Toxicology in Vitro 28 (2014) 1449–1460

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Numerical simulations of *in vitro* nanoparticle toxicity – The case of poly(amido amine) dendrimers

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ARTICLE INFO

Article history:
Received 4 March 2014
Accepted 28 July 2014
Available online 23 August 2014

Keywords:
Nanotoxicology
Polyamidoamine dendrimer
Reactive oxygen species
Cytotoxicity
Numerical simulation
Quantitative structure activity relationships

ABSTRACT

A phenomenological rate equation model is constructed to numerically simulate nanoparticle uptake and subsequent cellular response. Polyamidoamine dendrimers (generations 4–6) are modelled and the temporal evolution of the intracellular cascade of; increased levels of reactive oxygen species, intracellular antioxidant species, caspase activation, mitochondrial membrane potential decay, tumour necrosis factor and interleukin generation is simulated, based on experimental observations.

The dose and generation dependence of several of these response factors are seen to well represent experimental observations at a range of time points. The model indicates that variations between responses of different cell-lines, including murine macrophages, human keratinocytes and colon cells, can be simulated and understood in terms of different intracellular antioxidant levels, and, within a given cell-line, varying responses of different cytotoxicity assays can be understood in terms of their sensitivities to different intracellular cascade events.

The model serves as a tool to interpolate and visualise the range of dose and temporal dependences and elucidate the mechanisms underlying the *in vitro* cytotoxic response to nanoparticle exposure and describes the interaction in terms of independent nanoparticle properties and cellular parameters, based on reaction rates. Such an approach could be a valid alternative to that of effective concentrations for classification of nanotoxicity and may lay the foundation for future quantitative structure activity relationships and predictive nanotoxicity models.

1. Introduction

Research involving nanoparticles has seen a huge increase in recent years. This is undoubtedly due to the novel properties these particles possess and their potential uses in a variety of fields including: medicine, electronics, engineering, cosmetics, food, textiles, packaging and many more (PEN, 2013).

Medical applications is a sector where nanomaterials have shown great potential and have already been used in several areas including: *in vivo* and *in vitro* diagnostic tools, biocompatible materials for implants, nutraceuticals, cancer therapy and, in particular, drug delivery (Jain, 2007; Balasundaram et al., 2006; Nishimura et al., 2007; Nair et al., 2010; Bharali et al., 2009; Koo et al., 2005; Hans and Lowman, 2002). In terms of drug delivery, this interest is due to properties such as the ability to cross biological barriers easier than some more traditional delivery vehicles and the potential to escape from intracellular compartments such as lysosomes and endosomes (Watson et al., 2005). A full list of properties and potential uses for nanoparticles in drug delivery is somewhat outside the scope of this paper and has been reviewed elsewhere (De Jong and Borm, 2008). While the avenue of new medical applications does look promising, it has been found that some nanoparticles, when exposed to mammalian cells, elicit a toxic response (Jain et al., 2010).

In vitro studies indicate this toxicity to be the result of oxidative stress, manifest as increased Reactive Oxygen Species (ROS) production shortly after endocytosis (Xia et al., 2006), with subsequent trafficking of the nanoparticle seen to occur through endosomes and lysosomes (Nel et al., 2006; Salvati et al., 2011). The oxidative stress leads to a release of inflammatory factors

Abbreviations: ROS, Reactive Oxygen Species; EC_{50}, effective concentration 50%; QSAR, quantitative structure activity relationship; PAMAM, polyamidoamine; GSH, glutathione; MMPD, mitochondrial membrane depolarisation; TNF-α, tumour necrosis factor-alpha; IL-8, interleukin-8; IL-6, interleukin-6.

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http://dx.doi.org/10.1016/j.tiv.2014.07.014
0887-2333/

(Kennedy et al., 2009; Mukherjee et al., 2010a; Naha et al., 2010; Rothen-Rutishauser et al., 2007; Schins et al., 2000; Shvedova et al., 2003), reviewed by Medina et al. (2007) and potential activation of apoptotic pathways (Lee et al., 2009; Mukherjee et al., 2010b; Naha and Byrne, 2013; Jeng and Swanson, 2006). It is also important to consider processes such as endosomal rupture (endosomolysis) Watson et al., 2005; Salvati et al., 2011, which can enhance the toxic response as released nanoparticles have been shown to localise in and subsequently damage organelles such as the mitochondria (Kennedy et al., 2009; Mukherjee et al., 2010a). If medical applications are to remain a viable option for nanomaterials, then it will be essential to explore and better define the mechanisms involved in the toxic response.

However, in the majority of current studies, *in vitro* toxicity is quantified using the effective concentration for 50% loss of viability (EC_{50}) endpoint as an indicator of overall toxic effect. The EC_{50}, is the result of a complex cascade of events which occur between the initial exposure and cell death; it gives no indication of the mechanisms, kinetics or efficacy of the interim processes. Additionally, the measured EC_{50} is dependent on the assay employed and the responses of different cell lines to the same exposure conditions have been shown to vary significantly (Mukherjee et al., 2010a). Coupled with the broad range of nanoparticle compositions, structures, sizes and possible surface functionalisations, the result is a vast array of studies from which it is difficult to derive clear systematic trends.

A more rationalised approach to nanotoxicity classification is becoming increasingly important because, as more and more nanoparticles are made available, testing via a case-by-case approach will not be sufficient (Oomen et al., 2014; Clark et al., 2011). If nano-toxicology, and by extension nanomedicine, is to advance, the focus should be on:

(i) Identification of the particle based properties which induce the cellular response,
(ii) the cellular parameters which result in variations in that response, and
(iii) the variability introduced by the use of different assays.

By doing this, it may be easier to elucidate processes and events which are common to a large set of nanoparticles. Initial screening methods should be conducted *in vitro*, as there is a drive for a reduction in the use of animal models for evaluating toxicity, due to regulatory developments in both the EU and US (EU Directive-2010/63/EU and US Public Law 106–545, 2010, 106th Congress) (European Union, 2010; United States, 2000) generally based on the 3 R's of Russell and Burch (Russell and Burch, 1959) to replace, reduce and refine the use of animals used for scientific purposes. Therefore, there is currently much promotion of the development of *in vitro* models which can accurately infer *in vivo* results. One strategy to help meet these requirements, recently endorsed by the OECD (OECD, 2013), is the analysis of Adverse Outcome Pathways (AOPs), by which a sequential chain of causally linked events at different levels of biological organisation that lead to an adverse health or ecotoxicological effect is identified. The aim of the development of AOP is to (i) guide the development of (*in vitro*) Test Guidelines, (ii) provide a basis for the design of Integrated Approaches to Testing and Assessment (IATA), and (iii) guide the development of molecular profilers for the QSAR toolbox. A QSAR (OECD, 2007) (Quantitative Structure Activity Relationship) can be used to identify and model traits which are common to entire sets of nanoparticles and hopefully elucidate how these properties/traits impact the overall toxicity. By advancing the knowledge of these models, it is hoped that, eventually, it will be viable to predict the full toxic profile of a cell which has taken up a nanoparticle (Puzyn et al., 2009).

In the field of nano-toxicology (*in vitro*), the endpoint is usually the median EC_{50} of a colorimetric assay, but the choice of both assay and cell-line is large and little consideration has been given to the different modes of action of the assay within the cell. Many studies have explored the mechanisms underlying the toxic response, but little attention has been devoted to quantifiable comparison between different particles or cell lines, and relationship between endpoint and nanoparticle properties. The domain of nanoparticles is vast, but the paucity of systematic studies renders it difficult to establish domains of applicability of any structure–activity paradigm, and therefore to define unambiguous algorithms or to validate based on statistical goodness-of-fit, robustness and predictability.

In the drive to develop a better understanding of the structural dependence of toxicity and mechanisms, studies using a homologous series of nanoparticles with systematically varied physico-chemical properties can play a vital role. Systematic variations of cellular uptake and mechanisms of response, such as oxidative stress and inflammatory responses can be compared to the systematic changes in the physico-chemical properties of the nanoparticle. A better quantitative understanding of the mechanisms of response allows a better insight into the function of the cytotoxicity assays, and how the endpoints vary systematically with nanoparticle properties, but also with cell line, ultimately laying the foundation for the development of quantitatively predictive models for cellular response (Clark et al., 2011).

Polyamidoamine (PAMAM) dendrimer nanoparticles are a homologous series of nanoparticles of well defined physico-chemical properties which are systematically variable and elicit systematically variable cellular responses. PAMAM dendrimers are branched in conformation and consist of three main parts, (i) the initiator core, (ii) the interior branches and (iii) the exposed branch termini (Tomalia and Fréchet, 2002). Each set of these branches is called a Generation (G) and the generation determines the number of surface amino groups according to the formula:

$$N_{\text{amg}} = N_{BP(\text{G0})} \cdot 2^{G} \quad (1)$$

where N_{amg} is the number of surface amino groups, $N_{BP(\text{G0})}$ is the number of initial branching points at generation zero (G0) and G denotes the generation number. Thus, the diameter and number of surface amino-groups increases systematically with increasing generation.

This well defined branched system allows for the variation of parameters such as: Terminal modification via addition of cationic, anionic or neutral molecules, zeta potential and in particular generation, which will govern: number of amino groups and therefore effective surface charge, diameter and overall particle size. Ultimately this system lets us examine how these characteristics impact the cell-nanoparticle interaction and therefore may shed light on the processes involved in toxicity. Previous studies of exposure of aquatic species (Naha et al., 2009) and *in vitro* mammalian cell cultures (Mukherjee et al., 2010a, 2010b) have demonstrated that the polymeric dendrimer series with systematically varied structures elicits toxic responses which are well correlated with the variations in physico-chemical properties.

Uptake of these PAMAM dendrimers occurs via endocytosis, where the nanoparticle is enveloped in cellular membrane and transported into the cell (Mukherjee et al., 2010b; Kitchens et al., 2007; Hong et al., 2004). The toxic response has been shown to derive from an increased production of intracellular Reactive Oxygen Species (ROS) after endocytosis (Naha et al., 2010). The ROS production is counteracted by cellular anti-oxidants, one example of these being Glutathione (GSH), a thiol based tri-peptide (Mukherjee and Byrne, 2013). After this, a cascade of different events and the release of several characteristic cytokines and