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Anionic linear-globular dendrimers: biocompatible hybrid materials with potential uses in nanomedicine

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Abstract The use of dendrimers as *nano-sized excipients/vectors* in biological and pharmaceutical systems is dependent on the investigation of their toxicological profiles in biological media. In this study, a series of mechanistic in vitro structure-associated cell toxicity evaluations was performed on the two generations of an anionic linear-globular dendrimer G1 and G2 (where PEG is the core, and citric acid is the periphery) each of which has a different size, charge, and MW. In vitro cytotoxicity behavior of the dendrimers with the methods like crystal violet staining, methyl thiazolyl tetrazolium (MTT), and lactate dehydrogenase (LDH) assays was analyzed. The cell death mechanisms (apoptosis–necrosis) induced by the dendrimers were also evaluated in HT1080 cell line. The impact of the dendrimers on the release of the pro-inflammatory cytokines like TNF-α (tumor necrosis factor alpha) and IL1-β (interleukin 1 beta) was assessed in THP-1 cell line. Hemolysis assay and coagulation studies such as PT (prothrombin time) and APTT (activated partial thromboplastin time) on human blood samples were conducted to examine the interactions of the dendrimers with such bioenvironments. The results of cell cytotoxicity experiments and the amounts of IL1-β and TNF-α secretions from THP-1 cell line were consistent with the hemoglobin release from the erythrocytes and the results gained from the coagulation studies. In fact, no significant harmful effect was observed for the dendrimers up to the concentration of 0.5 mg/ml. Both apoptosis and necrosis were ascribed to cell death. The G1 with more flexibility, less negative charge, and greater poly dispersity in size versus the G2 displayed more toxicity than the G2 at the concentration of 1 mg/ml and above in most of the experiments. As a whole, these results suggest a biocompatible range for these hybrid structures up to the concentration of 0.5 mg/ml. Therefore, the potentiality for these structures to be employed in the different and numerous realms of nanomedicine will be very great.

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1 Introduction

Dendritic structures are a new class of polymers first reported by Vögtle and co-workers [1]. Dendritic architectures

contrary to regular polymers have well-defined structural and physicochemical properties. These polymers are normally prepared using a unique branching synthetic methodology, divergent/convergent method, which results in the production of materials with controllable size (nm), shape, molecular weight, and sometimes symmetric structures [2]. These structures have been showing promising applications in a large number of scientific fields such as physics, biochemistry, biotechnology, drug delivery, and biomedical applications [3]. Numerous publications have been devoted only to the different synthetic methods of these almost new materials leading to the development and creation of various kinds of such polymers. For example, globular or semi-globular-shaped structures or cationic, anionic, or neutral-charged dendrimers with different chemical end groups have been synthesized so far. Yet, little is known about the complex types of behavior of these new entities in biological systems, which is very crucial and vital for the future use of these materials in biomedical sciences. Understanding the behavior or interaction of these polymeric structures in/with biological systems, i.e. biocompatibility issues, involves a wide range of bio-toxicological analyses depending on their final applications. Non-toxicity of materials in biological systems has a broad definition which comprises the investigation of all the toxicological aspects of the materials under use. In other words, it is necessary to clarify the toxicity profile of these structures based on in vitro and in vivo experiments [4]. In this study, two generations of a negative-charged dendritic structure G1 and G2 composed of poly (ethylene glycol) with the molecular weight of 600 Dalton as the core and citric acid as the end groups were synthesized using method of Namazi and Adeli [5]. The high potential of these hybrids—two biocompatible components—as new drug delivery systems has been demonstrated in some studies thus far [6, 7]. Then, as the first screening step, a series of in vitro biological toxicity evaluation experiments was carried out on the two generations of this dendritic structure to examine the biocompatibility behavior of these entities. The toxicity studies included crystal violet staining (CVS), methyl thiazolyl tetrazolium (MTT), and lactate dehydrogenase (LDH) assays which were performed on a fibrosarcoma HT1080 cell line. This cell line was selected because of its stable metabolism besides its homogeneous growth process [8]. Secondly, the stimulation of the secretions of the pro-inflammatory cytokines such as TNF-α and IL1-β by the dendrimers was investigated in THP-1 monocyte cell line. Thirdly, the mechanisms of cell death induced by the dendrimers were studied at nontoxic concentrations based on apoptosis–necrosis assays, because the approval or rejection of a material for biomedical applications will be determined by both the degree and type of the either mechanism. Furthermore, hemolysis assay and coagulation studies such as prothrombin time (PT) and activated partial thromboplastin time (APTT) were carried out in order to scrutinize the impact of physicochemical features of the dendrimers on the release of hemoglobin from the intact erythrocytes or activation/deactivation of coagulation pathways, respectively. These negative impacts must be minimal to prevent clot formation and occurrence of unwanted coagulation effects in the blood circulation. In addition, there was a correlation found between the results of these experiments and the physicochemical structures of the dendrimers such as size distribution, aggregation behavior, and net charge density. Finally, biocompatible concentration levels for the possible applications of these materials in biological systems were determined according to the results of the in vitro experiments. These relations can be employed to facilitate the design of biocompatible dendrimers and to smooth the way for further applications of these structures in various and increasing fields of nanomedicine in the near future.

2 Experimental

2.1 Materials

The G1 (MW: 1,000 Da) and the G2 (MW: 2,000 Da) were prepared according to Namazi method. HT1080 cell line (Human fibrosarcoma cell line, ATCC, CCL-121) and THP-1 cell line (Human monocyte cell line, NCBI, C563) were purchased from Pasteur Institute (Tehran, Iran). Annexin-V FLUOS staining kit from Roche (Roche, Basel, Switzerland); crystal violet dye, MTT powder, and DMSO were obtained from Sigma (Sigma-Aldrich, MO, USA). LDH kit, RPMI-1640 and FCS were provided by Promega (Promega^R, WI, USA) and Biosera (Biosera^R, East Sussex, UK), respectively. TNF-α, IL1-β assay kits and PT, APTT coagulation assay kits were supplied from U-CyTech (U-CyTech^R, Utrecht, Netherlands) and Stago (Stago^R GmbH, Albershausen, Germany), respectively.

2.2 Methods

2.2.1 Synthesis

Two generations of the dendrimers (G1, G2) were prepared by Namazi and Adeli methodology and used in the experiments. In brief, diacid poly(ethylene glycol) was chlorinated using thionyl chloride ($SOCl_2$) and reacted with citric acid as the monomer to generate the G1. Then, the G1 was coupled to citric acid to produce the G2 with the aid of dicyclohexylcarbodiimide (DCC) in the pyridine medium [5].

2.2.2 Potentiometric titration

Potentiometric titrations were performed manually using a Mettlertoledo pH meter at room temperature (23 ± 1) for determination of the number molecular weights. The dendrimers [G1 (6 mg); G2 (4 mg)] were dissolved in 0.1 M NaCl solutions to give the final concentrations of 1 mg/ml. The dendrimer solutions were titrated by standard NaOH (0.01 N) and back titrated by standard HCl (0.01 N), respectively.

2.2.3 Size and zeta potential distributions

All the experiments were conducted by means of the DLS (Malvern, Zetasizer Nano ZS, Worcestershire, UK) instrument in D.D.W. (double deionized water) at the two different concentrations (0.1 and 1 mg/ml).

2.2.4 Blood sample preparation

Anti-coagulated blood samples (3.2% sodium citrate) were obtained from healthy, un-medicated volunteers. Platelet poor plasma (PPP) was isolated by centrifuging the whole blood at 3,000 rpm (revolution per minute) for 15 min at room temperature. The blood samples were used just as prepared [9].

2.2.5 Degradation study

Degradation study was qualitatively carried out by a dew point osmometer (WESCOR^{R}5520, Utah, USA). The changes in their initial osmolalities during the time periods (0 and 7 days) and different pHs (5.4 and 7.4) were measured according to the morse rule—$\pi = inRT$; $n =$ molality, $i =$ activity coefficient, $R =$ gas constant, $T =$ temperature in electrolyte solutions. These changes are indicators of the production of new particles (colligative property).

2.2.6 Cell culture

Human fibrosarcoma epithelial like cell line (HT1080) and human monocyte cell line (THP-1) were obtained from Iran Pasteur Institute and used for cell cytotoxicity and inflammation studies, respectively. Cells were grown in 25 cm^2 culture flasks using complete RPMI-1640. Cell culture media were supplemented with 10% FCS and 1% penicillin–streptomycin; the cells were kept at 37°C and 5% CO_2 in an incubator. The cells were sub-cultured every 72 h and harvested from sub-confluent cultures (70% using 0.05% Trypsin–EDTA for adherent cells). The cells were cultured at 10^4 cells/well in 96-well plates in all the experiments except for the apoptosis–necrosis assays, TNF-α and IL1-β secretion assays for which 12-well plates at 10^6 cells/ml were used, respectively [8].

2.2.7 Crystal violet staining assay

Crystal violet staining (CVS) has been used as a test for the viability or functionality of adherent cells [8, 10]. After the dendrimers had been incubated with HT1080 cell line at different concentrations, the media were aspirated. Then, the cells were washed with cold PBS buffer three times to remove non adherent cells. Afterwards, the remaining adherent viable cells were fixed with 5% v/v formaldehyde/water about 5 min. Then formaldehyde was removed quickly, and the wells were washed three times with distilled water and stained with 100 µl of 1% v/v crystal violet solution at room temperature for 5 min. Next, the plates were completely washed with water, and crystal violet dyes were dissolved in 33% v/v acetic acid in water to form homogeneous colors which were ready for spectrophotometric determination. The absorbance of the dissolved dyes, proportionate to the number of viable cells, was measured in an automated plate reader at 570 nm; the results were compared to untreated negative control cultures.

2.2.8 MTT assay

MTT assay is one the current techniques which is widely used for cell viability measurements. This assay is based on the reduction of MTT to formazan by viable cells [8, 11]. After the end of the incubation times (24 and 48 h), the supernatants of the cells (HT1080) were removed, MTT solutions were added to each well of the plates at the final concentration of 0.5 mg/ml, and the cells were incubated for an additional 4 h. Thereafter the solutions were removed, the cells were lysed, and the dye was dissolved in 100 µl dimethyl sulfoxide. Then, the plates were kept in a dark place for another 1 h in order to be ready for spectrophotometric determination. The amounts of absorption in each well, i.e. the conversion of MTT to formazan by metabolically viable cells, were determined by an automated microplate reader at 570 nm. The results were compared to the untreated control cultures.

2.2.9 LDH assay

For LDH release measurement, a colorimetric assay that quantitatively measures the amount of LDH (a stable cytosolic enzyme released due to necrosis/or secondary necrosis in the cells) in the supernatants of the cells was used. To measure the released LDH in the culture supernatants, an LDH kit was used (Cytotox^{R} 96 Non-Radioactive, Promega, WI), the action of which is based on the

conversion of a tetrazolium salt (INT) into a red formazan product by the released LDH. The amount of color formed is proportionate to the number of lysed cells [8, 12]. Twenty-four hours after the seeding, the cells were treated with different concentrations of the dendrimers up to the final concentration of 2 mg/ml and incubated for another 24 h with the cells (HT1080). After removing 50 µl of the cell culture media from each well of the plates, and the remaining contents were diluted to a 1:1 ratio with fresh media and plated into new microtiter plates. Next, 50 µl of substrate solutions were added to each well and the plates were incubated for additional 30 min at room temperature. Samples from each well were read with a microplate reader at wavelength of 492 nm. The released LDH from the treated cells were compared to the DMSO (0.1% v/v) treated negative control cells, because this concentration is considered nontoxic to cells and has been used in different fields of pharmaceutical industry.

2.2.10 Apoptosis–necrosis assays

Apoptotic and necrotic cells were identified by an AnnexinV-PI staining kit (PI: Propidium Iodide). Apoptosis and necrosis have been considered as the two different mechanisms of cell death. By these techniques, differentiation between these two mechanisms will be feasible [8, 13]. Following 24-h incubation period of HT1080 cell with two different concentrations of the G1 and G2 (0.25 and 0.5 mg/ml, being nontoxic concentration after the toxicity assessment with MTT, crystal violet staining, and LDH assays) with the HT1080 cells, they were washed three times with PBS, trypsinized, and transferred into a new tube. Next, the cells were centrifuged at 200 g for 5 min, counted with trypan blue, and about 10^6 cells analyzed for each flow cytometry run test. Following that, the cells (treated and untreated ones) were incubated with AnnexinV-PI reagent buffer for 15 min at room temperature. Finally, samples were diluted with 500 µl incubation buffer for flow cytometry analysis. The percentage of the treated apoptotic and necrotic cells in comparison with the untreated control cells was calculated by the flow cytometry (FACS Calibur, BD) method with WinMDI version 2.9 at suitable excitation and emission wavelengths (excitation at 488 nm, emission at 518 nm for Annexin-V detection and a band pass filter greater than 600 nm for PI detection) according to the suggested protocols in the kit for FITC-PI detections, respectively.

2.2.11 TNF-α and IL1-β assay

The amounts of TNF-α and IL1-β secretions were measured using a U-CyTech^R ELISA kit. After 24-h incubation period of the dendrimers with THP-1 cell line at the final concentration of 0.5 mg/ml, the cells were pelleted; and the supernatants were collected and used for spectrophotometric quantitative determination of TNF-α and IL1-β secretions according to their specific protocols by the supplier at wavelength of 450 nm with a microplate reader. The negative controls only contained complete RPMI-1640 [14].

2.2.12 Hemolysis assay

Blood samples were drawn from un-medicated volunteers (anti-coagulated samples). The final hematocrit fraction was about 5% v/v after centrifuging the whole blood at 1,000 rpm (revolution per minute) for 5 min and discarding the supernatant. Then, the dendrimers were dissolved in PBS and incubated (1 and 6 h) with the blood sample mixtures composed of 1:5 v/v dendrimer solutions to blood to make the final, specified concentrations. After this time, the mixtures were centrifuged at 1,000 rpm for 5 min and the supernatants were removed and read with a spectrophotometer at the wavelength of 413 nm. The results were compared to the positive control (0.1% v/v Triton-X100 in PBS) which causes 100% hemolysis [15].

2.2.13 PT and APTT coagulation assays

The dendrimers at different concentrations were tested for their impacts on coagulation processes. PT (prothrombin time) test is used to investigate the extrinsic and common coagulation pathways. On the other hand, APTT (activated partial thromboplastin time) assay is considered useful for the assessment of the intrinsic and common coagulation pathways. To perform the experiments, different concentrations of the dendrimer solutions in PBS were incubated for 5 min before adding the kit reagents. Once the tissue thromboplastin was added to the anti-coagulated blood samples, the required times for formation of fibrin clots were measured by visual inspections [9]. The study results were expressed in seconds as compared to the negative controls which only contained PBS (1:9; i.e. the ratio of dendrimer solutions to the blood samples).

2.2.14 Microscopic observation

After 24-h incubation of the dendrimers with HT1080 cell line, changes in cell morphology were observed using an inverse phase contrast microscope (HUND WETZLAR, Wilovert^R).

2.2.15 Statistical analysis

All the experiments were conducted in triplicate ($n = 3$). One-way ANOVA and Post-hoc studies were performed

for mean and multiple comparisons with SPSS[16] software, respectively. The results were reported as significant for $P < 0.05$ or highly significant for $P < 0.01$.

3 Results

3.1 Potentiometric titration

The results gained for the determination of the number molecular weights via potentiometric titration method were in accordance with the previous NMR spectroscopic data for the MW calculations. Based on the results, the approximate MWs of 1,000 and 2,000 Da (number MW) were calculated (Table 1) for the G1 and G2, respectively. The chemical structures and the synthesis method of the dendrimers are drawn in Fig. 1.

3.2 Size and zeta potential distributions

As can be observed from the data in Table 2, there were marked changes in the sizes and zeta potential distributions of the dendrimers with the concentration rates ranging from 0.1 to 1 mg/ml. The more the concentration is, the larger the size and the greater the poly dispersity in both of the dendrimers will be. The zeta potential measurements for the G1 at various concentrations showed an increment by increasing the concentration up to 1 mg/ml. In contrast, the opposite behavior was seen for the G2 when the concentration was raised. (Complementary file about the original diagrams can be found in the supplementary Fig. 1.)

3.3 Degradation study

As can be drawn from Fig. 2, there can be seen osmotic pressure changes (mosmol/kg) when the concentrations and pHs of the G1 and G2 were changed. The G1 showed the maximum osmotic pressure change when the concentration was 1 mg/ml, and the pH was about 7.4 (32.6% relative to initial osmotic pressure at time zero). In other concentrations (0.5 and 2 mg/ml) and pH (5.4), a relative change between 11 and 15% was observed. The maximum change for the G2 was seen when the concentration was 2 mg/ml, and pH was 7.4 (38.3%). At the other concentrations (0.5 and 1 mg/ml) and pH (5.4), the relative changes were between 13 and 19%.

Table 1 Potentiometric titration of the dendrimers in order to determine the number MWs

Dendrimers	G1	G2
Amount of NaOH (ml)	≈3.6 ml	≈3.6 ml
Approximate MWs	≈1,000 Da	≈2,000 Da
pKa_3	$pKa_3 \approx 6.4$	$pKa_3 \approx 6.5$

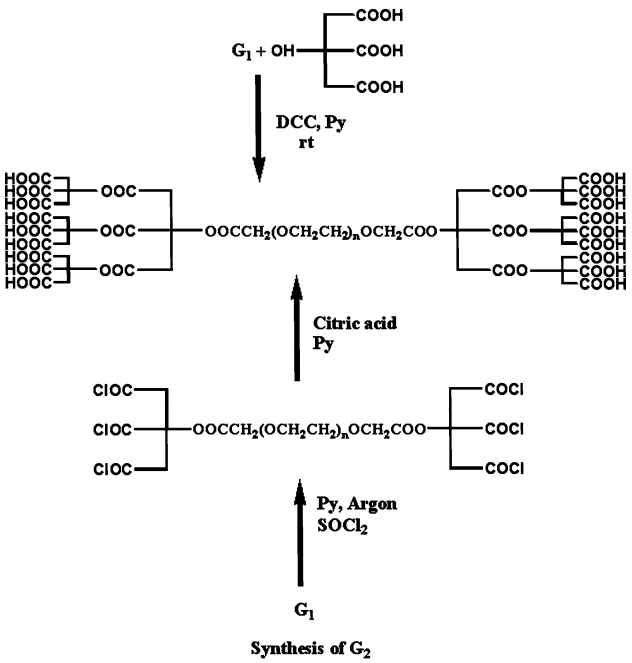

Fig. 1 The synthesis method and chemical structures of the dendrimers (G1, G2)

3.4 Crystal violet viability assay

As observed in Fig. 3, after 24-h incubation of the cells with different concentrations of the dendrimers from 0.0125 to 1 mg/ml, there was a rise in the cellular growth (i.e. functional adherent cells) for the G1 at the concentrations of 0.125 mg/ml (112%, $P < 0.05$, relative to the negative control), 0.250 mg/ml (122%, $P < 0.01$), and 0.5 mg/ml (109%, $P < 0.05$). The fall in the functionality of the cells was observed at the concentration of 1 mg/ml (66%, $P < 0.01$). Any significant difference ($P > 0.05$) was not seen in the cellular functionality for the G1 at the concentration of 0.0125 mg/ml. After 24 h of treatment, there were not any significant changes in the amounts of cellular viability for the G2 over the concentration ranges versus the negative control culture cells except at the concentration of 0.0125 mg/ml. This concentration of the G2 (0.0125 mg/ml) caused a lessening (92%, $P < 0.05$) in the cellular growth. After 48 h of treatment period, a marked growth in the cellular viability was observed for the G1 at the concentrations of 0.0125 mg/ml (120%, $P < 0.01$), 0.125 mg/ml (108%), and 0.250 mg/ml (116%, $P < 0.05$). On the other hand, a diminution was seen in the growth power of the cells at the concentrations of 0.5 mg/ml (85%, $P < 0.05$) and 1 mg/ml (40%, $P < 0.01$) in comparison with the negative control. The G2 showed a

Table 2 Size and zeta potential distributions of the dendrimers at two different concentrations (0.1 and 1 mg/ml)

Dendrimer generations	Size distribution (0.1 mg/ml)	Size distribution (1 mg/ml)	Zeta potential (0.1 mg/ml)	Zeta potential (1 mg/ml)
G1	40.3 ± 2.94 nm	4,850 ± 707 nm (14%) 340 ± 163 nm (86%)	−4.72 ± 6.26 mv	−8.70 ± 5.98 mv
G2	33 ± 2.03 nm	812 ± 225 nm (92.3%) 176 ± 28.1 nm (7.7%)	−31 ± 4.21 mv	−20.6 ± 3.58 mv

The size and zeta potential distributions of either dendrimer were given in the percentage and variance of each main peak

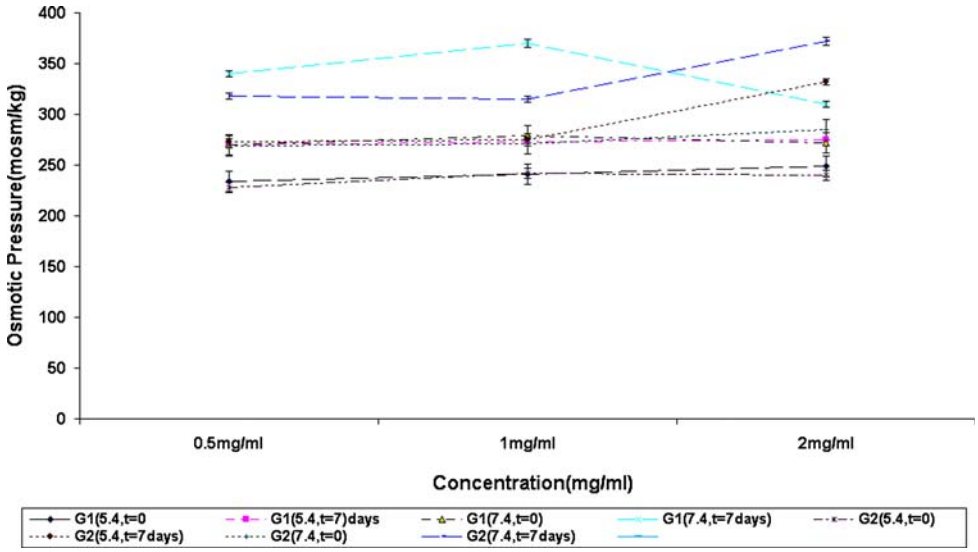

Fig. 2 The changes in the osmolality of the dendrimers in two different pHs were measured during the experiment time. Mean ± SE of the data ($n = 3$) are shown for each point of the graphs

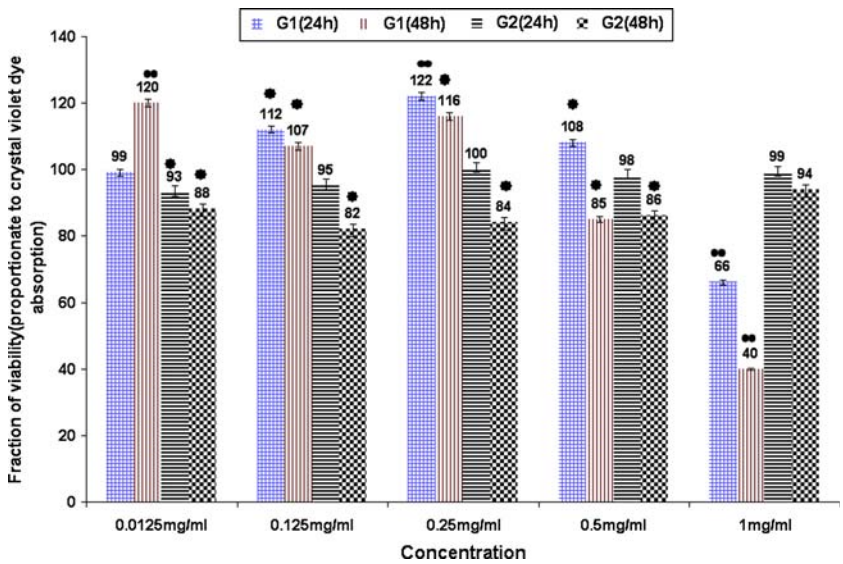

Fig. 3 The impact of the concentration, time, and generations of the dendrimers (G1, G2) on the functionality of HT1080 cell line (CVS assay) after 24 and 48 h of incubation times. Mean ± SE ($n = 3$) of the data are presented for each bar $^{*}P < 0.05$ (significant), $^{**}P < 0.01$ (highly significant) versus the group of the negative control (only culture media)

significant ($P < 0.05$) reduction in the growth at the concentrations of 0.0125 mg/ml (88%), 0.125 mg/ml (82%), 0.250 mg/ml (83%), and 0.5 mg/ml (86%). No significant change ($P > 0.05$) was noticed in the cellular viability for the G2 at the concentration of 1 mg/ml as compared to the negative control.

3.5 MTT assay

Based on MTT results drawn in Fig. 4, a time- and concentration-dependent viability behavior was seen for the dendrimers after 24 h of treatment. The G1 showed a significant rise in the cellular growth (i.e. mitochondrial

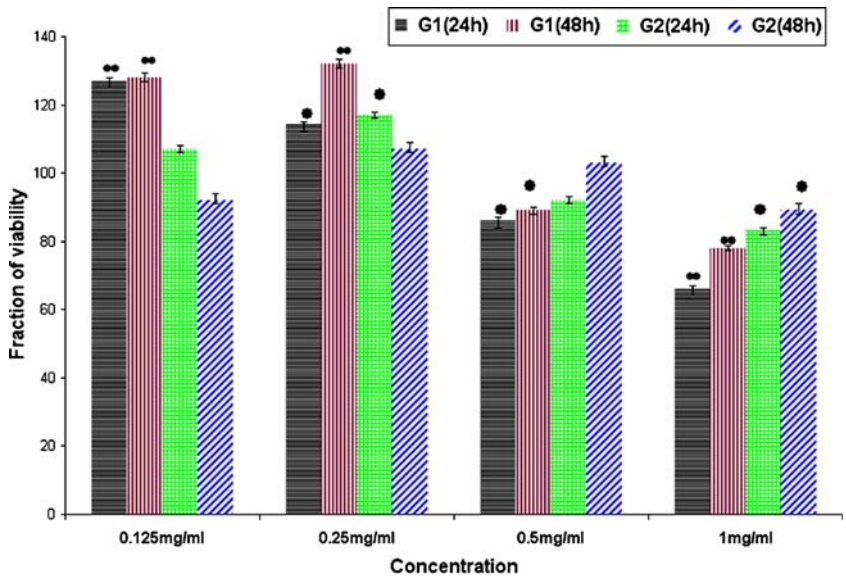

Fig. 4 The influence of the concentration, time, and the generations of the dendrimers (G1, G2) on the viability of HT1080 cell line (MTT assay) after 24 and 48 h of treatments. Mean ± SE ($n = 3$) of each group are calculated for each bar $^{*}P < 0.05$ (significant), $^{**}P < 0.01$ (highly significant) versus the group of negative control (only culture media)

activity) at the concentrations of 0.125 mg/ml (127%, $P < 0.01$, as compared with the negative control) and 0.250 mg/ml (114%). A decrement was observed in the mitochondrial reductive power of the cells at the concentrations of 0.5 mg/ml (86%, $P < 0.05$) and 1 mg/ml (66%, $P < 0.01$). For the G2, a significant enhancement was seen in the mitochondrial activity of the cells at the concentration of 0.25 mg/ml (117%), but a fall in the mitochondrial function was viewed at the concentration of 1 mg/ml (83%). No significant changes were found in the cellular growth for the G2 at the other concentrations as compared to the negative control cells. After 48 h of incubation period, a rise was noticed in the mitochondrial activity of the cells for the G1 at the concentrations of 0.125 mg/ml (128%, $P < 0.01$) and 0.250 mg/ml (132%, $P < 0.01$) as compared with the negative control. There was a significant fall in the growth power of the cells for the G1 at the concentrations of 0.5 mg/ml (88%, $P < 0.05$) and 1 mg/ml (78%, $P < 0.01$) versus the negative control. Meanwhile, the G2 did not show any significant statistical differences ($P > 0.05$) in the growth ranging up to the concentration of 0.5 mg/ml versus the negative control cells. On the other hand, a significant reduction (88%) in the mitochondrial activity was observed for the G2 at the concentration of 1 mg/ml.

3.6 LDH assay

On the basis of LDH assay data shown in Fig. 5, a concentration-dependent toxicity behavior for both of the dendrimers was seen. Decreased significant levels ($P < 0.05$) of LDH in the supernatants were observed for the G1 and G2 at the concentration ranging between 0.01 and 0.1 mg/ml versus the DMSO control (the absorbance of samples/the absorbance of DMSO ~80%). Almost the same LDH levels for the dendrimers were detected at the concentration of 0.5 mg/ml as compared to the DMSO control ($P > 0.05$). Increased levels of LDH ($P < 0.01$) were observed for the G1 and G2 at the concentrations of 1 mg/ml (154%, 128%) and 2 mg/ml (183%, 152%) as compared with the DMSO control, respectively. Totally, the G1 was more toxic than the G2 at the concentrations of 1 mg/ml and above.

3.7 Apoptosis–necrosis assays

At the end of the treatment of the THP-1 cell line with the dendrimers at two nontoxic concentrations, both apoptosis and necrosis mechanisms were found to have contributed to cell death (Fig. 6). Also, a concentration-dependency was observed for both processes. The amount of necrosis—necrosis and also apoptosis ≤2%—was low; as a result, lower unwanted inflammatory reactions would be seen due to the little amount of necrosis in the body. These observations were in accord with the other cell toxicity data.

3.8 TNF-α and IL1-β secretion assays

TNF-α (tumor necrosis factor alpha) and IL1-β (interleukin 1 beta) are pro-inflammatory cytokines which are secreted from the cells upon facing trauma or infection. These cytokines are regulators of inflammation and apoptosis processes. The unfavorable release of these cytokines can cause subsequent inflammation and tissue destructions, which will not be good indicators for the biocompatibility issues of materials. Based on the results of TNF-α release in Fig. 7, no significant differences in the TNF-α release

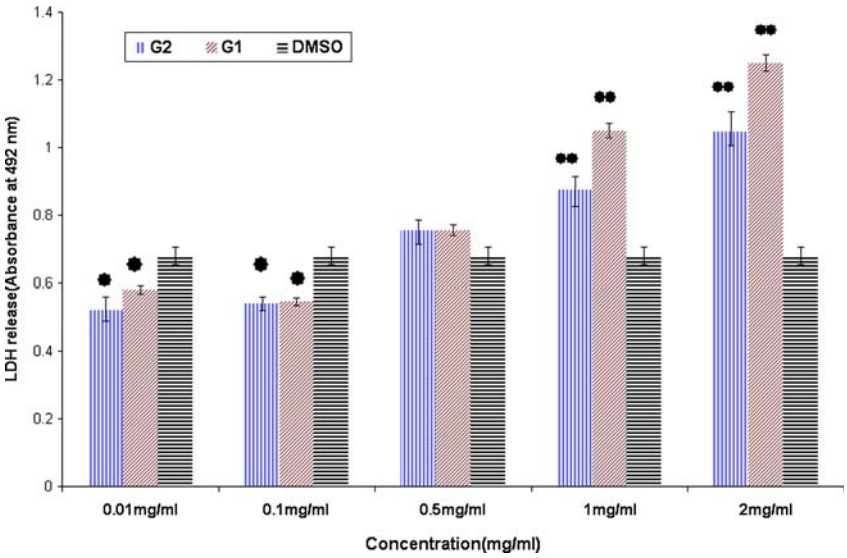

Fig. 5 The influence of the concentration and generations of the dendrimers (G1, G2) on cell membrane integrity (LDH assay) after 24-h incubation period in comparison with DMSO (0.1% v/v) as the negative control. Mean ± SE ($n = 3$) of the data are presented for each bar $^{*}P < 0.05$ (significant), $^{**}P < 0.01$ (highly significant) versus the DMSO control

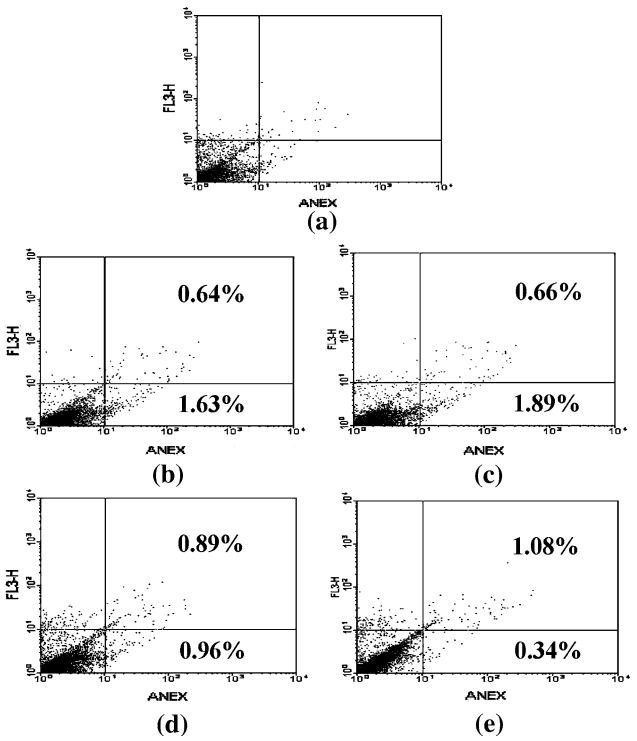

Fig. 6 Dot plots of AnnexinV-FITC/PI staining of HT1080 cells. Three phenotypes can be observed: viable (*lower left quadrant*); apoptotic (*lower right quadrant*), and necrotic or secondary apoptotic (*upper right quadrant*). **a** Untreated cells; **b** cells treated to 0.25 mg/ml of the G1; **c** cells treated to 0.5 mg/ml of the G1; **d** cells treated to 0.25 mg/ml of the G2; **e** cells treated to 0.5 mg/ml of the G2 after 24-h incubation time

were seen up to the concentration of 0.250 mg/ml for the G1 and G2 as compared to the negative control. A little increment (significant, $P < 0.05$) in the secretions of TNF-α was seen at the concentration of 0.5 mg/ml for the G1 (i.e. ~10 pg/ml) and G2 (i.e. ~12 pg/ml) as compared to the negative control (~6 pg/ml). In Fig. 8, the amounts of IL1-β secretions for both dendrimers were equal to those of the negative control even at the higher concentration (0.5 mg/ml).

3.9 Hemolysis assay

As seen in Fig. 9, a time- and concentration-dependent behavior was observed for both of the dendrimers. After 1 h of incubation, the amounts of hemoglobin release for the G1 and G2 were less than 2% (the absorbance of the samples/the absorbance of Triton-X100) up to the concentration of 1 mg/ml relative to the positive control (Triton-X100, 0.1% v/v). At the concentration of 2 mg/ml the amounts of hemoglobin release for both of the dendrimers were less than 13% (G1, 13%; G2, 11%) as compared with the positive control. At the end of 6-h treatment with the dendrimers, the amounts of hemoglobin release from the erythrocytes were less than 4% up to the concentration of 0.1 mg/ml for both dendrimers while the hemoglobin released from the erythrocytes were about 16% for the G1 and 8% in the case of the G2 at the concentration of 1 mg/ml. Moreover, the amounts of hemoglobin release were about 29% for the G1 and 21% for the G2 at the concentration of 2 mg/ml as compared with the positive control. Totally, the G1 was more hemolytic than the G2 at the concentrations of 1 mg/ml and above.

3.10 PT and APTT assays

According to PT coagulation data in Fig. 10, no significant ($P > 0.05$) differences in PT times for the G2 at both concentrations and for the G1 at the concentration of 0.5 mg/ml were observed. At the concentration of 1 mg/ml, the PT time for the G1 was more than 60 s ($P < 0.01$), which indicates

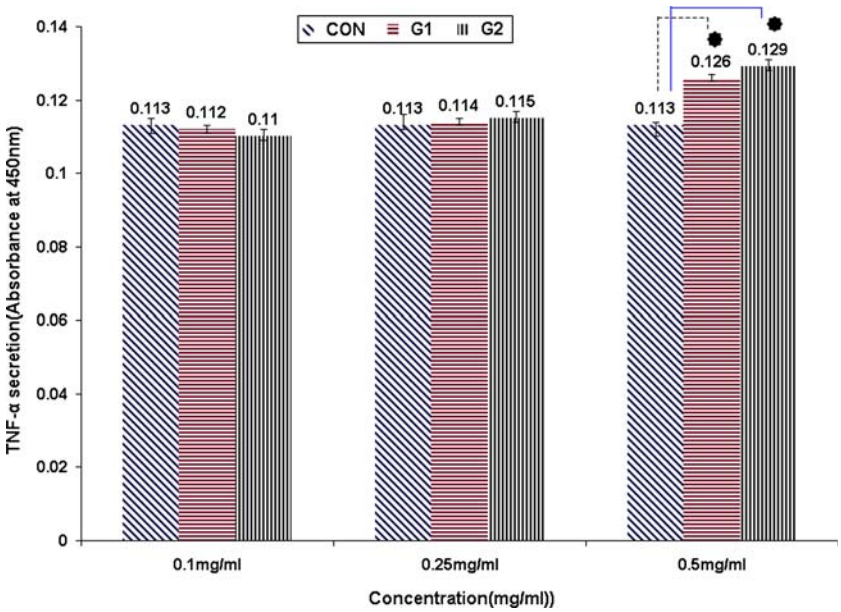

Fig. 7 The amount of TNF-α secretion from the THP-1 cell line at different concentrations of the dendrimers versus the negative control which contained only culture media after 24 h. Mean $\pm$ SE ($n = 3$) of the data in each group are presented for each bar $^*P < 0.05$ (significant) versus the negative control

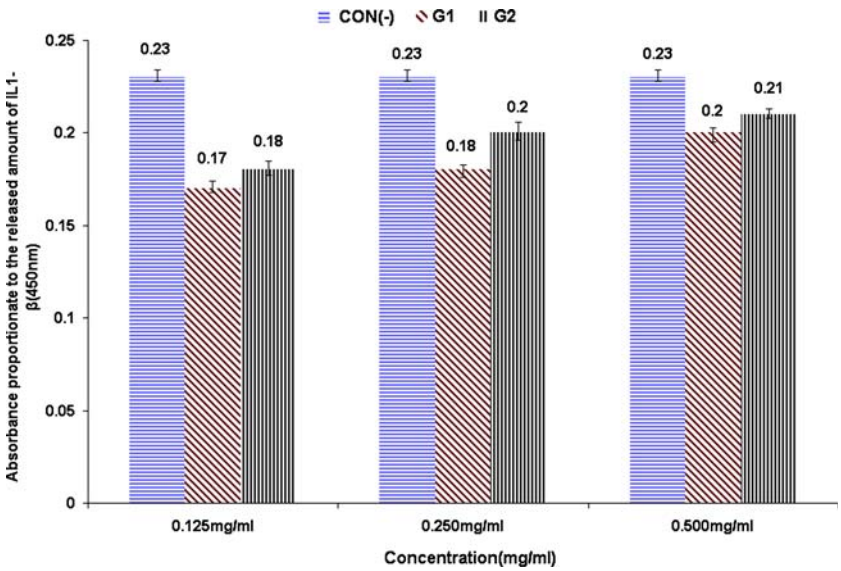

Fig. 8 The secretion of IL1-β from THP-1 cell line at different concentrations of the dendrimers compared to the negative control which contained only culture media after 24 h of treatment. Mean $\pm$ SE ($n = 3$) of the data are shown in each bar. There were no statistical differences between the dendrimers and the negative control at both concentrations

that this concentration of the G1 had a negative effect on the coagulation pathway. According to the APTT data which are shown in Fig. 11, no harmful effects were seen for both dendrimers at the higher concentration (1 mg/ml) as compared to the negative control. In contrast, at the lower concentration (0.5 mg/ml) the G1 showed less coagulation time about 47 s, and the G2 exhibited a higher APTT coagulation time about 59 s ($P < 0.01$) in comparison with the negative control, i.e. 51 s.

3.11 Microscopic observation

Any detaching cells (HT1080) or unnatural changes were not seen up to the concentration of 0.5 mg/ml for both dendrimers while at the concentration of 1 mg/ml small, condensed, and unnatural cells were observed especially for the G1, which could be a sign of apoptosis (Fig. 12).

4 Discussion

According to the degradation results, it is certain that there is production of more new particles due to the hydrolytic degradation process during the experimental condition which leads to the changes in the initial osmolality. Therefore, it would be reasonable to say that the dendrimers are also biodegradable in enzymatic conditions inside the cell environment. Consequently, they may be metabolized and excreted by the other detoxifying processes in the living organisms [16]. As a result, there can not be the risk

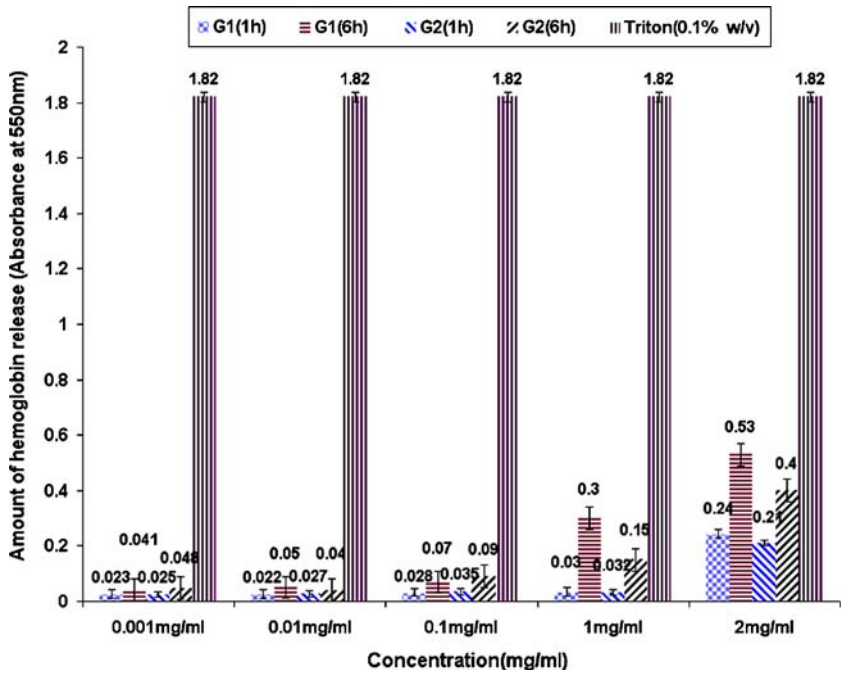

Fig. 9 The effects of different concentrations, times, and generations of the dendrimers (G1, G2) on hemoglobin release from the erythrocytes after 1 and 6 h of incubation. Mean ± SE ($n = 3$) of the data in each group are shown in each bar. There were highly significant statistical differences ($P < 0.01$) between the dendrimers at all concentrations and the positive control group. Triton-X100 (0.1% v/v) was considered as the positive control

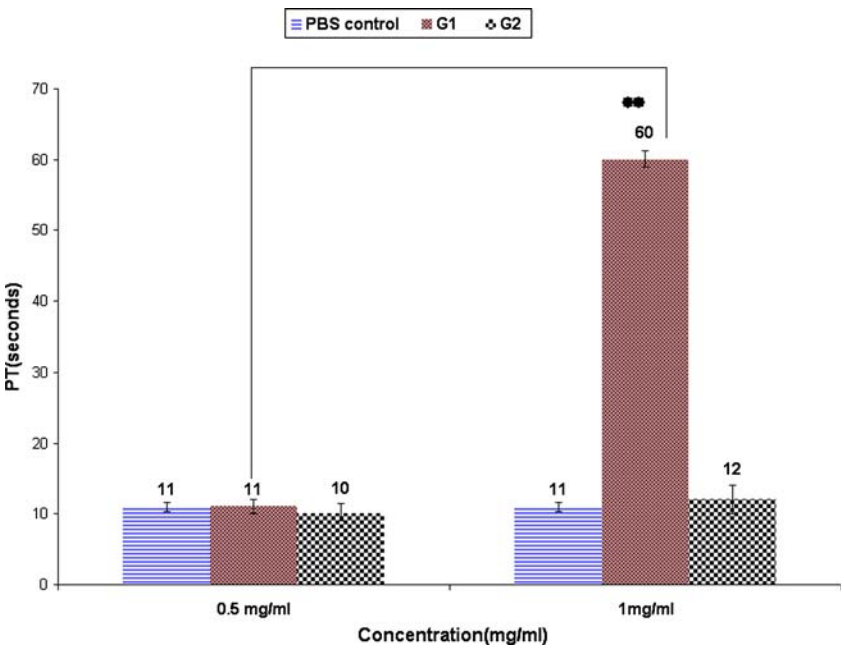

Fig. 10 The impact of the concentration and generations of the dendrimers on PT time. Mean ± SE ($n = 3$) of the data are drawn in each bar. The connector line shows two groups with statistical significance (post-hoc study) $^{**}P < 0.01$ (highly significant) versus the negative control

of unfavorable toxic accumulations of these materials in the cells and organisms as well. Almost the same parallel cytotoxicity results were observed for the dendrimers at different times and concentrations based upon the in vitro cytotoxicity assays like CVS, MTT, and LDH assays. Generally, the results confirmed each other harmoniously; that is to say, a nontoxic concentration up to the concentration of 0.5 mg/ml was achieved for both dendrimers. On the whole, the G1 was more toxic than the G2 at the concentrations of 0.5 mg/ml and above in most of the experiments except in some studies and concentrations which variable outcomes were seen. For example, after 24 h of incubation according to CVS assay, no toxicity was observed for the G1 up to the concentration of 0.5 mg/ml in comparison with the negative control (even a cellular growth about 108% was seen at this concentration); but based on MTT assay, a reduction in viability about 86% was found for the G1 as compared with the negative control. Similar cytotoxicity results were obtained for the G2 based upon MTT and CVS methods except at the

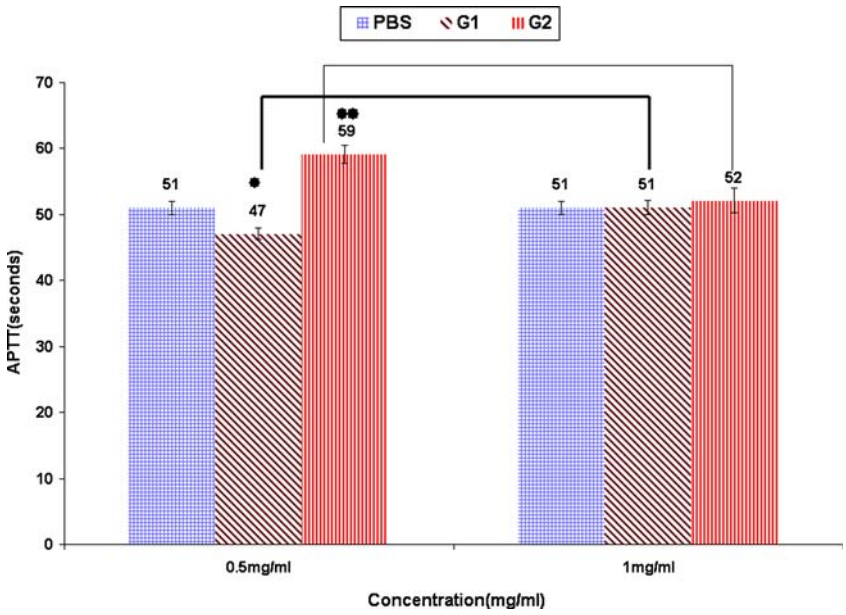

Fig. 11 The influence of the concentration and generations of the dendrimers on APTT time. Mean ± SE ($n = 3$) of the data are depicted in each bar, the two connector lines show the two groups with statistical significance (post-hoc test) $^{*}P < 0.05$ (significant), $^{**}P < 0.01$ (highly significant) versus the negative control

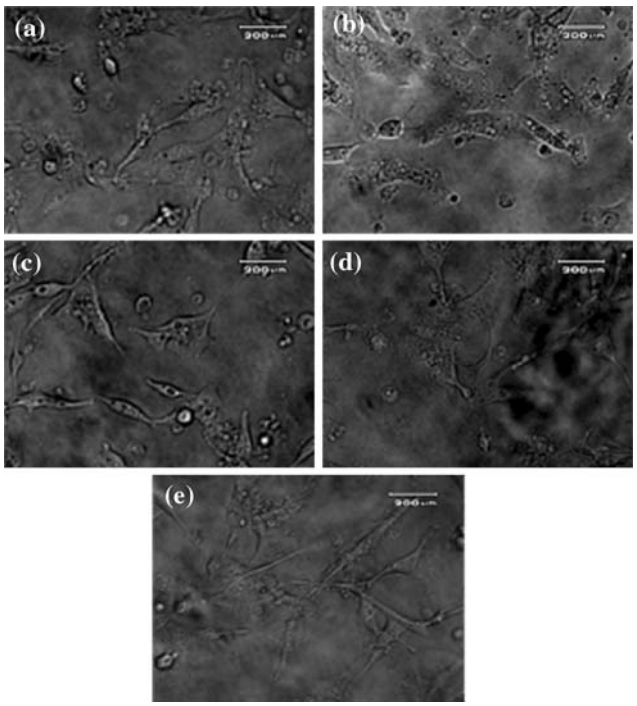

Fig. 12 The impact of concentration and generations of the dendrimers on the morphology of the HT1080 cells after 24-h incubation. **a** G1 (0.5 mg/ml); **b** G1 (1 mg/ml); **c** G2 (0.5 mg/ml); **d** G2 (1 mg/ml); **e** untreated control

concentration of 1 mg/ml which viabilities around 83% and 99% were found for the dendrimer according to the assays, respectively. After 48 h of treatment, based on MTT assay, a growth around 10% in the mitochondrial reductive power of the cells was observed versus 24-h treatment for the G1 and G2 at all concentrations. On the contrary, a decrease in the cellular growth about 10% was seen at the concentrations of 0.25 mg/ml and above for the G1 and G2 based on CVS assay. These differences in the cell viability at the mentioned concentrations and times between CVS and MTT assays can be elucidated based on the following explanations. Based on MTT assay an increase about 10% in the mitochondrial activity was noticed after 48 vs. 24 h of incubation. Yet, this seemingly increased viability must not be extended to the good functionality of the cells only based on the increment in the mitochondrial activity. This can be due to the malfunction of the cell respiratory system (reductive strength) which finally may lead to cell death. Also, given the fact that CVS method counts only the number of adherent viable cells which are able to uptake the dye, it seems logical to assume some relations between CVS assay and mitochondrial activity indirectly (functionality or malfunction). But, the results of either method must not be regarded to be completely identical. Thus, the other parameters must also be taken into account for the viability power of the cells. The more toxicity of the G1 than that of G2 at the concentrations of 0.5 mg/ml and above in most of the experiments can be explained by some reasons and hypotheses which were also mentioned for the other polymers with different structures. These hypotheses were corroborated by our observations as well. First, the G2 had a more globular and possibly less flexible structure than the G1 which causes a less likelihood of harmful interaction sites with the cell membrane components [17]. Secondly, the G2 showed a more net negative charge—as an example, G2, −20.6 mv, 1 mg/ml vs. the G1, −8.70 mv, 1 mg/ml—than the G1 which generally leads to more electrostatic repulsion between the negative charge of the cell membrane and the G2 negative surface charge. Hence, less possible harmful

interactions with the positive parts of the cell membrane components are formed, as were mentioned for the other dendrimers [18, 19]. Moreover, the G2 displayed less poly dispersity in the size than the G1 according to the dynamic light scattering data when the concentration was varied from 0.1 to 1 mg/ml. This condition may make a more homogeneous system for the G2 which will be interacting with the cell compartments more evenly. Considering this homogeneity, the cells can adapt themselves more easily with this new compartment. In other words, it can be detoxified or utilized gradually as a source of energy in comparison with the more heterogeneous structure/compartment, i.e. the G1 [20]. Based on apoptosis–necrosis assays at nontoxic concentrations, it was observed that both of the mechanisms (apoptosis and necrosis) had contribution to cell death like some other nanoscale structures [21]. Moreover, a time- and concentration-dependent behavior was seen for them like some of the other polymeric structures [22]. The amount of apoptosis for the G1 was a little more than that of G2 (two folds); in contrast, the percentage of necrosis for the G2 was a little more than that of G1. Yet, the quantities were very low, and these low degrees, especially low quantity of necrosis, are good marks for biocompatibility issues. So, there would be less chance of unwilling inflammatory reactions following the necrosis process. The induced secretions of TNF-α and IL1-β cytokines from THP-1 cell line were very low, which were in agreement with the other toxicity results. In consequence, subsequent inflammation and cell damage following the use of these materials can be very negligible. This would be another advantage of the biocompatibility of these structures [14]. Hemolysis assay showed no significant hemoglobin release for both dendrimers up to the concentration of 1 mg/ml after 1 and 6 h of incubation. Overall, the G1 was more hematotoxic than the G2 at the concentrations of 1 mg/ml and above, which was in accordance with the other cell toxicity data. This behavior might be due to the similar impacts of these structures on the membrane of the cells or the erythrocytes at these concentrations. Only after 6 h of treatment, a significant hemoglobin release was observed for the dendrimers which could be due to the more direct physicochemical interactions with the red blood cells over the time. Coagulation tests showed a change in APTT time, i.e. the intrinsic and common coagulation pathways, for both dendrimers at low concentration (0.5 mg/ml) in comparison with the high concentration (1 mg/ml). These effects could occur more as a matter of molecular shape or topological structure than as a matter of concentration only. Based on the APTT results the G1 displayed a pro-coagulatory impact (47 vs. 51 s for the negative control), and the G2 showed an anti-coagulation effect like sodium citrate (as a chelator). No effects on extrinsic and common pathways (PT time) were detected for the G2 while an anti-coagulation impact (60 s) was seen for the G1 at the concentration of 1 mg/ml. These observations show that each dendrimer has its own complex and different mechanisms on either of the two coagulation pathways. To put it totally, based on the in vitro experiments a concentration that ranges up to 0.5 mg/ml could be considered safe and biocompatible. However, complementary in vitro and in vivo studies will confirm our outcomes and clarify the toxicity profiles of these materials more for further potential usages of these materials in various fields of nanomedicine in the future.

5 Conclusion

Regarding the results obtained from all the in vitro cytotoxicity and hematotoxicity assays, a nontoxic concentration range up to 0.5 mg/ml was found for both dendrimers. However, according to APTT assay, a negligible anti-coagulation impact for the G2 (59 s vs. the control, i.e. 51 s) and a little coagulatory effect for the G1 (47 s vs. the control 51 s) were observed at the concentration of 0.5 mg/ml. It was expected for the dendrimers to show such nearly high biocompatibility since the dendrimers were composed of two known biocompatible components—that is, PEG and citric acid. Thus, it is hoped that with the aid of these outcomes the optimized, safe, and biocompatible dendrimers be screened and synthesized—for the potential applications of these structures in the different fields of biomedicine and drug delivery systems. Besides the experiments carried out, it should be taken into account that additional in vitro and in vivo studies will reveal the toxicity profiles of these dendrimers with greater details and pave the way for further applications of these structures in biomedical sciences.

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