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Ligand anchored dendrimers based nanoconstructs for effective targeting to cancer cells

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ABSTRACT

Dendrimers are considered versatile carriers especially for the treatment of diseases like cancer, AIDS, malaria etc. Cancer is a worldwide threat particularly in developing countries. A breakthrough research in this regard is a prime requirement. In the present study, folic acid was conjugated to fifth generation polypropylene imine (PPI) dendrimers and characterized through IR, NMR (^{13}C and ^{1}H), ESI mass spectroscopy as well as electron microscopic studies. Doxorubicin (DOX), an effective anticancer drug, was used in the present study to develop and explore the anticancer potential of the dendrimer based formulations. DOX was loaded (approximately 26 and 65%) to the PPI dendrimers as well as folate conjugated PPI (PPI–FA) dendrimers, respectively. These ligand conjugated dendrimers displayed very less (approximately 3 and 4%, respectively, for PPI–FA and PPI–FA–DOX) hemolysis. The developed formulation PPI–FA–DOX was stable enough. *In vitro* drug release of the formulation was found to be faster in the acidic media than at the higher pH. The prepared formulation displayed a higher cell uptake in MCF-7 cancer cell lines as evidenced by fluorescence studies. The results suggested that, in future, folic acid conjugated PPI dendrimers may emerge as a better choice for anticancer drug targeting.

1. Introduction

Attempts to deliver drug to the desired site in the body are a thrust area of biomedical research today. Nanotechnology based drug delivery approaches have proven some exciting results in this perspective (Brannon-Peppas and Blanchette, 2004; Farokhzad and Langer, 2009; Gupta and Jain, 2010). Diseases like AIDS, diabetes, tuberculosis and cancer are among the most disastrous diseases worldwide today. A recent fact sheet of WHO reports that cancer is a leading cause of death worldwide. It accounted for 7.9 million deaths (around 13% of all deaths) in 2007 which is an alarming figure. Among the different types of cancer lung, stomach, liver, colon and breast cancers cause the most cancer deaths each year. The projected death figure due to cancer was estimated to be 12 million in 2030. Deaths due to cancer are more prevalent in low and middle income countries (WHO). Tumor is a morbid state of tissue growth, which encompasses its own morphogenesis and after proliferation termed as cancer. The unorganized rapid growth and widened inter-endothelial junctions with higher permeability are the key features of tumor (Carmeliet and Jain, 2009; Jain, 1987). Complete cure of the cancer is still a challenge to the biomedical research. Available anticancer drug delivery approaches invariably attack healthy cells along with the cancerous cells, which is an important issue. Recent reports of the last decade showed that nano-particulate carriers displayed higher accumulation behavior at the tumor site. Higher accumulation of the anticancer drugs can be achieved by enhanced permeation and retention effect (EPR) and/or by avoiding reticulo-endothelial system (RES) and/or through utilization of ligand mediated targeting. Polymeric nanocarriers in this context are among one of the best choices for this purpose. These include nanoparticles, dendrimers, liposomes etc (Papahadjopoulos and Gabizon, 1990; Kommareddy et al., 2005).

Dendrimers in the last decade have emerged as a promising nano-polymeric tool for the drug delivery and targeting. Dendrimers are the mono-dispersed, three dimensional, highly branched, macromolecular nanocarriers (1–100 nm). Higher drug loading capacity, easy synthesis, stability, tailor made functionality, transdermal ability are the important advantages of the dendrimers (Tomalia et al., 1985; Sevenson and Tomalia, 2005). The carrier has been successfully used for the solubilization of insoluble drugs, diagnosis, controlled and sustained as well as transdermal drug delivery approaches and for other nonmedical

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purposes (Chauhan et al., 2003; Satija et al., 2007). Tailor made surface functionality of the dendrimers has been explored for targeting of anticancer and anti-HIV drugs etc (McCarthy et al., 2005; Agarwal et al., 2009). Polyamidoamine (PAMAM) and polypropylene imine (PPI) dendrimers are among the most studied classes of dendrimers especially in the drug delivery. Surface primary amine group of dendrimers has been successfully conjugated with the ligands like galactose and mannose for liver targeting and other potential body areas in the earlier studies (Bhadra et al., 2003, 2005).

Ligand mediated active targeting of the anticancer drugs through dendrimers have proven well in the past studies. However, studies based on PPI dendrimers in this regard as compared to PAMAM dendrimers are scarce. Earlier reports suggest that the encapsulation capacity of the PPI dendrimers is comparable to the PAMAM dendrimers (Richter-Egger et al., 2001). Cationic toxicity is still an important issue, which creates hurdle in the possible drug delivery potential of the dendrimers *in vivo* (Malik et al., 2000). Surface engineering of the dendrimers can be a solution in this regard, and if we engineer the surface of dendrimers with some biocompatible anticancer ligand it would be a beneficial approach, both in terms of targeting and reduced toxicity. In the present study, we report design, synthesis and characterization of folic acid conjugated PPI dendrimers encapsulated with DOX, an effective anticancer drug, and these conjugated dendrimers were assessed for their anticancer potential *in vitro* using human mammary cancer MCF-7 cells. It was envisaged that the folic acid conjugation will shield the cationic charge associated toxicity of the dendrimers *in vivo* in general and additionally will target the dendrimeric conjugates to the tumor, specifically.

2. Material and methods

2.1. Materials

Raney Nickel was purchased from Fluka (USA), while ethylene diamine (EDA) and acrylonitrile were purchased from CDH (India). DOX was obtained as a generous gift from Dabur India Ltd. Nylon membrane filter (0.45 μm) was obtained from Pall Gelman Sciences, USA. Folic acid, NHS (*N*-hydroxy succinimide), DCC (*N*,*N*-dicyclohexyl carbodiimide), and DMSO (dimethylsulfoxide) were purchased from Hi Media (India). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] and FITC (fluorocein isothiocynate) were purchased from Sigma (Germany). All the other reagents and chemicals were of analytical grade and were used as received.

2.2. Synthesis and characterization of EDA core PPI dendrimers up to 5.0 G

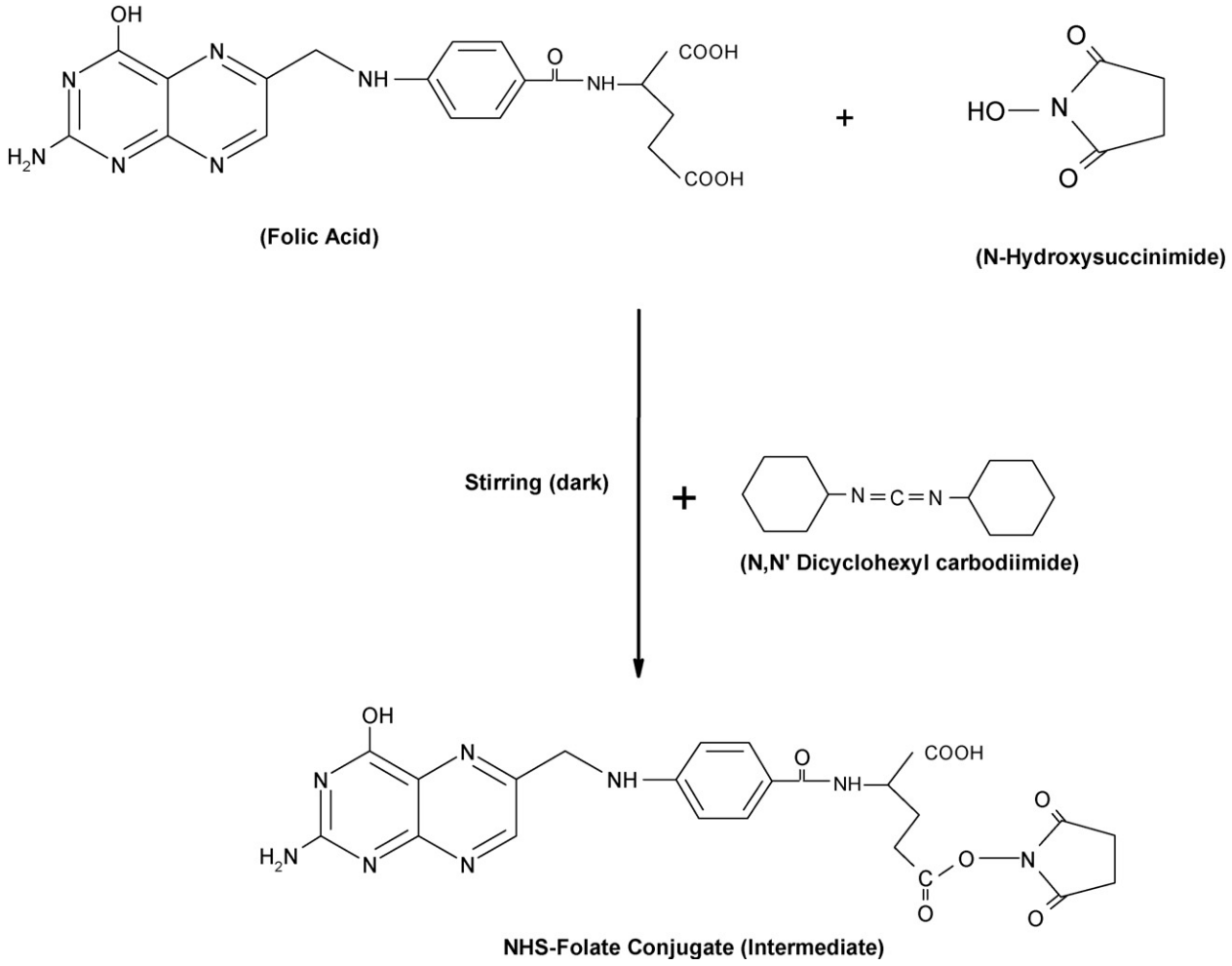
Synthesis of the EDA core PPI dendrimers was according to the classical report by Meijer and coworkers in 1993 with some modifications (Brabander-van den Berg and Meijer, 1993). Meijer and coworkers have used diaminobutane core in dendrimer but in the present study EDA was selected as dendrimer core for the synthesis of PPI dendrimers (Brabander-van den Berg and Meijer, 1993). The detailed synthesis and characterization of PPI dendrimers has been reported in our previous study (Gupta et al., 2007). Briefly, EDA core PPI dendrimers up to 5.0 G were synthesized using repetition of double Michael addition of acrylonitrile to primary amines, followed by heterogeneously catalyzed hydrogenation of the nitriles, resulting in double number of primary amines. Acrylonitrile (8.35 mol, 443 g; 2.5 molar times per NH_2) was added to a solution of ethylenediamine (1.67 mol, 100.34 g) in 1.176 kg water. The exothermic reaction caused the temperature to rise to 38 ± 2 °C. After this exothermic effect the reaction mixture was heated at 80 °C for 1 h to complete the addition reaction. The excess of acrylonitrile was removed as a water azeotrope by vacuum distillation (16 mbar, bath temperature 40 °C; Superfit, India). Obtained crystalline solid was 0.5 G PPI dendrimer. Conversion of half to full generation dendrimers (1.0 G) was carried out in a laboratory scale catalytic hydogenator (Superfit, India). The reaction was repeated in the same sequence up to fifth generation (5.0 G). PPI dendrimers were characterized at each step using different spectroscopic techniques like IR, ^{1}H NMR as well as transmission electron microscopy (TEM). IR spectroscopy of dendrimers was carried out using KBr pellet method after adsorption of smaller amount of dendrimer on KBr pellets in PerkinElmer IR spectroscope. IR analysis of 5.0 G PPI dendrimers revealed peaks at C–N stretching of CH_2–NH_2 (1118.3 cm^{-1}), C–H stretching (2957.5 cm^{-1}, 2824.2 cm^{-1}), N–H bending vibrations of amine (1597 cm^{-1}) and, most importantly, N–H stretching of primary amine (3396.5 cm^{-1}). ^{1}H NMR spectroscopic studies of dendrimer samples were carried out at 300 MHz, after dissolving in D_2O (Bruker DRX 300 MHz, USA). Important chemical shifts obtained were between 0.6 and 1.25 ppm (alkane); 1.3 and 1.9 ppm (secondary alkane); 2.3 and 2.9 ppm (alkyl amines); 7.933 ppm (primary amines). TEM was performed after drying dendrimeric sample on 3 mM forman (0.5% plastic powder in amyl acetate)-coated copper grid (300 mesh) at 60 kV (Morgani, 268D; Holland) after staining negatively using uranyl acetate (4%) and photomicrographs were taken at suitable magnifications (Morgani, 268D; Holland). This analysis was mainly performed to confirm the size of the synthesized dendrimers.

2.3. Folic acid conjugation

Folic acid conjugation was achieved using NHS activated folic acid and its direct reaction with the PPI dendrimers.

2.3.1. Preparation and characterization of NHS ester of folic acid

Active ester of folic acid was synthesized using slightly modified method reported earlier (Lee and Low, 1994). Briefly, folic acid (1 g) was dissolved in 50 ml of dimethylsulfoxide (DMSO) along with 1.1 molar excess of NHS; 1.1 molar excess of DCC was then added, and the reaction mixture was stirred overnight in dark at room temperature. The insoluble byproduct, dicyclohexylurea, was removed by filtration through glass wool (Scheme 1). The filtrate containing DMSO solution of the NHS–folate product was stored at −20 °C until its use in further synthesis. FA–NHS was characterized through NMR (both ^{13}C and ^{1}H), IR and ESI mass spectrosocopy. In IR spectroscopic analysis FA–NHS samples were analyzed by the KBr pellet method in a PerkinElmer 783 IR spectrophotometer. Important peaks obtained in the IR spectra of folic acid were at 3543.5, 3420.6 cm^{-1} (N–H stretch of primary amine and amide); 3324.9 cm^{-1} (alkyl C–H and C=C stretch); 1696.0 cm^{-1} (aromatic C=C bending and stretching); 1485.3 cm^{-1} (CH–NH–C=O amides bending); 838.1 cm^{-1} (aromatic C–H bending and benzene 1,4-disubstitution); and for the NHS conjugated folic acid 3761.0 cm^{-1} (amide N–H and C=O stretching); 3446.0 cm^{-1} (primary aliphatic amine N–H stretching); 2927.1 cm^{-1} (carboxylic acid C=O and O–H stretching unconjugated); 1631.8 cm^{-1} (ketones C=O unconjugated stretch); all of which confirmed the synthesis of NHS ester of folic acid. Further, FA–NHS ester was also characterized with the ^{1}H NMR and ^{13}C NMR. ^{1}H NMR (D_2O, Bruker DRX300, Germany) obtained at 400.1300184 MHz, d 6.99 (d, C=C), d 1.9–2.0 (m, CH—C=O—), d 2.4 (t, —CH—CH—, conjugated NHS ring), d 7.6 (d, C=C, benzene ring). ^{13}C NMR revealed important shifts at 166.43 (CH_2COO, conjugated portion of the NHS to folic acid); 169.16 (NC=O; NHS ring); 174.16 (HO—C=C; folic acid ring); 148 (CH=N—; folic acid ring).



Scheme 1. Synthesis of NHS ester of folic acid (intermediate conjugate).

2.3.2. Conjugation of NHS–folic acid ester to EDA core PPI dendrimers

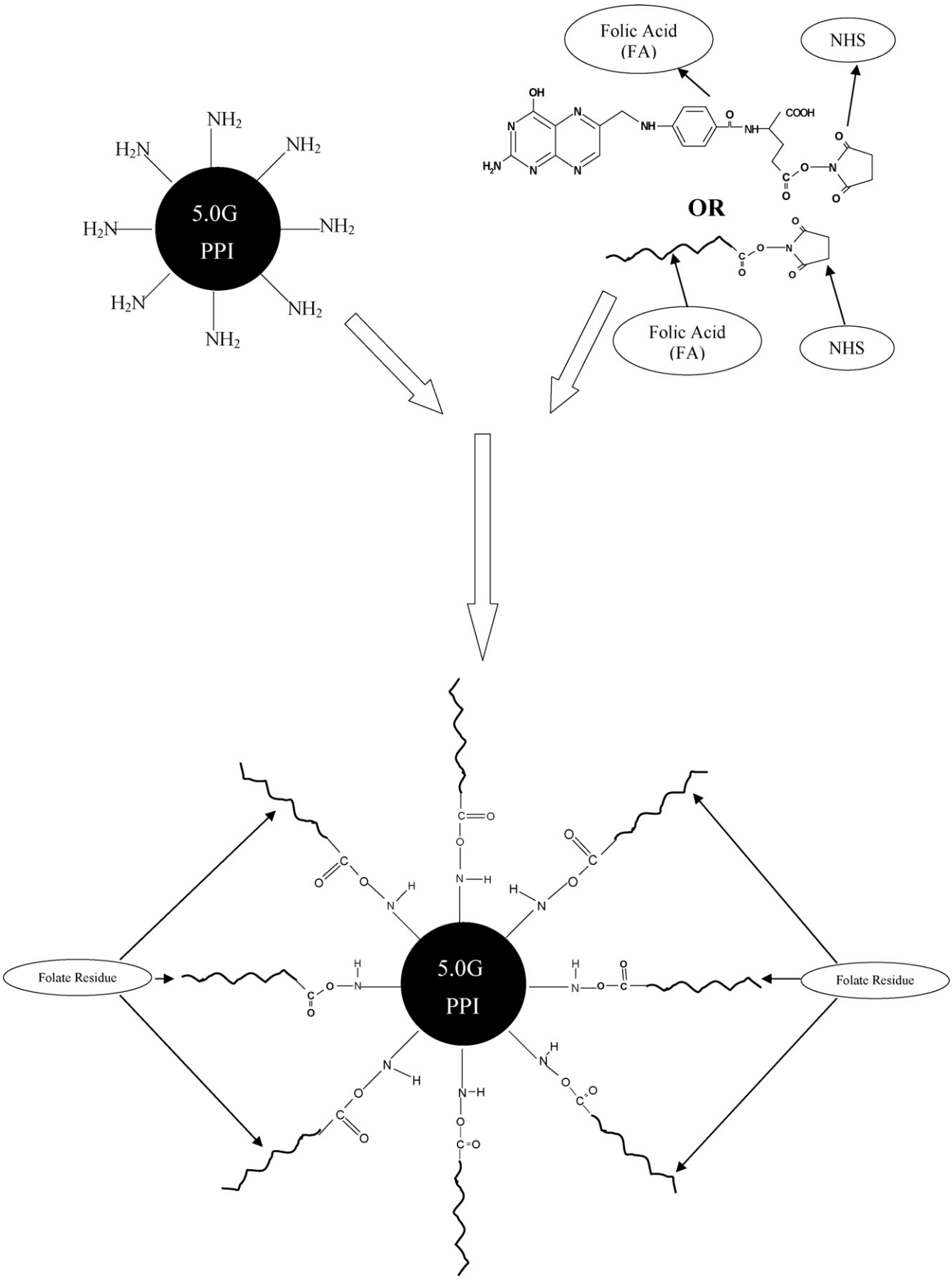
The active ester of folic acid in DMSO (25 mg/ml) was mixed with EDA core PPI dendrimers in DMSO (10 mg/ml) and stirred for 5 days at room temperature (20–30°C) in a metabolic shaker (Superfit, India). Acetone was added to the reaction mixture, and a yellow precipitate thus formed was collected by filtration and dried under vacuum. The crude product was dissolved in deionized water, and the pH was adjusted to 8–10 using 1 N NaOH solution. The solution was purified using Sephadex G-25 column and deionized water as effluent. Fractions containing the conjugate were collected, and the pH of the solution was adjusted to 3 using 1 N HCl (Kono et al., 1999). The product was precipitated out, collected by filtration, and dried under vacuum (Scheme 2).

2.4. Characterization of folic acid conjugates

Folic acid conjugated PPI dendrimers (PPI–FA) were characterized using different spectroscopic techniques like IR, NMR (^{13}C and ^{1}H) and ESI mass spectroscopy. IR spectrum of the PPI–FA revealed important peaks at 771.61 cm^{-1} (aromatic C—H bending); 1221.9 cm^{-1} (ester unconjugated C=O stretching) at 1262.5 cm^{-1}; 1653.3 (aromatic C=C bending and stretching due to attachment of folic acid). ^{1}H NMR of PPI–FA displayed newer shift of R–(C=O)–NH–CH_2–CH_3 of folic acid linkage appeared at δ 1.5–2.5 ppm. Synthesis of the folic acid conjugated dendrimers was further confirmed by ^{13}C NMR. ESI mass spectra were recorded on a Micromass Quattro II Triple Quadrupole mass spectrometer. The samples (dissolved in suitable solvents such as methanol/acetonitrile/water) were introduced into ESI source through a syringe pump at 5 μl/min. The ESI capillary was set at 3.5 kV and the cone voltage was 40 V. Spectra were collected in 6 s scans, and the printouts were averaged spectra of 6–8 scans. Spectra recorded at high mass units were computer deconvoluted. Transmission electron microscopic studies were performed similar to the procedure explained for the characterization of dendrimers. All other spectroscopic studies were performed as explained in earlier section.

2.5. Drug loading and in vitro release studies

DOX was loaded in the PPI dendrimers through equilibrium dialysis method as reported earlier with slight modifications (Singhai et al., 1997; Agarwal et al., 2009). Briefly, DOX was dissolved in acetone (10 mg/ml) and to this solution approximately 1.4 ml aqueous triethylamine (TEA) was added in a molar ratio of 2:1 (DOX:TEA). The solution was stirred overnight and was mixed with the aqueous solution of PPI dendrimers (25 mg/ml). The mixture was magnetically stirred (Remi, India) (50 rpm) for 72 h using teflon bead and subsequently dialysed through dialysis membrane (MWCO 3000; Sigma, Germany) for 15 min to remove the unentrapped drug under strict sink conditions (sink condition ensures the complete dissolution of the drug it refers to the excess solubilizing capacity of the dissolution medium), which was then



Scheme 2. Schematic synthesis of PPI–FA.

estimated spectrophotometrically at 490 nm (UV-1601, Shimadzu, Japan) to determine indirectly the amount of drug loaded within the system. Similar procedure was followed subsequently for drug loading to PPI–FA conjugates (aqueous solution of PPI–FA was prepared in a concentration of 25 mg/ml equivalent of PPI dendrimers). Finally, two different formulations were prepared i.e. PPI–DOX (DOX encapsulated PPI dendrimers) and PPI–FA–DOX (DOX loaded folate conjugated PPI dendrimers).

Both PPI–FA as well as PPI–FA–DOX formulations were subjected to *in vitro* release studies using dialysis tube diffusion technique in phosphate buffer saline (pH 7.4, 6.4) as well as in double distilled water (DDW) as recipient medium. Two-milliliter solution of the above-prepared formulation was placed in the dialysis sac (MWCO 6000–7000 Da, Sigma), hermetically tied and immediately suspended in 50 ml of aqueous recipient medium under perfect sink conditions. The volume of recipient compartment was maintained by replenishing with 1 ml of sink solution, after each withdrawal of 1 ml aliquot. The same procedure was followed for both the recipient media.

2.6. Hemolytic toxicity

Hemolytic study was performed with a view to monitor reduction in toxicity of PPI dendrimers due to folic acid conjugation. The study was performed according to previous reported studies (Bhadra et al., 2003; Singhai et al., 1997). Whole human blood was collected using heparin as anticoagulant in HiAnticlot blood collecting vials (Hi Media, India) and centrifuged at 3000 rpm for 15 min in an ultracentrifuge (Z36HK, HERMLE LaborTchnik GmbH, Germany). RBCs collected from the bottom were washed with normal saline (0.9%, w/v) in reverse osmosis water (Ultra Max^{TM} 370 Series, Younglin, Korea) until a clear, colorless supernatant was obtained above the cell mass. Cells were re-suspended in normal saline. This RBC suspension was used further for hemolytic studies. To 1 ml of RBC suspension, 5 ml distilled water was added, which was considered to be 100% hemolytic. Similarly, 5 ml of normal saline was added to 1 ml of RBC suspension in another tube assumed to produce no hemolysis, hence acting as control. Half milliliter of formulation was added to the mixture of normal saline (4.5 ml) and RBC suspension (1 ml). Similarly, 0.5 ml of drug solution and 0.5 ml of dendrimer solution were taken in separate tubes and mixed with 4.5 ml of normal saline and 1 ml of RBC. Drug (i.e. DOX) and PPI dendrimer in separate tubes were taken in such amount that the resultant final concentrations of drug and dendrimer were equivalent in all the cases. The tubes were allowed to stand for half an hour with gentle intermittent shaking and were centrifuged for 15 min at 3000 rpm in an ultracentrifuge (Z36HK, HERMLE LaborTchnik GmbH, Germany). The supernatants were taken and diluted with an equal volume of normal saline, and absorbance was taken at λ_{max} 540 nm, against supernatant of normal saline diluted similarly, as control. The percent hemolysis was calculated for each sample by taking the absorbance of water as 100% hemolytic sample, using the following equation:

$$\%\,\text{Hemolysis} = \frac{AB_S}{AB_{100}} \times 100$$

where AB_S is the absorbance for the sample and AB_{100} is the absorbance for control.

Similar procedure was followed to determine the hemolytic toxicity for the PPI–FA, PPI–DOX and PPI–FA–DOX.

2.7. Hematological studies

Hematological parameters were analyzed in male albino rats (SD strain) of uniform weight and size. Fifteen animals were selected and divided into five groups consisting of three rats in each group. Plain drug (DOX), PPI–FA, PPI–DOX and PPI–FA–DOX formulations were administered, containing 250 mg/ml equivalent DOX (wherever required), intravenously into first, second, third and fourth groups of animals, respectively, daily up to 7 days. The fourth group was kept as control, which was maintained on same regular diet for 7 days. After 15 days blood samples were collected from the animals and analyzed for RBC count, WBC count and differential count of monocytes, lymphocytes and neutrophils at a pathology laboratory (Agarwal et al., 2009). This study was performed only on the DOX and both the formulations.

2.8. Stability studies

These studies were performed to assess the stability aspects of the developed formulations in terms of color change, drug leakage etc. It was anticipated that the conjugated formulation (PPI–FA–DOX) would release the drug slowly compared to plain dendrimer formulation (PPI–DOX) (Agarwal et al., 2009; Singh et al., 2008). The samples (5 ml) of PPI–FA–DOX formulations were kept in dark in amber colored vials and in colorless vials at 0 °C, ambient room temperature (RT) (20–30 °C) and 60 ± 2 °C in controlled ovens for a period of 5 weeks and analyzed every week for any visual changes like precipitation, turbidity, crystallization, color, consistency and drug leakage. Drug leakage was determined by monitoring the release of drug from the formulation after storage at accelerated condition (60 ± 2 °C). Formulation samples (2 ml) were dialysed across cellulose tubing. The external medium (50 ml) was analyzed for the drug content, spectrophotometrically. The procedure was repeated weekly for up to 5 weeks.

2.9. Cancer cell inhibition assay

MCF-7, an estrogen receptor positive human breast cancer cell line derived from pleural effusion, the most commonly used

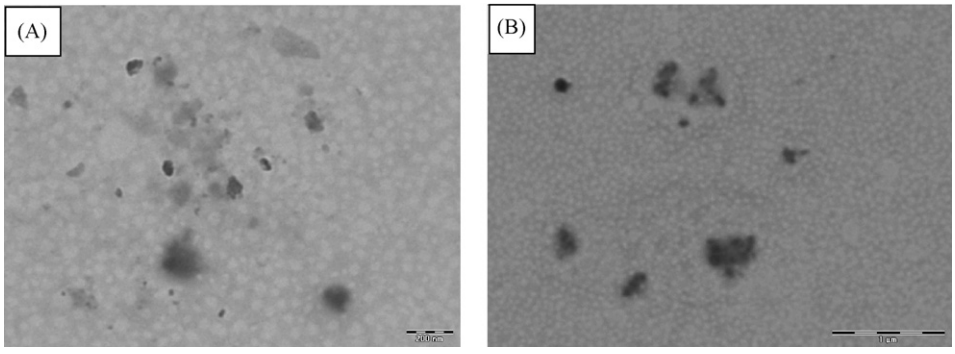


Fig. 1. Transmission electron micrograph of (A) PPI dendrimers, where bar shows 200 nm, and (B) folic acid conjugated PPI dendrimers, where bar shows 1 μm.

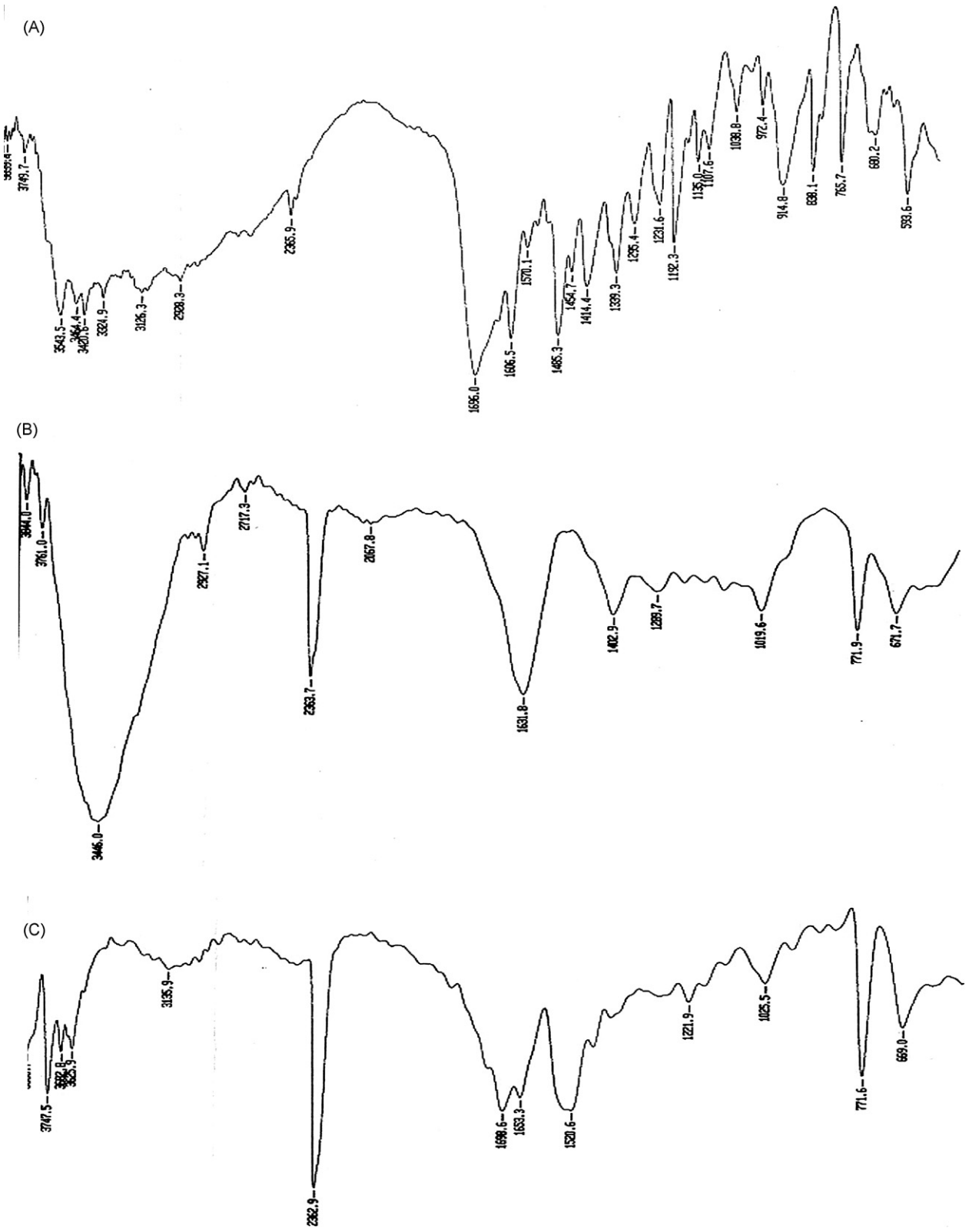


Fig. 2. Infra red spectra of (A) FA, (B) FA–NHS and (C) PPI–FA.

cell line for screening of anticancer breast agents (Soule et al., 1973), was used for evaluation of comparative anticancer activity of the prepared formulations. The cytotoxic activity of the compounds was determined using MTT assay (Sashidharan et al., 2007). 1×10^4 cells/well were seeded in 96-well micro culture plates in 200 μL DMEM, supplemented with 10% FBS and incubated for 24 h at 37 °C in CO_2 incubator. Formulations (PPI, PPI–FA, PPI–FA–DOX, PPI–DOX) and DOX alone were diluted to the various concentrations in culture medium and added to the wells along with vehicle treated wells as control group. After 18 h of incubation,

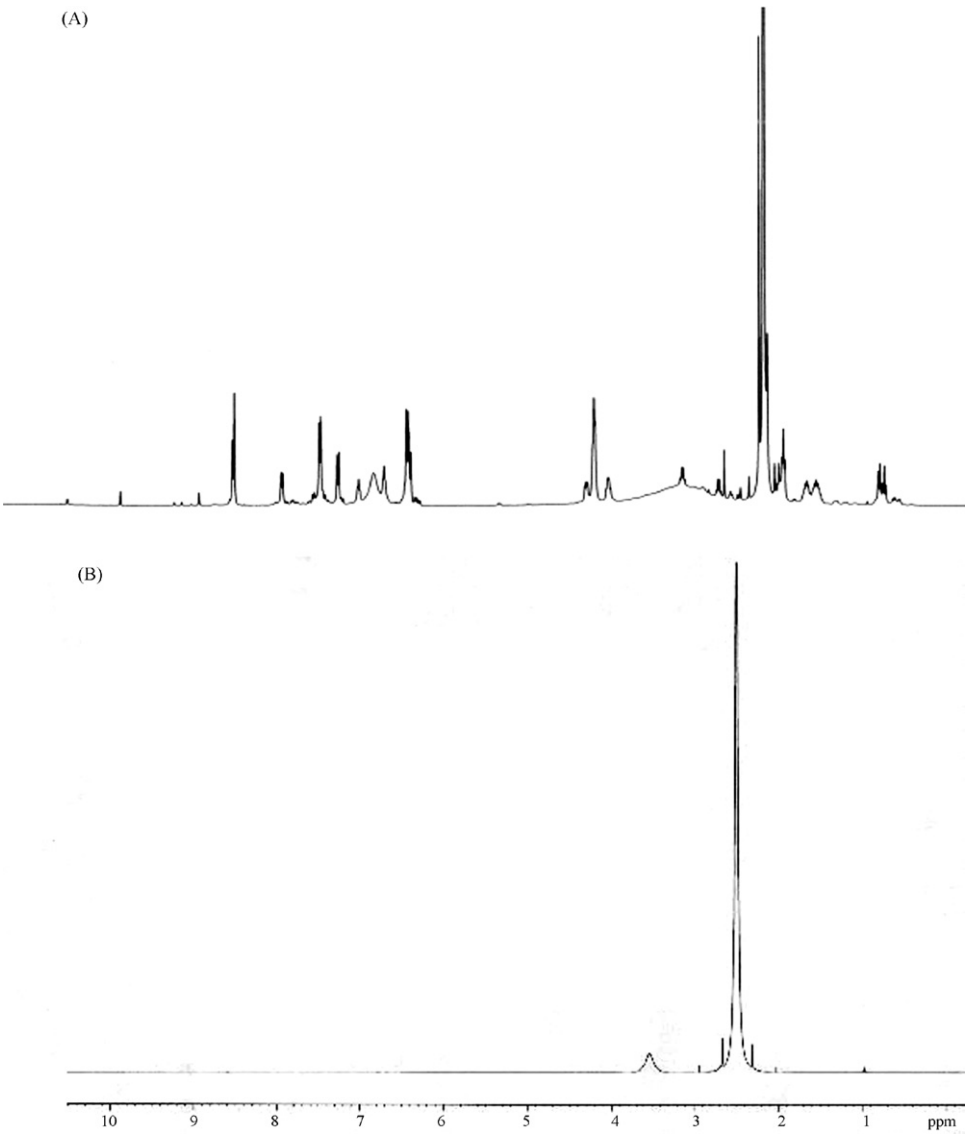


Fig. 3. ^{1}H NMR spectra of (A) FA–NHS and (B) PPI–FA.

10 μl MTT (5 mg/ml) was added to each well and the plates were further incubated for 4 h. Then the supernatant from each well was carefully removed, formazon crystals were dissolved in 100 μl of DMSO and absorbance at 540 nm wavelengths were recorded (Molecular Devices, USA). The percentage cell growth inhibitions for each treatment were calculated by comparing with vehicle treated group.

2.10. Cell uptake assay

The cellular uptake of DOX conjugates was determined with the following procedure. Briefly, each formulation was labeled (PPI, PPI–DOX, PPI–FA–DOX, DOX) in 5:1 ratio with FITC solution (10 mg/ml in DMSO). The suspension was incubated at RT for 8 h with intermittent mixing every 20 min. 2.5×10^5 cells/well were seeded in six-well plate and incubated for 24 h at 37 °C with 5% CO_2, and then the medium in each well was replaced with 2 ml of phenol red-free, serum-free, and antibiotic-free medium containing various concentrations of labeled formulations. The cells were incubated for 4 h and then the medium was removed and cells were washed three times with PBS. Then the fluorescence due to uptake of fluorescent labeled formulation of suspension was qualitatively analyzed under inverted fluorescent microscope (Leica, Germany).

2.11. Statistical analysis

All the statistical analyses were performed with Graph Pad Instat Software (Version 3.0, Graph Pad Software, CA, USA) using either unpaired *t* test or one-way ANOVA followed by Tukey–Kramer multiple comparison test. Difference $p < 0.05$ was considered as extremely significant difference.

3. Results and discussion

3.1. Synthesis and characterization of the EDA core PPI dendrimers up to 5.0 G

Dendrimer synthesis was performed as reported earlier by De Brabander and Meijer with some modifications (Brabander-van den Berg and Meijer, 1993). EDA was used as dendrimer core. Synthesis was monitored at every step (or generation) up to fifth generation through the spectroscopic methods. IR and ^{1}H NMR spectroscopy confirmed the synthesis of PPI dendrimers. The important peaks as

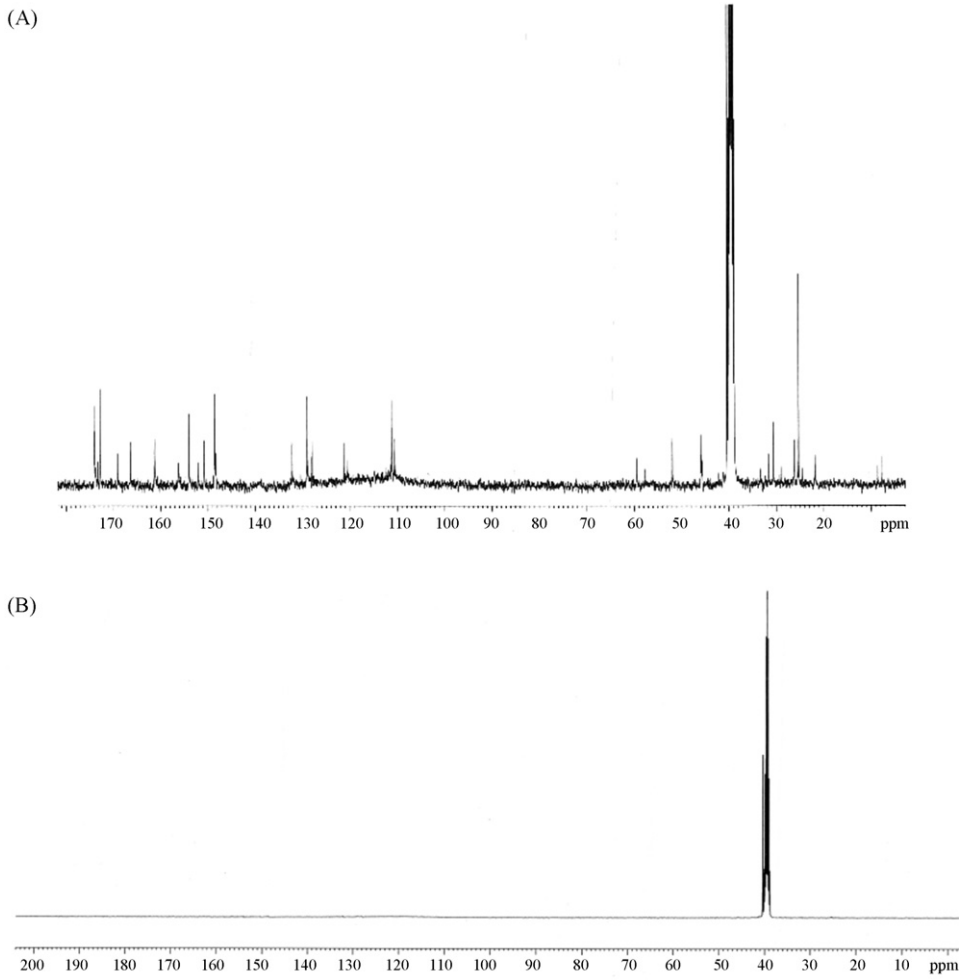


Fig. 4. ^{13}C NMR spectra of (A) FA–NHS and (B) PPI–FA.

mentioned in Section 2 of the manuscript indicate that half generation dendrimers displayed strong peak of –CN (1100–1200 cm^{-1}) terminals, while the full generation dendrimers displayed strong peak of $-NH_2$ (3300–3400 cm^{-1}). ^1H NMR of the different generations also supported the synthesis of PPI dendrimers as evident from the mentioned chemical shift. The detailed synthesis was reported in our earlier study (Gupta et al., 2007). Results of all the spectroscopic data were similar and comparable to the synthesis of PPI dendrimers reported by Brabander-van den Berg and Meijer (1993). Electron microscopy was carried out to further authenticate the size of dendrimes (nanometric range or otherwise?). It was found that the size of the dendrimers was in the nanometric range and TEM photograph displayed aggregates (Fig. 1A) of the dendrimers, which was similar to the earlier reports (Gupta et al., 2007; Bhadra et al., 2005). All these parameters confirmed the synthesis of PPI dendrimers. The dendrimers synthesized were in the

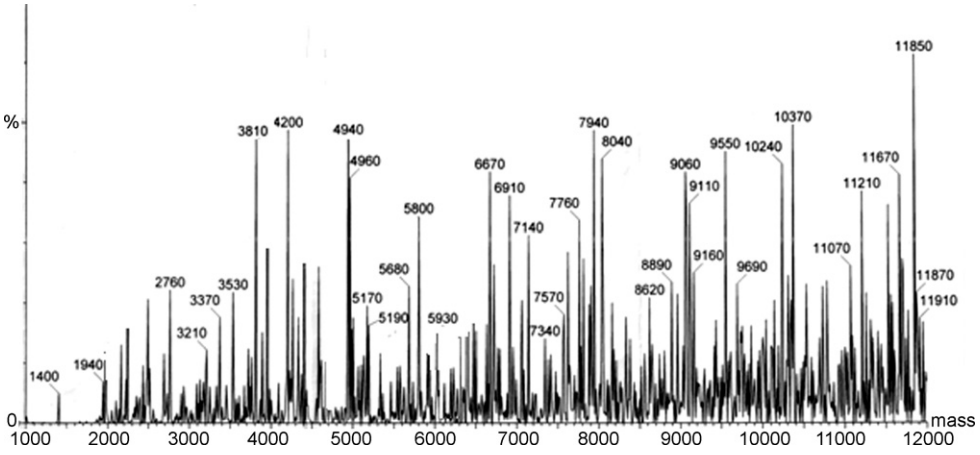


Fig. 5. ESI mass spectra of PPI–FA.

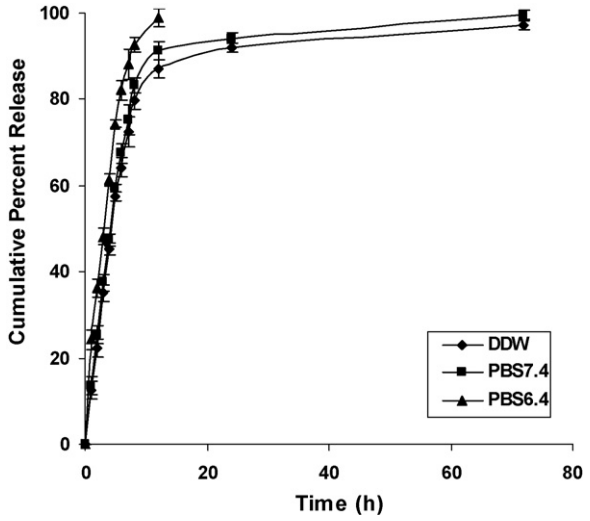


Fig. 6. *In vitro* drug release from PPI–DOX formulation in double distilled water (DDW), PBS 6.4 and PBS 7.4 as recipient medium. Values represent mean ± S.D. ($n = 6$).

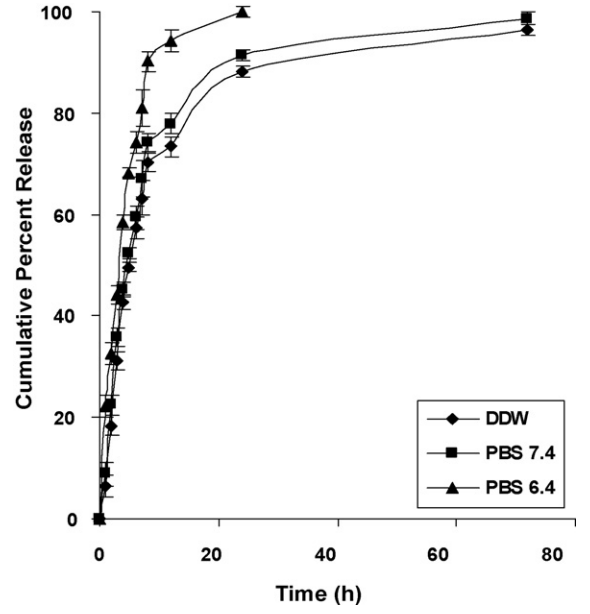


Fig. 7. *In vitro* drug release from PPI–FA–DOX formulation in double distilled water (DDW), PBS 6.4 and PBS 7.4 as recipient medium. Values represent mean ± S.D. ($n = 6$).

physical state of honey like consistency with dark brownish color having high viscosity.

3.2. Folic acid conjugation

Similar to the characterization of PPI dendrimers in our earlier study, folic acid conjugated dendrimer was characterized through various spectroscopic studies (Singh et al., 2008). IR (Fig. 2), ^{1}H NMR (Fig. 3) and ^{13}C NMR confirmed (Fig. 4) the synthesis of PPI–folic acid conjugate synthesis. ESI mass spectra of the conjugated PPI dendrimers revealed the mass of 11,850 (Fig. 5). The mass of 5.0 G PPI dendrimers as reported earlier was approximately 7140. Increase in mass further confirmed the conjugation of folic acid molecules (about 5–6 folic acid molecules per dendrimer molecule). Electron microscopy of the PPI–FA conjugate clearly demonstrated the increase in size which supports (Agarwal et al., 2009) the conjugation as indirect evidence (Fig. 1B).

3.3. Drug loading, entrapment efficiency and formulation development

The drug carrying capacity of the formulation was determined through calculation of the quantity of drug loaded therein and hence determining the moles of the drug entrapped per mole of the dendrimer. Drug encapsulation was accomplished through equilibrium dialysis method. UV–visible spectrophotometry (UV-1601, Shimadzu, Japan) was used to confirm indirectly the drug loading in PPI dendrimers as well as PPI–FA conjugates, which was evident from a slight shift in the λ_{max} of DOX (495 nm) (Jansen et al., 1994). Percent drug encapsulation was measured indirectly by estimating the un-entrapped drug spectrophotometrically during equilibrium dialysis method. Percent DOX encapsulation in both i.e. PPI–DOX and PPI–FA–DOX was found to be 26 ± 0.7 and 64.78 ± 1.3% (data not reported), respectively. The higher drug loading in case of PPI–FA–DOX conjugate was possibly due to increased dendritic architecture of folic acid conjugated dendrimers (Agrawal et al., 2007; Bhadra et al., 2003). Both these formulations i.e. PPI–DOX and PPI–FA–DOX were selected for the subsequent studies.

3.4. In vitro release kinetics

In vitro drug release studies were carried out in three different media (i. e. DDW, PBS 7.4 and PBS 6.4) for both the formulations (i.e. PPI–DOX, PPI–FA–DOX). Drug release from both the formulations was relatively rapid initially followed by sustained and slow release pattern in the later half of the experiment. A possible mechanism of delayed release of the drug molecule may be the encapsulation of the drug in the hydrophobic cavities of the dendrimer that act as a sink to retain the drug molecules for extended duration than the surface of dendrimer molecules. In case of PPI–DOX approximately 57.46± 1.4%, 59.46± 0.9% and 74.36 ± 1.04% drug was released, respectively, in DDW, PBS 7.4 and PBS 6.4 within initial five-hour 5 h (Fig. 6). While in the case of PPI–FA–DOX, the drug release was 49.55 ± 0.88, 52.36 ± 1.44 and 68.24 ± 1.21, respectively, in DDW, PBS 7.4 and PBS 6.4 (Fig. 7). It can be observed that the drug release was slower and sustained in case of PPI–FA–DOX compared to PPI–DOX. This may be possibly due to the steric hindrance of the conjugated folic acid on the PPI dendrimer surface limiting the release of drug from dendritic framework. *In vitro* release studies at pH 6.4 were carried out in the view that the tumor vicinity is relatively acidic in nature. The rapid release behavior of the drug from dendrimeric formulations at pH 6.4 supports the hypothesis that the dendrimeric formulation will release the drug in higher concentration, whenever it will reach the target site i.e. tumor, as the pH in such environment is generally below 7. The results of the *in vitro* release as well as drug encapsulation studies indicate a higher drug loading at the same time delayed release pattern due to folate conjugation (Singh et al., 2008), our target of higher drug loading and longer time release pattern is thus accomplished.

3.5. Hemolytic (ex vivo) and hematological (in vivo) studies

Hemolytic toxicity associated with the cationic charge of the dendrimers poses a hurdle in its usage as drug delivery carrier. Polycationic charge on the dendrimer surfaces due to primary amine groups limits their possible applications *in vivo* as a drug carrier (Malik et al., 2000). The hemolytic toxicity in the present study was measured in terms of percent RBC hemolysis. It was found that free drug DOX exhibited 16.37 ± 0.88%, while PPI and PPI–DOX displayed 14.38 ± 0.25%, and 18.35 ± 0.63% RBC hemolysis, respectively. Surprisingly the folic acid conjugation on the PPI dendrimer surfaces drastically reduced the RBC hemolysis. PPI–FA

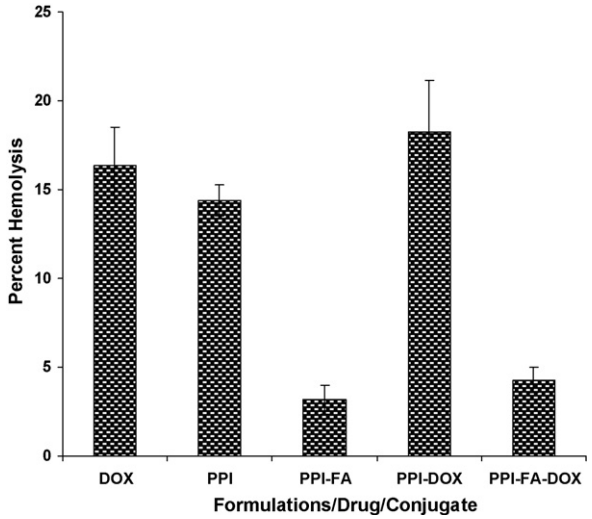


Fig. 8. Percent hemolytic toxicity of drug, PPI dendrimers and different formulations ($n = 4$). Values represent mean ± S.D.

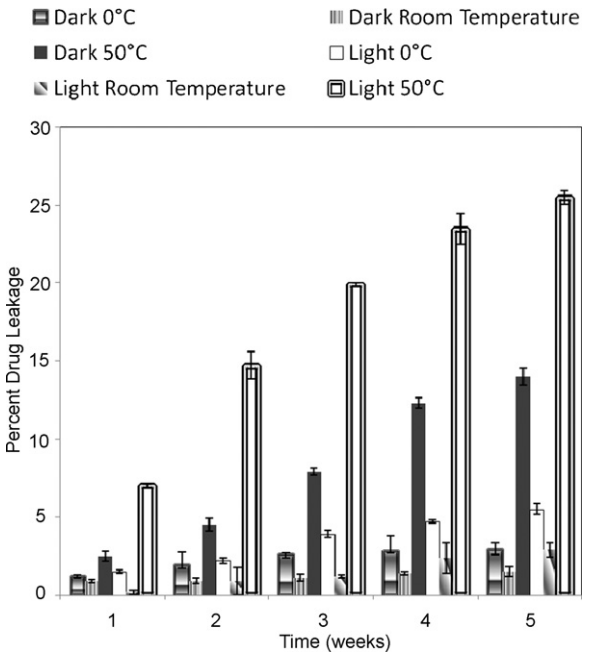


Fig. 9. Percent drug leakage from PPI–FA–DOX in conditions of temperature and light up to 5 weeks.

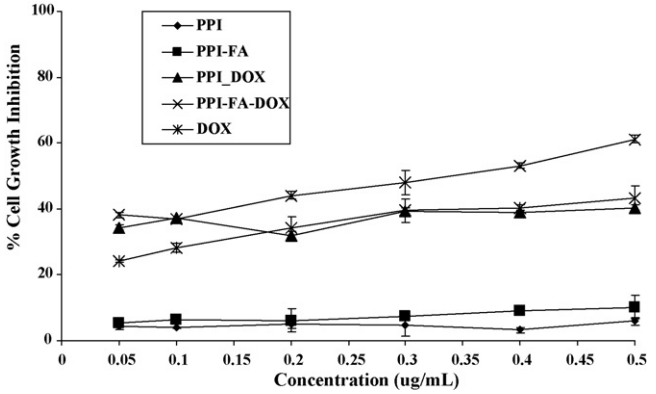


Fig. 10. Cell growth inhibition assay in MCF-7 cells treated with various formulations.

and PPI–FA–DOX displayed only 3.19 ± 0.08 and 4.28 ± 0.03% of RBC hemolysis, respectively, which is about 4 and 3.5 times lesser than PPI–DOX and PPI dendrimers (Fig. 8). The results clearly indicate that the folic acid conjugates at the surface of the dendrimers interferes with the RBC's biocompatibly, which might be responsible for the reduced RBC hemolysis. The results are also similar to the previous reports of surface conjugated dendrimers (Agarwal et al., 2009; Agrawal et al., 2007). Hemolytic toxicity of PPI–FA–DOX was approximately 3, 4 and 5 folds less then PPI, pure DOX and PPI–DOX, respectively. The reduction in the toxicity was possibly due to shielding/locking of DOX and primary amine groups in the dendritic architecture due to surface conjugation with folic acid, making its surface more biocompatible. The dramatic reduction in the hemolytic toxicity gives a possibility that these may be used in future as a tool for drug delivery.

Hematological parameters were determined to study the effect of formulations on the different components of blood. The study was conducted to analyze the toxic manifestations of the prepared formulations on the other blood components like WBC, monocytes etc. Blood samples were analyzed for RBC, WBC and differential lymphocyte counts at a pathology laboratory (Table 1). It is clear from the table that RBC count was almost similar for control group and PPI–FA–DOX receiving animals. However, the RBC count decreased below normal (i.e. of control group) in case of DOX and PPI–DOX. Similarly, the WBC count of PPI–DOX increased significantly ($p < 0.05$) compared to normal values. In the case of PPI–FA–DOX, the WBC count increased but to a lesser extent than for PPI–DOX. Similarly, a relative increase in lymphocyte count was observed with PPI–DOX than PPI–FA–DOX. The results clearly indicate the superior biocompatible behavior of the folate conjugated than the plain dendrimers. The results were similar and in accordance with the earlier hematological studies carried out with nanoparticles and dendrimers (Agrawal et al., 2007). The study was performed on PPI–DOX and PPI–FA–DOX formulations exclusively with the objective of exploring their anti-tumor activity *in vitro* as well as *in vivo*.

Table 1
Haematological parameters of dendrimer formulations/drug.

Formulations	RBC count ($\times 10^6/\mu L$)	WBC count ($\times 10^3/\mu L$)	Differential count ($\times 10^3\ \mu L$)		
			Monocytes	Lymphocytes	Neutrophils
Control	8.9 ± 0.4	10.2 ± 0.4	1.3 ± 0.6	7.9 ± 0.4	1.6 ± 0.3
DOX	7.6 ± 0.3[a]	11.6 ± 0.4[a1]	1.1 ± 0.3[a2]	9.7 ± 0.4[a3]	1.9 ± 0.3[a4]
PPI–DOX	7.1 ± 0.4[b]	17.4 ± 0.3[b1]	1.8 ± 0.5[b2]	14.3 ± 0.3[b3]	3.1 ± 0.3[b4]
PPI–FA–DOX	8.7 ± 0.3[c]	11.6 ± 0.3[c1]	1.2 ± 0.3[c2]	8.6 ± 0.8[c3]	3.1 ± 0.5[c4]

$n = 6$ albino rats per group, tabulated values represent mean ± S.D.
[a,a1,a2,a3,a4] $P < 0.05$ (comparison of DOX with PPI–DOX).
[b,b1,b2,b3,b4] $P < 0.05$ (comparison of DOX with PPI–FA–DOX).
[c,c1,c2,c3,c4] $P < 0.05$ (comparison of PPI–DOX with PPI–FA–DOX).

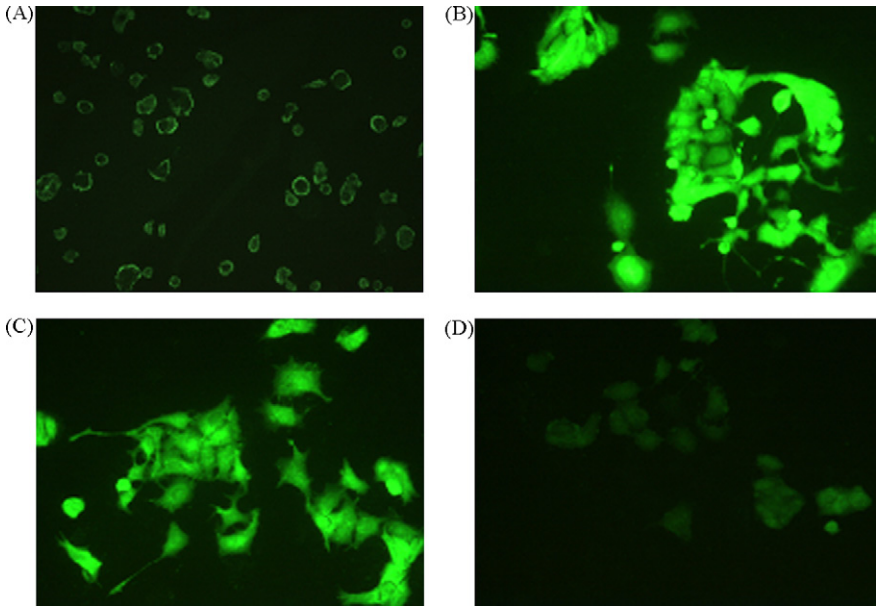


Fig. 11. MCF-7 cells (40×) treated with FITC-labeled formulations for 4 h showing representative cellular uptake. (A) PPI–DOX, (B) PPI–FA–DOX, (C) PPI–FA, (D) DOX alone.

3.6. Stability studies

Stability studies are prerequisite in the development and characterization of a successful pharmaceutical formulation. In the present study, stability was assessed in terms of turbidity, color change, crystallization and other visual changes (Fig. 9). Another important parameter, which was measured to assess the stability, was drug leakage. The drug leakage was measured for the formulation PPI–FA–DOX, at various conditions of temperature [0 °C, room temperature (20–30 °C) and 60 °C] after storage in dark (amber color glass vials) as well as light (colorless vials) for a period of 5 weeks. PPI–FA–DOX was most stable in dark, at RT (Fig. 9). In general, drug leakage was higher in light than in dark, which may be attributed to structure cleavage due to temperature and light. Percent drug leakage was found to be least at RT compared to at 0 and 60 ± 2 °C. At 0 °C shrinking of dendrimeric architecture may be the possible reason leading to decreased cavity enclosing drug molecules and hence higher leakage. However, the greater leakage at 60 ± 2 °C may be due to increased solution kinetics. It can be inferred from the above results that folate conjugated dendrimer based formulation is more stable in dark at RT than at 0 and 60 ± 2 °C (Bhadra et al., 2003, 2005).

3.7. Cancer cell inhibition assay

MCF-7 cells were treated with six equivalent doses ranging from 0.05 to 0.5 μg/ml. The result of the cell inhibition assay exhibited significant differences. PPI–FA–DOX exhibited highest percent cell growth inhibition compared to other formulations as well as drug itself (Fig. 10). However, all three formulations showed dose-dependent inhibition of MCF-7 cells. IC50 value of PPI–FA–DOX was found to be 0.45 μg/ml while it was 0.7, 0.75 μg/ml, respectively, for DOX and PPI–DOX. The IC_{50} values of PPI–FA and PPI alone were undetectable up to the concentration of 0.9 μg/ml. The higher uptake in the case of the PPI–FA–DOX was possibly due to the ligand specific targeting of dendrimers due to surface conjugation of the folic acid. Human mammary carcinoma cells (MCF-7) were selected for the analysis as it has been investigated several times in a number of studies. MCF-7 over expresses folate receptors and this was the reason we selected MCF-7 cell line for our studies (Li et al., 2009).

3.8. Cell uptake assay/fluorescence technique

Similar to the results of the percent cell growth inhibition assay fluorescence study also showed higher uptake of PPI–FA–DOX (Fig. 11). The higher uptake was possibly due to the folate residues on the surface of the dendrimer compared to PPI–DOX ($p < 0.005$). Among the free DOX and PPI–DOX the higher uptake was observed with the PPI–DOX, which might be due to higher drug loading resulting in a higher release. The results again support the strategy that the ligand mediated anticancer delivery can result in higher uptake of drug to the MCF-7 cells. The study to develop any possible *in vitro–in vivo* correlation is in progress.

4. Conclusion

The major hurdle with the dendrimer applicability in the drug delivery is their inherent toxicity due to cationic surface. Surface engineering of dendrimers can lead to improved properties, especially in the context of biomedical applications. The shielding of the surface charge, however, drastically reduces hemolytic behavior. The present study was aimed to develop and characterize a ligand conjugated dendrimer based carrier for the delivery of DOX. The prepared conjugate was further assessed to target DOX to MCF-7 cancer cell lines. The toxicity profile in terms of hematological as well as hemolytic was ensured before its *in vivo* fate. Results clearly indicate that the folic acid conjugation has drastically reduced the toxicity. The *in vivo* result indicates that folic acid conjugation has provided higher uptake and better targeting.

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