From *in vitro* to *ex vivo*: subcellular localization and uptake of graphene quantum dots into solid tumors

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Abstract

Among various nanoparticles tested for pharmacological applications over the recent years, graphene quantum dots (GQDs) seem to be promising candidates for the construction of drug delivery systems due to their superior biophysical and biochemical properties. The subcellular fate of incorporated nanomaterial is decisive for transporting pharmaceuticals into target cells. Therefore a detailed characterization of the uptake of GQDs into different breast cancer models was performed. The demonstrated accumulation inside the endolysosomal system might be the reason for the particles’ low toxicity, but has to be overcome for cytosolic or nuclear drug delivery. Furthermore, the penetration of GQDs into precision-cut mammary tumor slices was studied. These constitute a far closer to reality model system than monoclonal cell lines. The constant uptake into the depth of the tissue slices underlines the systems’ potential for drug delivery into solid tumors.

Supplementary material for this article is available online

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(Some figures may appear in colour only in the online journal)

1. Background

Nanoparticle based drug delivery systems have gained a lot of research interest due to their ability to control the release of...
drugs and improve their selectivity and potency [1]. Possible applications range from tuberculosis [2] and inflammation [3] to cancer [4], where nanoparticle-based drug delivery systems have the potential to overcome platinum treatment resistance [5]. Recently, a liposomal nanoparticle formulation of cytarabine and daunorubicin showed impressive clinical benefit in AML patients with subsequent FDA approval [6].

Among the nanomaterial platforms already established in research are liposomes, carbon nanotubes, gold nanoparticles, dendrimers, polymeric nanoparticles and superparamagnetic iron oxides [7]. Graphene quantum dots (GQDs) present a promising next-generation nanomaterial due to their extremely large surface to mass ratio. They consist of one or few layers of graphene and emit strong fluorescence due to quantum confinement and edge effects [8, 9]. As GQDs enter the cytoplasm of various cell types in vitro without significant toxicity [10–12], they are used in biomedical research not only for drug delivery applications, but also for long-term and deep tissue imaging, cancer diagnostics and intracellular sensing [13–16]. Regarding drug delivery, it has been shown that GQDs improve the anti-cancer activity of cisplatin [17] and doxorubicin [18, 19], while it is possible to monitor the release of doxorubicin due to their fluorescence properties [20]. For the use of GQDs as a carrier of anti-cancer drugs a detailed knowledge about their subcellular localization, their differential uptake into different tumor models and their penetration into the depth of solid tumor tissues is vital.

Breast cancer represents the most common cancer among women worldwide [21] and incident rates are still predicted to increase [22]—a reason for the unaltered urgent demand for new therapeutic concepts. Breast cancer cell lines are commonly applied as easy-to-use laboratory model systems, but may display geno- and phenotypic changes from the original tissue due to longstanding in vitro cultivation [23]. Moreover monoclonal cell lines can hardly depict the heterogeneity presented by the human breast cancer disease [24].

In contrast, ex vivo cultivated precision-cut tissue slices (PCTS) of defined thickness contain all cell types of an organ and therefore allow to examine multicellular biochemical processes like metabolism, bio-transformation and transport of drugs in a nearly natural environment [25]. Possible applications regarding tumor tissue reach from exploring signal transduction pathways [26, 27] and response to pharmacotherapy [27–29] to the recently established examination of gene therapy [30] and oncolytic viruses [30, 31]. Moreover, the decisive role of communication between tumor cells and their unaltered microenvironment for the settling and proliferation of metastasized tumor cells renders PCTS a potential in the basic examination of metastasis [32].

To evaluate the potential of GQDs as nanocarriers targeting human breast cancer cells, we characterized their uptake into benign MCF-10A breast epithelial, malignant luminal MCF-7 and triple-negative MDA-MB-231 breast cancer cell lines in detail. As especially for the development of nanocarriers a high degree of consistency between an experimental model system and the target tumor tissue is vital [33], we extended our analysis to the ex vivo model of MMTV-PyMT precision-cut mammary tumor slices (PCMTS). Compared to the human disease, the model presents various similarities on histological and tumor biological levels, especially regarding prognostic biomarkers [34–36]. For a detailed analysis the complete penetration of GQDs into the depth of the ex vivo cultivated PCMTS was demonstrated constituting a highly important prerequisite for an application as drug delivery system.

2. Methods

A detailed description of all experimental procedures can be found in the supplementary material available online at stacks.iop.org/NANO/30/395101/mmedia.

2.1. Synthesis of GQDs

GQDs were synthesized from citric acid and diethylene-triamine in accordance to the method published by Qu et al [37].

2.2. Fluorescence analysis

FACS analysis on single cells was performed using a Beckmann Coulter CytoFLEX flow cytometer. Photoluminescence (PL) properties of cell suspensions and GQD solutions were recorded using a Horiba FluoroMax-4 spectrophotometer at an excitation wavelength of 360 nm.

2.3. Cultivation and incubation of GQDs on cell lines and primary cells

MCF-7, MDA-MB-231, MCF-10A monoclonal and MMTV-PyMT primary cells were cultured in selected nutrition media. Sterile GQDs were added to the cell suspensions for the indicated incubation periods. Counter-staining of various cell organelles was performed using Thermo Fisher Scientific CellLight fluorescent proteins.

2.4. Cultivation and incubation of GQDs on PCTS

PCTS were cut and cultured following the protocol by de Graaf et al [25] for handling precision cut liver slices (PCLS). For cutting a Leica VT1200 S vibrating blade microtome was used. Sterile GQDs were added to the nutrition media for the incubation period. Fine slices were cut using a Leica Frigomobil sliding microtome.

2.5. Confocal fluorescence microscopy

Confocal imaging on living single cells and fixed tissue slices was performed using a Zeiss LSM 710 confocal laser scanning microscope evaluating the DAPI or Hoechst 33342 channel (405 nm excitation, 410–495 nm emission), the GQD channel (488 nm excitation, 495–530 nm emission) and the RFP channel (543 nm excitation, 550–700 nm emission).
3.2. GQD uptake into benign and malignant human breast epithelial cell lines

Starting with the most primitive in vitro breast cancer model, we studied the uptake of GQDs into MCF-7 cells, representing the estrogen receptor (ER) positive, progesterone receptor (PgR) positive, HER2 negative luminal A-subtype, into MDA-MB-231 cells as an example for triple negative (ER negative, PgR negative, HER2 negative) breast cancer and into MCF-10A cells as a model for non-tumorigenic mammary epithelial cells. To establish a GQD concentration and a time frame, that is tolerable for the cells, we conducted the MTT viability assay at concentrations ranging from 50 μg ml⁻¹ to 1 mg ml⁻¹ over a period of 24 h, 48 h and 72 h (left part of figures 2(A)–(C)). After 24 h incubation time, no significant changes (statistically significant defined as \( p < 0.05 \) as compared to the untreated controls) in cell viability was detected for all cell lines and concentrations, while after 48 h a statistically significant decrease in cell viability to 67% compared to control cells was measured for MCF-7 cells at a concentration of 1 mg ml⁻¹. The viability of MDA-MB-231 cells and MCF-10A cells decreased to 74% at the same time point and concentration, but did not reach statistical significance. In contrast, a clear concentration dependent decrease in cell viability was observed for all three cell lines following 72 h of incubation with a statistically significant decrease at concentrations of 750 μg ml⁻¹ and 1 mg ml⁻¹ to minimum values of 64% for MCF-7 cells, 56% for MDA-MB-231 cells and 66% for MCF-10A cells. Summarized, all three cell lines show a similar MTT pattern and the GQDs seem to be well tolerated up to concentrations of 500 μg ml⁻¹ over a time period of 48 h.

Therefore, we decided to use a GQD concentration of 500 μg ml⁻¹ and a maximum time of 48 h for the following uptake studies. The uptake of the GQDs into the cells after 48 h incubation time at a concentration of 500 μg ml⁻¹ was visualized by confocal fluorescence microscopy on living cells stained with Hoechst 33342. The right parts of figures 2(A)–(C) show the merged fluorescence images of the cells, representing the nuclei in blue (405 nm excitation, 410–495 nm emission, referred to as Hoechst 33342 channel) and the GQDs in olive (488 nm excitation, 495–630 nm emission, referred to as GQD channel). GQDs appear as bright spots as they also emit in the Hoechst 33342 channel. The observed distinct and localized bright areas near the nucleus indicate, that the GQDs accumulate in the same regions for all three cell lines.

To assess the uptake into the cell lines quantitatively, we measured the change of the cells’ mean fluorescence intensity by flow cytometry (figure 2(D)) using the PB450 channel. The MDA-MB-231 and MCF-10A cells showed a similar behavior over time with a signal change of 7500 following 24 h incubation time and a change of 10 000 after 48 h of incubation, which indicates a saturation over time. In contrast, the MCF-7 cells showed a three times higher signal change, namely 22 000 after 24 h and of 30 000 after 48 h incubation time, implying a similar saturation pattern just at a higher level. Additionally, we analyzed the forward scatter (FSC).
data as a measure for the cells’ size. The MCF-7 cells appeared to be larger than MDA-MB-231 and MCF-10A cells with a mean FSC value of $5.1 \times 10^5$ for MCF-7 cells, $3.9 \times 10^5$ for MDA-MB-231 cells and $4.3 \times 10^5$ for MCF-10A cells.

In order to explore the regions of accumulation, we counter-stained various cell organelles in typically perinuclear localization using organelle specific RFP-tagged proteins (see figure 3). To depict the cell borders we first analyzed the GQDs’ fluorescence channels in combination with transmitted light. In all experiments (except for the mitochondrial counter-staining) the nuclei were co-stained, as well. Again, the GQD accumulations were uniquely identified by a fluorescence signal in both the Hoechst 33342 channel and the GQD channel. A further merging with the cell organelles’ fluorescence signal recorded in the RFP channel allowed the identification of the GQD-containing subcellular structures: endoplasmic reticulum (figure 3(B)) and mitochondria (figure 3(F)) were depicted close to the GQD accumulations, but no superimpositions of all signals could be determined. Instead the GQDs accumulated in late endosomes (figure 3(D)) and, to a larger extent, in lysosomes (figure 3(E)). Slight shifts between the different signals that keep the morphology of the stained structures can be explained by motions of the living cells. Moreover, these structures were localized next to the Golgi apparatus, which itself did not display a direct merging of fluorescence signals with the GQDs (figure 3(C)). The localization inside late endo- and lysosomes next to Golgi apparatus and nucleus indicates that the nanoparticles are taken up via endocytosis [38, 39].

### 3.3. GQD uptake into PCTS

Next, we extended our examination towards PCTS presenting the probably closest-to-reality in vitro cultivation system. We started analyzing the ex vivo uptake of GQDs into mouse liver tissue of C57BL/6 mice, as PCLS constitute a well-established model system with pre-defined methods of cultivation available. The homogeneous tissue facilitates precise cutting under defined conditions leading to comparable slices of high similarity [40]. These are optimally suited to establish incubation and analyzing methods before extending the experiment to more difficult-to-handle tumor tissue.

In order to characterize the uptake of GQDs into PCLS we performed various incubation experiments modifying slice thickness, incubation time and analyzing methods. All incubation steps were carried out using the GQD concentration of $500 \mu g ml^{-1}$ and incubation period of 48 h as previously established.

We first evaluated the GQD uptake analyzing 20 μm fine slices cut from 300 μm PCLS via confocal microscopy. As all recorded images were massively overlayed with the cells’ auto-fluorescence signal in both detection channels (probably enhanced by PFA fixation [41]), a detailed subcellular localization was not possible. Exemplary images are presented in figure S1.
Figure 3. Confocal microscopy images of MCF-7 cells incubated with GQDs after counter-staining of perinuclear cell organelles. (A) Superimposition of the transmitted light, nuclei are represented in blue, GQD emissions in blue and olive. (B)–(F) Cell organelles are represented in red, nuclei in blue, GQDs in blue and olive. Merging of the blue and olive color results in a white signal, merging of the red and olive color in an orange signal and merging of all three colors in a dark yellow signal. For the mitochondrial counter-staining (F) the nucleus was not stained due to overlapping fluorescence channels of the applied dyes. All images were taken using a 63× oil objective. Scale bars: 10 μm.
Therefore, we evaluated the fluorescence properties of dissolved complete or fine-cut PCLS using an excitation wavelength of 360 nm (corresponding to the GQDs’ maximum of absorption). Generally, the incubation with GQDs led to an increase in the fluorescence intensity and a shift of the wavelength of maximum fluorescence towards the evaluated emission channel of the applied GQDs. The evaluation reached statistical significance and the results were further verified by a flow cytometric evaluation. They indicate that the GQD fluorescence dominated the cells’ fluorescence spectra outstripping the native and fixative-induced autofluorescence as a consequence of the incubation experiments. This demonstrates a cellular uptake of the applied GQDs by the PCLS. Typical examples for GQD incubated and control group slices can be found in figure S2.

We further evaluated the uptake of the GQDs into the depth of PCTLS. Analyzing the fluorescence of fine-cut PCLS both by fluorescence spectroscopy and by flow cytometry, we did not detect a decreasing uptake over the penetration depth (examples are presented in figures S2(C) and (D)). This indicates a homogeneous uptake into all layers of the PCLS.

After establishing culture and incubation techniques using liver tissue we extended our study to PCMTS from the mouse MMTV-PyMT model, which is widely used in experimental breast cancer research due to the spontaneous induction of multiple tumors early in life.

Already in the macroscopic examination the MMTV-PyMT tumors presented as inhomogeneous and irregularly formed, especially in comparison to the homogeneous liver tissue. Again, all uptake experiments of GQDs on PCMTS were performed for 48 h using the GQD optimized concentration for incubation experiments of 500 μg ml⁻¹ established before.

After cultivation, a histological evaluation of the PCMTS showed that about 90% of the cells were vital while 10% presented necrosis without significant differences when the slices were cultivated with or without GQDs (figure 4(A)). Furthermore, an M30 apoptosis staining was performed. Neither in the control nor in the GQD incubated PCMTS we determined a relevant amount of apoptosis (see figure S4). This indicates that the tissue slices tolerate both cultivation and incubation with GQDs without major cellular damage. For the incubation with GQDs, this observation resembles the outcome of the MTT viability assay formerly performed on monoclonal cell lines and primary tumor cells.

To verify the GQD uptake into MMTV-PyMT PCMTS we performed a statistical evaluation of the integrated fluorescence intensity signal from dissolved 30 μl h fine slices cut from 300 μl PCMTS. For each group (N_{GQDs} = 44, N_{control} = 43) the slices were cut from four different tumors explanted from three mice. Following the GQD incubation we observed an increase in the integrated fluorescence intensity from 1.9 × 10⁵ to 3.9 × 10⁶ (see figure 4(B)) at an excitation wavelength of 360 nm reflecting the GQDs’ maximum emission; the evaluation reached statistical significance indicated by a p-value of p = 1.15 × 10⁻¹. We continued analyzing the wavelength of maximum fluorescence intensity by a statistical evaluation. Here, the incubation with GQDs led to a shift in wavelength from λ_{max} = 444.3 nm to λ_{max} = 450.5 nm. A p-value of p = 6.0 × 10⁻⁷ demonstrated statistical significance (see figure 4(C)). The elevated fluorescence intensity and the wavelength shift towards the maximal emission wavelength of the applied GQDs clearly
demonstrated a cellular uptake of the GQDs into the depth of the PCMTS. This was further validated by an increase in the mean fluorescence intensity of \textit{in toto} dissolved PCMTS recorded via flow cytometry from \( \langle I \rangle = 0.5 \times 10^{5} \) to \( \langle I \rangle = 4.1 \times 10^{5} \) following the incubation with GQDs (not shown).

To analyze a possible dependency of the GQD uptake on the penetration depth inside the slices we first regarded the integrated fluorescence intensity data as a function of the fine slices’ position inside the PCMTS. In all four individually performed experiments we did not detect a significant dependency of the fluorescence intensity on the penetration depth (see figure S3 for an example comparing a GQD incubated PCMTS with a native control slice). We validated this finding by changing the analyzing method to flow cytometry. Using the flow cytometer’s PB450 channel we did not detect any correlation between the mean fluorescence intensity of the single suspended cells and the position inside the PCMTS (an example is presented in figure 4(D)).

To conclude, as for the liver tissue, we could demonstrate a cellular GQD uptake into MMTV-PyMT PCMTS. As again for this tissue type no decrease of the uptake over the penetration depth was detectable applying two different methods of analysis, we assume a homogenous uptake even into deeper tumor cell layers similar to the liver tissue.

3.4. GQD uptake into MMTV-PyMT primary cells

To verify the method of uptake for the particular cell types present into the MMTV-PyMT tissue, we finally analyzed MMTV-PyMT primary cells. Primary cell cultures established from a small tumor piece after collagenase D digestion constitute a more realistic model than monoclonal cell lines, as their percentage distribution of cell types may be compared to solid tissue.

Again, we started the analysis performing the MTT viability assay. As for the monoclonal cell lines, we did not see a significant decrease in cellular viability. For GQD concentrations and incubation periods up to the formerly applied parameters of \( 500 \mu \text{g ml}^{-1} \) and 48 h the vitality stayed >90% (see figure 5(A)).

For the analysis of the subcellular GQD uptake we used confocal microscopy, applying the same conditions as for the MCF-7 cells (48 h incubation time at a concentration of \( 500 \mu \text{g ml}^{-1} \)). The counter-staining of cell organelles was limited to an examination of late endosomes (figure 5(C)) and lysosomes (figure 5(D)) representing the organelles in which the GQDs localized in MCF-7 cells. In general, we could demonstrate the same subcellular accumulation of the nanoparticles as in the former experiments. Variations in the subcellular distribution of endosomes and lysosomes between MCF-7 and MMTV-PyMT cells may be explained by morphological differences between the two cell types.

To quantitatively investigate the GQD uptake by flow cytometry, we used concentrations of \( 200 \mu \text{g ml}^{-1} \) and \( 500 \mu \text{g ml}^{-1} \) and an incubation period for up to 48 h. For both concentrations the changes in mean fluorescence intensity (using the flow cytometer’s PB450 channel) were examined as a function of time setting the zero point to the cells’ auto-fluorescence signal and the starting point of the incubation period to \( t = 0 \) h (figure 5(E)).

After 24 h of incubation we observed a signal change (with the error bars indicating the standard deviation) to 16 000 for the higher and to 7000 for the lower concentration. After 48 h the signal changed to 28 000 and 13 000, respectively. These values are comparable to the results from the former measurements on monoclonal breast epithelial and adenocarcinoma cells.

![Figure 5](image-url)
4. Discussion

In this study the uptake of GQDs into various breast cancer tumor models was studied in detail. All cell types analyzed in our experiments (in particular benign epithelial and adenocarcinoma cells of different malignancy) depicted a cellular uptake of GQDs, which did not reach complete saturation over the incubation period of 48 h. MCF-7 cells showed a three fold higher uptake as compared to MDA-MB-231 and MCF-10A cells. This can only partially be explained by the cells’ size, as the forward scatter data only translates into a 1.5 fold higher surface area of MCF-7 cells. Therefore luminal MCF-10A cells. This might be related to an endocytotic uptake, as MCF-7 are known to have a high endocytosis rate as compared to other cell lines [42].

The method of uptake is decisive for a nanoparticle’s intracellular fate [43], which in consequence may influence the applicability for drug delivery. Moreover, the uptake mechanism is strongly dependent on the physical properties of the applied particle [44].

The model of in vitro cultivated cell lines allowed for a subcellular localization of the nanoparticles in living cells via confocal fluorescence microscopy. Inside the cells the particles were localized in late endosomes and lysosomes, which is consistent with a mechanism of uptake via endocytosis. Similar observations were made analyzing primary breast cancer cells. These may be regarded a superior model system compared to tumor cell lines, as long-term cultivated immortalized monoclonal cells are prone to genotypic and phenotypic drifting [45].

An endocytotic uptake has been described for different types of nanoparticles [43] and is connected with a membranous encapsulation inside the endolysosomal system [38]. This might be the reason for the low cytotoxicity of the applied GQDs. For constructing a drug delivery system, however, an endosomal escape has to be achieved to render cytosolic and nuclear access to the particles. Otherwise, an application stays limited to the specific field of pharmaceuticals targeting structures inside the endolysosomal system such as pH-dependent pro-drugs, lysosomal enzymes and inhibitors of β-secretase [46].

To evaluate a more close to reality model system, we transferred our analysis to the examination of PCTSs. We started analyzing the ex vivo uptake of GQDs into mouse liver tissue of C57BL/6 mice as a well established model system. Regarding gene expression PCLS show the highest degree of similarity in comparison to liver cell lines and hepatocyte cultures [47].

We found a homogeneous uptake into all layers of the PCLS, which is of relevance as the liver plays an important role for the bio-transformation, metabolism and toxicity of nanoparticles. For example, carbon nanotubes showed a long-term accumulation in this organ, which underlines its importance for toxicology studies [48]. More recently, carbonate apatite nanoparticles were used to deliver the pro-drug cyclophosphamide into murine livers, where it was activated for a later treatment of breast cancer [49].

Especially for the evaluation of pharmaceutical effects on tumors the ex vivo system can represent a vital tool, as the sensitivity of breast cancer cells to cytostatics depends on dynamic interactions between tumor cells and a microenvironment [50]. Moreover, PCTSs can depict the intra- and inter-tumoral variability of solid tumors to a larger extent [51].

The ex vivo breast cancer model represents the most sophisticated tumor model we used and may depict the in vivo situation in breast cancer to a large extent. For tumor biology studies, cultivation of PCTS offers the chance to allow deeper insights into the underlying pathogenic processes than highly reductionist cell lines, as alterations in biology and pharmacology regarding signal dynamics within a tumor’s microenvironment can be studied [52]. Thus, they might be regarded as one of the most realistic model systems to perform basic uptake analysis for building up a nanoparticle based drug delivery system, before extending the examinations towards animal experiments necessitating unlikely larger cost and regularly effort. In our experiments the GQDs had no obvious negative effects on the viability of the tissue as assessed by M30 CytoDEATH assay and a histological evaluation. Unfortunately, we were not able to perform a subcellular localization of the applied GQDs inside the tissue slices, as confocal microscopy on fixed cells was hampered by a massive auto-fluorescence and live experiments are not possible due to the necessity of cutting the tissue to thinner slices. We therefore changed our method of analysis to evaluating the fluorescence of dissolved PCMTS. We were not only able to verify the cellular uptake, but did also detect a homogeneous uptake into the depth of the tissue. This result is consistent with the distribution of the fluorescent dyes rhodamine B and lucigenin in rat precision-cut liver slices [53] and fluorescence marked taxol in human mammmary breast cancer slices [50].

In conclusion, the complete penetration of the GQDs into the solid tumor slices without obvious negative effects on the viability of the tissue is promising for a usage as core of a drug delivery system, since for that purpose a distribution of anti-cancer drugs throughout complete tumors has to be ensured. First studies of such a system should concentrate on the luminal type, as MCF-7 cells appear to have a distinct higher uptake of GQDs. Nevertheless, the GQDs appear to be encapsulated in endosomes and lysosomes. Therefore endosomal escape has to be achieved to target the cytoplasm or the nucleus.

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