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Biocompatible hybrids based on nanographene oxide covalently linked to glycolporphyrins: Synthesis, characterization and biological evaluation

Carla I.M. Santos [a, b, **], Gil Gonçalves [c], Mónica Cicuéndez [c, d], Inês Mariz [a], Virgília S. Silva [e], Helena Oliveira [d, e], Fábio Campos [e], Sandra I. Vieira [f], Paula A.A.P. Marques [c, ***], Ermelinda M.S. Maçôas [a, *], M.Graça P.M.S. Neves [b], José M.G. Martinho [a]

[a] CQFM, Centro de Química-Física Molecular, IN—Institute of Nanosciences and Nanotechnology, CQE, Centro de Química Estrutural, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal
[b] QOPNA, Chemistry Department, University of Aveiro, Campus Universitario de Santiago, 3810-193 Aveiro, Portugal
[c] TEMA-Nanotechnology Research Group, Mechanical Engineering Department, University of Aveiro, Campus Universitario de Santiago, 3810-193 Aveiro, Portugal
[d] CICECO-Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Campus Universitario de Santiago, 3810-193 Aveiro, Portugal
[e] CESAM & Department of Biology, University of Aveiro, Campus Universitario de Santiago, 3810-193 Aveiro, Portugal
[f] iBiMED-Institute of Biomedicine, Department of Medical Sciences, University of Aveiro, Agra do Crasto, 3810-193 Aveiro, Portugal

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ABSTRACT

The major limitation in the development of hybrids based on graphene oxide (GO) and porphyrins is their dispersibility and stability in aqueous systems due to the hydrophobic character induced by porphyrins. Most of the previous approaches reported the direct functionalization of GO with polyethylene glycol (PEG) chains followed by the self-assembly of porphyrins by π-π interactions. Here, new hybrids were prepared using porphyrins previously functionalized with different number/types of glycol branches to be covalently attached through esterification to the carboxyl groups of GO sheets of nanometric dimensions. The number of the glycol chains and its relative position in the porphyrin core showed to be fundamental to improve the hybrids dispersion and stability in aqueous solutions. The best performing hybrids were characterized by transmission electron microscopy, X-ray photoelectron spectroscopy, Fourier transform infrared, UV-Vis absorption and fluorescence spectroscopy. The *in vitro* biocompatibility assessment of these hybrids was conducted using human Saos-2 cells. Their effects on cell proliferation and viability, the generation of reactive oxygen species as well as the cell morphology after cell uptake were analysed. The results demonstrate the biocompatibility of these hybrid nanomaterials with human Saos-2 cells, which is very promising for future application in biomedicine namely in cancer therapy.

1. Introduction

Nano-graphene oxide (nano-GO) is a graphene derivative with nanometric dimensions (<100 nm) [1], and in general, it is obtained by a controlled fragmentation of graphene oxide (GO) promoted by ultrasonication [2]. This carbon nanomaterial has been explored for applications in a large variety of fields including catalysis [3], nanoelectronic devices [4], nanocomposite materials [5], energy devices [6] and nanomedicine [7]. The chemical versatility of nano-GO confers a large potential for the establishment of covalent bonds with other molecules with high relevance for biological applications [8]. In the tailoring of GO for cancer therapy, several surface coating strategies have been reported in the literature over the last

* Corresponding author.
** Corresponding author. QOPNA, Chemistry Department, University of Aveiro, Campus Universitario de Santiago, 3810-193 Aveiro, Portugal.
*** Corresponding author.
E-mail addresses: cims@ua.pt (C.I.M. Santos), paulam@ua.pt (P.A.A.P. Marques), ermelinda.macoas@tecnico.ulisboa.pt (E.M.S. Maçôas).

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few years, such as covalent and non-covalent approaches [9–11], with polymers, biomolecules and nanoparticles for the development of innovative nanoplatforms able to provide, new cancer targeting strategies [12], controlled drug delivery systems [13,14], phototherapies trigged by external stimulus [15,16] and more efficient biomedical imaging probes [17,18].

Polyethylene glycol (PEG) is an example of a hydrophilic biocompatible polymer which has been extensively applied to functionalize nano-GO. It has been shown that PEGylation of GO with branched PEG improved GO stability and its dispersibility in aqueous solutions [19,20]. For instance, Liu and co-workers [21] functionalized nano-GO with PEG and grafted poly(maleic anhydride-*alt*-1-octadecene) yielding nanoGO–PEG with excellent physiological stability and ultra-long blood circulation half-life, useful to perform cancer photothermal treatment (PTT). Recently, we reported that the PEGylated nano-GO cell internalization mechanism, concentration and kinetic uptake were dependent on the characteristic of each cell type [22]. Furthermore, it was also described that the number of PEG branches has a strong influence on cell viability and cell uptake kinetics [23].

The functionalization of GO with tetrapyrrolic macrocycles, such as phthalocyanines, chlorins and porphyrins is also deserving some attention from the scientific community. These molecules are well-known by their role as photosensitizers (PS) in photodynamic therapy (PDT) an emerging therapeutic modality that has been successfully used in the treatment of neoplastic and non-malignant diseases [24]. Part of PDT success rely on the photophysical properties of these macrocycles like high molar absorption coefficient in the visible spectral region, high intersystem-crossing yield, long-lived triplet excited state and low cytotoxicity in dark [25]. The development of porphyrin/GO hybrid materials for cancer therapy and bioimaging is a recent topic of research. From the best of our knowledge the first report for the synthesis of hybrid GO with porphyrins [*e.g.* 5-(4-aminophenyl)-10,15,20-triphenylporphyrin (NH_2-TPP)] dates from 2009 [26]. The authors observed that the photoinduced electron- and/or energy-transfer mechanisms played a significant role in the superior optical limiting performance of the NH_2-TPP/GO hybrid material. However, it was reported that this graphene hybrid materials just formed stable dispersions in organic solvents. Wang et al. recently reported similar findings on the preparation of covalent bonded porphyrin–reduced graphene oxide (rGO) hybrids via two different strategies using 1,3-dipolar cycloaddition reactions [27]. Su *et al.* proposed the development of new targeted porphyrin/GO hybrids by non-covalent functionalization for brain cancer therapy with good stability in aqueous solutions. They report a higher photothermal conversion when irradiated with 808 nm laser light when compared with graphene counterparts, able to cause significant ablation for *in vitro* brain cancer cells [28]. In another work it was reported the assembly of the cationic porphyrin derivative 5-(p-(4-trimethylammonium)-butoxyphenyl)-10,15,20-triphenylporphyrin bromide (MitoTPP) onto the PEG-functionalized and folic acid-modified nano-GO [29]. The results showed that this dual target nanosystem was able to release its cargo MitoTPP at lower pH, which subsequently accumulates in mitochondria of cancer cells over expressed with folate receptor (FR). After irradiation with 650 nm light singlet oxygen was generated causing oxidant damage and promoting higher cellular cytotoxicity on those FR-positive cells. Graphene quantum dots (GQDs) produced from hydrothermal treatment of GO were also recently used as a platform for the development of multifunctional theranostic agents [30]. The conjugation of porphyrins into PEGylated and aptamer functionalized GQDs enabled the specific labelling of A549 lung cancer cells that could be detected by *in vitro* bioimaging due to the intrinsic blue fluorescence of GQDs. Moreover, an excellent PTT/PDT therapeutic efficiency was observed for both *in vitro* A549 cancer cells and *in vivo* multicellular tumor spheroids (MCTS) with laser light irradiation at 635/980 nm.

The preparation of new hybrids based on porphyrins and nano-GO remains a great challenge to obtain key materials that can fulfil all the biological requirements such as aqueous dispersibility and stability during acceptable periods of time. Herein, we report, for the first time, the synthetic access and full characterization of nano-GO covalently linked to porphyrins bearing glycol branches. Additionally, taking into account the potential biomedical applications of these graphene-based hybrid nanomaterials, their *in vitro* biocompatibility with human Saos-2 osteoblasts was studied.

2. Experimental section

2.1. Chemicals

Pyrrole, 2,3,4,5,6-pentafluorobenzaldehyde, nitrobenzene, chloroacetic acid, sodium hydroxide, synthetic graphite flakes and all oxidizing and reducing agents were purchased from Sigma–Aldrich. Ethylene glycol and tetraethylene glycol were obtained from Alfa Aesar. All these chemicals were used without further purification. The solvents were obtained from Panreac and Riedel-de Haen and used as received or distilled and dried using standard procedures.

2.2. Samples preparation

2.2.1. Synthesis of nano-GO

GO was obtained from exfoliation of high purity graphite in acidic medium by a modified Hummers method [31]. Briefly, a mixture of 2.5 g of graphite,1.9 g of $NaNO_3$ and 85 mL of H_2SO_4 was placed in a flask cooled in an ice bath, and the mixture was kept stirring until total homogenization. After that, 11.25 g of $KMnO_4$ was gradually added to the solution while stirring. After 2 h, the solution was removed from the ice bath, and further stirred for 5 days. Finally, a brown-coloured viscous slurry was obtained. The mixture was washed with an aqueous solution (500 mL) of 3 wt % H_2SO_4 and 0.5 wt % H_2O_2. The solid product obtained after the rigorous cleaning process was rinsed using copious amounts of distilled water. Finally, the resulting GO was dried by lyophilisation in order to obtain a non-agglomerated dried powder.

The nano-GO preparation was based on the breakage of GO by combined mechanical aging and tip sonication processes [4]. Briefly, GO was re-suspended in water with a concentration of 1 mg/mL and subjected to ultra-sonication treatment (Vibra Cell Bioblock Scientific model 75,043 at 225 W) during 3 h at room temperature.

2.2.2. Functionalization of nano-GO with extra carboxylic groups (nanoGO-CO_2H)

The nano-GO obtained by ultrasonic treatment with an average lateral size of *ca* 50 nm was further functionalized with carboxylic groups to improve its capability to be esterified with the porphyrins bearing glycol units. For that purpose, a nano-GO dispersion in NaOH (1.25 M) was prepared (1 mg/mL). The carboxylic groups were inserted by adding 2.5 g of chloroacetic acid to 50 mL of the dispersion and the reactional mixture was maintained in an ultrasonication bath (Bandelin SONOREX Digital 10P) for 180 min at 25 °C. The resulting nanoGO-CO_2H dispersion with extra carboxylic groups was neutralized and purified by repeated rinsing and filtrations (further details in Supporting Information- Figs. S1 and S2) [32]. For simplicity this sample will be just labelled as nanoGO-CO_2H.