

# PEGylated Dendritic Architecture for Development of a Prolonged Drug Delivery System for an Antitubercular Drug

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**Abstract:** The present study was aimed at developing and exploring the use of PEGylated poly (propylene imine) dendritic architecture for the delivery of an anti-tuberculosis drug, rifampicin. For this study, PEGylated poly(propylene imine) dendritic architecture was synthesized and loaded with rifampicin. Various physicochemical and physiological parameters UV, IR, NMR, TEM, DSC, drug entrapment, drug release and hemolytic toxicity 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. The systems were found suitable for prolonged delivery of rifampicin.

**Keywords:** Dendrimers, PEGylation, micelles, rifampicin, prolonged release.

## INTRODUCTION

Dendrimer represents a novel type of polymeric material. It is also known as starburst [1] or cascade [2] or molecular trees [3] or arborols, or polymers. They attract the increasing attention of all because of their unique structure, high degree of control over molecular weight and the shape that has led to the synthesis of unimolecular micelles [4-7].

Among the plethora of possible dendrimers, poly (propylene imine) dendrimers have been synthesized on a relatively large scale and characterized-both experimentally and by molecular simulation only in recent years [8-10]. The potential applications of the poly(propylene imine) dendrimers are generally based on one or more of the following characteristics: regular size and shape; larger number of readily accessible end groups; either nitrile or amine; possibility of end group modification in order to tailor properties such as solubility, reactivity, toxicity, stability and glass transition temperature, polyelectrolyte character; and possibility of encapsulating guest molecules [11-16].

Considering the use of dendrimers for drug delivery, it is necessary that they are nontoxic and biocompatible. However, it has been demonstrated that widely used dendrimers, such as PAMAM and poly(propylene imine) (PPI) dendrimers bearing primary amino group termini, are quite cytotoxic, and also these dendrimers were cleared rapidly from the circulation when administered intravenously [17-21].

Poly ethylene glycol (PEG) is typically a clear, colorless, odorless substance that is soluble in water, stable to heat, inert to many chemical agents, that does not hydrolyze or deteriorate, and is generally non-toxic, PEG is considered to be biocompatible, which is to say that PEG is capable of coexistence with living tissue or organisms without causing harm, as reviewed earlier [22, 23].

It has been shown that covalent attachment of poly(ethylene glycol) to proteins decreases their immunogenicity and increases their circulation time [24, 25]. Moreover, a number of studies have demonstrated that poly (ethylene glycol) chains grafted to surface of polymer micelles and liposomes suppress their interaction with plasma proteins and cells and prolong their blood elimination half-life [26-31]. On the basis of these findings, it seems that dendrimers covered with poly(ethylene glycol) grafts are attractive compounds as drug carriers in *in vivo*. Such molecules are expected to encapsulate drugs in their dendrimer moiety and reveal biocompatibility due to their hydrophilic shell consisting of poly(ethylene glycol) grafts [19, 32-34].

The present study was aimed at developing and exploring the use of PEGylated newer EDA-PPI dendrimers for delivery of anti-tuberculosis drug, rifampicin (RIF). RIF was selected for incorporation into PEGylated ethylene diamine (EDA)-PPI dendrimers based on its antitubercular activity, short biological half-life and solubility characteristics. PEGylation of EDA-PPI dendrimers establishes suitability of PEGylated dendrimer as a drug delivery system for RIF. It was observed from the hemolytic study that this delivery system could be safely administered through i.v. route. We expect that this approach will improve the management of drug therapy in tubercular patients by delivering the drug at a controlled rate for a prolonged period of time.

## MATERIALS AND METHODS

### Materials

MPEG<sub>2000</sub>, Raney Nickel (Sigma, Germany), Raney Nickel (Merck, India), triethylamine, dioxan, ethylene diamine, acrylonitrile (CDH, India), succinic anhydride, N, N dicyclohexyl carbodiimide (DCC), Cellulose dialysis bag (MWCO 12-14 Kda, Himedia, India), 4 dimethyl amino pyridine (sd-fine chemicals, India), Rifampicin was a benevolent gift from Lupin laboratories, Aurangabad, India.

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All other chemicals were reagent grade and used without further modification.

## Methods

### *Synthesis of 5.0G EDA-PPI Dendrimers*

EDA-PPI dendrimers were synthesized by the previously reported and established procedure [8,35]. The half generation EDA-dendrimer-(CN)<sub>4n</sub> (where *n* is generation of reaction or reaction cycle) was synthesized by double Michael addition reaction between acrylonitrile (2.5 molar times per terminal NH<sub>2</sub> group of core amine moiety) and aqueous solution of ethylenediamine or previous full generation dendrimers. After the initial exothermic phase, the reaction mixture was heated at 80°C for 1 h to complete the addition reaction. The excess of acrylonitrile was then removed by vacuum distillation (16 mbar, bath temperature 40°C). The full generation EDA-dendrimer-(NH<sub>2</sub>)<sub>4n</sub> was obtained by hydrogeneration in methanol at 40 atm hydrogen pressures and 70°C for 1 h with Reney Nickel (pretreated with hydroxide and water) as catalyst. The reaction mixture was cooled, filtered and the solvent was evaporated under reduced pressure. The product was then dried under vacuum. EDA-PPI dendrimers up to 5.0G were prepared by repetition of all the above steps consecutively, with increasing quantity of acrylonitrile.

### *Synthesis of PEGylated 4.0G and 5.0G EDA-PPI Dendrimers*

MPEG<sub>2000</sub> (8g, 8m mol), succinic anhydride (500 mg, 10 m mol), 4 dimethyl amino pyridine (488 mg, 8 m mol) and triethylamine (404 mg, 8 m mol) were dissolved in dioxane and the resulting solution was stirred over night at room temperature. Dioxane was evaporated in vacuum and the residue was dissolved in dichloromethane, filtered and concentrated. MPEG-COOH<sub>2000</sub> was precipitated out with ether and dried in oven to remove final traces of solvent [36].

To a solution of 4G EDA-PPI dendrimer (0.01 mmol) in dimethyl sulfoxide (DMSO) (10 ml), MPEG COOH<sub>2000</sub> (0.32 mmol) in DMSO (10 ml) and N, N dicyclohexyl carbodiimide (DCC) (0.32 mmol) in DMSO (10 ml) were added and the solution was stirred for 5 days at room temperature. The product was precipitated by addition of water, filtered and dialyzed (MWCO 12-14 Kda, Himedia, India) against double distilled water for 24 h to remove free MPEG COOH<sub>2000</sub>, DCC and partially PEGylated dendrimers followed by lyophilization (Heto drywinner, Germany). A similar procedure was followed for the preparation of the PEGylated 5G EDA-PPI dendrimer except for the amount of the dendrimer used (0.005 m mol).

### *Drug Loading in Formulations*

The known molar concentrations of EDA-PPI dendrimer-(NH<sub>2</sub>)<sub>32&64</sub> and PEGylated 4.0G and 5.0G EDA-PPI dendrimers were dissolved separately in methanol and mixed with methanolic solution of RIF (100 mol). The mixed solutions were incubated with slow magnetic stirring (50 rpm) using teflon beads for 24 h. These solutions were twice dialyzed in cellulose dialysis bag (MWCO 1000 Da Sigma, Germany) against double distilled water under sink conditions for 10 min to remove free drug from the formulations,

which was then estimated spectrophotometrically ( $\lambda_{\max}$  475 nm) (UV-1601, Shimadzu, Japan) to determine indirectly the amount of drug loaded within the system. The dialyzed formulations were lyophilized and used for further characterization.

### *Morphology of the Dendrimers*

Transmission electron microscopy (TEM) was performed to investigate particle size and provide information on nanoparticle morphology. Prepared and dialyzed RIF loaded dendrimer formulations were used for Transmission electron microscopic studies. The TEM studies were carried out using 3mm Forman (10.5% plastic powder in amyl acetate) coated copper grid (300 mesh) at 60 Kv using negative staining by 2% phosphotungstic acid (PTA) for whole generation of dendrimers at 150,000X magnification on Philips CM-10 TEM and Fei-Philips Morayagni 268D with digital TEM image analysis system at 50-60 Kv.

### *Differential Scanning Calorimetry*

Differential scanning calorimetry (DSC) was performed to study the thermal stability and changes in crystallinity over a range of temperatures. The carriers and manufactured drug particles were studied by this method. A known mass of powder was placed in an aluminum pan, and a lid was crimped onto the pan. The pan was then placed in the sample cell of a DSC module (DSC Q10 V9.0 Build 275, TA Instruments, USA). The temperature of the DSC module was equilibrated at 35°C and then increased at a rate of 10°C/min under a N<sub>2</sub> gas purge until the material began to degrade. The temperatures were obtained for each peak in the resulting curve and provided indications of temperature stability and phase transitions.

### *In Vitro Release*

Drug release from known amounts of RIF loaded PEGylated 4.0G and 5.0G EDA-PPI-dendrimers were determined using a modified dissolution method. The medium comprised of a 0.05 mol phosphate buffer solution (PBS) (pH 7.4) with 200 µg/ml ascorbic acid, which was added as an antioxidant to prevent oxidative degradation of RIF. The dialysis bags were filled with a known mass of RIF loaded PEGylated dendritic architectures (MWCO 1000 Da) and the dialysis bags were placed in 50 ml of PBS (pH 7.4) at 37°C with slow magnetic stirring under sink conditions. Aliquots of 1 ml were withdrawn from the external solution and replaced with the same volume of fresh PBS. The drug concentration was detected in a spectrophotometer at 475 nm  $\lambda_{\max}$ .

### *Hemolytic Toxicity of Dendrimer-Drug Systems*

The RBC suspension was obtained following the reported procedure for hemolytic studies [37]. Briefly, the RBC suspension (5% hematocrit) of the human blood collected in HiAnticlot blood collection vials (Himedia Labs, India). 0.5 ml of suitably diluted RIF encapsulated PEGylated and non-PEGylated formulations were added to 4.5 ml of normal saline and incubated for 1h 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 incubated for 1h with RBC suspension. The drug and dendrimers in sepa-

rate tubes were taken in such amount that the resultant final concentrations of drug and dendrimer were equivalent in all the cases. The PEGylated system of dendrimer–drug complex was taken in amount such that the resultant final concentrations of drug and dendrimer were equivalent to that in non-PEGylated systems. This allowed comparison of the hemolysis data of the drug, dendrimer, RIF loaded EDA-PPI dendrimers and PEGylated dendritic architectures to assess the effect of PEGylation on hemolysis. After centrifugation, supernatants were taken and diluted with an equal volume of normal saline and absorbance was measured at 540 nm. To obtain 0 and 100% hemolysis, RBC suspension was added to 5 ml of 0.9% NaCl solution (normal saline) and 5 ml distilled water, respectively. The degree of hemolysis was determined by the following equation:

$$\text{Hemolysis (\%)} = \frac{\text{Abs} - \text{Abs}_o}{\text{Abs}_{100} - \text{Abs}_o} \times 100$$

where *Abs*, *Abs*<sub>100</sub>, and *Abs*<sub>o</sub> are the absorbance of sample, a solution of 100% hemolysis, and a solution of 0% hemolysis; respectively.

### Stability Studies of PEGylated Dendrimer Formulations

#### Physical Stability

RIF loaded PEGylated 4.0G and 5.0G EDA-PPI-dendrimers were stored for three months at 40±2°C in the dark. The appearance of the dendrimers was monitored by transmission electron microscopy.

#### Chemical Stability

After storage for three months at 40°C the drug content and release rate of RIF loaded PEGylated 4.0G and 5.0G EDA-PPI-dendrimers were determined.

#### Statistical Analysis

The results were expressed as mean±standard deviation (S.D.) (n=3) and statistical analysis was performed with SPSS 10.1 for Windows® (SPSS®, Chicago, USA). The differences between the percentage RIF loading of 5.0G PEGylated EDA-PPI-dendrimers and EDA-PPI-dendrimers-(NH<sub>2</sub>)<sub>64</sub> were observed by pair-wise comparisons using unpaired t test performed in GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, California, USA.

## RESULTS AND DISCUSSION

### Synthesis of PEGylated 4.0G and 5.0G EDA-PPI Dendrimers

4.0G EDA-PPI dendrimer-(NH<sub>2</sub>)<sub>32</sub> and 5.0G EDA-PPI dendrimer-(NH<sub>2</sub>)<sub>64</sub> were synthesized by the procedure reported by De Brabender-Van Den Berg and Meijer [8] using ethylene diamine as initiator core [35]. Synthesis of 0.5G PPI was confirmed by IR peaks, mainly of nitrile at 2249 cm<sup>-1</sup>. All the nitrile terminal 0.5G PPI got converted into EDA-(NH<sub>2</sub>)<sub>4</sub>, which was confirmed by IR of PPI (1.0 G) that gave major peak at 3432 cm<sup>-1</sup> of amine (N—H stretch). Synthesis of EDA-PPI dendrimers-(NH<sub>2</sub>)<sub>32&64</sub> were similarly confirmed by IR peaks for C—C bend (1103 cm<sup>-1</sup>); C—N stretch (1230 cm<sup>-1</sup>, 1326 cm<sup>-1</sup>); C—H bend (1423 cm<sup>-1</sup>, 1477 cm<sup>-1</sup>); N—H deflection of amine (1643 cm<sup>-1</sup>) and primary amine at 3400 cm<sup>-1</sup>(N—H stretch), confirming nitrile terminal groups of dendrimer were converted to amine terminals. The results matched with the reported synthesis of PPI dendrimers [8, 35]. The synthesized dendrimers were PEGylated using DCC and MPEG COOH<sub>2000</sub>. IR and NMR data proved the synthesis shown in Table 1, 2 and Fig. (1).

#### Drug Loading

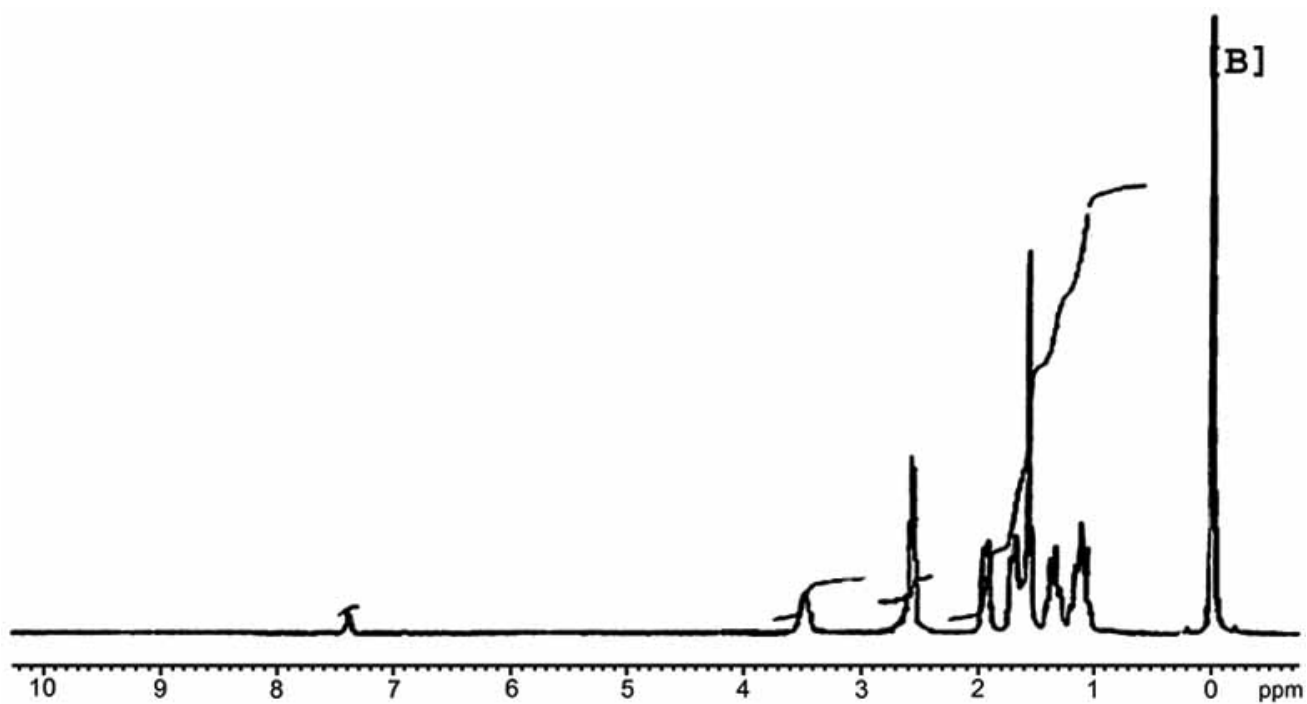
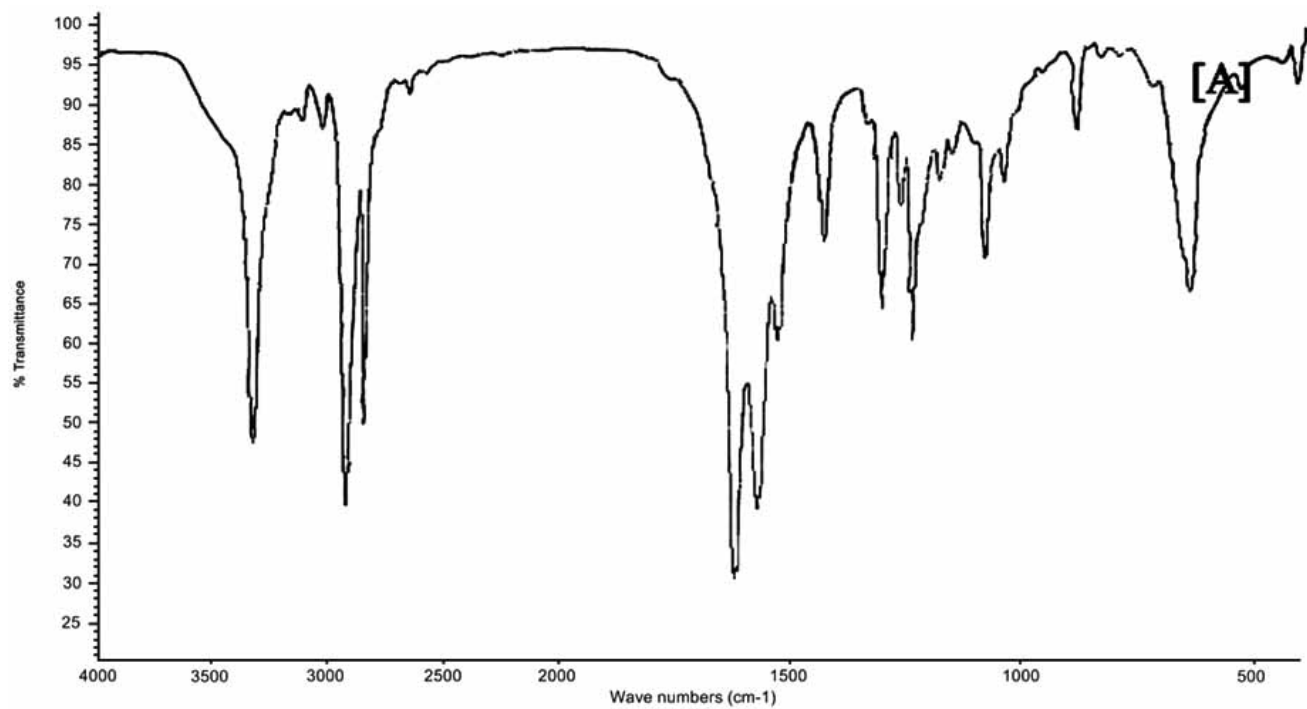
Noncovalent interactions among RIF and PEGylated dendrimers, such as hydrophobic interaction and hydrogen bonding, contribute to the physical binding of drug mole-

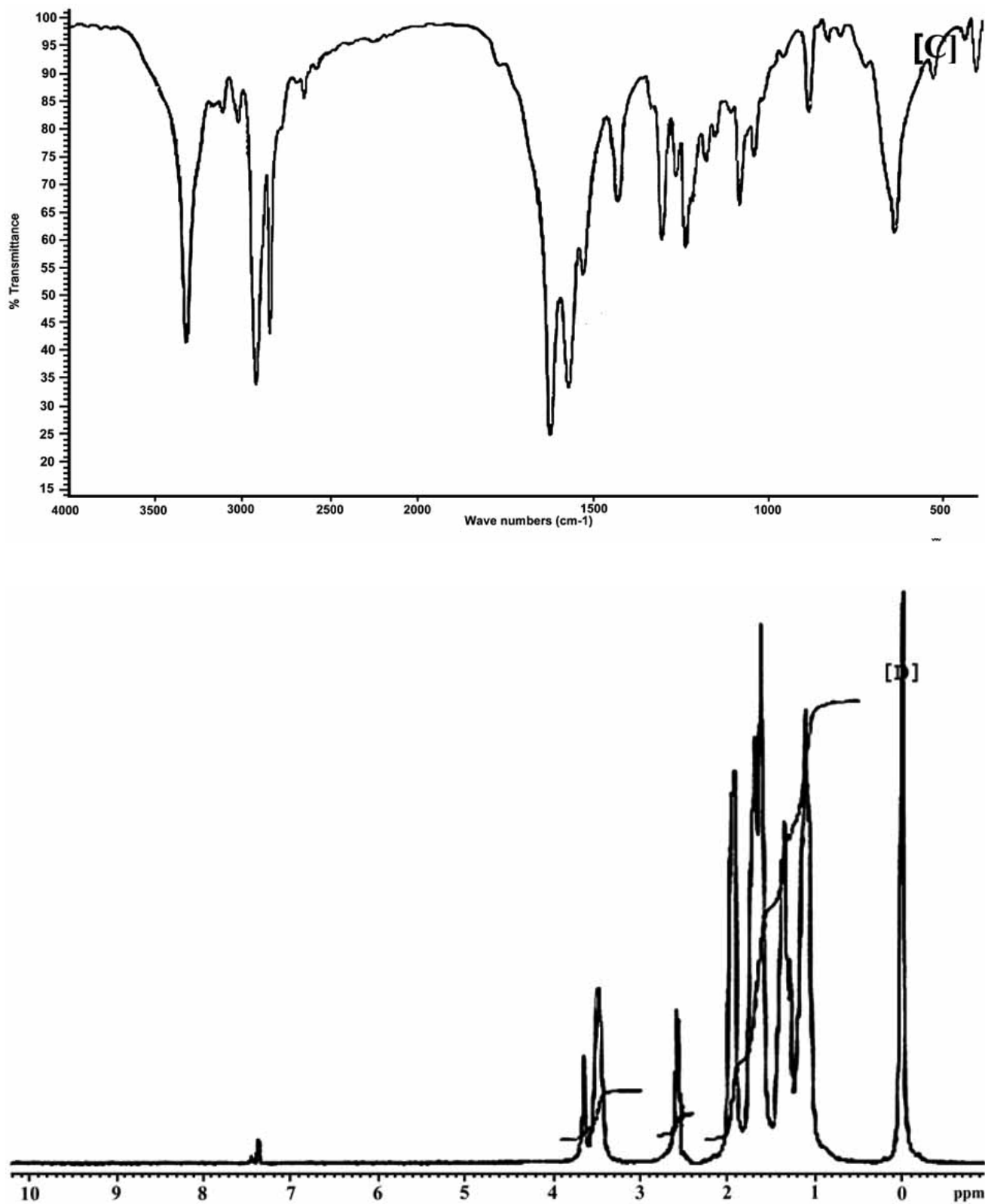
Table 1. Selected values of IR Spectroscopic Data (KBr) for Dendrimers

Compound	C=O stretch of carbonyl group (cm <sup>-1</sup> )	C—H stretch (cm <sup>-1</sup> )	Secondary amide (cm <sup>-1</sup> )
PEGylated 4.0 G EDA-PPI dendrimer	1626.07	2928.34, 2850.37	3326.56
PEGylated 5.0 G EDA-PPI dendrimer	1626.11	2928.22, 2850.41	3326.65

Table 2. Selected δ Values of <sup>1</sup>H NMR Spectroscopic Data (300 MHz in DMSO) for Dendrimers

Compound	(s, CONH)	(s, CH <sub>2</sub> -CH <sub>2</sub> -O)	(m, correspond to the presence of CH <sub>2</sub> of EDA-PPI dendrimers)
PEGylated 4.0 G EDA-PPI dendrimer	7.41	3.49, 3.47	1.95 to 1.03
PEGylated 5.0 G EDA-PPI dendrimer	7.52	3.64, 3.47	1.94 to 1.07





**Fig. (1).** IR and <sup>1</sup>H NMR spectrum of (solvent DMSO) : PEGylated 4.0G [A and B] and 5.0G [C and D] EDA-PPI dendrimer.

cles inside dendritic micelles and surface MPEG layers. As shown in Table 3 the percentage RIF loading of 5.0G PEGylated EDA-PPI-dendrimers was significantly increased (p value is 0.0003, considered extremely significant) compared

to EDA-PPI-dendrimers-(NH<sub>2</sub>)<sub>64</sub>. PEGylation increases the RIF loading capacity of the EDA-PPI-dendrimers-(NH<sub>2</sub>)<sub>32&64</sub> due to more interaction of drug and MPEG at the peripheral portions of dendrimers.

**Table 3. Nomenclature, Physicochemical and Biological Characterization of RIF Loaded Formulations**

Formulation Code	Actual number of terminal amine groups	% drug entrapment*	% hemolytic toxicity <sup>a</sup> *
EDA-PPI dendrimer-(NH <sub>2</sub> ) <sub>32</sub>	32	28.21±1.56	14.6±1.33
EDA-PPI dendrimer-(NH <sub>2</sub> ) <sub>64</sub>	64	39.34±2.71	20.39±0.82
PEGylated 4.0G EDA-PPI dendrimer	-	47.19±1.21	1.43±0.59
PEGylated 5.0G EDA-PPI dendrimer	-	61.23±1.51	2.71±1.12

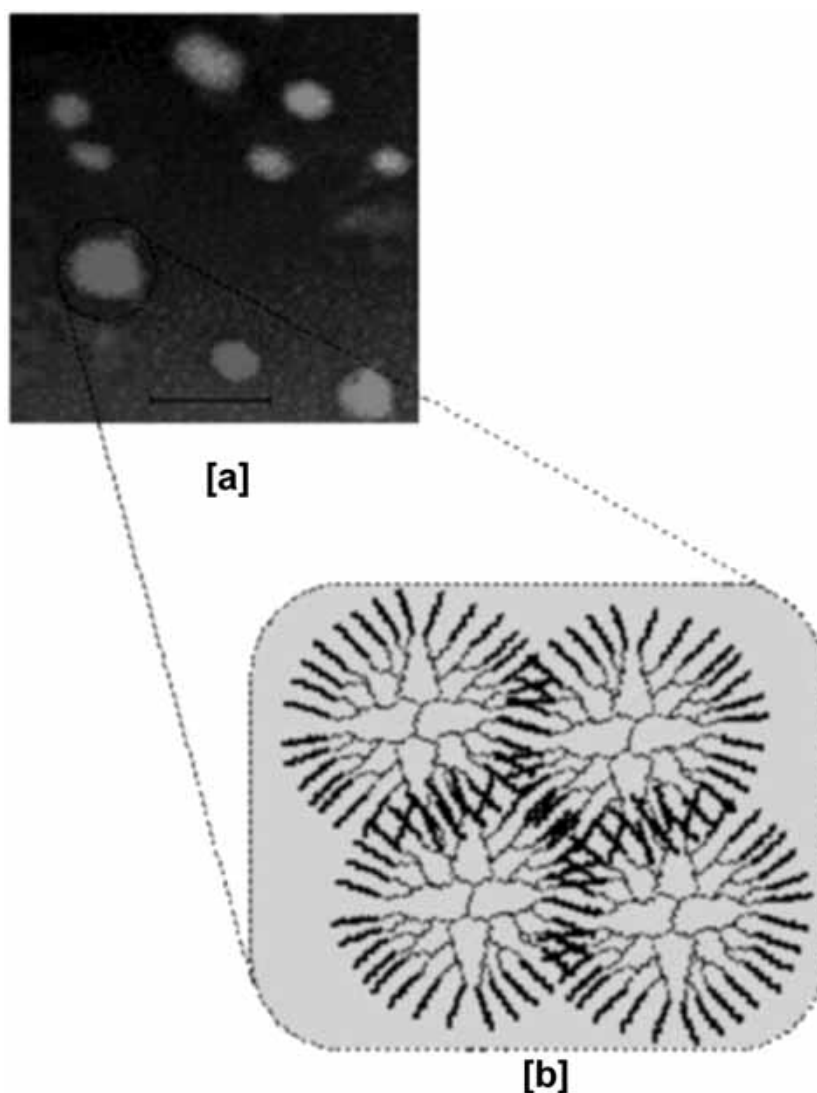
<sup>a</sup> % hemolysis produced by 5 mg/ml formulations on 5% hematocrit RBCs on incubation for 1h.  
\* mean ±SD. (n = 3).

### Morphology of the Dendrimers

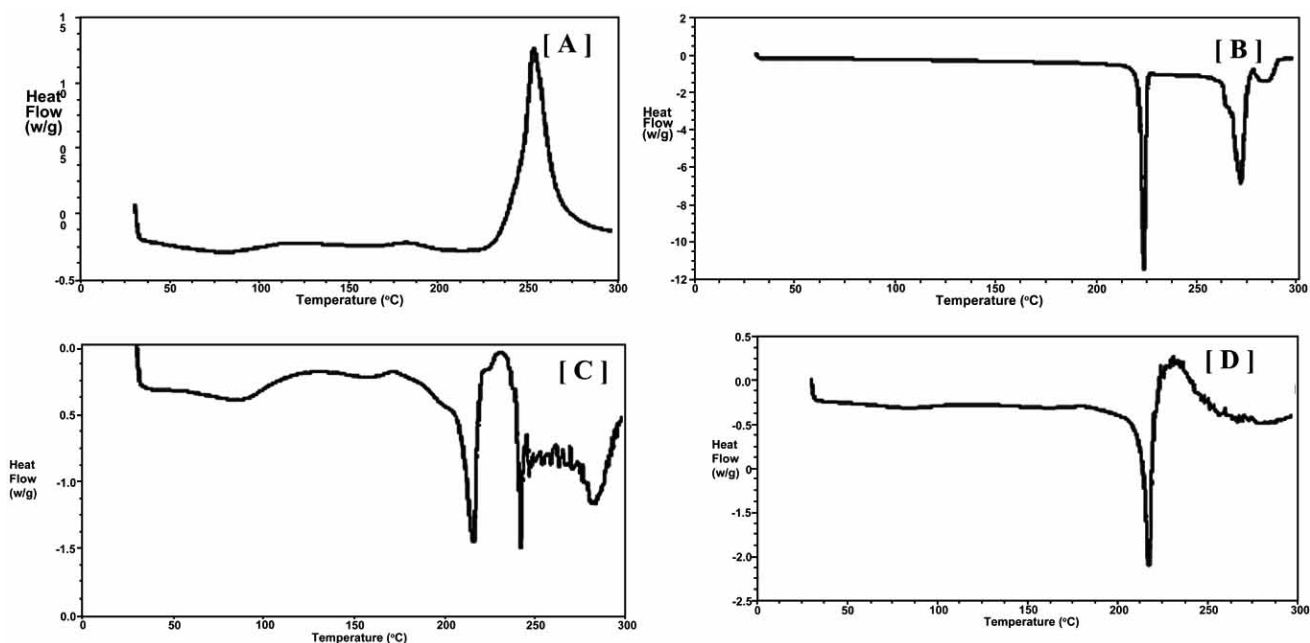
TEM micrographs (Fig. 2a and b) show that the drug loaded dendrimers were more or less spherical in shape (PEGylated 5.0G EDA-PPI dendrimers) and that the dendrimers were agglomerated.

### Differential Scanning Calorimetry

Curves of DSC (Fig. 3) suggest that RIF loaded PEGylated EDA-PPI dendrimers was not a physical mixture. Upon heating to 187°C, RIF experienced an endothermic transition immediately followed by an exothermic transition. This was



**Fig. (2).** (a) TEM of RIF loaded PEGylated 5.0G EDA-PPI dendrimers at 150,000X where |——| shows 10nm on negative staining with 2% phosphotungstic acid. (b) A two-dimensional schematic diagram of agglomerations of dendritic micelles.



**Fig. (3).** Curves of differential scanning calorimetry: (A) RIF; (B) 5.0G PEGylated EDA-PPI dendrimers; (C) 5.0G PEGylated EDA-PPI dendrimers + RIF physical mixture; (D) RIF loaded 5.0G PEGylated EDA-PPI dendrimer.

previously described as melting followed by recrystallization [38]. Two endothermic peaks near 223.66°C and 271.33°C were found for PEGylated 5.0G EDA-PPI dendrimers. The characteristic peaks of RIF and 271.33°C of blank PEGylated 5.0G EDA-PPI dendrimers, respectively, both almost disappeared in the curve for RIF loaded PEGylated EDA-PPI dendrimer. The DSC curve of the physical mixture also differed from that of RIF loaded PEGylated EDA-PPI dendrimers.

### In vitro Release

A comparative evaluation of the effect of PEGylation on the release of RIF from EDA-PPI dendrimer-(NH<sub>2</sub>)<sub>32&64</sub> was performed. RIF release from various formulations is shown in Fig. (4). Among the four formulations PEGylated dendrimers gave relatively slower release of RIF when compared with non-PEGylated dendrimers. While the 4.0G EDA-PPI dendrimers-(NH<sub>2</sub>)<sub>32</sub> released 97.3% of RIF in 36 h, PEGylated 4.0G EDA-PPI dendrimers released only 46.3 % in 36h and 93.1% in 96h. The 5.0G EDA-PPI dendrimers-(NH<sub>2</sub>)<sub>64</sub> released 94.6% of RIF in 48 h while PEGylated 5.0G EDA-PPI dendrimers released only 43.3% and 93.4% in 48 and 120 h, respectively. The cumulative % release of RIF in the PEGylated EDA-PPI dendrimers was decreased with increase of dendrimer generation. This may be due to greater hydrophobic interaction between the drug and the core of higher generation dendrimer (5.0G). The difference in number of terminal PEG groups also contributes to the slower drug release profile whereby both dissolution as well as diffusion of drug occur through small channels in the PEGylated dendrimers.

### Hemolytic Toxicity

The whole generation of amine-terminated charged dendrimers showed hemolytic toxicity (~14.6–20.3%). But PE-

Gylation of the dendrimers was found to have decreased the hemolysis of the RBCs significantly to less than 3% (Table 3) 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.* [37]. The toxicity was due to the polycationic nature of the EDA-PPI dendrimers, which was also responsible for their cytotoxicity, particularly in case of whole generation of amine-terminated charged dendrimers [18]. This is comparable and similar to the effects produced by surface modification of PAMAM dendrimers and similar to the cations in earlier studies [23, 39, 40].

### Stability

After storing the RIF loaded PEGylated 4.0G and 5.0G EDA-PPI-dendrimers at 40±2°C for three months neither change in its appearance and redispersing ability nor significant difference in potency ( $p > 0.05$ ) and cumulative % release were detected (Table 4 and Fig. 5).

**Table 4.** Stability of RIF Loaded PEGylated Dendrimers at 40±2°C

Compounds	Storage time (3 months)*
<b>PEGylated 4.0G EDA-PPI-dendrimer</b>	
Appearance	Pale red powder
RIF content	46.3±2.73
<b>PEGylated 5.0G EDA-PPI-dendrimer</b>	
Appearance	Pale red powder
RIF content	60.6±3.24

\*mean ±SD. (n = 3).

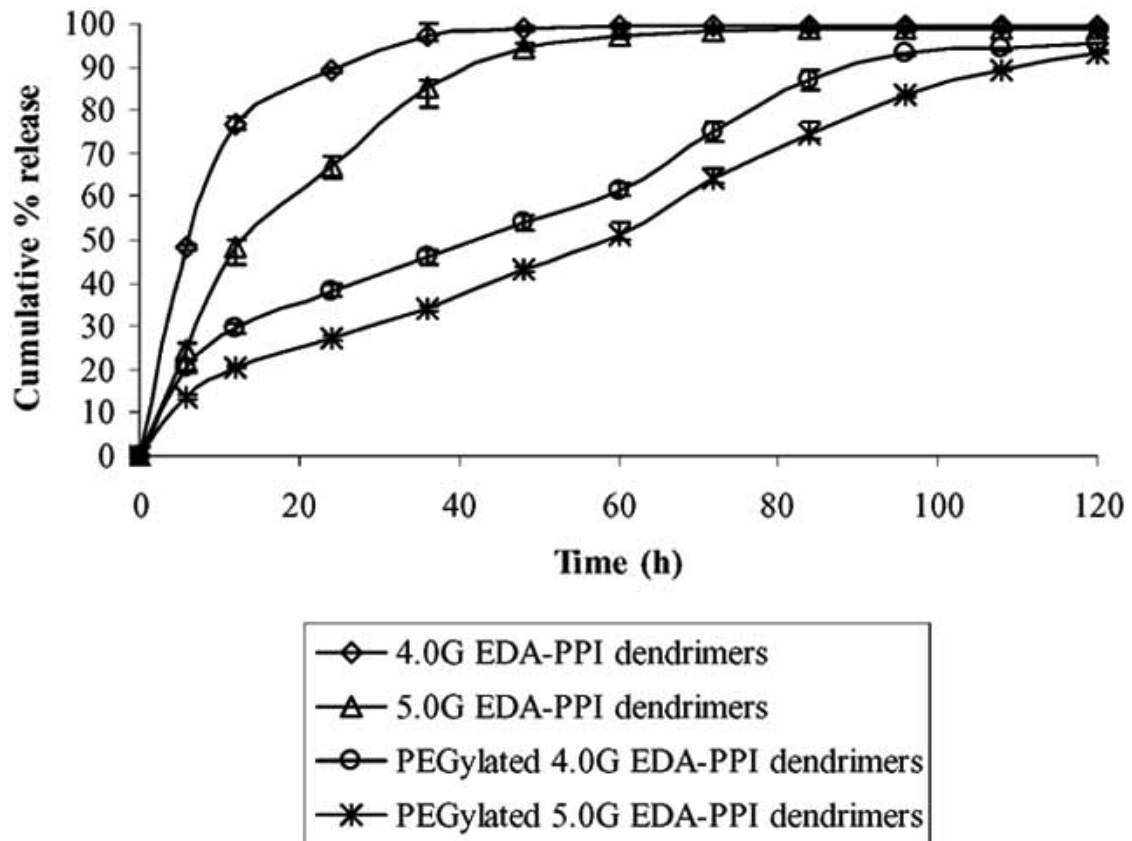


Fig. (4). Drug release in phosphate buffer pH 7.4 at 37°C (n=3).

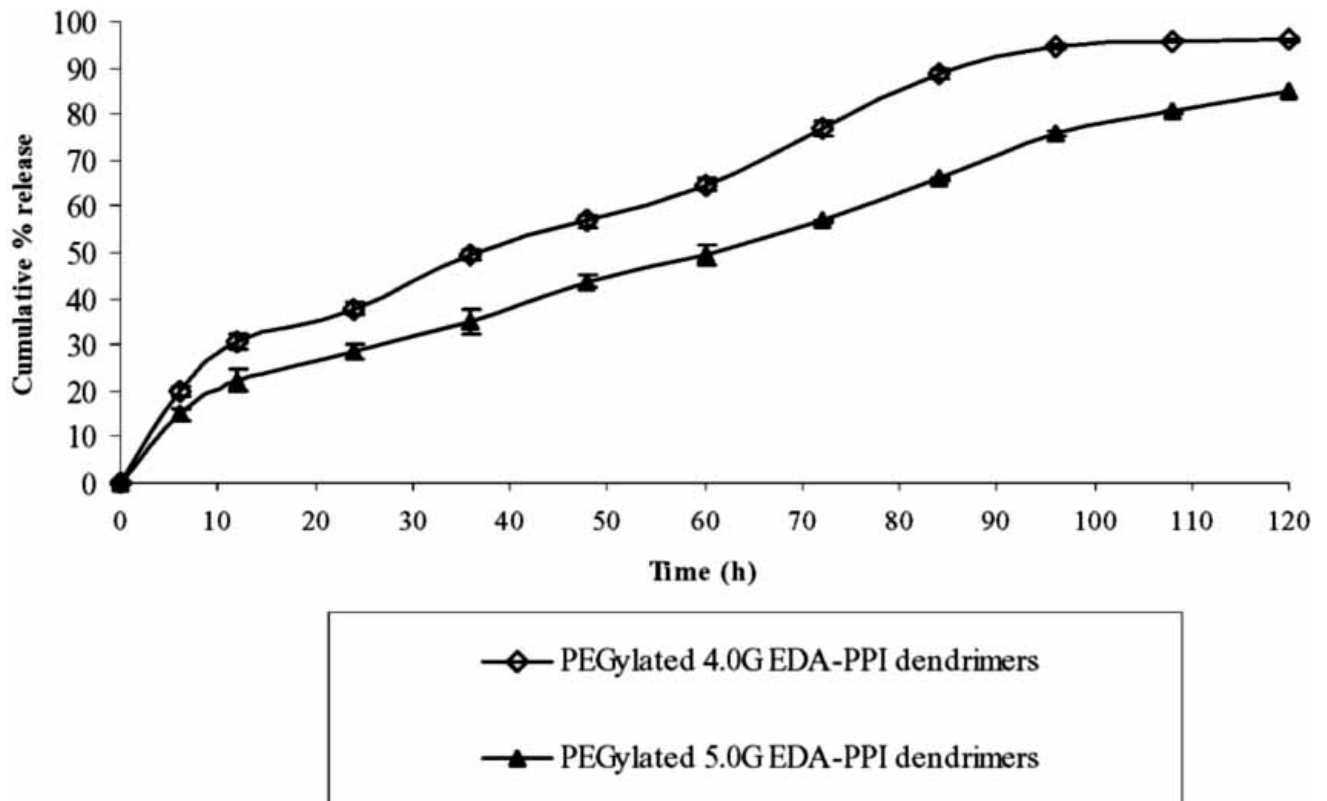


Fig. (5). Drug release in phosphate buffer pH 7.4 at 37°C (n=3) after storage for three months.

## CONCLUSION

4.0G EDA-PPI dendrimer-(NH<sub>2</sub>)<sub>32</sub> and 5.0G EDA-PPI dendrimer-(NH<sub>2</sub>)<sub>64</sub> were PEGylated using methoxy poly(ethylene glycol). PEGylation has been found to be suitable for modification of ethylene diamine initiator core EDA-PPI dendrimers-(NH<sub>2</sub>)<sub>32&64</sub> for prolonged release of RIF. The result of hemolytic toxicity studies revealed that these systems are relatively safer and hold potential as delivery system for rifampicin for parenteral administration. Pharmacokinetic and pharmacodynamic aspect of this dendritic architecture in animal models is under study and we expect that this approach will improve the management of drug therapy in tubercular patients by delivering the drug at a controlled rate for a prolonged period of time thus optimizing the efficacy by minimizing fluctuations in plasma drug concentration.

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## LIST OF ABBREVIATIONS

PPI	=	Poly(propylene imine)
EDA	=	Ethylene diamine
DCC	=	N, N dicyclohexyl carbodiimide
MWCO	=	Molecular weight cut off
RIF	=	Rifampicin
PEG	=	Poly ethylene glycol
MPEG	=	Methoxy Poly ethylene glycol

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