

Oxidized Graphene Nanoribbons as a Delivery System for the Bioactive Sphingolipid Ceramide

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ABSTRACT

Ceramides are a group of sphingolipids that have garnered a growing interest as possible anticancer agents due to their chain-length-dependent, potent pro-apoptotic effects. However, as lipids, they have virtually no solubility in aqueous solution. Consequently, there is currently no clinically approved delivery method for *in vivo* use of ceramide as a therapeutic agent. To this end, we have developed a novel method for loading the short-chain C₆ ceramide onto oxidized graphene nanoribbons (O-GNRs). Mass spectrometry revealed a loading efficiency of 51% C₆ onto O-GNRs. Using a Prestoblue viability assay, we determined that that 100 µg/mL of C₆-loaded O-GNRs reduced HeLa cell viability by 97% compared to controls. We confirmed that C₆-loaded O-GNRs were actually entering cells using transmission electron microscopy. Taken together, these data show that O-GNRs are a promising delivery agent for ceramide.

Keywords: graphene, cancer, drug delivery, ceramide, sphingolipid

1 INTRODUCTION

Ceramide, a type of sphingolipid, has been increasingly recognized for the important roles it plays in cell metabolism and certain diseases. For example, aberrant ceramide metabolism has been shown to play key roles in cancer survival and cell proliferation[1, 2]. As such, these metabolic pathways are promising targets for inducing apoptosis in cancer cells via externally-delivered ceramide. One candidate for targeting these pathways is the synthetic, short-chain C₆ ceramide, which has been shown to inhibit cell growth and induce apoptosis when delivered to cancer cells[1]. However, as it extremely hydrophobic, it requires a delivery system to have sufficient solubility in aqueous solution. Current methods for increasing ceramide solubility for delivery into cells, such as solubilization in DMSO and

liposomes, have issues such as low efficacy, complex and error-prone loading processes, or would not be suitable for therapeutic use. Here we present a facile method for loading ceramide onto oxidized graphene nanoribbons (O-GNRs) derived from carbon nanotubes, and we demonstrate highly-reduced HeLa cell viability after exposure to ceramide-loaded O-GNRs. We also demonstrate high levels of uptake of our ceramide-loaded O-GNRs (referred to as Cer-O-GNRs) into HeLa cells via TEM.

2 METHODS

2.1 Materials

Ceramide C₆ was purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other materials and reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) unless noted otherwise.

2.2 O-GNR Synthesis and C₆ Loading

O-GNRs were synthesized from carbon nanotubes using the longitudinal unzipping method[3]. O-GNRs (1 mg/mL) and C₆ ceramide (200 µg/mL) were dispersed in 100% ethanol using bath sonication. 500 µL of each of these stock solutions were added to a 20mL glass scintillation vial and the mixture was sonicated for 15 minutes. The mixture was then bubbled vigorously with nitrogen gas and covered with parafilm in order to prevent oxidation of ceramide. In order to force hydrophobic interaction between the O-GNRs and ceramide, 9 times the volume (9mL) of double-distilled H₂O was added to the solution containing O-GNRs and ceramide using New Era NE-300 syringe pump (New Era Pump Systems Inc. Farmingdale, NY, USA). Water was added in two phases: an initial slow phase for 1 hour at 2 mL/hr, and then a faster phase at 7 mL/hr until completion, for a total addition time of 2 hours. After completion of loading, the mixture was centrifuged for 1hr at 13000RPM and the supernatant with unloaded ceramide removed, leaving

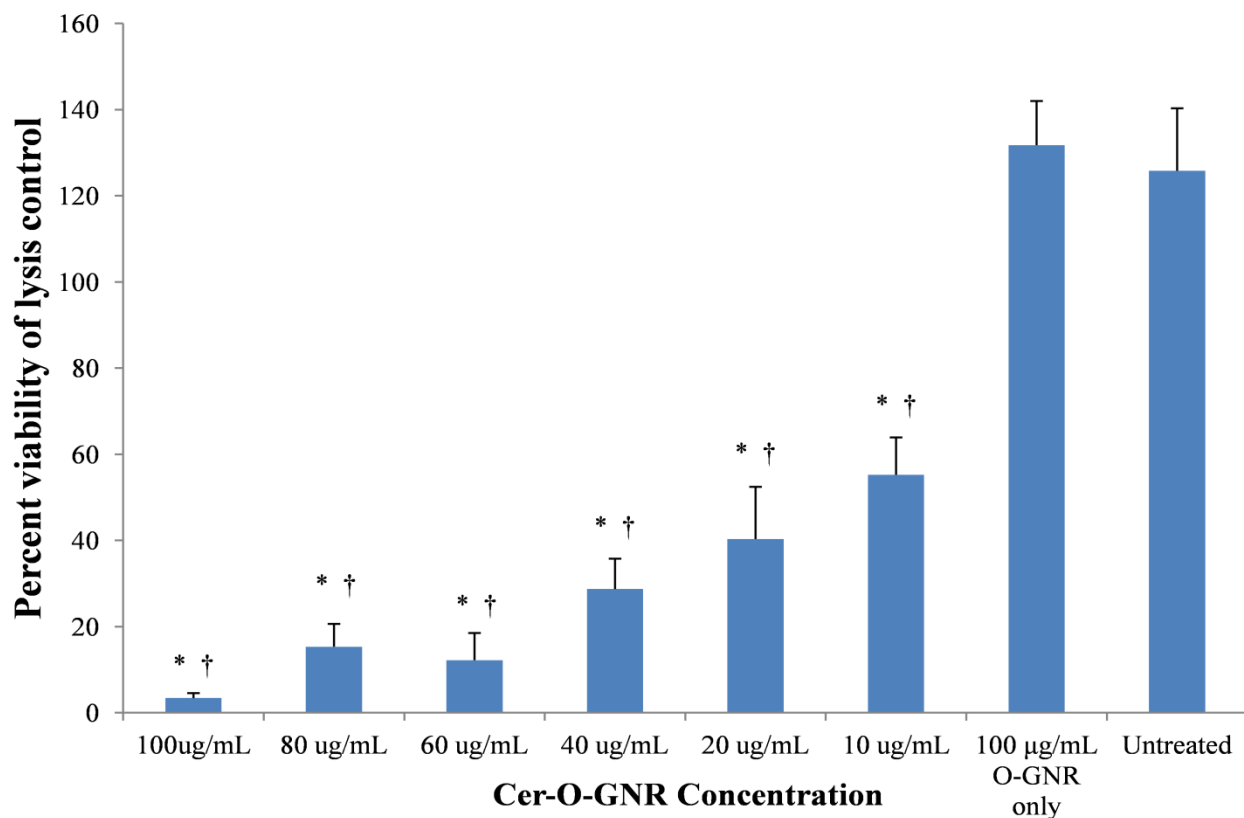


Figure 1: HeLa cell viability measured using Prestoblue after incubation with Cer-O-GNRs for 24 hours. Ceramide loading onto O-GNRs was 255 ng per microgram of O-GNRs. * indicates significant difference from “100 µg/mL O-GNRs only.” † indicates significant difference from “Untreated.”

ceramide-loaded O-GNRs (Cer-O-GNRs). In order to calculate loading efficiency, Cer-O-GNRs were resuspended in 100% ethanol and sonicated for 1 hour, to separate ceramide from the O-GNRs. The ceramide was isolated using centrifugation, and the concentration of ceramide in the supernatant was determined using mass spectroscopy.

2.3 Prestoblue Assay for Cell Viability

In order to determine the efficacy of Cer-O-GNRs as an anticancer agent, we incubated HeLa cells with Cer-O-GNRs and measured cell viability using Prestoblue, a resazurin-based viability dye. HeLa cells were grown at 37 °C, 5% CO₂ in DMEM supplemented with 10% fetal bovine serum and 1% penicillin streptomycin. Cells were seeded in 96 well plates at a density of 5000 cells per well and allowed to adhere overnight. The following day, HeLa cells were incubated with Cer-O-GNRs at concentrations ranging from 10-100 µg/mL. In order to ensure that any observed reduction in viability was not due to the loading process or O-GNRs themselves, O-GNRs were sent through the loading and purification processes without any ceramide, and incubated with cells at the highest concentration tested (100 µg/mL). After 24 hours the media was removed from each well and washed twice with Phosphate Buffered Saline. 100 µL of the viability reagent mixture was added to each well and placed back in the incubator. After 2 hours, the

fluorescence intensity of each well was measured with an excitation wavelength of 530nm and emission wavelength of 580nm using a Molecular Devices SpectraMax M2e (Sunnyvale, CA, USA). The cell viability is expressed as a percent of the lysis control with the formula $((I_{test} - I_{blank}) / (I_{control} - I_{blank}) \times 100\%) - 100\%$, where I_{test} is the fluorescence intensity of cells exposed to Cer-O-GNRs or untreated/O-GNR only controls, $I_{control}$ is the fluorescence intensity of lysed cells, and I_{blank} is the fluorescence intensity from empty wells. Statistical analysis was performed using one-way ANOVA with Tukey’s post-hoc analysis.

2.4 Cellular Uptake of O-GNRs via TEM

HeLa cells were seeded on ACLAR® film (Electron Microscopy Sciences, Hatfield, PA) at a density of approximately 20000 cells per well in 6 well plates and allowed to adhere overnight. Cells were then exposed to 100µg/mL Cer-O-GNRs for one hour. After this incubation time, cells were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M PBS for 15 minutes at room temperature. The films were then placed in 2% osmium tetroxide in 0.1 M PBS and dehydrated through graded ethanol washes. The films were then embedded using durcupan resin. The embedded specimens were examined for areas with high cell densities, and these areas were cut into

80 nm sections using an Ultracut E microtome (Reichert-Jung, Cambridge, UK), and placed on formvar-coated copper grids. Sections were imaged using a Tecnai Bio Twin G transmission electron microscope (FEI, Hillsboro, OR), at 80 kV. Images were obtained using an XR-60 CCD digital camera system (AMT, Woburn, MA).

3 RESULTS AND DISCUSSION

3.1 Loading of C₆ onto O-GNRs

Using mass spectroscopy, we calculated the loading efficiency to be approximately 51% of our initial concentration, indicating a high loading efficiency despite the concentrations of ceramide and O-GNRs being diluted 20-fold by the loading process. This is a significant improvement in efficiency over the maximum possible incorporation of C₆ into liposomes[4].

3.2 Viability of HeLa Cells Exposed to Cer-O-GNRs

After exposure to 100 µg/mL of Cer-O-GNRs for 24 hours, HeLa cells had their viability reduced their viability by approximately 97% compared to the untreated or O-GNRs control (Figure 1). Even at the lowest concentration used (10 µg/mL), HeLa cell viability was reduced by 56% compared to the untreated controls. In order to ensure that this toxicity could not be attributed to either the loading process or the concentrations of O-GNRs used we put O-GNRs through the loading process without ceramide, and incubated them with HeLa cells at the highest concentration used (100 µg/mL). This control was significantly different from all Cer-O-GNR conditions tested while not being significantly different from the untreated control, meaning that the reduction in viability observed could not be attributed to the loading process or the O-GNRs themselves.

3.3 TEM of Cer-O-GNR Uptake in HeLa Cells

We have previously demonstrated that O-GNRs get taken into HeLa cells in great quantities via a macropinocytotic-like, EGFR-dependent process[5, 6]. However, since we were able to load significant quantities of C₆ onto O-GNRs, it was not clear whether this loading would interfere with the uptake of O-GNRs into HeLa cells. Figure 2 illustrates that loading O-GNRs with C₆ does not appear to interfere with this uptake process. There are many O-GNRs of different sizes and shapes present in vacuoles, as well as uptake of more particles in progress. Furthermore, these regions of uptake along the outer edge of the cell demonstrate the characteristic "blebbing" of the cell membrane previously observed in the uptake of O-GNRs into HeLa cells[6]. This suggests that loading ceramide onto O-GNRs does not affect their ability to enter cells, and that they can actually deliver their cargo inside the cell itself rather than merely releasing it in close proximity.

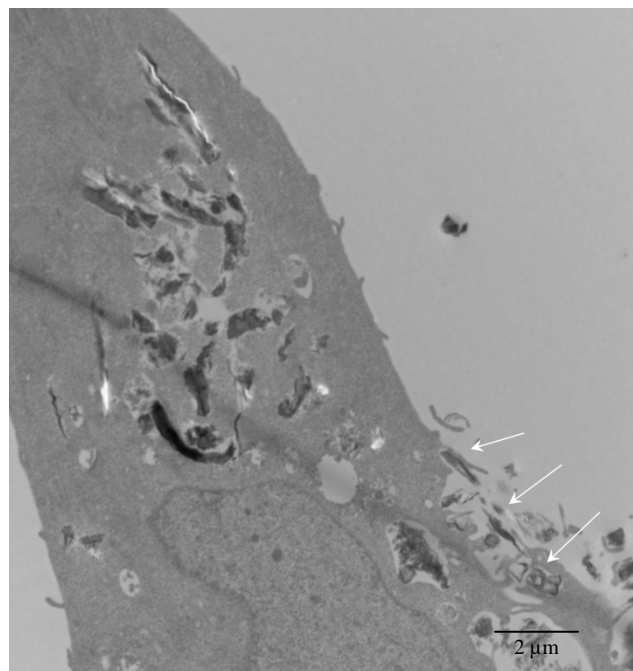


Figure 2: Transmission electron microscopy images of HeLa cells incubated with Cer-O-GNRs after one hour. There are many nanoparticles contained within vacuoles, as well as edge regions with "blebbing" of the membrane (white arrows) characteristic of the macropinocytotic-like process.

4 CONCLUSIONS

O-GNRs are a promising delivery system for the short-chain ceramide C₆. Using a fast, simple method for loading, we were able to induce high levels of toxicity and uptake in HeLa cells at low concentrations using our Cer-O-GNRs. Future studies will look at ceramides with longer variable chains such as C₁₆ ceramide.

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