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Letter

Culture Media Critically Influence Graphene Oxide Effects on Plasma Membranes

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In their article, "Graphene Oxide Nanosheets Stimulate Ruffling and Shedding of Mammalian Cell Plasma Membranes," Sun et al.[1] described how the early-stage interaction between graphene oxide (GO) flakes and rat basophilic leukemia (RBL) cells perturbed their plasma membrane without causing cytotoxicity. The formation of peripheral membrane structures detaching from the RBL cells (within 4 hr) was reported for two other mammalian cell types, MDS-MB-231 (human breast cancer) and NIH/3T3 (mouse fibroblast) cells. Such membrane fragments were formed almost immediately upon exposure to GO (within 2 hr) as a result of plasma membrane ruffling and then shedding of large membrane fractions. These membrane ruffles and fragments did not present the characteristic morphology of exosomal microvesicles or blebs[2,3] and could represent a new mechanism of early-stage cellular responses from interaction with the GO flakes.

Such novel cellular responses have not been previously reported in the literature. However, they were not observed in any of our own investigations or in those of collaborating laboratories using similar cell models and GO flakes of comparable lateral dimensions.[4–7] Finding such cellular processes intriguing, we reproduced the cell-culture model described in Sun et al. and initially exposed NIH/3T3 fibroblasts to our home-made GO suspensions under the same serum-free HEPES-buffered salt solution (BSS) for 4 hr at 100 μg/mL. GO suspensions were produced under endotoxin-free-like conditions[8] by a modified Hummers' method starting from graphite flakes (Nacional de Grafite) as previously described.[5] The GO flakes had a lateral size ranging from 35 to 300 nm (by transmission electron microscopy) and were one or two sheets thick.[7] The buffer solution was HEPES-BSS (also known as Tyrode's basal salt mixture: 135 mM NaCl, 5.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 5.6 mM glucose [pH 7.4, room temperature]; reference no. T2397, Sigma-Aldrich) completed with 20 mM HEPES (Sigma-Aldrich). Cells were initially seeded in 6-well plates at 300,000 cells/well in normal cell-culture media (containing 10% fetal bovine serum [FBS]). After 24 hr of incubation in a humidified CO_2 incubator, cell monolayers were washed once with the BSS. Then, cells were exposed to 100 μg/mL GO flakes suspended in serum-free HEPES-BSS.

4 hr after GO treatment was initiated, cellular responses in terms of plasma membrane fragmentation or visible modifications, such as shape or surface adhesion, were recorded in phase-contrast mode with a Primovert inverted microscope coupled with an Axiocam ERc 5 camera and ZEN lite software (ZEISS). As described by Sun et al.,[1] ruffling and shedding of the plasma membrane in the exposed NIH/3T3 cultures were observed with live-cell microscopy, even though we used a different GO sample (of a different starting graphitic material and produced by a different method). In agreement with Sun et al., we did not observe large material agglomerates in BSS over the 4 hr exposure.

We then compared the effects of GO exposure between NIH/3T3 fibroblasts suspended in BSS and those suspended in Dulbecco's phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 2 mM KH_2PO_4, 1.8 mM $CaCl_2$, and 1.0 mM $MgCl_2$ [pH 7.4, room temperature]; reference no. D8662, Sigma-Aldrich) or in the cell-culture medium recommended by the American Type Culture Collection for NIH/3T3 cells (Dulbecco's modified Eagle's medium [DMEM], reference no. D6429, Sigma-Aldrich). In all cases, no FBS was used, and 20 mM HEPES (extra buffering agent) was added as described by Sun et al. Surprisingly, we observed that the membrane ruffling and shedding effects occurred in both BSS and PBS, but not in DMEM (Figure S1). In DMEM—the recommended cell-culture medium for these fibroblasts—no cellular response at the plasma membrane was obtained within the 4 hr timescale. Instead, we observed the appearance of intracellular vesicles (attributed to cellular stress) as previously reported.[9] In addition, untreated cells (i.e., cells not exposed to GO flakes) behaved similarly in all three media conditions (BSS, PBS, and DMEM) and showed no response even after incubation for 4 hr without 10% FBS (Figure S1).

The cellular responses to GO exposure observed for fibroblasts cultured in BSS

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and PBS, but not in their normal cell-culture medium (DMEM), were studied in two other cell lines (commonly used to mimic pulmonary exposures). A549 (human lung epithelial carcinoma) and BEAS-2B (immortalized human lung bronchial epithelial) cell responses to GO were again found to be more pronounced in BSS and PBS than in the normal media recommended for these cell lines (F12 for A549 cells [reference no. N6658, Sigma-Aldrich] and RPMI for BEAS-2B cells [reference no. R8758, Sigma-Aldrich]). However, these cell responses were different than the plasma membrane ruffling and shedding and formation of membrane fragments observed with NIH/3T3 cells (Figure S1). These observations, particularly the differences between cell types, will require further investigations to accurately determine the cellular and plasma membrane processes in action.

Overall, these findings raise interesting questions regarding the critical roles of the suspension and culture media and how these affect the interaction between GO flakes and cells, possibly by modifying the way GO flake dispersions are affected by the chemical components of the media, as previously observed for other nanomaterials.[10] The main difference between the three suspension solutions used (BSS, PBS, and the respective cell-culture media) is their electrolyte (salt) composition. The aggregation, bundling, stacking, or other colloidal and structural effects on highly oxygenated, negatively surface-charged nanoparticles (such as GO flakes) will be critical. This also suggests that the same GO suspension might interact with cells differently depending on the composition of salts and ions present in the aqueous environment in which they are suspended and tested.

The last few years have provided interesting insights into the importance of the protein corona on toxicological responses, given that it alters the nanomaterial surface. Consideration of the chemical composition of the solutions and media into which nanomaterials are suspended and allowed to interact with cells is also critically important yet largely undervalued and rarely reported. The composition of buffer and culture media can shape and form the way in which nanomaterials interact with the biological milieu and systems, as well as interfere with the readout of the assays used. We believe that carefully controlling the content of ions and salts in the interaction between nanomaterials (particularly highly surface-charged ones, such as GO) and cells and how these alter cellular responses and intracellular signaling is imperative. More critically, further studies are needed to confirm whether such early-stage cellular responses translate into long-term cytotoxic effects. Equally important is the question of the relevance of such observations to hazard assessment of 2D materials, particularly the critical issue of the common adoption of standardized protocols for assessing the toxicology of such nanomaterials. Accurate and thorough reporting of the experimental conditions and details in performing such biological investigations cannot be overemphasized before generalizations are made and potentially misleading conclusions are reached.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at http://dx.doi.org/10.1016/j.chempr.2017.01.015.

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