Supplementary Material:

Materials and Methods

Test materials

Polyamidoamine (PAMAM) dendrimers, G4, G5 and G6, were purchased from Sigma Aldrich Ltd. (Wicklow, Ireland). All the particles have an ethylenediamine core and PAMAM G4, G5 and G6 have respectively 64, 128, and 256 functional primary amino groups on the surface. The molecular weights of PAMAM G4, G5, and G6 are 14,215 Da, 28,826 Da and 58,048 Da respectively. The nominal diameters of the PAMAM G4, G5, and G6 dendrimers are 4.5, 5.4, and 6.7 nm respectively (http://www.dendritech.com/index.html). Full physicochemical characterization has been reported in Mukherjee et al., 2010a. The diameters of G4, G5, and G6 measured in PBS by dynamic light scattering (DLS) were 3.7 ± 0.9 nm, 6.8 ± 0.6 nm, and 8.8 ± 1.3 nm, respectively, in good agreement with manufacturer’s specifications.

Reagents

Cell culture media and supplements were purchased from Sigma-Aldrich and Bioscience (Dublin, Ireland). Rhodamine-123 and CellLytic™ MT Cell Lysis Reagent were purchased from Sigma-Aldrich. In addition, 5-(and-6)-carboxy-2’,7’-dichlorofluorescein diacetate (carboxy-H₂DCFDA), ThiolTracker™ Violet, LysoSensor™ Green DND-189 and MitoTracker® Orange CM-H₂TMRos were purchased from Invitrogen (Dublin, Ireland). Caspase-3 and caspase-8 assay kits were purchased from BD Biosciences (Oxford, United Kingdom). The Human TNF ELISA kit was purchased from BD Biosciences. Human IL-6 and IL-8 ELISA development kits were purchased from PeproTech (London, United Kingdom).
Cell culture

HaCaT cells, an immortal noncancerous human keratinocyte cell line, were kindly provided by Prof. Dr. Boukamp, Heidelberg, Germany. HaCaT cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) Nutrient Mixture F-12 HAM with 2 mM L-glutamine supplemented with 10% fetal bovine serum (FBS), 1 µg/ml hydrocortisone, 45 IU ml⁻¹ penicillin, and 45 IU ml⁻¹ streptomycin at 37°C in 5% CO₂.

Preparation of dendrimer solutions

Dendrimer test solutions of different concentrations were prepared in 5% FBS, 1µg/ml hydrocortisone, 45 IU ml⁻¹ penicillin, and 45 IU ml⁻¹ streptomycin supplemented DMEMF-12 HAM media. The dendrimers were readily soluble in the media at 37°C and were dispersed uniformly by low-speed vortex. A slight increase in the particle size was observed in this medium in comparison with that in PBS, whereby the diameters for G4, G5, and G6 were found to be 6.2 ± 0.3 nm, 6.5 ± 0.1 nm, and 10.2 ± 0.5 nm, respectively.

ROS study

Carboxy-H₂DCFDA dye was used to study the intracellular ROS generation in HaCaT cells. The study was performed in black 96-well microplates (Nunc, Denmark), wherein the cells were seeded at a density of 1 × 10⁵ cells/ml in 100 µl of 10% FBS supplemented medium. After 24 hours of cell attachment, the cells were washed twice with 100 µl/well PBS, carboxy-DCFDA dye was added at a concentration of 10 µM in 100 µl/well, and the plates were kept in a 37°C, 5% CO₂ incubator for 1 hour. Following incubation, the dye solution was removed; the cells were washed twice with 100 µl/well PBS and were treated with 6 concentrations of each generation of dendrimer, prepared in 5% FBS containing media. The fluorescence measurement of the oxidized DCFDA dye was measured between 30 minutes to
4 hours and after 24-hour exposures in HaCaT cells at the respective excitation and emission wavelengths of 490 nm and 545 nm in a TECAN GENios (Grodig, Austria) plate reader. The results are calculated and presented as a percentage of ROS generation in comparison with the negative control. The negative controls are the unexposed cells grown in 5% FBS supplemented media, parallel to the PAMAM exposure in the same media. The concentration ranges of PAMAM G4, G5, and G6 chosen in this experiment were the EC\textsubscript{50} concentrations from MTT, Alamar blue (AB) and Neutral red (NR) assays and the other three concentrations were between or below those concentrations. The EC\textsubscript{50} concentrations of PAMAM G4, G5, and G6 from MTT, AB, and NR assays were derived from Mukherjee et al., 2010a and are presented in Table S1.

**ROS localization by Confocal Fluorescence Microscopy**

For the study of the intracellular ROS localization by confocal fluorescence microscopy (CFM), the time evolution of ROS generation in HaCaT cells, as indicated by the fluorescence of the Carboxy-H\textsubscript{2}DCFDA dye, upon exposure to G6 PAMAM dendrimers at a concentration of 1 µM was monitored. HaCaT cells were seeded at a density of 1 × 10\textsuperscript{5} cells/ml in glass-bottom Petri dishes in 10% FBS-supplemented media and incubated at 37°C in 5% CO\textsubscript{2} for 24 hours for cell attachment. After 24 hours of cell attachment, the cells were washed twice with 2 ml of PBS per Petri dish, carboxy-DCFDA dye was added at a concentration of 10 µM in 100 µl/well, and the plates were kept in a 37°C, 5% CO\textsubscript{2} incubator for 1 hour. Following incubation, the dye solution was removed, the cells were washed twice with 100 µl/well PBS, were treated with 1 µM of PAMAM G6 dendrimer, prepared in 5% FBS supplemented media, and the cells were exposed for 1 hour and 24 hours. This concentration (1 µM) was identified from a previous study as the concentration where
maximum ROS generation was observed at those time points of exposure. Following the exposure period, the cells were washed twice with pre-warmed PBS (37°C). Mitotracker dye solution was prepared at a concentration of 250 nM in pre-warmed PBS. Cells were stained with the Mitotracker dye solution for 30 minutes in a 37°C, 5% CO₂ incubator. Following staining, the cells were washed three times with pre-warmed PBS to ensure complete removal of unloaded dyes. Confocal microscopic images were then taken using a Zeiss CFM (LSM 510 META, Version 3.2 SP2, Carl Zeiss, Germany). For the carboxy-H₂DCFDA dye, the excitation wavelength used was 488 nm and the fluorescence emission was detected using a 505 nm – 530 nm band pass filter. For the Mitotracker dye, the excitation wavelength used was 543 nm and the fluorescence emission was detected using a 560 nm long pass filter.

Intracellular reduced glutathione level measurement

ThiolTracker™ Violet was used to estimate the levels of intracellular reduced glutathione (GSH) in HaCaT cells upon PAMAM exposure. The fluorescent dye can be used for intracellular GSH detection in flow cytometry or by fluorescent imaging in living cells (http://products.invitrogen.com/ivgn/product/T10096?ICID=search-product). In this study, the method was adapted using a fluorescence microplate reader to quantify the fluorescence intensity of the dye and therefore the intracellular GSH levels in control and exposed HaCaT cultures. The study was performed in black 96-well microplates (Nunc, Denmark), wherein the cells were seeded at a density of $1 \times 10^5$ cells/ml in 100 µl of respective media containing 10% FBS. After 24 hours of cell attachment, the cells were washed twice with 100 µl/well PBS and were exposed to 1 µM of PAMAM G4, G5, and G6 in 5% FBS-supplemented media for periods of 1, 2, 3, 4, 6, 12, and 24 hours. Following exposure, the cells were washed with 100 µl/well PBS and exposed to the ThiolTracker™ Violet dye, prepared in PBS at a concentration of 20 µM in 100 µl/well and the plates were kept in a 37°C, 5% CO₂ incubator for 30 minutes. Following incubation, the dye solution was removed, the cells were
washed thrice with 100 µl/well PBS and the fluorescence was measured at excitation and emission wavelengths of 405 nm and 535 nm respectively in a VICTOR3™1420 Multilabel Counter plate reader (Perkin-Elmer, Dublin, Ireland). In Figure 2 the results are expressed as the fluorescence intensity of ThiolTracker™ Violet dye in the PAMAM exposed and negative control cells. The negative controls are the un-exposed cells grown in 5% FBS-supplemented media, parallel to the PAMAM exposure in the same media. In Figure 7 the maximum GSH level was normalized to 1. Note that because of the staining procedure following the exposure time points, there is a delay of 30 – 60 minutes between the exposure time points and the fluorescence measurements of the dye.

*Caspase-8 and -3 assays*

Caspase-8 and -3 activation in HaCaT cells upon PAMAM G4, G5, and G6 exposure was studied in 96-well black microplates (Nunc, Denmark). First, the cells were seeded at a density of $1 \times 10^5$ cells/ml in 100 µl of medium containing 10% FBS. After 24 hours of cell attachment, the cells were washed twice with 100 µl/well PBS and were exposed to 1 µM of PAMAM G4, G5, and G6 in 5% FBS-supplemented medium for 2, 4, 6, 12, and 24 hours. After the exposure times, the percentages of caspase-8 and -3 activity in the cells were quantified using the caspase-8 and caspase-3 assay kits of BD Biosciences by following the manufacturer’s instructions. In brief, after different exposure times, the cells were lysed with 50 µl of cold CelLytic™ MT Cell Lysis Reagent on a shaker at 240 rpm for 15 minutes. The cell lysate was then collected and centrifuged at 12,000 rpm at 4°C for 10 minutes to pellet-out the cellular debris. The supernatants were then transferred to the respective vials and were kept at -80°C for further analysis. Caspase-8 fluorogenic substrate (Ac-IETD-AFC) at a concentration of 10 µl/ml and caspase-3 fluorogenic substrate (Ac-DEVD-AMC) at a concentration 25 µl/ml of were prepared in protease assay buffer (1X HEPES buffer).
200µl/well of the caspase-8 and caspase-3 fluorogenic substrate solutions were added separately to the respective 96-well black-bottom microplates and then 100 µl/well test supernatants were added. The microplates were then incubated for 1 hour at 37°C in dark conditions. Following incubation, the fluorescence was measured at an excitation and emission wavelength of 405 nm and 460 nm respectively in a VICTOR3V™ 1420 Multilabel Counter plate reader (Perkin-Elmer). The results are presented as percentage increase or decrease of the fluorescence intensity of the substrate in the PAMAM exposed cells in comparison with the negative controls. The negative controls are unexposed cells grown in 5% FBS-supplemented media, in parallel to the PAMAM exposure in the same media.

Mitochondrial membrane potential study

Rhodamine-123 was used to study the mitochondrial membrane potential of HaCaT cells upon PAMAM exposure. Rhodamine-123 is an organic dye, the fluorescent emission from which is employed as a measure of the mitochondrial membrane potential. The study was performed in black 96-well microplates (Nunc, Denmark), wherein the cells were seeded at a density of $1 \times 10^5$ cells/ml in 100 µl of medium containing 10% FBS. After 24 hours of cell attachment, the cells were washed twice with 100 µl/well PBS and were exposed to 1 µM of PAMAM G4, G5, and G6 in 5% FBS-supplemented medium for 2, 4, 6, and 24 hours. Following the appropriate exposure times, the cells were washed with 100 µl/well PBS, then Rhodamine-123 was added in unsupplemented media at a concentration of 10 µg/ml in 100 µl/well, and the plates were kept in a 37°C, 5% CO$_2$ incubator for 30 minutes. Following incubation, the dye solution was removed, the cells were washed twice with 100 µl/well PBS and the fluorescence was measured at excitation and emission wavelengths of 485 nm and 535 nm respectively in a TECAN GENios (Grodig, Austria) plate reader. The results are presented as percentage of fluorescence of Rhodamine-123 dye in the PAMAM exposed cells compared to the negative controls. The negative controls are the unexposed cells grown in
5% FBS-supplemented media, in parallel to the PAMAM exposure in the same media. Because of the staining procedure following the exposure time points, a delay of 45 minutes to 1 hour exists between the exposure time points and the fluorescence measurements of the dye.

**TNF-α and IL8 expression study**

An enzyme linked immunosorbant assay (ELISA) was performed to quantify the pro-inflammatory mediators (TNF-α, IL-8, IL-6) after the exposure of the HaCaT cells to PAMAM dendrimers. The low-cost, routine technique is widely used to monitor and quantify the production of cytokines and is favoured over the relatively time-consuming and costly techniques of qRT PCR. It is noted that although RT-PCR quantifies the mRNA levels of the respective proteins inside the cells, all the mRNAs do not translate into their respective proteins. Therefore, to understand the effects of the respective caspases, ELISA is a better representation of the exact amount of respective final active proteins inside the cells upon their activation. The principle of the ELISA is based on the sandwich technique, in which the capture antibody (primary antibody) was coated in the 96 well plate (Nunc immuno plate, Roskilde, Denmark). The capture antibody of TNF-α was prepared in coating buffer (0.1 M sodium carbonate in dH₂O, pH 9.5) according to the manufacturer’s instruction and that for IL-8 was prepared in PBS at a concentration of 0.5 µg/ml. The plates were sealed and incubated overnight at 4°C for TNF-α and at room temperature (25 ° C) for IL-8. The wells were aspirated to remove the liquid, and the plates were washed four times with PBS-T (phosphate buffer saline with 0.05% of Tween 20) and then blocked with 10% FBS in PBS for TNF-α and 1% BSA in PBS for IL-8 at room temperature for 1 hour. The plates were again washed with PBS-T four times and 100 µl of different dilutions of supernatant were added to the respective wells. Standards of TNF-α and IL-8 at concentrations from 10 to 800 pg/ml, in duplicate, were added to the first two columns of the 96-well plates and the plates
were incubated for 2 hours at room temperature. The plates were aspirated and washed five times, whereupon 100 µl of the detection antibody against the respective marker (secondary antibody) and streptavidin-horseradish peroxidise conjugate were added to the 96-well ELISA plate together (for TNF-α) or separate (for IL-8) according to the respective manufacturer’s instructions. The plates were washed seven times with washing buffer and 100 µl of substrate solution (for TNF-α, tetramethylbenzidine and hydrogen peroxide and for IL-8, 2,2’-Azino-Bis(3- Ethylbenzthiazolin-6-Sulfonic acid)) were added to each well and the plates were incubated at room temperature to develop the color. The color development time was 30 minutes for TNF-α followed by the addition of stopping solution of 2 N H₂SO₄ and the absorbance was measured at 450 nm in a VICTOR3™1420 Multilabel Counter plate reader (Perkin-Elmer). The color development time was optimized to be 15 minutes for each assay of IL-8 using the standards and the absorbance was measured at 405 nm in a VICTOR3™1420 Multilabel Counter plate reader (Perkin-Elmer). The results are presented as percentage of absorbance (for TNF-α) and fluorescence (for IL-8) of the substrates in comparison with the negative controls. The negative controls are the unexposed cells grown in 5% FBS-supplemented media, in parallel to the PAMAM exposure in the same media.

Data analysis and statistics

All experiments were conducted in at least triplicate (three independent experiments). Fluorescence as fluorescent units (FUs) of all of the assays was quantified using microplate readers- VICTOR3™1420 Multilabel Counter plate reader (Perkin-Elmer) and TECAN GENios (Grödig, Austria). Raw data from the ROS study, GSH, all the ELISA data (TNF-α and II-8), casapase -8 and -3 data and mitochondrial membrane potential data (using rhodamine 123) were collected and analyzed using Microsoft Excel® (Microsoft Corporation, Redmond, Washington). All the data were expressed as mean percentage viability relative to the unexposed control (100%) ± standard deviation (SD). Statistical
analyses were carried out using one-way analyses of variance (ANOVA) followed by Dunnett’s multiple comparison tests. Statistical significance was accepted at $P \leq 0.05$ for all tests. Negative values obtained from different assays are indicative of levels which are less than those of negative controls, as a result of either reduction by quenching mechanisms, or the reduction in cell population due to apoptosis and cell death.

**Discussion and numerical simulation**

The sequential activation of caspase 8 and caspase-3 can be simulated using the rate equation model:

\[
\frac{dN_{C8}}{dt} = K_{C8}N_{ROS} - K_{C3}N_{C8} \quad \text{Equation S1 (5)}
\]

\[
\frac{dN_{C3}}{dt} = K_{C3}N_{C8} - K_{M}N_{C3} \quad \text{Equation S2 (6)}
\]

$N_{C8}$ and $N_{C3}$ represent the expression levels of caspase-8 and -3 respectively, while $K_{C8}$ and $K_{C3}$ represent their rates of generation. Equation S1 generates caspase-8 from the early stage of ROS generation, and Equation S2 caspase-3 directly from caspase-8. $K_{M}$ is a rate of quenching of caspase-3, resulting in the decay of mitochondrial membrane potential (Equation S3).

\[
\frac{dN_{M}}{dt} = K_{M}N_{C3} - K_{TNF}N_{M} \quad \text{Equation S3 (7)}
\]

\[
\frac{dN_{TNF}}{dt} = K_{TNF}N_{M} - K_{IL8}N_{IL8} \quad \text{Equation S4 (8)}
\]
The model assumes that, in the early stages, mitochondrial membrane potential decay is the result of excess levels of caspase 3 ($N_{C3}$) and occurs at a rate $K_M$. The amount of MMPD is represented by $N_M$. Similarly, the model simulates the generation of TNF-α represented by $N_{TNF}$, as a subsequent step to mitochondrial membrane potential decay at a rate $K_{TNF}$. Levels of IL8, $N_{IL8}$, are generated in a subsequent step, at a rate $K_{IL8}$. In Equations S4 and S5, generation of TNF-α from MMPD, and IL8 from TNF-α, act as quenching terms for the populations of MMPD and TNF-α. However, the recovery terms could be represented by independent rate constants. As it is not apparent experimentally over the 24 hour period, no decay term is included for $N_{IL8}$ in Equation S3.

\[
\frac{dN_{IL8}}{dt} = K_{IL8}N_{IL8}
\]

Equation S5 (9)

References


**Table legends:**

Table S1: EC$_{50}$ concentrations from AB, NR and MTT assays of PAMAM G4, G5, and G6 upon 24 h exposure to HaCaT cells (summarized from Mukherjee et al., 2010a).

<table>
<thead>
<tr>
<th>Cytotoxicity assays</th>
<th>EC$_{50}$ in µM [Confidence Interval]</th>
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<tbody>
<tr>
<td></td>
<td>PAMAM G4</td>
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<tr>
<td>MTT assay</td>
<td>3.21 [2.89-3.52]</td>
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</table>
**Figure Legends:**

Figure S1: ROS generation in HaCaT cells upon different concentrations of PAMAM exposure: A) G5, B) G4. C) shows the generation of ROS upon 1 h exposure to 1 µM of different generations (G4, G5, and G6) of PAMAM. The levels of ROS generation are correlated with their surface amino groups.

Figure S2: Localization of ROS upon 1 µM PAMAM G6 exposure
A) after 1 h exposure the ROS was in vesicles in the cytosol, B) after 24 h exposure ROS was co-localized in the mitochondria. Figure S2A is an overlay image whereby the red color represents mitochondria (Mitotracker dye) and the green color represents ROS (Carboxy-H$_2$DCFDA). Figure S2B is an overlay of Mitotracker dye stained (red color) mitochondria and Carboxy-H$_2$DCFDA (green color) for ROS detection, which produces orange color where they are co-localized.

Figure S3: Caspase-8 and -3 expression as a function of exposure time of 1 µM PAMAM: A) G4, B) G5, C) G6.

Figure S4: TNF-α expression in HaCaT cells at different exposure time points for different concentrations of PAMAM: A) G4, B) G5, C) G6.

Figure S5: IL-8 expression in HaCaT cells at different exposure time points of different concentrations of PAMAM: A) G4, B) G5, C) G6.

Figure S6: The simulations predict a linear dependence of the relative amounts of ROS generation on (A) generation number, and therefore number of surface amine groups, for a specific time and dose (1µM), (B) at different doses of PAMAM (G6).

Figure S7: Demonstration of the approximate linear dependence of the simulated maximum TNF-α levels as a function of surface amino groups per dendrimer generation.
Figure S8: Diagrammatic representation of PAMAM toxicity on HaCaT cells.

Figure S1

A)

B)
The graph shows a linear relationship between surface amino groups and ROS (arb. units). As the number of surface amino groups increases, the ROS levels also increase in a linear fashion, as indicated by the trend line and the error bars for the data points.
Figure S2

A)
Figure S3.

A) 

![Graph showing % of caspase activation over hours of exposure for caspase-8 and caspase-3.]

B) 

![Graph showing % of caspase activation over hours of exposure for caspase-8 and caspase-3.]

Figure S4.

A) % of TNF-α expression

B) % of caspase activation
Figure S5.

A)  

B)
C) % of IL-8 expression over hours of exposure for different concentrations:

- 0.5 µM
- 1 µM
- 1.3 µM
- 2.23 µM
- 3.17 µM
Figure S6.

A) Number of Amino Groups

B) Concentration (µM)
Figure S7.

[Graph showing the relationship between Number of Surface Amino Groups and TNF-α Max. The x-axis represents the number of surface amino groups ranging from 50 to 300, while the y-axis represents TNF-α Max ranging from 0.5 to 4.5.]
Figure S8.