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European Journal of Medicinal Chemistry 44 (2009) 1155–1166
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Original article

PEGylated PPI dendritic architectures for sustained delivery of H_2 receptor antagonist

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Received 17 July 2007; received in revised form 30 April 2008; accepted 13 June 2008
Available online 26 June 2008

Abstract

The present study was aimed at synthesizing and exploring the use of long circulating biocompatible PEGylated PPI 5.0G dendrimers for sustained delivery of a H_2 receptor antagonist, Famotidine. PPI 5.0G dendrimers were synthesized and PEGylated using dicarboxylic acid PEG 2000. PEGylation was confirmed by SEC, IR, NMR and MASS spectroscopies. Famotidine was loaded in PEGylated dendritic system and confirmed by IR and differential scanning calorimetry. The PEGylated dendritic system has shown an increased drug loading capacity, a reduced hemolytic toxicity and demonstrated a suitability of PEGylated PPI 5.0G dendrimer for prolonged delivery of Famotidine during in vitro release, in vivo blood level and tissue distribution studies in albino rats. The ulcer index after 5 h of treatment with different formulations was found to be 4.5 ± 0.28 in case of plain Famotidine solution, while ulcer index was significantly reduced to 0.5 ± 0.13 in case of Famotidine loaded PEGylated PPI 5.0G dendrimers, indicating sustained release of the drug from drug–PEGylated dendrimer complex. The results suggested that such PEGylated dendrimeric systems could serve as nanoparticulate depot for drugs in body.

Keywords: PPI dendrimers; PEGylation; Famotidine; Sustained drug delivery; Pharmacokinetic; Pharmacodynamic

1. Introduction

Dendrimers represent hyperbranched, monodisperse, three-dimensional macromolecules, with host-guest entrapment properties and defined molecular weight. They allow definite control of size, shape and position of functional groups [1–4]. For that reason, dendrimers have fascinated escalating attention for their applications in many fields. Among them, the use of dendrimers as a drug delivery carrier has been of enormous curiosity [5,6]. Dendritic architectures are one of the most novel emerging delivery systems with the potential of delivering hydrophobic agents with better prospective. These dendrimeric macromolecules possess a large number of peripheral end groups and interior cavities offering a better opportunity for delivery by becoming charged and by acting as static covalent micelles [7]. In the recent years, dendritic architectures have shown promising gallows in the field ranging from drug delivery to carbonyl metallo immunoassay including development of vaccines, antibacterial, antiviral and chemotherapeutics [8–11]. Prolonged residence time of the drug in the blood and protection of the bioactives from its environment with increased stability are other potential advantages of dendrimeric architecture. Previous reports also suggest the use of dendritic macromolecules for delivery of different bioactives. Cationic dendrimers have shown significant toxicity due to the presence of multiple cationic amine groups [12]. To avoid cytotoxicity and hemolytic toxicity, it is indispensable to modify the surface amine groups of cationic dendrimers with neutral or anionic moieties [12,13]. Even if dendrimers have vast applications in biomedical field, their use is restricted due to reticuloendothelial system (RES) uptake, drug leakage, immunogenicity, hemolytic toxicity, hydrophobicity, etc. PEGylation of dendrimers can generally overcome these limitations. Polyethylene glycol (PEG) conjugation or linking with the dendritic system is called PEGylation, which improves water solubility and non-statistical

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0223-5234/$ - see front matter © 2008 Published by Elsevier Masson SAS.
doi:10.1016/j.ejmech.2008.06.012

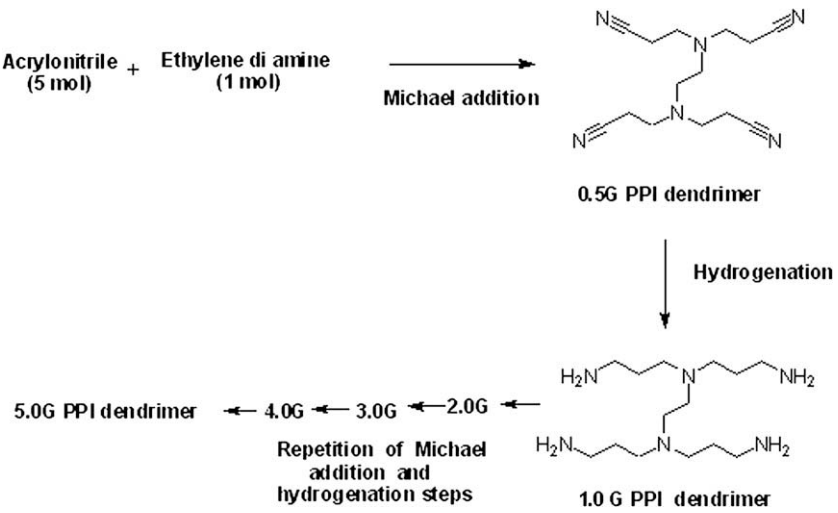

Fig. 1. Scheme for the synthesis of PPI 5.0G dendrimer.

attachment of drug molecules. The main purposes of PEGylation of dendrimers are to alter biodistribution and pharmacokinetics of dendrimers by increasing blood circulation due to decreased RES, liver, spleen and macrophageal uptake, to alter solubility profile of dendrimers making them more soluble systems, to decrease the toxicity by shielding the peripheral $-NH_2$ groups, to improve utilization as drug delivery system by increasing drug loading, sustained and controlled drug delivery safely and appropriately [14]. The present study was aimed at developing and exploring the use of long circulating biocompatible PEGylated PPI 5.0G dendrimers for sustained delivery of a H_2 receptor antagonist. Famotidine was taken as a model H_2 receptor antagonist.

2. Materials

Famotidine was a benevolent gift from IPCA LABS (Mumbai), India. Ethylenediamine, acrylonitrile and *N*-hydroxysuccinimide (NHS) were purchased from CDH, India. *N*′-Dicyclohexyl carbodiimide (DCC) and cellulose dialysis bag (MWCO 12–14 kDa) were purchased from Himedia, India. Raney Nickel was purchased from Merck, India. Polyethylene glycol 2000 (PEG 2000) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of reagent grade and purchased from CDH, India.

3. Methods

3.1. Synthesis of PPI 5.0G dendrimer

PPI 5.0G dendrimer were synthesized by earlier reported divergent method [15,16]. In brief, double Michael addition reaction method was used to produce half-generation (−CN terminated) by adding acrylonitrile to the initiator core, ethylenediamine (EDA). It was pursued by heterogeneous hydrogenation using Reney Nickel catalyst to synthesize full 1.0G generation ($-NH_2$) dendrimers. The reaction sequence was repeated intermittently to fabricate PPI dendrimers up to fifth generation (PPI 5.0G) as shown in Fig. 1. IR spectroscopy was carried out using Perkin–Elmer IR spectroscope (USA). NMR spectroscopy of the dendrimer sample was carried out at 300 MHz in $CDCl_3$ (Bruker DRX, USA).

3.2. PEGylation of PPI 5.0G dendrimer

The polyethylene glycol derivatization (PEGylation) was performed following the activation of end functional groups of PEG 2000. The activation of PEG 2000 was carried out by converting it to dicarboxylic acid derivatives and subsequently to NHS ester according to Veronese et al. [17] with slight modification. Chloroacetic acid was used to prepare dicarboxymethyl PEG 2000 diether (CM-PEG-CM). This generated two carboxylic acid functional groups on PEG 2000. PEG 2000 (4 mM) was dissolved in 50 ml of *tert*-butanol at 50 °C and to it 32 mM potassium *tert*-butanolate was added. The mixture was stirred at same temperature overnight. Chloroacetic acid (32 mM) was solubilized in *tert*-butanol and slowly added to the reaction mixture. The reaction was carried out under magnetic stirring for 24 h. The solvent was evaporated and 50 ml of dichloromethane was added to the precipitate. It was washed by stirring with 250 ml of water and the layers were separated using a separating funnel. The lower layer of dichloromethane was removed by allowing the mixture to stay for 1 h and concentrated to 10–15 ml. This was added to cold 200 ml of ether. Keeping in refrigerator for overnight with excess of ether further increased the precipitation. The precipitate was removed and re-precipitated by dichloromethane and ether precipitation repeatedly. Precipitate of dicarboxylic acid PEG 2000 was dried in a Petri dish and slightly heated in an oven. The dicarboxylic acid PEG 2000 was then converted to NHS ester by DCC and NHS. The NHS ester of dicarboxylic acid PEG 2000 (0.32 mM) was dissolved in 10 ml of dimethyl sulfoxide (DCM) and 0.01 mM of PPI 5.0G dendrimer was also dissolved in 10 ml of DCM separately. Both the solution were mixed and stirred for 5 days at room temperature (Fig. 2). The product was precipitated from cold

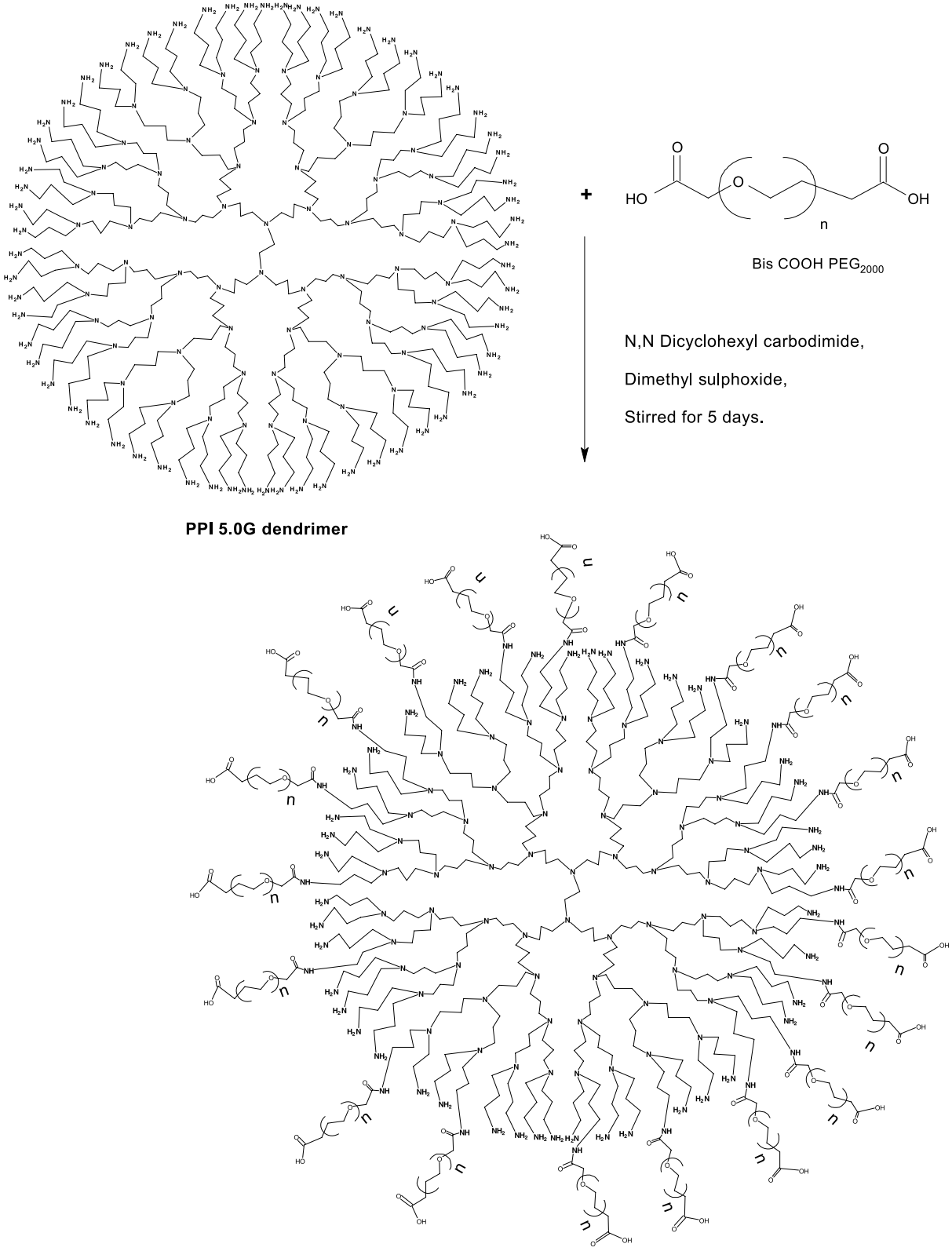

Fig. 2. Scheme for PEGylation of PPI 5.0G dendrimers (n = PEG 2000).

diethyl ether, filtered and dialyzed using dialysis membrane (MWCO 12–14 kDa, Himedia, India) to remove free bis-carboxylic acid PEG 2000, DCC and partially PEGylated dendrimers followed by lyophilization (Heto Drywinner, Germany). This is the first report of PEGylation of dendrimer using dicarboxylic acid PEG 2000.

3.3. Drug loading in formulations

The known molar concentrations of PPI 5.0G dendrimer and PEGylated PPI 5.0G dendrimers were dissolved separately in methanol and mixed with methanolic solution of Famotidine (100 mol). The mixed solutions were incubated with slow magnetic stirring (50 rpm) using teflon beads for 24 h. The methanol was evaporated and dialyzed twice in cellulose dialysis bag (MWCO 1000 Da Sigma, Germany) against methanol under sink conditions for 10 min to remove free drug from the formulations, which was then estimated spectrophotometrically ($\lambda_{max} = 265.2$ 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.

3.4. Characterization of the formulation

The drug-PEGylated dendrimer complex was characterized by IR spectroscopy (Perkin–Elmer, USA) and differential scanning calorimetry (DSC). IR spectra of Famotidine-PEGylated dendrimer complex, plain drug and plain PEGylated dendrimer were compared to confirm loading of drug in PEGylated dendrimer.

Differential scanning calorimetry (DSC) was carried out to analyze the thermal stability and transformation in crystallinity over a range of temperatures. Famotidine–PEGylated dendrimer complex, plain drug, plain PEGylated dendrimer and physical mixture of drug with PEGylated dendrimer were studied and compared by this method. Sample was placed in an aluminium pan, and a lid was crimped. The pan was then positioned 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 amplified at a rate of 10 °C/min under a N_2 gas flush out until the material began to degrade. The temperatures were obtained for each peak from the resulting curve, which provides indications of temperature stability and phase transitions.

Size exclusion chromatography (SEC) was used to determine the absolute molar mass of the PEGylated PPI 5.0G dendrimers. This experiment was performed using a SEC system consisting of a refractive index detector. The analysis was done at room temperature using two serially aligned TSK-GEL columns G3000PW and G4000PW. The isocratic mobile phase was PBS (pH 7.5) at a flow rate of 1 ml/min. Sample concentration was kept as 1 mg/ml in PBS, and 100 µl was injected. Molecular weight of PEGylated PPI 5.0G dendrimers was determined using Astra V software (Wyatt Technology Corporation).

3.5. In vitro release

Drug release from known amounts of Famotidine loaded PEGylated PPI 5.0G dendrimers was determined at different pHs using a modified dissolution method. The media comprised acid phthalate buffer (pH 4.0), phosphate buffer saline (pH 7.4) and borate alkaline buffer (pH 10.0). The dialysis bags were filled with a known amount of Famotidine loaded PEGylated dendrimeric nanostructures (MWCO 1000 Da) and were placed in 50 ml of different media at 37 ± 2 °C with slow magnetic stirring under sink conditions. Aliquots of 1 ml were withdrawn from the external solution and replenished with an equal volume of fresh media. The drug concentration was detected by UV spectrophotometer at 265 nm (λ_{max}).

3.6. Comparison of hemolytic toxicity of PPI 5.0G dendrimer and PEGylated PPI 5.0G dendrimer

The RBC suspension was obtained following the reported procedure for hemolytic studies [18]. In brief, the RBC suspension (5% hematocrit) of the human blood was collected in HiAnticlot blood collection vials (Himedia Labs, India). About 0.5 ml of suitably diluted PEGylated and non-PEGylated PPI 5.0G was added to 4.5 ml of normal saline and incubated for 1 h with RBC suspension. This allowed the comparison of the hemolysis data of the dendrimer 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. RBC suspension was added to 5 ml of 0.9% NaCl solution (normal saline) and 5 ml of distilled water, respectively, to obtain 0% and 100% hemolysis. The degree of hemolysis was determined by the following equation:

$$\text{Hemolysis}\ (\%) = \frac{\text{Abs} - \text{Abs}_0}{\text{Abs}_{100} - \text{Abs}_0} \times 100$$

Where Abs, Abs_{100} and Abs_0 are the absorbencies of sample, a solution of 100% hemolysis and a solution of 0% hemolysis, respectively.

3.7. Stability studies of PEGylated dendrimer formulations

PEGylated dendritic system loaded with Famotidine was exposed to accelerated conditions of temperature and light. The formulation was taken in different vials and stored in dark (amber color vials) and in light (colorless vials) at 0 °C, room temperature (25 ± 2 °C) and 50 ± 2 °C in thermostatically controlled oven for a period of 5 weeks. The samples were analyzed every week for any precipitation, turbidity, crystallization, color change, consistency and drug leakage. The data obtained were used for the analysis of any physical and chemical degradation, the required storage conditions

and the precautions required for storage. The samples were initially clear and transparent at 0 °C.

The loss of drug from the formulation was ascertained after storage at accelerated conditions. The known amount of formulation was kept in benzoylated cellulose tubing (Sigma, USA) and dialyzed across the tubing. The external medium (10 ml methanol) was monitored for the content of the drugs spectrophotometrically. The percentage increase in drug release from the formulation was used to analyze the effects of accelerated conditions of storage on the formulations.

3.8. In vivo studies

Male albino rats (Sprague Dawley strain, 120 ± 5 g) were utilized for in vivo experimental study. All the animal studies were conducted in accordance with the protocol approved by the Institutional Animal Ethical Committee of Dr. H.S. Gour University, Sagar (registration no. 379/01/ab/CPCSEA). Comprehensive in vivo kinetic studies were performed, which were later correlated using various pharmacokinetic parameters.

The ulcer protective efficiency of Famotidine loaded PEGylated dendrimer was compared with plain Famotidine solution dissolved in PBS (pH 7.4). The animals were divided into five groups, each group containing four animals. The first group was treated as control and was fed with PBS (pH 7.4) by i.v. route. Second, third and fourth group was treated with plain Famotidine solution, plain PEGylated dendrimers and Famotidine loaded PEGylated dendrimers, respectively. The fifth group was fed with PBS (pH 7.4) and treated as blank.

One milliliter of 80% ethanol was used orally to induce gastric ulcer [19] after 4 h except fifth group. The alcohol was given to dissolve the mucous coat of the stomach and so the condition was made to allow gastric acid to act on gastric walls. After 1 h, the animals were sacrificed and stomachs were removed and dissected carefully to observe the ulcer protective function of Famotidine loaded PEGylated dendrimer as compared to plain Famotidine solution. The incised stomachs were first washed with running tap water and placed on the watch glass and examined for severity of ulceration according to the following scale: 0 = normal gray colored stomach, 0.5 = pink to red coloration of stomach, 1 = spot ulcer, 1.5 = hemorrhagic streak, 2 = number of ulcer < 5, 3 = number of ulcer > 5, 4 = ulcers with bleeding. The ulcer index (U_I) was calculated by adding the total number of ulcers and severity of ulcer:

$$U_I = U_N + U_S$$

- U_N = number of ulcers in animal
- U_S = severity score

Male albino rats of Sprague Dawley strain were used for pharmacokinetic and tissue distribution study of plain Famotidine and Famotidine loaded in PEGylated PPI 5.0 dendrimer. Animals are divided into two groups. Here, 0.1 ml solution of Famotidine loaded PEGylated dendrimer and plain Famotidine (in PBS) was administered by i.v. injection into rats in each group. Famotidine levels in blood and various organs were estimated by HPLC method [20]. Separation was carried out on reversed-phase C18 column and the column effluent was monitored using UV detector at 267 nm. The mobile phase consists of 0.03 M disodium hydrogen phosphate buffer–acetonitrile (93:7, v/v) adjusted to pH 6.5 at a flow rate of 1.0 ml/min.

3.9. Statistical analysis

The results are 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 Famotidine loading in PEGylated PPI dendrimers and non-PEGylated PPI dendrimers 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.

4. Results and discussions

4.1. Synthesis of PEGylated PPI 5.0G dendrimers

PPI 5.0G dendrimers were synthesized by the procedure reported by De Brabender Van Den Berg [15] using ethylenediamine as initiator core [14]. Synthesis of 0.5G PPI was confirmed by IR peaks, mainly of nitrile at 2248 cm^{-1}. All the nitrile terminal 0.5G PPI got converted to $(NH_2)_4$, which was confirmed by IR of PPI 1.0G that exhibited major peak at 3431 cm^{-1} for amine (N–H stretch). Likewise, IR peaks also confirmed the synthesis of PPI 5.0G dendrimers. The main peaks are of C–C bend (1113 cm^{-1}); C–N stretch (1231 cm^{-1}, 1326 cm^{-1}); C–H bend (1422 cm^{-1}, 1477 cm^{-1}); N–H deflection of amine (1643 cm^{-1}) and primary amine at 3410 cm^{-1}(N–H stretch), confirming that nitrile terminal groups of dendrimer were converted to amine terminals. The results matched with the reported synthesis of PPI dendrimers [15,16].

The synthesized dendrimers were PEGylated using DCC and dicarboxylic acid PEG 2000. IR and NMR data proved the synthesis of PEGylated dendrimers. The IR spectrum of PEGylated PPI 5.0G dendrimer exhibited major peak of N–H stretch of amide at 3420.4 cm^{-1}. An important IR peak at 1254.9 cm^{-1} of ether linkage (C–O) appears in the spectrum of PEGylated dendrimers. C=O stretch of amide group has been found near 1657.3 cm^{-1}. The important peak of C–N stretch of amide also appears at 2920.6 cm^{-1} as shown in Fig. 3(a). NMR spectrum and shifts of PEGylated dendrimers as compared to that of simple dendrimers proved PEGylation. There was increase in integral value for the shift of secondary $-CH_2$ groups on PEGylation. This is due to the increase in number of secondary $-CH_2$ groups in PEG that are linked on PEGylation. Similarly, strong peak of ether linkage appears at 3.4–3.6 ppm due to the presence of ether linkages in PEG in high amount. The characteristic peak of amide linkage appeared near 7.82 ppm in NMR spectrum of PEGylated dendrimers as shown in Fig. 3(b).

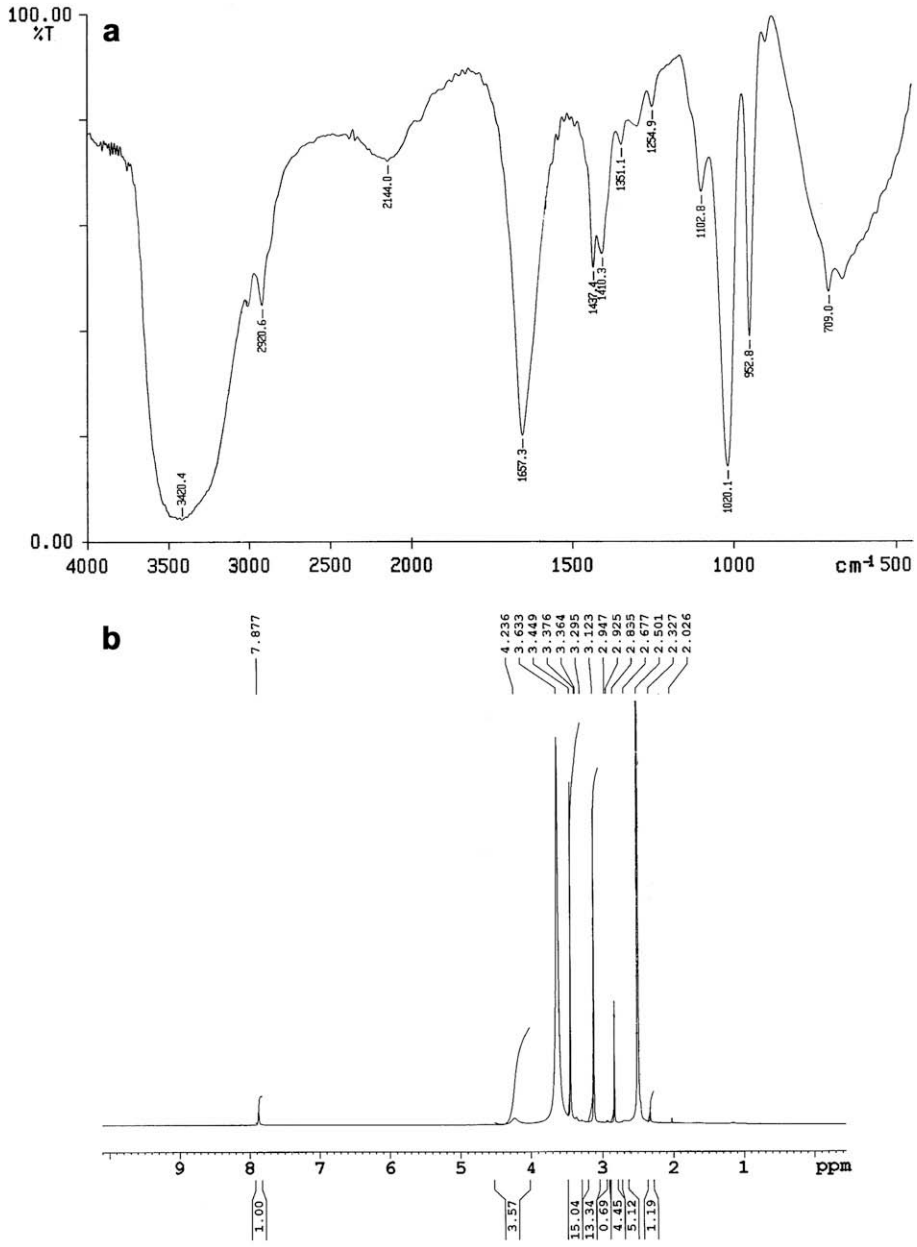

Fig. 3. (a) IR spectrum of PEGylated PPI 5.0G dendrimers, (b) NMR spectrum of PEGylated PPI dendrimers.

4.2. Drug loading

Non-covalent interactions between Famotidine and PEGylated PPI 5.0G dendrimers, such as hydrophobic interaction and hydrogen bonding, contributed to the physical binding of drug molecules inside dendritic micelles and surface PEG layers. The percentage loading of Famotidine in PEGylated PPI 5.0G dendrimers (Fig. 4) was significantly increased (p value 0.0001, extremely significant) compared to non-PEGylated PPI dendrimers. PEGylation increases the Famotidine loading capacity of the PPI 5.0G dendrimers due to more interaction of drug and PEG at the peripheral portions of dendrimers. Famotidine entrapment in PEGylated dendrimers increased significantly due to more sealing of dendrimeric structure by PEG at the peripheral portions of dendrimers as coat, which prevented drug release by enhancing complexation probably by increasing steric hindrance over dendrimer periphery. Number of moles of Famotidine entrapped in 1 mol of PEGylated dendritic architecture was found to be 56.6 ± 1.4 mol as compared to 27.59 ± 1.9 mol in non-PEGylated system.

4.3. Characterization of the formulation

The shifts in IR peaks of Famotidine–PEGylated dendrimer complex as compared to plain Famotidine and plain PEGylated dendrimers confirmed the drug loading. The characteristic peaks of plain Famotidine and PEGylated dendrimer were present in Famotidine loaded PEGylated dendrimer (Fig. 5).

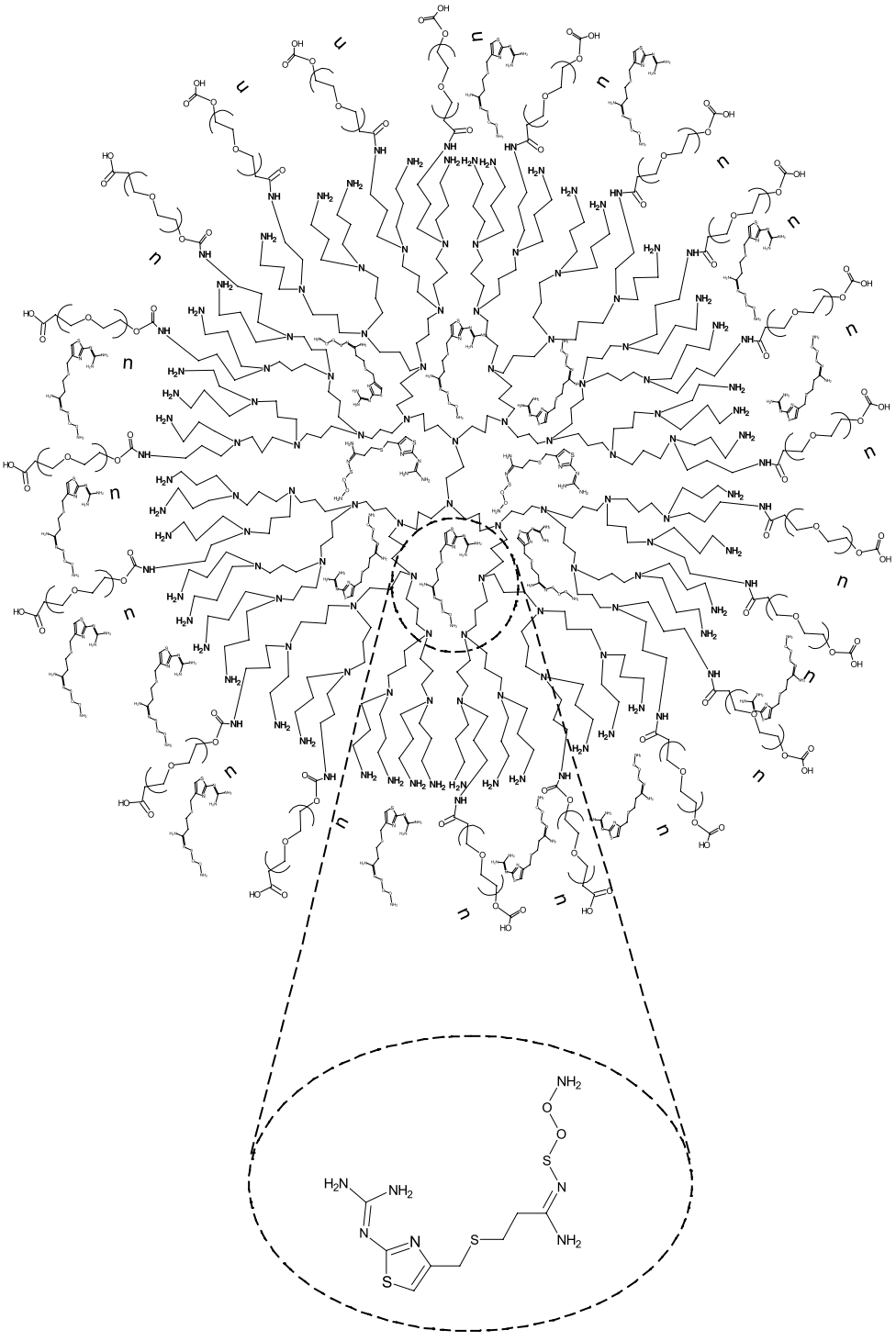

Fig. 4. Famotidine loaded PEGylated PPI 5.0G dendrimer (n = PEG 2000).

Curves of DSC clearly suggested the difference between the physical mixture of plain drugs and encapsulated drug–PEGylated PPI 5.0G dendrimer complex (Fig. 6). The plain Famotidine DSC curve showed an endothermic peak at its melting point near 165 °C. PEGylated PPI 5.0G dendrimers experienced an endothermic peak near 245 °C. In physical mixture of Famotidine with PEGylated PPI 5.0G dendrimers both the peaks of Famotidine and PEGylated PPI 5.0G dendrimer were found near 165 °C and 215 °C, respectively. In DSC curve of Famotidine loaded PEGylated PPI 5.0G dendrimers the peaks of plain Famotidine and PEGylated dendrimers almost disappeared and a very broad peak near 252 °C was observed. The DSC curves clearly demonstrated and confirmed the formation of drug dendrimer complex.

Size exclusion chromatography was used to determine the absolute molecular weight of the PEGylated PPI 5.0G dendrimers. Fig. 7 shows the mass fraction of PEGylated PPI 5.0G dendrimers. The calculated molecular weight obtained

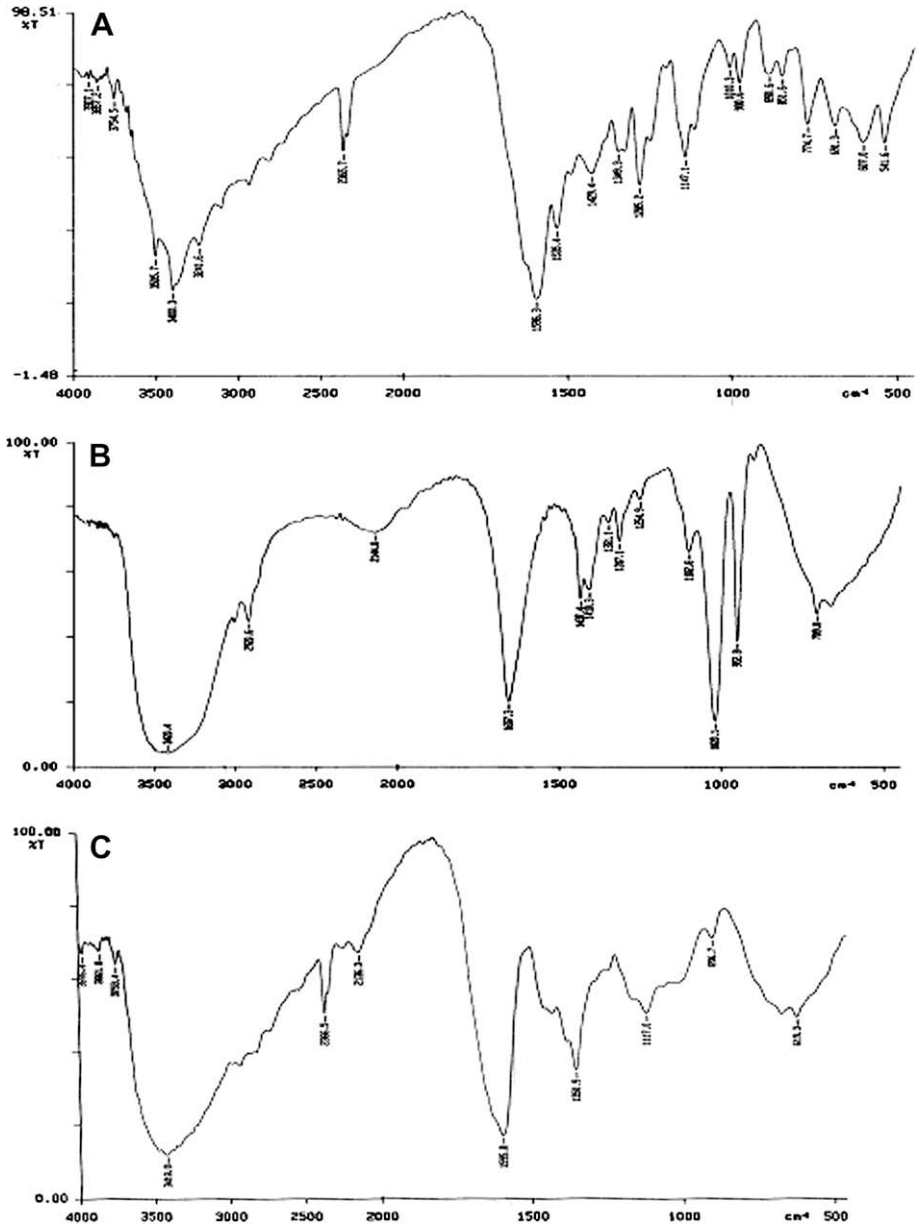

Fig. 5. Comparison of IR spectra of (A) plain Famotidine, (B) plain PEGylated PPI 5.0G dendrimer, and (C) Famotidine–PEGylated PPI 5.0G dendrimer complex.

is 48 125 slightly less than the theoretical molecular weight (48 140). The molecular weight indicates that 20 PEG chains were conjugated to one molecule of PPI 5.0G dendrimer. The SEC data match well according to MALDI-TOF mass spectra of PEGylated PPI 5.0G dendrimers. The polydispersity index was found to be 1.0009.

4.4. Hemolytic toxicity

The hemolytic toxicity of the dendrimers was enough to impose a constraint in its use as a drug delivery system. The toxicity is due to the polycationic nature of the PPI dendrimers. However, PEGylation of dendrimers was found to decrease the hemolysis of the RBC considerably at all concentrations due to the shielding or coating of the charged quaternary ammonium ion that is generally formed on the amine-terminated whole generations of PPI dendrimers, responsible for hemolysis. The whole generation of amine-terminated charged PPI 5.0G dendrimers. showed hemolytic toxicities of 9.2 ± 1.06, 12.2 ± 2.18, 14.7 ± 1.02 and $16.9 \pm 1.93\%$ at 0.1, 0.2, 0.3 and 0.4% w/v concentrations, respectively. But PEGylation of the dendrimers was found to have decreased the hemolysis of the RBCs significantly to 1.5 ± 0.35, 2.3 ± 0.52, 2.9 ± 0.42, and $3.4 \pm 0.33\%$ at 0.1, 0.2, 0.3 and 0.4% w/v concentrations, respectively (Table 1). This was due to the 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. [18].

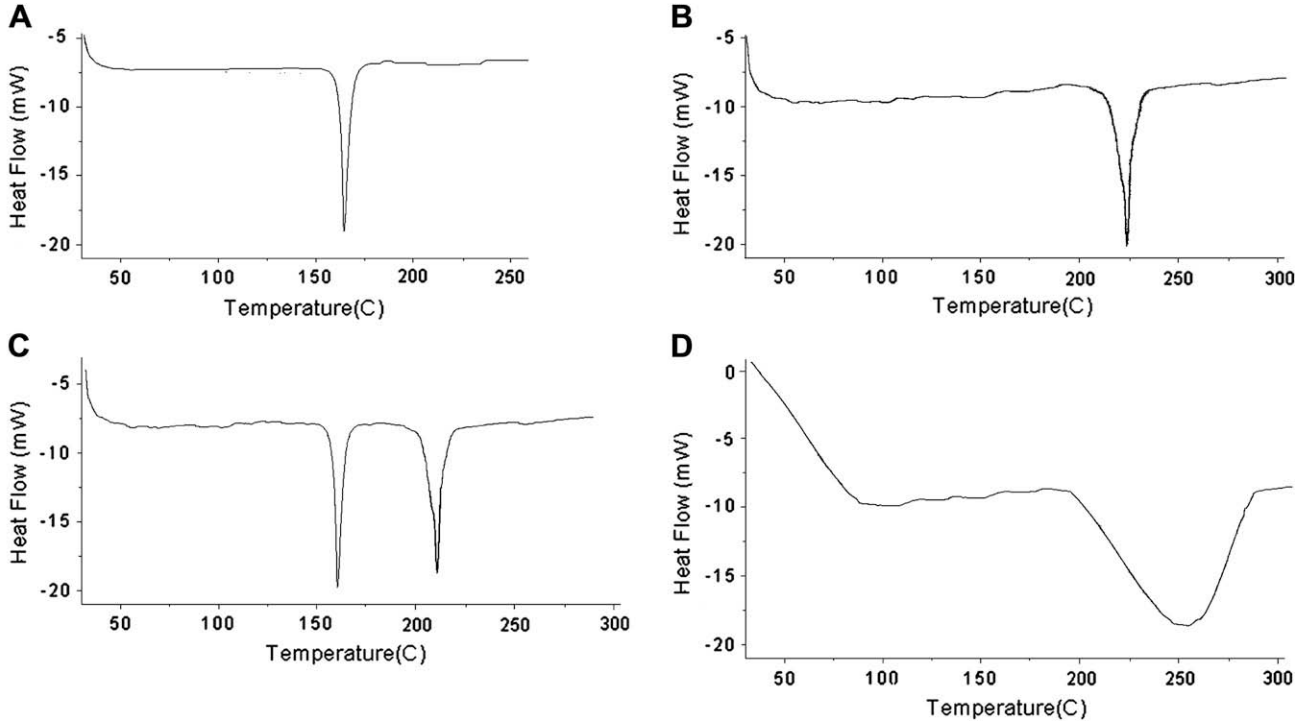

Fig. 6. Curves of differential scanning calorimetry: (A) plain Famotidine, (B) PEGylated PPI 5.0G dendrimers, (C) physical mixture of Famotidine and PEGylated PPI 5.0G dendrimers, and (D) Famotidine loaded PEGylated PPI 5.0G dendrimers.

4.5. Stability studies of PEGylated dendrimer formulations

The stability study carried out on the drug–PEGylated dendrimer complexes at various accelerated conditions of temperature (0 °C, RT, and 50 °C) and light showed that the dendritic formulations are stable even at higher temperature if kept in dark (amber color vials) but are unstable in the presence of light (colorless vials). There was change in color and precipitation noted after 5 weeks when kept at 50 °C in the presence of light but no such change was observed at the same temperature in dark (Fig. 8). However, there was drug loss from the formulation that was observed at higher temperature and it was found to be even greater in the presence of light. This may be due to higher reaction kinetics in the presence of light at high temperature. The dendritic structures are supposed to be more open at higher temperature and this change in surface characteristics might cause the conformational changes in the structure and release of the drug.

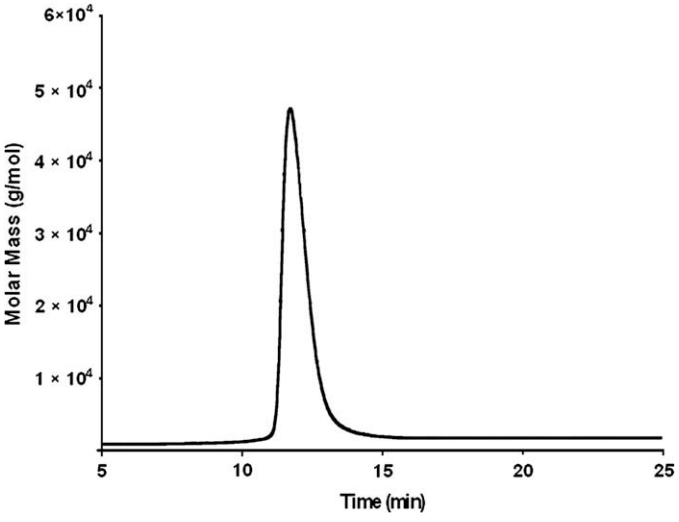

Fig. 7. Mass fraction profile of PEGylated PPI 5.0G dendrimers as measured by SEC.

4.6. In vitro release

The in vitro release of Famotidine loaded PEGylated PPI 5.0G dendrimer from the formulation was retarded at pH 7.4 and 10.0 as compared to pH 4.0. This is possibly due to the contribution of tertiary nitrogens within the dendritic cavities and primary nitrogens on the dendrimer periphery. Only $40.7 \pm 1.0\%$ and $55.2 \pm 1.9\%$ of Famotidine was released at pH 10.0 and 7.4, respectively in 24 h, while $96.8 \pm 1.5\%$ of Famotidine was released at pH 4.0 in 2.5 h (Fig. 9).

Table 1
Comparison of percentage hemolysis of plain and PEGylated dendrimers

PPI 5.0G dendrimers	%Hemolysis	PEGylated PPI 5.0G dendrimers	%Hemolysis
D_1	9.2 ± 1.06	PD_1	1.5 ± 0.35
D_2	12.2 ± 2.18	PD_2	1.9 ± 0.5
D_3	14.7 ± 1.02	PD_3	2.9 ± 0.42
D_4	16.9 ± 1.93	PD_4	3.4 ± 0.33

'D_1', 'D_2', 'D_3' and 'D_4' represent 0.1, 0.2, 0.3 and 0.4% w/v concentrations of plain dendrimer. 'PD_1', 'PD_2', 'PD_3' and 'PD_4' represent PEGylated dendrimer in 0.1, 0.2, 0.3 and 0.4% w/v concentrations ($n = 3$).

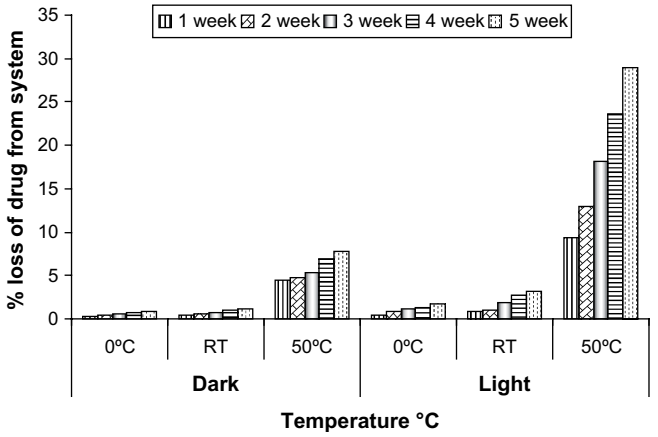

Fig. 8. Comparative stability study of Famotidine loaded PEGylated PPI 5.0G dendrimers at different temperatures in the dark and light conditions ($n = 3$).

The nitrogens of tertiary amines are strongly basic ($pK_a = 9.5$) [21]. These quaternized nitrogens bind to the counter ions, such as carboxylate ions in the case of Famotidine, and control their dissociation. Therefore they will be 2/3 protonated at pH 7.4. PPI 5.0G dendrimer shrunk the hydrophobic interior cavities of the dendrimer at pH 7.4 and caused sustained release of drug. The PEGylation will further retard the drug release. As the drug will be released from the dendrimer, it will be stuck in the PEG chains and slowly released into the environment. But as the pH lowered, the dendrimer became more and more protonated and the branches expanded and both the attached as well as the encapsulated drug showed burst release from the dendrimer within very short period of time. The size of the dendrimer increased at acidic pH as compared to pH 7.4 and 10.0. Lee et al. [22] have established that due to the presence of amine groups in PAMAM dendrimer there is difference between density and size of the dendrimer at different pHs. At higher pH, density and size of the PAMAM dendrimer are less but at low pH, density and size of dendrimer molecule are relatively higher. This is due to the protonation of the primary and tertiary amines. PPI dendrimer also have both the types of primary and tertiary amines and their protonation will affect the size and density of the dendrimer molecule, which contributed to sustained drug delivery at higher pH, but burst release at lower pH. The in vitro results

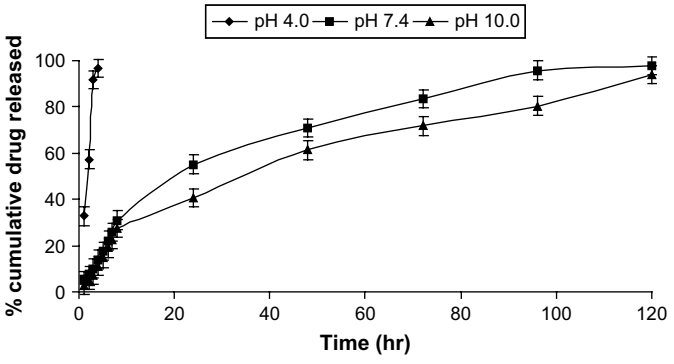

Fig. 9. Cumulative percentage of Famotidine release from PEGylated PPI 5.0G dendrimer at different pHs ($n = 3$).

Table 2
Effect of Famotidine loaded PEGylated PPI 5.0G dendrimer on ulcer index

Treatment group	(i)	(ii)	(iii)	(iv)	(v)
Ulcer index	18.50 ± 0.47	5.5 ± 0.28	14 ± 0.95	0.5 ± 0.53	ND

(i) Ethanol treated group, (ii) plain Famotidine, (iii) plain PEGylated PPI 5.0G dendrimer, (iv) Famotidine loaded PEGylated PPI 5.0G dendrimer, (v) control group, ND = not detectable.

were found to be similar to that previously reported by Kumar et al. [16].

4.7. In vivo studies

The in vivo investigations demonstrated that there was a marked difference in the percentage of drug distribution from the bound drug through PEGylated dendrimer when compared with the free drug ($P < 0.05$). The pharmacodynamic study of dendritic formulation gave an idea about the ulcer protective function with time. It was observed that the formulation under study not only decreased the ulcer index to a significant larger magnitude but also sustained this magnitude. In case of PEGylated dendrimer–drug formulation, the ulcer index was found to be only 0.5 ± 0.53 after 5 h of dosing. However, for plain drug the ulcer index was found to be 5.5 ± 0.28 after 5 h of dosing (Table 2). The possible reason for this result may be the drug concentration in the body that was maintained for a longer duration in case of Famotidine loaded PEGylated dendrimer as compared with that of plain Famotidine.

The maximum plasma concentration was observed for i.v. administration of Famotidine loaded PEGylated PPI 5.0G dendrimer as compared to plain Famotidine (Table 3 and Fig. 10). The C_{max} values attained after i.v. administration were 216 ± 6.1 and 247 ± 4.9 ng/ml for plain Famotidine and Famotidine loaded PEGylated PPI 5.0G dendrimer, respectively. The $AUC_{(0 \rightarrow \infty)}$ (ng/ml/h) for plain Famotidine and Famotidine loaded PEGylated PPI 5.0G dendrimer was found to be 1108.7 and 3790.64 ng/ml/h, respectively (Table 4). When

Table 3
The serum concentration of plain Famotidine and Famotidine loaded in PEGylated PPI 5.0G dendrimer, $n = 5$

S. no.	Time (h)	Concentration (ng/ml)	
		C	D
1	0.5	216 ± 6.1	247 ± 4.9
2	1	186 ± 3.8	231 ± 5.7
3	2	150 ± 5.3	219 ± 2.7
4	3	128 ± 5.4	205 ± 7.8
5	4	109 ± 2.1	181 ± 6.3
6	5	78 ± 7.6	165 ± 4.7
7	6	–	158 ± 7.4
8	7	–	142 ± 3.9
9	8	–	130 ± 3.3
10	12	–	121 ± 6.4
11	24	–	109 ± 5.3
12	36	–	87 ± 4.8
13	48	–	71 ± 7.3

Dose = 0.27 mg/kg body weight i.v. route, C = plain Famotidine, D = Famotidine loaded in PEGylated PPI 5.0G dendrimer.

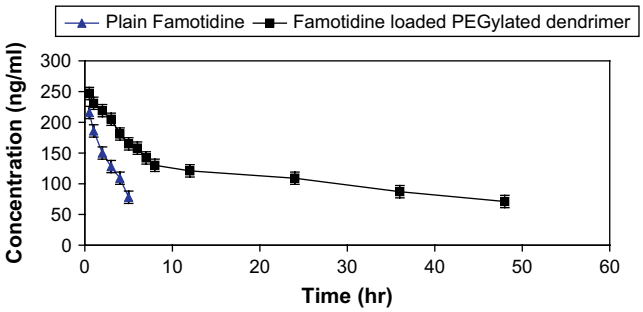

Fig. 10. The blood serum concentration of Famotidine loaded PEGylated PPI 5.0G dendrimer and plain Famotidine ($n = 5$).

apparent $t_{1/2}$ of formulations was compared, the parameter was 3.31 and 8.1 h for plain Famotidine and Famotidine loaded PEGylated PPI 5.0G dendrimer, respectively. After i.v. administration, $t_{1/2}$ increased in the case of Famotidine loaded PEGylated PPI 5.0G dendrimer as compared to plain Famotidine, which is attributed to the polyethylene glycol (PEG) coating of PPI 5.0G dendrimer that rendered the formulation more 'biostable' compared to plain Famotidine.

Further, PEGylated PPI 5.0G dendrimer formulations of Famotidine demonstrated highest $t_{1/2}$ probably because of dendrimer size and its ability to impart better 'Stealth' features as compared to free drugs. The higher AUC values of PEGylated PPI 5.0G dendrimer formulations also indicate that the formulations are of long circulating nature. The blood level studies also demonstrated the long circulating and sustained release property of PEGylated PPI 5.0G dendrimer. Previous reports also suggested the long circulating and sustained releasing property of PEGylated dendritic system [23,24].

In order to understand the fate of drug loaded PEGylated PPI 5.0G dendrimers in vivo, the biodistribution to various major organs was investigated. The amount of drug in the body depends upon its release, distribution, metabolism and excretion from the body. The biodistribution of Famotidine is generally more in bile and excretion is mainly through kidneys. But PEGylated PPI 5.0G dendrimers, due to its sustained drug delivery and long circulatory nature, made the drug molecules to be more available in blood than in bile for a longer period of time. The amount of plain drugs in different metabolic and excretory organs was found to be higher at 4 h as compared to PEGylated dendrimer formulation, which indicated slow release pattern from the PEGylated dendritic formulations. The amount of drug in the case of plain Famotidine was found to be 223 ± 8.5 ng/ml in kidneys at 4 h, which was higher as compared to PEGylated dendritic formulation (93 ± 9.8 ng/ml) but the position was reversed at 8 h. It indicates that most of the plain drug was eliminated out of the body. While in the case of PEGylated dendritic formulations at 8 h, higher amount of drugs was observed in excretory organs (Table 5), demonstrating slow release of drugs from them. Similar results were observed for lungs also. PEGylation has reduced the hepatic and bile accumulation of the drugs. These results well agree with the reports that the PEGylation of drug carriers such as nanoparticles and other polymer could improve their biodistribution characteristics of drugs by reducing non-specific interaction with biomolecules [25]. This finding is strongly suggesting that PEGylation is also able to improve the biodistribution properties of the dendrimeric drug carriers such as PPI 5.0G dendrimers. The result of these studies signified the ability of drug loaded PEGylated PPI 5.0G dendrimer to alter the pharmacokinetics of the drugs and may be used as sustained drug delivery system.

Table 4
Pharmacokinetic parameters of plain Famotidine and Famotidine loaded in PEGylated PPI 5.0G dendrimer in serum of male albino rats

S. no.	System	$AUC_{(0\rightarrow\infty)}$ (ng/ml/h)	$AUMC_{(0\rightarrow\infty)}$ (ng/ml/h)	C_{max} (ng/ml)	$t_{1/2}$ (h)	MRT (h)	K_{el}
1	C	1108.7	5159.5	216 ± 6.1	3.31	4.65	0.209
2	D	3790.64	56692.25	247 ± 4.9	8.1	14.95	0.085

AUC = area under curve, MRT = mean residence time, AUMC = area under movement curve, C_{max} = maximum concentration, $t_{1/2}$ = half-life, K_{el} = elimination constant.

Table 5
The drug level attained at various time intervals in different tissues, $n = 4$

S. no.	Organ	System	Concentration (ng/ml) at different time intervals	
			4 h	8 h
1	Liver	C	142 ± 6.3	129 ± 4.7
		D	72 ± 3.0	348 ± 5.9
2	Kidney	C	223 ± 8.5	159 ± 8.9
		D	93 ± 9.8	213 ± 8.7
3	Lung	C	175 ± 5.2	128 ± 9.3
		D	86 ± 7.4	232 ± 6.9
4	Spleen	C	136 ± 5.6	108 ± 9.8
		D	68 ± 8.3	216 ± 6.4

C = Plain Famotidine and D = Famotidine loaded in PEGylated PPI 5.0G dendrimer.

5. Conclusion

The in vitro release, blood level and tissue distribution data indicate the potential of the developed system for prolonged delivery of H_2 receptor antagonist, Famotidine. The in vitro results suggest that the PEGylated dendrimers can be used for targeted delivery of drugs to acidic pH sites like endosomes having pH similar to 5. The PEGylation has shown to amplify drug loading but has 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 release depot nanoparticulate systems for drug delivery producing least blood dyscrasias as against non-PEGylated systems.

Acknowledgement

Mr. Virendra Gajbhiye acknowledges the All India Council of Technical Education, New Delhi, India for the grant of Junior Research Fellowship and Inter University Consortium, Indore (India) for DSC facility.

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