

2012-05-15

Polyamidoamine Dendrimer Nanoparticle Cytotoxicity, Oxidative Stress, Caspase Activation and Inflammatory Response: Experimental Observation and Numerical Simulation

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Recommended Citation

Mukherjee, S.P., Byrne, H.J., 2012. Polyamidoamine dendrimer nanoparticle cytotoxicity, oxidative stress, caspases activation and inflammatory response: experimental observation and numerical simulation. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 9, 202-211 (2013) doi:10.1016/j.nano.2012.05.002

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Funder: This work was conducted under the framework of the INSPIRE programme, funded by the Irish Government's Programme for Research in Third Level Institutions, Cycle 4, National Development Plan 2007-2013, supported by the European Union Structural Fund.



ELSEVIER

Nanomedicine: Nanotechnology, Biology, and Medicine
xx (2012) xxx–xxx

Original Article

nanomedjournal.com

Polyamidoamine dendrimer nanoparticle cytotoxicity, oxidative stress, caspase activation and inflammatory response: experimental observation and numerical simulation

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Received 9 November 2011; accepted 7 May 2012

Abstract

Mechanisms underlying the in vitro cytotoxicity of Polyamidoamine nano-dendrimers in human keratinocytes are explored. Previous studies demonstrated a systematic, dendrimer-generation-dependent cytotoxicity, oxidative stress, and genotoxicity. The emerging picture is of dendrimer endocytosis, endosomal rupture and subsequent mitochondrial attack and cell death. To understand the underlying mechanisms, the evolution of reactive oxygen species, intracellular glutathione, caspase activation, mitochondrial membrane potential decay, and inflammatory responses have been examined. Early-stage responses are associated with endosomal encapsulation, later-stage with mitochondrial attack. In all cases, the magnitude and evolution of responses depend on dendrimer generation and dose. The early-stage response is modelled using a rate equation approach, qualitatively reproducing the time, dose and generation dependences, using only two variable parameters. The dependence of the response on the nanoparticle physicochemical properties can thus be separated from internal cellular parameters, and responses can be quantified in terms of rate constants rather than commonly employed effective concentrations.

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Key words: Polyamidoamine Dendrimer; Molecular mechanism of cytotoxicity; Rate equation model; Numerical simulation

The rapid advance of nanotechnology has rendered it imperative that possible hazardous effects of nanomaterials on humans and the environment are elucidated. Nanoparticles (NPs) with different chemical composition and size have been shown to induce different levels of injury to cells and organisms, and thus a fundamental understanding of the mechanisms of their interaction is critical.¹ In vitro studies have demonstrated that the generation of intracellular reactive oxygen species (ROS) by NPs is a key to their toxicity by triggering different cell-death pathways, including cytokine² and caspase-activation³ and nuclear-DNA damage.⁴

To elucidate the mechanisms underlying toxic responses and establish structure-activity-relationships, NPs of well-defined physicochemical properties that are systematically variable and elicit systematically variable cellular responses can play a key role. PAMAM dendrimers are widely explored, commercially available NPs of well-defined structure.⁵⁻⁹ They have a 2-carbon ethylenediamine core with terminal amidoamines attached,

yielding a highly branched radial structure having tertiary-amine branches and primary surface amino-groups. The diameter and number of surface amino-groups increases systematically with increasing generation.⁵

PAMAM dendrimers have been proposed for a range of biomedical applications, from MRI contrast agents,¹⁰ to targeted delivery of drugs,¹¹ DNA,¹² and siRNA,¹⁴ However, they have been reported to be toxic to mammalian cell lines⁵⁻⁹ and aquatic species.⁷ The polar surface amino groups impart an effective cationic charge, and endocytosis leads to oxidative-stress, mitochondrial and DNA damage, and ultimately apoptosis.⁵⁻⁹ PAMAM dendrimers have also been reported to activate expression of different cytokines, such as tumor necrosis factor (TNF- α), interleukin-6 (IL-6), and macrophage inflammatory protein-2 (MIP-2), in a mouse macrophage cell line.⁸ Previous studies have demonstrated the cytotoxicological responses to these species to vary systematically with increasing dendrimer generation and therefore number of surface amino groups.^{5,6,8} A similar systematic response was observed for the generation of ROS, onset of apoptosis, and levels of DNA damage.⁶ The mechanism of the toxic response has been at least partially elucidated, based on standard cytotoxicity assays including MTT, AB, and NR and microscopic co-localization studies.^{6,15}

No conflict of interest was reported by the authors of this article.

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doi:10.1016/j.nano.2012.05.002

The mechanistic model which has emerged is one of endocytosis, oxidative stress, endosomal rupture through the proton-sponge effect, followed by mitochondrial damage and the onset of apoptosis.^{6,16} The use of amine groups to induce endosomolytic behavior is a well-established strategy in drug delivery,¹⁷ and the endosomolytic activity observed for PAMAM dendrimers is consistent with this. The cellular toxicity thus has primarily two phases; an early-stage, in which oxidative stress is primarily due to the presence of particles in endosomes, and a later stage, in which the particles migrate to the mitochondria, generating further oxidative-stress.^{6,9} However, the toxicity of a specific NP to different cell lines can differ, due to the differences in intracellular constituent levels.⁶ Understanding the metabolic pathways in the target cell, and their dose and time dependencies, is therefore critical to understanding the toxic responses in vitro, and ultimately in vivo.

In this study, the mechanism of PAMAM toxicity to the human keratinocyte, HaCaT, cell line is further explored. ROS production upon exposure to different PAMAM generations and doses is monitored as a function of time. Intracellular levels of the antioxidant glutathione (GSH), representative of the natural cellular antioxidant defense mechanisms, are also monitored. Changes in caspase-8 and caspase-3 activity, mitochondrial membrane potential (MMP) and TNF- α , IL-8 expression, over an exposure period of 24 hours are also explored. Based on the observations, potential underlying pathways for the early- and late-stage cellular responses are proposed.

The early-stage responses are visualized with the aid of a phenomenological rate-equation model, which qualitatively reproduces the generation and dose dependence of the sequence of events. It is highlighted that, although simplistic, such a rate-equation approach is a valuable tool in visualizing and elucidating cellular responses. It identifies response rates as critical parameters in determining toxicity and potentially a more reliable route towards quantitative structure-activity relationships than commonly employed cytotoxicological endpoints.

Methods

Commercially available PAMAM dendrimers of generation 4 (G4), 5 (G5) and 6 (G6) were used in this study. The nominal diameters of the PAMAM G4, G5, and G6 dendrimers are 4.5, 5.4, and 6.7 nm respectively.⁵ Full physicochemical characterization has been reported.⁵ All studies were performed using HaCaT cells. Assays were performed to evaluate ROS generation, GSH depletion, caspase-8 and 3 activation, mitochondrial membrane potential decay (MMPD), and TNF- α and IL-8 expression upon PAMAM exposure at different doses and time points. A detailed description of the materials used and experimental methods is given in the Supplementary Material available online at <http://www.nanomedjournal.com>.

Experimental results

ROS measurement

The generation of ROS shows a complex behavior as a function of time and dose for all PAMAM dendrimer generations, although the response is somewhat systematic as

a function of generation. As a function of exposure time, a biphasic response is observed over a 24-hour time period at lower concentrations of PAMAM, as shown in Figure S1. At a fixed time point, the increase in ROS levels has been shown to increase monotonically with generation (Figure S1, C),⁸ and when expressed in terms of molar concentration of surface NH₂ groups, the dose dependences of ROS for the different generations are overlaid.¹³

For PAMAM G6, an initial or early-stage maximum in ROS levels is observed after \sim 1 hour, for doses of 0.5 μ M to 1.16 μ M, whereas for higher doses (1.3 μ M and 2.23 μ M), the maximum is observed at \sim 0.5-hour exposure (Figure 1, A). At \sim 4 hours, exposure at all concentrations results in a reduction of the ROS levels below those of the control, whereas a later increase in the ROS levels is observed at \sim 24 hours for doses of 0.5 μ M and 1 μ M.

A similar behavior is observed for exposure to PAMAM G5, although the early-stage maximum for the lower doses has shifted toward the longer time of \sim 2 hours (Figure S1, A). This trend is continued for G4, all but the highest exposure concentration exhibiting a maximum response after \sim 3 hours (Figure S1, B). After \sim 24 hours, only the lowest dose exposure results in ROS levels above the control, all others being significantly quenched in comparison with the control levels.

At a concentration of 1 μ M, the maximum amount of ROS was produced after a \sim 24-hour exposure, the levels increasing with increasing generation of PAMAM (G4 < G5 < G6). Notably, this concentration is close to the EC₅₀ of G5 and G6, as previously determined in HaCaT cells using MTT assay (Table S1), and at this concentration, increased lysosomal activity in comparison with control after \sim 24-hour exposure was also observed.⁶ It was found that, at this concentration, in early stages after exposure (e.g., 1 hour), ROS levels increased linearly with number of surface amino groups per generation, as shown in Figure S1, C. With increasing doses, for all generations, after initial increase in ROS levels, the levels are seen to be reduced significantly below those of negative controls and do not recover over a 4-hour period.

ROS localization by CFM

Confocal fluorescence microscopy (CFM) demonstrates that early-increased (1 – 2 hour) levels of ROS localized in sacs/vesicles in cytosol (Figure S2, A), consistent with previous observations of early-stage trafficking of endocytosed PAMAM dendrimers in endosomes.¹⁵ However, in the later stages (\sim 24 hours), ROS are co-localized in the mitochondria (Figure S2, B), potentially indicating that they are generated through differing mechanisms.

GSH measurement

In control cells, intracellular GSH level increases approximately linearly as a function of time over 24 hours, as shown in Figure 1, B. Such a linear increase is commonly observed when studying in vitro cell cultures.^{18,19} Upon exposure to PAMAM dendrimer solutions at a 1- μ M concentration, initial linear increase in GSH levels follows the trend observed for control, but an abrupt deviation from the levels of controls is observed

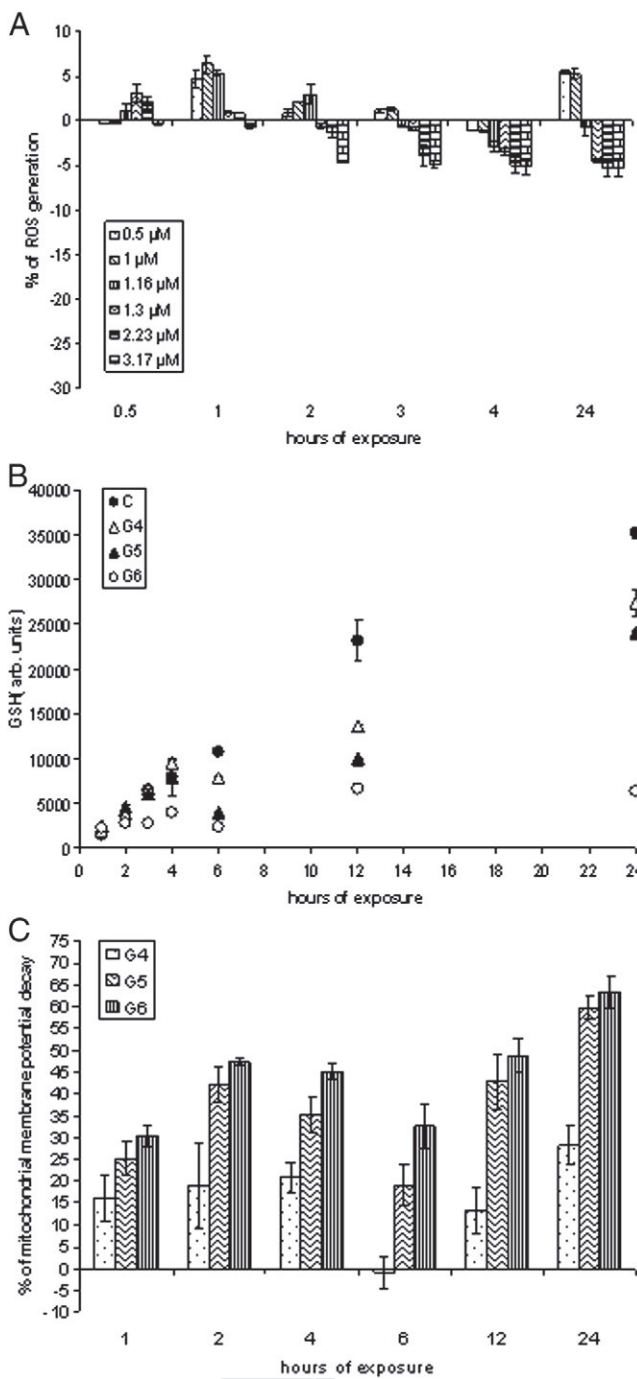


Figure 1. (A) ROS generation in HaCaT cells upon different concentrations of PAMAM G6 exposure. (B) GSH depletion upon 1 μ M PAMAM G4, G5, G6 exposure in HaCaT cells as a function of exposure time. The y-axis shows the fluorescence intensity of the ThiolTracker Violet dye measured in the plate reader. The values are represented as arbitrary units (arb. units). (C) Mitochondrial membrane potential decay of HaCaT cells as a function of exposure time to 1 μ M of PAMAM G4, G5, and G6.

172 within 1 – 5 hours. For G4, the deviation is observed after 4
 173 hours, for G5 3 hours, and for G6 1 hour. Following these
 174 timepoints, the degree of reduction of the GSH levels is also seen
 175 to be systematic in dendrimer generation (G4<G5<G6).

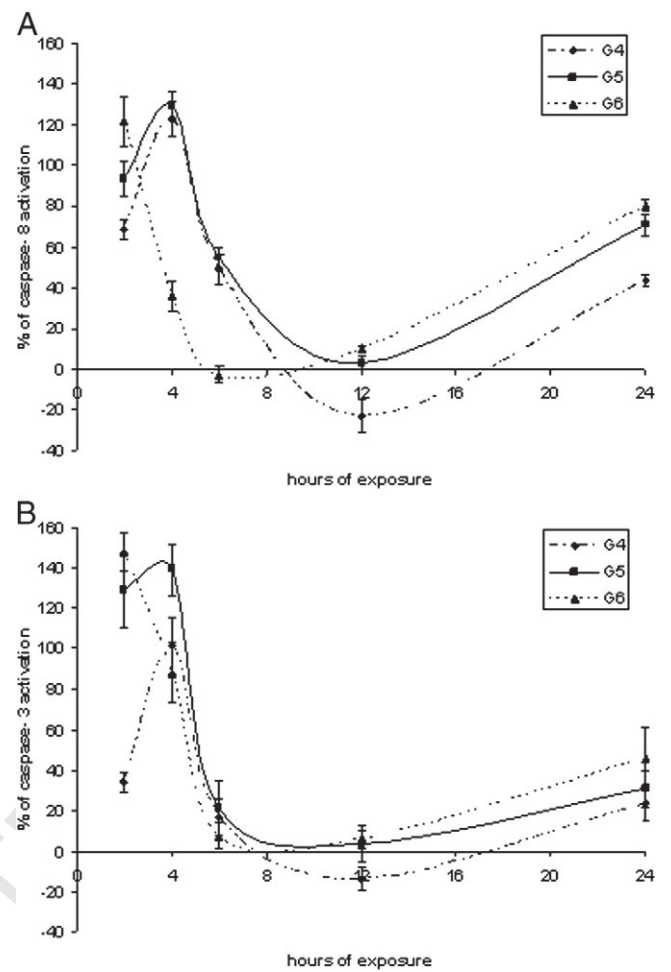


Figure 2. Expression of different caspases after exposure times of 1 μ M PAMAM G4, G5 and G6- (A) caspase-8, (B) caspase-3.

Caspase-8 and -3 activity

176

The activity of caspase-8 and 3 was studied at different time
 177 points for 1- μ M doses of PAMAM G4, G5, and G6. For both the
 178 caspases, a biphasic activity was observed (Figure 2). For
 179 PAMAM G4 and G5, an initial or early-stage maximum of
 180 caspase-8 levels was observed after \sim 4 hours' exposure,
 181 whereupon a minimum was observed before subsequent increase
 182 after 24 hours' exposure (Figure S3, A, S3, B). For G6, the early-
 183 stage maximum was found after \sim 2 hours' exposure (Figure
 184 S3C). Comparing the percentage increase of caspase-8 with
 185 control upon exposure to 1 μ M for the different PAMAM
 186 generations after 24 hours, a systematic increase is observed
 187 (G4<G5<G6).
 188

A similar behavior is observed for the time evolution of
 189 caspase-3 levels. For PAMAM G4 and G5, early-stage
 190 maximum were observed at \sim 4 hours' exposure and after an
 191 intermediate decrease, a late increase of caspase-3 activity was
 192 observed at 24 hours (Figure-S3, A, S3, B). For G6, early-stage
 193 increase of caspase-3 activity was observed at \sim 2 hours'
 194 exposure (Figure S3, C). Again, a systematic generation
 195 dependence of the percentage increase of caspase-3 levels in
 196

197 comparison with control upon 1- μ M exposure after 24 hours is
 198 observed (G4<G5<G6). Thus, although, in a similar way to the
 199 behaviour of ROS levels, the temporal evolution is complex, a
 200 clear systematic variation in the response with dendrimer
 201 generation is also evident.

202 Mitochondrial membrane potential decay

203 Upon exposure to 1- μ M solutions of the respective PAMAM
 204 dendrimer generations over a 24-hour period, the percentage of
 205 mitochondrial membrane potential decay (MMPD), in compar-
 206 ison with controls, also shows a biphasic response, as shown in
 207 Figure 1, C. For all generations, the degree of MMPD increases
 208 initially within early stages of exposure. It then decreases to a
 209 minimum after $\sim 6 - 7$ hours of exposure, after which a further
 210 increase is observed up to 24 hours of exposure. The early-stage
 211 MMPD was observed after $\sim 4 - 5$ hours for G4 and $\sim 2 - 3$
 212 hours for G5 and G6. At all time points, percentage of MMPD in
 213 comparison with control was seen to vary systematically with
 214 dendrimer generation (G4<G5<G6).

215 TNF- α and IL8 expression

216 An upregulation of TNF- α expression that is time, dose, and
 217 generation dependent was observed. For G4, the maximum
 218 amount of TNF- α was expressed at a concentration of 3.21 μ M
 219 after ~ 6 -hour exposure, as shown in Figure S4, A. For G5, the
 220 maximum amount of TNF- α expression was observed for 1 μ M
 221 after ~ 4 -hour exposure (Figure S4, B). For G6, the maximum
 222 amount of TNF- α is expressed at 1 μ M after ~ 4 -hour exposure
 223 (Figure S4, C). For a 1- μ M dose, maximum percentage of TNF-
 224 α expression in comparison with the controls increases with
 225 increasing PAMAM generation (G4<G5<G6), although the
 226 maxima occur at different exposure times (Figure 3, A).

227 Over a 24-hour period, IL-8 expression shows a monotonic-
 228 increase for all doses, for all dendrimer generations, and the
 229 maximum response was observed after a 24-hour exposure. For
 230 G4, the maximum amount of IL-8 was expressed at 3.21 μ M
 231 (Figure S5, A), for G5, at 1 μ M (Figure S5, B) and for G6 at 1 μ M
 232 (Figure S5, C). The percentage of IL-8 expression in comparison
 233 with controls increases with increasing generation of PAMAM
 234 (G4<G5<G6), as shown for 1- μ M dose in Figure 3, B.

235 Discussion and numerical simulation

236 The cellular responses upon exposure to PAMAM dendri-
 237 mers are a complex function of generation, dose, and time.
 238 Figure 4 summarizes the time evolution of the cellular responses
 239 for the case of 1- μ M exposure to the G6 dendrimer. The plot
 240 indicates a defined sequence of events, and the relative temporal
 241 evolutions of the different responses indicate some degree of
 242 interdependence of the responses.

243 Previous studies have demonstrated a two-phase response of
 244 cells to exposure to PAMAM dendrimers; early-stage localiza-
 245 tion in endosomes,¹⁵ followed by a later-stage localization in
 246 mitochondria.⁹ These two phases are well visualized in Figure
 247 S2, A and S2, B, whereby, at early stages, the ROS are primarily
 248 localized in smaller vesicles assumed to be endosomes, whereas

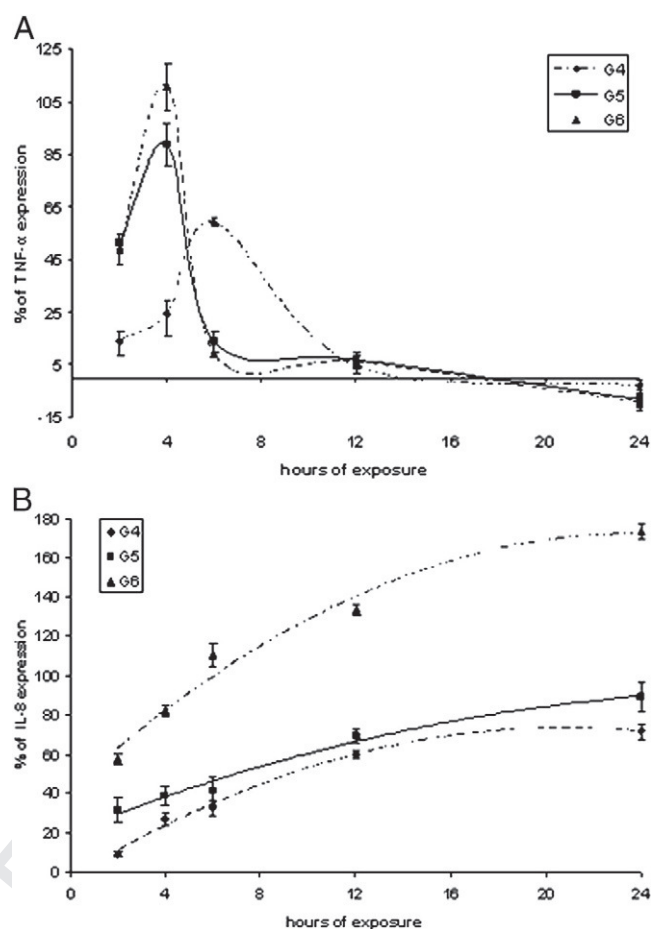


Figure 3. Inflammatory responses in HaCaT cells upon 1- μ M exposures to all dendrimer generations for different time points (A) TNF- α , (B) IL-8.

249 after 24 hours, the ROS are mostly localized in the mitochondria. 249
 250 This two-stage process is further manifest in the time-dependent 250
 251 profile of the ROS levels at low doses, for all generations, as 251
 252 shown in Figure 1, A. 252

253 Key to the onset of the toxic response is the increase in ROS 253
 254 levels upon PAMAM exposure and the concomitant changes in 254
 255 intrinsic cellular antioxidant levels. As shown in Figure 1, B, a 255
 256 linear increase in cellular GSH levels is observed in control cells. 256
 257 Upon exposure to PAMAM dendrimers, a generation-dependent 257
 258 reduction in the rate of increase of GSH levels is observed. The 258
 259 degree and rate of reduction is generation dependent, associating 259
 260 the phenomenon with early-stage increase of ROS levels as a 260
 261 result of exposure.²⁰ It should be noted, upon careful inspection, 261
 262 that there is an apparent time lag between the onset of ROS and 262
 263 the deviation of the GSH levels from linearity. This lag is 263
 264 understandable in terms of differing experimental protocols used 264
 265 to monitor the respective responses. To monitor ROS levels, 265
 266 carboxy-H₂DCFDA dye was uploaded in the cells before particle 266
 267 exposure, whereupon ROS levels were measured after different 267
 268 exposure times. For the measurement of TNF- α , IL-8, and 268
 269 caspases, the cells were lysed immediately after exposure and so 269
 270 the exposure time was equal to the measurement time. To 270
 271 monitor GSH levels, however, cells were stained with 271
 272 ThiolTrackerTM Violet for a period of ~ 30 minutes post 272

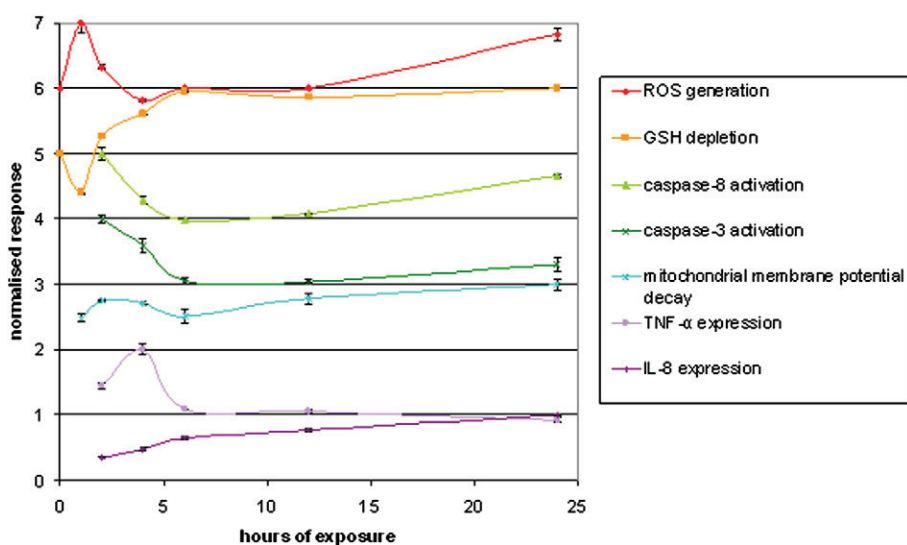


Figure 4. Representation of the different cellular responses as a function of time, for 1- μ M exposure to PAMAM G6. For visual purposes, the responses from different assays have been normalized to 1 and different responses are offset by 1. The normalized responses are all in respect of negative controls, whereby the response for ROS represents percentage of ROS generation in comparison with control, GSH represents percentage of depletion in comparison with control; caspase-8, caspase-3, TNF- α , and IL-8 represent percentage of expression in comparison with control; mitochondrial membrane potential decay represents percentage of MMPD in comparison with control.

273 exposure. Thus, including sample washing and preparation, there
274 is a gap of 30 – 60 minutes from exposure time.

275 Although the results indicate systematic dependences of
276 responses on dose and dendrimer generation, the four-dimensional
277 response/dose/time/generation system is not easily
278 visualized. To better visualize and elucidate the mechanisms of
279 response, the system can be modeled using a simple phenom-
280 enological rate-equation model, similar to those commonly
281 employed for modeling molecular photodynamics.²¹ The
282 particle dose and levels of ROS, GSH, etc., are described by
283 populations, and changes in populations are governed by rate
284 constants. The rate equations can be numerically integrated using
285 an iterative Euler approach²² to yield the temporal evolutions of
286 the populations.

287 Salvati et al have demonstrated that the cellular uptake of
288 polystyrene NPs occurs by endocytosis at a constant rate over a
289 time period of 24 hours and that the rate is dose dependent.²³ In
290 the case of PAMAM dendrimers, endocytotic process is assumed
291 to be generation dependent, and thus the number of particles in
292 the cells, N , increases as:

$$\frac{dN}{dt} = GK_{endo}D \quad \text{Equation 1}$$

293 where K_{endo} is an endocytosis rate constant, G is a generation-
294 dependent scaling factor, and D represents the dose. Once
295 endocytosed, particles continuously generate ROS, build-up of
296 ROS is counteracted by increased levels of GSH, and the
297 interaction quenches both the levels of ROS and GSH. Thus:

$$\frac{dN_{ROS}}{dt} = NGK_{ROS} - K_q N_{ROS} N_{GSH} \quad \text{Equation 2}$$

$$\frac{dN_{GSH}}{dt} = K_{GSH} - K_q N_{ROS} N_{GSH} \quad \text{Equation 3}$$

300 The first term in Equation 2 is a generation- (G) and dose-
301 (D) dependent term describing continuous ROS generation at
302 a rate K_{ROS} . This rate is independent of dendrimer generation,
303 but $DK_{ROS}G$ scales linearly with the number of surface
304 amino groups per generation and dose. The second term
305 describes the quenching of the ROS at a rate K_q , which
306 depends on both ROS levels, N_{ROS} , and GSH levels, N_{GSH} . In
307 Equation 3, the linear increase of the control levels of GSH, at
308 a rate of K_{GSH} , is described by the first term, and the second
309 term describes the quenching of the GSH levels. Thus, as a
310 function of generation, for the same molar concentration,
311 simply changing the parameter G for successive generations
312 should reproduce the generation-dependent behaviors observed
313 in Figures 1, A and 1, B, and, for a given dendrimer generation,
314 changing D should similarly mimic the dose dependences of
315 Figure 1, A.

316 Using a constant generation rate, however, the model predicts
317 a monotonic increase in ROS levels over the exposure time, in
318 contrast to early increase and decrease observed experimentally.
319 If, however, the ROS population is constrained to saturate, a
320 generation- and dose-dependent rise and fall is reproduced. To
321 simulate such saturation, the rate of generation is proposed to
322 be dependent on the number of ROS generated and thus time
323 such that:

$$\frac{dK_{ROS}}{dt} = -K_{ROS}N_{ROS} \quad \text{Equation 4}$$

324 Figure 5, A shows the predicted time and generation
325 dependence of the rate of generation of ROS. Based on such a
326 saturable ROS generation rate, Figure 5, B and 5, C show the

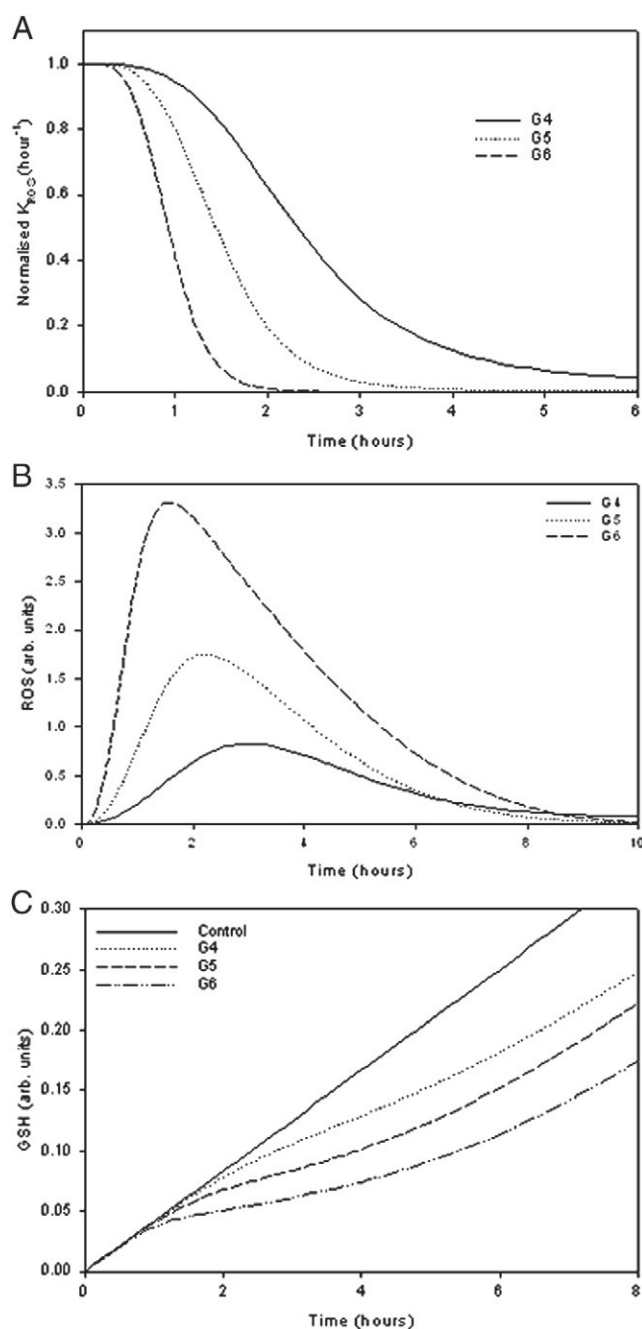


Figure 5. The simulated behaviors for all dendrimer generations of (A) the rate of ROS generation, K_{ROS} , (B) the levels of intracellular ROS, and (C) the levels of intracellular GSH as a function of time. In (C), the solid line depicts the linear increase of GSH levels in negative controls.

332 simulated behaviors for the levels of intracellular ROS and GSH
 333 as a function of generation. For G4, maximum ROS levels
 334 are observed at ~ 3 hours, for G5 at ~ 2 hours, and for G6 at
 335 ~ 1.6 hours, in good agreement with experimental observations.
 336 Notably, however, the evolution curves of Figure 5, B are not
 337 as sharply varying with time as the behaviors observed
 338 experimentally in Figure 1, A. This is a result of the simplistic
 339 representation of the rate of generation of ROS in Equations (2-
 340 4). This is further manifest in the smooth departure of the levels

of GSH from linearity in Figure 5, C, in comparison with rather
 abrupt behavior observed in Figure 1, B. The time delay between
 experimental ROS and GSH generation observed by comparing
 Figures 1, A and 1, B is further manifest here, as the modeled
 GSH follows the modeled increase in ROS levels. Because of the
 complexity of multiple processes leading to ROS generation and
 saturation, the simulation cannot therefore be considered to be a
 fit to the experimental data. Nevertheless, the simplistic approach
 qualitatively reproduces generation-dependent departure from
 linearity of the GSH levels in both extent and rate.

The simulations similarly faithfully predict an approximately
 linear dependence of the relative amounts of ROS on generation
 number, and therefore on number of surface amino groups for a
 fixed time and dose, as shown in Figure S6, A for the case of 1
 hour of exposure of G6. At the 6-hour time point, however, a
 notably different behavior is observed, highlighting the importance
 of experimentally monitoring the full-time evolution of the
 response. Moreover, as the maxima shift in time as a function of
 dose and generation, at specific time points, complex dose
 dependences similar to those in Figure 1, A, can be reproduced,
 as shown in Figure S6, B for the cases of 1 hour, 1.5 hours and 2
 hours of G6 exposure.

It should be noted that biphasic ROS generation is observed
 only at low doses in a generation-dependent fashion. At
 elevated doses, the levels of GSH are depleted such that the
 ROS levels are not quenched and no intermediate time-scale
 minimum in their levels is observed. In Figure 5, C, the
 generation dependence of this process is apparent in the
 prolonged timescales of GSH depletion, which further increase
 with increasing dose.

The activities of both caspase-8 and 3 upon 1- μM PAMAM
 exposure were also found to be biphasic over 24 hours for all
 generations (Figures 2, S3). The maximum percentages of early
 caspase-8 were activated after ~ 4 hours' exposure of G4 and G5,
 whereas those for G6 occurred after ~ 2 hours' exposure
 (Figure 2, A). The activity then decreased to, or below, the
 level of the control after ~ 12 hours for G4 and G5 and after ~ 6
 hours for G6, before subsequently increasing to a maximum after
 24 hours' exposure (Figure 2, A). Variations in activity of
 caspase-3 follow a trend similar to that of caspase-8 (Figure 2,
 B). In both cases, the activity profile and the percentage activity
 in comparison with control are dependent on generation.

In an attempt to understand the possible underlying response
 pathways, it is noted that apoptosis can be mediated through
 two major pathways, the death-receptor pathway (extrinsic)²⁴
 and the mitochondrial pathway (intrinsic).²⁵ Notably, caspase-
 8 and 3 activations are seen to occur almost instantaneously
 after ROS generation, both in early and late stages. The
 observation of the early-stage maximum activation of caspase-
 8 before the maximum early-stage MMPD and activation of
 TNF- α indicates that caspase-8 activation in the current study
 is via the Fas/FasL mediated FADD pathway, independent of
 the TNF- α mediated FADD or mitochondrial pathway²⁶
 (Figure 4). Previously it was also shown that intracellular
 ROS mediates Fas-ligation that leads to caspase-8 and 3
 activation.²⁷ It is therefore proposed that activation of early
 caspase-8 and 3 is mediated via extrinsic FasL mediated
 Fas-signaling pathway.^{27,28} Caspase-8 activation acts as an

399 upstream process for caspase-3 activation by activating pro-
400 caspase-3.²⁹ FADD pathway can be naturally inactivated by
401 caspase-8-like inhibitory protein (cFLIP)^{30,31} or can be inhibited
402 by the inhibitors of apoptosis proteins (IAPs), for example X-
403 linked inhibitor of apoptosis protein (XIAP).^{32,33} Such mecha-
404 nisms can potentially decrease the activity of caspase-8 and 3
405 after its early activation (Figures 2, S3).

406 The activation of caspase-8 by intrinsic mitochondrial
407 pathway, which is independent of the classical FADD pathway,
408 is also well known.^{34,35} Through this pathway, caspase-8 can be
409 activated either via mitochondrial p38-MAPK or mitogen- and
410 stress-response kinase 1 (MSK1), which are sequentially
411 activated upon mitochondrial oxidative-stress (Mchichi et al,
412 2007). The second-phase of caspase-activation follows the
413 second-phase of ROS, generated in the mitochondria, thereby
414 causing mitochondrial oxidative-stress. Therefore, its activation
415 could potentially occur through the mitochondrial p38-MAPK or
416 MSK1 pathway.

417 The expression of TNF- α , a pro-inflammatory cytokine, can
418 also be activated by p38-MAPK and other MAPKs upon
419 oxidative stress.³⁶ NPs have been shown to upregulate TNF- α
420 and CXCL8 via ROS and MAPK activation.³⁷ TNF- α activation
421 in turn activates the expression of the chemokine IL8.³⁸ It has
422 also been reported that TNF- α downregulates FasL expression in
423 the vascular endothelial cells.³⁹ Therefore, sequential TNF- α
424 and IL8 expressions following early-stage maximum in ROS
425 generation caspase activation, and MMPD is consistent with its
426 activation by a MAPK pathway. TNF- α activation before the
427 second-phase of caspase activation possibly activates Smac/
428 Diablo, which inhibit IAPs,⁴⁰ resulting in the second phase of
429 caspase activity with prolonged exposure time.

430 The mitochondrial membrane potential study reveals that the
431 decay rapidly follows the caspase activation, in both early and
432 later stages, with a delay of ~45 minutes to 1 hour (Figures 2 and
433 1, C). This delay comes in part from the staining of the cells with
434 rhodamine-123 for measuring mitochondrial membrane potential
435 after the exposure time point, whereas in caspase study the cells
436 were lysed immediately after exposure. Therefore, caspase
437 activation, which is an instantaneous effect of ROS generation,
438 probably via Fas pathway, results in rapid decay of MMP.²⁸ The
439 extent of MMPD and the time evolution is generation dependent
440 (Figure 1, C). The biphasic response is consistent with the model
441 of early-stage ROS generation by particles in subcellular
442 vesicles, most likely endosomes, which cause oxidative stress
443 to the mitochondria, followed by endosomal release and
444 localization of the dendrimer particles in the mitochondria,
445 leading to cell death via the mitochondrial injury pathway,⁴¹
446 generating further ROS as a result.^{41,9} The decrease in MMPD
447 after the early-stage increase could be due to the effects of
448 mitochondrial chaperones, e.g., prohibitin, which elevate the
449 synthesis of ATP and stabilize MMP, delaying the onset of
450 apoptosis.⁴²

451 The inflammatory study indicates a subsequent sequential
452 activation of TNF- α and IL-8. The maximum TNF- α
453 expression was observed after ~6 hours' exposure at 3.21 μ M
454 for G4 and after ~4 hours' exposure at 1 μ M for G5 and G6
455 (Figure 3, A). Having reached the maximum, the expression is
456 seen to decrease with exposure time to levels less than control.

The expression of IL-8 increases monotonically with exposure 457
time to a maximum at 24 hours' exposure at 3.21 μ M for G4, 458
and 1 μ M for G5 and G6 (Figure 3, B). Above and below these 459
concentrations, the expression of TNF- α and IL-8 decreases. 460
Notably, these concentrations are also the EC₅₀ values obtained 461
from the dose response from MTT assay in HaCaT cells⁵ 462
(Table S1). The maximum percentage of increase of TNF- α 463
and IL-8 expression was also seen to increase with increasing 464
generation of PAMAM dendrimer, and therefore number of 465
surface amino groups. 466

467 It is notable that TNF- α activation is only observed in the
468 early stages and does not follow the biphasic evolution of ROS 469
and caspase activation and MMPD. In the early stages, the NPs 470
are located in vesicles, proposed to be endosomes, and thus 471
oxidative stress is generated external to the mitochondria. At the 472
later stages, dendrimer NPs and generation of ROS have been 473
located in the mitochondria.^{9,6} Although derived from the 474
mitochondria, it has been demonstrated that acute, internal stress 475
can suppress the expression of pro-inflammatory cytokines, such 476
as TNF- α ,⁴³ without affecting IL-8 expression.⁴⁴ Similarly, 477
PAMAM generates acute stress when incorporated into 478
mitochondria (24 hours)⁶ and the second phase of TNF- α 479
expression is not observed in our study.

480 In the first phase of evolution, the rate-equation model can be
481 simply extended to include the ROS-dependent activation of
482 caspases, and subsequent MMPD and generation of TNF- α and
483 IL8, and thus visualize the generation and dose dependences,
484 as described in the Supplementary Material. Figure 6, A–C 485
illustrates the time evolution of the process as well as generation-
486 dependent behavior predicted by the model, simply by changing
487 the parameter G in Equations (1) and (2). All rates are kept
488 constant and the differing rates of evolution of subsequent stages
489 are the result of the early-stage generation- (or dose-) dependent
490 increase in ROS levels. For example, maximum TNF- α
491 expression is predicted for G4 at 5.1 hours, for G5 at 4.4 hours
492 and for G6 at 4 hours. Although the experimental time intervals
493 do not differentiate the maxima for G5 and G6, the model
494 predicts a sequence of activation of G6<G5<G4. Furthermore,
495 although the magnitudes of responses are normalized to the
496 maximum for graphical representation, at each step in the
497 cascade, the generation dependence of G6>G5>G4 is also
498 reproduced faithfully, as shown by the approximately linear
499 dependence of the maximum levels of TNF- α , as a function
500 of number of surface amino-groups per dendrimer generation
501 in Figure S7.

502 In all cases, only the early stage of the cellular responses has
503 been modeled. This stage is proposed to originate from initial
504 endocytosis of the particles and encapsulation in endosomes.
505 ROS is most likely generated via the proton-pump mechanism,
506 resulting in depletion of GSH and other antioxidants and the
507 onset of caspase activation, MMPD, and inflammatory re-
508 sponses via TNF- α activation. The second phase appears to be
509 spatially distinct, in that it is associated with localization of
510 dendrimer NPs and ROS generation in the mitochondria.
511 Although the second phase is not modeled here, a similar
512 rate-equation approach could be employed to simulate the
513 responses, their time evolution and dependences on dose and
514 dendrimer generation. A more complete understanding of

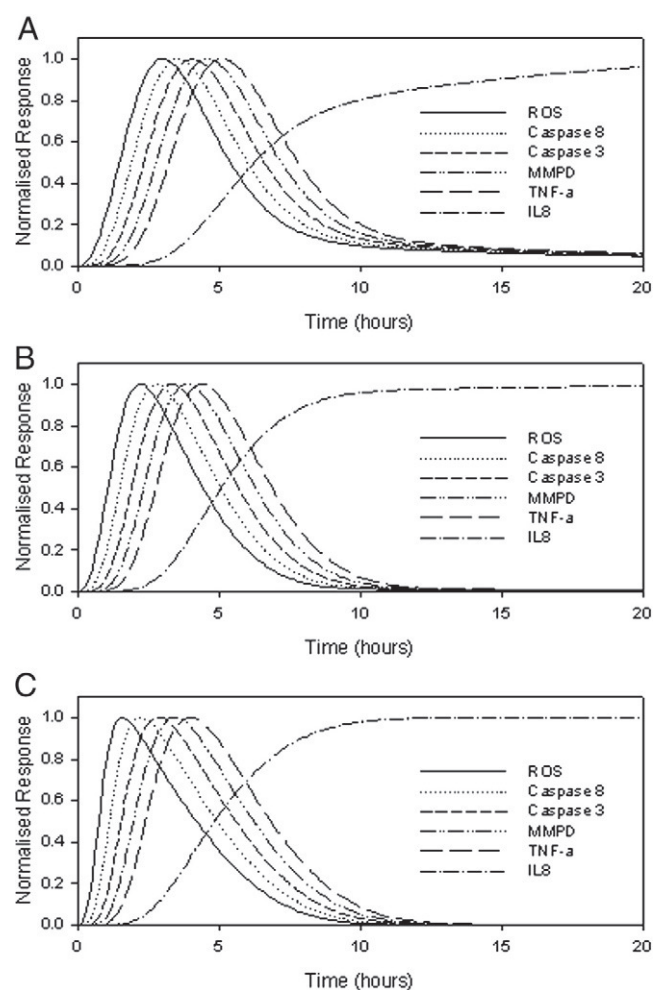


Figure 6. Illustration of the generation dependent behavior for (A) G4, (B) G5, and (C) G6, in terms of normalized ROS generation, MMPD and TNF- α and IL8 expression, predicted by the model, simply by changing the parameter G in Equation (2).

generation and dose dependence of the process of endosomal rupture, particle migration to and uptake by the mitochondria, and recovery of intracellular antioxidant levels would be required. Between the two phases, such phenomena as the quenching of ROS levels to below those of controls, the recovery of antioxidant levels, and migration of antioxidants to localised subcellular sites should be considered. Nevertheless, the phenomenological model is readily adaptable to include more complex phenomena, simply by adding additional terms to the rate equations (Equation S1–S5).

The overall mechanism that can be postulated from the cellular responses is diagrammatically represented in Figure S8. PAMAM dendrimers enter the cells by endosomal uptake or by rupture of the plasma membrane.^{15,6,45} Initial oxidative stress results from early-stage ROS generation whereas the dendrimers are encapsulated in early-stage subcellular vesicles, most likely endosomes.⁶ The generation dependence of the ROS generation rate and yield point to the reactive NH_2 surface groups as the origin of oxidative stress. Intracellular antioxidants result in a quenching of early-stage ROS and are

themselves quenched by the action.⁴⁶ The study of GSH levels and their time evolution, as an example of intracellular antioxidants, highlights the importance of intracellular defense mechanisms, and potentially points to a source of differentiation of different cellular responses to NP exposure. The ROS probably play a pivotal role in the possible FasL/Fas mediated activation of caspase-8 which further activates caspase-3, leading to the initial MMPD. Maximum TNF- α expression after early-stage maximum ROS, caspase-8 and 3 activities and MMPD suggests TNF- α activation by mitochondrial pathway. TNF- α subsequently induces IL-8 expression and therefore its expression gradually increases with exposure time. After reduction of early-stage ROS, it is proposed that the action of different caspase inhibitor proteins results in reduction of caspase-8 and 3 activities and MMP similarly recovers, possibly through the action of mitochondrial chaperones. In the second phase, PAMAM dendrimers rupture the endosomes by so-called “proton-sponge effect” and are released into the cytosol and interact directly with other cellular organelles. After ~ 16 hours they have been located in the mitochondria, whereupon the oxidative stress is increased, resulting in further MMPD. No further TNF- α expression is observed, but a second phase of activation of caspases is observed, which is possibly through the mitochondrial pathway. The sequential and potentially independent pathways of caspase activations that are associated with the early and late stage of ROS generation can be understood by further studies of the activation of Fas and mitochondrial p38MAPK or MSK1. The potential role of IAPs and cFLIP in the inhibition of caspase-8 and 3 after their early-stage activation and the contribution of Smac/Diablo activation profile should also be studied for further elucidation of the biphasic caspase activation. Following the activation of these different cell-death pathways and the activation of caspases, the cell enters apoptosis, its nuclear DNA undergoes fragmentation, and finally it dies.⁶

Although simplistic at this stage, the numerical modeling approach enables a visualization of the complex generation, dose and time dependences of the cellular responses. A fundamental understanding of in vitro cytological responses is becoming increasingly important, given the implications of EU Directive 2010/63/EU on reduction, replacement, and refinement of animal models for scientific experimentation. Consideration of the responses in terms of rate equations elucidates their sequence, interdependencies, and relative magnitudes. It is a potential route towards quantifying nanotoxicological responses in terms of response rates that are determined by NP properties and cellular and even cell-line-dependent parameters, independently. The overall cytotoxicological response, as frequently monitored by classic cytotoxicological assays and expressed, for example, as an EC_{50} , is a convolution of a cascade of events, which can potentially be better expressed as response rates that can be defined per NP and cellular system.

A nonlinear response and saturation are required to reproduce the experimental observations, but the origin and mathematical form of this nonlinearity and saturation requires clarification. In cytotoxicity, dose dependences are commonly empirically represented by the Hill function,⁴⁷ but this is not

593 easily represented in a form that indicates the rates of
594 contributing processes.⁴⁸ In pharmacokinetics, it is simply
595 acknowledged that to account for a nonlinear response to a
596 linear dose, at least one of the intermediate stages must be
597 nonlinear.⁴⁹

598 The endocytotic process is assumed to be linearly dependent
599 on dose, but a low-dose exposure of many cells to few NPs is
600 most likely significantly different from one in which there are
601 many particles per cell, and ultimately the capacity of a single
602 cell to endocytose NPs must be limited. In the simplistic
603 treatment presented here, no changes to cell population due to
604 cell proliferation or death have been included. The doubling time
605 for HaCaT cells is 23 hours.⁵⁰ The Alamar Blue assay shows
606 that, upon exposure to 1 μ M PAMAM G6 for 24 hours, the
607 percentage cytotoxicity in comparison with control was 28%
608 (data not shown). Therefore, it is predictable that after 6 hours'
609 exposure the effect of 1 μ M G6 on HaCaT cell proliferation and
610 viability was not significant.

611 Notably, the dose dependence of ROS generation is nonlinear
612 and potentially originates in its time evolution. However,
613 although the model demonstrates how a complex dose
614 dependence can arise, the results are by no means a fit with the
615 experimental data, and further work utilizing model NP systems
616 is required to accurately predict the dose dependence. Ultimate-
617 ly, however, such a rate-equation model may provide the basis
618 for quantification of NP toxicity and cellular susceptibility, and
619 thus quantitative structure-activity relationships.

Q3620 Appendix A. Supplementary data

621 Supplementary data to this article can be found online at
622 <http://dx.doi.org/10.1016/j.nano.2012.05.002>.

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Graphical Abstract

Polyamidoamine dendrimer nanoparticle cytotoxicity, oxidative stress, caspase activation and inflammatory response: experimental observation and numerical simulation

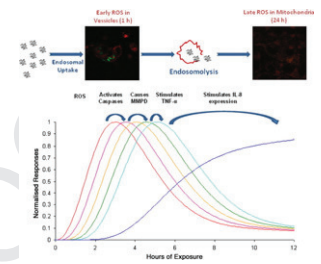
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Structural dependence of cytotoxic responses to PAMAM dendrimers derive from ROS generation and resultant cascades can be visualized and simulated mathematically.

Nanomedicine: Nanotechnology, Biology, and Medicine xx (2012) xxx



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Supplementary Material