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Stealth dendrimers for drug delivery: correlation between PEGylation, cytocompatibility, and drug payload

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Abstract It is advantageous to utilize low generation polyamidoamine (PAMAM) dendrimers for drug delivery because low generations (generation 4.0 or below) have more biologically favorable properties as compared to high generations. Nevertheless, modification of low generation dendrimers with PEG to create stealth dendrimers is still necessary to avoid potential side effects by long term accumulation. However, low generation dendrimers have much fewer surface sites for drug loading as compared to higher generations. To efficiently utilize low generation dendrimer-based stealth dendrimers for drug loading, PEGylation needs to be optimized. In this study, we synthesized a series of stealth dendrimers based on low generation Starburst™ PAMAM dendrimers (i.e., G2.5, G3.0, G3.5, and G4.0) and quantitatively assessed PEGylation efficacy in modulating cytocompatibility of low generation PAMAM dendrimers. Cytocompatibility of stealth dendrimers was examined using endothelial cells. The results showed that PEGylation degree on low generation dendrimers could be dramatically reduced to leave as many unoccupied surface groups as possible for drug loading, while maintaining the drug carrier cytocompatibility. 3PEGs-G3.0 and 10PEGs-G4.0 were considered initially optimized stealth dendrimers that would be further modified to deliver drugs of interest. Correlation of PEGylation, cytocompatibility, and drug payload allowed us to optimize low generation dendrimer-based stealth dendrimers for drug delivery and advance the understanding of structure-property relationship of stealth dendrimers.

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1 Introduction

Dendrimers provide an ideal platform for drug delivery as they possess a well-defined highly branched nanoscale architecture with many reactive surface groups [1–5]. Their highly clustered surface groups allow for targeted drug delivery and high drug payload to enhance therapeutic effectiveness. To avoid potential accumulated toxicity and immunogenicity, one approach that has proved successful is to create stealth dendrimers by conjugating biocompatible polyethylene glycol (PEG) to the dendrimer surface [6]. Stealth dendrimers have shown many promising structural advantages. Conjugated PEG not only reduces cytotoxicity and immunogenicity of dendrimers but provides dendrimers with excellent solubility, and favorable pharmokinetic and tissue distribution. Further, the inclusion of PEG as a shell on the dendrimer surface provides a means to protect delivered drugs against enzymatic degradation [7], encapsulate hydrophobic compounds [8], and facilitate gene delivery [6]. It has been well documented that high degree of PEGylation would ensure more acceptable cytocompatibility for dendrimers. For example,

12-branched and 36-branched poly (ethylene oxide) grafted carbosilane (CSi-PEO) dendrimers neither induced haemolysis nor exhibited toxicity at concentrations up to 2 mg/mL [9]. Further, 36-branched dendrimers allowed higher cell viability than the 12-branched dendrimers.

Starburst™ polyamidoamine (PAMAM) dendrimers have been extensively investigated since they were introduced in the early 1980s [10]. It is advantageous to utilize low generation PAMAM dendrimers for drug delivery because low generations (generation 4.0 or below) appear to have relatively low or negligible toxicity and immunogenicity as well as favorable biodistribution [11]. Nevertheless, modification of low generation dendrimers with PEG is still necessary based on the concern that in vivo use of low generation PAMAM dendrimers may still cause side effects due to long term accumulation. However, low generation dendrimers have much fewer surface sites for drug loading as compared to higher generations since the number of surface groups exponentially decreases along with decrease in dendrimer generation. High PEGylation degree would dramatically impair drug loading capacity of low generation dendrimers. Provided PEGylation is solely used to maintain the scaffold cytocompatibility, an optimal drug delivery system by stealth dendrimer should have a minimal degree of PEGylation for maintaining the scaffold cytocompatibility and a maximal drug payload for enhancing therapeutic effectiveness. To efficiently utilize low generation dendrimer-based stealth dendrimers for drug delivery, we synthesized a series of stealth dendrimers based on low generation Starburst™ PAMAM dendrimers (i.e., G2.5, G3.0, G3.5, and G4.0) and quantitatively assessed PEGylation efficacy in modulating cytocompatibility of low generation PAMAM dendrimers. Correlation of PEGylation, cytocompatibility, and drug payload allowed us to optimize low generation dendrimer-based stealth dendrimers for drug delivery and advance the understanding of structure-property relationship of stealth dendrimers.

2 Materials and methods

2.1 Materials

Starburst™ G2.5, G3.0, G3.5 and G4.0 polyamidoamine (PAMAM) dendrimers, methoxypoly (ethylene glycol) (MPEG-OH, MW=2000), *p*-nitrophenyl chloroformate (*p*-NPC), triethylamine (TEA), tetrahydrofuran (THF), dimethylformamide (DMF) and diethyl ether were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Deuterium oxide (D_2O, 99.9%) was purchased from Cambridge Isotope Laboratories (Andover, MA). Fresh distilled water was used throughout the experiment unless specified. Bovine brain microvascular endothelial cells (BBMVECs, passage two), bovine endothelial cell growth medium, attachment factor solution and subculture reagent kit including Hank's balanced salt solution, trypsin/EDTA, and trypsin neutralizing solution were purchased from Cell Applications Inc (CAI) (San Diego, CA). One cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Gaithersburg, MD).

2.2 Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy

^{1}H-NMR spectra were used to characterize the synthesized stealth dendrimers and estimate the actual average number of PEG arms per dendrimer. ^{1}H-NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer. The internal standard was tetramethylsilane. Each sample was dissolved in D_2O, filtered and degassed before measurement. The chemical shift of D_2O is 4.8 ppm.

2.3 Synthesis of stealth dendrimers

The synthesis of stealth dendrimers (#PEGs-G3.0/G4.0, i.e., an average of # PEGs per G3.0 or G4.0 dendrimer) of varying PEGylation degree and dendrimer generation followed the procedures described in our previous work [12, 13]. Particularly, G2.5- and G3.5-based stealth dendrimers were synthesized by coupling homo-bifunctional PEG (MW 2000) to the dendrimer surface [12]. The synthesis of G3.0- and G4.0-based stealth dendrimers was based on heterofunctional MPEG-OH and described below [13]. MPEG-OH (0.4 g) was dissolved in 5 mL of THF. To the THF solution were added 80.6 mg of *p*-NPC and 56 μL of TEA. After the mixture solution was stirred continuously for 24 h at room temperature, the salt that precipitated out of the reaction solution was filtered off. The filtrate was then concentrated and dropwise added to cold ether. The resulting MPEG-P-NPC was collected and purified by reprecipitation from THF into cold ether. MPEG-P-NPC was recovered by rotary evaporation under reduced pressure. To a 10 mL of DMF solution containing 4.8 mg of MPEG-*p*-NPC was added a certain amount of PAMAM dendrimers. After 24-h reaction while stirring at room temperature, the reaction solution was concentrated and then added to cold ether to precipitate out stealth dendrimers. Stealth dendrimers were further purified by extensive dialysis against water.

2.4 Cell culture

Culture and subculture of BBMVECs followed the protocol provided by the supplier. The growth medium was bicarbonate buffered, sterile-filtered, and fully supplemented with fetal bovine serum, growth factors and antibiotics. Basic fibroblast growth factors or platelet derived growth factors were not included in the growth medium. An atmosphere of 5% CO_2 and 95% relative humidity was maintained in the incubator for cell culture. To maximally retain the original morphologic and phenotypic characteristics of the cells, the cells at passage seven or below were used in this study.

2.5 Cytocompatibility study

In vitro cytocompatibility of stealth dendrimers with endothelial cells was investigated. Unmodified dendrimers G2.5, G3.0, G3.5, and G4.0 were used as control. Each well of the 96-well plates was pretreated with 35 μL of attachment factor solution and gently rocked to allow the solution to cover the whole well surface evenly. The plates were subsequently incubated for 30 min at 37 °C before the attachment factor solution was removed. Endothelial cells were seeded at 1,000 cells/well and allowed to grow in 100 μL of growth medium for two days. At the end of the 2nd day, the old medium was replaced with 100 μL of fresh growth medium containing polymers. After 4 h treatment with polymers, polymer-containing medium was completely replaced with 100 μL of fresh growth medium. Then, 10 μL of CCK-8 solution was added and incubated with polymer-treated cells for 2 h. The optical density of each well was read on a LP400 microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA), using a test wavelength of 450 nm and a reference wavelength of 620 nm. Cell viability was calculated as follows:

$$\text{Cell viability}\,(\%) = \frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100$$

where A_{test} is absorbance of a well with cells, CCK-8 solution and polymer solution; A_{blank} is absorbance of a well with medium and CCK-8 solution, without cells; A_{control} is absorbance of a well with cells and CCK-8 solution, without polymer solution. Similarly, cell viability after 12 h and 24 h treatment with polymers was measured using the above procedures.

Morphology of BBMVECs after being treated with polymers was observed on an inverted phase contrast microscope (Zeiss, Germany) at a total 100× magnification. Data are expressed as means ± SEM ($n = 4$). Statistical evaluation of the data was performed by analysis of variance (ANOVA) followed by Student-Newman-Keuls test for pairwise comparison of subgroups. Differences among means were considered statistically significant at a p value of ≤0.05.

3 Results and discussion

Mono-functional PEG (MPEG-OH) was employed to synthesize G3.0- and G4.0-based stealth dendrimers to avoid forming loops and crosslinks on the dendrimer surface. To achieve better coupling efficiency, the hydroxyl group of MPEG-OH was activated first by *p*-NPC to form an active intermediate that readily reacts with a primary amine group. Then, the *p*-NPC-activated MPEG was coupled to G3.0 or G4.0 PAMAM dendrimer through a stable carbamate bond. ^{1}H-NMR spectroscopy was used to examine the resulting stealth dendrimers. According the ^{1}H-NMR spectra, each type of stealth dendrimer showed the same chemical shifts because of the similarity of stealth dendrimers in their structural components. For example, the chemical shifts for 3PEG-G3.0 are: δ 3.67 ppm (PEG, -CH_2-); 2.40 ppm, 2.60 ppm, 2.80 ppm, 2.98 ppm, and 3.26 ppm (PAMAM, -CH_2-). The multiple peaks between 2.2 ppm and 3.4 ppm are assigned to the methylene protons of branching units within the dendrimer. The peak at 3.6 ppm is assigned to the methylene protons in the repeat units of PEG. Methyl protons at the end of PEG have a single peak at 3.4 ppm. The synthesized stealth dendrimers had relatively high purity as evidenced by the absence of interfering proton peaks of the reactants, the intermediates, and the reaction solvent. ^{1}H-NMR spectroscopy has demonstrated accuracy in determining PEGylation degree in our and other researchers' previous work and thereby was applied to determine PEGylation degree. According to the ^{1}H-NMR spectrum, stealth dendrimers of varying PEGylation degree and dendrimer generation, i.e., 3PEGs-G3.0, 8PEGs-G3.0, 10PEGs-G4.0, and 21PEGs-G4.0, were obtained. The impact of PEGylation on cytocompatibility of the stealth dendrimers were evaluated thereafter.

To study impact of PEGylation on cytocompatibility of stealth dendrimers, viability of endothelial cells after being exposed to stealth dendrimers was assessed. Trypan blue is a commonly used reagent to differentiate live cells and dead cells by staining dead cells, but it does not differentiate healthy cells and cells that are alive but losing cell functions. The enzyme-based assays that can provide such information about the functionality of the tested cells are preferred. The MTT assay is the most used method to measure cell viability based on the dehydrogenase activity inside the cell. However, MTT fromazan crystals are not water-soluble, thus requiring further treatment to solubilize the crystals prior to measurement. Moreover, MTT is toxic

to cells. To overcome the limitations of using trypan blue and MTT, we employed a more stable and sensitive cell counting kit-8 (CCK-8) recently developed by Dojindo to measure cell viability. Water-soluble tetrazolium salt-8 (WST-8) in CCK-8 reacts with most of the dehydrogenases in the cell, making the CCK-8 method more sensitive than the MTT assay. In addition, WST-8 does not require further treatment as it produces a highly water-soluble formazan dye during the assay [14, 15].

To compare cytocompatibility with PEGylated dendrimers and that of pure dendrimers, unmodified polyanionic G2.5 and G3.5 PAMAM dendrimers and polycationic G3.0 and G4.0 PAMAM dendrimers were evaluated as control. Endothelial cells were treated with dendrimers of final concentrations 0.2, 2, or 20 μM for 4, 12, or 24 h. Our results showed that G2.5 and G3.5 at 0.2 and 2 μM had negligible cytotoxic effect to endothelial cells up to 24 h. When the dendrimer concentration increased up to 20 μM, we noticed that cell viability dropped in the first 4 h and recovered over the longer incubation period (12-h and 24-h) (Fig. 1). We believe that the cell viability fluctuation during the observation period was because the cells were shocked when the dendrimers were first added and then quickly adapted to the addition of G2.5 or G3.5. The cell viability at 12 h and 24 h was not statistically different from each other. Not surprisingly, polycationic G3.0 and G4.0 PAMAM dendrimers evidently showed time, concentration, and generation-dependent cytotoxicity to endothelial cells, which was also observed in other work. As shown in Fig. 2, high concentration of G3.0 and G4.0 (e.g., 20 μM) and long incubation period (e.g., 24 h) caused significant decrease in cell viability. The same concentration of G3.0 was less cytotoxic than G4.0 to endothelial cells. We also found that G4.0 was most toxic to endothelial cells when its concentration was 20 μM and the incubation period was 24 h. The results were also similar to other previous work [11].

Since G2.5- and G3.5-based stealth dendrimers did not cause any cytotoxic effects to endothelial cells under the conditions we investigated, we only discuss the modulation of cytocompatibility of G3.0- and G4.0-based stealth dendrimers through PEGylation. Both 3PEGs-G3.0 (PEGylation degree 9%) and 8PEGs-G3.0 (PEGylation degree 25%) gained significantly improved cytocompatibility as compared to control according to Fig. 3. Further, the degree of the enhanced cytocompatibility of G3.0 by 3PEGs-G3.0 was comparable to that of G3.0 by 8PEGs-G3.0. Therefore, decreasing PEGylation degree while maintaining dendrimer cytocompatible was possible, by which more surface sites could be reserved for drug loading. The cytotoxicity of G4.0 was also significantly reduced through PEGylation (Fig. 4). Similarly, a low

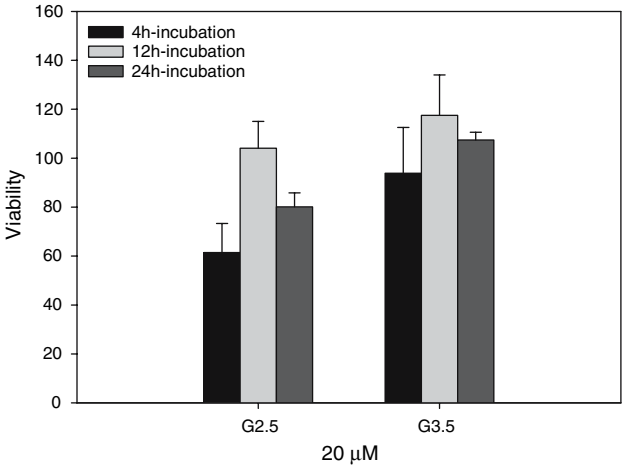

Fig. 1 Viability of endothelial cells after being treated with 20 μM G2.5 and G3.5 after 4-, 12-, and 24 h-incubation

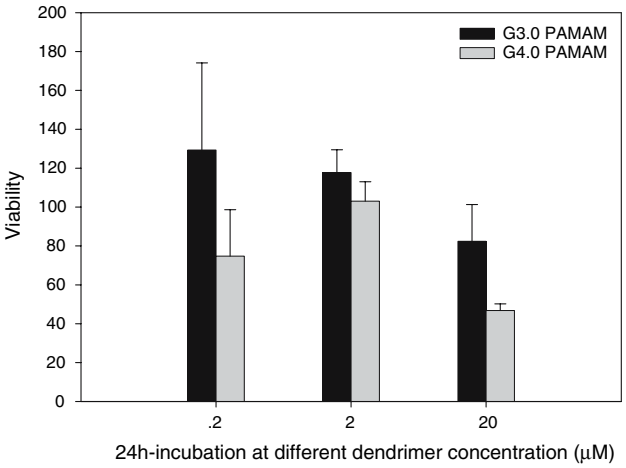

Fig. 2 Viability of endothelial cells after being treated with 0.2, 2, and 20 μM G3.0 and G4.0 after 24 h-incubation

PEGylation degree (i.e. 10 PEGs per G4.0, PEGylation degree 16%) could keep G4.0 cytocompatibility sufficiently. Cell morphology upon addition of dendrimers and stealth dendrimers was monitored. The images of the cells treated with 20 μM of G4.0 and 10PEGs-G4.0 for 4-h, 12-h and 24-h are presented in Figs. 5–7. The images indicate that the cells not having cytotoxic effect spread over; however, the cells having cytotoxic effect became rounded and started to die. The images of cell morphology were consistent with the cell viability results.

In summary, cytocompatibility is one of highly desired features for in vivo applications of dendrimers. Although low generation PAMAM dendrimers are believed to be non-toxic and non-immunogenic, long-term use of dendrimers may lead to accumulation with adverse affects. Therefore, the widely used PEGylation technique was applied to construct stealth dendrimers to enhance cytocompatibility of dendrimers. PEGylation on the dendrimer

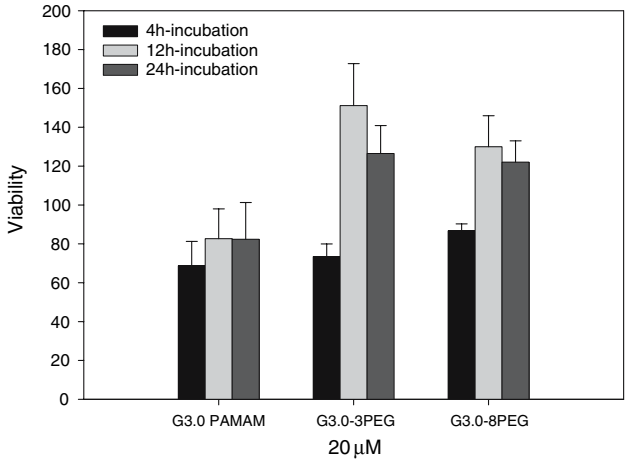

Fig. 3 Viability of endothelial cells after being treated with 20 μM G3.0-based stealth dendrimers after 4, 12 and 24 h incubation

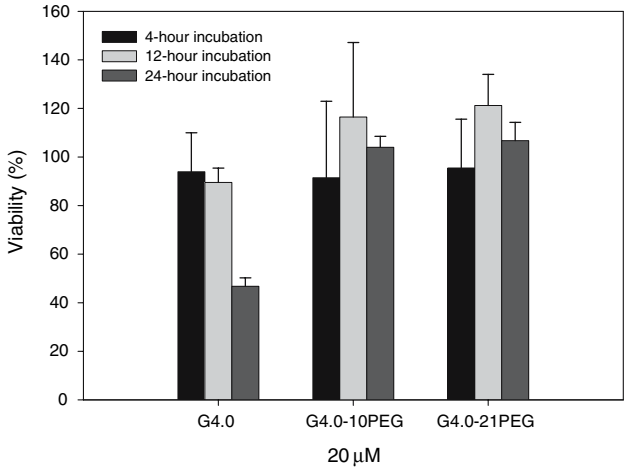

Fig. 4 Viability of endothelial cells after being treated with 20 μM G4.0-based stealth dendrimers after 4, 12 and 24 h-incubation

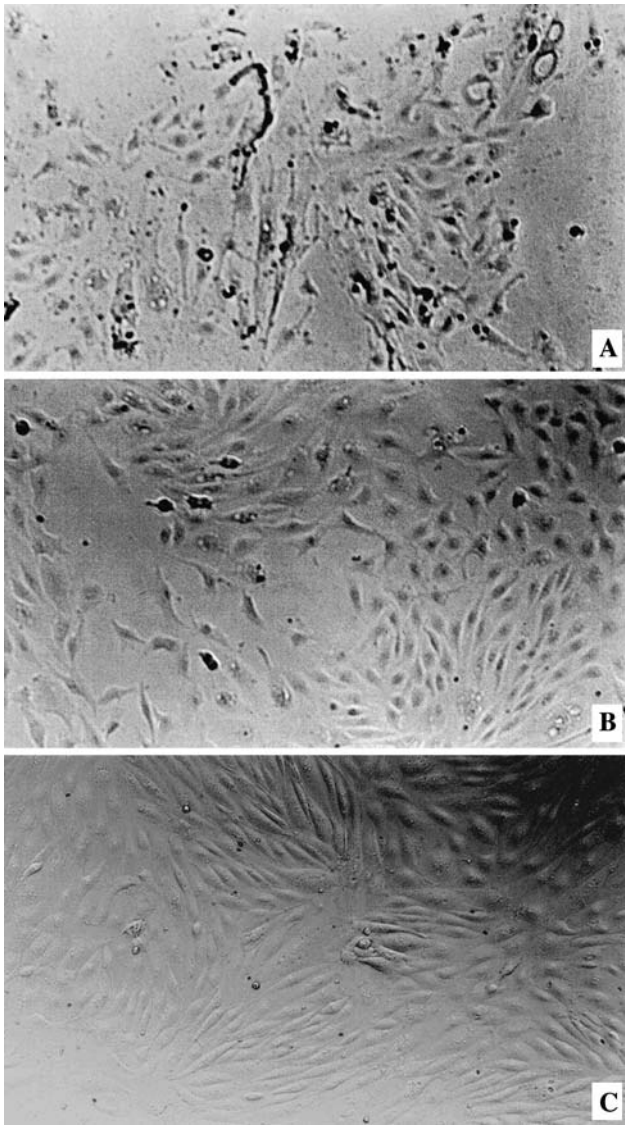

Fig. 5 Phase-contrast microscopic images (original magnification 100×) of endothelial cells after being treated with 20 μM G4.0 (**a**), 10PEGs-G4.0 (**b**), and medium without G4.0 or 10PEGs-G4.0 (**c**) after 4 h-incubation

surface results in the decrease in number of available surface sites for drug loading. A high PEGylation degree significantly reduces the drug loading capacity of low generation dendrimer-based drug carriers as low generation dendrimers don't have as many surface groups as high generation dendrimers. This study demonstrated that PEGylation degree on G3.0 and G4.0 could be dramatically reduced, while keeping the scaffold acceptable cytocompatibility. 3PEGs-G3.0 and 10PEGs-G4.0 were considered initially optimized stealth dendrimers that would be further modified to deliver drugs of interest following the synthetic chemistry methods we proposed previously [7, 12, 13, 16, 17]. It should be noted that prior to clinical application, the drug-containing stealth dendrimers must be examined in a more complex environment vigorously to determine drug release kinetics, biodistribution, and therapeutic efficacy. The cytocompatibility work presented here is the initial step that warrants the use of low generation PAMAM dendrimer-based stealth dendrimers for drug delivery.

4 Conclusions

This study revealed that PEGylation degree on low generation PAMMA dendirmers could be reduced to leave as many unoccupied surface groups as possible for drug loading, while maintaining the drug carrier cytocompatibility. 3PEGs-G3.0 and 10PEGs-G4.0 were considered

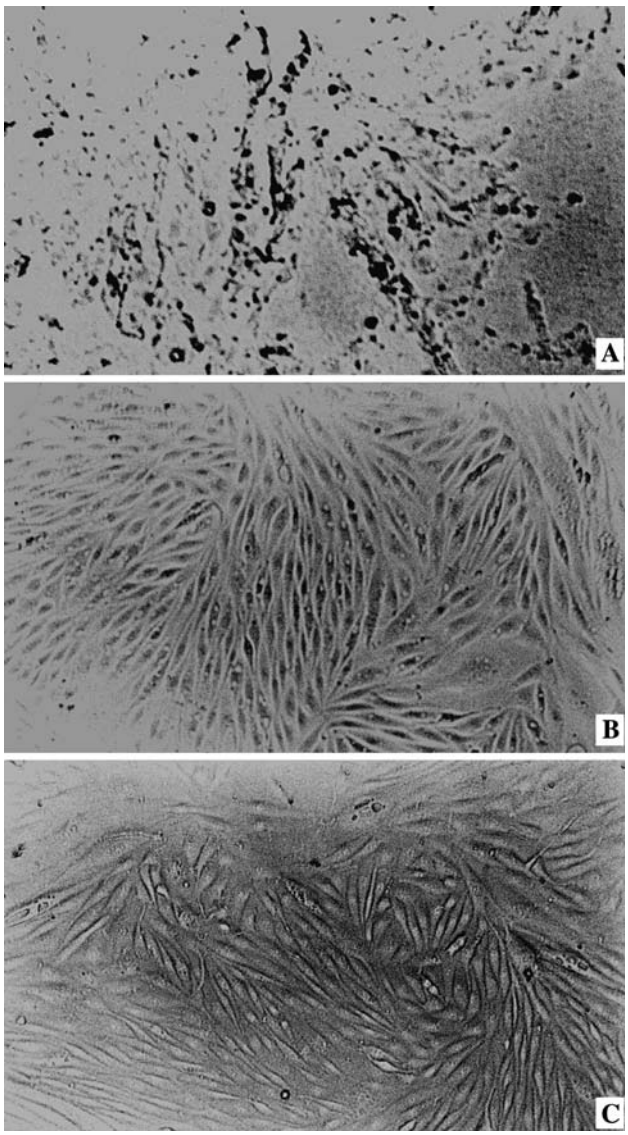

Fig. 6 Phase-contrast microscopic images (original magnification 100×) of endothelial cells after being treated with 20 μM G4.0 (**a**), 10PEGs-G4.0 (**b**), and medium without G4.0 or 10PEGs-G4.0 (**c**) after 12 h-incubation

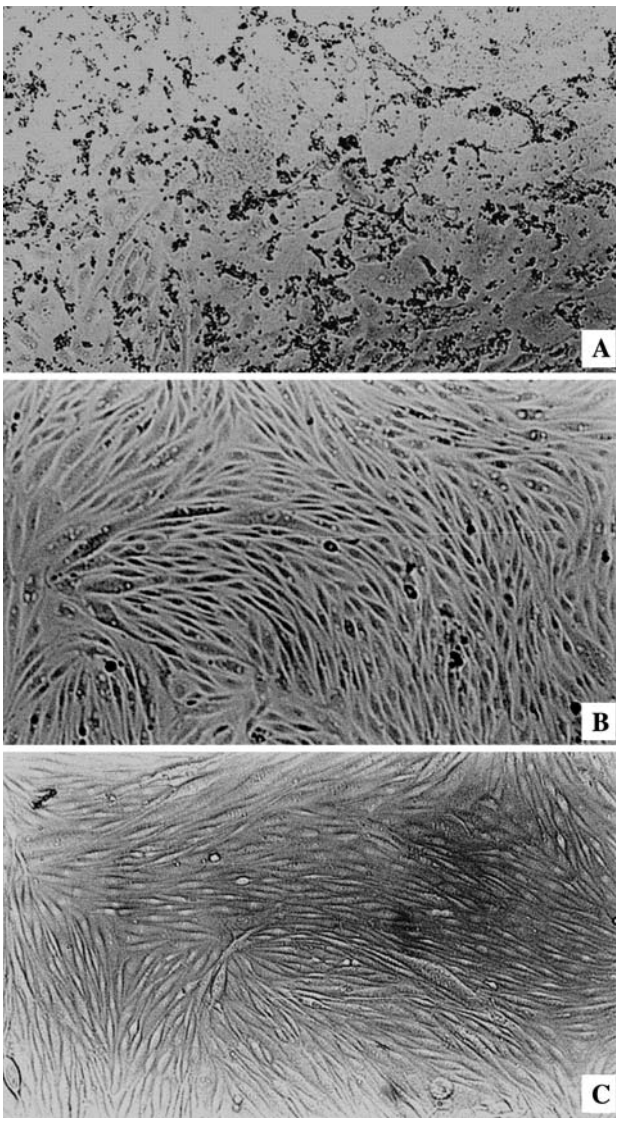

Fig. 7 Phase-contrast microscopic images (original magnification 100×) of endothelial cells after being treated with 20 μM G4.0 (**a**), 10PEGs-G4.0 (**b**), and medium without G4.0 or 10PEGs-G4.0 (**c**) after 24 h-incubation

initially optimized stealth dendrimers that would be further modified to deliver drugs of interest. Correlation of PEGylation, cytocompatibility, and drug payload allows us to optimize low generation dendrimer-based stealth dendrimers for drug delivery and advance the understanding of structure-property relationship of stealth dendrimers.

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