Interplay between material properties and cellular effects drives distinct pattern of interaction of graphene oxide with cancer and non-cancer cells

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Abstract

Understanding how graphene oxide (GO) interacts with cells is fundamental for its safe and efficient biomedical use. However, a systematic investigation involving a panel of cell lines, the use of thoroughly characterized, label-free nanomaterials and a range of complementary analytical techniques is still lacking. Herein, the uptake profile of two types of label-free, thin GO sheets with distinct lateral dimensions was established in thirteen cell lines: eight cancer (HeLa, A549, PC3, DU-145, LNCaP, SW-480, SH-SY5Y and U87-MG) and five non-cancer (BEAS-2B, NIH/3T3, PNT-2, HaCaT and 293T) cell lines, using a set of complementary analytical techniques (confocal microscopy, transmission electron microscopy, and flow cytometry).

We show a striking difference in the GO uptake profile between studied cancer and non-cancer cells, regardless of the lateral dimension of GO. In non-cancer cells, GO was interacting with the plasma membrane and was efficiently internalised by the cells. In contrast, GO was

predominately interacting with the plasma membrane of cancer cells, showing a minimal to no cellular uptake.

Using a range of nanomaterials with different properties (microspheres and graphene flakes), we confirmed that cancer cells could take up other nanomaterials in a manner comparable to non-cancer cells. We identified that the thickness of GO played a crucial role in the observed, distinct pattern of cellular interactions. Subsequently, we investigated the effect of GO on actin filaments, which are known to be crucial in the uptake process. We found that GO induced actin cytoskeleton reorganisation and impaired cellular migration in cancer but not in non-cancer cells. Taken together, we identified an interplay between the properties of GO and cellular effects elicited by GO that led to the reported striking differences in the uptake pattern. We propose that thin GO sheets acted as a cue upon interaction with the plasma membrane of cancer cell lines, subsequently inducing intracellular changes reflected in impaired endocytosis and migration activity of cancer cells, leading to reduced capacity towards GO uptake.

Introduction

Graphene oxide (GO), due to aqueous dispersibility, large surface area suitable for the loading of therapeutic cargos through surface functionalisation and excellent biocompatibility has received extensive research interest for various biomedical applications[1–3]. Examples include but are not limited to drug delivery platforms, contrast agents for bioimaging, and GO-enhanced photosensitising agents. However, to fully explore the biomedical potential of GO and assure its safe use, it is crucial to understand how GO interacts with biological systems at the cellular level.

Currently, a considerable number of *in vitro* studies have investigated interactions and uptake profile of GO. These studies mostly focused on cancer[4–26], normal[27–32], or immune cells[33–35], with the vast majority of the studies using immortalised, commercially available cell lines. Considering the surging interest in using GO in cancer therapy, especially as drug carriers, it is critical to understand the uptake pattern of GO in cancer and normal cells. Some studies have compared the uptake of GO between cancer and normal cells[36–39], cancer and immune cells[40–43], or a combination of cancer, normal, and immune cells[44–46]. However, these studies largely used only one of each cell type[36,40,41,44] or a total of no more than three cell lines for comparison[38,39,42,43,46]. One study examined the uptake of GO in six cell lines (three cancer, one normal, and two immune cell lines) and reported uptake of GO only in immune cells[45].

Thus, no existing studies have compared a large panel of cancer and normal cells, where commonalities and differences between GO interaction with diseased and healthy cells could be explored in-depth. Studies reporting GO uptake in cancer and normal cells have been contradicting[36–39,44–46]. Some studies showed the uptake of GO in both cancer and normal cells[38,39,44,46], and others showed no uptake of GO in cancer and/or normal cells[36,37,45]. In some cases, GO is modified with different moieties to enhance better cancer cell interaction compared to normal cells[37,39]. However, much uncertainty still exists about the uptake pattern of GO in cancer and normal cells. One factor that may have added to the inconsistent findings is that some studies involved the attachment of labels to enable GO tracing and uptake detection in cells [36,38,46]. Consequently, the intrinsic properties of GO can be altered by the labelling process, and the risk of misinterpretation due to potential label detachment is also increased. Thus, the understanding of the uptake of non-labelled, non-functionalised GO is still lacking.

Furthermore, the selection of analytical techniques for the uptake assessment of GO is critical. Confocal laser scanning microscope (CLSM), transmission electron microscopy (TEM), and flow cytometry are three of the most common techniques used for cellular uptake assessments. However, each of these techniques has its advantages and shortcomings. Briefly, CLSM allows efficient distinguishment between internalised and cell surface adsorbed material in a time-efficient manner, but fluorescent labelling of material is often needed. TEM is an extremely powerful technique that offers high-resolution images, but it is a low throughput technique, so assessment by TEM is often restricted to a small sample size of cells. Also, considering that cellular compartments and GO are built mainly of carbon atoms, clear distinguishment between the material and cellular compartments is not always easily achieved. Finally, flow cytometry quantifies the total cellular interaction of material but cannot distinguish between internalised material and those adsorbed on cells. Thus, CLSM, TEM, and flow cytometry are good complementary techniques, but ambiguity could arise due to over-reliance on only one of these techniques, particularly flow cytometry. However, surprisingly, flow cytometry has been frequently used as a stand-alone or main analytical technique for assessing cellular uptake of GO[20,21,33,42–46], and contradictory findings emerged. For example, while both Yue et al. and Paino et al. investigated the uptake of non-functionalised label-free GO (with similar sizes) by flow cytometry, the former reported that cancer and normal cells (MCF-7, Hep G2, LLC, and HUVEC) were not able to internalise the material, but the latter reported the uptake of GO in both cancer and normal cells (HeLa, L929)[44,45].

Herein, we systematically compared the uptake of label-free, non-functionalised GO in a large panel of cancer and normal cell lines using a mixed-method approach combining CLSM, flow cytometry and TEM. Furthermore, we identified the properties of GO and cellular effects that influence the way GO interacts with cancer and non-cancer cells.

Result and discussion

Interactions of GO with cancer and non-cancer cells.

Assessment by CLSM.

We first assessed the cellular uptake of GO by confocal imaging. We used small-GO (s-GO, 25 nm – 2.4 µm) and ultrasmall-GO (us-GO, 10 – 790 nm) in the present study (**Figure 1**, **Table S1**). All treatments were performed in the RPMI medium in the presence of foetal bovine serum (FBS, 10 %). As discussed in our previous studies, material gains new biological identities when dispersed in the biological medium[28,47]. Coating of various serum proteins and other supplements (e.g., electrolytes and biomolecules) on the material surface changes its properties, such as surface charge, size, and colloidal stability. This ultimately affects its interaction with the cellular system, and hence, comparable experimental settings for GO treatments were kept throughout the study.

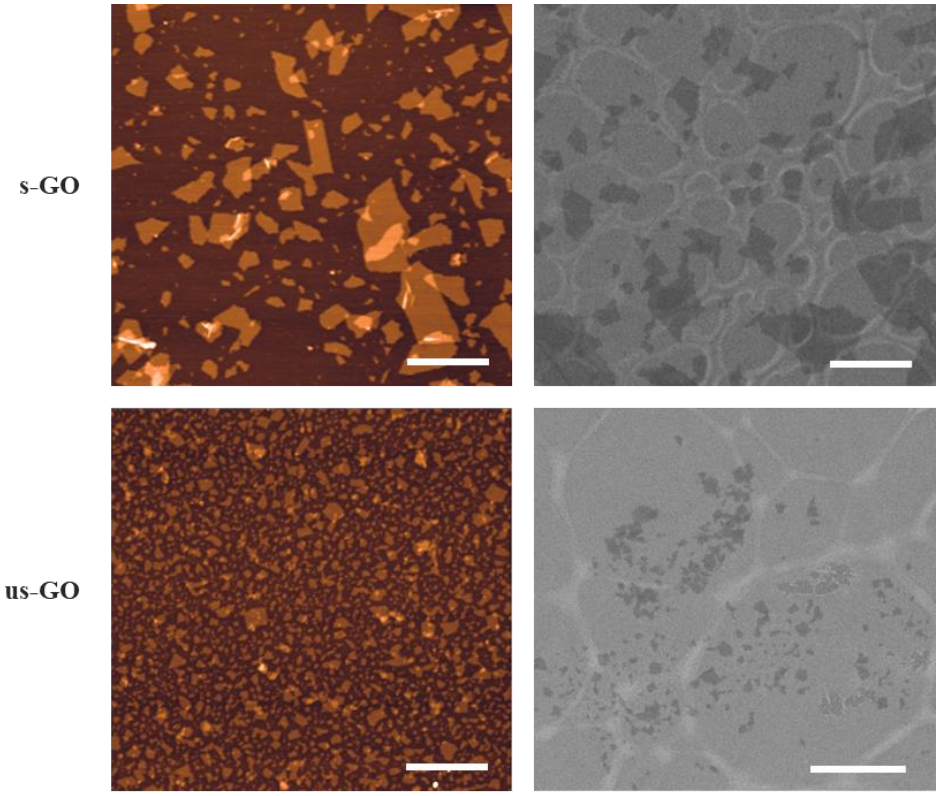

Figure 1: Characterisation of GO (s- and us-GO) by atomic force microscopy (left panels) and electron microscopy (right panels). Scale bar = 1 µm. See **Table S1** for the summary of the physico-chemical properties.

Figure S1 shows the interaction of GO (s- and us-GO, 50 µg/mL) in NIH/3T3 (non-cancer) and HeLa (cancer) cells after 24 h of incubation by confocal imaging. Cellular uptake of GO is indicated by the localisation of the GO signal (in red) within the plasma membrane (stained green); internalised GO is shown as concentrated red spots localised towards the centre of the cell. On the other hand, extracellular GO interacting with the plasma membrane is shown as dispersed clouds of red signals surrounding or localised on top of cells. Interestingly, we identified two distinct GO interaction patterns between the two cell lines. In NIH/3T3, both s- and us-GO were found internalised and interacting with the plasma membrane, with no obvious differences in the uptake quantity. Meanwhile, for HeLa cells, both s- and us-GO were found to interact with the plasma membrane, and no intracellular distribution of the materials was observed. This pattern agrees with the results from our previous study, where we observed the uptake of GO in the non-cancer cell lines[47]. The uptake of GO (s- and us-GO at 25, 50, and 75 µg/mL for 24 h) was then assessed in a total of eight cancer and five non-cancer cell lines by CLSM (**Figure 2**, **S2** – **S13**, and **Table S2**). Surprisingly, the non-cancer cells were repeatedly

found to take up both s-GO and us-GO, but none of the cancer cell lines were found to take up the two materials. Further analysis in cancer (HeLa, LNCap, PC3) and non-cancer (BEAS-2B, NIH/3T3, PNT-2) cells showed the same interaction pattern remained for large-GO (l-GO, 1.5 – 25.5 µm) (**Figure S14**).

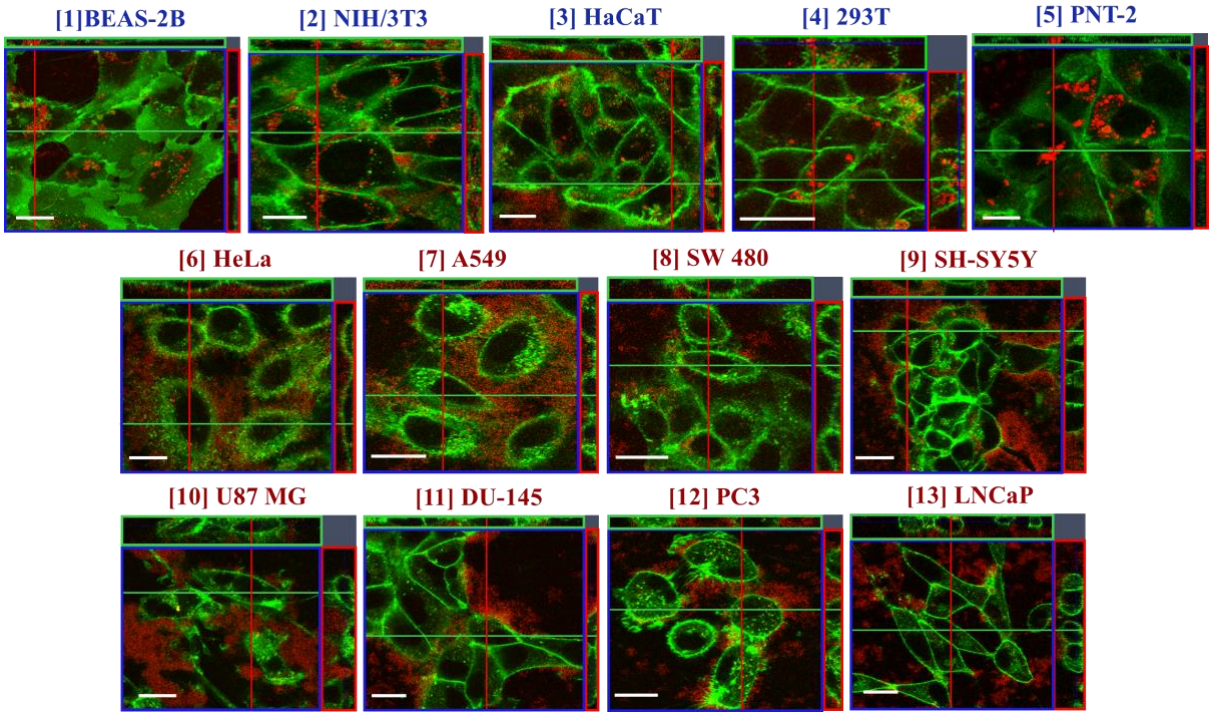

Figure 2: Interaction of GO (s- and us-GO) in cancer and non-cancer cells, assessed by CLSM (orthogonal projections of the middle section of the cell are shown). Non-cancer cells: **[1]** BEAS-2B (s-GO treated), **[2]** NIH/3T3 (us-GO treated), **[3]** HaCaT (s-GO treated), **[4]** 293T (us-GO treated), **[5]** PNT-2 (s-GO treated). Cancer cells: **[6]** HeLa (s-GO treated), **[7]** A549 (us-GO treated), **[8]** SW 480 (s-GO treated), **[9]** SH-SY5Y (us-GO treated), **[10]** U87 MG (s-GO treated), **[11]** DU-145 (us-GO treated), **[12]** PC3 (s-GO treated), and **[13]** LNCaP (s-GO treated). See **Figure S2** – **13** for the full panel of GO (s- and us-GO) interaction profiles in cell lines **[2]** – **[13]**, respectively. See Chen et al.[47] for in-depth GO (s- and us-GO) interaction profiles and uptake mechanisms in **[1]** BEAS-2B cells. See **Table S2** for the summary of GO (s- and us-GO) interaction profiles in all cell lines. Green = plasma membrane, Red = GO. Scale bar = 20 µm.

The optimal cell culture media for individual cell lines varies depending on the cell's growth requirements[48]. Herein, all studied cell lines were maintained in cell-type specific media

(supplemented with 10% FBS) either recommended by the American Type Culture Collection (ATCC) or previously determined experimentally. However, we used the same cell culture media (RPMI-1640 with 10% FBS) in all GO treatments. RPMI-1640 is one of the most widely used media for the maintenance of cancer and non-cancer cells[49]. The benefit of this approach is that the potential variability in GO cellular response induced by the differences in cell-culture media compositions can be excluded. Hence, this allows better comparisons of the GO cellular interactions between cancer and non-cancer cells. To establish if the GO uptake differences between cancer and non-cancer cells were due to the effect of the treatment media, we also examined the uptake profile using different treatment media. **Figure S11b** shows the exposure of human epithelial prostate carcinoma cells (DU 145) to GO treatment under the ATCC recommended medium for DU 154 cells (Eagle's Minimum Essential Medium [EMEM] with 10 % FBS) by CLSM. We observed no apparent changes in the GO (s- or us-GO) interaction pattern with DU 145 cells using RPMI-1640 (see **Figure S11a**) or EMEM (see **Figure S11b**). In addition, human epithelial glioblastoma cells (U87 MG) also showed comparable GO interaction patterns using RPMI-1640 (see **Figure S10**) or U87 MG cell-specific media, as shown in our previous study[50]. Therefore, the choice of treatment media was not the dominant factor driving GO uptake differences between cancer and non-cancer cells.

Assessment by TEM.

To confirm the observations by CLSM, we next examined the uptake of GO using TEM (**Figure 3a** – **d**). As expected, internalised GO was found within the vesicles of BEAS-2B and NIH/3T3 cells, with no obvious signs of cytosol localised GO flakes. In contrast, most HeLa and A549 cells showed no sign of GO internalisation. Thus, this finding confirmed the uptake difference of GO in cancer and non-cancer cells, as identified by CLSM. However, we noticed that cancer cells attempted to take up the materials, as indicated by plasma membrane ruffling and deformations near GO (**Figure S15**). Also, although most of the s- and us-GO were found interacting with the cancer cell plasma membrane without being taken up, very few cancer cells were found with internalised small amounts of us-GO, localised within intracellular vesicles (**Figure S15**).

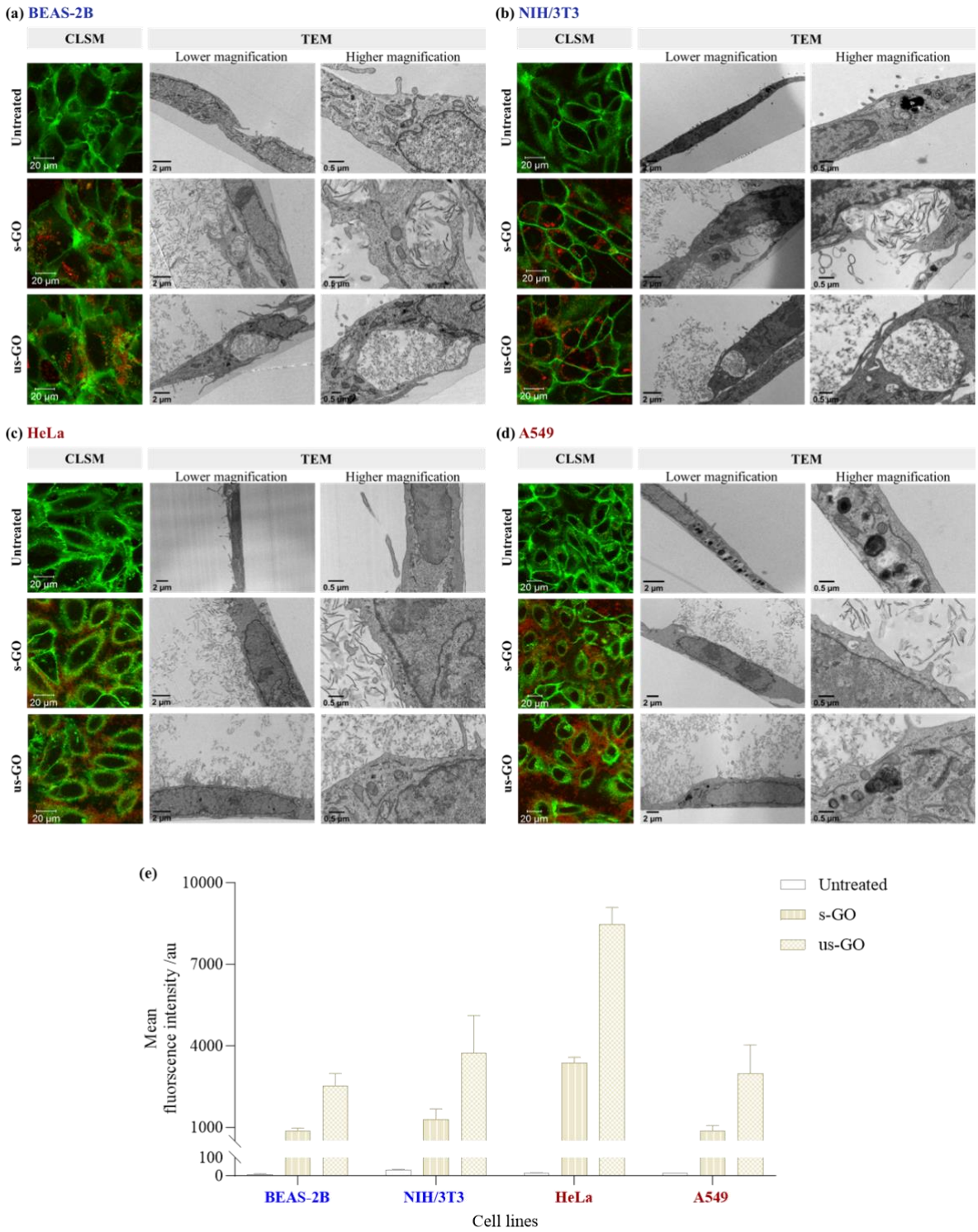

Figure 3: Interaction of GO (s- and us-GO, 50 μg/mL) with **(a)** BEAS-2B, **(b)** NIH/3T3, **(c)** HeLa and **(d)** A549 cells by CLSM, TEM, and **(e)** flow cytometry. For analysis by flow cytometry, the cells were collected with PBS (-/-) washing; see **Figure S16** for cells collected without PBS (-/-) washing. See **Table S3** for the corresponding statistical analysis of the interaction of GO with cells by flow cytometry. Green = plasma membrane, red = GO.

Assessment by flow cytometry.

We further examined the cellular interaction of GO using flow cytometry, which quantifies the total interaction of GO with cells (i.e., the sum of GO taken up and adhered to the cells). The interaction of GO (s- and us-GO) with cancer (HeLa, A549) and non-cancer (BEAS-2B, NIH/3T3) cell lines were compared, with (**Figure 3e**) and without (**Figure S16**) washing prior to flow cytometry examination. For both cancer and non-cancer cell lines, washing greatly reduced the mean fluorescent intensity (MFI) of GO (s- and us-GO), which confirmed the presence of GO bound to the cell surface (see **Figures 3e** and **S16**). However, regardless of the types of GO and whether washing was performed, the MFI for HeLa cells was significantly higher than for the other three cell lines. In contrast, no significant differences were observed between A549, BEAS-2B and NIH/3T3 cells (see **Table S3a** and **Figure S16** [**table a**]).

This is a rather interesting result because, by CLSM and TEM, both HeLa and A549 cells clearly showed minimal uptake of GO compared to BEAS-2B and NIH/3T3 cells. Thus, GO adhered to the surface of HeLa and A549 cells would greatly contribute to the fluorescent signal measured by flow cytometry. The difference in MFI between HeLa and A549 cells suggests that GO adhered more strongly to the HeLa cell membrane than the A549 cell membrane (**Figure 3e**). A question raised from this result is whether the minimal uptake of GO across different cancer cell lines was due to a common or different mechanism. If cancer cells followed a common mechanism, this result suggests that the underlining mechanism, to a certain extent, has tolerances for a range of interaction forces between GO and the cell. Also, the MFI for us-GO was significantly higher than s-GO in all studied cell lines, with and without washing (see **Table S3b** and **Figure S16** [**table b**]). Considering the spectrofluorometric analysis of the emission spectra of GO showed that the intrinsic fluorescent intensity of s-GO was higher than us-GO (**Figure S17**), the finding suggests that us-GO was better internalised and/or adsorbed to the plasma membrane of the cells than s-GO.

The most unexpected finding so far is the consistent limited uptake of both GO materials by cancer cells. Regarding the uptake of non-functionalised label-free GO in cancer cells, most previous studies have reported successful uptake of GO using flow cytometry[20,21,44] and TEM[5,16,22,23,40]. However, this does not necessarily mean that our findings contradict the earlier studies. For example, based on flow cytometry results, several studies concluded the uptake of GO in A549 cells[20,21]. Although comparable observation is seen in this study (see **Figures 3e** and **S16**), unless the evidence for complete removal of extracellularly bound GO has been

provided, it is difficult to confirm the uptake solely based on flow cytometry. Another point to mention is that previous studies have often reported a higher uptake of GO in cancer than in non-cancer cells when assessed by flow cytometry[42–44,46]. This conclusion differs from the one made here, as we observed visually a much higher uptake of GO in non-cancer than in cancer cells (see **Figures 2**, **3**, and **S1** – **14**). However, in the present work, HeLa cells also showed the highest measured signal upon treatment with GO by flow cytometry. Thus, this further illustrates how potentially misleading conclusions might have been generated using a single analytical technique.

Using TEM, Chang et al. reported no uptake of GO (430 ± 300 nm) in cancer cells (A549)[4]. In contrast, as stated before, the uptake of GO in cancer cells by TEM has been widely reported[5,16,22,23,40]. However, what has been less considered is that the cell sample size analysed by TEM is relatively small compared to other techniques, such as confocal imaging. Therefore, some studies suffer from an overemphasis on the success or failure of GO uptake but are rarely concerned with the level of GO uptake and the scale of the cell sample. In fact, even for those who have performed the investigation using several techniques, the attention paid to the level of GO internalisation is still limited[24,41]. For example, based on prostate cancer cells (PC3), Zhu et al. characterised the cellular localisation of GO by TEM and fluorescently labelled GO by CLSM[24]. Using both TEM and CLSM, Zhu et al. clearly showed that GO adhered to the cell surface and was internalised by PC3 cells[24]. However, visual inspection of the uptake level of GO was far less compared to those internalised by non-cancer cells in this study (see **Figure S5** for the interaction of GO in prostate non-cancer cells). Although it may be argued that the lower uptake level of GO in Zhu et al.'s study was due to the lower tested concentration of GO (10 μg/mL, 24 h), it is clear from confocal images that most of GO was found interacting with the plasma membrane than being taken up by PC3 cells[24].

With regards to the uptake of GO in non-cancer cells, the results from the present work accord with our previous findings[28,47] and literature findings for both non-functionalised, label-free GO[31] and fluorescently labelled GO[32,36]. To date, not many studies have reported the lack of GO uptake in non-cancer cells. However, the uptake of GO in non-cancer cells has been shown to depend on the cell phenotype[31]. For example, Kucki et al. showed that GO was internalised by undifferentiated intestinal cells but not the differentiated intestinal cells with hair-like surface structures[31]. Thus, cell surface topography that permits the better association of GO

with the plasma membrane allows enhanced GO uptake. In another example, Yue et al. concluded that there was no uptake of GO in the non-cancer HUVEC cells[45]. However, the conclusion was reached solely based on flow cytometry.

Assessment of the uptake capacity of cancer and non-cancer cells.

Size, shape, and surface chemistry are the most extensively studied physico-chemical properties that play critical roles in nanomaterials (NMs) cellular interaction. In order to explain the observed differential uptake of GO by cancer and non-cancer cells, we aimed to understand the capacity and preferences of cancer and non-cancer cells towards the uptake of materials with various sizes, shapes, and surface chemistry. The NMs used were divided into two groups: **(1)** commercially available polystyrene microspheres of various sizes and functionality (negatively charged carboxylate-modified beads [0.1, 0.5, and 1 μm] and positively charged amine-modified beads [0.2 and 1 μm]), and **(2)** graphene flakes of various functionality (negatively charged 1-pyrenesulfonic acid sodium salt stabilised graphene dispersion [Gr-PS1] and positively charged trimethylammonium linked pyrene stabilised graphene dispersion [Gr-TMA_3]). Readers should refer to previous literature for detailed production and characterisation of Gr-PS1 and Gr-TMA_3[30,51,52].

The reasons for the use of these NMs were as follows. First, conventional NMs are three-dimensional, and those in the shape of spheres are commonly used[53]. Variability in dimensionality is one of the biggest differences between conventional and two-dimensional (2D) NMs like GO. Meanwhile, spheres have been reported to be better internalised than 2D sheets[36]. For example, Seemork et al. reported better internalisation of oxidised carbon nanosphere than oxidised carbon sheet in cancer (A549) and non-cancer cells (HEK293T)[36]. Thus, the microsphere represents a potentially less challenging material to internalise than 2D sheets. Therefore, by interrogating cells' capacity to take up microspheres of different sizes and functionality, we can confirm if cancer cells, in general, have a lower endocytic ability than non-cancer cells or if this applies only to materials with specific properties. Second, using graphene flakes of different functionalities and surface charges, we can confirm the ability of cancer cells to internalise 2D NMs and investigate if the uptake of 2D NMs can be affected by the surface charge and type of 2D NMs used.

Uptake of microspheres.

Figures 4 and **5** present the interaction of cancer and non-cancer cells with the FITC-labelled microspheres, respectively assessed by CLSM and flow cytometry. For simplification purposes, the surface charges of the beads (positively or negatively charged) are indicated by the positive (+) or negative (-) sign, respectively. Interestingly, **Figure 4** showed that for both cancer and non-cancer cells, the uptake of the -1 μm beads was comparable to the +1 μm beads in each cell line. Further inspection of the images for these two types of beads revealed that the BEAS-2B cells exhibited the highest uptake, followed by HeLa, NIH/3T3 and A549 cells. This was confirmed by flow cytometry (see **Figure 5**) in which the MFI of the -1 and +1 μm beads for each cell line were as follows: BEAS-2B (-1 μm = 20000, +1 μm = 18871), NIH/3T3 (-1 μm = 4735, +1 μm = 5174), HeLa (-1 μm = 5756, +1 μm = 6438) and A549 (-1 μm = 4270, +1 μm = 4531). The uptake of -0.5 μm beads in cancer and non-cancer cells exhibited a similar trend to the 1 μm beads: the MFI of the beads was highest in BEAS-2B cells (6341), followed by NIH/3T3 (2168), HeLa (1545) and A549 (866) (see **Figures 4** and **5**).

In contrast, comparing the uptake of the smaller-size beads with opposite surface charges, it is apparent from the confocal images that the +0.2 μm beads were taken up much better than the -0.1 μm beads (see **Figure 4**). The different cellular response toward the -0.1 and +0.2 μm beads was also reflected by flow cytometry (see **Figure 5**). Moving on to the comparison between different cell lines, CLSM showed that uptake of the +0.2 μm beads was slightly higher in HeLa and BEAS-2B cells compared to A549 and NIH/3T3 cells, as indicated by saturation of signal in HeLa and BEAS-2B cells. This agrees with the result generated by flow cytometry, which showed the highest MFI of +0.2 μm beads in HeLa (22614), followed by BEAS-2B (12707), A549 (11472) and NIH/3T3 (8356) cells. Also, the measurement by flow cytometry for the -0.1 μm beads agrees with the observation by CLSM: BEAS-2B (1843) and NIH/3T3 (662) cells showed higher uptake of the -0.1 μm beads than Hela (545) and A549 (344) cells.

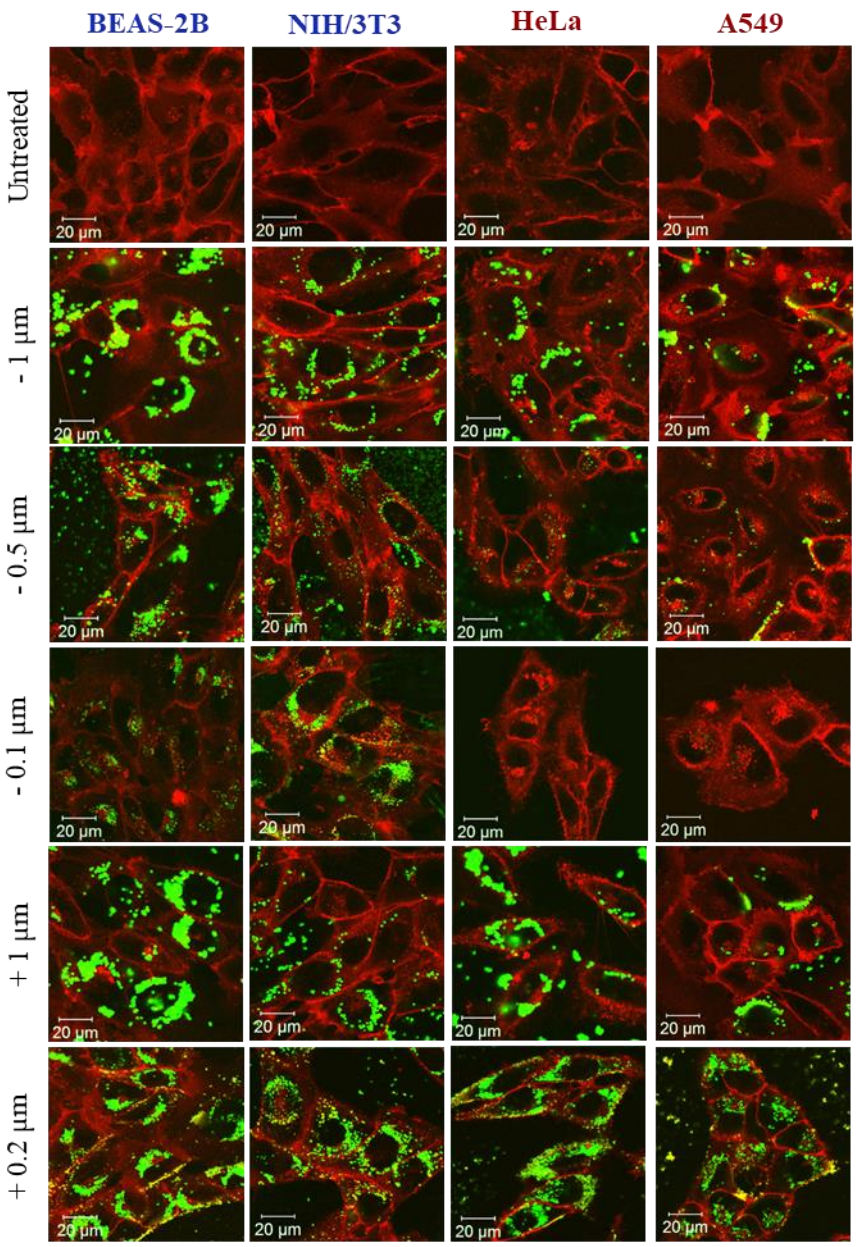

Figure 4: Interaction of BEAS-2B, NIH/3T3, HeLa and A549 cells with negatively charged (–) 0.1, 0.5, and 1 µm carboxylate-modified, and positively charged (+) 0.2 and 1 µm amine-modified beads by CLSM. Red = plasma membrane, green = FITC-labelled beads. Scale bar = 20 µm.

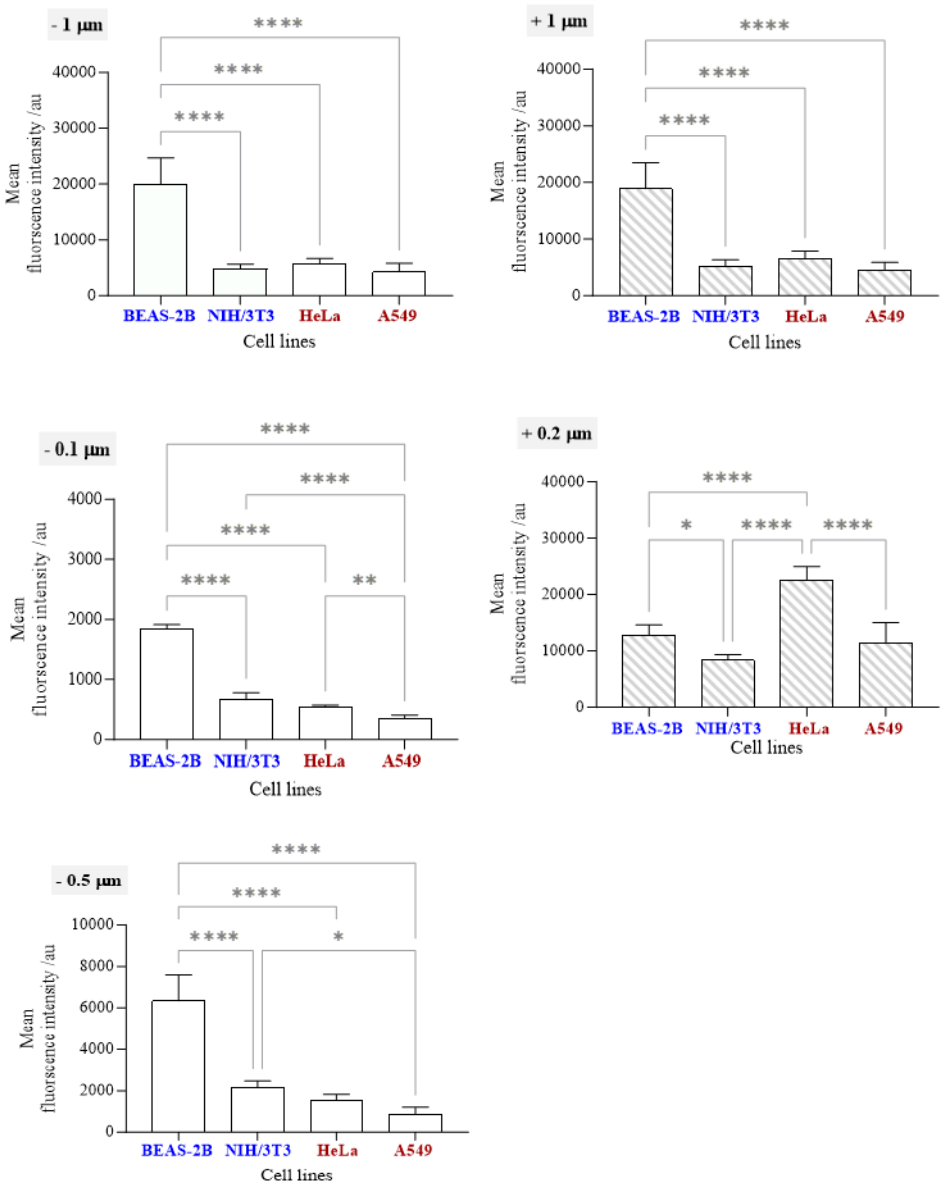

Figure 5: Interaction of BEAS-2B, NIH/3T3, HeLa and A549 with negatively charged (–) 0.1, 0.5, and 1 µm carboxylate-modified, and positively charged (+) 0.2 and 1 µm amine-modified beads by flow cytometry. The data were statistically analysed using analysis of variance (one-way ANOVA) with Tukey's multiple comparisons test. n = 3 with duplicates. *Statistically different: $*p < 0.05$, $**p < 0.01$ and $****p < 0.0001$.

These findings provide important insight into the effect of material properties on its uptake in cells. First, although certain types of beads were taken up better than others, and this varied in different cell lines, both cancer and non-cancer cells could take up all the beads irrespective of their size or charge. This means cancer cells can take up negatively charged material of various sizes. Furthermore, the fact that the NIH/3T3 cell showed no significant difference in the

uptake of -1 μm beads compared to the A549 and HeLa cells suggests the cancer cells do not, intrinsically, exhibit a lower uptake capacity toward negatively charged material. This view is further supported by the comparable uptake of -1 and +1 μm beads in each cell line. However, both cancer and non-cancer cells were found to take up the small positively charged (+0.2 μm) beads better than the negatively charged (-0.1 μm) beads. Yet, what stands out for the cancer cells is that the +0.2 μm beads were not only taken up better than the -0.1 μm beads but were also internalised the most out of the five types of beads. This suggests that the role of surface charge on the uptake of material in cancer cells may become more significant when the material is below a certain size.

Uptake of Graphene flakes.

From the investigation of the uptake of microspheres, we understand that surface charge may have a significant role in the uptake of material in cancer cells when the material is below a certain size. In this section, we investigated the uptake of Gr-TMA_3 and Gr-PS1 in cancer (HeLa) and non-cancer (BEAS-2B) cells. In **Figure S18**, CLSM was used to examine the uptake of graphene flakes in BEAS-2B and HeLa cells. As shown in **Figure S18**, due to the light absorption properties of graphene flakes, the material appears black in the bright field channel. On the other hand, the cell was stained green with the fluorescein diacetate (FDA) dye, and where the cell had taken up the material, the flakes quenched the signal of the dye (see **Figure S18**). Hence, co-localisation of the black material (in the bright field channel) with the absent intracellular fluorescent signal (in the FDA channel) indicates uptake of the graphene flakes. It is apparent from **Figure S18** that both Gr-TMA_3 and Gr-PS1 were internalised in BEAS-2B and HeLa cells, which confirms that cancer cells could take up positively or negatively charged 2D NMs. However, in both BEAS-2B and HeLa cells, the positively charged Gr-TMA_3 were internalised better than the negatively charged Gr-PS1 flakes. Considering that graphene flakes had an average size of approximately 200 nm[30], this is in line with those observed from the uptake study of the microspheres, where the small positively charged microspheres were taken up better than the negatively charged ones. In addition, BEAS-2B cells were noticeably taken up Gr-PS1 better than HeLa cells. This is also in line with those observed from the uptake study of the microspheres, where the small negatively charged microspheres were taken up better in BEAS-2B than HeLa cells.

So far, findings from the uptake study of microspheres and graphene flakes suggest that the material surface charge affects its uptake in cancer and non-cancer cells, but not in an “all or

nothing" manner. As is well-known, the material surface charge affects the electrostatic interactions between the material and cell plasma membrane[54]. Positively charged NMs have been reported to internalize better than their negatively charged counterparts due to a more energetically favourable interaction with the cell surface that enhances the endocytosis process[54,55]. Indeed, it has been suggested that the lack of GO uptake in non-phagocytic cells may be due to the electrostatic repulsion between the material and plasma membrane[45]. However, we observed GO interaction with both cancer and non-cancer cell plasma membranes, and our result by flow cytometry suggested that GO adhered strongly to the cancer cell surface (see **Figures 2**, **3**, and **S1** – **16**). In fact, the adhesion of GO on the plasma membrane of cancer cells has been widely acknowledged by different research groups[21,24,32,41].

It is worth noting here that GO may interact more with the cancer cell plasma membrane than the microspheres/graphene flakes. As shown by CLSM (see **Figure S1**, the middle section of cells), GO was displayed as a uniform cloud of signal surrounding the HeLa cell plasma membrane, outlining the shape of HeLa cells. In comparison, substantially fewer microspheres/graphene flakes were found surrounding the HeLa cell plasma membrane (see **Figures 4** and **S18**). The difference in material thickness may have contributed to the difference between GO and the microspheres/graphene flake in the plasma membrane interaction. Being mostly monolayer, over 90 % of the GO sheet had a thickness of 2 nm or less (see **Table 1**). In contrast, while the microspheres had a thickness range of 0.2 - 1 μm, the graphene flakes existed mostly as few-layers graphene (70 and 61 % for Gr-TMA_3 and Gr-PS1, respectively) with a thickness range of 2 – 20 nm[30,51,52].

Previous research into the effect of Gr thickness by simulation showed that few-layer graphene (3 or 9 layers) exhibited a minor increase in the energy barrier for piecing the lipid bilayer compared to the single-layer graphene[56]. Yet, our understanding of the effect of thickness on the cellular uptake of graphene-based material is lacking. It can be expected that thickness influences the specific surface area by volume of the material, and these differences can directly affect the colloidal behaviour of the material, which ultimately results in the formation of different biological interfaces when interacting with cells[57]. Thus, despite its two-dimensionality, the colloidal behaviour of graphene flakes may share commonality with the three-dimensional microspheres, contributing to a more similar pattern of cellular interaction. In contrast, the thinner GO possess greater two-dimensionality and remains as individualised 2D sheets when interacting with cells, as shown by TEM (see **Figure 3**).

Taken together, we understand that cancer cells could take up materials with diverse properties, including shapes, sizes, or surface charges comparable to GO, to an extent. However, neither the uptake of microspheres nor the graphene flake occurs in the cell-type specific manner as observed for GO. We believe that the difference in thickness between GO and microsphere/graphene flake may have contributed to the cell-type specific uptake of GO.

Assessment of the effects of GO on endocytosis-relevant biological properties of the cells.

The effect of GO on actin filaments.

It was reported that GO could induce biological changes in cells, such as alteration of the plasma membrane composition[58], cytoskeleton[13,20,41,59] and migratory ability of cells[19,60]. Therefore, we asked if GO could induce differential changes in endocytosis-relevant biological properties of cancer and non-cancer cells, ultimately contributing to the observed differences in GO uptake.

The effect of GO on cytoskeleton was examined by actin filament staining. Remodelling of the cell cytoskeleton networks is a prerequisite for endocytosis[61,62]. In particular, among the three major constituents of the cytoskeleton network (microtubule, intermediate filament, and actin filament), actin filaments are crucial in all three major uptake pathways[63]; actin filaments disruption agents such as latrunculins and cytochalasin D are well-known pharmaceutical inhibitors of endocytosis[64].

Figure 6 (and **Figure S19**) show the actin filament network for BEAS-2B and HeLa cells with and without GO (s- or us-GO, 100 μg/mL, 24 h), stained using the Alexa-Fluor 488-labelled phalloidin (see **Figure S20** for NIH/3T3 and A549 cells). In addition, methyl-beta-cyclodextrin (MβCD), which can disrupt the cytoskeleton network by depletion of the cholesterol in the cell membrane, was used as the positive control. Images in **Figure 6** (and **S19**, **S20**) are shown in the range indicator mode to better compare the actin filament network. The images are displayed in black and white, with red and blue areas. Red and blue represent areas with saturated and zero intensity, respectively. At the same time, black and white colours represent lower and higher intensity areas, respectively. The settings were adjusted to display the background in blue, and the same intensity settings were applied in each cell line.

For all four cell lines, it is apparent that M βCD induced significant disruption to the actin filament network, resulting in an altered cell morphology compared to the untreated cells (see **Figure 6**, **S19** and **20**). In general, M βCD treated cells appeared smaller than the untreated cells. In addition, features including the loss of the parallel distribution of the filaments, formation of actin spots, fragmented fibres, and increased stress fibres can also be identified in M βCD treated cells. On the other hand, the result showed that GO (s- and us-GO) could induce changes to the actin filament network in cancer cells, but no apparent differences were noted in the non-cancer cells (see **Figure 6**, **S19** and **20**). One observed similarity in the effect of GO (s- and us-GO) on HeLa and A549 cells was that the actin filament density appears to be reduced in the GO treated cells compared to the untreated cells, shown by the increased lower intensity areas in GO treated cells. However, neither s-GO nor us-GO in HeLa or A549 cells could induce changes to a level comparable to M βCD, where a pronounced reduction in the shape and size of cells was observed.

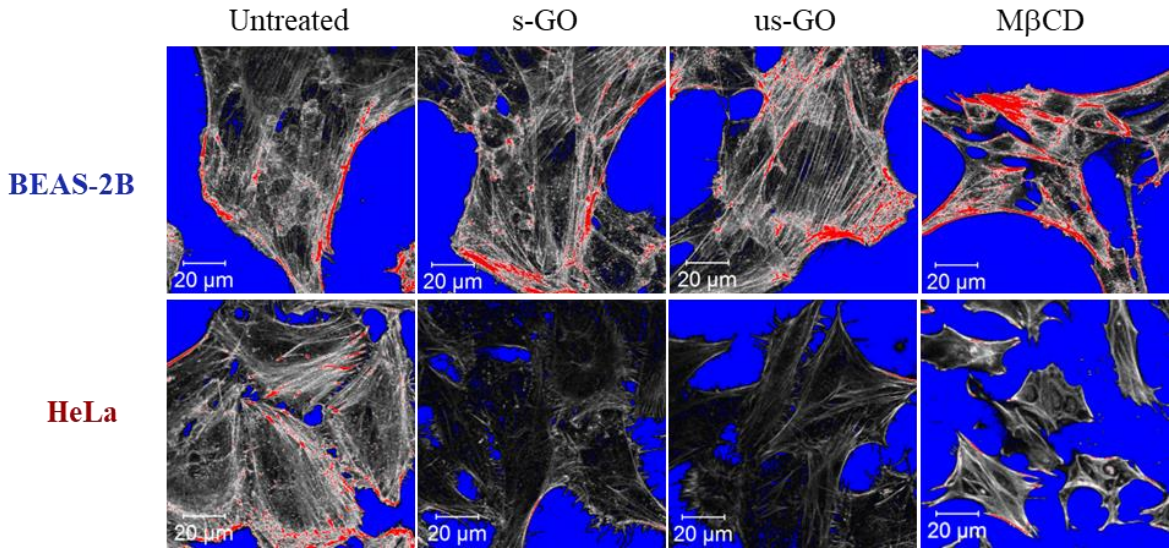

Figure 6: Staining of actin filament in BEAS-2B and HeLa cells, treated with and without GO (s-GO or us-GO at 100 μg/mL, 24 h) or MβCD (5 mM, 4 h, positive control). Cells were stained with the phalloidin 488 dye for actin filament, and the images are shown in the range indicator mode: red and blue represent saturated and zero intensity areas, whereas black and white represent lower and higher intensity areas, respectively. See **Figure S19** for more BEAS-2B and HeLa cell images at different magnifications. See **Figure S20** for actin filament staining in NIH/3T3 and A459 cells.

As is well known, the dynamic actin cytoskeleton is heavily involved in many endocytic pathways, including clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis

(CavME) and macropinocytosis[64]. Notably, we previously confirmed GO internalisation in BEAS-2B cells *via* these three pathways[47]. Furthermore, disruption to the organisation and dynamic of the actin cytoskeleton has been widely recognised as the effect of various endocytosis inhibitors[64]. Hence, this result provides some tentative initial understanding of the observed GO uptake difference between cancer and non-cancer cells. Yet, the finding raised the intriguing question regarding the underlying mechanism for the remodelling of the actin cytoskeleton by GO and the selectivity toward cancer cells.

In agreement with the present results, previous studies have reported the reduction of the actin filament density in cancer cells with GO treatment[20,41]. It has been suggested that GO can impair the cytoskeleton by direct interaction[20,69] or indirectly through suppression of the cell adhesion system[41]. Tian et al. and Bera et al. suggested the direct interference of GO with the actin filament and microtubule network in A549 and HCT116 cancer cells, respectively[20,69]. However, in these studies, the main evidence for GO's direct interference with the cytoskeleton was based on GO incubation with the isolated actin filaments and tubulin protein. In our study, for cancer cells, GO was found predominantly surrounding the plasma membrane and not being available to directly interact with actin filaments in the cytosol.

In the study by Zhu et al., GO impaired the plasma membrane and lowered integrin expression in A549 cells, which subsequently affected the cell adhesion complex and the cytoskeleton network[41]. A growing body of evidence demonstrated that changes in cell biomechanical properties induce actin cytoskeleton reorganisation and modulate the uptake of various nanoparticles[70–73]. For example, higher uptake of nanoparticles was observed for cells seeded on stiffer substrates[71,72]. Furthermore, substrate stiffness has been reported to regulate cell stiffness, with cell softening and reduced actin polymerisation observed for cells seeded on softer substrates[74]. Considering the increasing amounts of evidence to suggest that cancer cells are softer than normal cells in 2D cultures[75], and the recent evidence that shows GO can modulate cancer cell mechanical properties by reducing cell stiffness and inducing actin cytoskeleton remodelling[76]. One possible hypothesis is that GO acts as mechanical cues that prompt further softening of cancer cells, resulting in cytoskeletal reorganisation, and ultimately affecting the activity of endocytosis toward GO.

The effect of GO on cell migration.

Subsequently, the effect of GO on cell migration was investigated to validate its effect on actin filaments. Like endocytosis, regulation of the cytoskeleton network is essential for cell migration[65,66]. Therefore, the disruption of the actin cytoskeleton will affect cell migration. For

instance, pharmaceutical inhibitors such as cytochalasin B, which disrupt actin filament polymerisation, can inhibit cell migration as well as endocytosis[67,68]. We employed time-lapse tracking to assess the effect of GO on cell migration.

Herein, HeLa and BEAS-2B cells were used as representatives of cancer and non-cancer cell lines. This is because HeLa has been one of the most studied cancer cell lines for GO cellular interaction. On the other hand, BEAS-2B was chosen because we have previously established the uptake mechanism of GO in BEAS-2B cells[47].

In **Figure 7**, we compared migratory differences between untreated cells and cells treated with s-GO (50 μg/mL), for BEAS-2B and HeLa cells. Using cell trajectories, we observed that for both BEAS-2B and HeLa cells, untreated cells moved in a larger spread area than cells treated with s-GO (**Figure 7a**). However, the difference between the observed effect was greater for HeLa than BEAS-2B cells (**Figure 7a**). This observation was further supported by the mean square displacement (MSD) measurement (**Figures 7b** and **7e**), where the average surface area used by cells was plotted over time[77].

To understand better the effect of s-GO on BEAS-2B and HeLa cells, we further examined two key parameters that account for cell migration efficiency: directionality persistence and cell speed[77]. In **Figures 7c** and **7f**, the average directionality ratio over time is shown. The directionality ratio (d/D) for a set time point is the ratio of the straight-line distance (d) from the cell's initial to end positions at the set time point over the actual distance (D) the cell travelled. Hence, if the directionality ratio equates to one, it suggests the cells have followed a perfectly straight path, whereas if the ratio approaches zero, it means the cells have followed a highly curved path. However, it should be noted that, in the absence of migration cues, directionality persistence for randomly moving cells will decay and vary considerably over time[77]. Therefore, instead of comparing the directionality ratio at one set time point (e.g., at the endpoint), assessing the directionality ratio over the time course better represents directional persistence.

As shown in **Figure 7c**, the BEAS-2B untreated cells followed a straighter path than s-GO treated BEAS-2B cells. This is shown by the rapid decay of the directionality curve in s-GO treated cells (mean d/D: 0.236 ± 0.008) compared to untreated cells (mean d/D: 0.402 ± 0.008). Whereas for HeLa untreated and s-GO treated cells, no apparent differences in the directionality ratio were observed over time (see **Figure 7f**). The directionality curve for HeLa untreated (mean d/D: 0.359 ± 0.006) and s-GO treated (mean d/D: 0.372 ± 0.006) cells stay

overlapped over the entire time course of cell trajectory. On the other hand, the HeLa cell speed reduced significantly with s-GO treatment, but comparable cell speeds were found for BEAS-2B cells with or without s-GO treatment (see **Figure 7d** and **7g**, respectively). For example, the average speed for HeLa untreated cells was 0.091 ± 0.050 μm/min, which was reduced by 50.5 % in s-GO treated cells (0.045 ± 0.021 μm/min). In contrast, only an 8 % difference was found in the average speed between BEAS-2B untreated (0.438 ± 0.170 μm/min) and treated (0.403 ± 0.124 μm/min) cells.

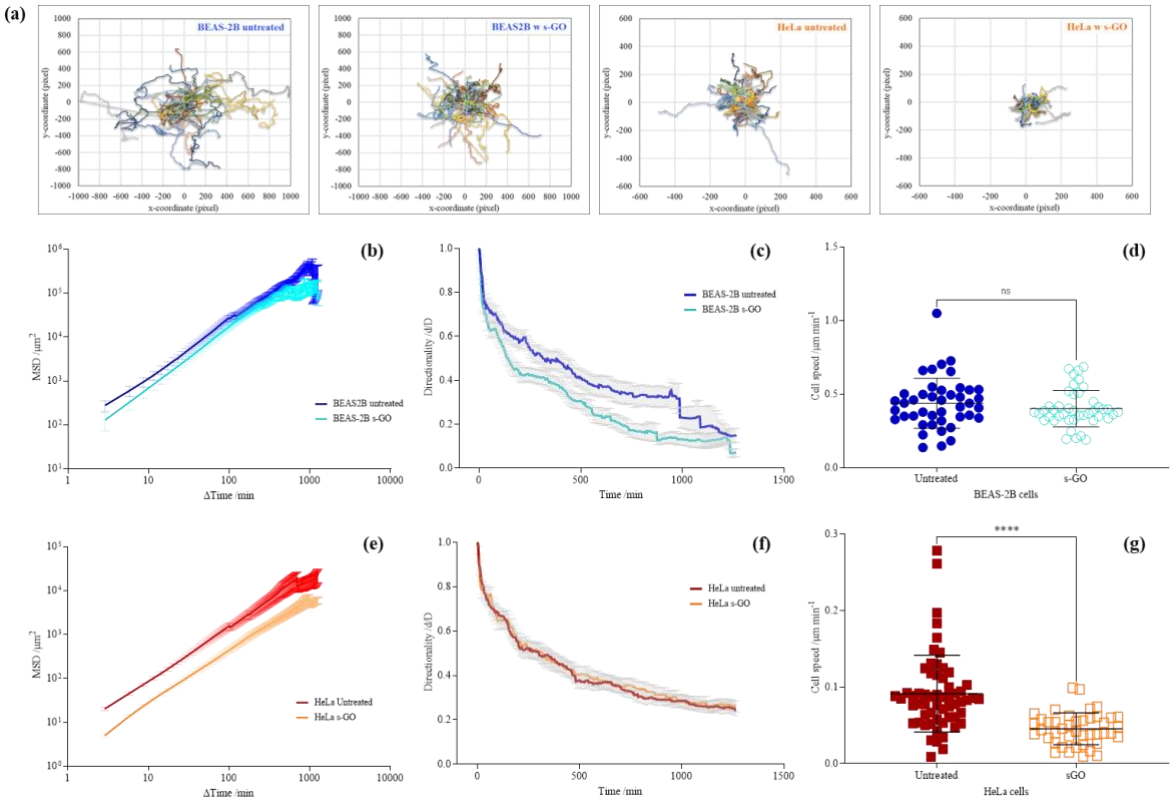

Figure 7: The effect of GO (50 μg/mL) on **(a – d)** BEAS-2B and **(a**, **e – g)** HeLa cell migration, assessed by live cell time-lapse tracking. Cell trajectories are visualised *via* **(a)** plot at the origins and further analysed *via* **(b**, **e)** MSD, **(c**, **f)** directionality analysis, and **(d**, **g)** cell speed measurement. Cell speeds are statistically analysed using an unpaired T-test. Number of cells analysed per condition = 43. *Statistically different: ****$p < 0.0001$, ns = not significant.

The observed effect on the speed and spread area of s-GO treated HeLa cells supports the finding that GO induced actin filament disruption in this cell line. Also, what stands out from the result is that s-GO affected the migration of BEAS-2B and HeLa cells differently, with the directional persistence lowered in the former and the cell speed reduced in the latter. The result

showed that the effect of s-GO on cell migration was also cell-line specific, further supporting the idea that GO induces differential biological changes in cancer and non-cancer cells.

In addition, as shown in **Figure 8**, we directly compared the migratory ability between BEAS-2B, NIH/3T3, HeLa and A549 cells when interacting with s-GO (50 µg/mL). The non-cancer cells exhibited a larger spreading area compared to the cancer cell lines, with BEAS-2B cells exploring the widest territory, followed by NIH/3T3, A549 and HeLa cells (**Figure 8a**). This agrees with the MSD plot (**Figure 8b**). Furthermore, HeLa and A549 cells displayed a higher directional persistence than BEAS-2B and NIH/3T3 cells. For example, the mean directionality ratio for HeLa and A549 cells was 0.382 ± 0.005 and 0.322 ± 0.006, whereas for BEAS-2B and NIH/3T3 cells was 0.263 ± 0.010 and 0.260 ± 0.008, respectively (**Figure 8c**). In contrast, BEAS-2B and NIH/3T3 cells displayed a higher cell speed than the A549 and HeLa cells when interacting with s-GO (**Figure 8d**). For example, the average speed for BEAS-2B and NIH/3T3 cells was 0.640 ± 0.280 and 0.327 ± 0.093 µm/min, respectively. In comparison, the speed for HeLa and A549 cells was 0.117 ± 0.068 and 0.114 ± 0.056 µm/min, respectively.

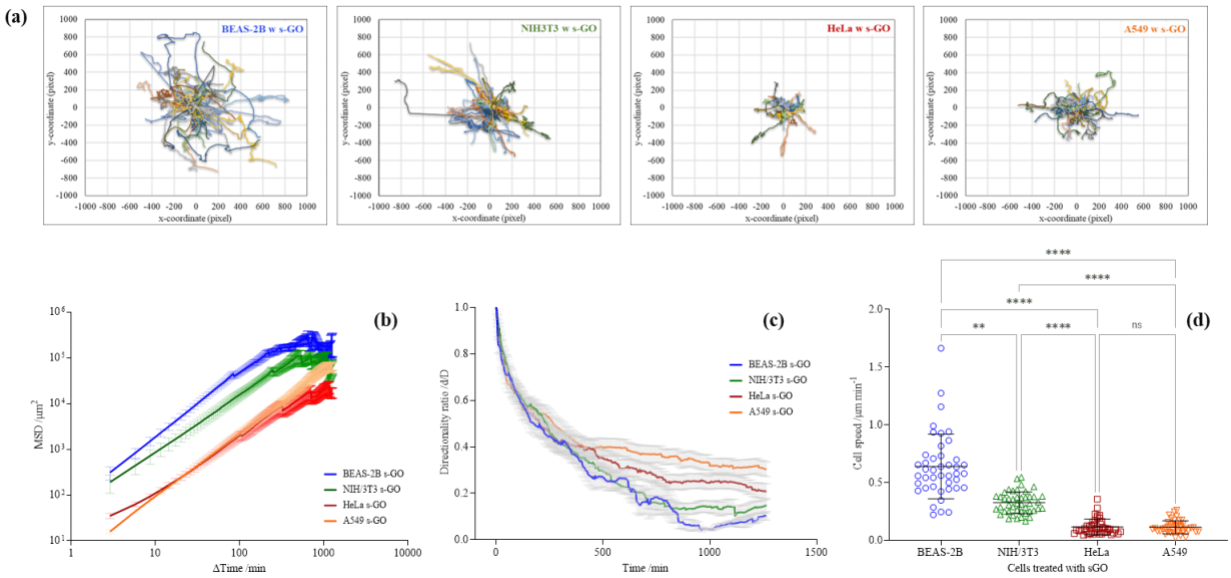

Figure 8: Comparison of cancer (HeLa, A549) and non-cancer cells (BEAS-2B, NIH/3T3) migration when interacting with s-GO (50 µg/mL). Cell trajectories are visualised *via* **(a)** plot at the origins and further analysed *via* **(b)** MSD, **(c)** directionality analysis, and **(d)** cell speed measurement. Cell speeds were statistically analysed using analysis of variance (one-way ANOVA) with Dunn's multiple comparisons test. Number of cells analysed per condition ≥ 43. *Statistically different: ** $p < 0.01$, ****$p < 0.0001$, ns = not significant.

Taken together, direct comparisons of the migratory ability between BEAS-2B, NIH/3T3, HeLa and A549 cells confirmed that a greater effect of GO on cancer cell migratory ability than non-cancer cells (see **Figure 8b**). BEAS-2B and NIH/3T3 non-cancer cells followed a more curved cell path and moved faster than the HeLa and A549 cancer cells when interacting with s-GO. This result supports GO induced actin filament disruption in cancer cells and further illustrates differences between cancer and non-cancer cells when interacting with the material. Consistent with our result, studies have reported the ability of GO to retard cell migration[13,20,41,60]. However, most of these studies were performed only on cancer cells and lacked systematic comparison with non-cancer cells. Considering GO localised mostly extracellularly to cancer cells but was able to induce intracellular (see **Figure 6**, **S19** and **20**), and behaviour (see **Figure 7**) changes in cancer cells, elucidating the complex linkage between GO and cancer cells provides a novel opportunity to advance the understanding of manipulating cancer cells with extracellularly localised GO.

Conclusion

We systematically compared interactions of non-labelled, thin GO flakes with eight cancer and five non-cancer cell lines using a mix-method approach (CLSM, TEM, flow cytometry). Large, small and ultra-small GO flakes were found to predominately interact with the plasma membrane of cancer cells, rather than being internalised. Efficient uptake of GO is reported in all studied non-cancer cell lines.

We explored factors that drive cell-type preference towards GO uptake, including material properties and the effect of GO on endocytosis-relevant biological properties of the cell. Uptake of other materials, including polystyrene microspheres and graphene flakes with various sizes and surface charges, reveals that all were internalised by cancer (HeLa, A549) and non-cancer (Beas-2B, NIH3T3) cells. This confirmed that cancer cells could take up different NMs, including thicker graphene flakes, revealing the role of GO thickness in the observed uptake differences. Furthermore, we show that GO induced actin cytoskeleton (a key player in endocytosis) disruption in cancer but not in non-cancer cells, is leading to differential changes in migratory behaviour of cancer cells.

Our results reveal that an interplay between properties of GO (in particular, thickness of the material) and actin cytoskeleton disruption contributed to the observed uptake differences between cancer and non-cancer cells. This work invites further studies to explore the effects of GO on plasma membrane of cancer cells, potentially translating into an opportunity towards extracellular cancer cell manipulation using GO and plasma membrane targeted biomedical applications.

Experimental

Materials characterisation.

Aqueous suspensions of large (l-GO), small (s-GO), and ultrasmall (us-GO) GO sheets were produced in house, and fully characterised, as previously described in Rodrigues et al.[3]. GO stock suspensions prepared in water for injection and under endotoxin-free conditions, and the same batch has been already used in Loret et al.[78]. Emission spectra of GO (s- and us-GO, 2 mg/mL) were acquired using Cary Eclipse fluorescence spectrophotometer (Varian Inc., Agilent Technologies, UK) at room temperature. GO was prepared in Milli-Q water or cell culture medium (RPMI with 10 % FBS). GO prepared in cell culture medium was washed by centrifugation (30 min, 13 000 rpm, x 2, re-suspension in Milli-Q water) and re-suspended in Milli-Q water for measurement (excitation wavelength = 525 nm, blank = Milli-Q water). Graphene dispersion (Gr-TMA_3 and Gr-PS1) were kindly provided by Professor Ciniza Casiraghi (University of Manchester). Full production and characterisation of Gr-TMA_3 and Gr-PS1 can be found in Shin et al. and Yang et al.[30,51,52].

Cell culture.

Thirteen immortalised cell lines were used in this study. This includes five normal cell lines and eight cancer cell lines. (1) Human epithelial bronchial cells (BEAS-2B, CRL-9609, ATCC, LGC standards, UK), (2) human prostate epithelial cells (PNT-2, kindly provided by Professor Anna Nicolas, University of Manchester), and (3) human prostate carcinoma cells (LNCaP, kindly provided by Professor Anna Nicolas, University of Manchester) were maintained in RPMI-1640 cell culture medium (R8758, Sigma-Aldrich, Merck Sigma, UK). (4) Human epithelial colon adenocarcinoma cells (SW 480, CCL-228, ATCC, LGC standards. UK) were maintained in advanced RPMI-1640 cell culture medium (R8785, Sigma-Aldrich, Merck Sigma, UK). (5) Mouse fibroblast embryonic cells (NIH/3T3, CRL-1658, ATCC, LGC standards, UK), (6) human epithelial keratinocyte cells (HaCaT, PCS-200-011, ATCC, LGC

standards, UK), (7) human epithelial embryonic kidney cells (293T, CRL-2316, ATCC, LGC standards, UK), (8) human epithelial cervical adenocarcinoma cells (HeLa, CCL-2, ATCC, LGC standards, UK), and (9) human epithelial glioblastoma cells (U87 MG, HTB-14, ATCC, LGC standards, UK) were maintained in DMEM cell culture medium (D6429, Sigma-Aldrich, Merck Sigma, UK). (10) Human epithelial lung carcinoma cells (A549, CCL-185, ATCC, LGC standards, UK), and (11) human epithelial prostate adenocarcinoma cells (PC3, CRL-1435, ATCC, LGC standards, UK) were maintained in F-12 Ham cell culture medium (N6658, Sigma-Aldrich, Merck Sigma, UK). (12) Human epithelial prostate carcinoma cells (DU 145, HTB-81, ATCC, LGC standards. UK), and (13) human epithelial bone marrow neuroblastoma cells (SH-SY5Y, CRL-2266, ATCC, LGC standards, UK) were maintained in EMEM cell culture medium (M4655, Sigma-Aldrich, Merck Sigma, UK). All the cell lines except for LNCaP cells, were supplemented with 10 % FBS, 1000 units Penicillin, and 1 mg/mL Streptomycin. LNCaP cells were supplemented with 10 % hyclone FBS, 1 mM sodium pyruvate and 10 mM of HEPES solution. The cells were maintained at 37 ^{o}C in a humidified 5 % CO_2 incubator. Cells were split at 80 % confluence with 0.05 % Trypsin-EDTA (Sigma-Aldrich, Merck Sigma, UK), and 10 % FBS (or hyclone FBS for LNCaP cells) was used to stop the activity of Trypsin-EDTA.

Cell culture treatments.

Cells were seeded in Cellview^{TM} dishes, 12-well plates, or 24-well plates with Aclar for experiments by confocal microscopy, flow cytometry, and TEM, respectively. Cells were always seeded in the cell-type specific medium for at least 24 h before treatments and treated when the cells reached 60 – 80 % confluence (except for the wound healing assay). In all treatment condition, RPMI-1640 cell culture medium supplemented with 10 % FBS, 1000 units Penicillin, and 1 mg/mL Streptomycin were used as the pre-treatment and treatment medium and maintained at 37 ^{o}C in a humidified 5 % CO_2 incubator.

Confocal microscopy.

Uptake of GO. Cells were treated with GO (l-GO at 50 µg/mL, s-GO and us-GO at 25, 50, and 75 µg/mL, 0.5 mL/well, 24 h), after removal of cell supernatant, stained with the CellMask^{TM} green plasma membrane dye (C37608, Thermo Fisher Scientific, UK) in the fresh treatment medium with a dilution of 1:2500, and imaged by Zeiss 780 CLSM using the 40X objective. Zeiss microscope software ZEN was used to process all the images. Excitation/emission wavelength: CellMask^{TM} green = 488/520, GO = 594/620-690 nm.

Uptake of the beads. Cells were treated with the positively charged (+1 µm and +0.2 µm) or negatively charged (–1 µm, –0.5 µm and –0.1 µm) beads (1.5 µL/mL, 0.5 mL/well, 24 h, F8765, F8764, F8823, F8813, F8803, Thermo Scientific, UK, respectively), after removal of cell supernatant, stained with CellMask™ deep red plasma membrane dye (C10046) , Thermo Fisher Scientific, UK) in the fresh treatment medium with dilution of 1:2500, and imaged by Zeiss 780 CLSM using the 40X objective. Zeiss microscope software ZEN was used to process all the images. Excitation/emission wavelength: CellMask™ deep red = 649/666, beads = 505/515 nm.

Uptake of graphene flakes. Cells were treated with the positively charged (Gr-TMA_3) or negatively charged (Gr-PS1) graphene flakes (50 µg/mL, 0.5 mL/well, 24 h), just before imaging, cell supernatant removed and stained with Fluorescein Diacetate live cell dye (100 nM, 0.5 mL/well, Thermo Fisher Scientific, UK) in the fresh treatment medium, and imaged by Zeiss 780 CLSM using the 40X objective. Zeiss microscope software ZEN was used to process all the images. Excitation/emission wavelength: FDA = 488/520 nm.

Time-lapse tracking: untreated vs s-GO treated cells. BEAS-2B or HeLa cells were seeded in the Cellview™ dish and treated with s-GO (50 µg/mL, 0.5 mL/well) containing CellMask™ green plasma membrane stain (C37608, Thermo Scientific, UK). Live-cell time lapse was recorded within 1 h after the addition of GO treatment for 21 h, using Zeiss 780 CLSM (40X objective, time lapse mode). The videos were then extracted using Zeiss microscope software ZEN with the compression ratio of 20 and frames per second of 10. Excitation/emission wavelength: CellMask™ green = 488/520, GO = 594/620-690 nm.

Time-lapse tracking: cancer vs non-cancer cells when interacting with s-GO. BEAS-2B, HeLa and A549 cells were seeded in the same Cellview™ dish and treated with s-GO (50 µg/mL, 0.5 mL/well) containing CellMask™ green plasma membrane stain (C37608, Thermo Scientific, UK). Live-cell time lapse was recorded within 1 h after the addition of GO treatment for 21 h, using Zeiss 780 multiphoton CLSM (40X objective, time lapse mode). The videos were then extracted using Zeiss microscope software ZEN. Excitation/emission wavelength: CellMask™ green = 488/520, GO = 594/620-690 nm.

Time-lapse tracking: video processing and analysis. To obtain the cell trajectory information, cells were tracked manually using the CellTracker (version 1.1) software[79]. Briefly, the extracted video of live-cell time lapse by Zeiss 780 multiphoton CLSM was loaded onto CellTracker as Bio-Format images. Then, using the manual tracking option, the position of individual cell was manually defined by user clicking on the cell at every image frame until cell leave the frame; ≥ 43 cells were analysed in every conditions. Afterward, the statistic tap in CellTracker was selected, user defined manually the pixel size of frame (0.209 μm/pixel) and the time elapsed between subsequent frames (2.88 min/frame) according to the pre-defined setting of the recorded time lapse. The average cell speeds (μm/min) and individual cell trajectory (defined as *xy*-coordinate in each image frame) were extracted using the statistic function from CellTracker. Subsequently, the computed *xy*-coordinates data from the CellTracker were further analysed using the DiPer programme[77] to generate the plot at the origin, MSD curve, and directionality curve. Briefly, the pre-written program codes for DiPer were downloaded and saved as Maro-Enabled Workbook using Excel[77]. Then the *xy*-coordinates data from the CellTracker were copied onto the Maro-Enabled Workbook and further analysed using the corresponding Macros for the plot at origin, MSD curve, and directionality curve[77].

Staining of the actin filament. Cells were treated with GO (s-GO and us-GO, 100 μg/mL, 0.5 mL/well, 24 h) or M βCD (5 mM, 0.5 mL/well, 4 h, C4555, Sigma), washed with pre-warmed PBS (0.5 mL/well, D8662, Sigma-Aldrich, Merck Sigma, UK), fixed with formaldehyde (3.7 %, 0.5 ml/well, 10 min; 28908 Thermo Fisher Scientific, UK), permeabilized with Triton-X (0.1 % in PBS (-/-), 0.5 mL/well, 10 mins), re-washed with PBS (-/-) (0.5 mL/well, x 2, D8537, Sigma-Aldrich, Merck Sigma, UK), stained with Alexa Fluor™ 488 Phalloidin (1:1500 dilution prepared in PBS (-/-), 0.5 mL/well, 20 min, A12379, Thermo Fischer, UK). After staining, cells were washed with PBS (-/-) (0.5 mL/well, x 2) and ProLong™ Gold Antifade Mountant (P36930, Thermo Fisher Scientific, UK) was added to the cells. The cells were exposed to a higher concentration of GO (100 μg/mL) because the potential role of GO as an actin filament disrupting agent was investigated. Following the working principle for pharmaceutical inhibitors, the highest tested non-toxic concentration of inhibitor was used[28].
Cells were imaged using Zeiss 780 CLSM with the 40X objective. Images were processed using Zeiss microscope software ZEN. Excitation/emission wavelength: Phalloidin = 495/518.

Flow cytometry.

Cellular interactions with GO. Cells were treated with s-GO or us-GO (50 µg/mL, 1 mL/well, 24 h). After treatment, cells were collected with and without washing. The cells without washing were detached with 0.05 % Trypsin-EDTA (300 uL/well, 10 min), neutralised with FBS (30 uL/well), collected in 1.5 mL Eppendorf tube, and stored in ice until analysis. The cells with washing were washed with PBS (-/-) (1 mL/well, x 2), detached with 0.05 % Trypsin-EDTA (300 uL/well, 10 min), neutralised with FBS (30 uL/well), collected in 1.5 mL Eppendorf tube, re-washed by centrifugation (500 RPM, 5 mins, 4 ^{O}C, x 2), re-suspended in PBS (-/-) (300 uL/tube), and stored in ice until analysis. The samples were sampled using FACSVerse flow cytometry using PE-Cy7-A channel (band pass: 488 780/60). Excitation/emission band pass: GO = 594/620-690.

Cellular interactions with beads. Cells were treated with the positively charged (+1 µm and +0.2 µm) or negatively charged (–1 µm, –0.5 µm and –0.1 µm) beads (1.5 µL/mL, 1 mL/well, 24 h, F8765, F8764, F8823, F8813, F8803, Thermo Scientific, UK, respectively). After treatment, detached with 0.05 % Trypsin-EDTA (300 uL/well, 10 min), neutralised with FBS (30 uL/well), collected in 1.5 mL Eppendorf tube, stored in ice, analysed by FACSVerse flow cytometry (bandpass: 488 530/30) using the FITC channel. Excitation/emission band pass: +1, +0.2, -1, -0.5, -0.1 µm beads= 505/515.

TEM.

Cells were seeded on sterilised Aclar placed in 24-well plate. Subsequently, cells were treated with s-GO or us-GO (50 µg/mL, 0.5 mL/well, 24 h) and fixed at room temperature (4 % formaldehyde, 2.5 % glutaraldehyde in 0.1 M HEPS budder, 0.5 mL/well, 1 h). Samples were postfixed with 1 % osmium tetroxide + 1.5 % potassium ferrocynaide in 0.1M cacodylate buffer (pH 7.2) for 1 hour, then in 1 % uranyl acetate in water overnight. Then they were dehydrated in ethanol series infiltrated with TAAB LV resin and polymerised for 24h at 60C degree. Sections were cut with Reichert Ultracut ultramicrotome and observed with FEI Tecnai 12 Biotwin microscope at 100kV accelerating voltage. Images were taken with Gatan Orius SC1000 CCD camera.

Statistical analysis.

Flow cytometry, cell speed, and directionality data were statistically analysed using GraphPad Prism (version 9). The analysis of variance (one-way (**Figure 5**) or two-way (**Figures 3e**, **S16**) ANOVA) with Tukey's multiple comparisons test was used for flow cytometry data (n = 3 with duplicates). Cell speeds data were statistically analysed using unpaired T-test (**Figure 7**) or analysis of variance (one-way ANOVA) with Dunn's multiple comparisons test and descriptive statistics (**Figure 8**), and the result was reported as mean ± SD (number of cells analysed per condition ≥ 43). Directionality data were analysed using descriptive statistics, and the result was reported as mean ± SEM. *Statistically different: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and ****$p < 0.0001$. ns = not significant.

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Supporting information

Table S1: Summary of the physico-chemical characterisation of s-GO and us-GO.

Parameter	Technique	s-GO	us-GO
Lateral dimension	Optical microscopy	Non detectable (< 2 µm)	Non detectable (< 2 µm)
	SEM	25 nm – 2.4 µm 95 % < 1.2 µm Mean = 232 nm [n = 742]	10 nm – 503 nm 95 % < 250 nm Mean = 48 nm [n = 636]
	AFM	25 nm – 1.3 µm 95 % < 625 nm Mean = 84 nm [n = 775]	20 nm – 790 nm 95 % < 210 nm Mean = 59 nm [n = 4268]
Thickness	AFM	1 - 2 nm	1 - 2 nm
Optical properties	Absorbance	ε_{230} (mL µg^{-1} cm^{-1}) = 0.038	ε_{230} (mL µg^{-1} cm^{-1}) = 0.041
	Fluorescence λ_{600} (λexc_{525})	1.056*C_{GO} (µg/mL)	0.915*C_{GO} (µg/mL)
Degree of defects (I_D/I_G)	Raman	1.19 ± 0.02	1.18 ± 0.03
Peak (2θ) Interlayer distance (nm)	XRD	12.81^o 0.70	11.53^o 0.76
Zeta potential	Electrophoretic mobility	-50.8 ± 1.1 mV	-53.5 ± 2.2 mV
Functionalisation degree	TGA	30 – 75 ^{o}C: 7 % (water) 200 – 250 ^{o}C: 24 % 250 – 950 ^{o}C: 18 % Total: 42 %	30 – 75 ^{o}C: 7 % (water) 200 – 250 ^{o}C: 23 % 250 – 950 ^{o}C: 13 % Total: 36 %

Chemical composition Purity (%C + %O), C:O ratio, π-π*, *O-C=O*, *C=O*, *C-O*, *C=C*	XPS	C: 70.7 %, O: 25.5 %, N: 1.8 %, S: 1.4 %, B: 0.6 % 96.2 %, 2.8, 0.2 %, 4.1 %, 3.2 %, 46.5 %, 46.0 %	C: 73.0 %, O: 24.8 %, N: 0.4 %, S: 1.4 %, B: 0.4 % 97.8 %, 2.9, 0.2 %, 5.8 %, 7.5 %, 37.3 %, 49.1 %

SEM = scanning electron microscopy, TEM = Transmission electron microscopy, AFM = atomic force microscopy, ε_{230} = molar absorption coefficient at 230 nm, λ_{600} = emission at 600 nm, λexc_{525} = excitation at 525 nm, C_{GO} = graphene oxide concentration, XRD = X-ray diffraction, TGA = thermogravimetric analysis, XPS = X-ray photoelectron spectroscopy.

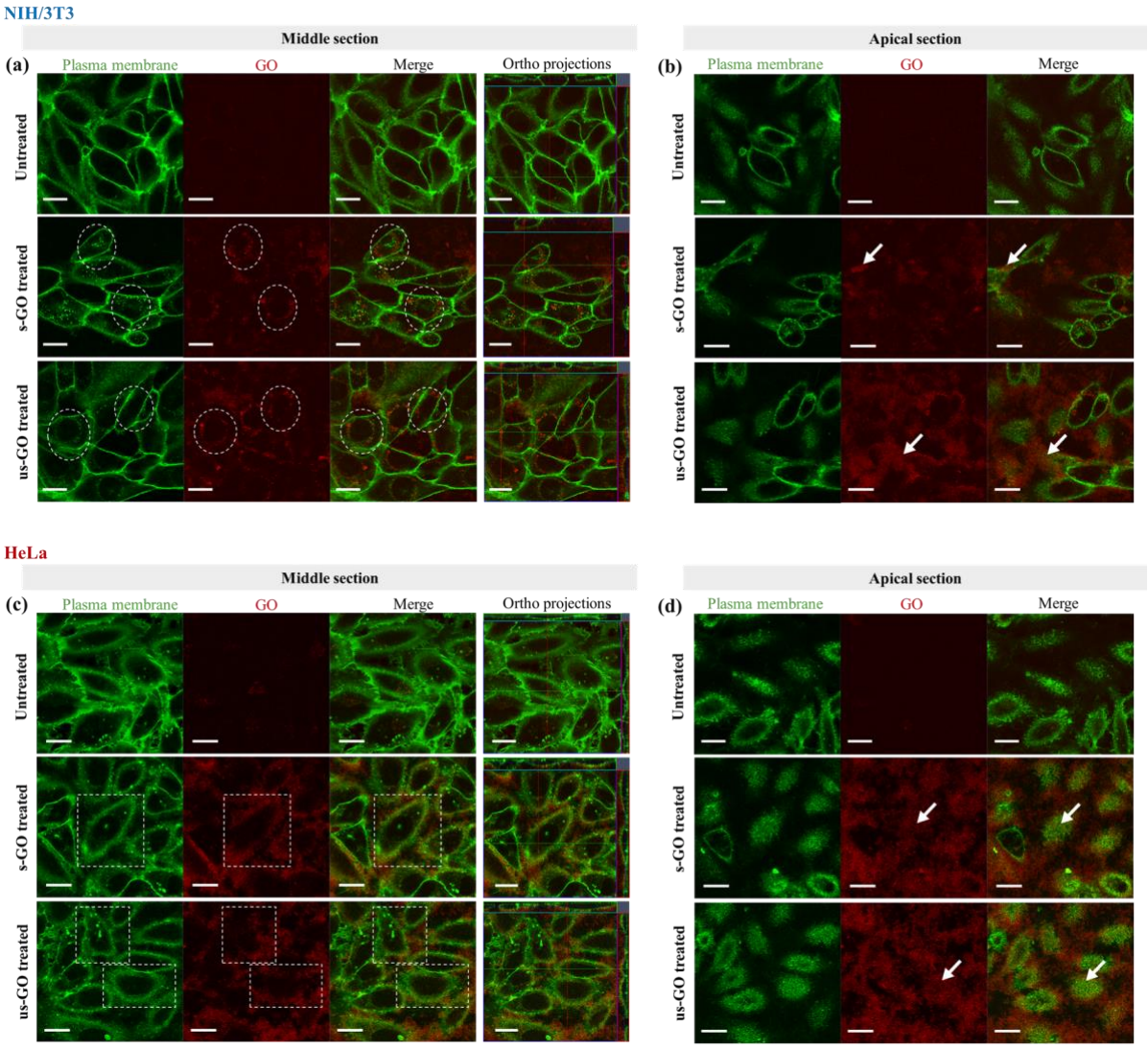


Figure S1: Representative images (middle and apical section) of GO (s- and us-GO) interaction profiles in **(a – b)** NIH/3T3 and **(c – d)** HeLa cells by CLSM. Both s-GO and us-GO (50 µg/mL) were found internalised in NIH/3T3 cells (indicated by white circles) but predominately surrounded the plasma membrane in HeLa cells (indicated by white rectangles). The materials were also found on the plasma membrane (indicated by white arrows) in both cell lines. Green

= plasma membrane, red = GO. Scale bar = 20 µm. See **Figures S2** and **S6** for the interaction of s-GO and us-GO at different concentrations with NIH/3T3 and HeLa cells, respectively.

Table S2: Summary of GO (s- and us-GO) interaction profile in cancer (HeLa, A549, SW 480, SH-SY5Y, U87 MG, DU-145, PC3 and LNCaP) and non-cancer (BEAS-2B, NIH/3T3, HaCaT, 293T and PNT-2) cells by CLSM.

Cell line	Tissue/morphology	s-GO and us-GO uptake
BEAS-2B	Normal human lung/epithelial	+
NIH/3T3	Normal mouse embryonic/fibroblasts	+
HaCaT	Normal human skin/epithelial	+
293T	Normal human embryonic kidney/epithelial	+
PNT-2	Normal human prostate/epithelial	+
HeLa	Adenocarcinoma human cervix/epithelial	–
A549	Carcinoma human lung/epithelial	–
SW 480	Adenocarcinoma human cervix/epithelial	–
SH-SY5Y	Neuroblastoma human bone marrow neuroblastoma/epithelial	–
U87 MG	Glioblastoma human brain/epithelial	–
DU-145	Carcinoma human prostate/epithelial	–
PC3	Adenocarcinoma human prostate/epithelial	–
LNCaP	Carcinoma human prostate/epithelial	–

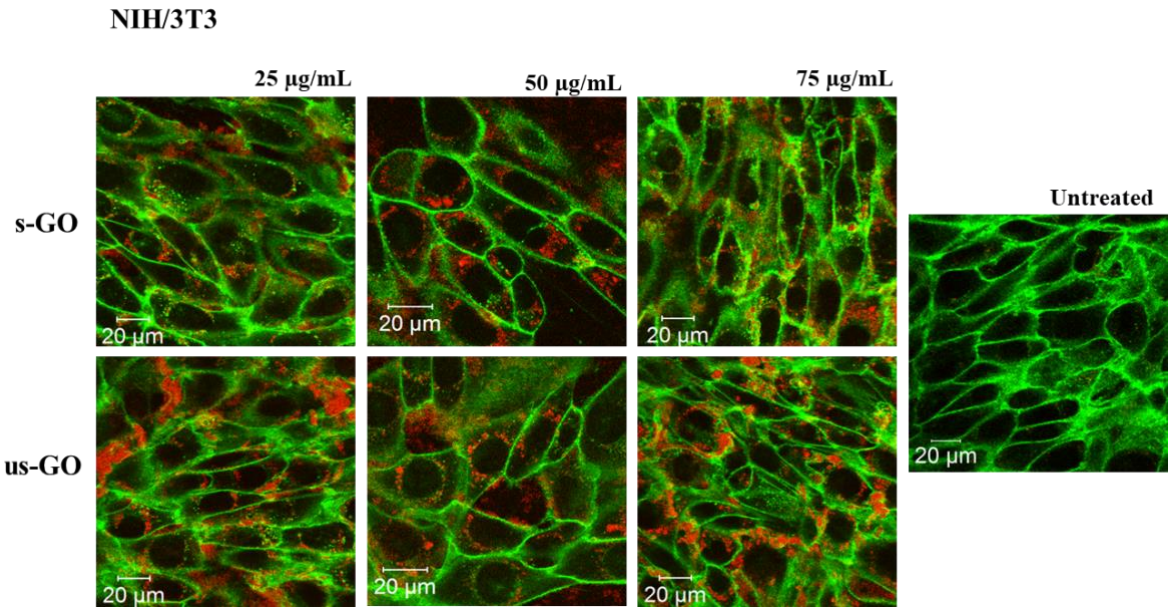


Figure S2: Interactions of s-GO and us-GO at 25, 50 and 75 µg/mL with NIH/3T3 cells. Green = plasma membrane, red = GO.

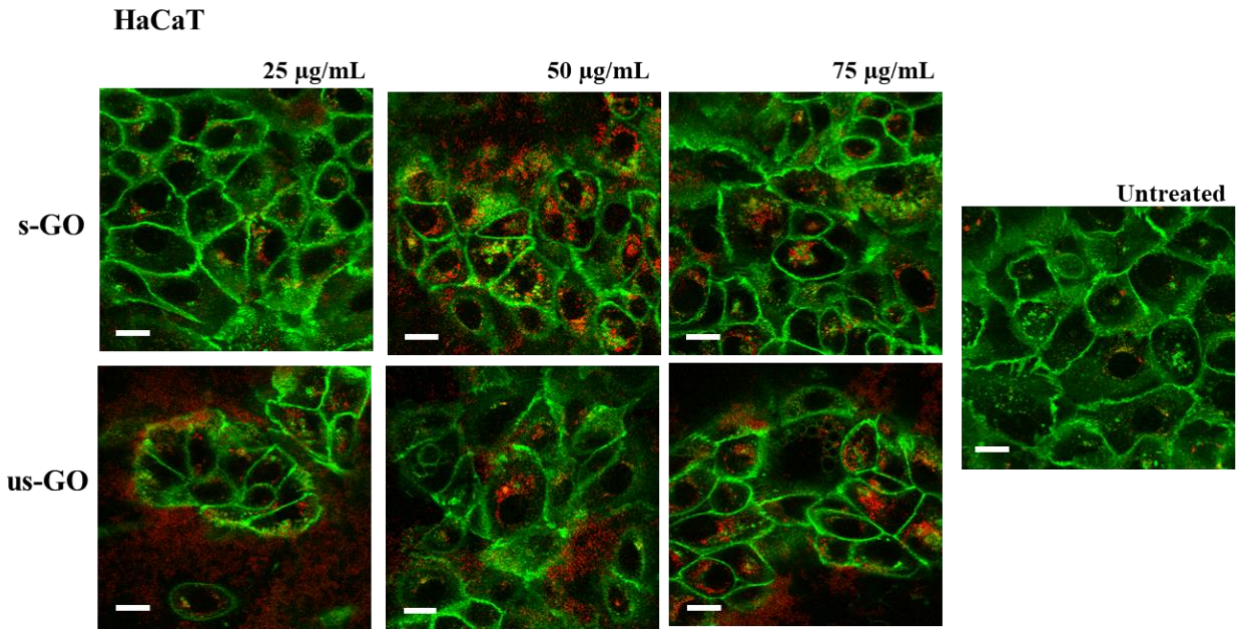


Figure S3: Interactions of s-GO and us-GO at 25, 50 and 75 µg/mL with HaCaT cells. Green = plasma membrane, red = GO. Scale bar = 20 µm.

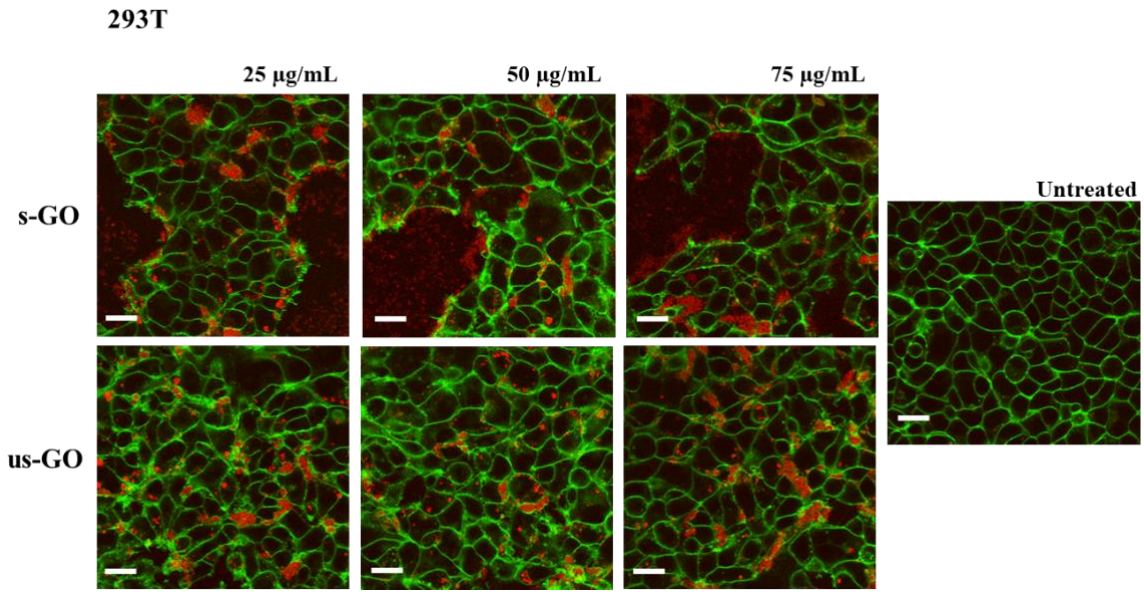


Figure S4: Interactions of s-GO and us-GO at 25, 50 and 75 µg/mL with 293T cells. Green = plasma membrane, red = GO. Scale bar = 20 µm.

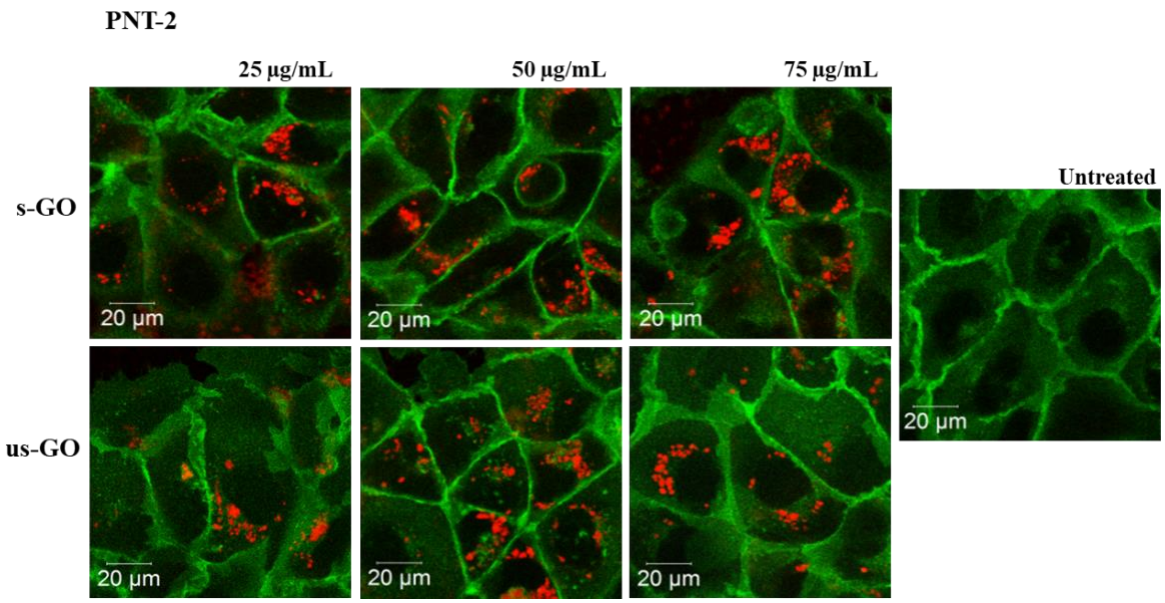


Figure S5: Interactions of s-GO and us-GO at 25, 50 and 75 µg/mL with PNT-2 cells. Green = plasma membrane, red = GO.

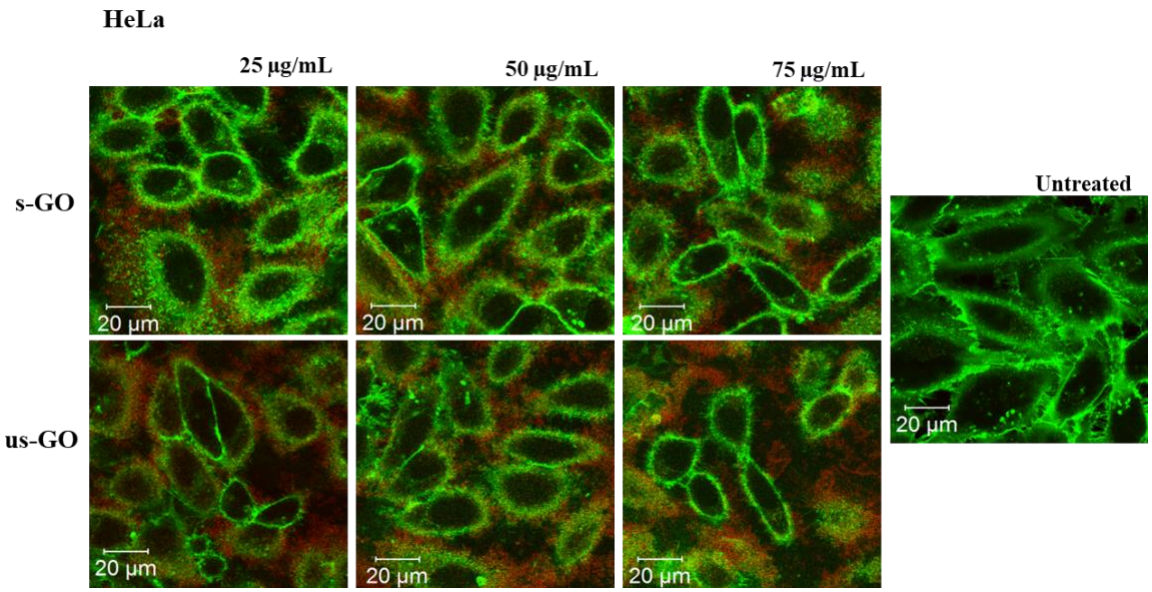


Figure S6: Interactions of s-GO and us-GO at 25, 50 and 75 µg/mL with HeLa cells. Green = plasma membrane, red = GO.

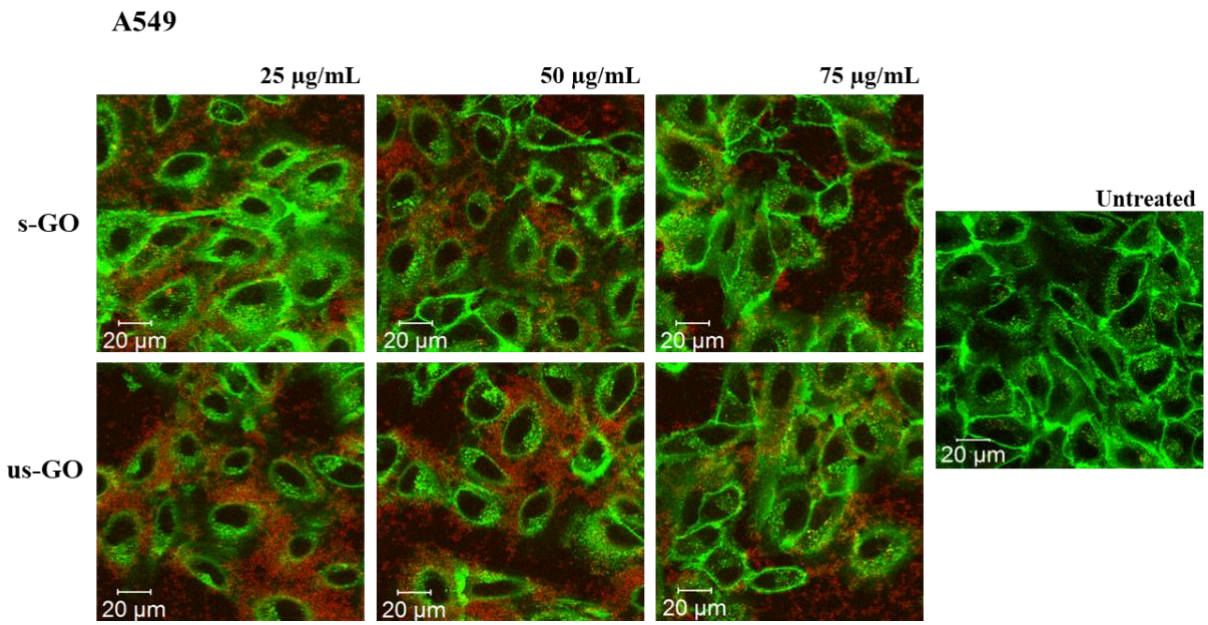


Figure S7: Interactions of s-GO and us-GO at 25, 50 and 75 µg/mL with A549 cells. Green = plasma membrane, red = GO.

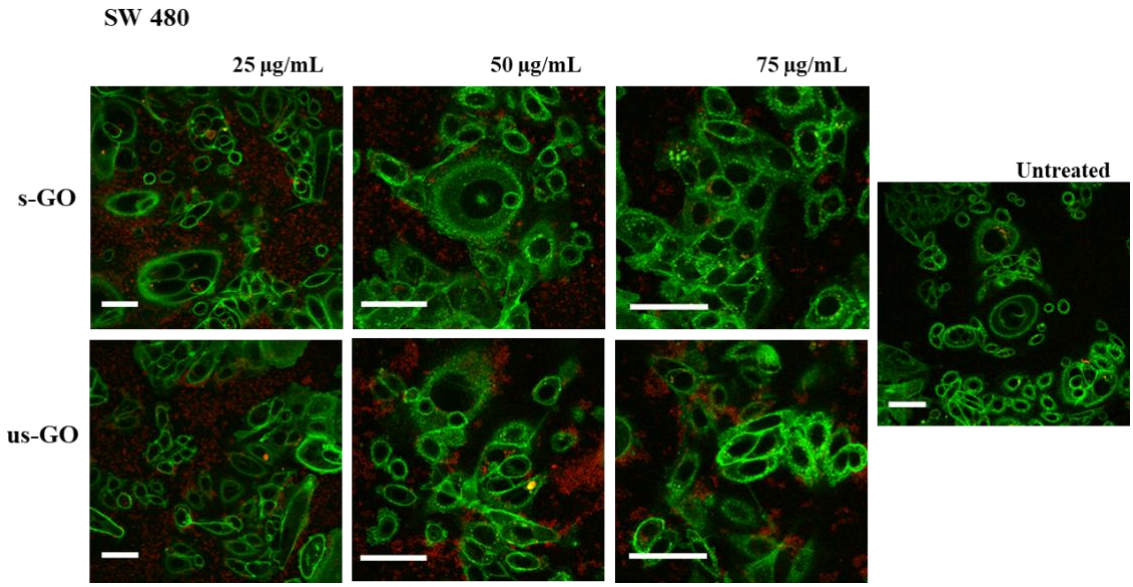


Figure S8: Interactions of s-GO and us-GO at 25, 50 and 75 µg/mL with SW 480 cells. Green = plasma membrane, red = GO. Scale bar = 50 µm.

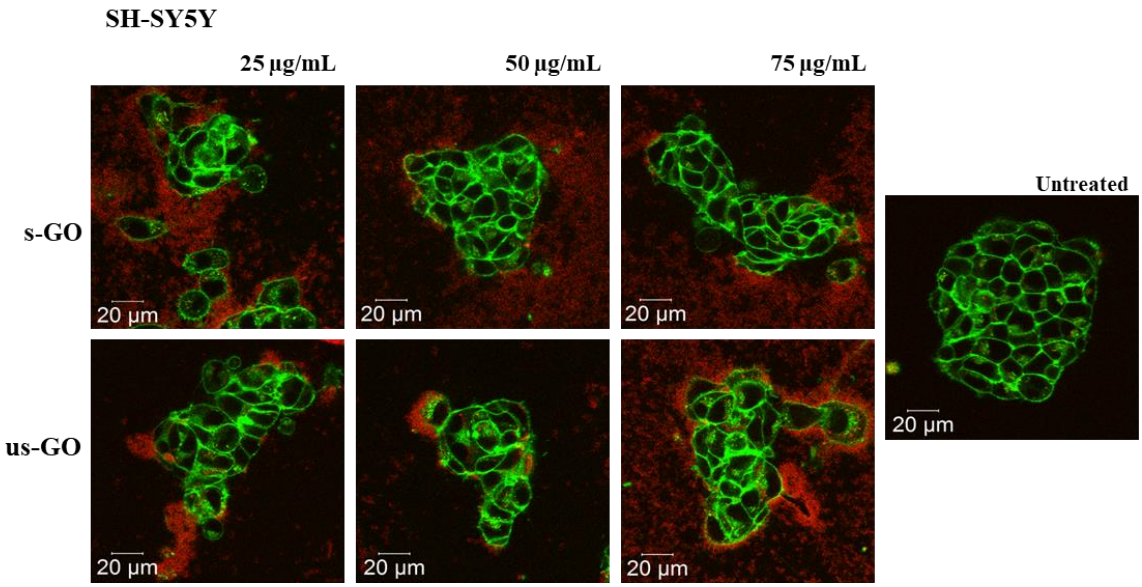


Figure S9: Interactions of s-GO and us-GO at 25, 50 and 75 µg/mL with SH-SY5Y cells. Green = plasma membrane, red = GO.

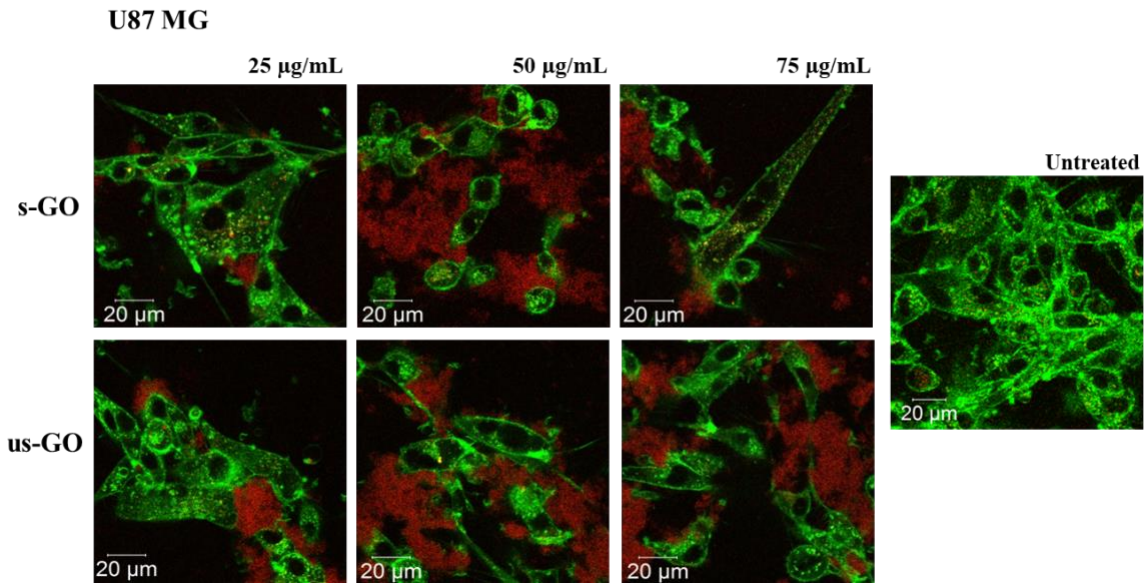


Figure S10: Interactions of s-GO and us-GO at 25, 50 and 75 µg/mL with U87 MG cells. Green = plasma membrane, red = GO.

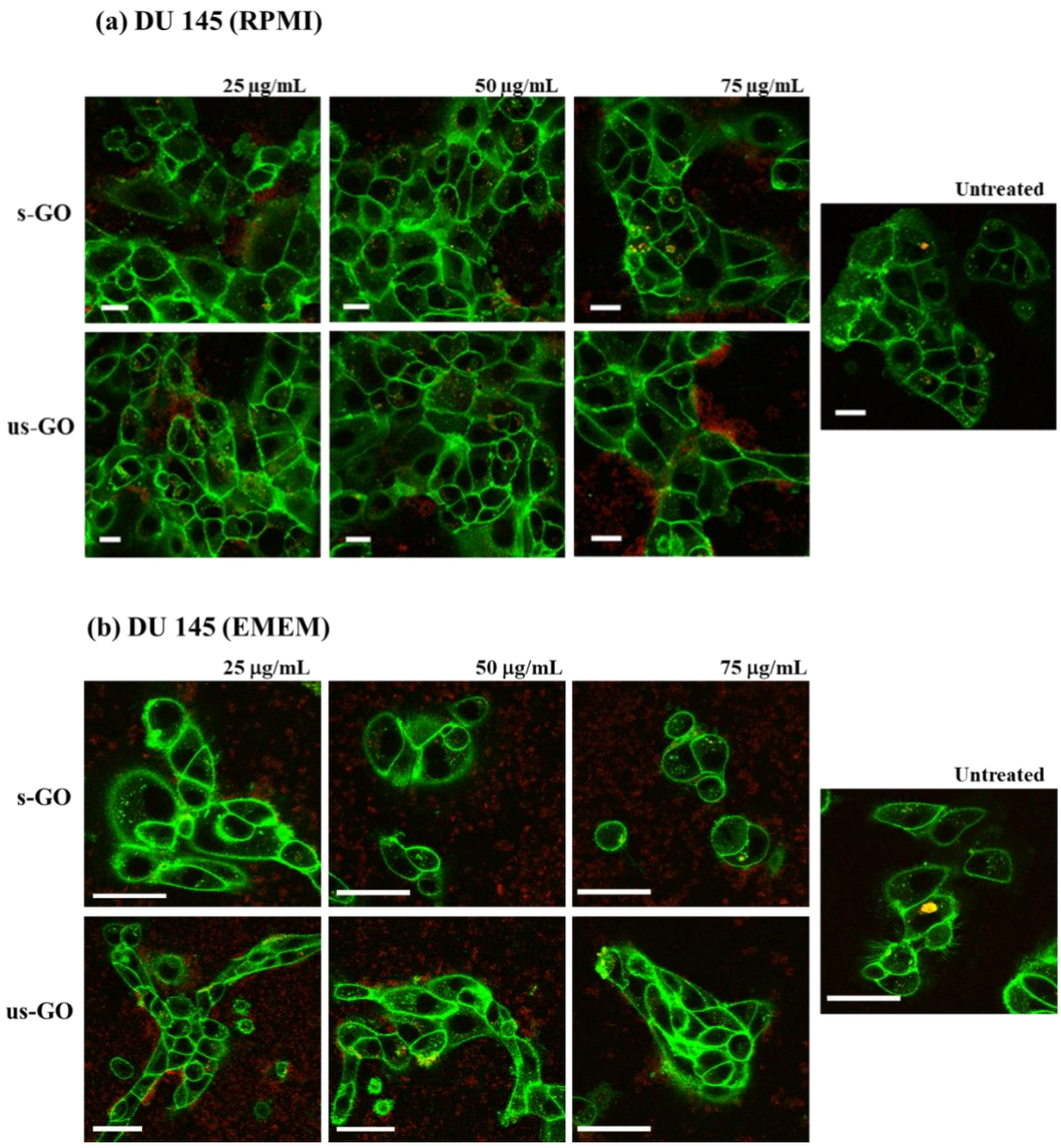


Figure S11: Interactions of s-GO and us-GO at 25, 50 and 75 µg/mL with DU 145 cells [in **(a)** RPMI with 10% FBS and **(b)** EMEM with 10 % FBS]. Green = plasma membrane, Red = GO. Scale bar = **(a)** 20 µm or **(b)** 50 µm.

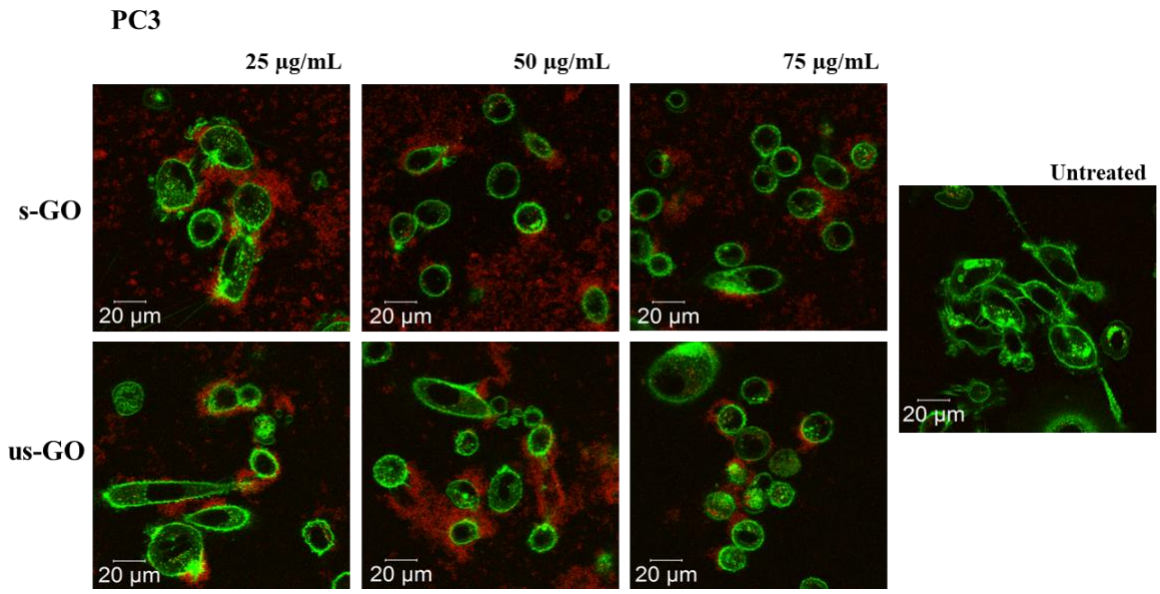


Figure S12: Interactions of s-GO and us-GO at 25, 50 and 75 µg/mL with PC3 cells. Green = plasma membrane, red = GO.

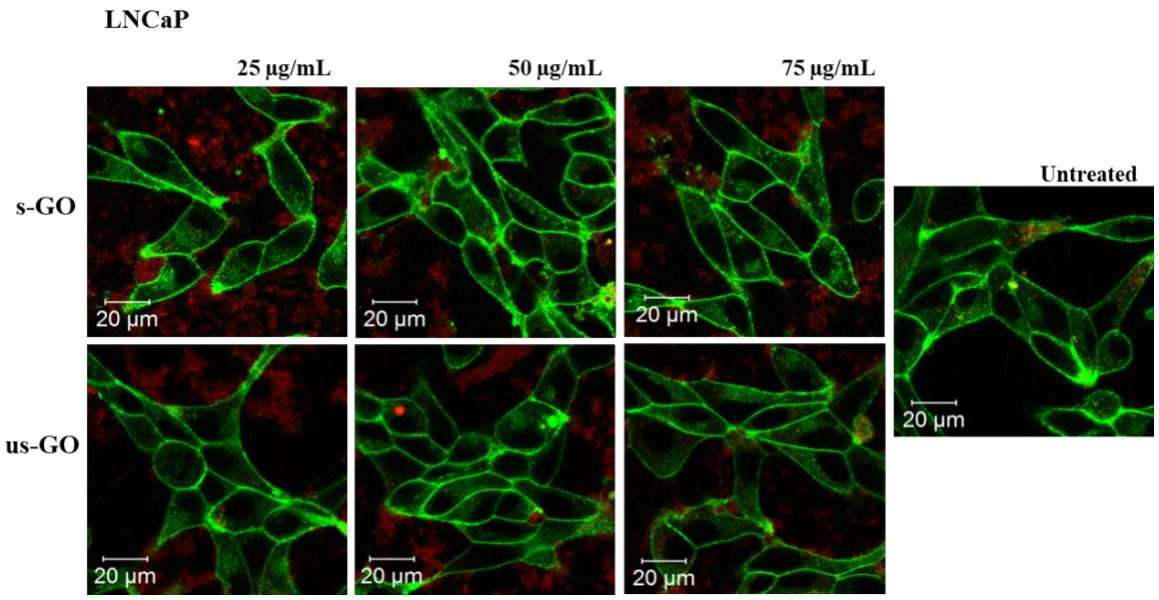


Figure S13: Interactions of s-GO and us-GO at 25, 50 and 75 µg/mL with LNCaP cells. Green = plasma membrane, red = GO.

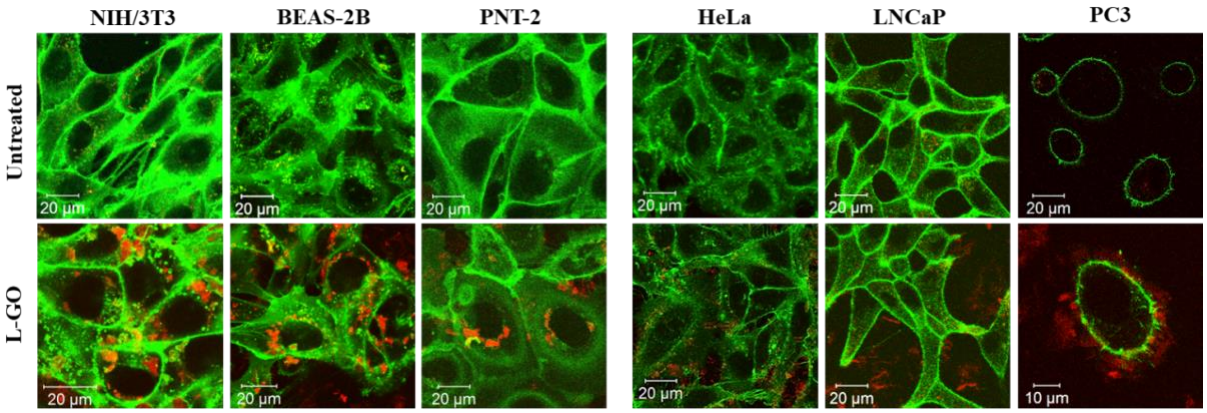


Figure S14: Interaction l-GO (50 µg/mL) with non-cancer (NIH/3T3, BEAS2B, PNT-2) and cancer (HeLa, LNCaP and PC3) cell lines by confocal imaging. Green = plasma membrane, red = GO.

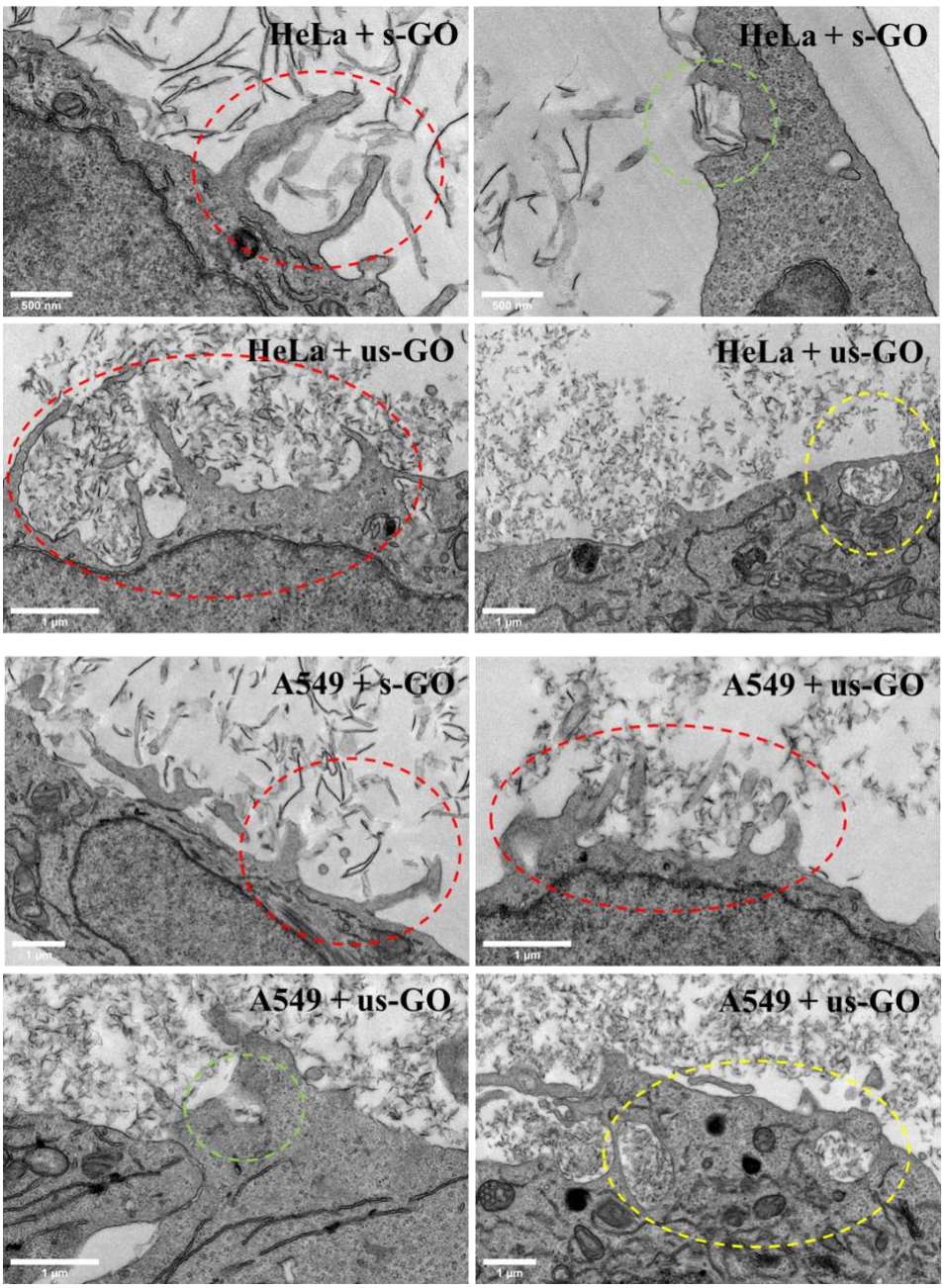
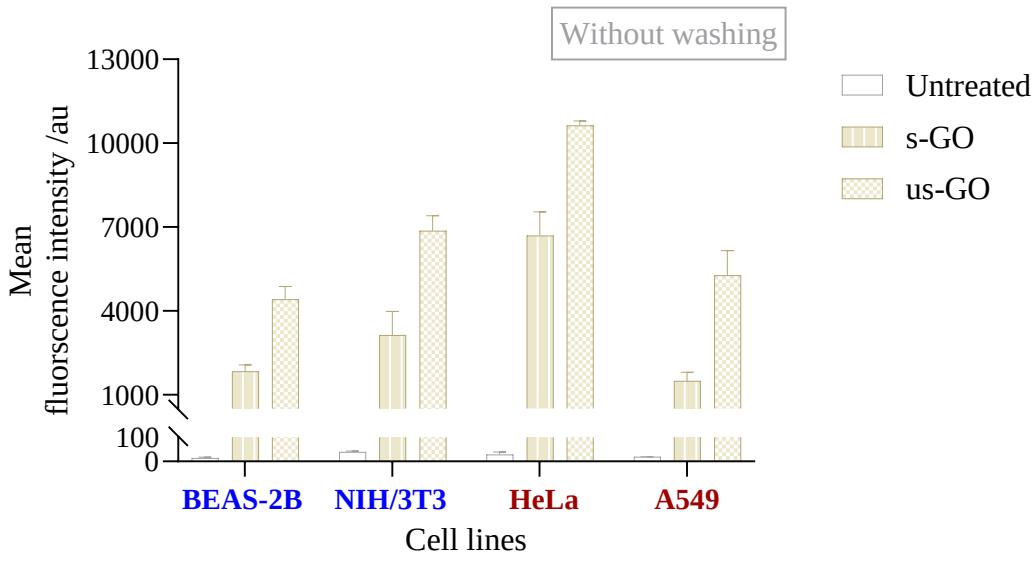


Figure S15: Sign of membrane ruffles (indicated by red circles) and membrane invagination (indicated by green circles) was observed in HeLa and A549 cells by TEM imaging. Also, the uptake of us-GO is found in a few cancer cells (indicated by yellow circles).

Table S3: Statistical analysis for the interaction of GO (s- and us-GO) with BEAS-2B, NIH/3T3, HeLa and A549 by flow cytometry (cells collected with PBS (-/-) washing); see **Figure 3e** for the corresponding graph. Tables showed the statistical analysis for the interaction of GO (s- and us-GO): **(a)** compared across the four cell lines, and **(b)** within each cell line. The data were statistically analysed using analysis of variance (two-way ANOVA) with Tukey's multiple comparisons test. n = 3 with duplicates *Statistically different: NS = non-significant, ****$p < 0.0001$.

(a)	s-GO	us-GO
HeLa vs A549	****	****
HeLa vs NIH/3T3		
HeLa vs BEAS-2B		
A549 vs NIH/3T3	NS	NS
A549 vs BEAS-2B		
NIH/3T3 vs BEAS-2B		

(b)	**HeLa**	**A549**	**NIH/3T3**	**BEAS-2B**
s-GO **vs** us-GO	****			



(a)	s-GO	us-GO
HeLa vs **A549**	****	****
HeLa vs **NIH/3T3**		
HeLa vs **BEAS-2B**		
A549 vs **NIH/3T3**	NS	NS
A549 vs **BEAS-2B**		
NIH/3T3 vs **BEAS-2B**		

(b)	HeLa	A549	NIH/3T3	BEAS-2B
s-GO **vs** us-GO	****			

Figure S16: Interaction of GO (s- and us-GO) with HeLa, A549, BEAS-2B and NIH/3T3 cells by flow cytometry (cells were collected without PBS (-/-) washing). Tables showed the statistical analysis for the interaction of GO **(a)** compared across the four cell lines and **(b)** within each cell line. The data were statistically analysed using analysis of variance (two-way ANOVA) with Tukey's multiple comparisons test. n = 3 with duplicates *Statistically different: NS = non-significant, ****$p < 0.0001$.

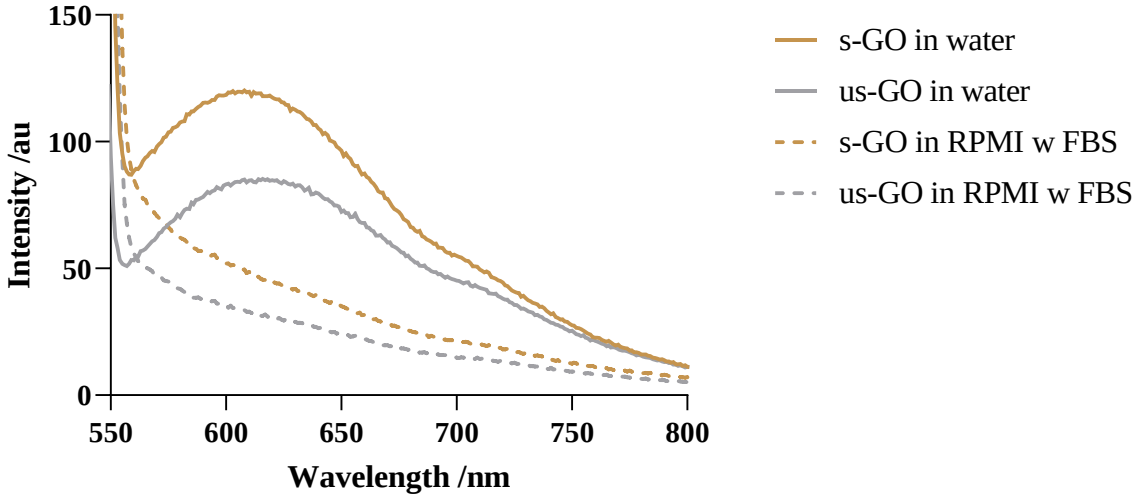


Figure S17: Emission spectra of s-GO and us-GO (2 mg/mL) in water (solid lines) and RPMI with 10 % FBS (materials freshly prepared in complete medium and re-suspended in water for measurement). Excitation wavelength of 525 nm is used.

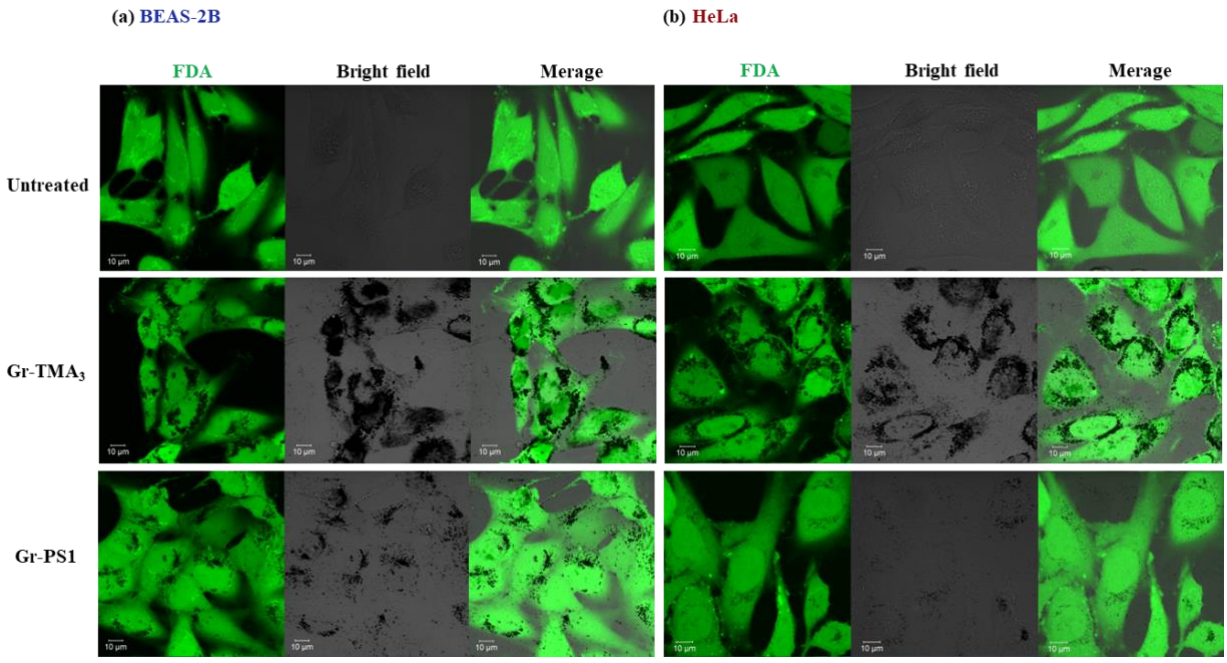


Figure S18: Interaction of Gr-TMA_3 and Gr-PS1 (50 µg/mL, 24 h) in **(a)** BEAS-2B and **(b)** HeLa cells by confocal imaging. Green = FDA dye labelled cells, black = graphene flake.

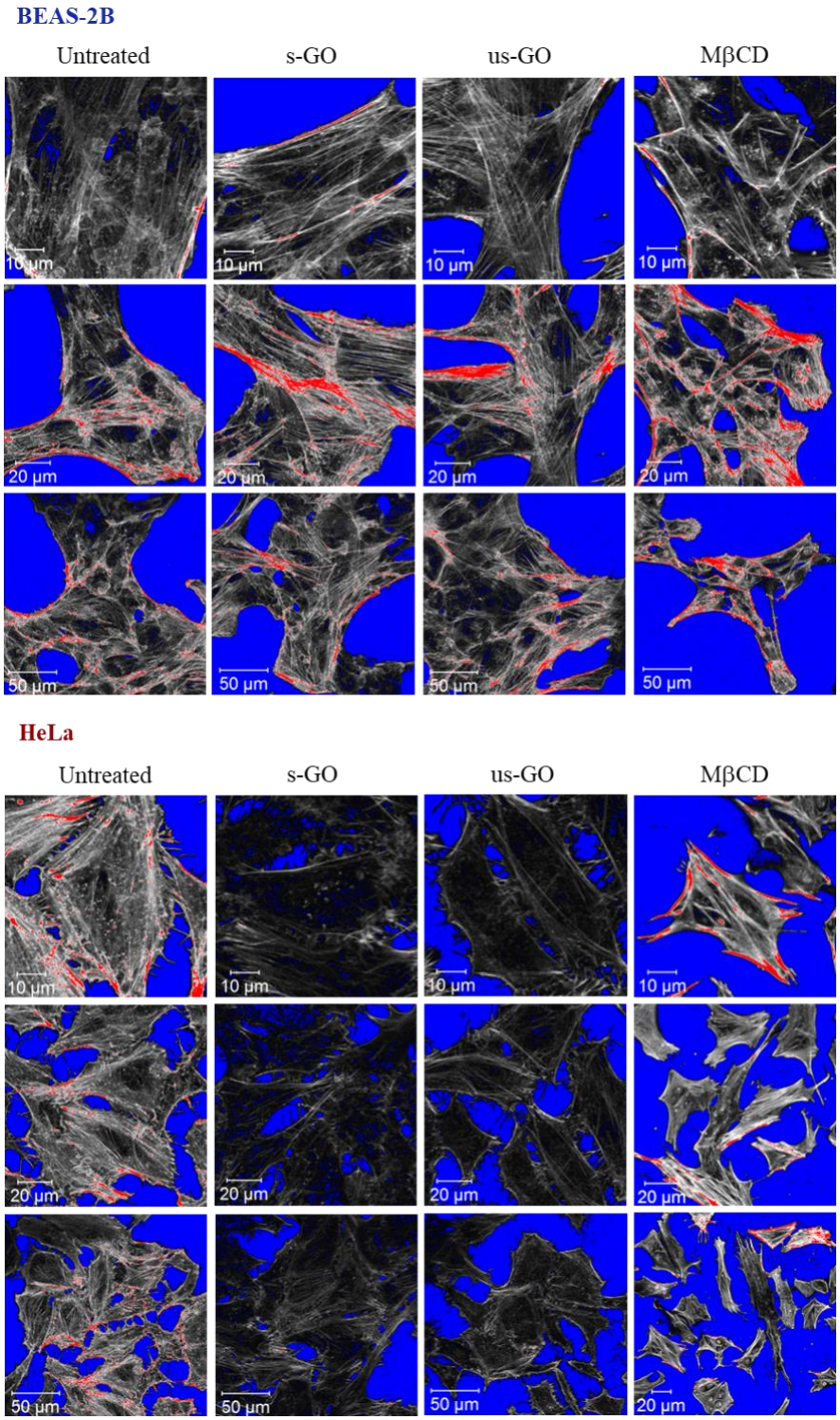


Figure S19: Actin filament staining for BEAS-2B and HeLa cells with and without treatment of GO (s-GO or us-GO, 100 µg/mL, 24 h) or M *β*CD (5 mM, 4 h). Images are shown in range indicator (pixels with zero, low, high, and maximum intensity are shown in blue, black, white and red, respectively).

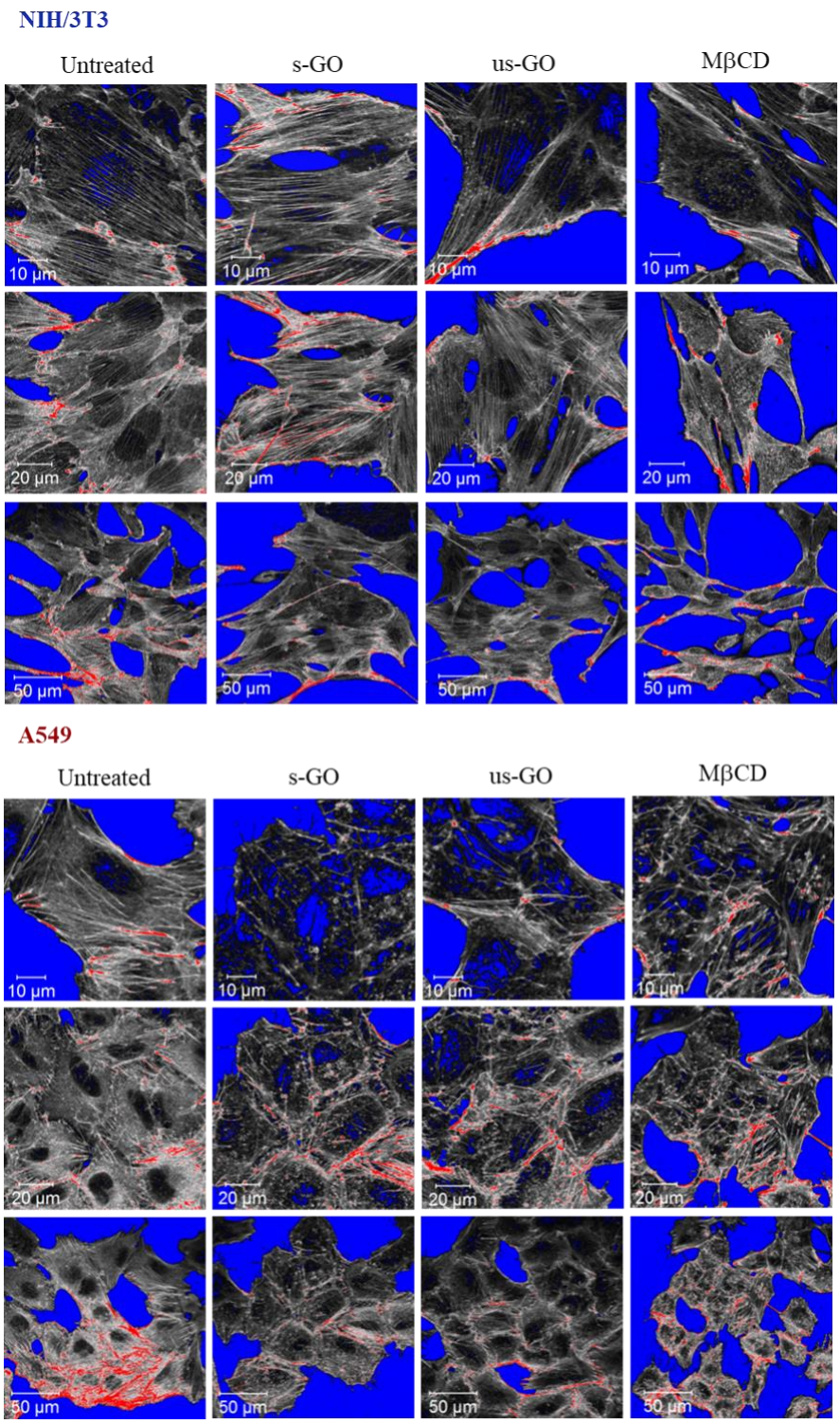


Figure S20: Actin filament staining for NIH/3T3 and A549 cells with and without treatment of GO (s-GO or us-GO, 100 µg/mL, 24 h) or M *β*CD (5 mM, 4 h). Images are shown in range indicator (pixels with zero, low, high, and maximum intensity are shown in blue, black, white and red, respectively).

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