1. Introduction

Photothermal Therapy (PTT) mediated by nanomaterials has been showing promising results in cancer treatment.\(^1\)\(^-\)\(^5\) This type of approach explores the ability of photo-responsive nanostructures to passively accumulate at the tumor site (through the Enhanced Permeability and Retention (EPR) effect), and to convert light into heat.\(^6\)\(^-\)\(^7\) For this purpose, the use of Near Infrared (NIR; 750–1000 nm) light is essential due to its minimal interactions with biological components (e.g. water, melanin, collagen) and high tissue penetration depth, enabling a spatio-temporal controlled effect.\(^6\)\(^-\)\(^10\)

Among the different NIR light-responsive nanomaterials, Graphene Oxide (GO) has been receiving great interest for application in cancer therapy.\(^11\)\(^-\)\(^13\) Upon interaction with NIR light, GO can produce a temperature increase capable of causing damage to cancer cells.\(^8\)\(^,\)\(^11\)\(^-\)\(^15\) Furthermore, the aromatic lattice of GO can encapsulate a wide variety of therapeutics, enabling its use in NIR-responsive drug delivery applications.\(^8\)\(^,\)\(^16\)\(^,\)\(^17\)

Despite its potential, the direct application of GO in cancer PTT is limited by its poor colloidal stability in biologically relevant media.\(^11\)\(^,\)\(^18\) As-synthesized GO precipitates in biological fluids, hindering its ability to reach the tumor site.\(^8\)\(^,\)\(^18\) This limitation has been solved by functionalizing the surface of GO with poly(ethylene glycol) (PEG) derivatives.\(^19\)\(^,\)\(^20\) However, recent reports have disclosed that PEG based coatings can be immunogenic.\(^21\)\(^,\)\(^22\) In brief, anti-PEG antibodies are generated at the moment of the first intravenous administration of PEGylated nanomaterials.\(^23\) Then, these anti-PEG antibodies mediate the rapid clearance of the PEG coated nanostructures in subsequent administrations, impairing their tumor homing capacity (known as the accelerated blood clearance phenomenon).\(^24\) These findings highlight the importance of developing novel materials to functionalize GO.

On the other hand, GO presents a modest photothermal capacity, requiring the administration of high dosages or the use of intense radiation to exert an appropriate therapeutic
effect.\textsuperscript{25–27} To address this problem, GO has been chemically reduced using hydrazine hydrate.\textsuperscript{28,29} This chemical process restores the graphitic lattice of this nanomaterial, improving its NIR absorption and hence its photothermal capacity.\textsuperscript{28} However, due to the toxicity of hydrazine hydrate, the reduced GO attained using this reducing agent has a weak cytocompatibility even after functionalization.\textsuperscript{28,29} Alternatively, other NIR-responsive nanomaterials (e.g. gold nanorods, upconversion nanoparticles)\textsuperscript{30–33} have been incorporated on the surface of GO to improve its photothermal capacity. Nevertheless, this process is both laborious and complex, limiting the future translation of these hybrid-nanostructures. Therefore, it is also necessary to implement novel approaches to increase the photothermal effect mediated by GO.

In this work, GO was functionalized with an amphiphilic polymer containing 2-(methacryloyloxy)ethyl(dimethyl-3-sulfopropyl)ammonium hydroxide (SBMA) brushes and was loaded with IR780, for the first time, to improve its colloidal stability and phototherapeutic capacity, respectively. We recently demonstrated that the functionalization of protein based nanoparticles with SBMA can improve their colloidal stability.\textsuperscript{7} Furthermore, SBMA brushes are capable of improving nanomaterials’ blood circulation time and have not been reported to experience the accelerated blood clearance phenomenon.\textsuperscript{34–36} In turn, IR780 (a hydrophobic small molecule) was selected to be attached to GO due to its good photothermal capacity upon NIR laser irradiation.\textsuperscript{7,37–39} Moreover, we recently showed that the optical properties of IR780 are superior to those of other NIR dyes such as Indocyanine Green, IR783, or IR820.\textsuperscript{1}

In order to attain the amphiphilic polymer with SBMA brushes, SBMA was grafted into poly(ethyleneimine) (PEI). Then, the resulting polymer was conjugated with hydrolysed poly(maleic anhydride-alt-1-octadecene) (PMAO), yielding SBMA-PEI-PMAO (SPP). Subsequently, GO was functionalized with SPP and loaded with IR780 (IR780-SPP/GO) through a simple sonication process. The results revealed that the SPP/GO and IR780-SPP/GO display a neutral surface charge and that maintain their size distribution overtime when in contact with biologically relevant media, demonstrating an improved colloidal stability. In contrast, the non-SBMA functionalized GO (PEI-PMAO coated GO and IR780 loaded PEI-PMAO coated GO) promptly precipitated in the biologically relevant media. By loading IR780 into the SPP/GO, its NIR absorption increased by 2.7-fold, leading to a 1.2 times higher photothermal heating. In \textit{in vitro} cell studies, the combination of SPP/GO with NIR light only reduced breast cancer cells’ viability to 73%. In stark contrast, by combining IR780-SPP/GO and NIR radiation, the cancer cells’ viability decreased to 20%, hence confirming the IR780-SPP/GO potential for cancer PTT.

2. Materials and methods

2.1. Materials

Michigan Cancer Foundation-7 (MCF-7) cell line and Normal Human Dermal Fibroblasts (NHDF) were obtained from ATCC (Middlesex, UK) and Promocell (Heidelberg, Germany), respectively. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was bought from Promega (Madison, WI, USA). Fetal Bovine Serum (FBS) was acquired from Biochrom AG (Berlin, Germany). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) and methanol were acquired from Fisher Scientific (Oeiras, Portugal). Cell imaging plates were obtained from Ibidi GmbH (Munich, Germany). Cell culture plates and T-flasks were purchased from Thermo Fisher Scientific (Porto, Portugal). GO was obtained from NanoPoz (Umultowska Poznan, Wielkopolska). Branched PEI (Mn 1250 Da), SBMA, Dulbecco’s Modified Eagle’s Medium-F12 (DMEM-F12), IR780 iodide, N-hydroxysuccinimide (NHS), paraformaldehyde, PMAO (average Mn 30 000–50 000 Da) and trypsin were bought from Sigma-Aldrich (Sintra, Portugal). Calcein-AM, Hoechst 33342® and Propidium Iodide (PI) were obtained from Thermo Fisher Scientific (Porto, Portugal). Water used in all experiments was double deionized (0.22 µm filtered, 18.2 MΩ cm).

2.2. Methods

2.2.1. Synthesis and characterization of SPP. The synthesis of SPP was a tri-step process. Initially, SBMA was covalently attached to PEI, through a Michael addition, following the method described by Venault et al. with slight modifications.\textsuperscript{40} In brief, SBMA (1.176 g) and PEI (0.5 g) dissolved in 5 mL of water were reacted under reflux for 6 h at 90 °C. Then, the solution was dialysed against water (500–1000 Da molecular weight cut-off membrane) for 2 days and freeze-dried (ScanVac CoolSafe, LaboGene ApS, Lyngø, Denmark), yielding SBMA-PEI (SP).

Subsequently, the hydrolysis of PMAO maleic anhydride rings was performed as we have previously described.\textsuperscript{30} Briefly, an aqueous solution (4 mL) containing PMAO (0.2 g) and NaOH (2 N) was stirred for 5 h at room temperature. Then, the solution’s pH was adjusted to 7 using HCl, followed by dialysis against water for 1 day (14 000 Da molecular weight cut-off membrane). The recovered solution was freeze-dried, yielding hydrolysed PMAO.

Finally, to produce SPP, SP was attached to the hydrolysed PMAO carboxyl groups using the carbodiimide chemistry.\textsuperscript{29} First, hydrolysed PMAO (50 mg) was activated with EDC (6 mg) and NHS (3 mg) in 25 mL of DMSO. Subsequently, 50 mg of SP in water (25 mL) was added to the above solution. After reacting for 6 h at room temperature, the solution was dialysed against water for 3 days (14 000 Da molecular weight cut-off membrane) and freeze-dried, yielding SPP.

As a control, PEI-PMAO (PP) was also produced as described above using hydrolysed PMAO (50 mg), PEI (50 mg), EDC (6 mg) and NHS (3 mg).

The successful synthesis of hydrolysed PMAO, SP, SPP and PP was confirmed by Fourier Transform Infrared Spectroscopy (FTIR) using a Nicolet iS10 spectrometer (Thermo Scientific Inc., MA, USA) with a spectral width ranging from 4000 to 600 cm\textsuperscript{-1}. Moreover, proton nuclear magnetic resonance (\textsuperscript{1}H NMR) spectra of SBMA, PEI, SP, hydrolysed PMAO and SPP were acquired by using a Bruker Avance III 400 MHz spectrometer.
were performed to conduct the analysis. Spectroscopy (Evolution 201 spectrophotometer, Thermo Fisher) was used to determine IR780 concentration. For such, IR780-SPP/GO was resuspended in a water/methanol solution (1:1 (v/v)) and the absorbance was measured at 490 nm using a microplate reader (Bio-Rad xMark microplate spectrophotometer). Non treated cells were used as the negative control (K−) while cells treated with ethanol 70% were used as the positive control (K+).

2.2.5. In vitro evaluation of the phototherapeutic effect mediated by SPP/GO and IR780-SPP/GO. The photothermal effect mediated by SPP/GO and IR780-SPP/GO was evaluated as previously described by us. Briefly, MCF-7 cells were seeded at a density of 1 × 10⁴ cells per well in 96-well plates. After 24 h, the medium was replaced by fresh culture medium containing different concentrations of SPP/GO (1–50 µg mL⁻¹ of GO equivalents) or IR780-SPP/GO (at 30/1.44 and 50/2.40 µg mL⁻¹ of GO/IR780 equivalents). After 4 h of incubation, cells were irradiated with NIR light (808 nm, 1.7 W cm⁻², 8 min). After 24 h incubation, cells’ viability was determined using the MTS method as described in Section 2.2.4.

To visualize the different phototherapeutic effects, MCF-7 cells incu...
3. Results and discussion

3.1. Formulation and characterization of SPP/GO and IR780-SPP/GO

In order to improve the colloidal stability of GO, this nanomaterial was functionalized with a SBMA-based amphiphilic polymer (SPP). Then, the SPP-functionalized GO was loaded with IR780 with the intent to improve its photothermal capacity – Fig. 1(A). The successful synthesis of SPP was confirmed by FTIR and $^1$H NMR (data presented in the ESI – Fig. S2, S3, S5 and S6†).

SPP was used to functionalize GO through a simple sonication process, yielding SPP/GO. In this process, the alkyl chain of SPP adsorbs on the aromatic matrix of GO by taking advantage from hydrophobic–hydrophobic interactions. The functionalization of GO with SPP was confirmed by FTIR, which revealed that the spectrum of SPP/GO displays the S═O stretch peak characteristic of SPP (Fig. S7†). When compared to GO, the absorption spectrum of SPP/GO presented an augmented absorption in the 200–290 nm range (Fig. S8†). The analysis of SPP demonstrated that this SBMA-based amphiphilic polymer also presents a strong absorption between 200 and 290 nm (Fig. S8†). In this way, the increased absorption of SPP/GO in this range is also indicative of the functionalization of GO with SPP. The EDS analysis revealed that SPP/GO has a carbon : oxygen : sulfur ratio of about 53 : 46 : 1, which also supports the presence of SPP in SPP/GO (the carbon : oxygen ratio of GO was determined to be 66 : 34 being in agreement with literature reports$^{5,42}$). Moreover, the Dynamic Light Scattering (DLS) analysis indicated that GO maintained its nanometric size distribution upon functionalization with SPP (Fig. 1(B)). The lateral dimensions of SPP/GO were then confirmed by TEM (Fig. S9†), revealing that this nanomaterial has a size compatible with its application in cancer therapy.$^{6,8}$

Then, IR780 was loaded in SPP/GO by taking advantage from hydrophobic interactions and π–π stacking. Both SPP/GO and IR780-SPP/GO demonstrated a similar size distribution, indicating that the IR780 loading did not affect the nanomaterials’ size (Fig. 1(B)). The IR780 encapsulation efficiency in IR780-SPP/GO was of about 74$^{/C6}$1$.\%$Moreover, the water solubility of IR780 after being encapsulated in SPP/GO was 3.69 mg mL$^{-1}$. Considering that the water solubility of free IR780 is about 0.40 μg mL$^{-1}$$^{43}$ the IR780 loading into SPP/GO led to a 9.23-fold increase in its solubility. Furthermore, the IR780-SPP/GO adsorbed 0.048 $\pm$ 0.004 μg of IR780 per μg of GO, which is in line with the drug loading capacity of GO derivatives (please note that non-loaded GO is removed during the centrifugation step, leading to a higher drug loading capacity).$^{11}$

The zeta potential of SPP/GO ($\sim$7.7 $\pm$ 0.4 mV) and IR780-SPP/GO ($\sim$8.1 $\pm$ 0.7 mV) revealed that these formulations have a neutral surface charge (nanomaterials with a zeta potential between $\pm$10 and +10 mV are considered to display a neutral...
surface charge"). Taking into account that the zeta potential of GO was determined to be \( -10.4 \pm 0.6 \text{ mV} \), this data indicates that the SPP coating can induce the neutralization of the surface charge of these nanomaterials. In fact, several nanomaterials functionalized with SBMA brushes also revealed a neutral surface charge.

As importantly, neutrally charged nanomaterials (zeta potential between \(-10\) and \(+10\) mV) are considered in the literature as optimal due to their improved blood circulation time, which favours their tumor uptake.

Lastly, the colloidal stability of SPP/GO and IR780-SPP/GO overtime in water and in cell culture medium (DMEM-F12 supplemented with 10% (v/v) of FBS) was investigated (Fig. S10†). Both SPP/GO and IR780-SPP/GO maintained their size distribution overtime (Fig. 1(C)). As a control, the stability of PP/GO and IR780-PP/GO was also analysed, being verified that these non-SBMA grafted nanomaterials promptly precipitate in water and in culture medium (Fig. S11†). In this way, the presence of the SBMA in the SPP/GO and IR780-SPP/GO endows these nanomaterials an excellent colloidal stability. In fact, the SBMA functionalization can reduce the adsorption of proteins on nanomaterials’ surface, enhancing their stability during circulation and possibility favouring their tumor uptake.

Together, this data indicates that the SPP/GO and IR780-SPP/GO have suitable physicochemical properties for application in cancer therapy.

3.2. Phototherapeutic capacity of SPP/GO and IR780-SPP/GO

To analyse the ability of SPP/GO and IR780-SPP/GO to interact with the NIR radiation, their absorption spectra was acquired (Fig. 2(A)). As expected, SPP/GO demonstrated a broad absorption in the NIR region (750–1000 nm), which is a characteristic feature of GO derivatives. In turn, the absorption spectrum of IR780-SPP/GO (Fig. 2(A)) displayed the GO characteristic absorption band as well as an increased absorption in the 680–870 nm range due to the presence of IR780 in this nanomaterial (Fig. 2(A) and (B)). Compared to SPP/GO, the IR780-SPP/GO presented a 2.7-fold higher absorption at 808 nm. Since 808 nm light will be used in the photothermal experiments, the enhanced absorption of IR780-SPP/GO at this wavelength may produce a better therapeutic outcome.

Then, the photothermal capacity of SPP/GO and IR780-SPP/GO was investigated by exposing these nanomaterials to NIR light (808 nm, 1.7 W cm\(^{-2}\), 8 min) and recording the temperature changes (Fig. 2(C) and (D)). At the maximum concentration tested (50 \( \mu \text{g mL}^{-1} \) of GO equivalents), the SPP/GO could produce a temperature increase to about 14.9 \( ^\circ \text{C} \) after 8 min of irradiation (Fig. 2(C)). Such temperature variation is sufficiently high to cause damage to cancer cells. At the same concentration, the IR780-SPP/GO generated a temperature increase to 13.3 \( ^\circ \text{C} \) just after 2 min of irradiation (Fig. 2(D)). After 8 min of laser exposure, the IR780-SPP/GO produced a photoinduced heat to 18.4 \( ^\circ \text{C} \) (Fig. 2(D)). In this way, the incorporation of IR780 in GO derivatives can be pursued to improve their photothermal potential. Moreover, it was found that the NIR radiation does not increase meaningfully the water temperature (\( \Delta T < 3.6\ ^\circ \text{C} \)). Such result is explained by the minimal interactions of the NIR radiation with water, thus suggesting that SPP/GO and

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**Fig. 2** Optical and photothermal properties of SPP/GO and IR780-SPP/GO. Absorption spectra of SPP/GO and IR780-SPP/GO (20 \( \mu \text{g mL}^{-1} \) of GO equivalents; in water) (A). Absorption spectrum of free IR780 (2.5 \( \mu \text{g mL}^{-1} \) in methanol) (B). Temperature variation curves of SPP/GO (C) and of IR780-SPP/GO (D) at different concentrations (of GO equivalents) during 8 min of NIR irradiation (808 nm, 1.7 W cm\(^{-2}\)).
IR780-SPP/GO can produce a spatio-temporal controlled photothermal heating.

For instance, Ma et al. prepared gold clusters ingrafted into reduced GO (prepared using hydrazine hydrate),\textsuperscript{49} that could generate a temperature increase of about 13.7 °C after 5 min of irradiation (808 nm, 2.2 W cm\textsuperscript{-2}) at a concentration of 90 μg mL\textsuperscript{-1} (of nanohybrids). Herein, the IR780-SPP/GO produced a photoinduced heat of 18.2 °C after 6 min of irradiation (808 nm, 1.7 W cm\textsuperscript{-2}) using only 50 μg mL\textsuperscript{-1} of GO. In this way, the incorporation of IR780 in GO is a promising alternative to using hydrazine hydrate for improving the GO photothermal capacity. Together, these results confirm the photothermal potential of IR780-SPP/GO.

### 3.3. Cytocompatibility of SPP/GO

Before determining the phototherapeutic potential of SPP/GO, its cytocompatibility when non-irradiated with NIR light was determined (Fig. 3). For such, MCF-7 cells and NHDF were used as models of breast cancer cells and healthy cells, respectively. Both MCF-7 cells (Fig. 3(A)) and NHDF (Fig. 3(B)) incubated with SPP/GO (up to 50 μg mL\textsuperscript{-1} of GO equivalents) did not suffer meaningful alterations on their viability, even after 24 and 48 h of incubation (cell viability > 86%).

Some studies have reported that nanomaterials functionalized only with PEI can be cytotoxic due to the highly positive surface charge of this polymer.\textsuperscript{49-51} For instance, Kievit et al. showed that PEI coated nanoparticles (zeta potential ≈ +37 mV) are highly cytotoxic (cell viability reduced to about 10%).\textsuperscript{49} Herein, even though SPP contains PEI in its composition, the surface charge neutralization mediated by the SBMA grafting may have played a critical role in rendering SPP/GO cytocompatible. In fact, this data is also in line with the excellent biocompatibility of SBMA-functionalized nanomaterials.\textsuperscript{7,52,53}

#### 3.4. Phototherapeutic effect mediated by SPP/GO and IR780-SPP/GO

Finally, the phototherapeutic effect mediated by SPP/GO and IR780-SPP/GO towards MCF-7 cells was investigated (Fig. 4(A)).

As expected, non-irradiated SPP/GO and IR780-SPP/GO did not induce cytotoxicity towards MCF-7 cells (Fig. 4(B)). Such is in agreement with the cytocompatible profile of SPP/GO and with the fact that non-irradiated IR780-based nanomaterials are generally non-cytotoxic.\textsuperscript{7,37,54}

At the highest concentration tested (50 μg mL\textsuperscript{-1} of GO equivalents), the combination of SPP/GO with NIR light (808 nm, 1.7 W cm\textsuperscript{-2}, 8 min) only caused a reduction of breast cancer cells’ viability to 73% (Fig. 4(B)). In stark contrast, the photothermal effect mediated by IR780-SPP/GO induced a reduction in the viability of cancer cells to about 20% (Fig. 4(B)). Therefore, by incorporating IR780 in the SPP/GO, its photothermal capacity was increased by 3.65-times (Fig. 4(B)). The enhanced therapeutic capacity of IR780-SPP/GO is related with its higher photothermal capacity when compared to that of SPP/GO (Fig. 2(C) and (D)). As importantly, cancer cells treated with only NIR light did not suffer any meaningful alteration in their viability, which is in agreement with the weak/minimal interactions of this radiation with biological components.

To further confirm these results, the Calcein-AM (labels live cells) and PI (labels dead cells) staining of MCF-7 cells after the different treatments was performed (Fig. 4(C) and (D)). In agreement with the cell viability results, the CLSM images of cells exposed to SPP/GO, SPP/GO plus NIR light, and IR780-SPP/GO revealed that these were broadly stained with Calcein-AM and PI (Fig. 4(D)). Furthermore, a very high number of PI stained cells was imaged on the IR780-SPP/GO plus NIR light group (Fig. 4(D)). The ability of IR780-SPP/GO to become internalized by MCF-7 cells was also confirmed by CLSM by taking advantage from the IR780 intrinsic fluorescence (Fig. S12†). Together, these results suggest that IR780-SPP/GO may produce an on-demand therapeutic effect upon NIR laser irradiation.

For instance, PEGylated polypyrrole-GO-gold nanohybrids, when administered at 60 μg mL\textsuperscript{-1}, could reduce the viability of cancer cells to 48% upon NIR laser irradiation (808 nm, 1.75 W cm\textsuperscript{-2}, 10 min).\textsuperscript{55} In another work, the photothermal effect mediated by PEGylated GO/CuS hybrids induced a reduction of
cancer cells’ viability to 44% at a concentration of 500 μg mL\(^{-1}\) of nanohybrids (980 nm, 1 W cm\(^{-2}\), 10 min).\(^{33}\) In this study, the photothermal effect mediated by IR780-SPP/GO led to a decrease on cancer cells’ viability to 20%, using a low dose of photothermal agents (50/2.40 μg mL\(^{-1}\) of GO/IR780 equivalents) and a shorter irradiation time (808 nm, 1.7 W cm\(^{-2}\), 8 min). In this way, IR780-SPP/GO are promising nanomaterials for the PTT of breast cancer cells.

### 4. Conclusion

In this work, GO was functionalized with an amphiphilic polymer containing SBMA brushes and was loaded with IR780, for the first time, with the intent to improve its colloidal stability and phototherapeutic capacity. The results revealed that the SPP/GO and IR780-SPP/GO display a neutral surface charge and that maintain their size distribution overtime when in contact with biologically relevant media, demonstrating an improved colloidal stability. In contrast, the non-SBMA functionalized GO (PP/GO and IR780-PP/GO) promptly precipitated in this media. By loading IR780 into the SPP/GO, its NIR absorption increased by 2.7-fold, leading to a 1.2-times higher photothermal heating. In \textit{in vitro} cell studies, the combination of SPP/GO with NIR light only reduced breast cancer cells’ viability to 73%. In stark contrast, by combining IR780-SPP/GO and NIR radiation, the cancer cells’ viability decreased to 20%, hence confirming the IR780-SPP/GO phototherapeutic potential. Overall, the IR780-SPP/GO presents an improved colloidal stability and enhanced photothermal capacity, rendering it a great potential for future application in cancer PTT.

### Abbreviation list

- ANOVA: Analysis of variance
- CLSM: Confocal laser scanning microscopy
- DLS: Dynamic light scattering
- DMEM-F12: Dulbecco’s modified eagle’s medium-F12
- DMSO: Dimethyl sulfoxide
- EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
- EDS: Energy-dispersive X-ray spectroscopy
- EPR: Enhanced permeability and retention
- FBS: Fetal bovine serum
Conflicts of interest

There are no conflicts to declare.

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