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Modification of the In Vitro Uptake Mechanism and Anti-Oxidant Levels in HaCaT Cells and Resultant Changes to Toxicity and Oxidative Stress of G4 and G6 Poly (Amido Amine) Dendrimer Nanoparticles

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1 Modification of the *in vitro* uptake mechanism and anti-oxidant levels in
2 HaCaT cells and resultant changes to toxicity and oxidative stress of G4
3 and G6 Poly (amido amine) dendrimer nanoparticles.

4
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11
12
13 **Keywords:**

14 Dendrimer nanoparticles

15 PAMAM nanoparticles

16 Endocytosis

17 Passive diffusion

18 Oxidative stress

19 Membrane permeability

20 Abstract:

21 The mechanism of cellular uptake by endocytosis and subsequent oxidative stress has been
22 identified as the paradigm for the toxic response of cationically surface charged nanoparticles. In
23 an attempt to circumvent the process, the effect of increased cellular membrane permeability on
24 the uptake mechanisms of poly (amidoamine) dendrimers generation 4 (G4) and 6 (G6) *in vitro*
25 was investigated. Immortalised, non-cancerous human keratinocyte (HaCaT) cells were treated
26 with DL-Buthionine-(S,R)-sulfoximine (BSO). Active uptake of the particles was monitored
27 using fluorescence microscopy to identify and quantify endosomal activity and resultant
28 oxidative stress, manifest as increased levels of reactive oxygen species, monitored using the
29 carboxy-H₂DCFDA dye. Dose dependent cytotoxicity for G4 and G6 exposure was registered
30 using the cytotoxicity assays Alamar Blue and MTT, from 6 to 72 hours.

31 Reduced uptake by endocytosis is observed for both dendrimer species. A dramatic change,
32 compared to untreated cells, is observed in the cytotoxic and oxidative stress response of the
33 BSO treated cells. The significantly increased mitochondrial activity, dose dependent anti-
34 oxidant behaviour and reduced degree of endocytosis for both dendrimer generations, in BSO
35 treated cells, indicates enhanced permeability of the cell membrane, resulting in the passive,
36 diffusive uptake of dendrimers, replacing endocytosis as the primary uptake mechanism. The
37 complex MTT response reflects the importance of glutathione in maintaining redox balance
38 within the mitochondria. The study highlights the importance of regulation of this redox balance
39 for cell metabolism, but also points to the potential of controlling the nanoparticle uptake
40 mechanisms, and resultant cytotoxicity, with implications for nanomedicine.

41

42 Introduction

43 Nanoparticle science is a rapidly advancing field which holds much promise in areas such as
44 targeted drug and gene delivery.^{1,2,3} However, nanoparticle uptake into a cell has been
45 demonstrated, particularly for nanoparticles with an effective cationic surface charge, to give rise
46 to cytotoxic responses, raising concerns about the potential health and environmental impact of
47 the proliferation of nanomaterials in consumer products.⁴ A systematic understanding of the
48 mechanisms of toxicity and their dependence on nanoparticle physico-chemical properties on a
49 cellular level is therefore required.⁵ In the context of nanomedical applications, understanding
50 and controlling the uptake process and subcellular trafficking of the delivery vehicle and the
51 bioavailability of the cargo are critically important.

52
53 Cellular uptake of nanoparticles principally occurs via endocytosis, whereby the nanoparticle is
54 invaginated by the cellular membrane and is transported into the cell.⁶ As the low pH
55 environment of the endosome attempts to digest the nanoparticle, the redox balance of the cell is
56 disrupted, and, in the case of nanoparticles with an effective cationic surface charge, the process
57 gives rise to an increase in the production of Reactive Oxygen Species (ROS), localised mainly
58 around the endosome (or later lysosome).^{6,7,8,9} Although intra cellular anti-oxidants attempt to
59 neutralise the imbalance, ROS production can be sufficient to lead to oxidative stress.¹⁰
60 Subsequently, a cascade of events and the release of several characteristic cytokines and
61 chemokines occurs, ultimately leading to cell death, a process which is the accepted paradigm of
62 nanotoxicity of many nanoparticles *in vitro*.⁶ The process has been well demonstrated for model
63 nanoparticle system such as amine functionalised polystyrene,^{11,12,13} amorphous nanosilica,¹⁴ and
64 nanomeric polymeric dendrimers.¹⁰

65
66 Aminated molecules are intrinsic antioxidants, however, and generally regarded as ROS
67 scavengers. As examples, spermine and spermidine have both been shown to reduce Fe^{3+} to Fe^{2+}
68 ¹⁵ and the ferric reducing activity of these molecules has been identified as a measure of anti-
69 oxidant potential.¹⁶ Carnosine, an endogenous dipeptide, has been shown to scavenge both
70 reactive oxygen and nitrogen species.¹⁷ Notably, in a study by Khalid *et al.* (2015), while the
71 larger, higher generations of the aminated nanoscale dendrimers Poly (propylene imine) (PPI)
72 were demonstrated to elicit oxidative stress and significant toxicity, the smaller, lower
73 generations exhibited intracellular antioxidant behaviour and low toxicity.¹⁸ Examination of the
74 uptake mechanisms indicated a transition from cellular uptake by passive diffusion at low
75 generations to active endocytosis for higher generations. The study suggests that, in the case of
76 catatonically charge nanoparticles, the endocytotic uptake and trafficking process is, in itself, a
77 source of cellular toxicity. In the context of drug delivery, invagination of the delivery vehicle
78 and cargo in this harsh environment may also be undesirable, and although escape by
79 endosomolysis is a potential strategy,¹⁹ this too can be a harsh process, causing significant
80 damage to the cell.^{20,21} For these reasons, circumventing endocytosis appears to be a valid
81 strategy for the reduction of toxicity associated with aminated nanoparticles as well as
82 nanoparticle drug or gene delivery.
83
84 Guarnieri *et al.* (2015), demonstrated that functionalization of aminated polystyrene
85 nanoparticles with the viral peptide gH625 (derived from *Herpes simplex virus – 1*), which has a
86 membrane perturbing domain, enables translocation of particles to the cytoplasm, avoiding
87 endocytosis and thus, dramatically reducing the cytotoxicity.²² An alternative strategy to increase

88 the permeability of the cellular membrane, *in vitro*, is through the application of DL-Buthionine-
89 (*S,R*)-sulfoximine (BSO). BSO has previously been employed to study the effects of the
90 reduction of levels of the intracellular antioxidant glutathione (GSH); and therefore oxidative
91 stress^{23,24,25,26} and has been shown to cause membrane permeabilisation.^{27,28} BSO works as an
92 inhibitor of the enzyme Glutamate Cysteine Ligase (EC 6.3.2.2) (historically known as gamma-
93 glutamylcysteine synthetase) which is the first step in the production of GSH in the cell.²⁹ This
94 reduction in GSH causes several different effects, one being the induction of the membrane
95 permeability transition in the mitochondria.^{30,31,32} The opening of this pore and the depletion of
96 GSH allows ROS to diffuse from the mitochondria to the cell and via lipid peroxidation, cause
97 damage to the cell membrane,²⁸ leading to increased permeability.

98
99 The reduction of GSH in the cell can have other adverse effects, however, mainly due to the fact
100 that GSH is one of the main antioxidants involved in maintaining the redox balance of the
101 mitochondria.³³ In the mitochondria, endogenous ROS is produced as a by-product of normal
102 metabolism and therefore completely eliminating GSH can leave the cell susceptible to damage
103 from this ROS.³⁴ Furthermore, reductions in cellular GSH can also lead to changes in the
104 regulation of Ca²⁺ distribution^{35,36,37} and the activation/deactivation of signalling pathways
105 involved with growth, differentiation and apoptosis.^{33,38,39,40} The loss of GSH and subsequent
106 changes in the cell have been implicated in several disease states which is reviewed elsewhere.³⁴

107
108 This study explores the impact, for HaCaT cell, of BSO treatment on the cellular uptake of, and
109 subsequent oxidative stress and toxic response to, poly (amido amine) (PAMAM) dendrimers.
110 These nanoscale aminated dendrimers have a systematically variable molecular structure, and the

111 homologous series of increasing generation, and concomitant size and number of surface amino
112 groups, has been demonstrated to be ideal to study the dependence of nanoparticle cellular
113 interactions on the physico-chemical properties of the particles. Previous studies have examined
114 the structurally dependent toxicity, and underlying mechanisms of endocytosis, oxidative stress,
115 immune responses and consequent toxicity^{7,8,9,10} and the responses have been numerically
116 modelled, as a guide towards a predictive toxicology approach.^{10,41} For consistency and to allow
117 comparisons with the previous work, the *in vitro* studies reported here were also carried out
118 using the immortalised human keratinocyte cell line, HaCaT, and identical oxidative stress and
119 cytotoxic assays. Furthermore, PAMAM dendrimers of Generation 4 and 6 were chosen as the
120 extremes of the previously reported cytotoxicological response. In comparison, it is
121 demonstrated that treatment with BSO results in a significant change in the nanoparticle uptake
122 mechanisms and cytotoxicity.

123 Materials and Methods

124 Materials

125 DMEM F12 HAM growth medium, Penicillin, Streptomycin, fluorescently labelled, Polystyrene
126 nanoparticles, with amine surface modification (PSNP-NH₂) 100nm, DL-Buthionine-(*S,R*)-
127 sulfoximine(BSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye
128 were purchased from Sigma-Aldrich, Ireland. The PAMAM dendrimer nanoparticles,
129 generations 4 (molecular weight: 14,214 g/mol) and 6 (molecular weight: 58,046 g/mol), were
130 purchased from Sigma-Aldrich and manufactured by Dendritech Inc. ThiolTracker™ Violet
131 (TTV), CellLight® Early Endosomes-RFP, BacMam 2.0, Fetal Bovine Serum (FBS), L-
132 Glutamine, Alamar Blue (AB) and 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate
133 (carboxy-H₂DCFDA) dye were purchased from Life Technologies™, Bio-Sciences, Ireland.
134 HaCaT cells were purchased from Cell Line Services (CLS), Eppelheim, Germany. TrueLine 96-
135 well cell culture plates were used for all viability and ROS studies.

136 All fluorescence and absorbance readings were taken on a Molecular Devices SpectraMax M3
137 Spectrometer. Confocal Laser Scanning Fluorescence Microscopy (CLSM) images were taken
138 on a Zeiss LSM 510 Confocal Laser Scanning Microscope and processed using ImageJ software
139 (with co-localisation analysis performed with the JaCoP plugin for ImageJ). All viability, ROS
140 and GSH data analysis was performed using SigmaPlot v10.0 software.

141

142 Methods

143 Cell culture

144 HaCaT cells are an immortalised, non cancerous human keratinocyte cell line and were used for

145 these experiments. The cells were cultured in DMEM F12 HAM supplemented with 10% FBS,
146 45 IU/mL penicillin, 45 IU/mL streptomycin and 2mM L-glutamine at 37°C in 5% CO₂.
147 All assays carried out in this set of experiments were performed in 96 well plates, with cells
148 plated at a concentration of 1x10⁴ cells/well in 100µL of DMEM medium. Cells were allowed 24
149 hours to attach and were then treated with BSO for an additional 18 hours (at a concentration of
150 200µM), after which cells were exposed to PAMAM G4 or G6 dendrimers (in DMEM F12
151 HAM, with 5%FBS, 45 IU/mL penicillin, 45 IU/mL streptomycin, 2mM L-glutamine and
152 200µM BSO) at various concentrations for the specified time points. Six replicates of each
153 concentration were performed per plate and each plate was repeated in triplicate.

154

155 ThiolTracker™ Violet (TTV)

156 ThiolTracker™ Violet (TTV) is a GSH detection agent. Cells were plated as described above and
157 a concentration gradient of BSO was applied. Cells were left for 18 hours at 37°C in 5% CO₂ to
158 allow for reduction of the amount of intracellular GSH. Cells were then washed twice with PBS,
159 100µL of TTV dye (at a final concentration of 20µM) were added to each well and the plates
160 were allowed to incubate at 37°C in 5% CO₂ for 30 minutes, after which the TTV solution was
161 removed and replaced with PBS. The fluorescence of each well was then read using the
162 SpectraMax M3 spectrometer with λ_{EX} = 404nm and λ_{EM} = 526nm. GSH values were calculated
163 as compared to the unexposed control.

164

165 Viability assays

166 Alamar Blue and MTT assays were used to determine the changes in cell viability, after
167 treatment with BSO as described above, as a result of exposure to both PAMAM G4 and G6

168 dendrimers. Both Alamar Blue and MTT were performed on the same plate. The PAMAM G4
169 concentrations used were: 0.16, 0.32, 0.65, 1.3, 2.6, 5.2, 7.8 and 10.4 μ M, while the PAMAM G6
170 concentrations were: 0.08, 0.16, 0.32, 0.65, 1.3, 2.6, 3.9 and 5.2 μ M. The lower initial value of
171 the PAMAM G6 dendrimers was used due to their reported EC₅₀ value being much lower than
172 their G4 counterparts.^{7,8,9} Dose dependent viability percentages were calculated at time points: 6,
173 12, 24, 48 and 72 hours. Percentage viability was calculated as compared to a control which had
174 been exposed to 200 μ M BSO, but had no nanoparticle treatment; this was to ensure any changes
175 were caused by the nanoparticle and were not the result of BSO treatment. A separate control
176 where no BSO was present was also performed and showed there was little difference between
177 cells with no BSO exposure and cells which were exposed to BSO (Supplementary Material,
178 Figure: S1).

179

180 Alamar Blue (AB)

181 The Alamar Blue assay was made up from 10X stock solution in medium (DMEM F12 HAM,
182 with no additional supplements). At the specified time point, the plates were removed from the
183 incubator and the medium containing PAMAM dendrimer was removed, the cells were washed
184 with 100 μ L PBS and then 100 μ L of DMEM F12 HAM(unsupplemented) containing Alamar
185 Blue were added to each well. The plates were incubated for 3 hours at 37°C in 5% CO₂ to allow
186 for conversion of the dye. The fluorescence of each well was then read using the SpectraMax M3
187 spectrometer with λ_{EX} = 555nm and λ_{EM} = 585nm.

188

189 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

190 A stock solution of MTT was made at a concentration of 0.5mg/mL. 500 μ L of this stock were
191 added for every 10mL of medium (DMEM F12 HAM, with no additional supplements). At the
192 specified time point, the plates were removed from the incubator and the medium containing
193 PAMAM dendrimer was discarded, the cells were washed with 100 μ L PBS and then 100 μ L of
194 DMEM (unsupplemented) containing MTT was added to each well. The plates were incubated
195 for 3 hours at 37°C in 5% CO₂ to allow for conversion of the dye. After 3 hours, any remaining
196 dye was removed and each well was again washed with 100 μ L PBS, after which 100 μ L of
197 DMSO was added and the plates were placed on a shaker for 10 minutes to allow for the dye to
198 solubilise. The absorbance of each well was then read using the SpectraMax M3 spectrometer
199 with $\lambda_{\text{ABS}}=595\text{nm}$.

200

201 Reactive Oxygen Species (ROS)

202 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) dye was used
203 for the detection of ROS. The dye was made up to a final concentration of 10 μ M in sterile PBS.
204 Before addition of PAMAM dendrimer, this dye was added to the cells and allowed to incubate
205 for 1 hour, after which, the dye was removed, the cells were washed thrice with PBS and the
206 medium containing PAMAM dendrimer was added. At the specified time points the fluorescence
207 was read by the SpectraMax M3 spectrometer with $\lambda_{\text{EX}}=488\text{nm}$ and $\lambda_{\text{EM}}=535\text{nm}$.

208

209 Confocal Laser Scanning Microscopy (CLSM)

210 Cells were plated onto MatTek 35mm glass bottom dishes at a concentration of 20,000 cells/dish
211 in DMEM F12 HAM (supplemented with 10% FBS, 45 IU/mL penicillin, 45 IU/mL
212 streptomycin and 2mM L-glutamine) and allowed to attach for 4 hours, at which point the

213 medium was removed and replaced with medium containing Celllight[®] Early Endosomes-RFP,
214 BacMam 2.0 at a concentration of 20 particles per cell. Early endosome formation was tracked
215 with the Celllight[®] Early Endosome – RFP kit, which transfects, into the cell, a version of Rab5a
216 with a bound Red Fluorescent Protein. The cells were allowed to incubate (37°C, 5% CO₂) for 16
217 hours to ensure transfection with the early endosome reagent. After this, the medium was
218 removed and cells were washed twice with PBS. For cells being tested without BSO, medium
219 was added for 18 hours (these samples are referred to as untreated cells/untreated controls in the
220 text), while for cells being tested with BSO, medium containing 200µM BSO was added for 18
221 hours. Cells were again washed with PBS and carboxy-H₂DCFDA was added for 1 hour (10µM
222 in 2mL PBS), after which cells were again washed twice with PBS. PAMAM dendrimers were
223 added at a concentration of 3.21µM (G4) and 1µM (G6) and cells were allowed to incubate for 3
224 hours (G4) or 1 hour (G6) and were then washed twice with PBS and imaged with the Zeiss
225 LSM 510 Confocal Laser Scanning Microscope. 100nm PSNP-NH₂ with attached Green
226 Florescent Protein was used as a positive control to ensure the Celllight[®] Early Endosomes-RFP
227 was functioning as expected; the results of this test are available in the supplementary material
228 section, Figure S2. Negative controls were also performed with cells which were not exposed to
229 any nanoparticles, and little to no fluorescence was noted (data not shown). For ROS monitoring,
230 doses and time points noted above were chosen based on the maximum responses previously
231 reported in literature.⁸ All confocal images were analysed using ImageJ and co-localisation
232 studies were performed using Manders split coefficients calculated with the JaCoP plugin for
233 ImageJ.⁴²

234

235 Data Analysis and Statistics

236 Data analysis was performed using SigmaPlot™ v10.0 and fluorescence was calculated based on
237 the values of BSO controls (which were unexposed to nanoparticles, but had been treated with
238 200µM BSO for 18 hours).

239 “The cytotoxicity, GSH and ROS experiments were performed in 96-well microplates with six
240 replicates per plate and each plate repeated three times. Therefore, data points shown represent
241 the mean of 18 points, with error bars representing \pm the standard deviation (as calculated by
242 SigmaPlot™ v10.0). Confocal Images were taken on a Zeiss LSM 510 and processed using
243 ImageJ software. Images were taken of eight cells/groups of cells and the images presented in the
244 manuscript are representative of the sampled cell population.”

245

246 Results

247 BSO treatment

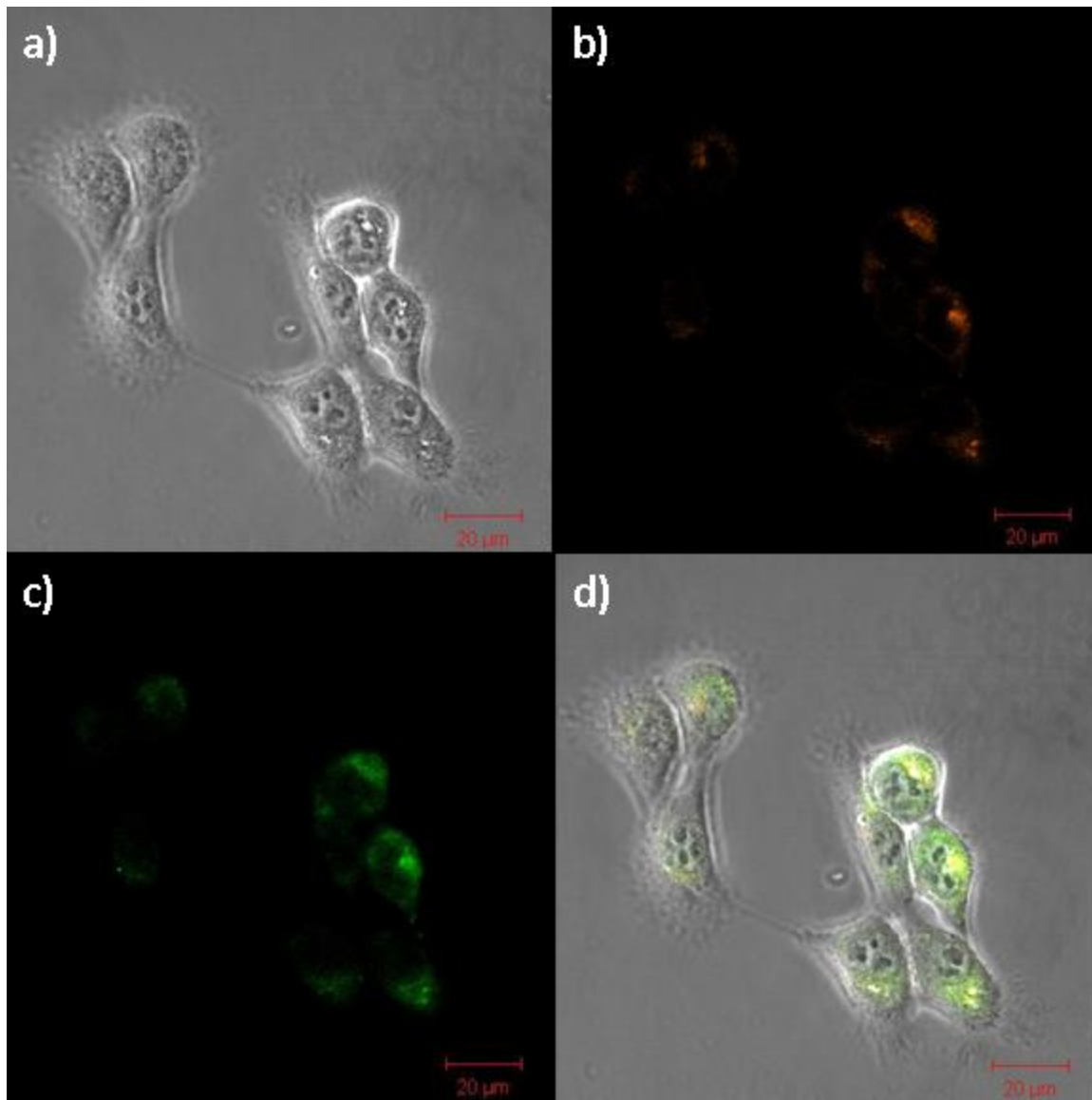
248 While maximising the desired effect of the BSO on the cells, it is important that the viability of
249 the cells is not affected. TTV showed a 40% reduction in intracellular levels of GSH for the
250 HaCaT cells upon 200µM BSO exposure for 18 hours (adapted from the methods of: He *et al.*
251 (2003)).²⁴ This dose and time point were found to have a minimal impact on cellular viability as
252 measured by the AB and MTT assays (data available in Supplementary Material, Figure: S3).
253 Higher concentrations were found to have an effect on cellular viability, although a more
254 pronounced effect was observed by CLSM, where signs of cellular stress were noted (data not
255 shown). Therefore 200µM BSO exposure was chosen as optimal.

256

257 PAMAM G4 dendrimers

258 CLSM was employed as a way to examine the effect of BSO treatment on the cellular uptake
259 mechanisms and subsequent oxidative stress. Early endosome formation was tracked with the
260 Celllight[®] Early Endosome – RFP kit and the formation of ROS was also tracked using the
261 carboxy-H₂DCFDA dye. Fluorescently labelled PSNP-NH₂ of 100nm diameter were employed
262 as positive controls, and the results for those are shown in the Supplementary Material: Figure
263 S2.

264

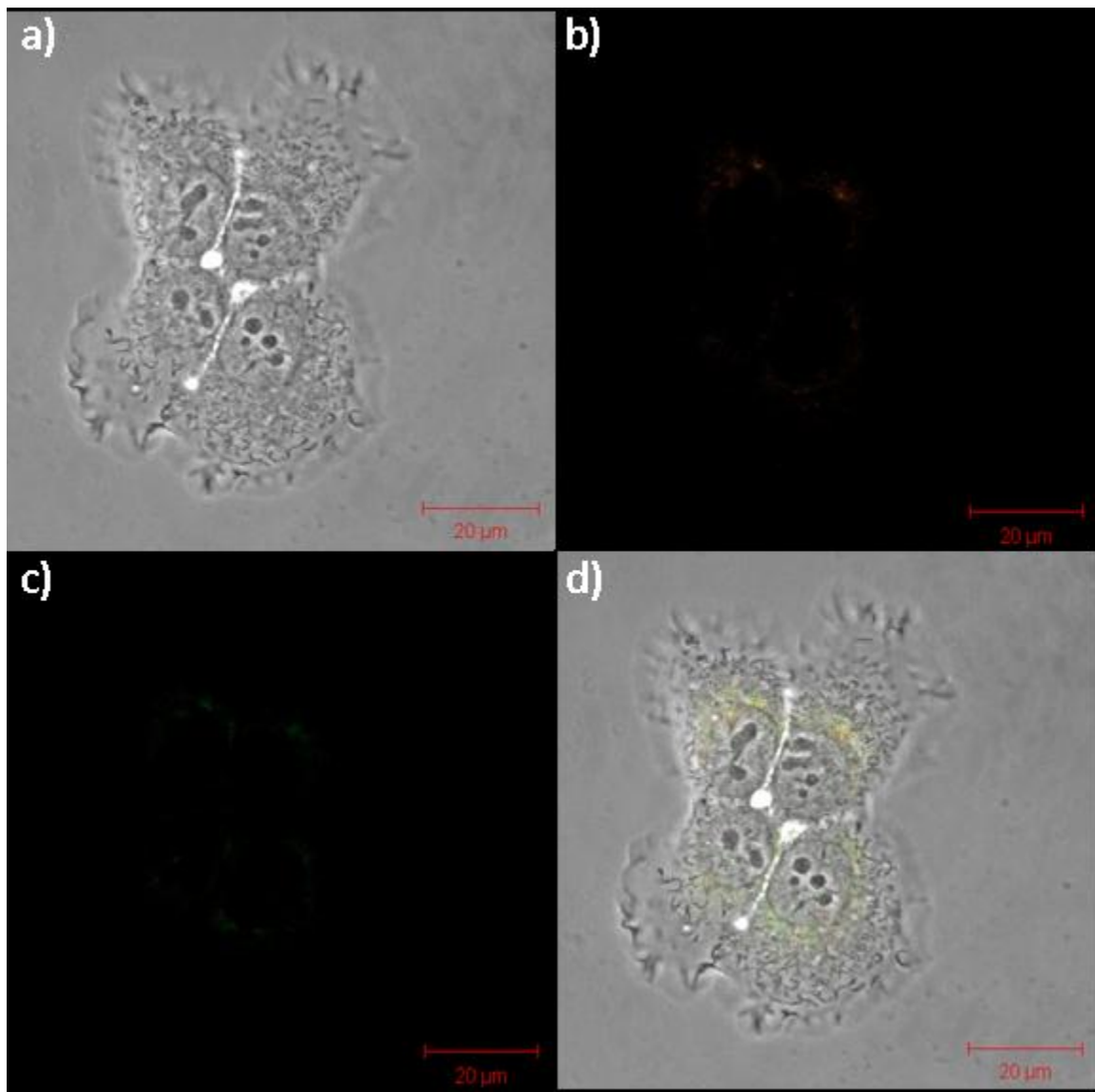


265

266 Figure 1: CLSM images of HaCaT (live) cells, upon exposure to 3.21 μM PAMAM G4
267 dendrimer at 3 hours (no BSO is present in this sample). Image a) shows the bright field image
268 of the cells, Image b) shows the fluorescence generated by the early endosomal red fluorescent
269 protein, Image c) shows the fluorescence generated by the ROS (interacting with the carboxy-
270 H₂DCFDA dye) and Image d) shows the overlay of images a-c, where yellow coloured areas
271 indicate simultaneous endosomal and ROS activity.

272

273 Figure 1 shows the HaCaT cells following the reported paradigm of PAMAM G4 endocytosis
274 and subsequent ROS generation at the endosomal sites.^{6,7,8,9} Co-localisation (performed on the
275 images in Figure 1) shows that 91(\pm 3)% of the generated ROS occurs in the neighbourhood of
276 endosomes, and that 71(\pm 4)% of endosomal formation resulted in increases in ROS production
277 (the other 30% of endosomal activity is most likely due to endocytosis which would be routinely
278 done by the cell and would not involve the dendrimers and therefore not produce ROS).
279



280

281 Figure 2: Confocal images of HaCaT (live) cells, upon exposure to 3.21 μ M PAMAM G4
282 dendrimer at 3 hours, with pre-treatment of BSO: 200 μ M for 18hours. Image a) shows the bright
283 field image of the cells, Image b) shows the fluorescence generated by the early endosomal red
284 fluorescent protein, Image c) shows the fluorescence generated by the ROS (interacting with the
285 carboxy-H₂DCFDA dye) and Image d) shows the overlay of images a-c, where yellow coloured
286 areas indicate simultaneous endosomal and ROS activity.

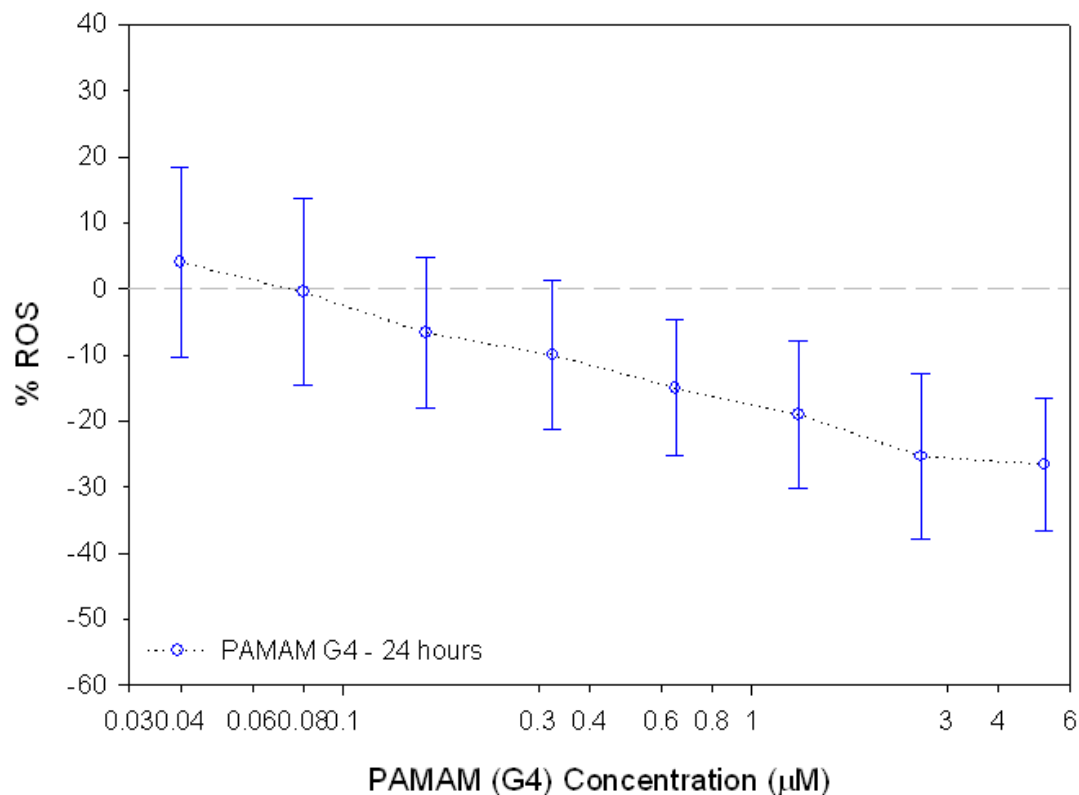
287

288 In contrast, Figure 2 shows HaCaT cells which have been treated with BSO for 18 hours prior to
289 PAMAM G4 exposure (BSO was still present in the media upon exposure, to ensure conditions
290 were consistent). In images (b) and (c), the intensity of the endosomal RFP and ROS dye have
291 been significantly reduced. Again co-localisation analysis was performed (on images in Figure 2)
292 and showed that 30(\pm 6)% of the generated ROS was happening around the endosomes, and that
293 46(\pm 14)% of endosomal formation resulted in increases in ROS production. Intensity analysis of
294 the red fluorescent protein produced by endocytosis, on average, showed a reduction in intensity
295 of 70(\pm 3)% for cells treated with BSO.

296

297 Endosomal uptake was clearly reduced, although a decrease in the intensity of response of the
298 ROS detection dye, carboxy-H₂DCFDA, was also noted, prompting a quantitative analysis of the
299 ROS contents of the HaCaT cells: Figure 3.

300



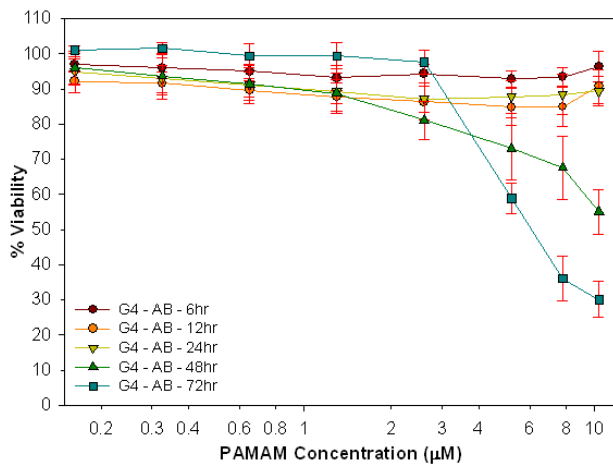
301
 302 Figure 3: Dose dependant generation of ROS in HaCaT cells (shown for 24 hours for the
 303 PAMAM G4). ROS is compared to BSO control which was set to equal 0%. The X-axis is
 304 plotted logarithmically to allow for better visualisation of lower concentrations. Data points are
 305 the mean of 18 samples, with error bars showing (\pm) the standard deviation.
 306
 307 The analysis of ROS showed a net decrease at all doses tested; for clarity, only the dose
 308 dependence of the 24 hour test has been shown, the full data-set is available in the supplementary
 309 material section (Figure S4). Over the dose range tested, the ROS response followed a
 310 monotonically decreasing trend. This result, is again, in contrast with previous observations, in
 311 which ROS levels were seen to increase above control levels in a dose dependant fashion, upon
 312 G4 dendrimer exposure.⁸

313

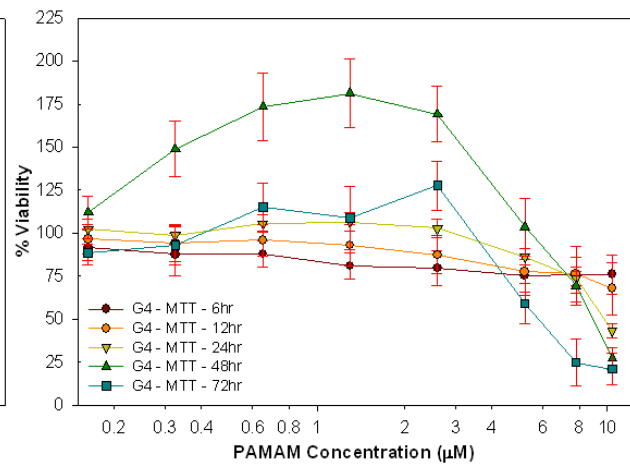
314 To examine how these changes in both endocytosis and ROS production affected the viability of
 315 the cell AB and MTT assays were performed. HaCaT cells were exposed to 200 μ M BSO for 18
 316 hours and subsequently exposed to varying concentrations of PAMAM G4 nanoparticles.
 317 Viability was measured at 6, 12, 24, 48 and 72 hours (Figure 4 (a) and (b)).

318

319 (a)



(b)



320

321 Figure 4: Alamar Blue (a) and MTT (b) dose dependant viability results for PAMAM G4

322 dendrimers in HaCaT cells after 6, 12, 24, 48 and 72 hours. Viability is calculated as the

323 percentage of living cells as compared to BSO control. Data points are the mean of 18 samples,

324 with error bars showing (\pm) the standard deviation.

325

326 The AB assay shows little or no toxicity of PAMAM G4 at time points 6, 12, and 24 hours,

327 whereas the viability is reduced to 50% at 48 hours and 30% at 72 hours, for the higher dose

328 exposures. In HaCaT cells which have not been treated with BSO, the EC_{50} obtained from AB is

329 around 10 μ M at 24 hours,⁷ whereas, in Figure 4, at the same time point, it is clear that there is no

330 significant toxicity. The MTT results show a dramatically different dose dependent cytotoxicity

331 profile for the BSO dosed cells compared to un-dosed.⁷ At 6 hours exposure (Figure 4b), the

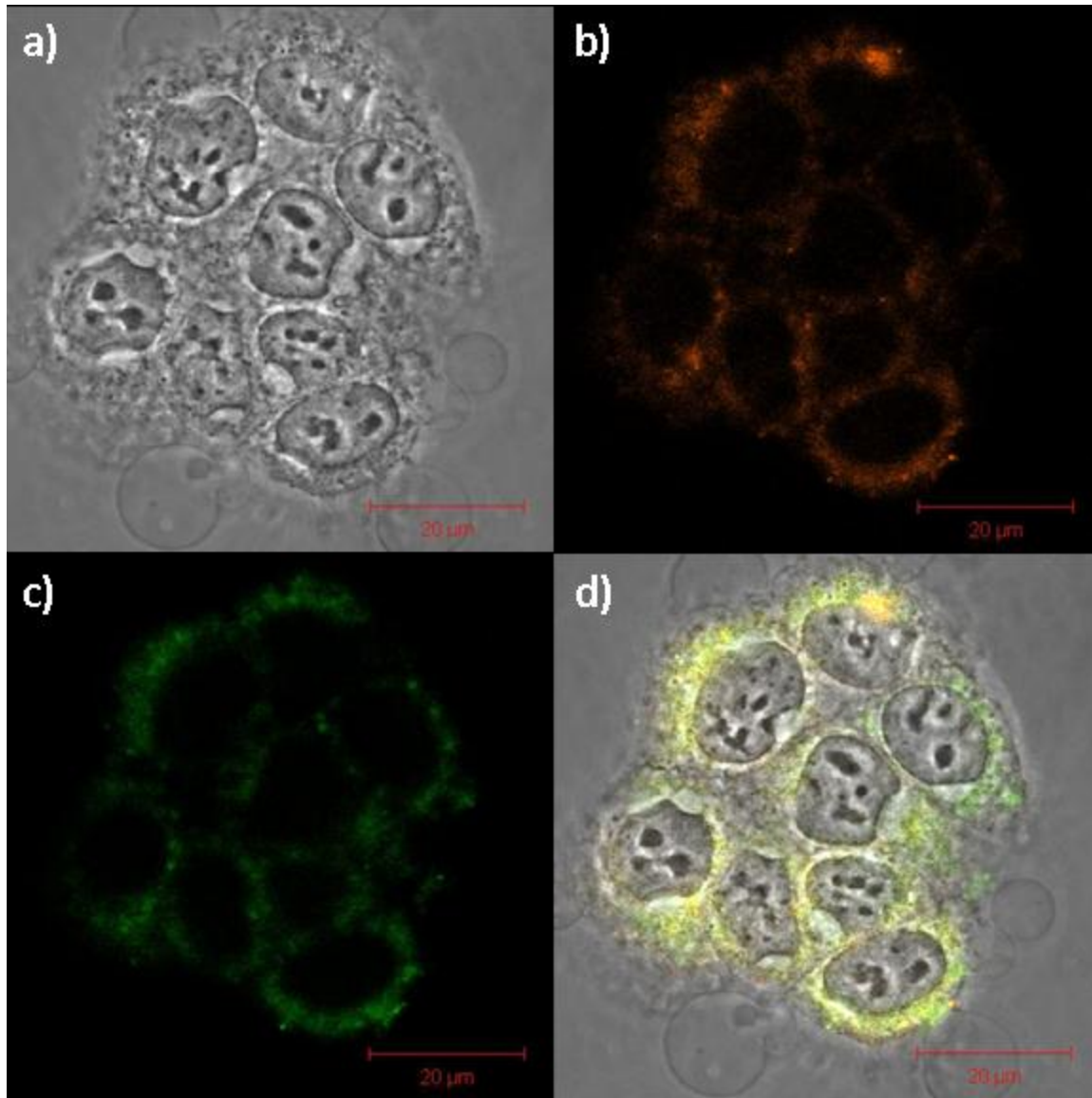
332 viability is seen to be reduced to around 80% over the entire dose range. However, at exposure
333 times of 12, 24, 48 and 72 hours, increases in viability are seen for the low-medium dose range.
334 The effect is particularly pronounced at the 48 hour time point, at which the recorded MTT
335 response is ~175% of control, for an exposure dose of ~1 μ M. For doses of ~2.6 μ M and higher,
336 the MTT assay response registers a decrease in viability, which is more pronounced in the longer
337 time point exposures.

338

339 PAMAM G6 dendrimers

340 Analysis of uptake and ROS production was again carried out using CLSM (with the same
341 method as the PAMAM G4) incorporating the carboxy-H₂DCFDA dye and Celllight[®] Early
342 Endosome – RFP kit (Figure 5 and 6).

343



344

345 Figure 5: Confocal images of HaCaT (live) cells, upon exposure to 1 μ M PAMAM G6 dendrimer

346 at 1 hour (no BSO is present in this sample). Image a) shows the bright field image of the cells,

347 Image b) shows the fluorescence generated by the early endosomal red fluorescent protein,

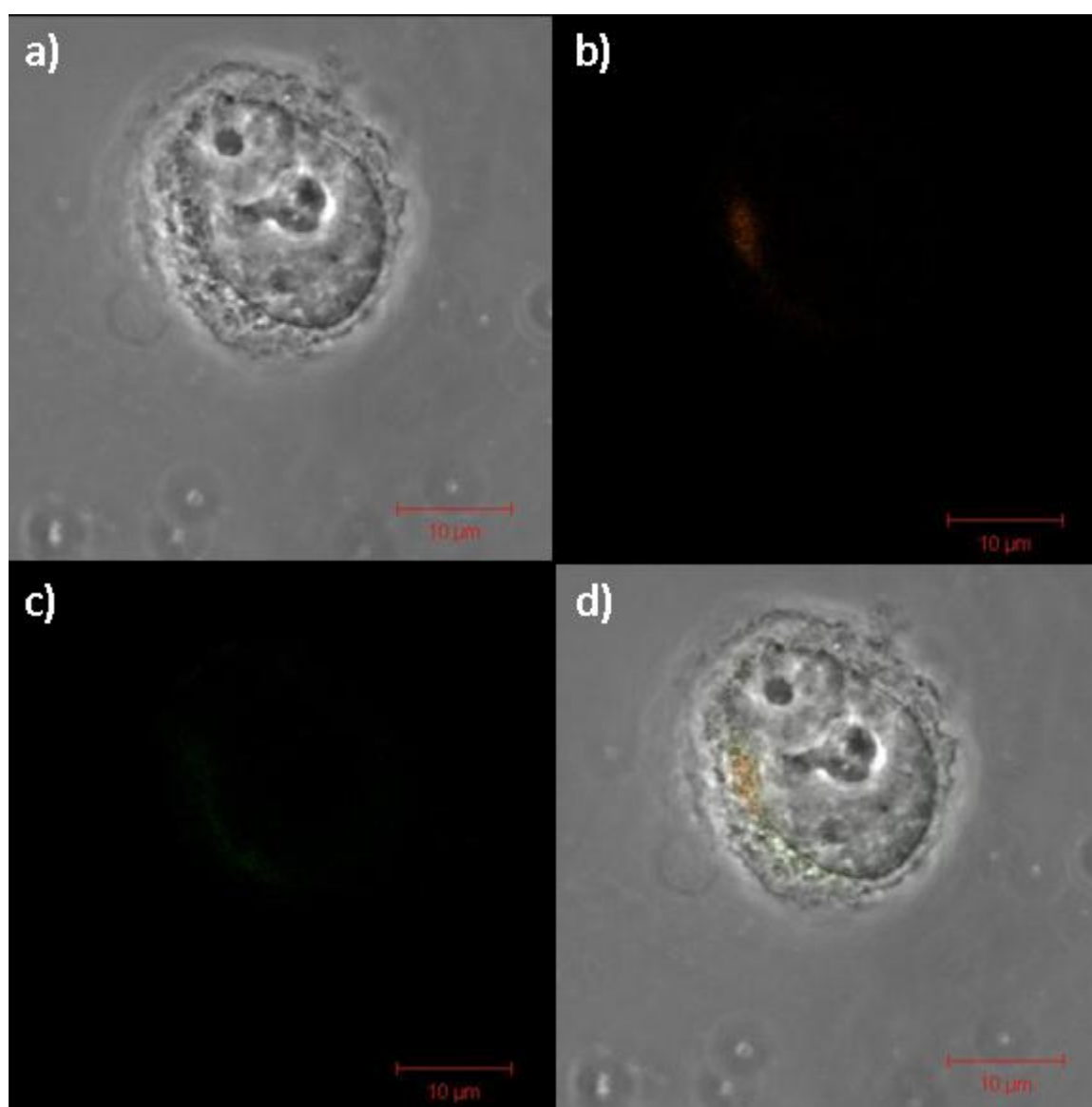
348 Image c) shows the fluorescence generated by the ROS (interacting with the carboxy-H₂DCFDA

349 dye) and Image d) shows the overlay of images a-c, where yellow coloured areas indicate

350 simultaneous endosomal and ROS activity.

351

352 In the absence of BSO, PAMAM G6 exposure, resulted in strong red fluorescence indicating a
353 high level of endocytosis, complemented by strong green fluorescence indicating increased ROS
354 production. Co-localisation analysis (of images in Figure 5) found that $75(\pm 2)\%$ of the generated
355 ROS occurred in the region of the endosomes, and that $92(\pm 1)\%$ of endocytosis resulted in
356 increases in ROS production. This strong level of co-localisation is indicative of the accepted
357 paradigm of nanoparticle uptake by endocytosis, and ROS production at the site of endosomes.
358



359

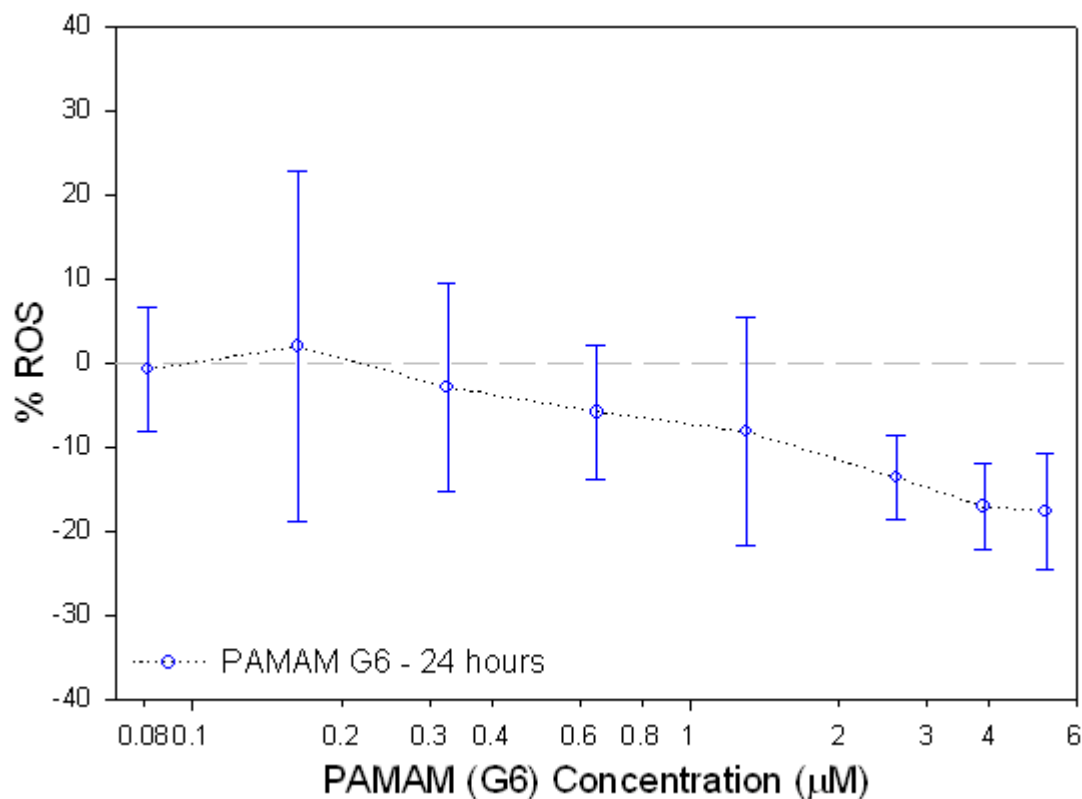
360 Figure 6: Confocal images of HaCaT (live) cells, upon exposure to 1 μ M PAMAM G6 dendrimer
361 at 1 hour, with pre-treatment of BSO: 200 μ M. Image a) shows the bright field image of the cells,
362 Image b) shows the fluorescence generated by the early endosomal red fluorescent protein,
363 Image c) shows the fluorescence generated by the ROS (interacting with the carboxy-H₂DCFDA
364 dye) and Image d) shows the overlay of images a-c, where yellow coloured areas indicate
365 simultaneous endosomal and ROS activity.

366

367 In comparison to exposure of PAMAM G6 in cells without BSO treatment (untreated cells),
368 there is a sparsity of endosomal activity and oxidative stress response in cells which had been
369 pre-treated with BSO for 18 hours. Analysis (of the images in Figure 6) showed decreased levels
370 of co-localisation between the ROS and endosomal formation: only 41(\pm 18)% of ROS
371 production was recorded in areas where endosomes had formed and 55(\pm 10)% of endocytosis
372 resulted in increases in ROS production. Intensity analysis of the red fluorescent protein
373 produced by endocytosis, on average, showed a reduction in intensity of 61(\pm 1)% for cells
374 treated with BSO when compared to the untreated cells.

375

376 Again, in light of the reduced ROS response, quantitative analysis, using carboxy-H₂DCFDA
377 was performed: Figure 7.



378
 379 Figure 7: Dose dependant generation of ROS in HaCaT cells (shown at 24 hours for the
 380 PAMAM G6). ROS is compared to BSO control which was set to equal 0%. The X-axis is
 381 plotted logarithmically to allow for better visualisation of lower concentrations. Data points are
 382 the mean of 18 samples, with error bars showing (\pm) the standard deviation.

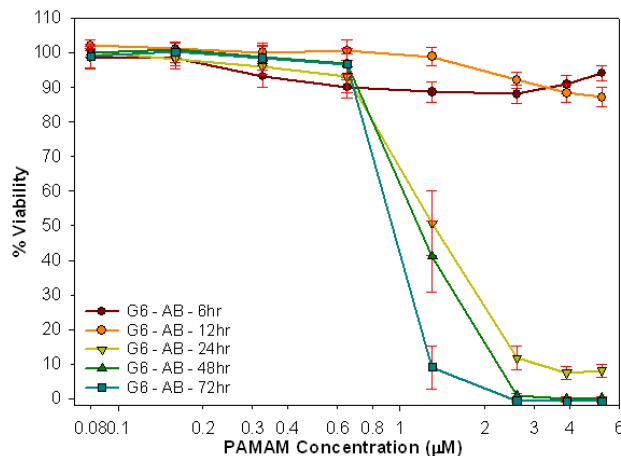
383
 384 The dose response of the ROS generation shows similarities with the G4 in the overall
 385 progression of the response, pointing to a trend where increasing concentration leads to
 386 decreasing amounts of ROS, contrary to what has been observed in previous studies without
 387 BSO exposure.⁸ As with the G4 analysis, for clarity, only one of the eight concentrations tested
 388 have been shown, the full data-set is available in the supplementary material section (Figure S5).
 389 At all doses tested, the ROS response followed the same trend as shown in Figure 7.

390

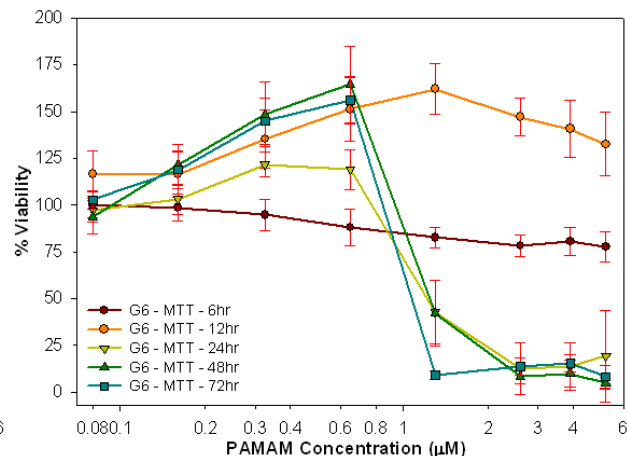
391 To analyse the effect this had on viability, AB and MTT assays were performed: Figure 8.

392

393 (a)



(b)



394

395 Figure 8: Alamar Blue (a) and MTT (b) dose dependent toxicity results for PAMAM G6

396 dendrimers in HaCaT cells after 6, 12, 24, 48 and 72 hours. Viability is calculated as the

397 percentage of living cells as compared to a BSO control. The X-axis is plotted logarithmically to

398 allow for better visualisation of lower doses. Data points are the mean of 18 samples, with error

399 bars showing (\pm) the standard deviation.

400

401 The viability results of the PAMAM G6 dendrimers are similar to those observed in the G4

402 analysis. In Figure 8, for 6 and 12 hour exposure, little toxicity is recorded by AB, although the

403 response is significantly stronger at the later time points. The previously reported EC₅₀ obtained

404 from the AB assay for PAMAM G6 dendrimers is in the range of 1µM-1.6µM at 24 hours,⁸

405 which would appear to agree with the values obtained in this study. However, as was the case for

406 PAMAM G4 exposure, the MTT assay registers increased percentage viability, compared to

407 BSO control, for the intermediate doses, for all but the 6 hour exposure time point. A cytotoxic

408 response is elicited for doses greater than 1 μ M, for the 24, 48 and 72 hour time points and the
409 response is consistent with the previously reported EC₅₀ values of 0.92 μ M-1.13 μ M.⁷

410

411 PAMAM G4-G6 Comparative Analysis

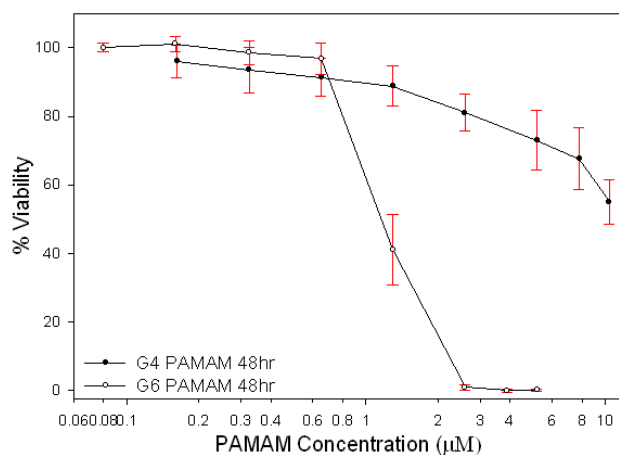
412 PAMAM nanoparticle toxicity is a generation dependant process,^{7,8,9} therefore, a comparison of
413 the response of BSO treated cells to G4 and G6 dendrimer exposure was performed, to establish
414 whether a similar generation dependence of the cellular responses is still evident.

415

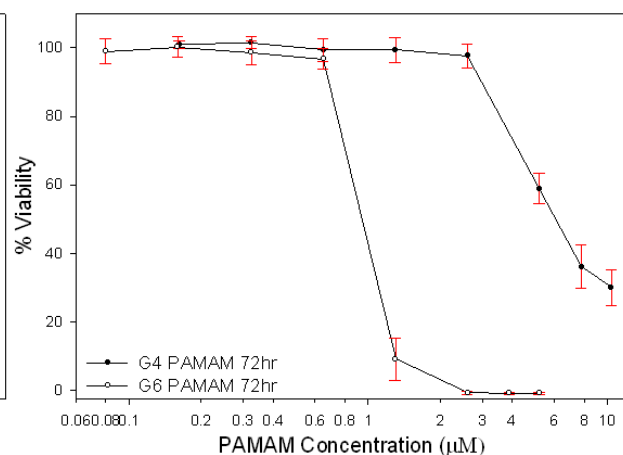
416 The AB and MTT assays were compared for PAMAM G4 and G6 dendrimers, and the results
417 are shown in Figures 9 (AB) and 10 (MTT). For both dendrimers, approximately equivalent
418 concentration ranges were used, although, for the PAMAM G6 dendrimers, a slightly lower
419 initial concentration was used due to the higher associated toxicity.^{7,8,9,10}

420

421 a)



b)



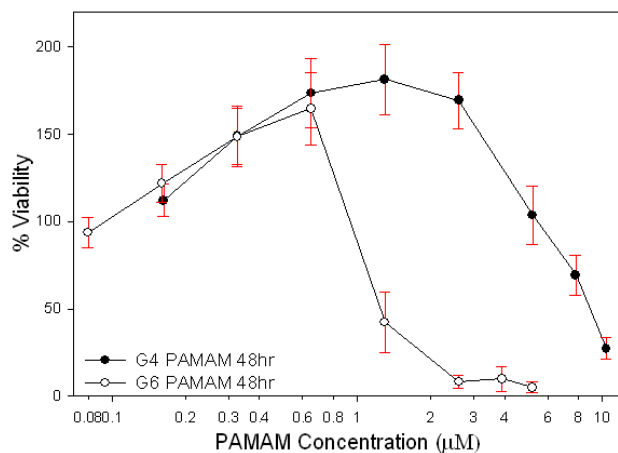
422

423 Figure 9: Alamar Blue (AB) dose dependant toxicity results comparing the PAMAM G4 and G6
424 dendrimers in HaCaT cells after 48 hours (a) and 72 hours (b). Viability is calculated as the
425 percentage of living cells as compared to BSO control. The x-axis is plotted logarithmically to

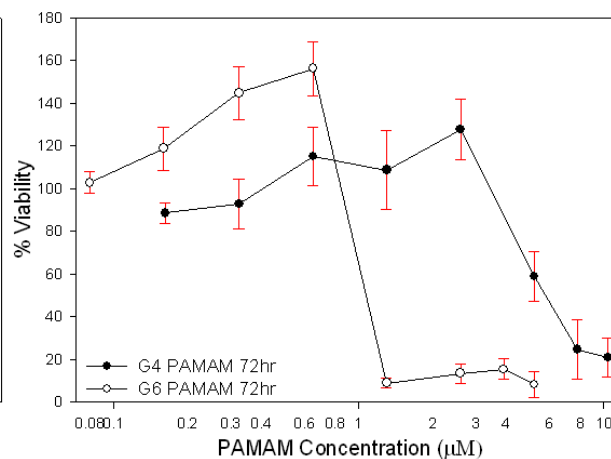
426 allow for better visualisation of lower doses. Data points are the mean of 18 samples, with error
 427 bars showing (\pm) the standard deviation. 6, 12 and 24 hour graphs can be seen in the
 428 supplementary material section (Figure S6).
 429
 430 Little or no significant cytotoxicity was registered by the AB assay for either dendrimer at the 6
 431 and 12 hour time points. At 24 hours, the higher toxicity associated with the G6 dendrimers
 432 becomes apparent, while the G4 still shows no significant change (Supplementary Material:
 433 Figure S6, c). At 48 hours (Figure 9(a)) the G4 dendrimers begin to elicit a significant toxic
 434 response and finally, at 72 hours (Figure 9(b)), the G4 toxic profile is beginning to match that of
 435 G6, although the reduction of viability is much higher for G6.

436

437 a)



b)



438 **Figure 10: MTT dose dependant toxicity results comparing the PAMAM G4 and G6 dendrimers**
 439

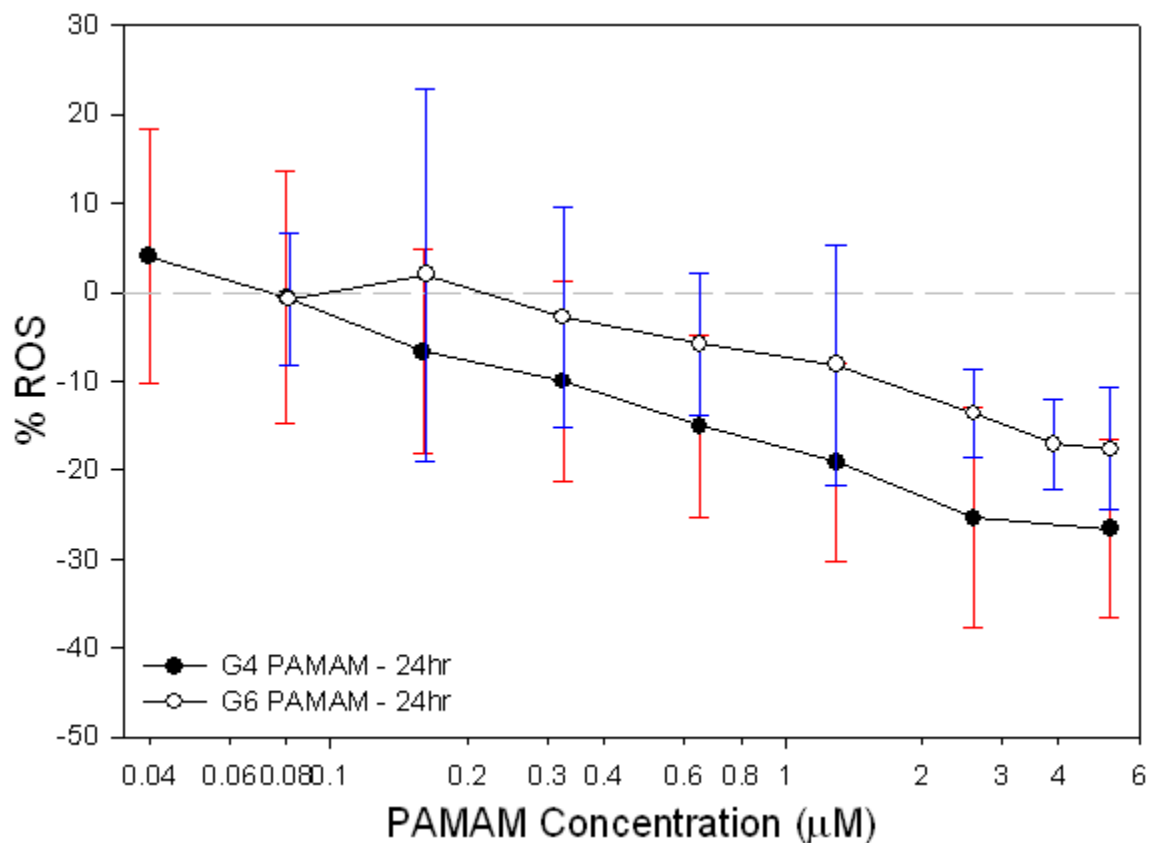
440 in HaCaT cells after 48 hours (a) and 72 hours (b). Viability is calculated as the percentage of
 441 living cells as compared to BSO control. The X-axis is plotted logarithmically to allow for better
 442 visualisation of lower doses. Data points are the mean of 18 samples, with error bars showing (\pm)

443 the standard deviation. 6, 12 and 24 hour graphs can be seen in the supplementary material
444 section (Figure S7).

445

446 In the MTT, similar to AB response, no significant difference was seen in the 6 hour analysis for
447 the two dendrimers, although differences become apparent at 12 hours (Supplementary Material:
448 Figure S7, b), at which point the MTT response increases above that of controls for the G6
449 dendrimer. This increase in MTT response also occurs for the G4 dendrimers, although, it is not
450 manifest until the later time point of 48 hours (Figure 10(a)). Similar to the AB response,
451 cytotoxicity is registered by the MTT assay for both dendrimers beginning at 24 hours; however,
452 the AB only reaches about 50% viability. At 48 and 72 hours a more complete toxic profile for
453 both assays is seen and distinct generation dependence is observable, more consistent with that
454 observed for untreated cells.⁷

455



456
 457 Figure 11: Dose dependant generation of ROS in HaCaT cells (shown at 24 hours for PAMAM
 458 G4 and G6). Data is compared to BSO control which was set to equal 0%. The X-axis is plotted
 459 logarithmically to allow for better visualisation of lower concentrations. Data points are the mean
 460 of 18 samples, with error bars showing (\pm) the standard deviation.

461
 462 To further investigate any generation dependence, the dose response of the ROS was compared
 463 for the G4 and the G6 (Figure 11). It is notable that the generation dependence of the trends is
 464 reversed in Figure11 where G4 elicits a more pronounced reduction in ROS levels than G6.

465

466 Discussion

467 The confocal images of Figures: 1 and 2 (PAMAM G4) and Figures: 5 and 6 (PAMAM G6)
468 show that, for cells treated with 200 μ M BSO for 18 hours prior to PAMAM exposure, the rate of
469 endocytosis has been markedly reduced. This reduction in endocytosis is accompanied by a
470 similar reduction in intracellular ROS and a dramatic change in the responses of the cytotoxic
471 assays. Nevertheless, the observed reduction of intracellular ROS and cytotoxic responses are
472 systematically dependent on dendrimer exposure time, dose and generation, consistent with the
473 intracellular action of the dendritic nanoparticles.

474

475 The demonstrated mechanism of PAMAM dendrimer toxicity is one of endocytosis,^{8,43,44} ROS
476 production,^{7,8,9,18} subsequent endosomolysis, whereby the nanoparticle bursts out of the
477 endosome/lysosome into the cytosol,¹⁹ and localisation in the mitochondria.⁴⁵ Mukherjee and
478 Byrne (2013) identified two apoptotic pathways, the death-receptor pathway (extrinsic, Fas
479 mediated FADD pathway⁴⁶) and the mitochondrial pathway (intrinsic, TNF- α mediated
480 FADD⁴⁷).¹⁰ The former is initiated by the earlier stage ROS generation in the region of the
481 endosomes, while the latter is initiated by the localisation of the dendrimers in the mitochondria.
482 It has been proposed that the early stage ROS production is due to the action of NADPH
483 oxidase⁶ (producing superoxide anions (O₂⁻)⁴⁸) and the v-ATPase proton pump (providing
484 protons,⁴⁹ ultimately leading to production of H₂O₂⁵⁰ in and around the endosome. In cells
485 treated with BSO, however, PAMAM dendrimers elicit a dramatically different cytotoxicity
486 profile, as registered by the AB and MTT assays, compared to that of untreated cells.^{7,8}

487

488 Studies by Khalid *et al.* (2015) of cellular uptake of PPI dendrimers have demonstrated that,
489 although the larger generation PPI dendrimers are endocytosed and elicit similar responses to
490 PAMAM equivalents in HaCaT cells, for smaller generation PPI dendrimers, uptake by passive
491 diffusion occurs and, when the dendrimers enter the cell in this way, they were observed to act as
492 antioxidants and elicit a significantly reduced cytotoxic effect.¹⁸ BSO exposure has been shown
493 to permeabilise the cell membrane,²⁸ and as a result, it is proposed that the PAMAM G4 and G6
494 dendrimers are able to circumvent the endocytotic process, are uptaken by passive diffusion, and,
495 as PAMAM dendrimers are similar in structure to PPI dendrimers and have comparable surface
496 chemistry, similarly behave as antioxidants in the cytosol, eliciting substantially reduced
497 cytotoxic responses.

498

499 Alamar Blue is a non-specific assay and measures cellular viability based on the overall activity
500 of the cytosolic environment.⁵¹ The significant reduction, rather than expected increase, of
501 toxicity as registered by this assay reflects the reduction in the endocytosis process (which
502 initiates the Fas mediated FADD (death-receptor) apoptotic pathway), in favour of the passive
503 diffusion of nanoparticles across the membrane, and consequent reduction in ROS generation in
504 the region of endosomes. However, endocytosis is not fully eliminated for either generation, and
505 therefore, the activation of the Fas mediated pathway, on a much reduced scale, would in part
506 explain why the generation dependent response is still observed for the PAMAMs.

507

508 The MTT assay measures mitochondrial activity as an indicator of cellular viability^{52,53,54} and, in
509 the case of the studies described here, the mitochondria are implicated in at least two different
510 processes and changes in MTT responses reflect the dose and generation dependence of these

511 processes at several time points. The first process is the loss of GSH from the cell, which has
512 been shown to cause activation of mitochondrial signalling pathways and expression of genes
513 associated with apoptosis, growth and differentiation.^{33,38,39,40} This overall increase in
514 mitochondrial activity (observed in the low dose regime), is seen as the initial increase in MTT
515 values above controls, associated with dose and generation dependent decrease in ROS below
516 controls, due to the antioxidant effect of the passively uptaken dendrimer nanoparticles.

517
518 The second effect gives rise to a sharp decrease in mitochondrial activity (observed in the higher
519 dose regime); consistent with PAMAM dendrimer localisation and disruption of the
520 mitochondria,^{7,8} initiating the mitochondrial apoptotic pathway, leading to cell death in a dose
521 dependant fashion, as observed for untreated cells.^{7,8} This process may be accelerated via the
522 opening of the mitochondrial membrane permeability transition pore.^{30,31,32} Whether passively
523 diffused into the cell, or released into the cytosol by endosomolysis after endocytosis, the result
524 of free PAMAM dendrimers in the cytosol and subsequent localisation to the mitochondria
525 should be equivalent, both resulting in disruption of the mitochondria, a second phase increase in
526 ROS within the cell, subsequent decay in the mitochondrial membrane potential and finally the
527 initiation of a cascade leading to apoptosis.^{7,8,9,10} It should also be noted that in both cases the
528 opening of the mitochondrial membrane transition pore occurs, either by the action of
529 BSO,^{30,31,32} or due to the release of the endosomal/lysosomal contents causing intracellular
530 release of Ca²⁺, leading to calcium dependant opening of the pore.²¹ This would, in both cases,
531 facilitate the entry of the dendrimer to the mitochondria. As a result, in the high dose regime, the
532 observed toxic response of the BSO treated cells, as registered by the MTT assay, is not very
533 different to that observed for untreated cells.

534

535 The passive diffusion of dendrimers across the cell membrane is a size dependant process¹⁸ and,
536 the generation dependence of the cellular responses to the two PAMAM dendrimer generations,
537 G4 and G6, (Figures: 1, 2, 5 and 6), is consistent with a higher uptake rate for the G4 dendrimer
538 than the G6. The greater reduction of intensity of Rab-5a-RFP compared to controls (with no
539 BSO treatment), G4 dendrimers (70%) compared to G6 (60%), indicates a higher diffusion rate
540 for G4 dendrimers, leaving fewer available for endocytosis. This further explains the higher rate
541 of anti-oxidative activity exhibited by the G4 dendrimers when compared to G6 (Figure 11).
542 Overall, it would appear that the membrane has become more permeable to an extent that favours
543 passive uptake, although not completely eliminating active endocytosis.

544

545 Within the framework of Adverse Outcome Pathways (AOPs), recently endorsed by the
546 OECD^{55,56} as a method to simplify the representation of the mode of action of a toxicant or
547 agonist, the generation of ROS can be considered the key Molecular Initiating Event (MIE) of
548 the AOP, which ultimately leads to the AO of loss of cell viability. The treatment with BSO
549 causes a depletion of GSH, which would lead to the expectation of much increased ROS levels
550 after endocytosis. However, that was not observed and the result of the reduction of intrinsic
551 GSH levels by BSO treatment was predominantly the increased permeability of the cell
552 membrane, resulting in an increased rate of uptake of the PAMAM dendrimers by passive
553 diffusion, making it a favoured uptake mechanism, more so for the smaller G4 dendrimer than
554 the larger G6. The co-existence of the parallel uptake mechanisms increases the complexity of
555 any model to describe the *in vitro* system, although it could prove a useful model to develop
556 networks of AOPs, initiated by different MIEs. However, to fully examine the effect of reduction

557 of GSH in terms of decreased anti-oxidant activity alone, an assay which did not cause decrease
558 of the permeabilisation of the cell membrane would be necessary.

559

560 Considering the potential for PAMAM dendrimers in nanomedical applications, it is important to
561 note that, when diffused into the cell, the aminated surface chemistry of the dendrimers lend
562 them antioxidant activity, similar to small molecular anti-oxidants, such as N-acetylcysteine
563 (NAC) and NAC amide (NACA - a more bio-available version of NAC). NACA has been
564 extensively studied as an antioxidant in the cell, due to its ability to diffuse across the membrane
565 and the presence of a terminal proton donor group.^{57,58} Interestingly, it has also been shown to
566 completely reverse the damage caused to the cell by depletion of GSH.^{59,60,61} NACA, due to this
567 strong anti-oxidant ability, has been proposed in the treatment of several disorders and diseases,
568 such as: HIV,⁶² Alzheimer's and Parkinson's disease,^{60,63} cataract formation,⁵⁹ retinal
569 degeneration⁶⁴ and essentially any disease where ROS is identified as the potential MIE.^{(See: 61 and}
570 ^{references therein)} If PAMAM dendrimers are seen to act in a similar way, it may potentially allow for
571 their use in a plethora of different nano-medical applications. As a strategy for drug release,
572 endosomolysis can be extremely disruptive to the cell²⁰ and therefore, in the case of cationic
573 nanoparticles for intracellular nanomedical applications, avoiding the process of endocytosis
574 may be a valid strategy to pursue.²² In terms of therapeutic applications, direct entry into the
575 cytosol may be a more convenient route for drug or gene delivery.

576

577 Conclusions

578 Although PAMAM dendritic nanoparticles are known to elicit significant cytotoxic responses *in*
579 *vitro*, the cellular response mechanisms can be notably altered by treatment of the cells with
580 BSO. The treatment increases the cell membrane permeability, enabling uptake of the particles
581 by passive diffusion, where after, they act as antioxidants in the cytosol, rather than producing
582 oxidative stress in the region of endosomes. The ability to tune the cellular uptake mechanism
583 allows direct entry into the cytosol and may have important implications for nanotoxicity as well
584 as drug and gene delivery using nanovehicles.

585

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589

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594

595 Declaration of interest

596 The authors declare no conflict of interest related to the work presented in this manuscript.

597

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