

2016-02-01


Structural Dependence of the In Vitro Cytotoxicity, Oxidative Stress and Uptake Mechanisms of Poly(propylene imine) Dendritic Nanoparticles

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Recommended Citation

Byrne, H. et al. (2016) Structural dependence of the In vitro cytotoxicity, oxidative stress and uptake mechanisms of Poly(propylene imine) dendritic nanoparticles", *Journal of Applied Toxicology*, 36, 464-473 (2016). doi:10.1002/jat.3267

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Funder: HEA

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1 **Structural dependence of the *In vitro* cytotoxicity, oxidative stress and** 2 **uptake mechanisms of Poly(propylene imine) dendritic nanoparticles.**

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10 **Abstract**

11 The *in vitro* cytotoxic and intracellular oxidative stress responses to exposure to poly
12 (propylene imine) (PPI) dendritic nanoparticles of increasing generation (number of
13 repeated branching cycles) (G0-G4) were assessed in an immortal non-cancerous human
14 keratinocyte cell-line (HaCaT). Confocal fluorescence microscopy with organelle
15 staining was used to explore the uptake and intracellular trafficking mechanisms. A
16 generation and dose dependent cytotoxic response was observed, increasing according to
17 generation and therefore number of surface amino groups. A comparison of the cytotoxic
18 response of G4 PPI and the related G4 Poly (amido amine) dendrimer indicates that the
19 PPI with the same number of surface amino groups elicits a significantly higher cytotoxic
20 response. The trend of cytotoxicity versus dendrimer generation and therefore size is
21 discontinuous in the region of G2, however, indicating a difference in uptake mechanism
22 for higher compared to lower generations. Whereas the higher generations elicit an
23 oxidative stress response at short exposure times, the lower generations indicate an
24 antioxidant response. Confocal microscopy indicates that, whereas they are prominent at
25 early exposure times for the larger PPI dendrimers, no evidence of early stage endosomes
26 was observed for lower generations of PPI. The results are consistent with an alternative
27 uptake mechanism of physical diffusion across the semi-permeable cell membrane for the
28 lower generation dendrimers and are discussed in terms of their implications for
29 predictive models for nanotoxicology and design strategies for nanomedical applications.
30
31

32 **Keywords:** Poly(propylene imine) dendrimers, Poly(amido amine) dendrimers,
33 cytotoxicity, oxidative stress, *in vitro* uptake mechanisms, structure property relationships
34

35 **1. Introduction**

36 The production of engineered nanoparticles has significantly increased over the last
37 decade, in line with the prediction of a shift from basic nanoparticle research and
38 development to mass production by 2015 (Project on Emerging Nanotechnologies, 2015).
39 Today, nanoparticle applications encompass every aspect of our lives, from fertilizers
40 (Liu and Lal, 2015) and fuel (Dahle and Arai, 2015) to medicine (Parat et al., 2015).
41 Concomitant with the increase in nanoparticle applications is the continuing study of their
42 potential impacts on the environment and human health and the recognition of the need
43 for systematic testing strategies and a greater understanding of the relationship between
44 nanoparticle physico-chemical structures and biological activity (Lynch et al., 2013,
45 Oomen et al., 2014). Understanding the mechanisms of cellular interactions will aid the

46 development of nanoparticles with properties that will maximise efficacy and minimise
47 non-specific toxicological responses. Furthering our knowledge of intracellular
48 mechanisms will assist the betterment of nanomedicine and more specifically drug
49 delivery.

50 The last decade of research has identified the beneficial application of polymers on the
51 nanoscale of 5-100 nm and, in this context, dendritic nanostructures feature highly
52 (Duncan and Izzo, 2005). The ability to control the size, shape and surface functionality
53 has attracted many to exploit these characteristics and potential usage in nanomedical
54 applications (Dear et al, 2006, Guillot-Nieckowski et al., 2007, Swanson et al., 2008, Na et
55 al., 2008, Sha et al., 2011). Dendrimers are 3-D branched polymeric particles of
56 nanometer scale. With increasing generation (number of repeated branching cycles), both
57 the size and surface structure is systematically varied. The dendrimer poly (propylene
58 imine) (PPI) is a well-defined, highly branched molecule (Tomalia, 2005). It possesses a
59 diaminobutane core and successive generations have increasing number of surface
60 functional groups. In the case of poly (propylene imine) (PPI) and the related family poly
61 (amido amine) (PAMAM) dendrimers (Tomalia, 2005), the surface functional groups are
62 polarised primary amino groups, resulting in an effective cationic surface charge The
63 surface groups provide excellent solubility that ensures their stability and dispersion in
64 aqueous solutions (Tomalia, 2005). Notably, increasing dendrimer generation provides a
65 route towards systematic variation of nanoparticle physico-chemical properties.

66 Polymeric dendrimer systems have been proposed for a range of biomedical applications,
67 from magnetic resonance imaging contrast agents (Bourne et al., 1996), to targeted-
68 delivery of drugs (Ywyman et al., 1999), DNA (Guillot-Nieckowski et al., 2007) and small
69 interfering RNA (siRNA) (Zhou et al., 2006). In the field of therapeutics, dendrimers
70 constitute an important class of drug delivery vehicles (Kannan et al., 2006, Pignatello et al.,
71 2009, Kesharwani et al., 2014, Kesharwani and Iyer 2015). They may potentially be used to
72 covalently bind and physically entrap drug molecules to improve their water solubility,
73 decrease their toxicity, increase their permeability and the affinity for their target (Najlah
74 et al. 2006).

75 Notably, in terms of understanding nanoparticle cell interactions which can govern
76 cytotoxic responses and intercellular trafficking mechanisms, important for optimised
77 drug delivery, such homologous series of structurally well-defined nanoparticles can also
78 play a critical role (Mukherjee and Byrne, 2010, Mukherjee et al., 2010, Naha et al, 2010,
79 Mukherjee and Byrne, 2013). Although it has been demonstrated that dendrimer toxicity
80 can be minimised by appropriate surface modification (Cheng et al., 2011, Wang et al.,
81 2012), study of the precise and systematically variable basis structures can add much to
82 the understanding of the dependence of the cellular interactions and responses on the
83 physico-chemical properties of nanoparticles, which in turn may lay the foundation for
84 quantitative structure property relationships and predictive models.

85 In this context, PAMAM dendrimers have been extensively studied to understand the
86 mechanisms of cellular interaction *in vitro* (Kitchens et al., 2008, Lee et al. 2009,
87 Mukherjee and Byrne, 2010, Mukherjee et al., 2010, Naha et al, 2010, Mukherjee and
88 Byrne, 2013). Mukherjee et al. identified that the toxic response is dependent on
89 generation and dose, the nanoparticles becoming more toxic with increasing generation
90 (size) (Mukherjee and Byrne, 2010). A strong correlation has been identified between
91 toxicity and intracellular reactive oxygen species (ROS) production (Naha et al, 2010,

92 Mukherjee and Byrne, 2013). A biphasic increase in ROS was observed, the initial
93 increase of ROS being attributed to the active uptake of the dendrimer by the process of
94 clatherin mediated endocytosis (Kitchens et al., 2008, Mukherjee et al., 2013). The
95 second increase of ROS is due to localization of the PAMAM at the mitochondria via the
96 mitochondrial injury pathway (Xia et al, 2006). The early stage responses to PAMAM
97 exposure have been modelled using a rate equation response, demonstrating that the time,
98 dose and generation dependent cytotoxic responses could be predicted using the single
99 parameter of number of surface amino groups, while the dependence on cytotoxic assay
100 and cell type was faithfully reproduced by considering the different timescales of the
101 endpoints, and the protective effect of intracellular antioxidants, respectively (Mukherjee
102 et al., 2013, Maher et al., 2014).

103 While it has been identified that polymeric dendrimer nanoparticles elicit a significant
104 cytotoxic response (Nel et al. 2006, Naha et al, 2010, Mukherjee and Byrne, 2010,
105 Mukherjee et al., 2010, Mukherjee and Byrne, 2013), precise structure activity
106 relationships are still to be elucidated. PPI and PAMAM dendrimers have similar outer
107 structures of surface amino groups but different cores, and therefore different overall
108 dimensions. Comparison of the chemically analogous but physically distinct
109 nanoparticles can add to the understanding of the dependence of the uptake on the
110 particle size. Furthermore, extension of the study from the higher and intermediate
111 dendrimer generations to lower generations addresses the fundamental questions of the
112 difference between large molecules and nanoparticles in terms of cellular uptake
113 mechanisms.

114 In this study, the toxic response to and interaction mechanisms of PPI dendrimers G0 –
115 G4 (using the nomenclature of Tomalia and Rookmaker, 2009, and Kesharwani et al.,
116 2015) were examined in HaCaT, human keratinocyte cells, as a model for dermal
117 interactions as well as for intercomparison with previous studies. Cellular toxicity was
118 determined by the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)
119 assay, which determines the mitochondrial activity of viable cells, and which has
120 previously been demonstrated to be the most sensitive amongst tested cytotoxicity assays
121 (Neutral Red, Alamar Blue, MTT) (Mukherjee and Byrne, 2010, Naha et al, 2010, Maher
122 et al., 2014). To further understand the mechanism of toxicity of PPI dendrimers, ROS
123 production was assessed on a subcellular level, as previous mechanistic studies which
124 monitored oxidative stress, mitochondrial membrane potential and inflammatory markers
125 (caspase 3, 8, TNF- α), have indicated that ROS generation is an early quantitative
126 indicator of toxic response (Naha et al., 2010, Mukherjee and Byrne 2013, Naha and
127 Byrne 2013, Maher et al., 2014). The results are qualitatively and quantitatively
128 compared to previous observations of HaCaT cells exposed to PAMAM dendrimers
129 (Mukherjee et al., 2010, Mukherjee and Byrne 2013), in order to evaluate the importance
130 of the structural variations between the two dendrimer series. The results indicate a
131 similarity of uptake mechanism and cytotoxic response, via a biphasic oxidative stress
132 profile, for the larger generations of PPI and PAMAM. However, a transition from a
133 regime of active uptake of the larger dendrimers by endocytosis, to one of passive uptake
134 of the smaller ones, by physical diffusion across the semi-permeable membrane, is
135 indicated. It is shown that the passive uptake of the smaller dendrimers results in
136 significantly lower oxidative stress and cytotoxicity, suggesting that understanding how

137 nanoparticles cross cell barriers e.g. cell membrane, can greatly benefit the targeting of
138 nanoparticles as drug delivery vehicles.

139 **2. Methods and Materials**

140 **2.1 Test Materials**

141 PPI dendrimers were obtained from Symo-Chem BV (Eindhoven, Netherlands). PPI
142 dendrimers generation 0 (G0), 1 (G1), 2 (G2), 3 (G3), 4 (G4) have 4, 8, 16, 32 and 64
143 primary surface amine group, respectively. The molecular weights of PPI G0, G1, G2, G3
144 and G4 are 316.5 Da, 773.3 Da, 1686.8 Da, 3513.9 Da and 7168.1 Da, respectively. The
145 dendrimers are readily soluble in water and G0, G1, G2, G3 and G4 stock solutions of
146 respective concentrations 6000 μ M, 500 μ M, 30mM, 9mM and 3.5mM were prepared in
147 Milli Q deionised water and cell culture medium. These stock solutions were then serially
148 diluted to generate the concentration ranges for the dose dependent measurements of cell
149 viability and oxidative stress. PPI nanoparticles were tested for interferences with the
150 respective assays, by acellular tests of the assays in the presence of nanoparticles at their
151 highest concentrations and none were found. Fluorescently labelled 40nm polystyrene
152 nanoparticles (PSNPs) were purchased from BioSciences (Dun Laoghaire, Ireland) and
153 made up to 1×10^{12} particles/ml in cell culture medium as a control for endosomal
154 detection.

155 Both the PPI and PAMAM dendrimers used are commercially available, and their
156 synthesis is well established. Size analysis was conducted using dynamic light scattering
157 and atomic force microscopy as a confirmation of particle dispersion and aggregation
158 state. The details of the methods are described in detail in the Supplemental Information.

159 **2.2 Cell culture**

160 The HaCaT cell line, an immortal non-cancerous human keratinocyte cell line, was used
161 to evaluate the cytotoxicity of the PPI dendrimers. HaCaT cells were obtained from Cell
162 Line Services (Eppelheim, Germany). They were passaged at 70-80% confluence and
163 cells did not exceed 15 passages. HaCaT cells were cultured in Dulbecco's modified
164 eagle medium (DMEM, Sigma Aldrich, Arklow, Ireland), Nutrient Mixture F-12, with 2
165 mM L-glutamine supplemented with 5% Foetal Bovine Serum (FBS), 45 IU ml⁻¹
166 penicillin, 45 IU ml⁻¹ streptomycin (all from BioSciences, Dun Laoghaire, Ireland) and
167 1 μ g/ml hydrocortisone (Sigma Aldrich, Arklow, Ireland) at 37 °C in 5% CO₂.

168 **2.3 Cytotoxicity**

169 The MTT (Sigma Aldrich, Arklow, Ireland) assay was used to measure cell viability. The
170 assay is based on measurement of the activity of the mitochondria, which is constant in
171 viable cells and therefore an increase or decrease in viability as a result of nanoparticle
172 exposure correlates to a change in the mitochondrial activity (van Meerloo et al., 2011).
173 Cells were seeded at a density of 1×10^5 cells/ml in a 96 well plate in 100 μ l 5% FBS
174 medium/well. After 24hr incubation at 37 °C in a 5% CO₂ incubator, the medium was
175 removed and the cells were washed with 100 μ l phosphate buffered saline (PBS) and
176 treated with dendrimer solutions of varying concentrations. Negative (medium without
177 PPI) and positive controls (exposed to H₂O₂, an oxidizing agent used to cause oxidative
178 damage within the cell) were also prepared. Three individual experiments were
179 performed, each including six replicates for both controls and treatments. After 24hr of
180 dendrimer exposure, the medium was removed from all wells and the cells were washed

181 with 100µl PBS. 100µl of 5mg/ml MTT dye in medium (without FBS or supplements)
182 were added to each well. After 3hr incubation at 37 °C in an atmosphere of 5% CO₂, the
183 dye was removed and the cells were washed with 100µl PBS and 100µl dimethyl
184 sulphoxide (Sigma Aldrich, Dublin, Ireland) were added and the samples were shaken at
185 240 rpm for 10mins. The MTT absorbance was measured at 595nm using a TECAN
186 GENios (Grodig, Austria) plate reader to determine the cell viability compared to the
187 control.

188 **2.4 Oxidative Stress**

189 As a measure of oxidative stress, changes in ROS production upon exposure to PPI
190 dendrimer nanoparticles were monitored using the Carboxy-H2DCFDA dye assay
191 (BioSciences, Dun Laoghaire, Ireland) (Kehrer and Paraidathathu, 1992). The study was
192 performed in black 96 well plates, and cells were seeded at 1×10^5 cells/well. The plates
193 were incubated at 37 °C in a 5% CO₂ atmosphere. After 24hrs, each well was washed
194 with 100µl PBS. Carboxy-H2DCFDA dye was added at a concentration of 10µM and
195 plates were incubated at 37 °C in a 5% CO₂ for 1hr. Plates were then removed and
196 washed 3 times with PBS at 100µl/well. Wells were treated with controls, positive
197 (medium without PPI) and negative (H₂O₂), and treatments of G0- G4 PPI dendrimers
198 prepared in 5% FBS containing media at varying concentrations. Experiments were
199 performed in three independent experiments and each included six replicates of each
200 treatment concentration. The fluorescence of the Carboxy-H2DCFDA dye was measured
201 at time intervals 1-4, 6, 12 and 24hrs, at an excitation wavelength of 488nm and emission
202 wavelength of 535nm using a TECAN GENios (Grodig, Austria) plate reader.

203 **2.5 Confocal Microscopy**

204 Commercially available PPI dendrimers are not fluorescent, and although several studies
205 using fluorescently labelled dendrimer nanoparticles as intracellular probes have been
206 reported, it is not clear that the transport mechanisms of (cationic) dendrimers
207 fluorescently labelled with anionic moieties (FITC in the case of Kitchens et al., 2007 or
208 Alexafluor in the case of Thomas et al., 2009) are the same as their unlabelled
209 counterparts. For example, Gajraj and Ofoli (2000) have reported that extrinsic labelling
210 of fluorescein-5-isothiocyanate (FITC) to bovine serum albumin (BSA) in a ratio of 2:1
211 changes its adsorption and diffusion properties. Confocal fluorescence microscopy was
212 therefore employed to visualize and locate the increased ROS production, as well as the
213 presence of early endosomes in HaCaT cells as a result of exposure to PPI dendrimers.
214 To monitor ROS production, cells were seeded by adding 100µl of 1×10^5 cell
215 suspension to the centre of a glass bottom Petri-dish. After 2hrs incubation at 37 °C in an
216 atmosphere of 5% CO₂, 3mls of 10% FBS supplemented medium were added. Cells were
217 incubated for 24hrs at 37 °C in 5% CO₂, where after they were treated with 2mls of
218 10µM Carboxy-H2DCFDA dye. The Petri-dishes were incubated for a further 45mins,
219 and then the cells were treated with the appropriate concentration of PPI dendrimer
220 (chosen to be close to the respective EC₅₀ value) and incubated for a duration of 5hr or
221 24hr. At each time point, the Petri-dish was removed and the cells were washed twice
222 with PBS. Mitotracker dye solution (BioSciences, Dun Laoghaire, Ireland)
223 (Bhattacharyya et al., 1995) was prepared at 2µM in pre-warmed PBS and added to the
224 cells for 30mins incubation at 37 °C in a 5% CO₂. After the staining, the cells were
225 washed with PBS three times and 3mls of warm PBS was added. Experiments were

226 performed in three independent experiments and each included six replicates of each
227 treatment concentration.
228 To examine the uptake mechanisms, fluorescently labelled PSNPs were used as a control,
229 as they have previously been demonstrated to be taken up in cells by endocytosis and
230 subsequently trafficked through endosomes and lysosomes (Ekkapongpisit et al. 2012,
231 Sandin et al., 2012, Monti et al., 2015). The CellLight reagent (BioSciences, Dun
232 Laoghaire, Ireland), a fluorescent protein-signal peptide fusion, was used to visualize the
233 early endosomes. It uses BacMan technology, which uses an insect cell virus
234 (baculovirus) coupled with a mammalian promoter (Kost and Patrick, 1999). Cells were
235 seeded by adding 100µl of 1×10^5 cell suspension at the centre of a glass bottom Petri-
236 dish. After 2hrs incubation at 37 °C in 5% CO₂, 3mls of 10% FBS supplemented medium
237 were added. Cells were incubated for 24hrs at 37 °C in a 5% CO₂, after which they were
238 treated with a solution containing 30 CellLight particles per cell (PPC). The Petri-dishes
239 were incubated for a further 24hrs. The following day, the cells were washed with PBS
240 and treated with the required concentration of PPI dendrimer, or PSNPs for control, and
241 incubated for a duration of 5hrs. After 5hrs, cells were removed, washed twice with PBS
242 and 3mls of warm PBS were added. Confocal images were taken using a Zeiss Confocal
243 Fluorescence Microscope (LSM 510 META, Carl Zeiss, Jena, Germany).

244 **2.6 Statistics**

245 All experiments were conducted in triplicate (three independent experiments), each
246 containing 6 replicates. Fluorescence as fluorescent units (FUs) of all of the assays was
247 quantified using a TECAN microplate reader (TECAN GENios, Grödig, Austria).
248 Control values were set at 100%.

249 Toxicity data was fitted by a sigmoid curve and a four parameter logistic model was used
250 to calculate EC₅₀ values. This analysis was performed using Xlfit3™, a curve fitting add-
251 in for Microsoft® Excel (ID Business Solutions, Guildford, UK). ROS data was analysed
252 using Microsoft Excel® (Microsoft Corporation, Redmond, USA). ROS data was
253 expressed as mean percentage viability in comparison to the unexposed control (100%) ±
254 standard deviation (SD).

255 **3. Results**

256 **3.1 Physico chemical properties**

257 As recommended, the physico-chemical properties of the test species were characterised
258 (Bouwmeester et al., 2011). Full details of the characterisation of PPI G0 – G4 determined
259 by Atomic Force Microscopy are presented in the supplementary information. The
260 particle size measurements for the PPI dendrimer series are tabulated in Table 1. The
261 measured particle sizes are consistent with manufacturer's specifications. However, PPI
262 dendrimers G0 and G1 are clearly present as aggregates in aqueous suspension at
263 concentrations above of >1000µM for G0 and >500µM for G1.

264

265 **3.2 Cytotoxicity**

266 As shown in figure 1, a dose and generation dependant cytotoxic response in HaCaT
267 cells, as determined using the MTT assay after 24 hr exposure was observed, G4
268 producing the highest toxic response and G0 the lowest response. This correlates well
269 with the variation in the number of surface amino groups present on the surface of the

270 dendrimer and strongly suggests a systematic structure activity relationship governing the
271 toxic response of PPI dendrimers. For all generations, a dose dependent response is
272 apparent, and the degree of toxicity increases systematically with generation, consistent
273 with a mechanism which is dependent on the number of surface amino groups, as
274 indicated for the related PAMAM dendrimer series (Mukherjee and Byrne, 2010,
275 Mukherjee et al., 2010, Naha et al, 2010, Mukherjee and Byrne, 2013, Maher et al.,
276 2014). For PPI G1, negligible cytotoxic response is observed at doses $<100\mu\text{M}$, above
277 which dose, the toxic response is seen to increase rapidly. A similar behaviour is
278 observed for PPI G0, above $1000\mu\text{M}$. In comparison with the dose dependent cytotoxic
279 response of the higher dendrimer generations, G2-G4, the dose dependence of the
280 response is very steep. It should be noted that aggregation of particles, itself a dose
281 dependent phenomenon, was observed at concentrations of $>1000\mu\text{M}$ for G0 and $>500\mu\text{M}$
282 for G1, indicating that the rapid decrease in cell viability is due to exposure to aggregates
283 of PPI G0 and G1 (supplemental material, Figure S7, S8).

284

285 **3.3 Oxidative Stress**

286 In the case of the PAMAM dendrimer series, the generation dependent toxic response has
287 origin in a similarly generation dependent oxidative stress response and this response has
288 a complex dependence on the dose and time (Mukherjee and Byrne, 2010). Studies of
289 PAMAM G4-G6, revealed a dose and time dependent biphasic ROS generation process
290 (Mukherjee and Byrne, 2010, Mukherjee et al., 2010, Naha et al, 2010, Mukherjee and
291 Byrne, 2013, Maher et al., 2014). The early phase (2-6hr) increase in ROS levels
292 compared to control was associated with early stage intracellular trafficking in
293 endosomes, whereas the later stage has been associated with localisation of the
294 dendrimers in mitochondria (Xia et al., 2006, Lee et al., 2009, Mukherjee et al., 2010). To
295 better understand the structural dependence of the PPI toxic responses, it is thus
296 important to consider the time and dose dependence of the ROS generation in the HaCaT
297 cell line.

298 The ROS study is consistent with a similar, although less pronounced, biphasic increase
299 of ROS in HaCaT cells for the higher generations, as exemplified for the case of G4 in
300 figure 2. For the case of $1.5\mu\text{M}$ exposure, an initial increase was observed after
301 approximately 5hrs of exposure. This initial increase was seen to subside within
302 approximately 6hrs exposure, after which a further continuous increase was observed, up
303 to the maximum exposure time of 24hrs. Generations G3 and G2 exhibit similar
304 behaviour to G4 (not shown). In contrast, PPI generations G0 and G1 are seen to induce a
305 strongly antioxidant response, reducing the intrinsic ROS levels to as low as $<60\%$ of the
306 control values in the case of PPI G0. The level of quenching of the intrinsic ROS reduces
307 with increasing time, however. The response to G2 and G3 exposure appears to be
308 intermediate between the extremes of oxidative stress for G4 and antioxidant behaviour
309 for G0 (data not shown).

310 In the case of PPI G0 and G1, the longer time increase in ROS levels may be similarly
311 associated with localisation of the dendrimers in the mitochondria, but the absence of the
312 early stage endosomal ROS suggests an alternative uptake mechanism to endocytosis, by
313 which the internalised G0 and G1 dendrimers act as antioxidants.

314

315 **3.4 Confocal Microscopy**

316 The study of the cytotoxic and oxidative stress responses of HaCaT cells to exposure to
317 PPI dendritic nanoparticles indicated a clear similarity to those previously documented
318 for PAMAM dendrimers (Mukherjee et al., 2010, Mukherjee and Byrne 2013). Notably,
319 the biphasic response of the ROS for the higher generations is similar to that observed for
320 PAMAM dendrimer exposure, attributed to a process of endocytosis and early stage ROS
321 production due to the endosomal proton pump mechanism, followed by endosomolysis
322 and subsequent localisation of the dendrimers and ROS production in the mitochondria
323 (Nel et al., 2009, Mukherjee et al., 2010). In order to confirm a similar mechanism as
324 origin for the biphasic ROS response to PPI exposure, an investigation of colocalisation
325 of the early and late stage ROS with the mitochondria was performed. An overlap of
326 Carboxy H₂DCFDA (yellow) and the mitotracker dye (red) producing an orange shade
327 indicates the presence of ROS in the mitochondria.

328 Figure 3 depicts exposure of 20µM PPI G2 at the early maximum ROS of approximately
329 5hrs and late stage of ROS at 24hrs. The dominance of the red fluorescence of the
330 mitotracker dye in (Figure 3a) indicates that no ROS was present in the mitochondria at
331 5hr exposure, whereas a significant ROS production was seen to be located in the
332 mitochondria at 24hrs (Figure 3b). In general, after 24hrs exposure of HaCaT cells, a
333 significant production of ROS was observed in the mitochondria for the case of each PPI
334 dendrimer generation. Figure S10 shows a similar image for 24hr exposure for the
335 example of 2µM of PPI G4.

336 The presence of early endosomes after the treatment of HaCaT cells with PPI dendrimers
337 was examined using the CellLight reagent. Fluorescently labelled 40nm PSNPs were
338 used as a positive control, as previous literature has shown that they are taken into cells
339 by the active uptake of endocytosis (Nel et al., 2009, Sandin et al., 2012, Ekkapongpisit
340 et al. 2012, Monti et al., 2015). Figure S11 confirms the endocytosis of polystyrene
341 nanoparticles after 5hr exposure; the fluorescently labelled nanoparticles are shown in
342 green and the red dye shows the presence of early endosomes. The significant overlap of
343 the two indicated by the orange colour in Figure S11 (d), confirms that the polystyrene
344 nanoparticles are localised in early endosomes.

345 In a similar way, early endosomes are clearly detectable by the red fluorescence of the
346 CellLight dye after 5hr exposure of HaCaT cells to PPI dendrimers, as shown in Figure 4,
347 for the example of 0.3µM PPI G4 dendrimer solution. Exposure of HaCaT cells after 5hrs
348 to PPI G0 and G1 showed no sign of early endosomes, however, as shown in Figure 5 for
349 the example of 1000µM PPI G0, in which no red fluorescent CellLight dye is detectable.
350 A similar examination of early stage exposure of HaCaT cells to PPI G1 dendrimer
351 solutions failed to detect any early stage endosomal activity (not shown), indicating the
352 absence of an active uptake of the smaller PPI dendrimers.

353

354 **4. Discussion**

355 The cytotoxicity of PPI dendrimers was determined by the MTT assay. A generation and
356 dose dependent toxic response was observed, in the order of G0<G1<G2<G3<G4. The
357 cytotoxicity of the related family of PAMAM dendrimers has been extensively studied
358 (Heiden et al., 2007, Lee et al., 2009, Mukherjee and Byrne, 2010, Mukherjee et al.,
359 2010, Naha et al., 2010, Mukherjee and Byrne, 2013, Naha and Byrne, 2013). The
360 responses have been similarly seen to increase with increasing generation and to be
361 correlated with the number of surface amino groups. Figure 6 compares the variation of

362 EC₅₀ of PAMAM G4 to G6 (values reproduced from Mukherjee et al., 2010) and PPI G0
363 to G4, in both cases measured by the MTT assay in the HaCaT cell line after 24hrs
364 exposure. As the degree of toxicity is inversely related to the magnitude of the EC₅₀
365 measured, the results are plotted as inverse (Ragnvaldsson et al. 2007). The EC₅₀ values
366 are listed along with physico-chemical parameters in Table 1.

367 In the case of each dendrimer series, a dependence of the toxic response, as represented
368 by the inverse EC₅₀ measured using the MTT assay at 24hrs, on the number of surface
369 amino groups is clearly seen. It has previously been proposed that the toxic response of
370 dendritic nanoparticles is governed primarily by the number of surface amino groups
371 (Mukherjee et al., 2010, Maher et al. 2014). A clear correlation between the toxic
372 response and the number of surface amino groups is evident for both the PAMAM and
373 PPI series, although the trend for the latter deviates sharply from linearity at the lower
374 generation, G2. The inverse EC₅₀ values of PPI show them to be relatively more toxic
375 than PAMAM. For example, PPI G4, with the same number of surface amino groups as
376 G4 PAMAM, is two to three fold more toxic.

377 Comparative studies between PPI and PAMAM have previously been presented.
378 Janaszewska et al. compared PPI G4 and PAMAM G4 and identified a rapid linear
379 decrease in cell viability in Chinese hamster ovary (CHO) and human ovarian carcinoma
380 cells lines over the concentration range of 0.1-10 μ M for both dendrimers (Janaszewska et
381 al., 2012). The dendrimers displayed an insignificant difference in reduction of cell
382 viability, although PPI G3 has half as many (32) surface amino groups as its PAMAM G4
383 counterpart (64). However, a previous cytotoxicity study by Shao et al. (2011), showed
384 PPI G2 with 16 surface amino groups to be more toxic than PAMAM G4 with 64 surface
385 amino groups, even though PAMAM G4 has 3 times more surface amino groups.

386 In considering differences in cytotoxicity endpoints, however, it is important to consider
387 the mechanisms of response, which in the case of PAMAM dendrimers is reported to be
388 due to oxidative stress after clathrin mediated endocytosis (Kitchens et al. 2008). A
389 biphasic increase in ROS has been observed, the first phase (1-6hrs) associated with the
390 endosomal proton pump mechanism and endosomolysis (Nel et al., 2006, Varkouhi et al.,
391 2011), the second (~16-24hrs) associated with dendrimer localization and ROS
392 production in the mitochondria (Lee et al., 2009, Mukherjee et al. 2010, Naha et al.,
393 2010). A similar biphasic ROS response is observed for exposure to the higher PPI
394 generations, G3 and G4, and the co-localisation of ROS in the mitochondria after 24hours
395 is a strong indication that a similar mechanism governs the oxidative stress and cytotoxic
396 response to PPI dendrimers. However, there is a significant disconnect between the trend
397 shown by the higher PPI generations, G3-G4, and the lower generations (G0-G2), the
398 latter group eliciting substantially lower responses. Notably, up to concentrations of
399 ~100 μ M, cells treated with PPI G1 showed little toxicity and only above ~100 μ M did the
400 viability decrease rapidly. Similarly, PPI G0 elicited little or no toxic response in cells
401 until concentrations above ~1000 μ M.

402 Figure 7 shows the %ROS compared to control measured using the Carboxy-H2DCFDA
403 dye assay at the time point of the early ROS maximum and a dose of 1.0 μ M, for both the
404 PPI and PAMAM dendrimer series (Mukherjee and Byrne 2010, Mukherjee et al., 2010).
405 The trend of ROS matches well that of the toxicity as a function of number of surface
406 amino groups, illustrated in figure 7, except for the lower generations of PPI, G0- G2. In
407 contrast, the trends of the late stage ROS versus number of surface amino groups show

408 little correlation to the toxicity, as shown in Figure S9. The correlation of Figure 8 for the
409 larger generations of PPI and PAMAM dendrimers indicates that, once internalised in the
410 cells, the early stage ROS is the primary source of the cytotoxic response after 24hrs, and
411 that for each dendrimer series, a similar degree of early stage oxidative stress results in
412 similar toxicity.

413 Nevertheless, figure 6 indicates a difference between the two series, the higher generation
414 PPI dendrimers eliciting a higher toxic response than the PAMAM series, and for the PPI
415 dendrimers, the lower generations elicit a lower toxic response. Recent studies have
416 explored the mechanism of PAMAM toxicity to the human keratinocyte, HaCaT, cell line
417 and modelled the observed responses according to a phenomenological rate equation
418 model (Mukherjee and Byrne 2013, Maher et al., 2014). The model was successful in
419 simulating the observed temporal and generation dependent responses, and was seen to
420 be extendable to murine macrophages (J774A.1) and human colon cells (SW480). The
421 simulated uptake of PAMAM dendrimers was seen to be generation (or size) dependent,
422 and translating the model to the PPI series, the observed higher levels of ROS generation
423 and toxic response are consistent with a higher rate of internalisation of the PPI G3 and
424 G4 dendrimers, which are substantially smaller in diameter than their PAMAM
425 counterparts, according to literature values (Crooks et al., 2001). However, within a
426 series, the diameter is only slowly varying as a function of generation. Once internalised,
427 the reactivity resulting in increased levels of ROS, is dependent on the number of surface
428 amino groups per dendrimer.

429 The lower PPI generations appear to show distinctively different response, both in terms
430 of toxicity and oxidative stress. Notably, ROS production by PPI G0-G2 was below that
431 of the control at 4hr exposure. Salvati et al. demonstrated that the uptake of smaller
432 molecules in a passive process, and occurs at rates significantly lower than that of
433 nanoparticles (Salvati et al., 2011). This may indicate that PPI G0-G2, due to their small
434 size, are taken up by an alternative internalization mechanism and avoid encapsulation in
435 endosomes in which early ROS is produced. The clathrin mediated endocytosis pathway
436 is generally accepted as the uptake mechanism for dendrimers (Kitchens et al., 2008).
437 However, it should be noted that in the study of Kitchens et al, for PAMAM G2
438 dendrimers, localisation correlations of only ~50% were observed with clathrin markers
439 after 0-1hr, indicating the co-existence of alternative uptake mechanisms. For example, in
440 their study, Saovapakhiran et al. (2009) concluded that that internalization of PAMAM
441 G3 dendrimers involved both caveolae-dependent endocytosis and macropinocytosis
442 pathways. Furthermore, chlorpromazine, an inhibitor of clathrin assembly-disassembly
443 (Wang et al., 1993) had no effect on the internalization of PAMAM dendrimers in A549
444 cells (Perumal et al., 2008).

445 Confocal microscopy of HaCaT cells exposed to PPI G0 and G4 for 5hrs provides strong
446 evidence that the smaller PPI dendrimers are not taken up by an active endocytotic
447 mechanism, as is the case for the larger PPI and PAMAM counterparts. Similar studies
448 have clearly demonstrated that such an endocytotic pathway is active of the uptake of PPI
449 and PAMAM dendrimers (Kitchens et al. 2008, Perumal et al., 2008), as well as a range
450 of nanoparticles, including PS NPs (Ekkapongpisit et al. 2012, Sandin et al., 2012, Monit
451 et al., 2015), used in this study as a control. In the case of cationic nanoparticles such as
452 PPI and PAMAM dendrimers within the acidifying environment of endosomes, it is
453 reported that the unsaturated surface amino groups sequester protons via the proton pump

454 mechanism, leading to early stage oxidative stress as a primary cause of cytotoxicity (Nel
455 et al., 2009).

456 However, in the absence of endocytotic uptake, molecules that possess positive amino
457 groups are generally regarded as scavengers of ROS. As examples, in a study conducted
458 by Mozdzan et al. (2006), spermine and spermidine both demonstrated the ability to
459 reduce Fe^{3+} to Fe^{2+} , and the ferric reducing activity of these molecules has been identified
460 as a measure of anti-oxidant potential (Lotito and Frei, 2004). Carnosine, an endogenous
461 dipeptide, has been shown to scavenge both reactive oxygen and nitrogen species
462 (Hipkiss, 2009).

463 The transport of molecules and particles into the cell can happen in many ways.
464 Generally it is considered that particles and nanoparticles are taken up by an active
465 endocytotic process which requires energy to be moved across the cell membrane.
466 Salvati et al. (2011) demonstrated, however, that the uptake rate and therefore mechanism
467 of dye molecules by cell is very different than that of nanoparticles, and notably does not
468 require energy activation. Passive uptake by physical diffusion across the semi-permeable
469 cell membrane is considered the mode of transport of molecules which can diffuse past
470 the cellular membrane due to their small size, without the need for an internal energy
471 source.

472 The study of the generation dependent response of the cytological responses to exposure
473 to different generations of the homologous PPI dendrimer series gives a clear indication
474 of the size dependent transition between realms of passive and active uptake of the
475 exogeneous agents, effectively the transition between molecule and particle. Notably,
476 although the basic chemical characteristics of the dendrimer generations are consistent
477 and continuously varied, an abrupt transition from anti-oxidant (G0-G1) to oxidant action
478 (>G2) is observed. G2 appears to be intermediate, as although early stage endosomes are
479 observable (Figure 3), the substantially reduced toxicity elicited suggests that this is not
480 the dominant uptake mechanism.

481 Elucidation of the critical importance of the endocytotic process for the cellular response
482 to nanoparticle exposure, at least in the case of cationic nanoparticles, has implications
483 for the development of predictive models and quantitative structure property relationships
484 (QSARS) for nanoparticles, and even strategic approaches for nanomedical applications.
485 There is a wealth of resources from the pharmaceutical field in terms of such models and
486 QSARS which could potentially guide strategies for nanotoxicology and nanomedicine,
487 many of which are available as open source tools (see for example
488 <http://www.opentox.org/>). However, the study of the homologous PPI series indicates
489 that, in considering the physico-chemical properties of the exposure species, the
490 thresholds governing the uptake mechanism of the cell must be considered. The current
491 study is a clear illustration that the initial, early phase oxidative stress observed for
492 cationic nanoparticles is a result of the mechanism of processing the uptaken
493 nanoparticles in endosomes, which struggle to digest them, causing extreme oxidative
494 stress which can lead to apoptosis (Heiden et al., 2007). In some cases, the burden on
495 endosomes can be extreme enough to cause endosomolysis (Kukowska-Latallo et al.,
496 1996, Zhou et al. 2006, Guillot-Nieckowski et al., 2007) which itself can be disruptive to
497 the cell (Mukherjee 2012). This strongly suggests that, in the case of cationic
498 nanoparticles for intracellular nanomedical applications, avoiding the process of
499 endocytosis may be a valid strategy to pursue (Guarnieri et al. 2014).

500 **5 Conclusion**

501 PPI dendrimers have been shown to elicit a systematic structurally dependent toxic
502 response in human cells *in vitro*. The toxicity is dose and generation dependent, and the
503 mechanism of response is consistent with that previously observed for PAMAM
504 dendrimers of initial endocytosis, giving rise to early stage oxidative stress, release into
505 the cytosol and a subsequent later stage of oxidative stress associated with the
506 mitochondria. Of importance for potential biomedical applications, the similarity of the
507 bi-phasic responses also indicates that, after initial encapsulation in endosomes, the
508 dendrimer species, and any active cargo, become bioavailable after the first few hours.
509 The observed correlation of the toxic responses for the higher generation dendrimers
510 points towards the basis of structure activity relationships, but the differences between
511 the responses of the PPI and PAMAM dendrimer series are indicative of a size dependent
512 uptake mechanism, which should be considered in a generalised model.
513 The anomalous behaviour observed for the smaller PPI dendrimer generations indicates
514 that alternative uptake pathways maybe accessible for the smaller species. Nanoparticles
515 and in particular dendrimers are generally considered to cross cellular membranes by
516 active transport mechanisms, particularly endocytosis, whereas smaller molecules can
517 enter the cell by passive mechanisms. Identifying the threshold between the active uptake
518 of the larger PPI generations G3 – G4, and the passive uptake of the smaller G0 and G1
519 dendrimers provides further insight into the mechanisms of nanotoxic responses and
520 potential design strategies for nanomedicine. Although cationic nanoparticles are
521 considered to be agents of oxidative stress, in this study it is demonstrated that the lower
522 generations intrinsically act as anti-oxidants and the oxidative stress elicited by the higher
523 generations is due to the endosomal and lysosomal pathway. The study suggests that
524 strategies which can bypass the intrinsic cellular uptake processes may be appropriate for
525 intracellular drug delivery. The study of the well-defined homologous PPI dendrimer
526 series also indicates that, in considering the development of QSARS, thresholds of
527 cellular uptake mechanisms must also be taken into consideration and that existing
528 molecular based toxicity databases and predictive models may not be easily extendible to
529 nanoparticle equivalents.

530

531 **Acknowledgement:**

532 This research was supported by the Integrated NanoScience Platform, Ireland (INSPIRE),
533 funded under the Higher Education Authority PRTL (Programme for Research in Third
534 Level Institutions) Cycles 4 and 5, co-funded by the Irish Government and the European
535 Union Structural fund.

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709

710 **Figure Captions**

711

712 Figure 1. Dose dependent cytotoxicity, as determined using the MTT assay after 24hrs
713 exposure of HaCaT cells to PPI G0, G1, G2, G3 and G4 dendrimers. Error bars indicate
714 the standard deviation of three individual measurements, each containing six replicates.
715 The solid lines are a fit to the experimental data, as described in Section 2.6.

716 Figure 2. ROS production compared to control in HaCaT cells treated with PPI
717 dendrimers G0, G1 and G4 over a 24hr period at EC_{50} concentrations determined by the
718 MTT assay.

719 Figure 3: Confocal image of HaCaT cells exposed to PPI dendrimers in the presence of
720 Carboxy H₂DCFDA dye (yellow) and mitotracker dye (red). (a) PPI G2 20 μ M after 5hrs
721 exposure, (b) PPI G2 20 μ M after 24hrs exposure. The dominance of the red mitotracker
722 dye indicates the absence of ROS in the mitochondria is (a) after 5hrs, whereas the
723 orange colour in (b) indicates colocalisation of ROS in the mitochondria after 24hrs.

724 Figure 4: Confocal image of HaCaT cells after 5hr exposure to 0.3 μ M PPI G4 in the
725 presence of CellLight dye (red). (a) CellLight fluorescence indicating the presence of
726 early endosomes, (b) phase contrast image. (c) overlap of early endosome fluorescence
727 and phase contrast image.

728 Figure 5: Confocal image of HaCaT cells exposed to 1000 μ M PPI G0 in the presence of
729 CellLight reagent after 5hrs exposure. (a) absence of CellLight fluorescence indicating
730 the absence of early endosomes, (b) phase contrast image. (c) overlap of early endosome
731 fluorescence and phase contrast image.

732 Figure 6. Comparison of EC_{50} values of PPI G0 – G4 dendrimers with those of PAMAM
733 G4- G6, as measured by the MTT assay in the HaCaT cell line after 24hrs exposure.
734 Error bars indicate the standard deviation of three individual measurements, each
735 containing six replicates. Solid lines are a guide to the eye.

736 Figure 7. Comparison of early maximum ROS in relation to surface amino groups of PPI
737 G0 – G4 and PAMAM G4 – G6. Error bars are the standard deviation of three
738 independent experiments, each including six replicates of each treatment concentration.
739 The solid line is a guide to the eye.

740 Figure 8. Early stage % ROS production vs Inverse EC_{50} (24hrs) of PPI and PAMAM in
741 HaCaT cells at EC_{50} values of PPI and PAMAM determined by the MTT assay. Error
742 bars (ROS) are the standard deviation of three independent experiments, each including
743 six replicates of each treatment concentration. The solid line is a guide to the eye.

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746 **Figure 1:**

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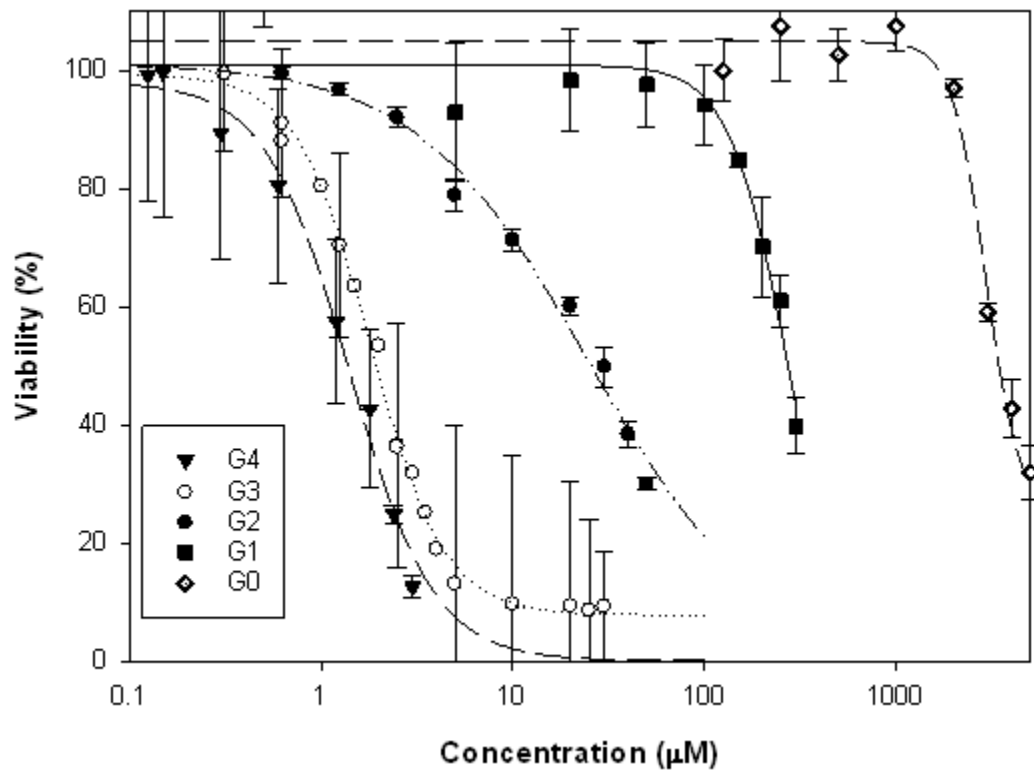
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778 **Figure 2:**

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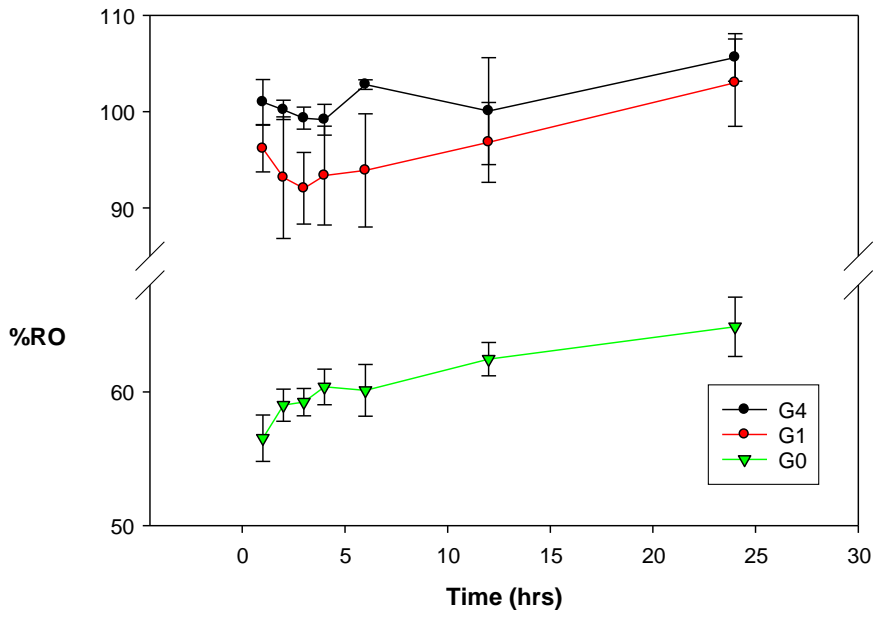
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791 **Figure 3:**

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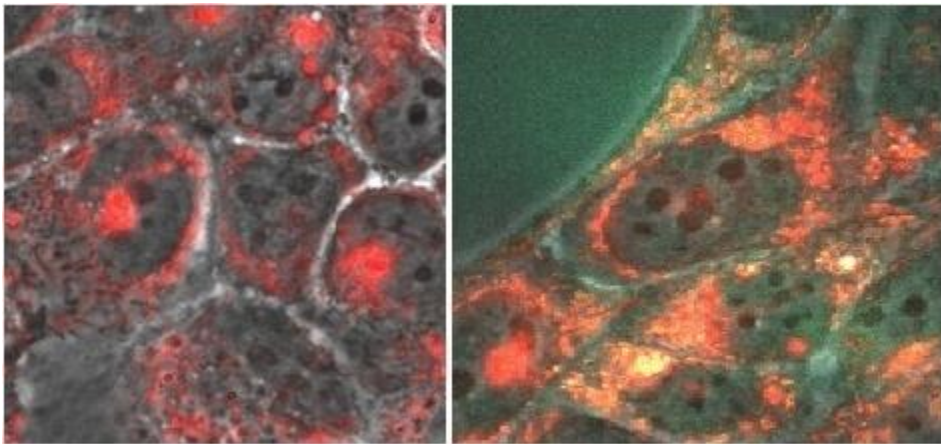
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799

(a)

(b)



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801

802

803

804 **Figure 4:**

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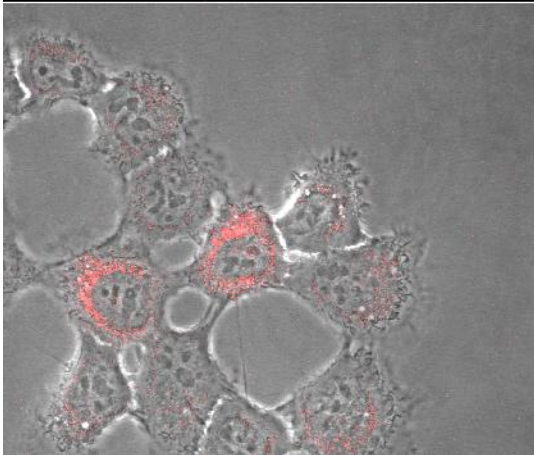
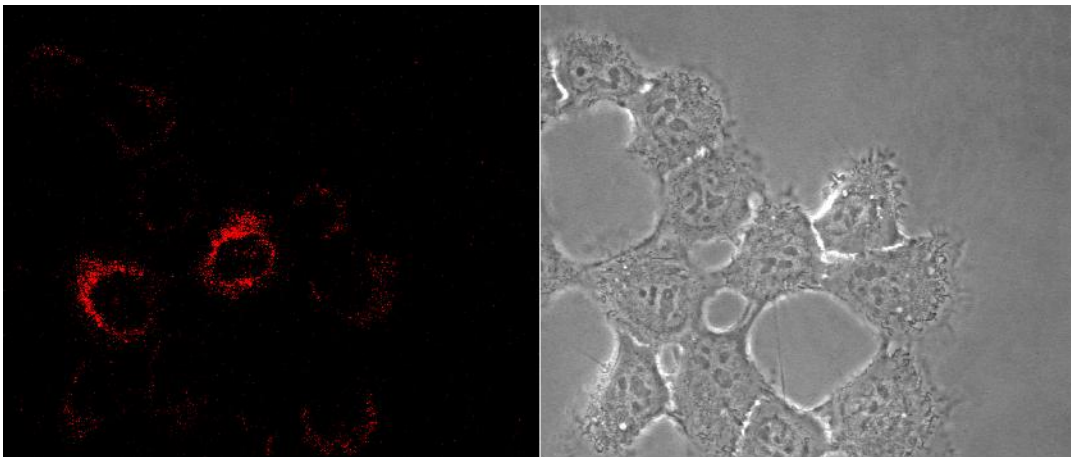
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810 (a)

(b)



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812 (c)

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815

816 **Figure 5:**

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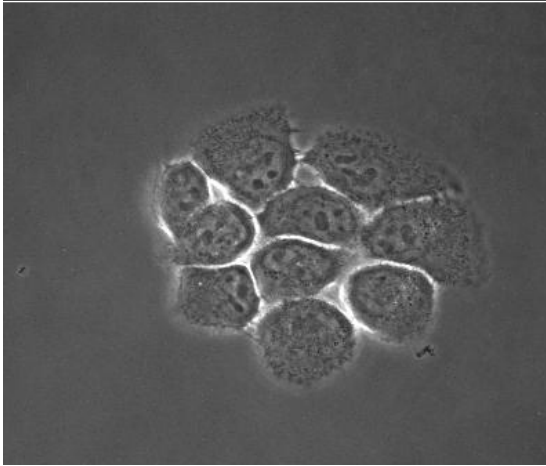
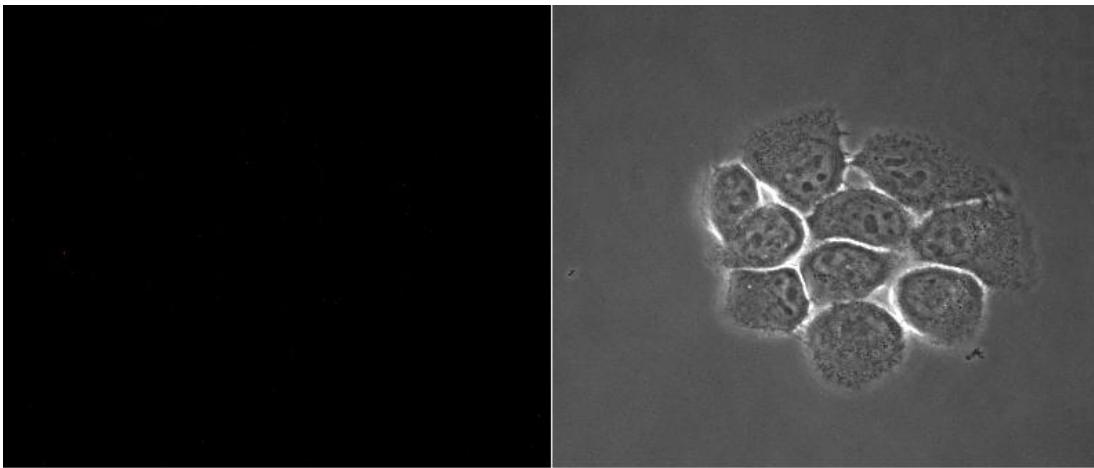
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821 (a)

(b)



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823 (c)

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825

826 **Figure 6:**

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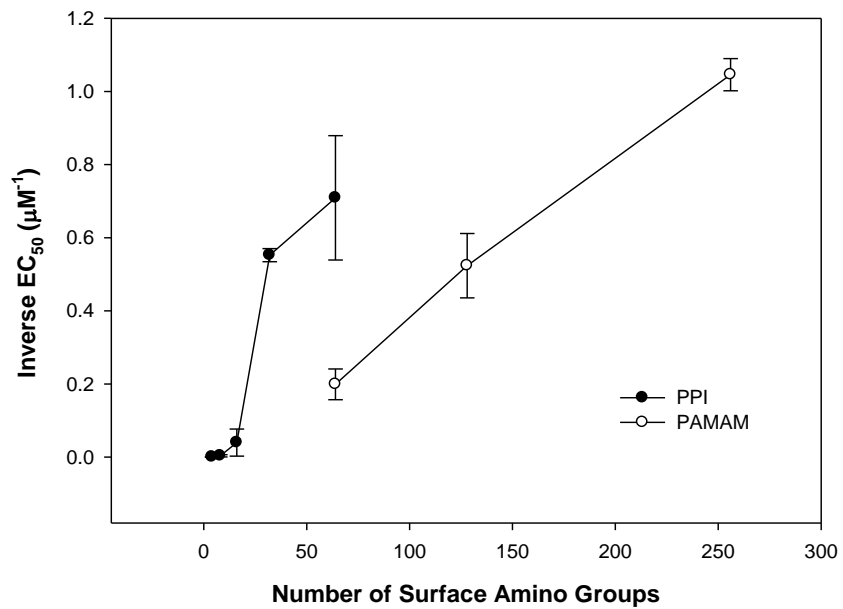
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841 **Figure 7:**

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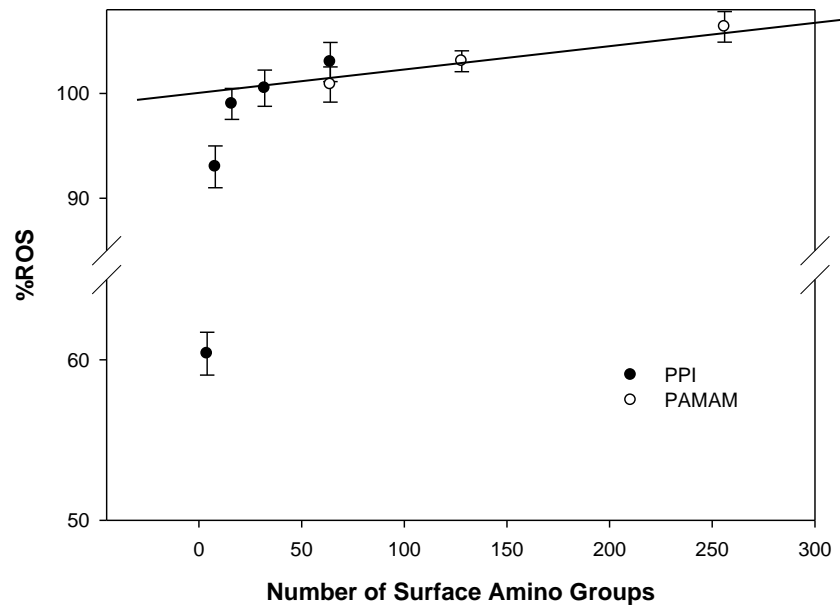
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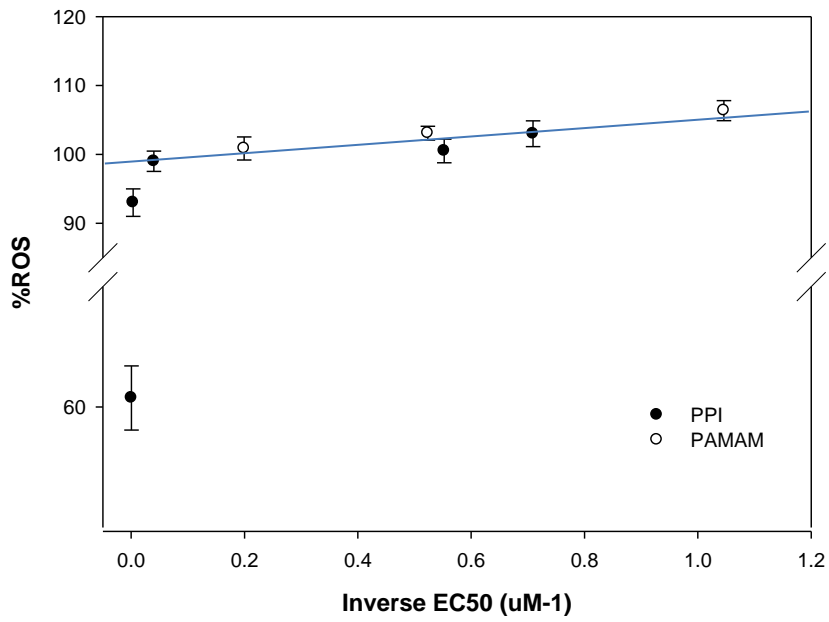
851 **Figure 8:**

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	Molecular Weight (grams per mole)	Number of Surface Amino Groups	Particle size (Crooks et al. 2001) (nm)	Particle size (AFM) (nm)	EC₅₀(μM)
PPI – G4	7,168	64	2.8	2.5-3.5	1.41 \pm 0.3
PPI – G3	3,514	32	2.4	1.8-2.6	1.81 \pm 0.3
PPI – G2	1,687	16	1.9 [2.4]	1.7-1.9	24.8 \pm 3.75
PPI – G1	773.3	8	[1.8]	2 – 8*	271.04 \pm 33.5
PPI – G0	316.5	4	[1.23]	4 – 10*	2939.72 \pm 191
PAMAM – G6	58,408	256	6.7	4.2-5.8	1.02 \pm 0.3
PAMAM – G5	28,826	128	5.4	4.1-4.6	1.91 \pm 0.28
PAMAM – G4	14,215	64	4.5	2.1-2.8	5.02 \pm 0.29

863

864 Table 1. Comparison of the structural characteristics and cytotoxic responses of PPI and

865 PAMAM dendrimers. [] indicates size as determined by the Hyperchem geometrical

866 optimised model (See supplemental Material). * indicates that particle aggregation was

867 observed

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