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International Journal of Pharmaceutics 257 (2003) 111–124
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

A PEGylated dendritic nanoparticulate carrier of fluorouracil

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Received 23 April 2002; received in revised form 11 November 2002; accepted 5 February 2003

Abstract

The present study was aimed at developing and exploring the use of uncoated and PEGylated newer PAMAM dendrimers for delivery of anti-cancer drug 5-fluorouracil. For this study, successive Michael addition and exhaustive amidation reactions were used to synthesize 4.0G PAMAM dendrimers, using ethylenediamine as core and methylmethacrylate as propagating agent. The dendrimer was PEGylated using *N*-hydroxysuccinimide-activated carboxymethyl MPEG-5000. IR and NMR data proved the synthesis. Various physicochemical parameters, SEM, TEM, λ_{max} values, hemolytic toxicity, drug entrapment, drug release and blood-level studies of both PEGylated and non-PEGylated systems were determined and compared. The PEGylation of the systems was found to have increased their drug-loading capacity, reduced their drug release rate and hemolytic toxicity. TEM study revealed surface properties of the systems. Stability studies had shown its stability at room temperature in dark. The systems were found suitable for prolonged delivery of an anti-cancer drug by in vitro and blood-level studies in albino rats, without producing any significant hematological disturbances. PEGylation has been found to be suitable for modification of PAMAM dendrimers for reduction of drug leakage and hemolytic toxicity. This, in turn, could improve drug-loading capacity and stabilize such systems in body. The study suggests use of such PEGylated dendrimeric systems as nanoparticulate depot type of system for drug administration.

Keywords: Poly(ethylene glycol); Polyamidoamine; Nanoparticle; Dendrimers; Fluorouracil; Anti-cancer

1. Introduction

Dendrimers are hyperbranched, uniformly distributed structures, having definite molecular weight, shape, size and host–guest entrapment properties. These have globular, highly branched, regular repeated molecular architecture that is constructed via stepwise procedures. There is therefore unprecedented control over structural units positioning (Tomalia et al., 1985). Dendrimers were recently reported as carriers for various chemotherapeutic agents, e.g. methotrexate (Gandhi, 1997), as metal complexes of dendrimer for delivery of anti-cancer drug 6-mercaptopurine (Khopade et al., 1999), fatty acid grafted dendrimers for delivery of anti-cancer drug 5-fluorouracil (5-FU) (Khopade et al., 2002).

Similarly, many PEGylated dendrimeric systems, which had been explored as potential drug-delivery agents, were reported earlier, like that consisting of water-soluble dendritic unimolecular micelles

Abbreviations: PAMAM, polyamidoamine; 4.0G, fourth generation of dendrimers; PEGylated, polyethylene glycol conjugated; MPEG-5000, methoxy polyethylene glycol of molecular weight 5000 Da; IR, infrared spectroscopy; NMR, nuclear magnetic resonance spectroscopy; SEM, scanning electron micrograph; TEM, transmission electron micrograph

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doi:10.1016/S0378-5173(03)00132-7

prepared by coupling with hydrophobic dendritic hypercores and surrounded by polyethylene glycol (PEG)-mesylates as hydrophilic shell. The monomeric core that was selected to build it was 4,4-bis (4′-hydroxy phenyl) pentanol, as this large monomeric unit provides flexibility to the dendritic structures, while contributing to the container capacity of the overall structure of four generations of dendritic unimolecular micelles. The container property was demonstrated by 'pyrene' solubilization in aqueous solution. This was demonstrated by indomethacin entrapment upto 11% and its sustained release characteristics (Liu et al., 2000). Another structure having polyether dendritic bromide with polyethylene oxide or PEG chains had been reported. In those copolymers, lower generation dendrons, especially G1 tend to form unimolecular micelle, whereas G2 and G3 formed multi-molecular micelles, presumably driven by hydrophobic effects and π–π interaction between dendritic blocks (Gitsov and Frechet, 1993).

Gitsov and Frechet (1996) also synthesized a novel class of amphiphilic star copolymers. To a four-armed PEG 'star' scaffolds derived from pentaerythritol core were attached four polyether dendrons. Results of SEC/VISC and ^{1}HNMR studies indicated the formation of unimolecular micelles in chloroform, tetrahydrofuran or methanol but with strikingly different structures. Thus, the star copolymers could self-organize into different micellar structures as a function of the environment. A potential application of these 'stimuli responsive copolymers' involved their uses as solvent-specific encapsulation agents. Chapman et al. (1994) also prepared amphiphilic copolymers derived from linear PEO and tBoC-terminated poly-α-ϵ-lysine dendrimers. The use of PEO as a platform for the dendrimers synthesis greatly facilitated separation because product upto fourth generation could be precipitated from reaction mixtures by ether. Surface tension method was employed to determine critical micelle concentration and micelle formation behavior. The surface of the aggregates was suggested to be highly compact. The existence of transition concentration for dye solubility of orange OT by G4 hydramphiphiles in water supported the micellar behavior.

There are a number of problems associated with PAMAM dendrimers like drug leakage due to relatively open network and hemolytic toxicity due to $-NH_2$ groups on the periphery. The PEG coating on the periphery of the amine-terminated whole generation dendrimers chemically can increase drug loading and overcome few drawbacks of drug-loaded dendrimeric system like hemolytic toxicity, drug leakage, macrophageal uptake, etc. and in case of 5-FU, like short half life and toxicity. Like for other drug-delivery systems, PEG conjugation (PEGylation) can greatly improve the stability and efficacy of dendrimers making them suitable conjugate for delivery of bioactive species in the body for much longer periods. This PEGylation can also help in targeting the system to the active sites of action suitably, with reduced immunogenecity, anti-genecity and toxicity by shielding the system from recognition by the detection and destruction mechanisms of the body. It prevents the sequestration and uptake of the system by reticulo-endothelial system as liver, spleen, etc. (Bhadra et al., 2002).

2. Materials and methods

2.1. Materials

Ethylenediamine and methylmethacrylate (E. Merck, India) were used after redistillation. Monomethoxy polyethylene glycol 5000 (MPEG-5000) was obtained from Sigma Chemical Co. (St. Louis, MO) and the drug 5-FU was obtained as gift sample from Roche, Switzerland. Cellulose dialysis tubing of MWCO 12000–14000 and pore size of 2.4 nm was purchased from HiMedia Lab, India. Rest all the chemicals were purchased from CDH, India.

2.2. Synthesis of PAMAM dendrimers

The dendrimers were synthesized on the basis of two consecutive chains forming reactions, the exhaustive Michael addition reaction and the exhaustive amidation reaction, repeating alternatively. Michael addition reaction was used for initiating the dendrimers using ethylenediamine molecule as initiator core. The exhaustive amidation reaction was used to link amine as ethylenediamine on to the carboxylic acid group of methyl methacrylate (taken in stoichiometric amounts) in dendrimers (Tomalia et al., 1985; Zhuo et al., 1999). The dendrimers so formed were lyophilized and used for further studies.

2.3. PEGylation

The polyethylene glycol derivatization (PEGylation) was done after activation of end functional groups of MPEG-5000. The activation of MPEG-5000 was done by first converting it to carboxylic acid derivatives and then to NHS ester (Veronese et al., 1989). One hundred milligram (6.3 μM) of lyophilized 4.0G PAMAM dendrimer having 64 terminal amine groups of theoretical molecular weight 15,932 g was dissolved in double distilled water. Sixteen molar times of MPEG-activated NHS ester (0.55 g) was added at basic pH (8–10) of dendrimer solution and stirred vigorously for 2 h at room temperature (RT) in dark. The final product was dialyzed to remove byproducts. This dialyzed product was then concentrated and lyophilized. The product was dissolved in dichloromethane and precipitated from cold diethyl ether as oily viscid lump, which was separated and dried.

2.4. Drug loading in formulations

The dendrimers so synthesized were dissolved in water and mixed with 100 molar times of 5-FU and allowed 5-FU to dissolve. The mixed solution was allowed to incubate with slow magnetic stirring (50 rpm) using teflon beads for 24 h. This solution was twice dialyzed under strict sink conditions at 10 min to remove free drug from the formulations, which was then estimated spectrophotometrically to determine indirectly the amount of drug bound with the system. The dialyzed formulations were lyophilized and used for further characterization.

2.5. Physicochemical characterization of the formulations

Every newer generation of dendrimers were confirmed for the completion of reaction at each step by copper sulfate aqueous solution. The degree of PEGylation was also evaluated using copper sulphate solution by determining ratio of decrease in absorbance of the violet color developed by 3 ml of copper sulphate solution (1%, w/v solution in water) on mixing with approximately equivalent amounts of 4.0G simple dendrimers and PEGylated dendrimers. TEM (Phillips) of PEGylated dendrimers were used to characterize surface and shape of these systems after drying on beryllium grid and staining negatively, at 180,000×.

The PEGylated and simple dendrimeric samples were analyzed by Infrared (IR, Perkin-Elmer) and NMR spectroscopy. For NMR study, 4.0G dendrimer was solubilized in D_2O using methanol as cosolvent and analyzed at 300 MHz (DRX 300). These were also analyzed in the range of 300–500 nm against water in UV1601 spectrophotometer (Shimadzu, Japan). Various drug-loaded formulations were scanned against same concentrations of unloaded dendrimer solution as blank to determine the shifts in peaks of drug on binding and loading in dendrimers.

One hundred milligram of dendrimer–drug complex formulation was filled in dialysis bag and the entrapped drug was allowed to pass out of the bag against perfect sink condition. Similarly, dialyzed and diluted dendrimer solutions without drug were taken as blank. The drug released was analyzed spectroscopically. This data was used for in vitro drug release study from dendrimeric systems at every 1-h interval for 3 days of both PEGylated and non-PEGylated dendrimer–drug solutions.

2.6. Hemolytic toxicity of dendrimer–drug systems

The RBC suspension was obtained as per the well-known and reported procedure for hemolytic studies (Singhai et al., 1997). For this, the RBC suspension was mixed with distilled water, which was considered as producing 100% hemolysis, and normal saline producing no hemolysis, hence acting as blank. 0.5 ml of suitably diluted PEGylated and non-PEGylated dendrimer–drug formulation was added to 4.5 ml of normal saline and interacted with RBC suspension. Similarly, 0.5 ml of drug solution and 0.5 ml of dendrimer solution were mixed with 4.5 ml of normal saline and interacted with RBC suspension. The drug and dendrimers in separate tubes were taken in such amount that the resultant final concentration of drug and dendrimer was equivalent in all the cases. The PEGylated system of dendrimer–drug complex was taken in amount such that the resultant final concentration of drug and dendrimer was equivalent to that in non-PEGylated systems. This allowed comparison of the hemolysis data of the drug, dendrimer, dendrimer–drug (5-FU) complex

and PEGylated systems to help understand the effect of PEGylation on hemolysis.

After centrifugation, supernatants were taken and diluted with an equal volume of normal saline and absorbance was taken at 540 nm against supernatant of normal saline diluted similarly as blank. The percent hemolysis was thus determined for each sample by taking absorbance of water as 100% hemolytic sample.

2.7. Stability studies of PEGylated dendrimer formulations

The PEGylated 4.0G dendrimer–drug formulation was kept in tightly closed amber colored and colorless glass vials at refrigerated condition (4 °C), RT (25 °C) and 50 °C for 5 weeks. The samples were analyzed initially and periodically at weekly intervals for any precipitation, turbidity, crystallization, change in color, consistency, drug leakage and chemical nature of formulations. The data obtained were used for the analysis of any physical or chemical degradation at the given storage conditions. Chemical stability was ascertained by comparison of the intensity of color developed by 1 ml of 1%, w/v solution of copper sulfate with 1 ml of formulation spectrophotometrically ($\lambda_{max} = 535$ nm). Increase in release of drug from the formulations after storage at accelerated conditions was also evaluated. The formulation samples (2 ml) were kept in cellulose dialysis tubing and dialyzed across the tubing. The external medium (10 ml) was analyzed for content of drug (5-FU) spectrophotometrically. The procedure was repeated every week for upto 5 weeks. The percentage increase in drug release from the formulation was used to analyze the effect of accelerated conditions on storage of the formulations.

2.8. Blood-level studies

Twelve male albino rats of Sprague–Dawley strain of similar weights were selected for in vivo studies. The animals were kept in well-spaced ventilated cages (Tarsons, India) and maintained on healthy and fixed diets prior to the studies and on minimal diet and feeding overnight prior to and on the day of blood-level studies. The animals were divided equally into four groups. The first group served as control, the second group was given 1000 μg 5-FU, intravenously (calculated at dose level of 12 mg/kg in human beings as reported in Reynolds, 1996) and the two remaining groups were given PEGylated and non-PEGylated dendrimer–drug formulations containing 5-FU equivalent to 1000 μg, intravenously through caudal vein. From the retro-orbital plexus of rats, 0.1 ml of blood samples were taken out at regular intervals, using uniformly tapered capillary at every 15 min for upto 2 h, then every half hour upto next 4 h, and at an hourly interval for next 6 h. The drug level in the blood was determined using spectroscopic method (Sawant and Murthi, 1993).

2.9. Hematological studies

The albino rats of uniform weight, size and sex were divided into four groups comprised of three animals in each group. First, second and third groups of animals were given simple plain drug (5-FU), non-PEGylated and PEGylated 5-FU–dendrimer complex (4.0G), respectively, containing 1000 μg of 5-FU (i.v.), every day. Fourth group was kept as control, which was maintained on same regular controlled minimal diet for 14 days. After 14 days blood was withdrawn from the animals and was analyzed for hemoglobin (Hb.) level, RBC, WBC, differential monocytes, lymphocytes and neutrophil counts.

3. Results and discussion

3.1. Synthesis of dendrimers and their physicochemical properties

A newer PAMAM series of dendrimers were synthesized on the basis of two consecutive chains forming reactions, repeating each alternatively (Fig. 1), using methyl methacrylate instead of methyl acrylate as branching monomer, as reported by Tomalia et al. (1985). All the reaction steps were confirmed for completion by the color developed by dendrimers on complexation with copper sulphate. The completion of reactions of whole generation dendrimers was confirmed by purple color and half generations were confirmed by deep blue color due to copper-chelation at the terminal groups of dendrimers. The peaks in IR and NMR further confirmed the progress of synthesis of dendrimers in each generation, which matched with theoretically reported peaks. The important peaks in

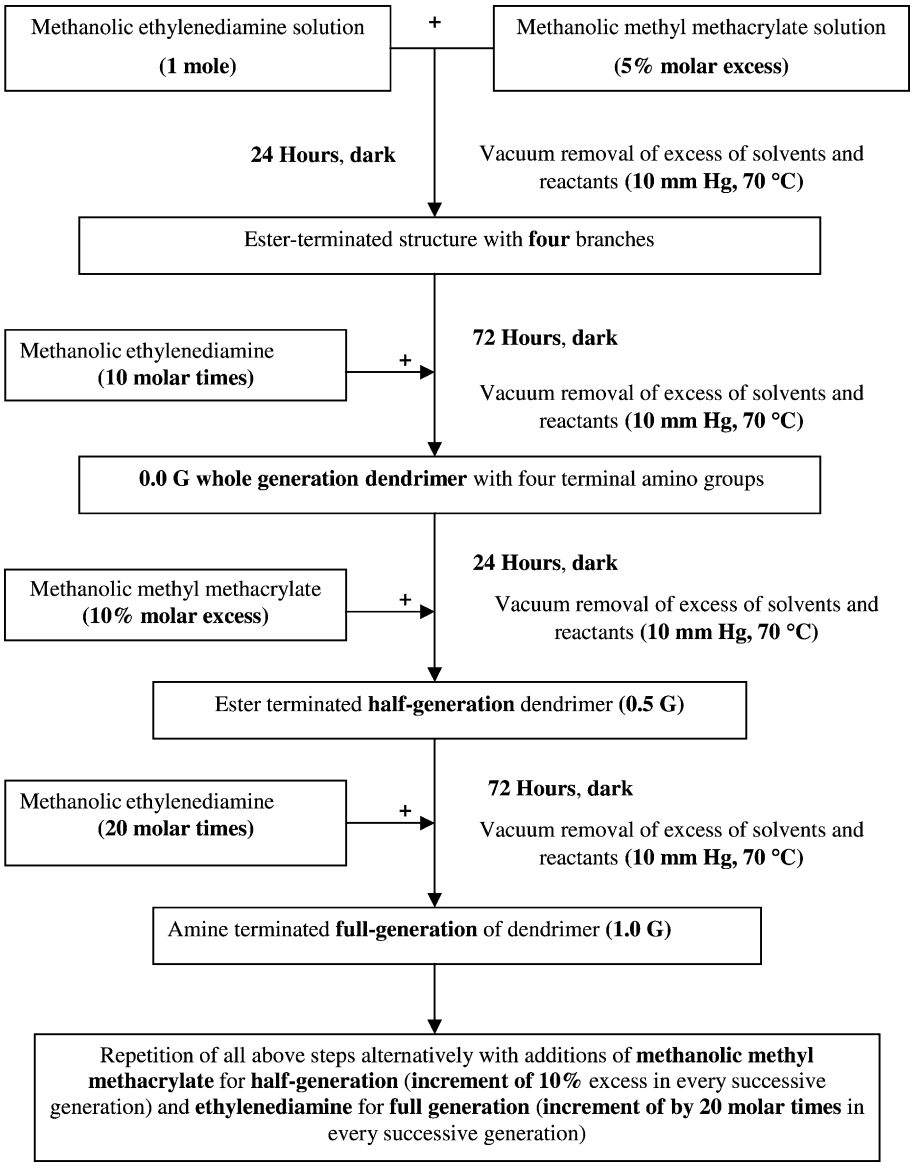


Fig. 1. Flow chart for synthesis of non-PEGylated PAMAM type of dendrimers.

IR spectra of 4.0G dendrimers were of N–H stretch for terminal primary amine at 3400 cm^{-1}; 3192 cm^{-1} for N–H stretch anti-symmetric type of substituted primary amine; 2883 cm^{-1} for C–H stretch; peak at 2179.4 cm^{-1} for quaternary ammonium ion (responsible for +ve charge); peak at 1620 cm^{-1} for C=O stretch of carbonyl group; peaks at 1278.7 and 1245.9 cm^{-1} for C–N stretch of primary amine, etc. Important shifts in NMR spectra of 4.0G dendrimer were 1.2–1.5 ppm (triplet and singlet) for secondary R_2CH_2 group; shifts at 2.4–2.6 ppm (triplet) for carbonyl ($CH_3C{=}O$) groups; doublet at 3.3–3.5 ppm for $–CH_2NH_2$ terminal groups; multiplet at 3.6–3.9 ppm for acidic–OH groups, etc.

The dendrimers obtained were light yellow to reddish yellow in color, oily, honey-like viscous liquids.

The lower generation dendrimers had lighter greenish yellow color and lesser viscosity. The viscosity increased and colors deepened as generations increased. The color change was found to be varying from yellowish to deep yellow to honey color and the higher generations were found to be nearing the state of glassy mass. The boiling point of the concentrated dendrimers was in the range of 168–220 °C. The dendrimers were found to be highly hygroscopic. They were stored as 10%, w/w solution in methanol, for maintaining the fluidity. The ester-terminated dendrimers had sweet smell but whole generation dendrimers had little odd, ammonia like odor. These were not soluble in diethyl ether and carbon tetrachloride but were found to be increasingly soluble in chloroform, dichloromethane, ethanol, methanol, and water with increase in generations of dendrimers. The refractive index of the dendrimers also decreased in case of higher generation of dendrimers from 1.463 to 1.355. The specific density of concentrated dendrimer was found to decrease from lower to higher generations in the range of 0.46 in case of 1.0G to 0.21 in case of 5.0G concentrated dendrimers. Intrinsic viscosity of whole generations of dendrimers increased with increase in generations, i.e. from 0.038 to 0.075 dl/g in case of 5.0G dendrimers. λ_{max} values were found not to be following so distinctive trend with increase in generations of dendrimers. These were found to be matching with the reported trends of similar PAMAM dendrimers (Tomalia et al., 1990).

3.2. Evaluation of PEGylation of dendrimers

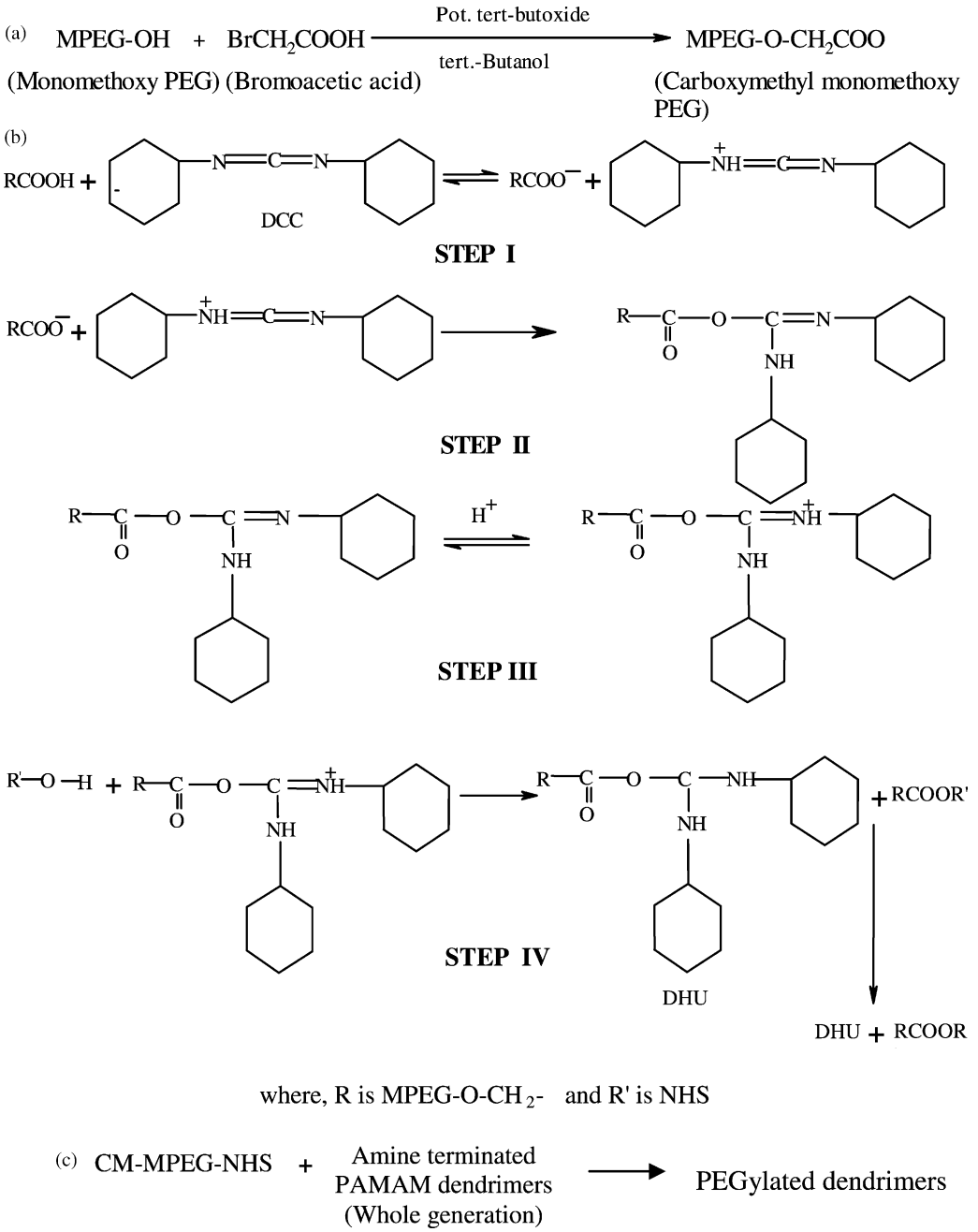
PEG coating was done after conversion of MPEG to carboxylic acid-terminated species followed by activation of the terminal –COOH group by DCC and NHS to *N*-hydroxysuccinimide ester derivatives (Fig. 2) (Royer and Anantharmaiah, 1979; Veronese et al., 1989). It was then hydrolyzed and amide formation, at the terminal $–NH_2$ groups of the dendrimers, had taken place at the basic pH (10–12). The impurities were removed by dialysis. The solvents as water and traces of methanol were removed by evaporation under vacuum. The dendrimers were found to have been PEGylated nearly 25% by percentage quenching of the intensity of violet color of copper-dendrimer chelate.

The PEGylation can well be confirmed by IR and NMR spectroscopic methods. The major peak of MPEG–COOH in IR spectra that undergoes major change is peak of carbonyl resonating symmetric and anti-symmetric peaks on linking by amide linkages at the dendrimeric ends. An important IR peak near 1100 cm^{-1} of ether linkage C–O appears prominently in the spectrum of the dendrimers 4.0G PEGylated species (Fig. 3). These two major changes in C–O linkages in dendrimers proved that the dendrimers had been well PEGylated. There is an upshift of C=O stretch of acid group of carboxymethyl MPEG on amide linkages.

The NMR spectrum (Fig. 4) and shifts of PEGylated dendrimers as compared to that of simple dendrimers provided the proof of PEGylation. There was a drastic increase in integral value for the shift of secondary $–CH_2$ groups on PEGylation. This might be due to increase in number of secondary $–CH_2$ groups in MPEG that are linked on PEGylation. Similarly, newer peak of ether linkage appears at 3.3–3.5 ppm in remarkably increased amount. This is due to the presence of ether linkages in MPEG in very high amount as compared to its total absence in 4.0G NMR. Thus, various peaks and shifts obtained were analyzed and interpreted (Table 1). The relative changes in peaks of PEGylated dendrimers as compared to non-PEGylated ones were used to confirm PEGylation. The extent of PEGylation was also confirmed by the decrease in intensity of violet color produced by dendrimers of various generations on interaction with copper sulphate. This served as the proof of approximately 25% PEGylation of the dendrimers of 4.0G generation.

3.3. In vitro evaluation of PEGylated dendrimers

The transmission electron microscopy (TEM) had shown surfaces of PEGylated dendrimers and increase in particle size on coating with MPEG-5000 (Fig. 5). This also showed increase in consistency of outer surface of non-PEGylated dendrimers, which had relatively open outer surface layer. The dendrimer–drug complexation and drug loading of the dendrimer can easily be accomplished by incubating drug with the dendrimer. The hydrophilicity and aqueous partitioning of the drug molecules facilitated complexation and hydrogen bonding type interaction of drug with dendrimers leading to binding of drug. This was evident by a sufficient change in the values of original



where, R is MPEG-O-CH$_2$- and R' is NHS

(c) CM-MPEG-NHS + Amine terminated PAMAM dendrimers (Whole generation) → PEGylated dendrimers

Fig. 2. Formation (a), activation (b), and reaction (c) of carboxymethyl-monomethoxy polyethylene glycol-NHS-activated ester (CM-MPEG-NHS) with dendrimers.

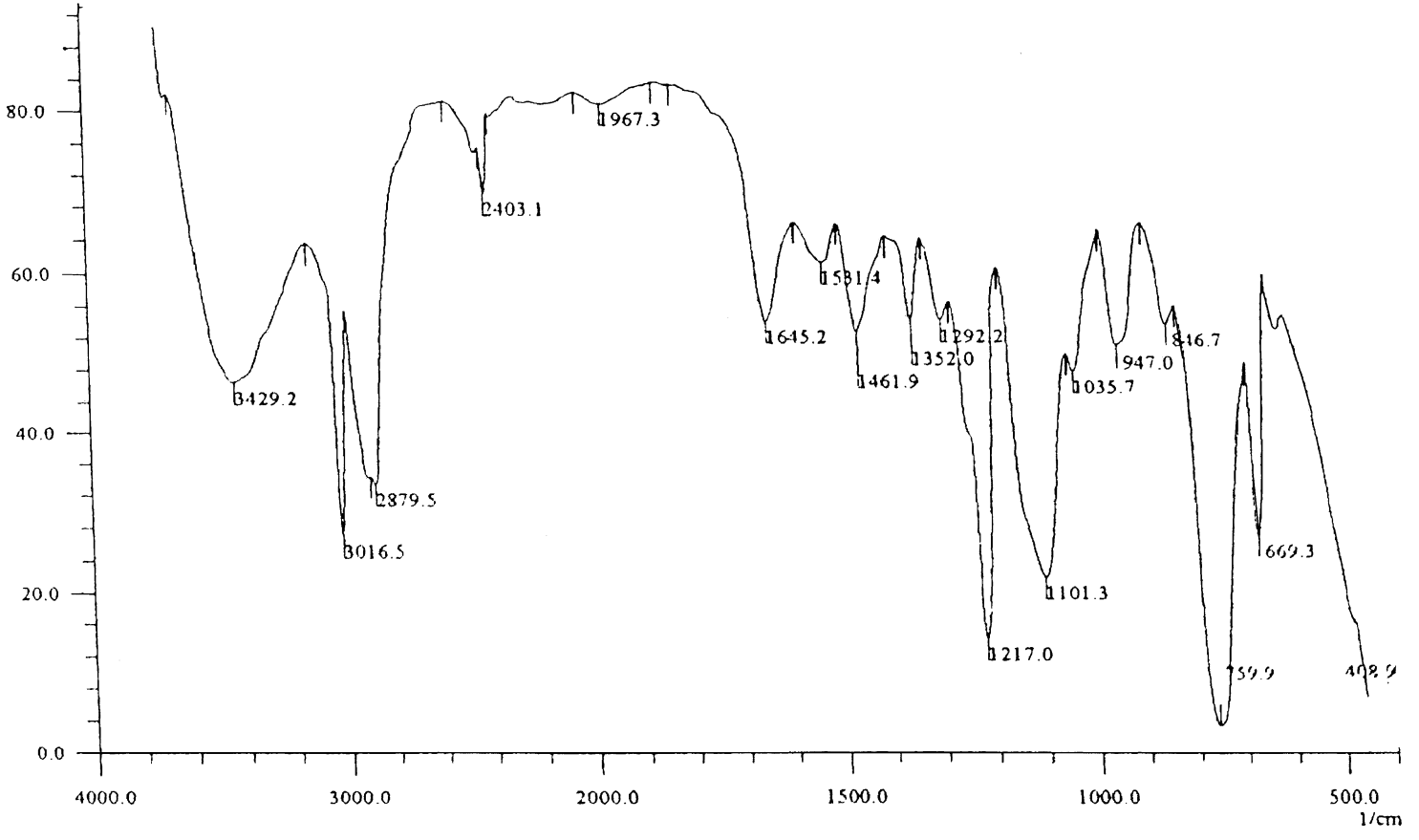


Fig. 3. IR spectroscopy of PEGylated 4.0G dendrimer.

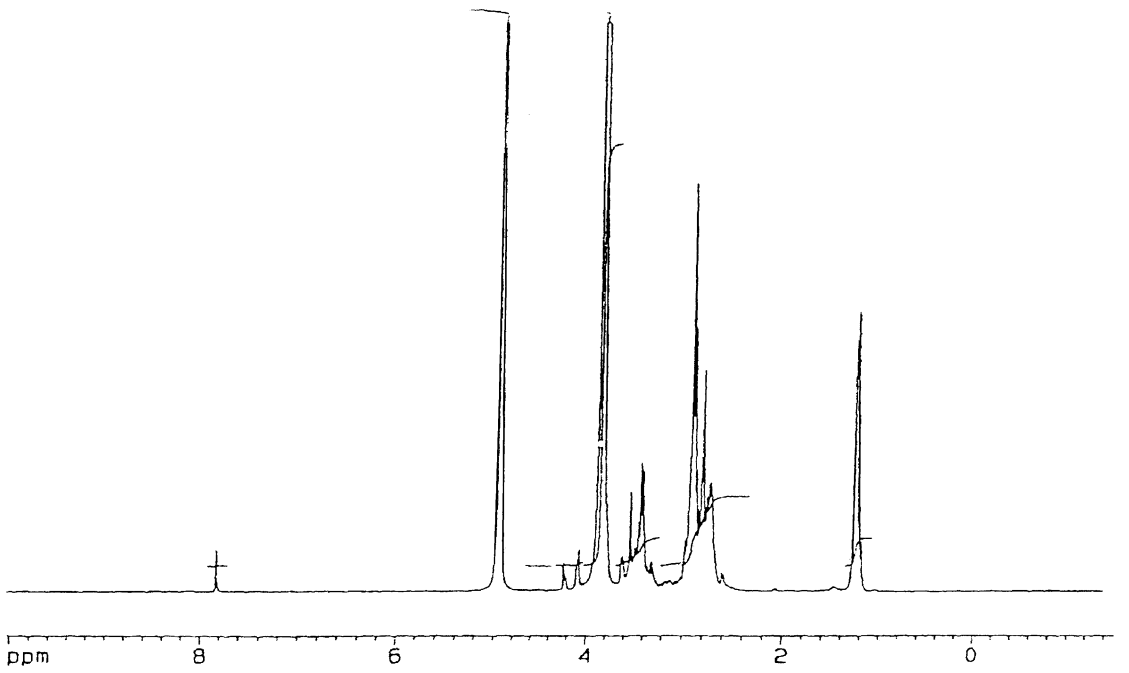


Fig. 4. NMR spectroscopy of PEGylated 4.0G dendrimer.

λ_{max} of FU (266.6 nm), which was found to be 266.0 nm in water and 270.0 and 307.0 nm for 5-FU entrapped in 4.0G dendrimers, when scanned against dendrimer of same concentration as blank. This was similar to that illustrated for eriochrome black-T with poly(propyleneimine) dendrimers (Jansen et al., 1994).

5-FU entrapment in PEGylated dendrimers increased significantly by 12 times due to more sealing of dendrimeric structure by MPEG at the peripheral portions of dendrimers as coat, which prevented drug release by enhancing complexation probably by steric and electronic effects of the additional functional groups made available by MPEG (Fig. 6). This, in turn, also reduced rate of drug release from such systems across dialysis membrane as observed during release profile studies, when the dendrimers were retained inside the membrane bag easily due to their higher molecular weight and hyperbranched polymeric spherical constitution.

The average release rate for PEGylated dendrimeric system was found to be 0.679%, which was nearly 1/6 times of that from non-PEGylated types (Fig. 7). Thus, release rates of drug from PEGylated systems and higher generations were found to have decreased due to dense chains of PEG (PEG coating) closing and covering the periphery (Fig. 6). Release of 5-FU from such dendrimers of PEGylated types continued

Table 1
NMR peaks of PEGylated dendrimeric formulations

δ value range (ppm)	Integral values	Interpretation
1.2–1.5 (triplet)	27.30	R_2CH_2 (secondary)
2.5–2.8 (mixed multiplet)	69.52	($-CH_3C{=}O$) carbonyl
3.6–3.8 (mixed multiplet)	27.45	Ether group of MPEG
3.3–3.5 (doublet large)	418.06	$-CH_2NH_2$ (remaining free amines)
4.8–5.0 (singlet large)	4.822	Amide ($-C{=}O-NH$)

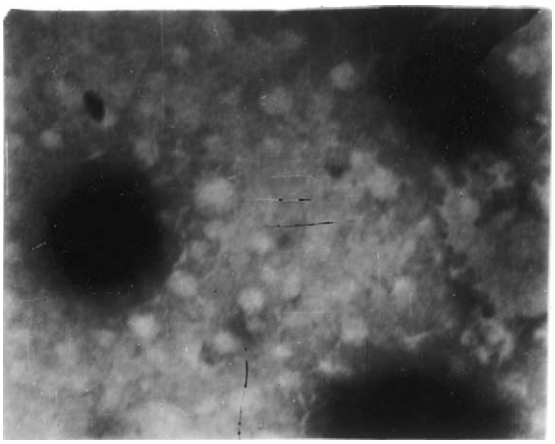


Fig. 5. TEM of 4.0G PEGylated dendrimers at 180,000×, where (—): 100 nm.

for upto 6 days across the membrane. This dendrimer on PEGylation thus behaved similar to spherical unimolecular polymer micelles with chains of hydrated MPEG on its surface as coat. This, in turn, increased the entrapment capacity of dendrimers and made them act as nanometric containers carrying drug. Such PEGylated dendrimers can be suggested for sustaining delivery of drug, 5-FU.

3.4. Hemolytic toxicity

Hemolytic toxicity was found in case of whole generation amine-terminated charged dendrimers (~15.3–17.3%), but negligible in case of half generations of carboxylic acid-terminated dendrimers. However, PEGylation of the dendrimers was found to have decreased the hemolysis of the RBCs significantly to below 5% due to inhibition of interaction of RBCs with the charged quaternary ammonium ion as determined by interaction with RBCs using the method suggested by Singhai et al. (1997). The hemolytic toxicity of the dendrimers was enough to preclude its use as drug-delivery system. The toxicity was due to the polycationic nature of the PAMAM dendrimers, which was also responsible for their cytotoxicity (Duncan and Malik, 1996), particularly, in case of whole generation amine-terminated charged dendrimers, but not in case of half generations of carboxylic acid-terminated dendrimers. However, PEGylation of the dendrimers were found to have

Table 2
Stability study of 4.0G PEGylated dendrimer–drug formulation

Parameters	Dark			Light		
	0 °C	RT	50 °C	0 °C	RT	50 °C
Turbidity (after 5 weeks)	+	–	++	++	+	+++
Precipitation (after 5 weeks)	+	–	++	+	+	+++
Crystallization (after 5 weeks)	+	–	+	+	–	+
Color change (after 5 weeks)	<	–	+	<	–	+++
Change in consistency (after 5 weeks)	+	–	<	++	–	<<
Change in chemical nature (%)						
1 week	1.1	0.2	4.5	1.9	0.6	9.8
2 weeks	1.3	0.3	5.8	2.7	0.8	13
3 weeks	1.7	0.4	6.9	3.4	0.9	18
4 weeks	1.9	0.4	8.2	4.2	1.1	21
5 weeks	2.2	0.5	9.5	4.8	1.8	25
Increase in drug leakage (%)						
1 week	0.3	0.2	5.1	0.6	0.8	8.5
2 weeks	0.6	0.3	5.9	0.8	1.1	12
3 weeks	0.7	0.5	6.7	1.1	1.3	18
4 weeks	0.9	0.6	7.5	1.3	1.7	23
5 weeks	1.1	0.6	8.4	1.6	2.5	26

(+) Indicates smaller change; (++) considerable change; (+++) enough change; (–) indicates no change; (<) and (<<) indicate extent of decrease as compared to initial.

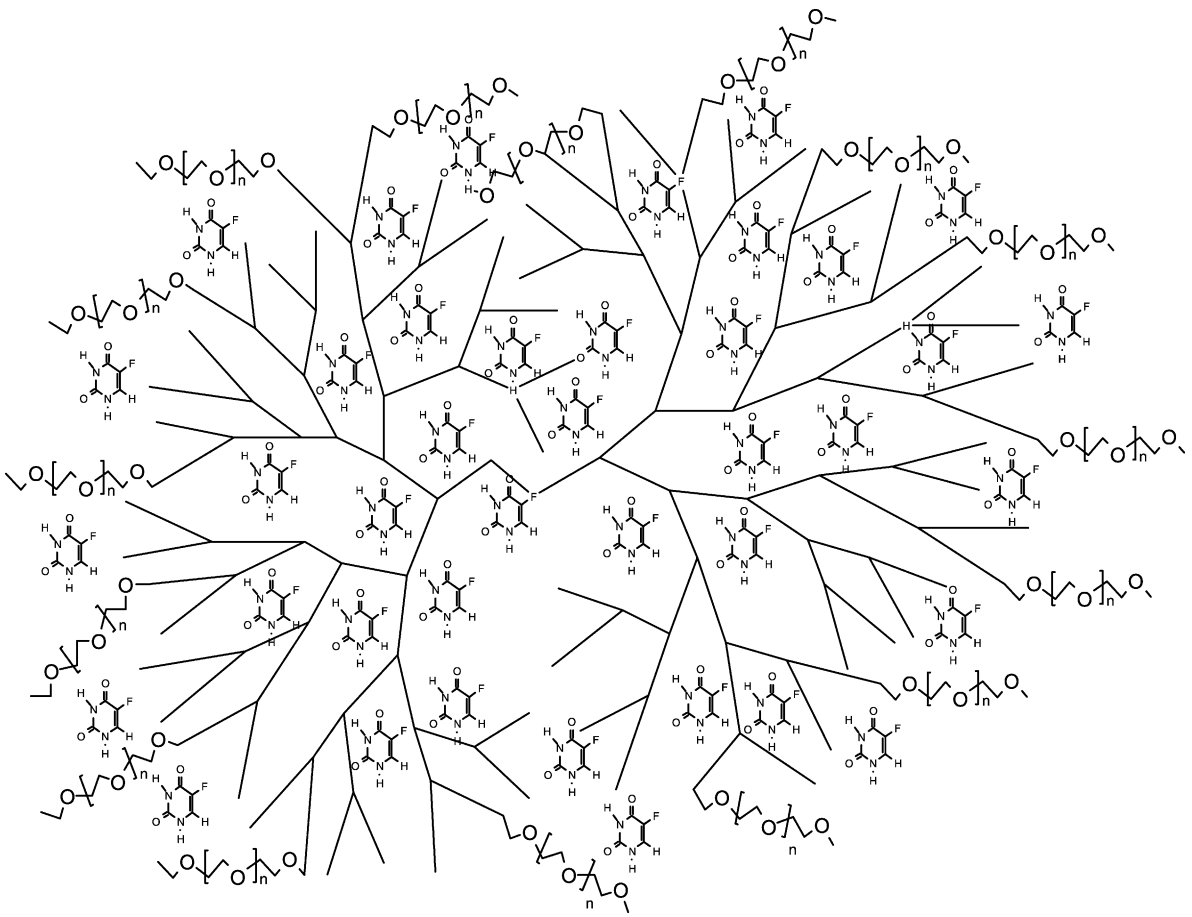


Fig. 6. Molecular sketch showing drug entrapped in PEGylated 4.0G dendrimer.

decreased hemolysis of the RBCs considerably due to significant inhibition of interaction of RBCs with the charged quaternary ammonium ion that is generally present on the amine-terminated whole generations of dendrimers.

3.5. Stability studies

The stability of the PEGylated 4.0G dendrimer–drug formulation (taken as representative formulation) was evaluated at various accelerated conditions of temperature (4 °C, RT, and 50 °C) after keeping both in dark (amber colored vial) and under fluorescent tube light (in colorless vials). These were evaluated every week, for a period of 5 weeks (Table 2). The formulation was found to be most stable in dark, at RT as concluded by observing the physical appearance of the formulations. The appearance of turbidity and precipitation was least in case of the formulations kept in dark and at RT. The crystallization of drug entrapped within the structures of dendrimers (PEGylated) was minimum at RT, when kept in dark. The drug leakage was found to be minimum at RT (∼0.6%) as compared to that at refrigerated conditions (∼1.1%), which may be due to the shrinking of the dendrimeric structure that leads to decrease in cavity entrapping the drug molecules. The coating by PEG produced greater ring closure leading to comparatively lesser or negligible leakage of the drug at higher temperature (∼8.4 and 26% at 50 °C in dark and in light, respectively). The percentage change in the intensity of the violet color by copper-chelation was used for

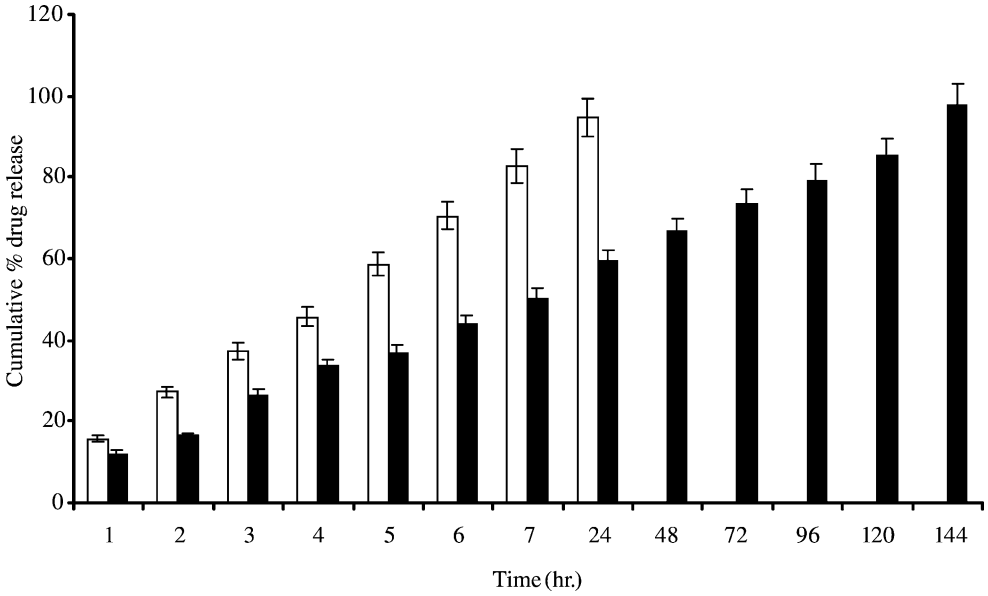


Fig. 7. In vitro cumulative drug release from 4.0G non-PEGylated (□) and PEGylated (■) dendrimer–drug complex.

the determination of free amino groups available at the periphery of PEGylated dendrimeric formulation. This change was found to be more when the formulations were stored in dark and light at 50 °C as compared to that when stored in dark at RT (20–50 times, respectively). This may be attributed to structure cleavage at higher temperature and light, leading to breakage of bonds due to higher reaction kinetics at higher temperature. There may be either cleavage of some bonds of PEG chains, or that between PEG and dendrimers, or the bonds constituted by drug with the dendrimers.

3.6. Blood-level evaluation of PEGylated dendrimers

Blood-level studies were taken up to determine the release and performance of these sustained release formulations in vivo. The formulations were predialyzed to remove unentrapped drug and the amount equivalent to 1000 μg 5-FU was administered to the rats by i.v. route. The blood serum was used to determine the concentration of the drug in blood samples at various time intervals, (Sawant and Murthi, 1993) (Table 3). The blood level of the drug was found to have reached its steady state value by 75–90 min. This period is closely equivalent to the period generally described for attainment of steady state i.e. 6–7 $t_{1/2}$. Similar to the release rate trend found in vitro, the blood level of the drug was found to be lower in case of PEGylated systems than that of non-PEGylated due to slower release rate of the drug. When given in dendrimeric system, the blood level of the drug was found to be lower and somewhat constant as compared to that found for the free drug solution, injected intravenously. The C_{max} from non-PEGylated dendrimers was found nearly 21–23 (μg/ml) and t_{max} occurred at third hour after i.v. injection and was detected upto 6–7 h, whereas, C_{max} from PEGylated dendrimers was found nearly one-third and at same time as from non-PEGylated dendrimers. The blood level was much prolonged and was detectable upto 12 h. The formulations were found to be following sustained release characteristics for 5-FU as shown by the relative increase in MRT (Table 4) for both non-PEGylated and PEGylated dendrimer–drug complexes as compared to plain drug solution (6.024 and 13.31 times, respectively). The release rate however, was found to have increased in vivo as compared to in vitro data, possibly due to the metabolism by the enzymes and hydrolysis in body. The blood level of the drug was found to be lower in case of PEGylated systems than

Table 3
Blood-level study of 5-fluorouracil in albino rats

Time (h)	Blood concentration (μg/ml)		
	5-FU	5-FU–dendrimer (non-PEGylated)	5-FU–dendrimer (PEGylated)
0.25	209.5 ± 10	10.2 ± 2	2.8 ± 0.4
0.5	106.6 ± 8	15.4 ± 2.4	4.1 ± 0.3
0.75	46.7 ± 5	17.5 ± 2.3	4.7 ± 0.6
1	22.3 ± 6	19.6 ± 2.4	5.2 ± 0.7
1.25	11.4 ± 3	20.1 ± 3.2	5.7 ± 0.4
1.5	4.1 ± 1	20.5 ± 1.9	6.1 ± 0.6
1.75	Undetectable	20.6 ± 2.1	6.2 ± 0.7
2	–	20.7 ± 2.1	6.2 ± 0.4
2.5	–	20.8 ± 2.1	6.4 ± 0.4
3	–	21.2 ± 2.4	6.3 ± 0.7
3.5	–	20.3 ± 2.4	6.2 ± 0.6
4	–	19.4 ± 2.3	6.3 ± 0.8
4.5	–	15.6 ± 1.2	6.4 ± 0.7
5	–	11.35 ± 0.6	6.5 ± 0.7
5.5	–	5.56 ± 0.6	6.3 ± 0.5
6	–	1.35 ± 0.2	6.3 ± 0.6
7	–	Undetectable	6.4 ± 0.5
8	–	–	6.6 ± 0.6
9	–	–	6.3 ± 0.3
10	–	–	6.4 ± 0.2
11	–	–	5.8 ± 0.3
12			1.6 ± 0.2
13			Undetectable

that of non-PEGylated due to slower release rate of the drug similar to the trend found in vitro for the drug release. This might also be attributed to better cellular penetration and adhesion properties of the PEG chains of PEGylated systems, which might lead to entanglement of such systems within capillary fenestration from which such systems might release drugs acting as nanoparticulate depot type carrier present in blood circulation. Further studies to explore such effects will be taken up for localized drug delivery.

Table 4
Various pharmacokinetic parameters of 5-fluorouracil as determined on administration of different formulations

Parameters	PDS	NPD	PD
C_{max} (μg/ml)	–	21.2	6.5–6.8
t_{max} (h)	–	3	5–8
AUC (μg h/ml)	99.6	96.2	70.3
AUMC (μg h^2/ml)	45.1	262.2	423.3
MRT (h)	0.45	2.72	6.02

PDS: plain drug solution; NPD: non-PEGylated dendrimer–drug formulation; PD: PEGylated dendrimer–drug formulation.

3.7. Hematological evaluation of formulations

The hematological study was undertaken to assess the relative effects of the PEGylated and non-PEGylated systems as compared to the plain drug on various blood parameters. The blood parameters undergoing major changes as to normal values of blood levels are RBC count, WBC count and differential lymphocytes count. The RBC count of non-PEGylated 5-FU–dendrimers was found to have decreased below normal values by $\sim 1 \times 10^6$ to $2 \times 10^6/\mu l$ RBCs as against similar PEGylated systems. The WBC count of non-PEGylated 5-FU–dendrimer complex increased by 3×10^3 to $4 \times 10^3/\mu l$ cells as compared to normal values. However, for 5-FU–PEGylated dendrimer complexes, the increase was by 1×10^3 to $1.5 \times 10^3/\mu l$ WBCs as compared to normal count in controlled group. The increase in WBC count is significant in case of non-PEGylated systems. Similarly, relatively greater increase in lymphocyte count was observed by non-PEGylated dendrimer–drug complexes, which

were by $\sim 2 \times 10^3/\mu l$ cells. This was similar to blood toxicity and cytotoxicity effects of acrylates nanoparticulates (Duncan and Malik, 1996). Acrylates are known to be stimulating the macrophage level and WBC count. This was found to be true in case of non-PEGylated dendrimer–drug systems than that of PEGylated systems conforming the trend of PEGylated systems (Veronese et al., 1989; Papahadjopoulus et al., 1991; Phillips et al., 1999) undergoing lesser phagocytic uptake.

4. Conclusion

The developed system had been found suitable for prolonged delivery of anti-cancer drug, 5-FU, by in vitro and blood-level studies. This can be termed as nanoparticulate drug-delivery depot type of preparation releasing the drug 5-FU and maintaining its concentration for longer period of time. The PEGylation has shown to increase drug loading but reduced drug release and hemolytic toxicity of the dendrimers. From the present study, it can be concluded that PEGylated systems can act as long circulatory sustained released depot nanoparticulate systems for anti-cancer drug delivery producing least blood dyscarsias as against non-PEGylated systems and anti-cancer drugs.

Acknowledgements

Financial assistance provided by the University Grants Commission, to D. Bhadra to carry out the present investigation is greatfully acknowledged.

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