Review of: "Joint Interactions of Graphene and Benzo[a]pyrene With Pulmonary Surfactant"

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Potential competing interests: The author(s) declared that no potential competing interests exist.

This manuscript summarizes data from a detailed study on the simultaneous interaction of a nanomaterial, graphene, and a potential environmental pollutant, benzopyrene (BP), with pulmonary surfactant (PS), the material that coats and protects the respiratory surface. The study combines computer simulation and a limited amount of experimental work to illustrate how much the encounter of different molecular entities at the nanoscale can mutually modulate their properties and potential effects in biological contexts. It is a very relevant example of the necessity to approach complex multimodal studies in order to understand the true impact of nanomaterials in health, both from the point of view of potential toxicology and of the development of biomedical (nano)applications. The study seems to have been carried out carefully, including the extensive analysis of numerous pertinent factors, which somehow reveal perhaps unexpected levels of complexity that are important to consider in future studies in the area.
Still, a few questions require further clarification before the full relevance of this work can be properly assessed.

1. A key concept, which is somehow related with the phenomenon studied here, is the “corona”. It refers to the modulation of the interactions, effects and fate of any given nanomaterial as a consequence of the association of different biomolecules from the (bio)environment where the nanomaterial enters. The current study extends somehow the idea suggesting that the association of nanomaterials with other environmental molecules could also originate “emerging” properties not exhibited by the independent entities. I would like to see a bit of more discussion on the corona idea connected with the main contribution of this study. By the way, the authors are referring and discussing very limited examples of recent relevant studies (most of them from the lab of Zuo) on the formation and effects of nanomaterials and coronas on PS (see for instance the work by Raesch et al (2015) in ACS Nano, or that from the lab of Beck-Broichsitter).
In an additional level of complexity, the combination of graphene and BP could induce formation of different coronas than occurring with pure materials, with distinct consequences.

2. The different simulations and experiments have approached and analyzed the combined or separated effect of graphene and BP on PS layers, including the possible effects of preferential interactions with some

of the PS components. Such preferential interactions could provide mechanistic information on the possible effects observed or predicted. However, very limited information is given with respect to those possible selected interactions. Only partial information regarding measured apparent diffusion of different molecules is given. One would expect a detailed examination of the possible association of components such as cholesterol with graphene or BP, as a consequence of potential entropic contributions derived from their planar structure. Also, it is particularly relevant the possibility of preferential interaction with the surfactant protein molecules, because they are critical for surfactant function and because other studies have revealed that interaction and sequestering of PS proteins is at the origin of surfactant inactivation by certain nanomaterials. Could the authors include some information in this line?

3. The effect of graphene to extract PS lipids is quite interesting, as well as the formation of apparent two-dimensional micelles at the surface of graphene that is exposed to air. What about the perturbation of the surfactant layer at the surface of graphene exposed to the lipid/water interface?

4. The induction of pores into surfactant layers as a consequence of the interaction of graphene or GO sheets is also of interest, because it may be related with the potential capabilities of these materials to disturb and penetrate cell membranes. However, one wonders how much this is an artifactual effect derived of the conditions selected for the simulations. Extraction of lipid molecules upon interaction with external graphene surfaces may generate holes in the PS layers because the simulations have been run under constant surface and a too low lipid density. In the real situation, surfactant layer would maintain a much more condensed state. Even at the end of expiration, the excess of material is thought to provide equilibrium surface tension values, in the order of 20-25 mN/m, far from 60 mN/m suggested by the authors. Furthermore, in vivo, the interfacial surfactant film would be connected with other surfactant membranes in the subphase, that would replenish the lipids lost upon interaction with the nanomaterials. Also pertinent with the formation of pores: which molecules and components partition into the pore edges at segments not occupied by the nanomaterial?

5. Another concern that requires further clarification refers to the PS models selected for both the simulations and the experiments. In some experiments, Curosurf, a clinical surfactant, has been used as a model of whole natural PS. Curosurf is an appropriate model, no doubt about it, however, it is far from having “similar compositions to human PS”. Curosurf is produced upon processing of an organic extract of whole porcine lung tissue, which as consequence, mixed lipids from surfactant and lipids from blood and pulmonary cell membranes. In the production procedure, some chromatographic steps depurate part of these spurious lipid components, including removal of most of cholesterol. Still Curosurf emulates reasonably the behavior of natural surfactant as it contains reasonable amount of surfactant lipids and hydrophobic surfactant proteins. Thus, instead of stating that Curosurf has similar composition to human PS, I suggest saying that Curosurf “is a reasonable surrogate of human PS”.

With respect to experimental simplified models with synthetic lipids, the authors have chosen preparing liposomes made of DOPC and DOPG. These molecular species are very unusual in surfactant where disaturated froms such as DPPC and monounsaturated species such as POPC and POPG are the most abundant. In contrast, the simulations have been approached using POPC and POPG. Why this discrepancy? The coexistence of saturated and unsaturated species in real PS is well know to induce segregation of ordered and disordered lipid phases, which may have an effect in the partition into the PS layers of different molecules. By the way, PC is not a “neutral lipid”, as stated for instance in page 18 (line 394), but a zwitterionic species, because it has no net charge but it is actually charged.
Curosurf, a clinical preparation from natural origin, contains real full-length SP-B and SP-C proteins. However, the system used to run computer simulations has been built by incorporating SP-C and a simplified form of SP-B, called mini-B, designed as a synthetic surrogate with only some motifs of the native protein. Some synthetic surfactant formulations have been tested containing mini-B bit they only mimic part of the properties of true SP-B-containing surfactants. All these extremes need to be clarified in the paper, for the readers to realize what the systems studied are really showing.

6. The procedure used to determine the ability of surfactant to solubilize BP, in the absence or presence of graphene, is not very clear. Please, rewrite the explanation of this procedure to make it clearer, adding, if necessary, an scheme to aid the readers to understand how these experiments have been carried out.

7. Liposomes of 100 nm prepared by extrusion are by no mean SUVs but LUVs (large unilamellar vesicles). Typically, the term SUVs is reserved for phospholipid vesicles with size smaller than 50 nm.
Hydrated lipid films produce lipid suspensions, not “lipid solutions” (lines 429-430).
Why GUVs are made in sucrose?
Why the lipids are hydrated at 40ºC? Typically, liposomes have to be prepared at temperatures above the phase transition temperature of the corresponding lipids, which in this case, is below 0ºC. That high temperature, for so long time, may cause oxidation and isomerization of the unsaturated phospholipid species.

8. In the QCM experiments, are the conditions applied allowing the liposomes to fuse and form supported bilayers? Or they maintain the closed, water-containing, liposomal structure?

9. I do not understand what the authors mean when stating that they have tested the interaction of graphene and graphene/BP complexes “under high inhalation concentrations”.

10. The results showing that molecules such as BP could partition more favorably into PS layers upon previous interaction with other materials such as graphene or other carbon nanoparticles, are very relevant. Could the authors some additional discussion on the time scale at which graphene interacts with

PS and BP goes from graphene into the lipids?

Other minor questions:

11. In the legend of figure 2, please indicate what is red in the fluoresce imaging of PS/graphene.

12. In Figure S3, pink spheres represent water molecules?

13. Please, check labels in Figure S11. It is confused as marked.

14. Please in figures such as Figure S12 include information regarding the color code informing about the level of perturbation.