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Cytotoxicity and biodistribution studies on PEGylated EDA and PEG cored PAMAM dendrimers

Mustafa U. Gürbüz[a], Kıvılcım Öztürk[b], Ali S. Ertürk[c], Digdem Yoyen-Ermis[d], Gunes Esendagli[d], Sema Çalış[b] and Metin Tülü[a]

[a]Faculty of Arts and Sciences, Department of Chemistry, Yıldız Technical University, Istanbul, Turkey; [b]Faculty of Pharmacy, Department of Pharmaceutical Technology, Hacettepe University, Ankara, Turkey; [c]Faculty of Pharmacy, Department of Basic Pharmaceutical Sciences, Adıyaman University, Adıyaman, Turkey; [d]Department of Basic Oncology, Hacettepe University Cancer Institute, Ankara, Turkey

ABSTRACT

Starting from *Ethylenediamine* (EDA) or poly(ethylene glycol) tetra amine (4-arm-PEG) cores, two different peripheral methylester (–$COOCH_3$) or amine (–NH_2) PAMAM dendrimers have been synthesized. In the growth phase of dendrimers, two important building blocks, methyl acrylate for the half generation and EDA for the full generations, have been used. In order to improve the yield and decrease the time for the aminolysis step, a microwave-assisted technique was applied. Both of these dendrimers with different cores were grown up to 4.5 generations where surface modification, i.e. PEGylation, with 10% *Poly(ethylene glycol) bis(amine)* was performed. In order to increase the solubility of dendrimers, esteric surfaces were converted to carboxylic acid groups. Accordingly, the dendrimers were soluble in water or in water–methanol mixture which enabled their purification by liquid-phase polymer-based retention in each step. Finally, the resulting products that were characterized with (NMR and FTIR) spectroscopy were evaluated *in vitro* and *in vivo*. The analytical grade dendrimers were not cytotoxic to mouse fibroblasts and their biodistribution was mainly determined in the site of injection (peritoneum), liver and kidneys.

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Dendrimer; PAMAM; PEGylation; cytotoxicity; biodistribution

1. Introduction

Efficacy of therapeutic agents may be limited due to the shortening of half-life and random distribution upon administration to the body.[1] Drug loading into carrier molecules such as nanoparticles, dendrimers, and liposomes has been considered as a solution to overcome these problems.[2,3] Certain types of dendrimers are also accepted as biopolymers which are biocompatible, physically and chemically stable. These easy-to-synthesize macromolecules possess capacity to be loaded with drug molecules and release them in a desired rate and quantity.[4,5] Unlike the classical polymers, functional groups of dendrimers can be modified depending on the biological necessities, so that they can be used as drug delivery

CONTACT Sema Çalış scalis@hacettepe.edu.tr; Metin Tülü mtulu@yildiz.edu.tr
Supplemental data for this article can be accessed here.

vehicles for transdermal or oral routes.[6,7] In recent years, due to multifunctionalities, dendrimers became a great potential for the mentioned purposes.[8] Among variety of dendrimers, poly(amidoamine) dendrimers (PAMAM) are well-defined macromolecules having modifiable surface functional groups and relatively hydrophobic interior cavities providing them with a unique feature to be used for drug delivery purposes.[9–11] One another advantage of PAMAM systems including monodispersity, controllable size, globular shape, improving the solubility of poorly water-soluble drugs, controlling drug release, improving the gene delivery are already known.[12,13]

Especially, the agents with low solubility in water are suitable for the encapsulation at the interior void of PAMAMs; making them available for transportation throughout the body. Accordingly, this drug delivery approach not only provides the target organ and/or tissue with an appropriate amount of therapeutic agents but also reduces systemic toxicity. Full generations of PAMAMs bear primary amino groups at the periphery which are easily *quarternized to get cationic functionality enabling dendrimers'* penetration through the cell membrane and intracellular release of the drug.[14,15] Conversion of ester terminal to carboxylic acid renders the dendrimers less toxic at cellular and systemic levels.[16] Malik et al. [17] have shown that anionic dendrimers were found to be less toxic than cationic dendrimers or not to be toxic against the cells like murine melanoma (B16F10), human lymphoblastic leukemia (CCRF), and human hepatoma (HepG2). At around the same time, Smith et al. [18] have also reported the carboxylic groups on the dendrimers have increased not only solubility of drug but also dendrimers itself. Alternatively, surface groups can also be modified with PEG (approved by Food and Drug Administration, U.S.A), specific peptides or antibodies. [19–21] These surface modifications decrease cytotoxicity, enhance the solubility of drugs and improve the biodistribution of dendrimers.[22,23] After the PEGylation of dendrimers, special ligands (e.g. special antibody, liposomes, etc.) are conjugated to PEG groups, so that drug loaded carriers can be targeted toward tumor tissues with a greater efficiency.[24–26] Therefore, if the drug is successfully encapsulated into PEGylated dendrimers with acid functionality, an ideal polymeric delivery system is theoretically prepared.[27]

EDA-based PAMAMs are of the first generation but later different types of dendrimers such as polymeric-cored PAMAMs have been synthesized.[28] A polymeric core provides greater internal void and retards the steric hindrance for further generations minimizing the starburst effect. In addition, if the selected polymer core is biocompatible, resulting dendrimers can have better reactivity, solubility, and/or amphiphilicity for *in vitro* and *in vivo* usage.[29–31]

Here we report: microwave-assisted [12] synthesis of five-generation EDA and PEG-cored PAMAMs and their PEGylations. We also report their biochemical properties in terms of cytotoxicity and biodistribution for the drug delivery purposes in further studies. So, we determined nontoxic concentrations, effect of PEGylation on cytotoxicity. With this information, we analyzed biodistribution of PEG-cored PEGylated PAMAMs on healthy mice. Advantages of this polymer in addition to the just listed above are found applicable in the active- or passive-targeted drug delivery to liver, kidney, and intraperitoneal organs.

2. Experimental part

2.1. Chemistry

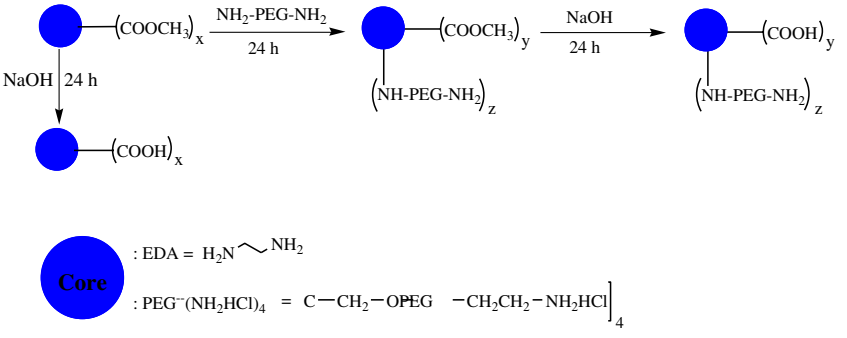
Tetra-amine PEG Mn 5000 (4arm PEG) and PEG bisamine HCI salt Mn 2000 were purchased from JemKem Technology, U.S.A. Methyl acrylate, EDA, n-butanol, NaOH were

purchased from Merck. All other chemicals are analytical grade and used without further purification. liquid-phase polymer retention (LPR) ultrafiltration membranes, Amicon 8000 Stirred Cell, and dialysis membranes having the molecular cut of size (MWCO) 1–10 kDa were supplied from Millipore. The CEM Focused Microwave™ Synthesis System, Model Discover (CEM Corporation, North Carolina, U.S.A) with a continuous microwave power delivery system with operator selectable power output from 0 to 300 W (±30 W) programmable in 1-watt increments, infrared temperature control system programmable from 25 to 250 °C, and 5 to 125 mL vessel capacity was used as microwave reactor. The FTIR spectra (4000–400 cm^{-1}) were recorded with a PerkinElmer Spectrum One in attenuated total reflectance (ATR). nuclear magnetic resonance spectroscopy (NMR) spectra were recorded on a Bruker Avance 500 MHz Spectrometer.

2.2. Synthesis of PAMAMs

EDA and PEG-cored dendrimers (EDAcPAMAM and PEGcPAMAM) first were grown up to 4.5 generations. For the half generations (0.5–4.5), methyl acrylate was used as dendron, same way for the full generations EDA was used to obtain amine-terminated dendrimer. When both cored dendrimers were grown up to 4.5 generations, their surfaces were modified with 10% PEG diamine (Scheme 1).

By repeating synthetics pathway described in experimental part, eight different types of dendrimers were synthesized. Numbers of terminal groups were assigned as *x* before PEGylation, *y* after the PEGylation and *z* for PEGamine functionality. (Table 1). Synthesized



Scheme 1. Stepwise growth and differentiations of synthesized dendrimers.

Table 1. PAMAMs having different functionality before and after PEGylation.

Dendrimer	*x*	*y*	*z*
E0.5–E4.5 Ester	4–64	–	–
E4.5 Acid (EDAcPAMAM)	–	65	–
P0.5–P-4.5 Ester	8–128	–	–
P4.5 Acid (PEGcPAMAM)	–	128	–
10% PEGylation			
E5 Ester- PEGamine	56	–	8
E5 Acid-PEGamine (EDAcPAMAM-PEG)	–	56	8
P5 Ester-PEGamine	118	–	10
P5 Acid-PEGamine (PEGcPAMAM-PEG)	–	118	10

E: EDA cored dendrimer, P: PEG cored dendrimer.

dendrimers were purified using special technique called LPR. According to LPR technique; selected size of ultrafiltration membrane disk having the range of MWCO 1–10 kDa was equipped with Amicon 8000 Stirred cell. (1:1) MeOH :Aqueous solutions of crude product was transferred into the cell. The solution was diluted to 200 mL inside the cell. Again methanol–water mixture was used as feeding solvent via special reservoir. Dialysis was performed under 15 psi bar nitrogen pressure for 24 h. Solvent was removed using rotary evaporator.

2.3. Synthesis of EDA and PEG-cored PAMAMs

EDA and PEG tetra-amine-cored half- (*Cn.5*) and full-generation (*Cn*) PAMAMs, where *C* and *n* refers to core type and generation number, respectively, were synthesized according to our previous study.[12] General experimental, detailed procedures, and characterization data were given in the supplementary file.[32] Synthesized E4.5 Ester and P4.5 Ester were used as precursors for the synthesis acidic (EDAcPAMAM, PEGcPAMAM and EDAcPAMAM-PEG, PEGcPAMAM-PEG) dendrimers.

2.4. General procedure for the hydrolysis of esteric PAMAMs

A methanolic solution of ester-terminated half-generation PAMAM was mixed with 1.5 M equiv. of NaOH per terminal ester. The final mixture was stirred for 24 h. Excess amount of solvent was removed under vacuum at bath temperature of 65 °C. Drying under vacuum resulted as white powder product. Yields were quantitative.

2.4.1. E4.5 acid (EDAcPAMAM)

A well-stirred solution of E4.5 Ester (1.0 g, 0.08 mmol) in 2 mL methanol was added to a stirred solution of NaOH (0.309 g, 7.72 mmol) in 2 mL methanol. The mixture was stirred for 24 h at room temperature. The product was a white powder (0.922 g, 100%). ATR-IR (cm^{-1}) 3450–3150 (COOH), 1648 (HNC=O), 1565 (HNC=O). ^{1}H NMR δH (500 MHz, CD_3OD) 2.38 (m, $NR_2CH_2CH_2COOH$), 2.58 (m, $NR_2CH_2CH_2COOH$). ^{13}C NMR δC (125 MHz; CD_3OD) 32.78 (CH_2CH_2COOH), 48.91 (CH_2CH_2COOH), 172.12 (*C*ONH), 181.23 (*C*OOH).

2.4.2. P4.5 acid (PEGcPAMAM)

A well-stirred solution of P4.5 Ester (0.5 g, 0.016 mmol) in 2 mL methanol was added to a stirred solution of NaOH (0.128 g, 3.2 mmol) in 2 mL methanol. The mixture was stirred for 24 h at room temperature. The product was a white powder (0.5 g, 100%). ATR-IR (cm^{-1}) 3450–3150 (COOH), 1651 (HNC=O), 1562 (HNC=O). ^{1}H-NMR δH (500 MHz; CD_3OD) 2.42 (m, CH_2CH_2COOH), 2.65 (m, CH_2CH_2COOH). ^{13}C-NMR δC (125 MHz; CD_3OD) 34.35 (CH_2CH_2COOH), 49.74 (CH_2CH_2COOH), 174.70 (*C*ONH), 181.36 (*C*OOH).

2.5. PEGylation process

Surface modification of PAMAMs with PEG bisamine was performed according to literature procedures.[24]

2.5.1. Synthesis of E5 Ester-PEGamine

PEG bisamine (1.94 g, 0.97 mmol) dissolved in 20 mL methanol was added dropwise to a solution of *E4.5 Ester* (1.487 g, 0.119 mmol) in 20 mL methanol in an ice/salt bath over 5 h of stirring. The mixture was stirred for 24 h at room temperature. Then the product was purified with LPR by dialyzing against methanol solution under 15 psi nitrogen (N_2) gas pressure for 24 h using Millipore ultrafiltration disks having a molecular cut of size 10 kDa. The product was a yellowish gel (2.45 g, 80%). ATR-IR (cm^{-1}) 3411, 3286 (NH), 1734 (C=O), 1650 (HNC=O), 1564 (HNC=O). ^{1}H NMR δH (500 MHz, DMSO-d_6) 2.42 (m, $NR_2CH_2CH_2COOCH_3$), 2.6 (m, $NR_2CH_2CH_2COOCH_3$), 2.66 (bm, $CONHCH_2CH_2NR_2$), 3.18 (bm, $CONHCH_2CH_2NR_2$), 3.50 (s, $COOCH_3$), 3.50–3.57 (m, OCH_2CH_2O, CH_2OCH_2), 4.22 (CH_2NH_2). ^{13}C NMR δC (125 MHz; DMSO-d_6) 31.49 ($CH_2CH_2COOCH_3$), 40.01 ($OCH_2CH_2NH_2$), 47.61 ($CH_2CH_2COOCH_3$), 48.36 ($CH_2CH_2NR_2$) 49.79 ($COOCH_3$), 68.43, 68.86, 69.28, 69.96, 70.11, 70.51, 70.98, (OCH_2CH_2O), 172.56 (CONH), 174.50 ($COOCH_3$).

2.5.2. Synthesis of P5 Ester-PEGamine

PEG bisamine (1.05 g, 0.52 mmol) dissolved in 20 mL methanol was added dropwise to a solution of *P4.5 Ester* (1.71 g, 0.05 mmol) in 20 mL methanol in an ice/salt bath over 5 h of stirring. The mixture was stirred for 24 h at room temperature. Then the product was purified with LPR by dialyzing against methanol solution under 15 psi nitrogen (N_2) gas pressure for 24 h using Millipore ultrafiltration disks having a molecular cut of size 10 kDa. The product was a yellowish gel (2.28 g, 70%). ATR-IR (cm^{-1}) 3434, 3367 (NH), 1733 (C=O), 1647 (HNC=O), 1548 (HNC=O). ^{1}H-NMR δH (500 MHz; CD_3OD) 2.39 (m, $CH_2CH_2COOCH_3$), 2.58 (m, $CH_2CH_2COOCH_3$), 2.79 (m, $CONHCH_2CH_2NR_2$), 2.88 (s, $OCH_2CH_2NH_2$), 3.28 (m, $CONHCH_2CH_2NR_2$), 3.37 (s, $CONHCH_2CH_2O$), 3.52–3.65 (m, OCH_2CH_2O, CH_2OCH_2), 3.68 (s, $COOCH_3$), 4.23 (s, CH_2NH_2). ^{13}C-NMR δC (125 MHz; CD_3OD) 33.64 ($CH_2CH_2COOCH_3$) 39.91 ($CONHCH_2CH_2O$), 40.43 (OCH_2CH2NH_2) 45.76 ($CH_2CH_2COOCH_3$), 50.52 ($COOCH_3$), 51.43 ($CONHCH_2CH_2NR_2$), 70.27, 70.74, 70.87, 71.43, 71.52, 72.09, 72.61, (OCH_2CH_2O), 169.19 (CONH), 174.69 ($COOCH_3$).

2.6. Synthesis of acidic PEGylated dendrimers

2.6.1. Synthesis of E5 Acid-PEGamine (EDAcPAMAM-PEG)

A well-stirred solution of *E5 Ester-PEGamine* (1.27 g, 0.049 mmol) in 2 mL methanol was added to a stirred solution of NaOH (0.169 g, 4.22 mmol) in 2 mL methanol. The mixture was stirred for 24 h at room temperature. The product was a white powder (1.22 g, 100%). ATR-IR (cm-1) 3450–3150 (COOH), 1653 (HNC=O), 1566 (HNC=O). 1H NMR δH (500 MHz, CD_3OD) 2.40 (m, $NR_2CH_2CH_2COOH$), 2.62 (m, $NR_2CH_2CH_2COOH$), 2.72 (bm, $CONHCH_2CH_2NR_2$), 3.61–3.73 (m, OCH_2CH_2O, CH_2OCH_2). 13C NMR δC (125 MHz; CD_3OD) 38.36 (CH_2CH_2COOH), 42.10 ($OCH_2CH_2NH_2$), 49.65 ($COOCH_3$), 69.78, 70.50, 71.02, 71.26, 71.51, 71.73, 72.22, (OCH_2CH_2O), 180.58 (CONH), 181.28 ($COOCH_3$).

2.6.2. Synthesis of P5 Acid-PEGamine (PEGcPAMAM-PEG)

A well-stirred solution of *P5 Ester-PEGamine* (1.19 g, 0.024 mmol) in 2 mL methanol was added to a stirred solution of NaOH (0.171 g, 4.27 mmol) in 2 mL methanol. The mixture was stirred for 24 h at room temperature. The product was a white powder (1.19 g, 100%). ATR-IR (cm^{-1}) 3400–3100 (COOH), 1652 (HNC=O), 1568 (HNC=O).

^{1}H-NMR δH (500 MHz; CD_3OD) 2.38 (m, CH_2CH_2COOH), 2.62 (m, CH_2CH_2COOH), 2.83 (m, $CONHCH_2CH_2NR_2$), 2.86 (s, $OCH_2CH_2NH_2$), 3.31 (m, $CONHCH_2CH_2NR_2$), 3.37 (s, $CONHCH_2CH_2O$), 3.52–3.65 (m, OCH_2CH_2O, CH_2OCH_2). ^{13}C-NMR δC (125 MHz; CD_3OD) 36.41 (CH_2CH_2COOH) 40.00 ($CONHCH_2CH_2O$), 40.14 (OCH_2CH2NH_2) 46.74 (CH_2CH_2COOH), 51.27 ($CONHCH_2CH_2NR_2$), 69.82, 70.31, 70.53, 71.05, 71.27, 71.55, 71.75, 72.29, (OCH_2CH_2O), 174.77 (CONH), 181.33 (COOH).

2.7. Cell culture and assessment of cytotoxic effects of the dendrimers

Mouse fibroblast cell line L929 (American Type Culture Collection, LGC Promochem, Rockville, MD, U.S.A), which is recommended by U.S. Pharmacopeial Convention (USP 26), was used for the evaluation of cytotoxic effects of the synthesized dendrimers. L929 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany) and 1% streptomycin and penicillin (Sigma, St. Louis, MI, U.S.A) in a 37 °C humidified incubator with 5% CO_2. Subcultivation was performed with 0.25 trypsin-EDTA solution (PAA, Pasching, Austria).

Toxicity of the EDA- or PEG-cored dendrimers was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. L929 cells were seeded in 96-well microplates (15 × 103 cells/well) in 50 µL cell culture medium and incubated for 24 h. Dendrimers (EDAcPAMAM, EDAcPAMAM-PEG, PEGcPAMAM, PEGcPAMAM-PEG) were diluted to various concentrations in serum-free culture medium and added into the wells (assays were performed in triplicates) along with vehicle solvent-treated wells as controls. Following 24 h and 48 h of incubation with dendrimer compounds, 25 µL MTT (5 mg/mL) solution was added into each well and the plates were further incubated for 4 h to allow formazan crystals to be formed by viable cells. Then, the cells were lysed (23% SDS in 80 µL 45% DMF lysis solution) during an overnight incubation. The optical density of each well read 570 nm using a microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). Cell viability was calculated as percentage change calculated in comparison to the OD values obtained from untreated control cells.

2.8. Preparation of fluorescent dye encapsulated inclusion complexes

Fifth-generation PEGcPAMAM-PEG dendrimers were synthesized as mentioned above. Rhodamine 123 (Rho123, Sigma) was dissolved in ultrapure water at 0.35 mg/mL concentration, PEGcPAMAM-PEG dendrimers were dissolved in methanol at 50 mg/mL concentration and then 500 µL Rho123 solution was added onto the 500 µL of PEGcPAMAM-PEG dendrimers in methanol. The solution was stirred overnight, then dried under vacuum in order to remove methanol. Lyophilization was applied to samples to remove water.

2.9. Biodistribution studies

Female Swiss albino mice (Hacettepe University Animal Breeding Facility, Ankara, Turkey) were housed under environmentally controlled standard conditions. The experimental procedures were approved by the local ethical committee (Approval No. 2015/79-4). Three mice were injected with Rho123-loaded PEGcPAMAM-PEG dendrimers which were (0.07 mg

Rho123 containing 5 mg PEGcPAMAM-PEG in 400 μL) suspended in 1x phosphate-buffered saline (PBS) solution via intreperitoneal (i.p.) route. Following a 16-h period, the mice were sacrificed and the organs were dissected. Tissues were cut and mechanically disaggregated to prepare cell suspensions which were prepared in 1xPBS, washed to remove cell debris and tissue lysate, and filtered through a 40-μm strainer. The cells were directly read on a flow cytometer (FACSAria II, Becton Dickinson, San Jose, CA, U.S.A) equipped with a 488-nm laser and 585/42 filter. Following the selection of singlet events through a doublet discrimination panel, the cells were gated on side scatter and forward scatter properties. Then, mean fluorescence intensity values were obtained. During all experiments, exposure of the samples to direct light was avoided.

2.10. Statistical analysis

All experiments were performed at least in triplicate. All values are expressed as arithmetic mean ± standard deviation (SD). Statistical significance between the groups was determined using the Student *t*-test or Mann–Whitney *U* test. p values < 0.05 were defined as significant.

3. Results and discussion

3.1. Characterization of PAMAMs

All the syntheses were monitored by ATR spectroscopy in each step and correlated by NMR. By examining Figure 1, appearance and disappearance of ester, amine, amide, and carboxylic acid peaks were the prominent peaks to observe the change in functional groups.

Formation of ester groups at ≈1732 cm^{-1}, amide I at ≈1640 cm^{-1} and amide II at ≈1540 cm^{-1} were easy ways of monitoring main reactions. Since both cores have the same type of amine functionality (1° amine), when they are alkylated, the amine peak at ≈3300 cm^{-1} disappeared and ester peak at ≈1732 cm^{-1} appeared. In amidation reactions characterization, disappearance of ≈1732 cm^{-1} peak stemming from the ester functional groups of PAMAMs, and the replacement of amide I (≈1640 cm^{-1}) and amide II (≈1540 cm^{-1}) peaks proved the fully conversion of ester-terminated PAMAM dendrimers to amine-terminated PAMAM dendrimers. So that, all the half generations through *E4.5 Ester – P4.5 Ester* were successfully followed. Coupling of PEG diamine to mentioned esters were monitored by observing first amide peaks (amide I, amide II ≈1640 cm^{-1} and ≈1540 cm^{-1}, respectively). Additionally, C-O ether bands at the backbone of PEG at ≈1100 cm^{-1}, methylene $-CH_2$ at ≈2900 cm^{-1} and again appearance of $-NH_2$ group at ≈3280 cm^{-1} were also a good way of observing aminolysis. Hydrolysis of ester groups to acidic groups was monitored by the disappearance of peak at ≈1732 cm^{-1} and appearance of new band at 3500–3100 cm^{-1} (most prominent 3320 cm^{-1})was assigned as OH part of carboxylic acid, which could readily make H-Bond resulting in broadening the spectrum. In the synthesis of P5 ester–PEG-amine, esteric band (≈1733 cm^{-1}) did not disappeared completely because esteric groups were PEGylated partially. At the third step, when esteric groups were hydrolyzed, esteric peak at ≈1732 cm^{-1} completely disappeared and acidic carbonyl peak formed at ≈1651 cm^{-1} These changes were attributed to clear transformations from ester to amine or ester to acid (see Figure 1).

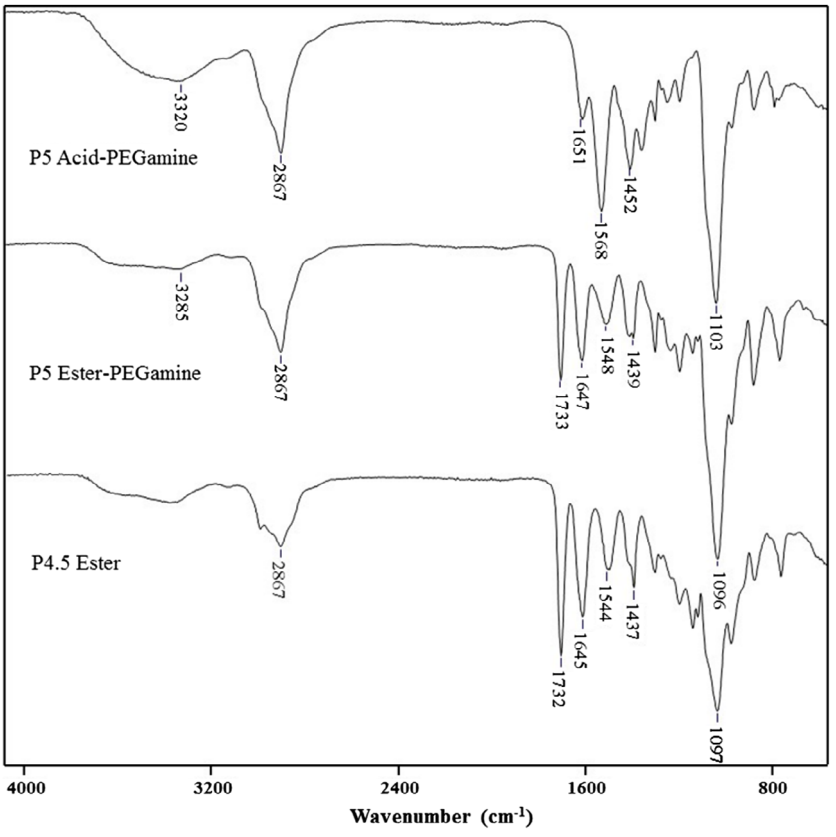


Figure 1. FTIR/ATR spectra of P4.5 Ester, P5 Ester-PEGamine and P5 Acid-PEGamine dendrimer.

By examining the ^{1}H NMR (Figure 2a) spectra of methyl, ester peaks of half generations were observed at 3.5–3.7 ppm. After partial PEGylation, rest of esteric protons and backbone protons of PEG overlapped and as a consequence of this, overlap peaks around 3.5–3.7 ppm became stronger. The peak around 4.3 ppm was attributed to terminal amino groups of PEG and its broadness was attributed to possible H-bond with the NMR solvent (Me-OD). Because of solubility difference, esteric dendrimers were measured in $CDCI_3$, while PEGylated dendrimers were taken in Me-OD. Same observations were also monitored during the hydrolysis of terminal esters into the carboxylic acids. Expected protons resulting from either amide or acidic hydroxide were not clearly observed because of H-bond with the deuterated solvent (Me-OD) (see Supplementary file).

Monitoring of PEGylation via ^{13}C-NMR was much easier (Figure 2b). In the ^{13}C NMR, esteric methyl carbons were observed at the range of 49–52 ppm. In the same way, the esteric carbonyls were observed at 171–174 ppm (small peaks were from inner carbonyls). In the partial PEGylation, some esters were converted to amides and new peaks appeared at 174–176 ppm (C=ONH). Ether groups (*–C–O–C–*) arising from backbone of PEG amine were resonanced at 68–72 ppm. Finally, after successful modification of both *E4.5 Ester* and *P4.5 Ester* with PEG amine, hydrolysis of esteric groups was monitored by resonance peaks at 181.28 ppm and 181.33 ppm. (Details are experimental part and supporting information)

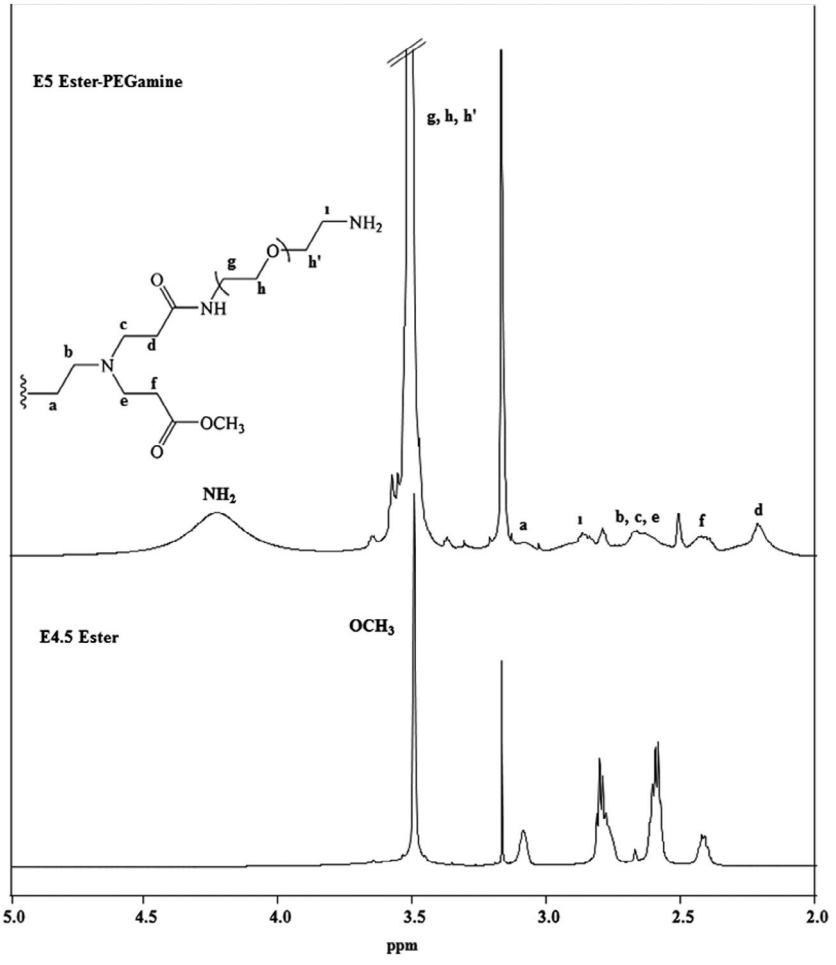


Figure 2a. Transformation from E4.5 Ester (in $CDCl_3$) to E5 Ester-PEGamine (in Me-OD) via ^1H NMR.

3.2. Cytotoxicity and biodistribution studies of the dendrimers

In general, even the highest concentration of either PEG- and EDA-cored PAMAMs did not severely hamper L929 fibroblasts' viability. Since MTT assay gives information on the metabolic activity of the viable cells, during 24 h incubation, a slight increase in EDAcPAMAM dendrimers was observed in the activity, whereas PEGylated derivative of these molecules exhibited no effect (Figure 3(A), upper panel). In contrast, in the absence of PEGylation, PEG-cored dendrimers displayed more adverse effects. On the other hand, there was no significant difference between cell viability upon treatment with the compounds for 48 h (Figure 3(A), lower panel) ($p > 0.05$). Basically, the initial metabolic effects were transient, and none of the dendrimers was prominently cytotoxic (cell viability >75%), *in vitro*.

There are numerous studies which have examined dendrimer cytotoxicity *in vitro*, which have different cell lines, a variety of incubation times (hours to days), and various assay methods. In Table 2, some of these studies which are similar to our material were summarized.

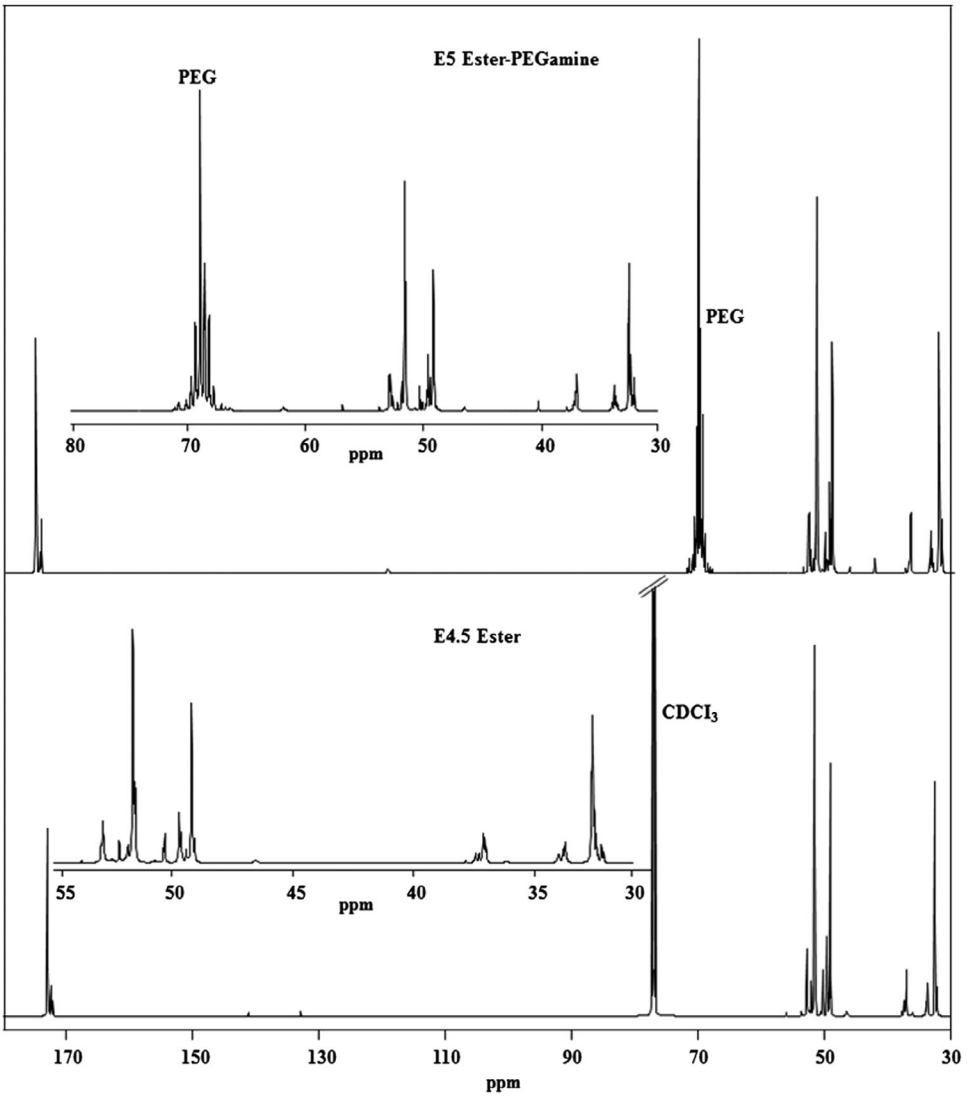


Figure 2b. Monitoring transformation of partial PEGylation via ^{13}C NMR: E4.5 Ester (in $CDCl_3$) and E5 Ester-PEGamine (in MeOD).

In this work, influence of PEGylation on cytotoxicity of dendrimers was shown. Due to the positive effects of PEGylation on PEGcPAMAM dendrimers, PEG-cored PAMAMs were selected for further studies. On the other hand, cytotoxicity of EDA-cored PAMAMs increased with PEGylation. Taking into account the flexibility of PEGylated dendrimers for serving as a platform for additional modifications and conjugations (i.e. with drugs or antibodies) that would provide them with functional features,[35] PEGcPAMAM-PEG dendrimers were preferred for biodistribution studies. Additionally, since the most convincing and stable results were obtained with PEGcPAMAM-PEG molecules, a promising candidate, *in vitro*, these dendrimers were tested for *in vivo* biodistribution. Expectedly, they were mostly retained in the site of injection (Figure 3(B)), peritoneal cavity which

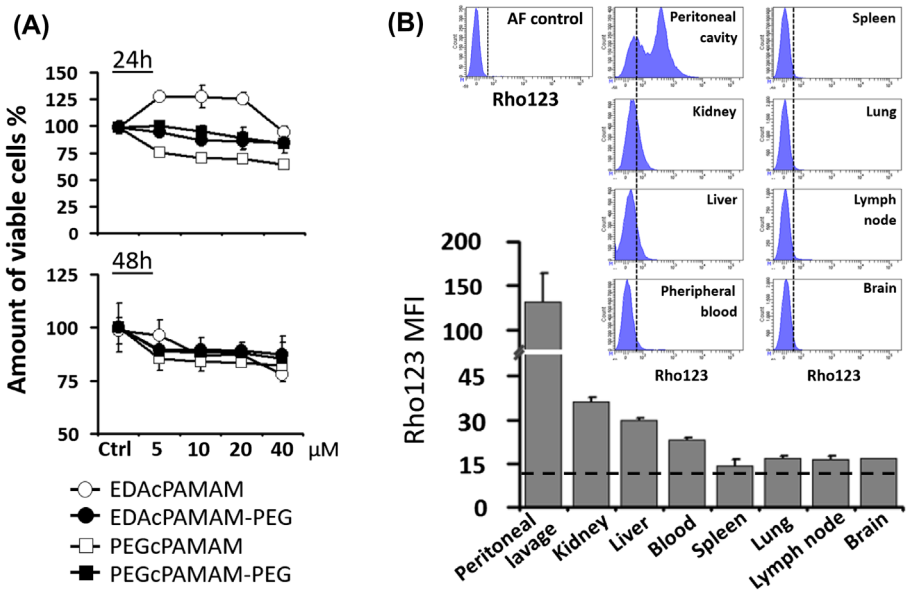


Figure 3. Effect of the dendrimers on the viability of fibroblast cells (A) was measured with MTT assay perofrmed for 24h and 48h. (B) Biodistribution of PEGcPAMAM-PEG dendrimers-loaded with the fluorescent dye rhodamine (Rho)123 through various organs of Swiss albino mice was analyzed at cellular level with flow cytometry. Representative flow cytometry histograms are shown for each tissue (upper panel). The dashed line marks the median autofluorescence (AF control) intensity threshold taken from control tissues obtained from untreated mice. The graphics were drawn with mean and standard deviation values obtained from three experiments, (Ctrl., untreated control cells; MFI, mean fluorescence intensity; AF, autoflourescence of untreated samples).

Table 2. Cytotoxicity of PAMAMs against different cells.

Dendrimer	Cell line	IC_{50} values	References
PAMAM (G4)	Caco-2	10 μM (24 h)	[19]
PAMAM (G3)	L929	>10 (mg/mL) (24 h)	[33]
PAMAM (G4)	B16F10	0.05 (mg/mL) (72 h)	[17]
PAMAM-OH (G4)	293	400 μg/mL (24 h)	[34]
PAMAM-COOH (G 4,5)	L929	>10 (mg/mL)	[13]

is rich in phagocytic cells that are capable of capturing dendrimers. The presence of high fluorescence signals in the liver and the kidney indicated that dendrimers were able to enter to the blood stream and reach to the organs which are specialized in capturing and clearing foreign molecules.[36,37] PEGcPAMAM-PEG dendrimers were not retained in the organs such as spleen, lung, mesenteric lymph node, or brain (Figure 3(B)).

4. Conclusion

In order to offer for the possible conjugation with an appropriate antibody, two different PAMAMs were synthesized using microwave-assisted technique. By means of MAS technique, we have accelerated and scaled up synthesis of commercial PAMAMs. A second group of PAMAM for the biochemical studies with similar syntheses has been made using FDA

administrated polymers. At this point, PEG tetra amine was used as polymeric core to attain bigger dendrimers, so that we aimed to retard the starburst effect of higher generations and to increase their biocompatibility for the biochemical studies. In each class of syntheses, MA and EDA were selected as dendritic building blocks. When both type of dendrimers were grown up to 4.5 generations, 10% surfaces were PEGylated by PEG bisamine, and the rest 90% esteric groups were converted to acidic group. In each step, synthesized dendrimers were purified by LPR method. After successful purification and characterization, their cytotoxicities and biodistribution were investigated. So, with the current study, we offer an accelerated dendrimer synthesis method and a new class of dendrimers as drug delivery materials.

As a conclusion, none of the dendrimers was prominently cytotoxic (cell viability > 75%), *in vitro*. The promising formulation successfully remained at the site of injection which easily reached the liver and kidney resulting in a successful transfer pattern, *in vivo*. Using this polymer in the active- or passive-targeted drug delivery applications especially liver, kidney, and intraperitoneal organ, damaged animal models will provide a safe and efficient therapy.

Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCiD

Metin Tülü http://orcid.org/0000-0001-9791-4922

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