Extended Abstract

Determination of advanced *in vitro* systems as valid, alternative test models to assess the potential genotoxicity of carbon nanotubes

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Due to their advantageous mechanical and physical properties, carbon nanotubes (CNTs) are a beneficial component for numerous applications (*e.g.* building materials, semi-conductors, car tyres, sporting equipment and drug delivery systems) (Robertson, 2004; Coleman *et al*. 2006; Pohls *et al*. 2012). Despite this, when considering their inevitable human exposure (*e.g. via* inhalation in an occupational setting), concerns have been raised as to the potential risk CNTs pose towards human health (Maynard *et al*. 2004; Maynard, 2007; Donaldson *et al*. 2006; 2010a). Although an increased research focus has been attributed to these carbonaceous nanofibers (ISO, 2008) over the last decade, understanding as to how CNTs may cause adverse health effects remains limited (Donaldson *et al*. 2010a; Grosse *et al.* 2014).

In 2008, Poland and colleagues published a seminal paper describing the potential for CNTs to elicit adverse biological responses *in vivo* (Poland *et al*. 2008). It was reported that when CNTs exhibit the specific characteristics of extreme length and heightened stiffness as well as, and importantly, being biopersistent that they can elucidate 'asbestos-like' effects (*i.e.* in comparison to long fibre amosite asbestos fibres) as previously seen *in vivo* (Davis *et al*. 1986). Notably, in this scenario, the CNTs were able to promote granuloma formation after 7 days exposure (Poland *et al*. 2008). These findings, in addition to a plethora of other research articles, highlighted that the specific physico-chemical characteristics of CNTs (*i.e.* number of walls (*e.g.* single-walled CNTs (SWCNTs) *vs*. multi-walled CNTs (MWCNTs)), chemical contaminants (*e.g.* Fe, Ni), length, width, aspect ratio and morphology) play a pivotal role in their ability to cause an adverse biological impact (Johnston *et al*. 2010). Yet, understanding of the biological mechanisms that contribute to such effects following CNT exposure

remains limited (Van Berlo *et al*. 2012; Moller *et al.* 2014). Therefore, in order to deduce such mechanisms and determine the specific cellular interaction and subsequent biochemical impact of CNTs, a wide panel of carbon nanotubes that differ in their physico-chemical properties (*e.g.* length, width, aspect ratio, morphology, chemical dispersants, number of walls and production route) must be investigated together, in the same manner (*i.e.* dose, exposure route/scenario, kinetics, *etc.*) and upon the same biological test system.

For an overview of the advantages and disadvantages of *in vitro* research (compared to *in vivo* strategies) please refer to Rothen-Rutishauser *et al*. (2008) and Hartung (2010; 2011). However, briefly, due to the extensive cost and laborious nature of *in vivo* research, as well as the ethical considerations that must be considered, to achieve the outlook put forward above, *in vitro* systems must be adopted. This is most notable since these are ideal for deducing the mechanistic pathways that could be associated with nanofibre exposure and their subsequent biological effects. However, their applicability and representative nature towards an *in vivo* environment is always questioned. In this context, advanced *in vitro* systems have received increased attention due to their ability, beyond that of monocultures, to mimic the important cell-to-cell interplay that occurs within any organ of the human body (Rothen-Rutishauser *et al*. 2008) and provide the basis for a next-level *in vitro* testing strategy when combined with realistic exposure systems (Endes *et al*. 2014; Chorterera *et al*. 2014). Yet, determination as to whether or not advanced, multi-cellular systems provide an improvement to the well-known and most commonly used monoculture systems in determining the hazard of *e.g.* nanomaterials however is equivocal, although of extreme importance. In this regard, in a recent study by Clift and colleagues (2014), it was shown that a multicellular system that mimics important cellular interplay within the human epithelial airway barrier (Rothen-Rutishauser *et al*. 2005) (consisting of an epithelial layer and human blood monocyte derived macrophages and dendritic cells), when compared to its independent monoculture systems, provided a heightened sensitivity in the biochemical factors produced from cells following CNT exposure (Clift *et al*. 2014). Despite this however, understanding as to whether or not this advanced *in vitro* system is able to predict the biological response to CNTs *in vivo* has not yet been investigated and thus must imminently receive increased research focus to determine its precise applicability and advantages. Recently however, such an outlook was reported by Snyder-Talkington and colleagues (2015). Investigating a co-culture of epithelial and endothelial cells exposed to MWCNTs, it was shown, when compared to a mouse model, that multi-cellular systems are able to significantly mimic specific responses at the gene-level also seen *in vivo* when exposed to a similar MWCNT dose (Snyder-

Talkington *et al.* 2015). Despite these important findings, there still remains need for extensive investigation to fully comprehend and prove the usefulness of multicellular models as alternative, representative biological systems to use within risk assessment.

Moving forward, and following on from the findings of Clift *et al*. (2014), where it was specifically shown that different CNT types can cause a significant increase in oxidative stress and activation of (pro-) inflammatory mediators over an acute period, a systematic evaluation of the panel of CNTs used in both Clift *et al.* (2014) and Poland *et al*. (2008), is currently being undertaken with the triple cell co-culture lung model of Rothen-Rutishauser *et al*. (2005). The objective of this is study is two-fold. Firstly, assessment is hypothesized to provide key information as to how the (intracellular) mechanisms responsible for the negative biological responses previously reported (Clift *et al.* 2014) may elucidate a genotoxic response. Secondly, it is the objective to assess the relevance of the co-culture system, in comparison with cell monoculture systems, to be used as a valuable, alternative model to investigate CNT-associated genotoxicity, and not simply determination of a cytotoxic or (pro-)inflammatory response. To achieve these objectives, a specific experimental approach to determine CNT-associated genotoxicity has been devised based on the outlook of Stone *et al.* (2009) and Donaldson *et al.* (2010b). Furthermore, the adopted approach has also been based on the key findings of Clift *et al*. (2014), in which CNTs were predominantly observed to be inside the macrophage cache on the apical side of the co-culture model following 24 hours exposure. Furthermore, the nanofibres were observed to locate within vesicles or be free in the cytoplasm and not found to be present within the cell nuclei. These findings highlight that CNTs could cause genotoxicity *via* secondary means (Schins and Knaapen, 2007), supporting previous research in the field. In this regard, there is consensus that CNTs can cause (pro-)inflammatory effects and that these can elucidate a genotoxic reaction *in vitro* and *in vivo* (Donaldson *et al*. 2010b). The observations on the preferential uptake by the macrophages by Clift *et al.* (2014) points towards the relevance of these secondary effects, and therefore further highlighting the fact that such mechanisms could never be identified from studies with mono-cultures (and thus also never be further mechanistically determined). Therefore, to subsequently comprehend the ability for the panel of CNTs to cause (secondary) genotoxicity *in vitro,* exposure of the triple cell co-culture of the human epithelial airway barrier at sub-lethal concentrations of CNTs to determine their causative ability for DNA damage, DNA repair, cell proliferation and cell cycle arrest is being undertaken. In addition assessment at the gene-level (*via* qRT-PCR) will allow for determination of the specific key, intracellular signaling cascades

that are switched-on or switched-off following CNT exposure and give specific insight into the mechanism(s) possibly at play when related to the specific physico-chemical characteristics of CNTs.

In summary, the findings from this research outlook will gain valuable understanding as to the specific physico-chemical properties of CNTs that mediate their potential genotoxicity *in vitro*, and, when compared to *in vivo*, as to whether or not advanced *in vitro* systems are a valid alternative for determining the potential genotoxicity of nanofibres.

Acknowledgements

The authors would like to acknowledge the funding received from the Swiss National Science Foundation, European Respiratory Society and the Adolphe Merkle Foundation that has enabled this research outlook to be achieved.

Conflict of Interest Statement

The authors declare no conflict of interest. The authors are entirely responsible for the writing and content of this manuscript.

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