalytic chemical vapor deposition (CCVD) from isobutane on a Fe/Al_2O_3 catalyst; synthesized MWCNTs were then subjected to purification, as reported previously, giving pristine MWCNT (p-MWCNTs) with purity > 95%.

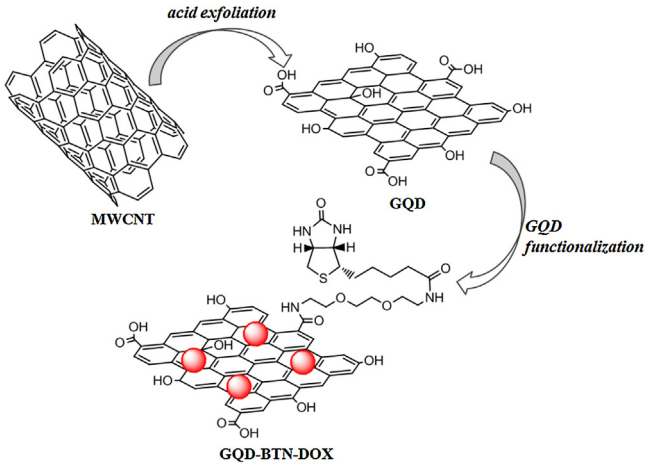

Fig. 1. GQD for Tumor Targeted Doxorubicin Delivery.

2.2. Synthesis of GQD

Pristine MWCNT were treated with a HNO_3/H_2SO_4 mixture in a 1:3 ratio (Donato et al., 2009); the mixture was placed in a reaction flask equipped with a condenser and the suspension was refluxed and sonicated in an ultrasonic water bath at 60 °C, for 4 days. The mixture was then diluted with deionized water and filtered under vacuum on 0,1 μm Millipore membrane. The filtrate was neutralized with NaOH and centrifuged at 3000 rpm. The resulting material was washed with deionized water until no salts were present in the washing solutions and dried at 60 °C under vacuum. The amount of carboxylic groups present on the nanomaterial was evaluated by Boehm titration, using $NaHCO_3$ as titrating agent (Oickle et al., 2010).

2.3. Synthesis of BTN

To a dispersion of biotin (0,409 mmol) in tetrahydrofuran, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl 0.491 mmol) and triethylammine (0.491 mmol) were added and the mixture was left under stirring for 1 h. 1-Hydroxybenzotriazole (HOBt, 66 mg, 0.491 mmol) was added and the mixture was stirred for 1 h more; then, tert-butyl-2-(2-(2-aminoethoxy) ethoxy) ethylcarbamate, synthesized according to a previously reported procedure (Iannazzo et al., 2012), was added and the suspension was left under stirring for 24 h at room temperature. After removal of the solvent, CH_2Cl_2 was added and the organic phase was washed with water, dried over sodium sulfate, filtered, and evaporated to dryness. The residue was purified by MPLC on a silica gel column using as eluent a mixture of CH_2Cl_2/MeOH (9:1) to afford the BTN module protected at the amino functionality, in 95% yield. The *N*-BOC protected BTN module (70 mg, 0.147 mmol) was dissolved in CH_2Cl_2 and added with trifluoroacetic acid (TFA, 0.295 mmol); the solution was left under stirring at room temperature for 1 h. Then, toluene was added to form a TFA azeotrope and the solvent was removed under vacuum. The residue was purified by MPLC on a silica gel column using as eluent a mixture of CH_2Cl_2/MeOH (9:1) to afford BTN in 98% yield (see NMR data in Supplementary material).

2.4. Synthesis of GQD-BTN

To a solution of GQD (30 mg) in CH_2Cl_2, EDC·HCl (0.134 mmol) and 4-dimethylaminopyridine (DMAP, 0.134 mmol) were added and the mixture was left under stirring at room temperature for 30 min. 1-Hydroxybenzotriazole (HOBt, 0.134 mmol) was added and the mixture was stirred for 30 min. Then, a solution of BTN (0.134 mmol) in CH_2Cl_2 (10 mL) was added and the suspension was left under stirring for 4 days at room temperature. The resulting material was washed several times with CH_2Cl_2 and water and centrifuged at 3000 rpm until no organic materials were present in the washing solutions and dried at 60 °C under vacuum.

2.5. Synthesis of GQD-BTN-DOX and GQD-DOX

A solution of GQD-BTN or GQD (30 mg) in deionized water was stirred with a solution of doxorubicin hydrochloride (10 mg) in 10 mL of basic buffer solution at pH 7.4, at room temperature for 48 h. The solution was then centrifuged at 3000 rpm and the resulting material was washed several times with water until no drug was present in the washing solutions and dried at 60 °C under vacuum. The amount of unbound DOX was determined by measuring the absorbance at 490 nm, relative to a calibration curve recorded under the same conditions. The drug loading for GQD-BTN-DOX and GQD-DOX were found to be 16.6 wt% and 17,8% respectively.