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Original article

Poly propyl ether imine (PETIM) dendrimer: A novel non-toxic dendrimer for sustained drug delivery

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ABSTRACT

In the present study, an attempt was made to study the acute and sub-acute toxicity profile of G3–COOH Poly (propyl ether imine) [PETIM] dendrimer and its use as a carrier for sustained delivery of model drug ketoprofen. Drug-dendrimer complex was prepared and characterized by FTIR, solubility and *in vitro* drug release study. PETIM dendrimer was found to have significantly less toxicity in A541 cells compared to Poly amido amine (PAMAM) dendrimer. Further, acute and 28 days sub-acute toxicity measurement in mice showed no mortality, hematological, biochemical or histopathological changes up to 80 mg/kg dose of PETIM dendrimer. The results of study demonstrated that G3–COOH PETIM dendrimer can be used as a safe and efficient vehicle for sustained drug delivery.

1. Introduction

Dendrimers have generated a great deal of interest as controlled and targeted drug delivery system due to their exceptional structural properties such as low polydispersity (≈1), high density of peripheral functional group, well defined globular shape (≈2.0 nm) and multivalency [1,2]. Dendrimers are synthesized from branched monomer units in a stepwise manner so it is possible to conduct precise control on molecular size, shape, dimension, density, polarity, flexibility and solubility by choosing different building/branching units and surface functional groups [1,3]. Till now dendrimers have been widely explored as carrier system in drug delivery [4]. Poly amido amine (PAMAM), Poly propylene imine (PPI), Polylysine and Triazine are most widely explored dendrimers for drug delivery and their major limitation is hematological toxicity and imperfect organ accumulation properties [5,6]. Due to this reason, no commercial dendrimer based formulation for systemic administration is available.

The present investigation was aimed at studying the possibility of using a novel dendrimer carrier system PETIM synthesized by Pharmaceutical company Bioplus Life Science Pvt. Ltd., India as a carrier for sustained delivery of drug [7–9]. PETIM was found to have good water solubility and stability as evaluated in the previous study [10]. This dendrimer system was also found to have a higher internal cavity diameter in comparison to PAMAM dendrimer as measured by Small angle x-ray diffraction techniques [11]. No work was carried out for evaluation of PETIM dendrimer as a drug carrier system for sustained delivery of drug. In the present study, an attempt was made to prepare drug-dendrimer complex using ketoprofen as a model drug and evaluating its sustained release potential.

Biocompatibility is a big issue in development of commercial viable formulation using dendrimer as carrier system [6]. Attempt was also made for evaluating the acute and short term sub-acute toxicity profile of PETIM dendrimer by studying the effect of PETIM administration on hematological, biochemical parameters and vital organ histopathology of mice. Cytotoxicity assay was also designed

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to study cytotoxicity of PETIM in comparison to commercially available PAMAM dendrimer.

2. Materials and methods

2.1. Materials

Polypropyletherimine (PETIM) dendrimer G3–COOH generation is synthesized by Bioplus Life Science Pvt Ltd, Hosur, India as per own patented procedure [7]. Ketoprofen, RPMI 1640 media, Fetal bovine serum (FBS), G4-OH PAMAM dendrimer and all other chemicals were procured from Sigma Aldrich Co. Ltd. USA. Dialysis membrane having molecular weight cut off 1 kD was purchased from Spectrapor, USA. Glucose, cholesterol, total protein content, urea, SGPT (Serum glutamic pyruvic transaminase), SGOT (Serum glutamic-oxaloacetic transaminase), ALP (Alkaline phosphate), creatinine and bilirubin estimation kit were purchased from Erba Ltd., India.

2.2. Synthesis of PETIM dendrimer

The PETIM series of dendrimers were synthesized by a divergent growth methodology as shown in Fig. 1. The 3-Aminopropane-1-ol was the monomer for the synthesis of PETIM dendrimer that possess tertiary amine as the branch point and ether as linker interconnected by propylene spacers. The details of synthetic procedure for preparation of different generation of PETIM dendrimers have been described in our previous report [7–10].

2.3. Formation of drug–dendrimer complex

Ketoprofen–PETIM complex was prepared as per method reported by Papagiannaros et al. [12]. Ketoprofen was dissolved in methanol and synthesized G3–COOH PETIM dendrimer was added. This solution was stirred overnight by magnetic stirrer (50 rpm), then dried under vaccum to remove methanol (Vaccum oven, Perfit, India). The complex was dissolved in distilled water and stirred for another 2 h to extract out the drug–dendrimer complex. The solution was then filtered through the 0.22 μm membrane filter and lyophilized (Heto, Allerod, Denmark) to remove the water completely. The drug–dendrimer complex was redissolved in PBS (7.4) and free drug was removed by dialysis method (molecular weight cut off 1 kD). Drug concentration was determined using HPLC assay. Characterization of drug–dendrimer complex was carried out by recording the FTIR spectra of drug–dendrimer complex.

2.4. Solubility study

Solubility study was carried out as per method reported by Higuchi and Connors [13]. Excess of drug (1, 2, 5 and 10 mg/ml) was added separately into the screw-capped vials containing dendrimer (20 mg/ml) in PBS (7.4). Vials were shaken for 24 h at 37 ± 1.0 °C on a horizontal rotary shaker (Remi equipments, Mumbai). The mixture obtained was filtered on a 0.45 μm membrane filter and drug content in filtrate was determined using HPLC assay.

2.5. In vitro drug release study

In vitro release behaviour of ketoprofen from the ketoprofen–PETIM (G3–COOH) dendrimer complex was investigated by dialysis method as described by Wang et al. [14]. Pure ketoprofen was dissolved in methanol at a concentration of 2 mg/ml and used as control. The complex was dissolved in PBS (pH 7.4) at a concentration of 2 mg/ml. This solution (2 ml in volume) was transferred to dialysis bag (molecular weight cut off 1 kD) immediately. The dialysis bag was placed in a 50 ml-beaker containing 40 ml PBS (pH 7.4). The outer phase was stirred continuously using magnetic stirrer at 50 rpm. At predetermined time intervals of 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 15, 20 and 24 h, 100 μl sample was withdrawn and

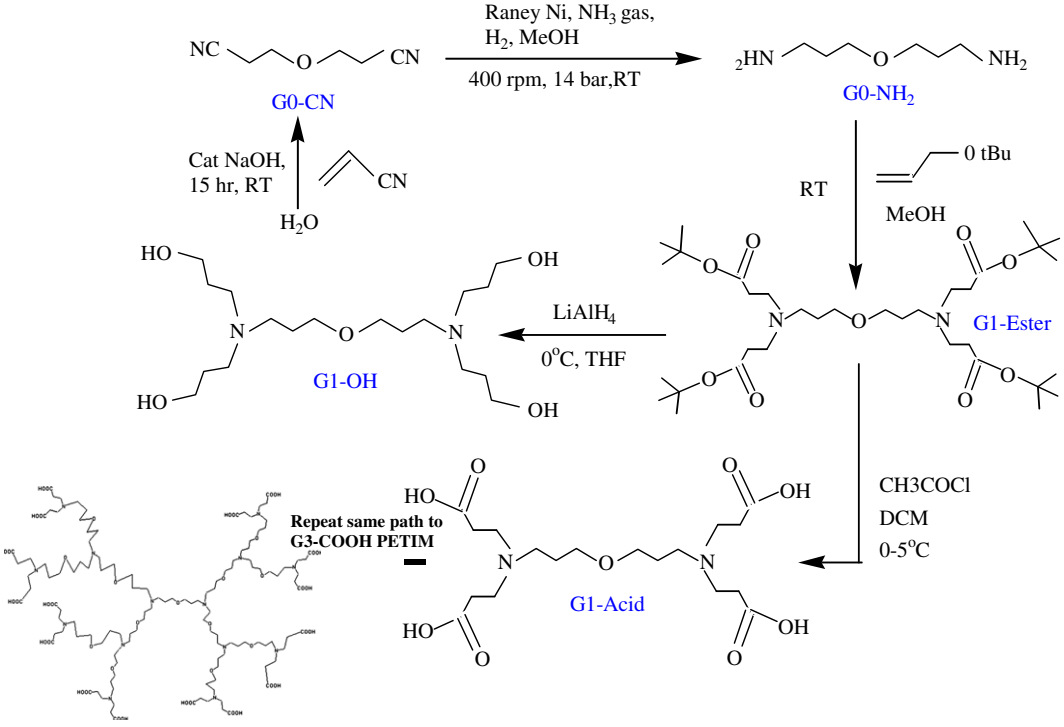

Fig. 1. Scheme for synthesis of PETIM dendrimer.

replenished with the same amount of receptor fluid. The concentration of ketoprofen present in the dialysate was monitored using HPLC and the percent drug release at various time intervals was calculated.

2.6. Hemolytic toxicity assay

The RBC suspension was obtained as per the well-known and reported procedure for hemolytic studies [15]. The RBC suspension was mixed with distilled water, which was considered as producing 100% hemolysis, as normal saline does not produce any hemolysis thus acting as a blank. The RBC suspension (0.5 ml) was added to 4.5 ml of PBS (pH 7.4) and then 1000, 100, 10, 1, 0.1 and 0.01 μg/ml of PETIM dendrimer solution in PBS were interacted with RBC suspension. After 1 h incubation at 37 ± 1.0 °C followed by centrifugation, the supernatant were taken and diluted with an equal volume of PBS (pH 7.4) and absorbance was taken at 540 nm against supernatant of normal saline. The percent hemolysis was determined for each sample by taking absorbance of water as 100% hemolytic sample.

2.7. Toxicity study

2.7.1. Acute toxicity

The acute toxicity of the PETIM dendrimer was evaluated in mice using the up and down procedure [16]. Thirty six mice (Swiss albino mice) of either sex (weight: 28–40 g; age: 12–16 weeks old) were randomly assigned into six groups ($n = 6$). Group of six mice of same sex were housed together. Dendrimer solution in PBS (pH 7.4) was administered intra-peritoneally. The first group of animals, serving as control, received PBS (pH 7.4) (5 ml/kg); the second, third, fourth, fifth and six group received G3–COOH dendrimer solution in PBS at dose 10, 20, 40, 80 and 100 mg/kg, respectively. Animals were observed for toxic symptoms continuously for the first 4 h after dosing. Finally, the number of survivors was noted after 24 h and these animals were maintained for further 13 days with observations made daily.

2.7.2. Sub-acute toxicity

Thirty mice (Swiss albino mice) of either sex (weight: 28–40 g; age: 12–16 weeks old) were randomly assigned into five groups ($n = 6$). Group of six mice of same sex was housed together. Dendrimer solution in PBS (pH 7.4) was administered intra-peritoneally once a day for 4 weeks. The first group of animals, serving as control, received PBS (PH 7.4) (5 ml/kg); the second, third, fourth and fifth group received G3–COOH dendrimer solution in PBS at dose 10, 20, 40 and 80 mg/kg, respectively. All animals were supplied with Fiel Purina Chow® and tap water during the testing periods. Animals were weighed and observed daily for physiological and behavioural changes. Animals were examined at the end of the test period for hematological and biochemical parameters. Body weight, water and food intake was measured daily. All investigations were performed after approval of the Institutional Animal Ethics Committee of the Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala and in accordance with the disciplinary principles and guidelines of committee for the purpose of control and supervision on experiments on animals (CPCSEA).

2.8. Blood analysis for hematological and biochemical parameters

Blood samples for hematological and biochemical estimations were collected on the 0 and 28th day from different groups of animals used for sub-acute toxicity assay. The blood samples were kept at room temperature for 30 min and then centrifuged at 4000 rpm for 15 min to separate serum. The blood samples containing the blood and anticoagulant were centrifuged at 4000 rpm for 15 min to separate the plasma. Both plasma and serum were used for evaluating the different biochemical parameters – glucose, cholesterol, urea, SGPT, SGOT, total proteins, ALP, Creatinine and bilirubin content with standard Erba estimation kit using Autoanlyzer (Erba, Chem 7, Germany). Standard procedure as specified in the kit literature was followed. The blood samples collected in the sodium citrate tubes were also used for analyzing red blood cell count (RBC), haemoglobin concentration (Hb), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin concentration (MCHC), Platelets (Plt), total leukocytes count (TLC), polymorphs, lymphocytes, monocytes and eosinophils count using auto hematolyzer (PooCH 100, Sys, Germany).

2.9. Histopathological study

Animals were sacrificed at the end of 28 days by cervical dislocation. Heart, kidney and liver were taken out and preserved in 10% formalin for histopathological examination. Sections were fixed and block was made using the conventional procedure [17]. Sections were cut at 4 microns with the rotary microtome. Paraffin wax was removed by warming the slide gently, until the wax melted and then was washed with xylene followed by washings with absolute alcohol and water respectively to hydrate the sections and stained with haematoxylin-eosin. Histological sections were examined using an optical microscope with the photographic arrangement (DX31, Olympus, Japan).

2.10. Cytotoxicity assay

Freshly thawed, actively growing small lung cancer cell line (A-549) was used. Twenty hours old, freshly seeded, actively growing cultures of the selected cell lines were harvested by trypsinization/shaking and centrifuged at 3000 rpm for 5 min. The cells were then washed with PBS and resuspended in RPMI containing 10% FBS at a concentration of 10^6 cells/mL. Micro titre plates were seeded by transferring 100 μL cells to each well and incubated at 37 ± 0.5 °C for 24 h under 5% CO_2 atmosphere (5% CO_2 incubator, New Brunswick Scientific, Ltd. Germany). The final number of cells seeded in each well was 5000. A stock solution (100 μl) was added in first well of the 96 well micro titre plate (B.D. Bioscience Ltd., USA) followed by serial dilution by 10× in subsequent well. The plate was incubated for 48 h and cell viability study was conducted using MTT assay. MTT solution (0.5% w/v in RPMI medium) was added to each well, and the plate was incubated at 37 ± 0.5 °C for 4 h. After 4 h the content of each cell was removed and the converted dye was solubilized in 150 μL isopropyl alcohol/dimethyl sulfoxide mixture (1:1). Absorbance of converted dye was measured at wavelength of 540 nm using ELISA plate reader (B.D. Bioscience Ltd., USA).

2.11. HPLC assay

Ketoprofen in the experimental protocol was estimated by the HPLC method as reported by Satterwhite et al. [18]. Orthophosphoric acid (0.1%) & Methanol (40:60) in isocratic mode was used as mobile phase and was delivered at 1.0 mL/min. The injected fluid (20 μL) was eluted in C18 column (Agilent Technologies, Inc., USA 250 × 4.6 mm) at room temperature and ketoprofen was monitored at 258 nm using a PDA detector (Agilent Technologies, Inc., USA). The calibration curve within a concentration range from 0.05 to 10.0 μg/mL was used to measure the ketoprofen concentration.

Table 1
Composition and characterizations of drug–dendrimer complex.

Characteristic Parameters	Value
Generation	G3–COOH
Terminal group	–COOH
Number of terminal group	16
Molecular weight (g/mol)	2667.3
Total no. of atoms (N)	421
Encapsulation efficacy	56.0 ± 2.2
[a]Size (nm)	4.14
[b]Solubilization	5.0 folds
Drug release after 24 h	79.9 ± 3.8

Values Represented as Mean ± SD ($n = 3$).
[a] Calculated by small angle x-ray scattering techniques as reported in previous publication [11].
[b] Ratio of saturation solubility of ketoprofen with PETIM dendrimer versus intrinsic water solubility.

2.12. Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of obtained results. The statistical analysis of data was performed using analysis of variance (ANOVA) (Graphpad, Version 2.01, San Diego, CA). A value of $p < 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. Preparation and in-vitro characterizations

PETIM dendrimer was synthesized using the previously described method as shown in Fig. 1 [7,9]. The theoretical details of synthesized dendrimer are summarized in Table 1. Formation of dendrimer structure was confirmed by FTIR and NMR spectroscopy. The various peaks and shifts obtained were analyzed, interpreted and matched with results reported in the previous study [7,9]. Drug–dendrimer complex was prepared by using constant amount of G3–COOH dendrimer (20 mg/ml) with varying amount of ketoprofen (1.0–10 mg/ml). Ketoprofen was selected as model drug due to its low water solubility (0.01% w/w), low bioavailability and G.I. toxicity [19]. PETIM dendrimer was found to have 5-folds increase in solubility of ketoprofen. Similarly, Yiyun et al. [20] studied the solubility enhancement effect of G2-G5 PAMAM dendrimer and found 2–4 folds increase in ketoprofen solubility. PETIM dendrimer was found to have a significant increase in ketoprofen solubility at lower generation probably due to the higher internal cavity diameter (14.14 Å) [11] in comparison to PAMAM dendrimer (8.0 Å) [21]. Another reason for solubilization ability of PETIM dendrimer is the presence of a number of hydrophilic groups in the periphery, leading to accommodate the larger number of water molecules as studied previously by water penetration study [11].

Fig. 2A–C shows the FTIR spectra of drug, dendrimer and drug–dendrimer complex, respectively. Peak around 2500 cm^{-1} shows the presence of tertiary amine, characteristic feature of dendrimer. Other peaks around 3100, 1650 and 1200 cm^{-1} shows the presence of –OH, –C=O and C–O–C functional groups, respectively [7,9]. These peaks are well correlated with G3–COOH structure as reported previously. FTIR spectra of pure ketoprofen showed the 2 characteristic sharp and symmetric carbonyl peaks at 1700 and 1655 cm^{-1}. These two peaks were ascribed to the diametric carboxylic and ketonic group stretching vibration, respectively. Results are well correlated with reported by Blasi et al. [22] for characterization of ketoprofen–PLGA interaction. The FTIR spectra of drug–dendrimer complex showed that characteristic carbonyl group peak of ketoprofen was slightly shifted

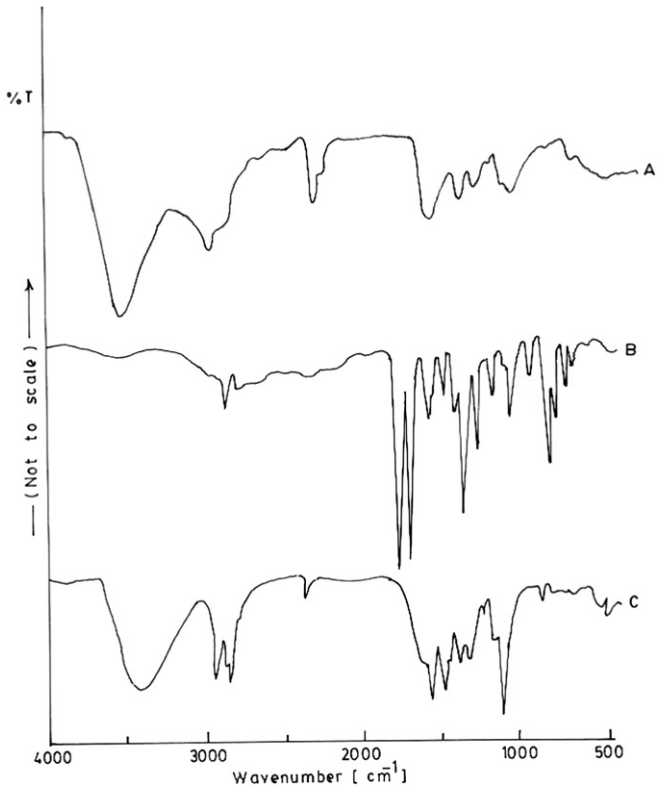

Fig. 2. IR-Spectra of PETIM dendrimer (A), ketoprofen (B) and drug–dendrimer complex (C).

towards lower side at 1600 cm^{-1} and sharpness was retained. Additionally, characteristic –C=O peak of dendrimer at 1700 cm^{-1} is also shifted and merged with the peak of the drug. This is due to of formation of drug–dendrimer complex. The dendrimer may complex the drug via various mechanisms i.e., hydrophobic interaction, hydrogen bonding or electrostatic attraction [23]. Low molecular weight drugs may encapsulate within the void space of the dendrimer. In the case of ketoprofen–PETIM complex, the encapsulation of ketoprofen may also be due to hydrophobic interaction. The bond between –COOH group of ketoprofen and internal cavities of dendrimer may also contribute for its encapsulation.

3.2. In vitro drug release study

The *in vitro* release behaviour of ketoprofen from the drug–dendrimer complex was examined in PBS 7.4 and results are summarized in Fig. 3. The release of ketoprofen from the drug–dendrimer complex was appreciably slower as compared to pure drug. After 2 h, 77.1% of the pure drug was permeated, whereas, only 19.5% was released from the drug–dendrimer complex. The drug release was prolonged to 24 h with drug–dendrimer complex, in comparison to 3 h with free drug, indicates the sustained release behaviour of PETIM dendrimer. The release rate was significantly slower than previously reported with ketoprofen–PAMAM dendrimer complex (76% in 10 h) [24].

3.3. Hemolytic toxicity measurement

Red blood cell (RBC) lysis measurement is a simple and widely used method to study polymer–membrane interaction. It gives

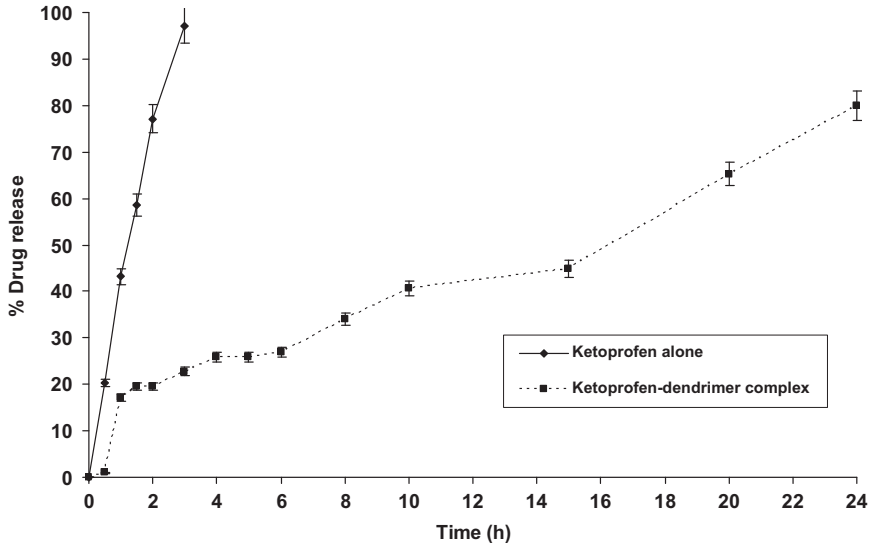

Fig. 3. Comparative % drug release of ketoprofen from drug–dendrimer complex and plain drug across the cellophane membrane. Mean ± SD ($n = 3$).

a quantitative measure of haemoglobin (Hb) release. The data obtained in such assay also give a qualitative indication of potential damage to RBC's of dendrimer administered. This is a good indicator of dendrimer toxicity in the *in vivo* conditions. Fig. 4 summarized the results of the hemolytic toxicity assay of G3–COOH PETIM dendrimer after 1 h incubation at concentrations from 0.01 to 1000 μg/ml. PETIM dendrimer showed concentration dependent hemolysis, 42 ± 1.4 and 47.6 ± 1.6% hemolysis was observed at 100 and 1000 μg/ml. Dendrimers are known for showing the concentration dependent hemolytic toxicity and this is its major limitation to use as carrier system in drug delivery. Investigated PETIM dendrimer showed significantly less hemolysis in comparison with PAMAM dendrimer [6]. Chen et al. [25] found 60 and 20% hemolysis at 10 μg/ml concentration of amine and carboxylate terminate Melamine dendrimer, respectively. In comparison, PETIM dendrimer at 10-folds higher concentration showed only 42 ± 1.4% hemolysis. This may be due to the chemical nature of PETIM dendrimer. Hemolytic toxicity of PAMAM dendrimer is reported due to interaction of positively charge NH_2 group of dendrimer with negatively charged surface of red blood cells. Electrostatic repulsion among red blood cells prevents their self aggregation and makes them suspended as colloids in blood. PAMAM dendrimer have net positive charge on the surface and interacted with negatively charged surface of red blood cells and causes hemolysis [26]. In comparison, G3–COOH PETIM dendrimer has net negative charge on the surface that minimized the interaction with RBCs.

3.4. Cytotoxicity assay

Cellular toxicity of PETIM dendrimer in comparison to commercial available PAMAM dendrimer was investigated using MTT assay, and results are summarized in Fig. 5. PAMAM G4-OH generation was selected for comparison purpose due to similar size (13 Å) [20] with PETIM G3–COOH dendrimer (14.14 Å). The MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay is simple nonradioactive colorimetric estimation to measure cell cytotoxicity, proliferation, or viability. MTT is a yellow, water-soluble, tetrazolium salt. Metabolically active cells are able to convert this dye into a water-soluble dark blue

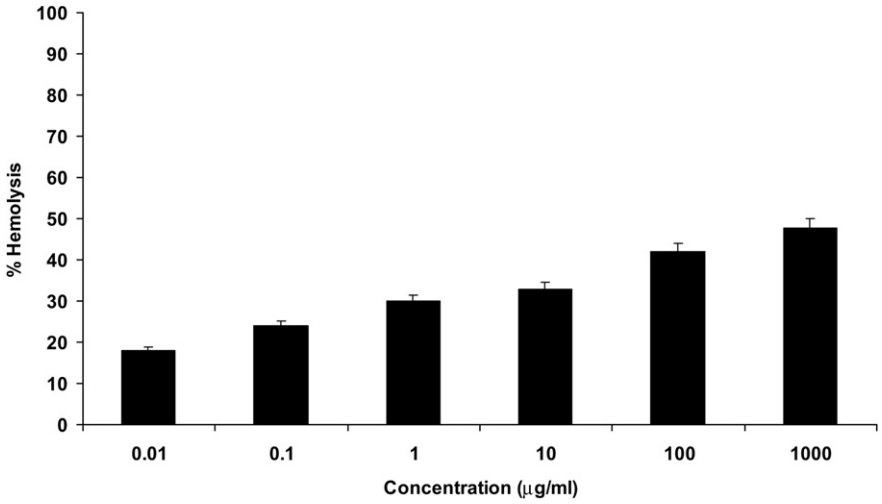

Fig. 4. Hemolysis induced by PETIM dendrimer at different concentrations incubate at 37 ± 1.0 °C for 1 h. Mean ± SD ($n = 3$).

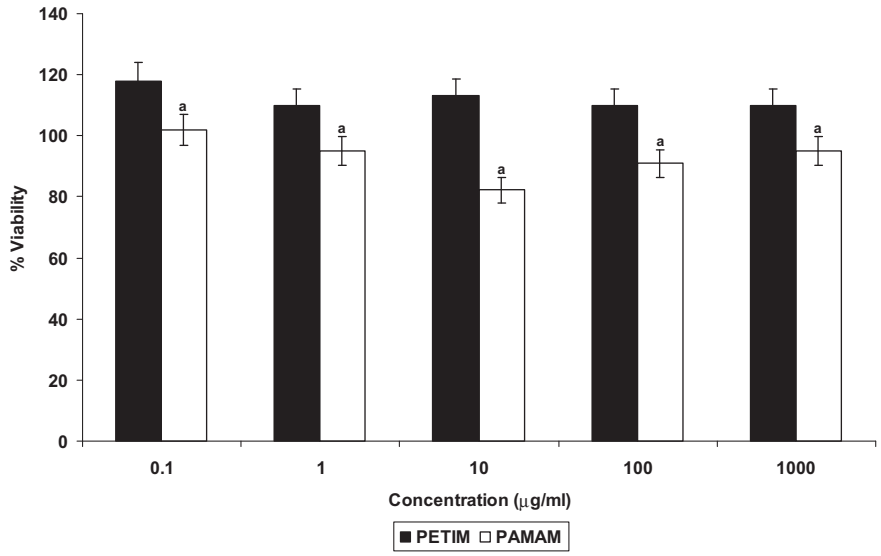

Fig. 5. Comparative cytotoxicity of the PETIM (G3–COOH) Vs commercial available PAMAM (G4-OH) dendrimer in small lung cancer cell line (A-549) after 48 h of incubation.

formazan by reductive cleavage of the tetrazolium ring. Formazan crystals, then can be dissolved in an organic solvent and quantified by measuring the absorbance of the solution at 550 nm, and the resultant value is related to the number of living cells. Results indicated that PETIM dendrimer are less toxic in comparison to commercially available PAMAM dendrimer in comparable concentration range (from 1000 to 0.1 μg/ml with dilution factor of 10×) (Fig. 5). PETIM dendrimer in concentrations range 1000 μg/ml to 0.1 μg/ml exhibited % cell viability as 110, 110, 113, 110 and 118%, respectively. PAMAM G4-OH dendrimer showed 95, 91, 82.2, 95 and 102% viability in comparative concentrations range, respectively. Results of cytotoxicity study revealed the significantly less toxicity of PETIM dendrimer in comparison to commercial G4-OH dendrimer. Similarly, Roberts et al. [27] showed G3 PAMAM dendrimer producing the 90% cell death at 7 ng/ml concentration.

3.5. Acute and sub-acute toxicity study

Dendrimer as drug carrier should be non-toxic in the *in vivo* conditions. However, many reports are available on *in vitro* cellular toxicity assay but only few studies reported for evaluation of chronic, acute and sub-acute toxicity of dendrimer. In the present study acute and 28 days sub-acute toxicity study of PETIM dendrimer was performed as per the OECD (The organization of Economic Co-operation Development) guidelines [15]. Table 2 shows the results of acute and sub-acute toxicity measurement at different doses i.e. 10, 20, 40, 80 and 100 mg/kg of PETIM dendrimer. Animals were observed daily for body weight, behavioral changes, food, water intake and mortality. No mortality or significant changes in general behaviour or other physiological activities were observed at any point in case of animals treated with 10–40 mg/kg PETIM dendrimer. In case of G3–COOH 80 mg/kg, no mortality was there up to 21st day but 83.3% survival was observed on 28th day. In the higher dose of 100 mg/kg, no death was observed up to 14th day but 83.3% and 66% survival was observed on 21st and 28th day, respectively. This mild toxicity is significantly ($p < 0.05$) less than the other dendrimers reported in the literature that are currently being considered as drug delivery vehicles [6]. Preliminary investigations of PAMAM found that toxicity commenced at 45 mg/kg i.p. [27]. The acute study of melamine dendrimer shows that 160 mg/kg is a lethal dose and 100% mortality was observed 6–12 h after i.p. injection [28].

3.6. Hematological toxicity evaluation

Table 3 shows the results of hematological measurements at dose 10, 20, 40 and 80 mg/kg of PETIM dendrimer. Dose was selected based on the results of acute toxicity measurement in which no mortality was observed to dose 80 mg/kg (Table 2). PETIM dendrimer treated group didn't show the significant ($p < 0.05$) difference in the hematological parameters as compared to value of control group and value at 0 day for same group of animals. At higher dose (80 mg/kg) haemoglobin content was found to have significant difference. Similarly, RBC, Platelet count and TLC value were also found to have significant differences. These results are well correlated with % survival/% changes in body weight results. Changes were observed only at higher doses of 80 and 100 mg/kg of PETIM dendrimer. Dendrimer formulation is known for causing the hematological toxicity. Robert et al. [27] also reported the significant changes in

Table 2
Acute and sub-acute toxicity study of PETIM dendrimer.

Group	Treatment	Days after administration				
		% Survival/% Change in body weight				
		1 Day	7 Day	14 Day	21 Day	28 Day
1	Control	100	100	100	100	100
		0	1.2	1.8	2.8	3.1
2	G3–COOH (10 mg)	100	100	100	100	100
		0	0	1.5	1.5	3.0
3	G3–COOH (20 mg)	100	100	100	100	100
		0	0	1.0	3.0	3.1
4	G3–COOH (40 mg)	100	100	100	100	100
		0	0	1.6	3.0	4.8
5	G3–COOH (80 mg)	100	100	100	100	83.3[a]
		0	0	1.6	5.0	7.5[a]
6	G3–COOH (100 mg)	100	100	100	83.3[a]	66[a]
		0	0	3.1	5.0	8.0[a]

The data obtained from various groups were statistically analyzed using one-way ANOVA followed by Tukey's Multiple Range Test. $P < 0.05$, is considered statistically significant.

[a] Data are represent of mean ± SD ($n = 6$). vs control.

Table 3
Effect of PETIM dendrimer on hematological parameters of mice.

Parameters	Control Group		G3–COOH 10 mg/kg		G3–COOH 20 mg/kg		G3–COOH 40 mg/kg		G3–COOH 80 mg/kg	
	0 Day	28 Day	0 Day	28 day	0 Day	28 Day	0 Day	28 day	0 Day	28 day
Body weight	32 ± 1.1	33 ± 1.3	33 ± 0.8	32 ± 0.5	28 ± 1.2	29 ± 1.8	30 ± 1.3	29 ± 1.2	29 ± 0.8	30 ± 0.5
Hb[c] (gm/dl)	10.6 ± 0.4	10.0 ± 1.2	10.5 ± 1.1	9.9 ± 0.1	10.8 ± 2.7	10.2 ± 0.7	9.5 ± 0.52	9.3 ± 0.8	9.7 ± 2.7	8.4 ± 0.2
RBC Million/Cu.m.m	6.1 ± 1.5	5.9 ± 0.9	6.7 ± 2.1	5.1 ± 0.4	6.7 ± 3.1	6.0 ± 2.5	5.2 ± 0.16	5.2 ± 0.2	7.1 ± 3.1	5.1 ± 0.3[b]
TLC[d]/Cu.m.m	7750 ± 494	4800 ± 524	6400 ± 707	8250 ± 494	8233 ± 908	7400 ± 900	6666 ± 2214	7450 ± 1767	6500 ± 173	8400 ± 2121[a]
DLC[e]										
Polymorphs%	32.5 ± 10.6	31.2 ± 4.0	36.5 ± 2.1	39 ± 2.0	34.7 ± 8.4	49.3 ± 7.5	46 ± 14.4	46 ± 5.6	45.0 ± 13.2	52.5 ± 28.9[a,b]
Lymphocytes%	63 ± 9.8	44 ± 8.4	58.5 ± 2.2	56 ± 2.7	58.3 ± 10.4	43 ± 6.5	49.7 ± 15	45.5 ± 2.12	48.3 ± 12.5	44.0 ± 31
Monocytes%	1.5 ± 0.7	2 ± 0.6	2.5 ± 0.7	3 ± 1.4	5.0 ± 1.4	3.0 ± 1.7	1.3 ± 0.5	2	3.0 ± 0.6	3.0 ± 0.3
Eosinophils%	3 ± 0.1	3 ± 0.6	2.5 ± 0.7	2 ± 1.4	3.7 ± 2.3	4.7 ± 0.4	3 ± 1	6.5 ± 3.5	3.3 ± 2.3	3.0 ± 1.4
Platelet count Lakhs/Cu.m.m	13.6 ± 2.2	12.6 ± 1.3	12.1 ± 0.5	16.6 ± 0.8	15.6 ± 4.1	16.6 ± 5.0	13.6 ± 9.06	15.02 ± 7.2	14.2 ± 6.0	18.2 ± 1.1[a]
MCV[f] fl	38.9 ± 12.3	50.9 ± 8.0	46.6 ± 6.6	62.1 ± 7.6	40.6 ± 6.9	61.9 ± 8.2	48.3 ± 1.9	45.6 ± 4.9	47.6 ± 27.8	53.9 ± 0.1
MCH[g] pg	13.7 ± 2.0	18.0 ± 2.4	13.1 ± 3.5	21.8 ± 2.1	12.0 ± 4.7	19.9 ± 1.4	9.3 ± 2.2	11.7 ± 2.0	16.1 ± 12.6	20.7 ± 0.8
MCHC[h] g/dl	26.2 ± 4.3	28.2 ± 5.1	25.6 ± 7.0	28.5 ± 0.7	25.4 ± 8.8	31.6 ± 11	18.9 ± 1.4	31.2 ± 1.1	26.6 ± 10.2	26.1 ± 1.1

The data obtained from various groups were statistically analyzed using one-way ANOVA followed by Tukey's Multiple Range Test. $P < 0.05$, is considered statistically significant.
[a] Data are represent of mean ± SD ($n = 3$). vs control group.
[b] Data are represent of mean ± SD ($n = 3$). vs 0 day value.
[c] Hemoglobin concentration.
[d] Total leukocyte count.
[e] Differential leukocyte count.
[f] mean corpuscular volume.
[g] mean corpuscular haemoglobin.
[h] mean corpuscular haemoglobin concentration.

hematological parameters of mice after treatment with G5 PAMAM dendrimer.

Table 4 shows the effect of PETIM administration on different biochemical parameters of mice blood. These biochemical parameters are the sign of systemic toxicity of carrier i.e. significant decrease in cholesterol level showed severe hepatotoxicity, alteration in SGPT and SGOT level also indicated the severe hepatotoxicity, alteration in urea and total proteins level indicated the nephrotoxicity or alteration in metabolic system and alteration in triglycerides level indicated atherosclerosis. This test is often carried out to determine the risk of developing heart diseases. There was no significant difference ($p < 0.05$) in biochemical parameters of treated groups compare to control group. At higher dose (80 mg/kg) SGOT, cholesterol and urea value of the treated group were found to have a significant difference with control group.

Biosafety profile of PETIM dendrimer was also established by performing the histopathology of vital organs liver, kidney and heart after 28 days of administration. Functional studies in toxicology along with biochemical and hematological studies were coupled with the histopathological study. Histopathological study is useful, especially during the anatomical localization of action of dendrimer and used to identify any alteration in gross morphology to determine the toxic effect of dendrimer [28].

Fig. 6A–F shows the eosin-haematoxylin stained photomicrographs of liver, kidney and heart of the control group and PETIM treated group at dose (80 mg/kg). There were no changes in normal histopathology of organs observed during dosing period with PETIM dendrimer. Photomicrographs of the liver of control group and G3–COOH treated group are comparable. Photomicrograph of the control group shows normal tabular architecture (Fig. 6A). Section of PETIM dendrimer treated group also shows normal tabular architecture of hepatocytes with a slight increase in the number of congested blood vessels (Fig. 6B). Photomicrograph of the kidney of the control group shows the presence of glomeruli and tubular along with congested blood vessels (Fig. 6C). Section of the treated group also shows the normal presence of glomeruli and tubules (Fig. 6D). Similarly, no histopathological changes were observed in the heart of control and treated group. Section of the control group shows the presence of the normal myocardial bundle

Table 4
Effect of PETIM dendrimer on biochemical parameters of mice.

Parameters	Control Group		G3–COOH 10 mg/kg		G3–COOH 20 mg/kg		G3–COOH 40 mg/kg		G3–COOH 80 mg/kg	
	0 Day	28 Day	0 Day	28 day	0 Day	28 Day	0 Day	28 day	0 Day	28 day
Glucose (mg/dl)	56.6 ± 2.1	57.1 ± 5.6	50.8 ± 1.7	52.8 ± 2.4	91.2 ± 8.0	93.7 ± 2.0	82.5 ± 1.9	75.7 ± 1.2	88.7 ± 1.7	73.9 ± 1.0[a,b]
Cholesterol (mg/dl)	80.6 ± 16	81.6 ± 9.2	92.0 ± 20	55.8 ± 1.4	93.6 ± 15	72.2 ± 15	97.4 ± 8.0	91.5 ± 2.5	78.0 ± 2.1	56.1 ± 1.4[a,b]
Urea (mg/dl)	62.4 ± 3.7	56.2 ± 4.9	52.3 ± 11	45.7 ± 9.3	48.2 ± 1.6	54.7 ± 9.7	62.2 ± 2.1	68.9 ± 8.9	53.9 ± 2.2	41.7 ± 5.6[a]
SGPT (IU/l)	48.5 ± 2.8	36.7 ± 9.5	32.5 ± 21	27.3 ± 7.2	48.9 ± 1.7	32.0 ± 5.2	45.6 ± 1.6	26.9 ± 13	38.2 ± 2.3	48.6 ± 6.4
SGOT (IU/l)	149.3 ± 86	132.5 ± 12	161.9 ± 14	147.2 ± 4.3	161.4 ± 4.8	113.0 ± 3.9	145.0 ± 8.5	134.4 ± 7.4	169.5 ± 12	127.6 ± 23[a]
Total proteins (g/dl)	4.9 ± 0.5	5.1 ± 0.9	5.3 ± 1.9	5.6 ± 0.6	5.4 ± 1.1	5.7 ± 0.7	5.2 ± 0.2	3.9 ± 0.6	6.1 ± 0.1	4.8 ± 3.3
ALP (IU/l)	200.6 ± 28	182.4 ± 19	199.2 ± 11	147.0 ± 33	173.7 ± 18.8	160.4 ± 39	178.0 ± 10.6	187.7 ± 14	148 ± 8.7	148.4 ± 14
Creatinine (mg/dl)	0.73 ± 0.05	0.95 ± 0.08	0.83 ± 0.1	1.0 ± 0.2	0.84 ± 0.1	0.55 ± 0.05	0.9 ± 0.1	1.0 ± 0.1	0.79 ± 0.02	0.97 ± 0.1
Bilirubin (mg/dl)	0.98 ± 0.02	0.98 ± 0.02	0.69 ± 0.2	0.79 ± 0.1	0.66 ± 0.2	0.71 ± 0.1	0.79 ± 0.1	0.71 ± 0.1	0.82 ± 0.02	0.81 ± 0.02

The data obtained from various groups were statistically analyzed using one-way ANOVA followed by Tukey's Multiple Range Test. $P < 0.05$, is considered statistically significant.
[a] Data are represent of mean ± SD ($n = 3$). vs control group.
[b] Data are represent of mean ± SD ($n = 3$). vs 0 day value.

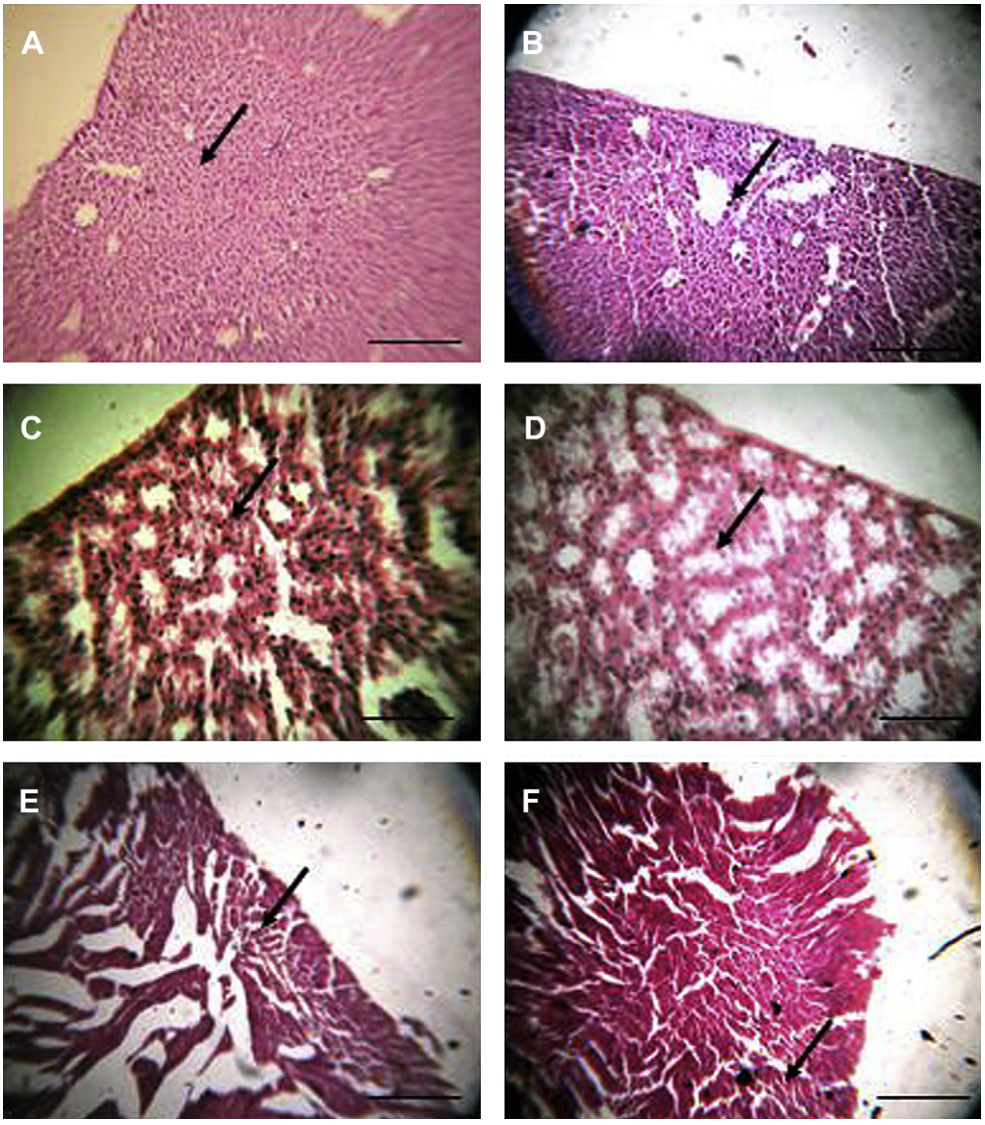

Fig. 6. Eosin-hematoxylin stained photomicrographs of liver, kidney and heart of control (A, C and E) and PETIM treated group (B, D and F) (magnification 450×) (Scale bar = 500 μm).

(Fig. 6E). Section of PETIM treated group also shows the presence of the normal myocardial bundle with increased congested blood vessels (Fig. 6F).

4. Conclusion

Dendrimer as carrier system has many advantages over other carrier systems like liposomes having the problems of stability and cost, nanoparticle having the problems of manufacturing process and batch to batch reproducibility. PETIM is a new development in the field of dendrimer. Results of present study revealed that this carrier system has good biocompatibility as determined by acute, sub-acute and cytotoxicity measurement assay. Cytotoxicity assay demonstrated significantly less cytotoxicity of PETIM dendrimer in comparison to commercial PAMAM dendrimer. PETIM dendrimer was also found to successfully encapsulate the model drug ketoprofen and *in vitro* drug release study showed its sustained release potential. PETIM dendrimer seems to be an attractive approach for sustained delivery of bioactive. Above studies showed that, PETIM dendrimer may be useful as a potentially safe drug delivery vehicle, having sustained release kinetics.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.ejmech.2010.08.006.

References

[1] M.J. Liu, J.M.J. Frechet, Designing dendrimers for drug delivery, Pharm. Sci. Technol. Today 2 (1999) 393–401.
[2] T. Shakthivel, A.T. Florence, Dendrimers and dendrons: facets of pharmaceutical nanotechnology, Drug Deliv. Technol. 3 (2003) 73–78.

[3] D.A. Tomalia, Birth of a new macromolecular architecture: dendrimers as quantized building blocks for nanoscale synthetic polymer chemistry, Prog. Polym. Sci. 30 (2005) 294–324.
[4] Y. Cheng, Z. Xu, M. Ma, T. Xu, Dendrimers as drug carriers: applications in different routes of drug administration, J. Pharm. Sci. 97 (2008) 123–143.
[5] D.M. Domanski, M. Bryszewska, G. Salamonczyk, Preliminary evaluation of the behavior of fifth-generation thiophosphate dendrimer in biological systems, Biomacromolecules 5 (2004) 2007–2012.
[6] R. Duncan, L. Izzo, Dendrimer biocompatibility and toxicity, Adv. Drug Deliv. Rev. 57 (2005) 2215–2237.
[7] N. Jayaraman, G.A. Jayamurugan, Dendritic macromolecules and a process therefore, Patent no. WO/2009/004639, 2009.
[8] T.R. Krishna, N. Jayaraman, Synthesis of poly (propyl ether imine) dendrimers and evaluation of their cytotoxic properties, J. Org. Chem. 68 (2003) 9694–9704.
[9] G. Jayamurugan, N. Jayaraman, Synthesis of large generation poly (propyl ether imine) (PETIM) dendrimers, Tetrahedron 62 (2006) 9582–9588.
[10] T.R. Krishna, M. Belwal, U.S. Tatu, N. Jayaraman, Synthesis of water soluble and non toxic poly(etherimine) dendrimers, Tetrahedron 61 (2005) 4281.
[11] C. Jana, G. Jayamurugan, R. Ganapathy, P.K. Maiti, N. Jayaraman, A.K. Sood, Structural of poly(propyl ether imine) dendrimer from fully atomistic molecular dynamics simulation and by small angle x-ray scattering, J. Chem. Phys. 124 (2006) 204719.
[12] A. Papagiannaros, K. Dimas, G.T. Papaioannou, C. Demetzos, Doxorubicin-PAMAM dendrimer complex attached to liposomes: cytotoxic studies against human cancer cell, Int. J. Pharm. 302 (2005) 29–38.
[13] T. Higuchi, A. Connors, Phase-solubility Techniques. in: C.N. Reilly (Ed.), Advances in Analytical Chemistry and Instrumentation. John Wiley, New York, 1965, pp. 117–212.
[14] F. Wang, T.K. Bronich, A.V. Kabanov, R.D. Rauh, J. Roovers, Synthesis and characterization of star poly(#-caprolactone)- *b*-poly(ethylene glycol) and poly(l-lactide)- *b*-poly(ethylene glycol) copolymers: evaluation as drug delivery carriers, Bioconjug. Chem. 19 (2008) 1423–1429.
[15] A.K. Singhai, S. Jain, N.K. Jain, Evaluation of an aqueous injection of ketoprofen, Pharmazie 52 (1997) 149–151.
[16] OECD (The organization of Economic Co-operation Development), The OECD Guideline for Testing of Chemicals: 420 Acute Oral Toxicity. OECD, Paris, 2000b, 1–14.
[17] S. Jain, P. Jain, R.B. Umamaheshwari, N.K. Jain, Transfersomes – a novel vesicular carrier for enhanced Transdermal delivery: development, characterization, and performance evaluation, Drug Dev. Ind. Pharm. 29 (2003) 1013–1026.
[18] H. Satterwhite, F.D. Boudinot, High-performance liquid chromatographic determination of ketoprofen and naproxen in rat plasma, J. Chromatogr. Biomed. Appl. 431 (1988) 444–449.
[19] Y.Y. Cheng, T.W. Xu, Dendrimers as potential drug carriers. Part I. Solubilization of non-steroidal anti-inflammatory drugs in the presence of polyamidoamine dendrimers, Eur. J. Med. Chem. 40 (2005) 1188–1192.
[20] C. Yiyun, X. Tongwen, F. Rongqiang, Polyamidoamine dendrimers used as solubility enhancers of ketoprofen, Eur. J. Med. Chem. 40 (2005) 1390–1393.
[21] M. Han, P. Chen, X. Yang, Molecular dynamics simulation of PAMAM dendrimer in aqueous solution, Polymer 46 (2005) 3481–3488.
[22] P. Blasi, A. Schoubben, S. Giovagnoli, L. Perioli, M. Ricci, C. Rossi, Ketoprofen Poly(lactide-co-glycolide) physical interaction, AAPS PharmSciTech 8 (2007) (Article 37).
[23] A.D. Emmanuelle, D. Attwood, Dendrimer drug interaction, Adv. Drug Delivery Rev. 57 (2005) 2147–2162.
[24] M. Na, C. Yiyun, X. Tongwen, D. Yang, W. Xiaomin, L. Zhenwei, C. Zhichao, H. Guanyi, S. Yunyu, W. Longping, Dendrimers as potential drug carriers. Part II. Prolonged delivery of ketoprofen by in vitro and in vivo studies, Eur. J. Med. Chem. 41 (2006) 670–674.
[25] H.T. Chen, M.F. Neerman, A.R. Parrish, E.E. Simanek, Cytotoxicity, hemolysis, and acute in vivo toxicity of dendrimers based on melamine, candidate vehicles for drug delivery, J. Am. Chem. Soc. 126 (2004) 10044–10048.
[26] D.M. Domański, B. Klajnert, M. Bryszewska, Influence of PAMAM dendrimers on human red blood cells, Bioelectrochemistry 63 (2004) 189–191.
[27] J.C. Roberts, M.K. Bhalgat, R.T. Zera, Preliminary biological evaluation of polyamidoamine (PAMAM) Starburst (TM) dendrimers, J. Biomed. Mater. Res. 30 (1996) 53–65.
[28] M.F. Neerman, W. Zhang, A.R. Parrish, E. Simanek, In vitro and in vivo evaluation of a melamine dendrimer as a vehicle for drug delivery, Int. J. Pharm. 281 (2004) 129–132.