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Evaluation of Surface Acetylated and Internally Quaternized Poly (Propylene Imine) Dendrimer as Biocompatible Drug Carrier for Piroxicam as a Model Drug

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Abstract

Two types of new surface acetylated and internally quaternized poly(propylene imine) (PPI) dendrimers with generation 2 and 3 QPPI-NHAc(G2)/(G3) were prepared and characterized with FT-IR, NMR and MALDI-TOF spectral techniques and then demonstrated as potential and biocompatible drug carriers using Piroxicam (PXM, an anti-inflammatory drug) as a model drug. The drug carrying potential of QPPI-NHAc (G2)/(G3) along with their commercial parent dendrimers PPI(G2)/(G3) and linear branched polyethyleneimine (PEI) were investigated in terms of aqueous solubility, in vitro release of PXM and cytotoxicity studies. Pharmacokinetic and biodistribution studies were also carried out in male albino wistar rats and the efficiency of dendrimer formulation was tested by carrageenan induced paw edema model. The observed results showed that aqueous solubility of PXM has significantly increased in the presence of higher generation modified dendrimer namely QPPI-NHAc(G3) to the tune of 50 fold compared with its intrinsic solubility and it is noted that the complexation of PXM with QPPI-NHAc(G3) is responsible for increased solubility. The degree of complexation was evidenced through UV-Vis and NMR (1H & 2D) spectral techniques. The in vitro release of PXM in the presence of modified dendrimers was remarkably slower as compared to PPI (G3)/(G2) as well as PEI and thus proves their ability for sustained/delayed release of PXM. The Cytotoxicity study on Vero cell line was performed through MTT assay and the results reveal that the QPPI-NHAc (G2)/(G3) showed appreciable increase in cell viability and thus indicate the reduced cytotoxicity and improved biocompatibility. Pharmacodynamic results reveal that in the case of QPPI-NHAc (G3)-PXM, 75% of inhibition was observed at 4th h and that was maintained above 50% until 8th h compared to plain PXM and proves that when the drug associated with dendrimer carrier, it retained for longer duration in the bio system. The 2nd, 4th and 8th h organ distribution data showed that higher recovery of PXM was observed at rat paw
with dendrimer-drug formulation QPPI-NHAc (G3)-PXM than plain PXM and it may be concluded that dendrimer-drug formulations not only enhanced the solubility but also controlled the delivery of bioactive with localized action at the site of inflammation.

**Keywords:**
Poly (propylene imine) dendrimer (PPI), Piroxicam (PXM), solubility, sustained release, Cytotoxicity, pharmacokinetics and biodistribution.

1. **Introduction**

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely prescribed medications in the world. As a therapeutic class, NSAIDs exhibit analgesic, anti-inflammatory, antipyretic and platelet inhibitory properties [1, 2]. However, these drugs have serious side effects such as gastrointestinal (GI) toxicities, gastric mucosal ulcerations and hemorrhage due to inhibition of prostaglandin production [3, 4]. The mechanism of action of NSAIDs has been attributed to their ability to inhibit the cyclooxygenase enzyme (COX). Out of the 2 isoforms of COX, COX-1 is responsible for mediating the production of prostaglandin while COX-2 is primarily associated with inflammation, pain and fever [5, 6]. The traditional NSAIDs are nonselective COX inhibitors. COX-2 selective NSAIDs are ideal anti-inflammatory drugs with minimum drug-related side effects, since they spare COX-1 activity. Piroxicam (PXM) is one such COX-2 selective acidic non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic properties. PXM is quite efficient in the short or long term treatment of rheumatoid arthritis, osteoarthritis and other painful inflammatory disorders [7-9]. However, there are number of aspects that limit its pharmaceutical application, the chief being its aqueous insolubility that immensely creates formulation tribulations [10]. Apart from this, oral administration shows low absorption and gastrointestinal tract associated adverse effects, which displays poor bioavailability [11]. This restricts their use in topical and parenteral applications.
Further, poorly water-soluble drugs often require high doses in order to reach therapeutic plasma concentrations after oral administration and hence its solubility has to be increased by suitable means.

Dendrimers are new class of artificial macromolecules with tree-like topological structure, nano-scale size, excellent monodispersity and dense peripheral groups [12]. Dendrimers have generated much interest in diverse areas more specifically for drug delivery due to their unique structure and chemical versatility [13, 14]. Surface functionalities of dendrimers can be functionalized with drugs, targeting moieties and biologically active components [15]. Drugs or genes can be either encapsulated within the dendrimers through non-covalent strategies such as hydrophobic, ionic and hydrogen bond interactions or conjugated to the peripheral groups of dendrimers via covalent methods [16]. Among the various dendrimers used for drug delivery, poly (amidoamine) (PAMAM) and poly (propylene imine) (PPI) dendrimers are the two commercially available and most extensively investigated for drug delivery applications [17]. A plethora of reports are available which evidence the drug carrying potential of dendrimers through properties like increased solubility of the hydrophobic drugs, sustained drug release behavior and increased efficiency of the drugs [18-20]. Dendrimer based drug-delivery systems provide an attractive platform for loading and release of conventional drug molecules which improve the pharmacodynamic and pharmacokinetic behaviors of several families of drugs. These reveal promising future of dendrimer based drug delivery systems. Dendrimers such as PAMAM and PPI possess cationic primary amine groups at the surface showed excellent drug delivery efficacy, but the cytotoxicity issue of these dendrimer is a serious setback which limits the clinical applications of most dendrimer based drug formulations [21]. Generally, the cytotoxicity of dendrimer is dependent on dendrimer generation, surface functionality/charge and concentration [22]. To overcome this, there are several studies
undertaken by various researchers to neutralize the cationic surface group through acetylation [23], PEGylation [24], glycosylation [25] and hydroxylation reactions [26]. Nair et al. described the Dendron based octa-guanidine appended molecular transport using poly(propylene imine) for efficient delivery of doxorubicin into cancer cells [27]. Saha et al. designed the peptide-docetaxel for targeted delivery with reduced toxicity and enhanced efficiency of docetaxel [28]. Surface acetylated dendrimers reduced the cytotoxicity significantly but at the same time such dendrimers failed to load the hydrophobic drugs due to the absence of cationic binding sites [29].

In a subsequent study, the neutral dendrimer namely, PAMAM-OH was employed to form Polyplex with DNA, with the analogy that the surface neutral PAMAM-OH dendrimer may be advantageous in terms of cytotoxicity. However, PAMAM-OH dendrimer is unable to form DNA Polyplex because of low pK_a value of its interior tertiary amines [30]. With a view to alleviate this, internal quaternary ammonium salt was introduced to the tertiary amines of PAMAM-OH dendrimers by methylation and thereby provides cationic binding sites for negatively charged plasmid DNA [31]. In continuation of this study, synthesis of surface modified and internally quaternized PAMAM dendrimer was carried out and employed as efficient siRNA delivery systems [32]. However, to the best of our knowledge there are no studies reported so far for synthesis of new dendrimer drug carrier having increased solubility, biocompatibility with reduced cytotoxicity and improved pharmacokinetic properties which obtained from surface acetylation and quaternization of commercial PPI (G2) and PPI (G3).

2. Materials and Methods

2.1 Materials

PPI dendrimer Diamino cored (DAB) with terminal amino groups of generation 2 and generation 3 was purchased from Symo Chem (Netherlands) and branched Poly ethylene imine (PEI) from Alfa aesar were used as received. Acetyl chloride, triethylamine, chloroform, methyl
iodide and dimethyl formamide were of analytical grade and were procured from SRL. MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) was purchased from Sigma-Aldrich, India. Amberlite IRA-402 Cl anion resin was from Sd fine Chem. Dialysis membranes (Float-A-Lyzer) were obtained from Spectra Pore. Membrane filter of pore size 0.45µm was obtained from Himedia lab. Double distilled water was used for solubility and *in vitro* release studies. Piroxicam was obtained as gift sample from Mesha Pharma Ltd, Mumbai, India.

UV-Vis spectra were recorded on TECHCOMP 8500 instrument. Fourier Transform Infrared (FTIR) spectra were recorded on a Bruker Tensor-27 FTIR spectrometer. $^1$H, $^{13}$C and 2D NMR spectra were recorded with Bruker 500 MHz UltraShield Plus instrument. MALDI-TOF spectra were taken in perceptive bio systems voyager-DE instrument in negative ion mode taking dithranol as the matrix. Cytotoxicity study was done in King Institute of Preventive Medicine, Chennai, India.

### 2.2 Synthesis and characterization of QPPI-NHAc (G2) and QPPI-NHAc (G3) dendrimer

Quaternised poly (propylene imine) dendrimers QPPI-NHAc (G2) and QPPI-NHAc (G3) was synthesized from generation 2 and generation 3 of Poly (propylene imine) dendrimer PPI (G2) and PPI (G3) by performing acetylation followed by quaternization reaction by adopting the reported procedure [32], with slightly modified and simplified. The typical synthetic route followed is shown in Fig. 1a. The commercial PPI (G2) (0.3 g, 0.388 mmol) and PPI (G3) dendrimer (0.3 g, 0.180 mmol) was taken in a 50 mL RB flask and dissolved in 10 mL chloroform. The temperature of this flask was reduced to 0 °C by placing it in freezing mixture. To this an excess amount of acetyl chloride (5 equivalents per surface primary amine) and triethylamine (1.2 equivalent of acetyl chloride) were added. The temperature of the container was brought back to room temperature. The reaction mixture was stirred for 36 h under N$_2$ atmosphere. The excess solvent from the container was evaporated under reduced pressure and
the resulting residue was dissolved in 5 mL water. The mixture was dialyzed extensively (MW Cutoff 1000Da) against double distilled water for 24 h with frequent replacement (once in 2 h) of outer aqueous medium to remove excess reactants and side products from the residual solution. The dialyzed solution was lyophilized to yield the corresponding reddish brown semi solid products i.e. PPI (G2)/ (G3) dendrimers attached with –COCH$_3$ as the surface group, viz., PPI-COCH$_3$ (G2)/ (G3). The acetylated product was further used for the quaternization reaction.

In this step, PPI-COCH$_3$ (G2) (0.1 g, 0.0917 mmol) and PPI-COCH$_3$ (G3) (0.1 g, 0.0428 mmol) was taken in a 50 mL RB flask and the same was dissolved in 2 mL DMF. To this, an excess amount of methyl iodide (1 mL) dissolved in DMF was added. The reaction mixture was stirred at 50 ºC for 48 h. The resulting reddish brown clear/transparent reaction mixture was precipitated by the addition of diethyl ether and the residue was dried in vacuum. It was dissolved in double distilled water and I$^-$ exchanged for Cl$^-$ ions. The eluted solution was lyophilized and named as QPPI-NHAc (G2) and QPPI-NHAc (G3). This product was characterized by FTIR, $^1$H & $^{13}$C NMR and MALDI-TOF spectral techniques. The observed spectral results strongly confirmed its structure. The degree of functionalization of the product was determined by $^1$H NMR and MALDI-TOF spectral techniques and the newly prepared quaternized dendrimers was assessed for its drug carrying potential using PXM as a model drug.

2.3 Solubility Study of PXM Using QPPI-NHAc (G2) and QPPI-NHAc (G3) Dendrimer Carrier

The newly prepared dendrimer based drug carrier viz., QPPI-NHAc (G2) and QPPI-NHAc (G3) was tested for the solubility study of PXM under identical conditions. Additional control experiment was also carried out using poly ethylene imine (PEI) to compare for highlighting the significance of modified dendrimers under identical conditions. The degree of solubility of PXM was quantitatively estimated by adopting the previously described Phase
The experimental procedure adopted for this solubility study involves 7 different concentrations of drug carrier in the range from 0.05mM to 0.35mM. Then from each concentration, 5 mL was taken in 20 mL vials and to which excess PXM (5 mg) was added. These vials were sonicated for 30 min and subsequently shaken mechanically for 24 h at 35 °C. In parallel, controlled experiment was also performed by taking 5 mg of drug in 5 mL of phosphate buffer solution (without dendrimer). The vials were allowed to stand for 24 h to attain equilibrium. Then these solutions were centrifuged at 5000 rpm for 20 min and filtered using cellulose acetate membrane filter having pore size 0.45 µm. The respective filtrate was diluted appropriately with PBS and analyzed spectrophotometrically using UV-vis spectrophotometer. The quantum of solubility of PXM was estimated by measuring the characteristic peak observed at 354 nm ($\lambda_{\text{max}}$). Though the increase in absorption of PXM is the indication for the extent of drug solubilised, but to get the exact amount of the drug solubilised in the respective dendrimer carrier solution, a calibration curve was constructed within the drug concentration range of 3.5 µM - 35 µM which is described by the equation, $y=(17,545\pm224.34)x$, ($R^2=0.998$). The directional coefficient of the equation is equal to the absorption coefficient of PXM, $\varepsilon_{\text{max}}=17,545\text{M}^{-1}\text{cm}^{-1}$. Using this calibration curve, the amount of drug solubilised in each test solution was determined. The blanks were performed on the same concentrations of dendrimer delivery solutions so as to cancel any absorbance that may be exhibited by the dendrimer molecules. The results of phase solubility studies are shown in Table-1. The solubility experiments were repeated three times. The loading efficiency of drug in dendrimers was calculated by below mentioned formula.

$$\text{Loading efficiency} (\%) = \frac{\text{Actual drug content (AC)}}{\text{Theoretical drug content (TC)}}$$

\text{AC - Actual quantity of drug present in the carrier}
TC - 100% theoretical quantity of drug present in the carrier

2.4 UV-Vis spectroscopy studies

The association of PXM to QPPI-NHAc (G3) dendrimer carrier was confirmed by UV-Vis spectroscopy where PXM gives maximum absorbance at its characteristic wavelength (354 nm). The saturated solutions obtained from the solubility studies were diluted to a proper concentration. Since the dendrimers in the diluted solutions give extremely low absorbance between 250 and 700 nm, the absorbance obtained from PXM-QPPI-NHAc (G3) dendrimer solution would be solely from PXM. The absorbance of PXM at its characteristic wavelengths was related with the amount/solubility of PXM in the dendrimer solutions or buffer solutions.

2.5 NMR spectral studies for interactions between PXM and QPPI-NHAc (G3) dendrimer carrier

2.5.1 $^1$H NMR studies

$^1$H NMR experiments were conducted on a Bruker Advance 500.149 MHz NMR spectrometer at 298.2 K for QPPI (G3) dendrimer/PXM complex (2 mg of QPPI (G3) and 1 mg of PXM dissolved in 1 mL of D$_2$O). The temperature was kept constant within (0.2 K by the use of a Bruker temperature control unit.

2.5.2 2D NMR Studies

NOESY experiments were obtained for a QPPI-NHAc (G3) dendrimer/PXM solution (2 mg of QPPI-NHAc (G3) and 1 mg of PXM dissolved in 1 mL of D$_2$O) acquired at 500.149 MHz, using a 300 ms mixing time and a 8.2 µs $^1$H 90° pulse width. The experiments were done with a 2s relaxation delay and 205 ms acquisition time. Eight transients were averaged for each 400×1024 complex $t_1$ increment. The data were processed with Lorentz-Gauss window function and zero filling in both dimensions to display data on a 2048×2048 2D matrix. All data were processed with NMR Pipe software on a Linux workstation.
2.6 *In vitro* drug release studies

The extent of drug released from the dendrimer carriers, QPPI-NHAc (G2), QPPI-NHAc (G3) and PEI were studied by an equilibrium dialysis method under *in vitro* conditions as described in the references. The drug-dendrimer formulation for *in vitro* study was as follows. That is, 2mg of PXM was dissolved in 5 mL of dendrimer carrier (0.02 mM) to get the formulation having drug concentration 1.3 mM. Using this formulation, *in vitro* release study was performed by taking 5 mL of respective drug-dendrimer solution in dialysis bag (MW cutoff 1kDa) and it was placed immediately in 150 mL beaker, containing 100 mL of phosphate buffer solution. The outer phase was stirred continuously to maintain sink conditions. At scheduled intervals, 3 mL of sample was withdrawn from outer phase and subsequently replenished with equal amount of fresh buffer solution. The withdrawn sample irrespective of type was analyzed spectrophotometrically with UV using the drug characteristic peak at 354nm. Similarly, *in vitro* release of PXM from their parent dendrimers viz., PPI-NH₂ (G2) & PPI-NH₂ (G3) and branched polyethyleneimine (PEI) was also studied by adopting the same experimental condition. The quantum of drug released by the parent dendrimer and PEI was determined as before. The control experiment was also carried out by taking 2 mg of PXM in 5 mL of phosphate buffer solution to get the same drug concentration as in the drug-dendrimer formulations. This was also studied for *in vitro* release by usual UV analysis. Three repeats were conducted for each sample. Irrespective of the dendrimer carriers, PEI and the control, plots were drawn for the % of drug released vs time.

2.7 Cytotoxicity Assay

The cytotoxicity of modified dendrimer drug complex QPPI-NHAc (G2)-PXM, QPPI-NHAc (G3)-PXM and its parent dendrimers PPI-NH₂ (G2)-PXM, PPI-NH₂ (G3)-PXM complexes along with PXM (without dendrimers) were evaluated by the well-established 3-(4,5-
dimethylthiazol-2-yl)2, 5-diphenyltriazolium bromide (MTT) colorimetric assay. Vero cell line (NCCS) was chosen for the MTT colorimetric assay. The Vero cell line used for this study is “normal cells” and it was isolated from kidney epithelial cells extracted from an African green monkey. One of the reasons for choosing the normal cell line is, while performing/discharging its assigned role; i.e. it should not disturb or cause toxicity to normal cells. Further, our objective in this study is to examine the compatibility of synthesized dendrimer carriers for the poorly soluble drugs (or) to prove that the therapeutic value for the formulation of QPPI-NHAc (G2)/(G3)-Piroxicam should be improved and thus have plan to recommend the same to human cell line. In this context, it is observed and known that the genetical similarities between human cell line and Vero cell line are very close and hence we decided and choose Vero cell line as a model for this study. The assay procedure involves the incubation of cell line at 37 °C in 5% CO2 and minimum essential medium (MEM) with Earle’s salts supplemented with 10% fetal calf serum. The cells were used when they reached 70-80% confluence. These cells were seeded in 96 well plates for 48 h before MTT assay to allow adherence of the cells to the plates. Then culture medium from the plates was replaced with fresh medium containing different concentrations of QPPI-NHAc (G3) and PPI-NH2 (G3). The control study was performed by treating the cells only with the medium without the dendrimer samples. Following the standard protocol for MTT, the mediums were removed after 24 h from each of the well and the cells were incubated with MTT in PBS at a concentration of 1mg/mL (100 µl) for 4 h. After this, in each well, the MTT containing medium was replaced with DMSO to dissolve the resulted purple formazan crystals which was generated from MTT by the living cells. Absorbance of the solutions in each well irrespective of carrier was recorded at 570 nm using a micro plate reader (Thermo multiscan EX). The viability of cells was calculated with regard to the untreated cell control, which was considered as 100% viability. The viability of cells treated with the different dendrimer carrier is expressed as a
percentage of the untreated cell control. The obtained cell viability was plotted against the concentration of the carrier.

2.8 In Vivo Anti-Inflammatory Activity.

The animal studies were performed in accordance with the protocol approved by the Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethical Committee Guidelines (Approval No:02.01.2012) for the investigation of experimental pain in conscious animals. In this study, a pharmacodynamic study was performed using nonimmunological carrageenan induced hind paw edema method [34, 35]. In the anti-inflammatory activity 0.1 mL of 1% carrageenan was taken as phlogistic agent. Acute inflammatory activity was determined by measuring the change in volume of inflamed paw produced by injection of carrageenan using a plethysmometer. On the basis of solubilization efficacy as well as better in vitro performance of PPI dendrimers compared to PEI (branched) it is selected for in vivo evaluation. Male Albino Wistar rats weighing around 160-180 g were procured from Tamil Nadu veterinary and Animal Sciences University, Chennai and were housed under standard husbandry conditions and the rats were fasted for 12 h before dose administration. The animals were numbered and marked on the right hind paw on each animal. Each time the paw was dipped in the plethysmometer up to the fixed mark to ensure constant paw volume and the study was carried out at daytime to avoid any variation due to circadian rhythms. Animals were divided into five groups (including one control group), each group comprising six animals. The dendrimer-drug test formulations namely PPI (G2)-PXM, PPI (G3)-PXM, QPPI-NHAc (G2) and QPPI-NHAc (G3) which in turn were solubilized in PBS solution (pH 7.4) and used for the study. The plain drug PXM (control) was solubilized in PBS pH 7.4 using DMSO as cosolvent. The dose of 2 mg/kg (equivalent to PXM) body weight was administered through an intravenous route in albino rats of the respective group, precluding the
control. A dose of 0.1 mL solution of carrageenan (1% w/v in normal saline) was injected in the right hind paw of the animals to be tested, 10 min post administration of the test formulation. The paw volume was measured every hour until the eighth hour, the last two observations were recorded at 12th h and 24th h, and from the result obtained a graph was plotted between percentage inhibitions of edema vs time (h). Percentage inhibition of edema was calculated for respective group by following the equation [36] % inhibition of edema = (V_{control} - V_{treated}/V_{control}) x 100, where \(V_{control}\) and \(V_{treated}\) are the mean edema volume of rats in control and test groups respectively.

2.9 Pharmacokinetics and Biodistribution Studies

Pharmacokinetic and organ distribution studies of dendrimer-drug complexes and free drug along with their parent dendrimers were carried out in male albino wistar rats with carrageenan-induced inflammation in the right hind paw. Albino rats were divided in five groups each comprising six rats were numbered and marked. The test formulations of dendrimer-drug complexes QPPI-NHAc (G2)-PXM & QPPI-NHAc (G3)-PXM and plain drug PXM in the doses of 2 mg/kg body weight were administered through an intravenous route. The blood samples were collected from the retro-orbital plexus at regular intervals of time duration and the blood samples were allowed to stay for 15 min and then centrifuged at 3000 rpm for 15 min to separate RBCs and serum. The supernatant (serum) was collected with the help of micropipet and were analyzed for drug content using HPLC (Waters 2489 UV/Vis detector, USA) for the determination of various pharmacokinetic parameters.

For organ distribution studies, animals were divided into five groups, and each group was administered with the same intravenous dose (2 mg/kg) of dendrimer-drug formulations and plain PXM with their corresponding parent dendrimers. Five rats from each group were humanly sacrificed at 2, 4, and 8 h, and the organs, viz., paw, kidney, liver and spleen were removed
immediately and weighed then the organs were refrigerated at -20 °C by wrapping them in aluminum foil. The organs were homogenized, centrifuged at 4000 rpm for 15 min followed by collection of supernatant and assayed for PXM by the HPLC method as reported earlier, with slight modifications [37, 38] employing a C18 column, 5 µm particle size (analytical column protected by a compatible guard column). The HPLC system (Waters 2489 UV/Vis detector, USA), and the elution was carried out by using water:methanol:acetic acid (44:50:6) as the mobile phase. The flow rate was kept at 1.0 mL/min throughout the process.

3. RESULTS AND DISCUSSION

3.1 Synthesis and characterization of QPPI-NHAc (G2) and QPPI-NHAc (G3) dendrimers

Earlier studies reveal that the commercial PAMAM and PPI dendrimer have been modified by surface functionalization using different moieties and explored the same for different applications and found convincing results [39, 40]. In connection to our expertise in this field, in this study, we have modified the commercial PPI (G2) and PPI (G3) into QPPI-NHAc (G2) & QPPI-NHAc (G3) via procedure followed in the earlier study. That is, in the first step acetyl chloride was surface functionalized on PPI (G2)/(G3) and the obtained surface acetylated product was then quaternized using methyl iodide and the product obtained in each reaction was characterized with FTIR, $^1$H and $^{13}$C NMR and MALDI-TOF techniques. The FTIR spectra of commercial PPI-NH$_2$ (G2) & PPI-NH$_2$ (G3) and hydroxylated PPI-NHAc (G2) & PPI-NHAc (G3) were shown in Fig. 2a & 2d and 2b & 2e respectively. The spectra for PPI-NH$_2$ (G2) (Fig. 2a) and for PPI-NH$_2$ (G3) (Fig. 2d) shows stretching vibration at 3356 cm$^{-1}$ & 3280 cm$^{-1}$ and 3334 cm$^{-1}$ and 3284 cm$^{-1}$ assigned for the surface amine groups. In the FTIR spectra of PPI-NHAc (G2) (Fig. 2b) & PPI-NHAc (G3) (Fig. 2e) these -NH$_2$ peaks disappeared and also individual peaks at 1717 cm$^{-1}$ and 1715 cm$^{-1}$ appeared corresponding to the characteristic C-O (str) vibrations of carbonyl group in acetyl chloride. This observation confirms the alkylation of
PPI (G2) and PPI (G3) with acetyl chloride. The FTIR spectrum of QPPI-NHAc (G2) (Fig. 2c) shows characteristic peaks at 2958 cm\(^{-1}\) and 2740 cm\(^{-1}\) for C-H (str), 1434 cm\(^{-1}\) for C-H (bend) and 1160 cm\(^{-1}\) for C-N (str) and the FTIR spectrum of QPPI-NHAc (G3) (Fig. 2f) shows characteristic peaks at 2991 cm\(^{-1}\) and 2773 cm\(^{-1}\) for C-H (str), 1454 cm\(^{-1}\) for C-H (bend) and 1162 cm\(^{-1}\) for C-N (str). The new intense peaks at 1160 cm\(^{-1}\) and 1162 cm\(^{-1}\) respectively which correspond to the C-N\(^{+}\)str due to the quaternization of tertiary amines in PPI-NHAc (G2) and PPI-NHAc (G3). The chemical shift value from the \(^1\)H \& \(^{13}\)C NMR spectra of QPPI- NHAc (G2) and QPPI- NHAc (G3) dendrimers further provide the convincing evidence for surface alkylation and internal quaternization. The \(^1\)H NMR for QPPI- NHAc (G2) (Fig. 3a) gives a sharp singlet at \(\delta 2.734\) due to \(-\text{COCH}_3\) (acetyl protons) and a sharp singlet at \(\delta 3.353\) which is due to (N-CH\(_3\)) methyl protons. Similarly, \(^1\)H NMR recorded for QPPI- NHAc (G3) (Fig. 3b) gives an intense singlet signal at \(\delta 2.673\) due to \(-\text{COCH}_3\) (acetyl protons) and a sharp singlet at \(\delta 3.241\) due to (N-CH\(_3\)) methyl protons. In \(^{13}\)C NMR for QPPI- NHAc (G2), methyl carbons (N-CH\(_3\)) show peak at \(\delta 49.321\), acetyl carbons (\(-\text{COCH}_3\)) show peak at \(\delta 38.837\) and carbonyl carbons (\(\text{COCH}_3\)) show peak at 176.96 respectively. Likewise, \(^{13}\)C NMR spectrum of QPPI- NHAc (G3), methyl carbons (N-CH\(_3\)) show peak at \(\delta 46.839\), acetyl carbons (\(-\text{COCH}_3\)) show peak at \(\delta 36.096\) and carbonyl carbons (\(\text{COCH}_3\)) show peak at 174.34 respectively.

The structures of the QPPI- NHAc (G2) and QPPI- NHAc (G3) dendrimers were also evidenced through MALDI-TOF analysis and the observed spectra are shown in Fig. S1a & Fig. S1b. The characteristic peaks corresponding to m/z values at 2154 and 4559 for QPPI- NHAc (G2) and QPPI- NHAc (G3). The obtained experimental m/z values agree well with the theoretical values for the modified dendrimers. The collective results observed from FT-IR, NMR (\(^1\)H \& \(^{13}\)C) and MALDI-TOF spectra strongly confirm the respective structure of surface alkylated and internally quaternized PPI (G2) and PPI (G3) dendrimers. This modified
dendrimer QPPI-NHAc (G2) and QPPI-NHAc (G3) was employed for drug delivery applications using PXM as a model drug.

3.2 Solubility Studies of PXM Using QPPI-NHAc (G2) and QPPI-NHAc (G3) Dendrimer Carriers

Drug solubility is one of the core factors, which influences the movement of a drug from the site of administration into the bloodstream. It is widely acknowledged that insufficient drug solubility can lead to poor absorption [41]. The poor solubility of PXM can be addressed with newly developed QPPI-NHAc (G2) and QPPI-NHAc (G3) dendrimers by employing it as solubility enhancer. Additionally in order to project the significance of modified dendrimers in drug delivery the PPI (G2) & PPI (G3) and branched PEI also employed and studied. Solubility studies were performed by keeping the seven different concentrations in the range from 0.05 mM to 0.35 mM. The amount of solubilised drug was estimated in each concentration of dendrimer carriers from the UV absorption and the calibration curve was drawn. The observed drug solubility with increasing trend is given in Table-1. To record precisely, at the maximum concentration (0.35 mM) the QPPI-NHAc (G2), QPPI-NHAc (G3) and PEI has promoted the solubility of PXM to the tune of 22, 50 and 27 folds respectively higher than with the intrinsic solubility of plain drug. In the case of commercial PPI (G2) & PPI (G3) under similar experimental conditions, the solubility of PXM has increased to 32 and 65 fold to its intrinsic solubility. However, the parent dendrimers are able to load the drug but at the same time its cytotoxicity is not tolerable. Therefore, the quaternized forms of modified dendrimers with reduced toxicity are reasonable in drug loading and releasing behaviour. This observation in turn ensures that the water solubility of PXM has been significantly increased proportionally to the concentration of dendrimer carriers. The comparative results reveal that the dendrimer derived from higher generation viz., QPPI-NHAc (G3) has increased the solubility of PXM to the tune of
50 folds compare to its intrinsic solubility and 2 fold than with their corresponding lower generation dendrimer viz., QPPI-NHAc (G2). Also it is clearly explained that the solubility of PXM in the presence of branched PEI is substantially lower than PPI (G3) and QPPI-NHAc (G3), due to poor ionic interaction and signifies that the higher generation of modified dendrimers is better way of delivering drug.

The linear equations for QPPI-NHAc (G2) and QPPI-NHAc (G3) carriers were obtained from their solubility plots (Fig. 4b) and it is calculated as \( y = (3.89 \pm 0.129)x + (1.452 \times 10^{-4} \pm 0.285 \times 10^{-4}) \) \((R^2=0.994) \) and \( y = (9.10 \pm 0.415)x + (4.216 \times 10^{-4} \pm 0.928 \times 10^{-4}) \) \((R^2=0.988) \) respectively. The directional coefficients of former one is \( n = 3.89 \pm 0.129 \) and for later one is \( n = 9.10 \pm 0.415 \) respectively and these values in turn can be interpreted as a number of drug molecules combined/bound by one molecule of dendrimeric unit. That is, the tentative number of drug molecules combined/bound by a single molecule of dendrimeric unit was determined as 3.89±0.129 for QPPI-NHAc (G2) and 9.10±0.415 for QPPI-NHAc (G3) carriers. Further, we also found that the drug loading efficiency of QPPI-OH (G2) is 64.3% and for QPPI-OH (G3) is 58.4% respectively. The increased solubility noticed in the presence of QPPI-NHAc (G2) and QPPI-NHAc (G3) is due to (i) the availability of internal positive binding sites with neutral outer surface. (ii) the multi charged cations viz., QPPI-NHAc (G2)/(G3), attracted the anion of PXM whose formation is facilitated by the generation of HCl, a stronger acid compared to PXM, (iii) thus PXM binds electro statically with QPPI-NHAc (G2)/(G3) in aqueous medium and thus forming the complex viz., \((\text{PXM-O}^-)_n(\text{QPPI-NHAc})^{n+}\), (iv) thereby PXM gets internalized within the QPPI-NHAc (G2)/(G3) dendrimer branches. This interaction substantially promotes the solubility of PXM. The increased solubility has directly helps to increase its bioavailability.

3.3 UV-vis spectroscopy for the interactions between PXM and QPPI-NHAc (G3) dendrimer carrier
The use of dendrimer as a drug delivery carrier usually depends on its ability to form a complex with the drug. Interactions between PXM and dendrimer molecules were determined by UV-vis spectroscopy. PXM gives maximum absorbance at its characteristic wavelength (354 nm). After the addition of increasing concentrations of QPPI-NHAc (G3) dendrimers, blue shifts were observed (Fig. 4a) (the $\lambda_{\text{max}}$ peak shifted about 8-10 nm from 354 nm). In general, any shift in $\lambda_{\text{max}}$ suggests the existence of interaction between the complex forming components [42]. In this study, electrostatic interaction between the anionic group of PXM and the cationic groups of QPPI-NHAc dendrimer can explain this blue shift. It is known that increase in polarity of the solvent generally shifts n-$\pi$ bands to shorter wavelength. The hypsochromic groups diminish the color of chromogen i.e. they cause displacement to shorter wavelengths. In our study, acetylation of NH$_2$ groups i.e.COCH$_3$ and NHCOCH$_3$ produces hypsochromic shift (blue shift). Further, saturated sulphoxide absorbs with high intensities. In our study also, SO$_2$ absorption is due to n-$\pi$ transition present in the PXM drug. Therefore, the occurrence of shift noticed in ($\lambda_{\text{max}}$) supports the formation of complex through electrostatic interaction.

3.4 NMR spectral studies for interactions between PXM and QPPI-NHAc (G3) dendrimer carrier

3.4.1 $^1$H NMR studies

It is well known that the $^1$H NMR spectroscopy is a useful technique to investigate the intermolecular interactions in solutions because it gives information pertinent to the formation of aggregates, ion pairing, encapsulation and size variations [43]. The $^1$H NMR technique was employed here to investigate the molecular interactions between QPPI-NHAc (G3) dendrimer and PXM drug molecules. The chemical shift assignment for each proton in the dendrimer, PXM and dendrimer-PXM complex is so critical because the shift of each signal is helpful to define the zone of interaction between the dendrimer and PXM [44]. The Fig. 3c shows the $^1$H NMR
spectra and chemical shift assignments for QPPI-NHAc (G3)/PXM complex. The protons of PXM and QPPI-NHAc (G3) dendrimer were labeled with numbers 1-11 and characters a-e, respectively as shown in Fig. 3c. It can be observed that PXM has scattered NMR peaks in the range of 2-8.5 ppm and that QPPI-NHAc (G3) dendrimer has seven NMR peaks in the range of 1.0-4.0 ppm. Having assigned the chemical shifts of the QPPI-NHAc (G3) dendrimer and PXM, we can analyze the complexation of the dendrimer and PXM by $^1$H NMR. The QPPI-NHAc (G3)-PXM complex has shown two types of peaks which correspond to PXM molecule and dendrimer scaffold respectively. It is observed that significant changes were noticed in chemical shifts of methylene protons (c, d and e) in QPPI-NHAc (G3) dendrimer induced by the addition of PXM. The downfield chemical shift of these protons have localized at the outermost layer of the QPPI-NHAc (G3) dendrimer and thus proves the ionic interactions between quaternized ammonium groups of the dendrimer and deprotonated group of PXM [45].

3.4.2 2D-NOSEY Analysis

It is well known that 2D NOESY technique is proved to be an effective method for providing insights into the molecular features of host-guest interactions [46-48]. It is capable of revealing a spatial relationship between nuclei in a molecule or in a complex of molecules [46]. If the host and guest are bound, they should be in close proximity to each other and NOE cross-peaks should be seen in the corresponding spectral region. In other words, the absence of a NOE cross-peak in the region can be used to rule out the interaction between related nuclei [48]. In this study, $^1$H-$^1$H NOESY experiments were performed to determine the dipolar contacts for both intermolecular and intramolecular interactions of QPPI-NHAc (G3) dendrimer with PXM. The $^1$H-$^1$H NOESY spectrum of a QPPI-NHAc (G3)/PXM/DMSO solution at a mixing time of 300 ms is shown in Fig. 5a. Strong NOE cross-peaks are observed between the hydroxyl protons of
PXM and scaffold protons of the QPPI-NHAc (G3) dendrimer, which indicates close proximity between these protons.

The partial $^1$H-$^1$H NOESY spectrum (the region of the dendrimer proton signals with chemical shifts ranging from 1.0 to 4.5 ppm) for QPPI-NHAc (G3) dendrimer-PXM complex (Fig. 5b) shows 5 peaks corresponding to three CH$_2$ protons (a-c) in the interior of the dendrimer, one N-CH$_3$ proton (d) and one COCH$_3$ proton (e) in the outermost layer of QPPI-NHAc (G3) dendrimer and these observations are in accordance with the $^1$H NMR spectrum of the QPPI-NHAc (G3)-PXM complex. Several cross peaks were obtained between the protons of QPPI-NHAc (G3) and the protons of PXM. Intermolecular NOE peaks such as (c-8, 9), (d-8, 9) and (e-8, 9) as well as similar intramolecular NOE peaks (a-c), (b-c), (a-e) and (b-e) were also noticed. In addition to this, some intramolecular cross peaks (8, 9-10, 11) and (1, 2-8, 9) were also observed and this in turn indicates the interaction between the aromatic protons of PXM. Therefore, all these observations strongly confirm the interactions of PXM molecules with the scaffold protons of QPPI-NHAc (G3) dendrimer.

3.5 In vitro drug release studies

The very purpose of drug carrier is to carry the drugs and deliver them at the right site of action. One of the prerequisite of a good carrier is it should deliver the drug in a sustained/delayed manner. Thereby the side effect of drug can be minimized by preventing the fluctuation of the therapeutic concentration of the drug in the body. It also eliminates the chance of over or under dosing. Normally, one of the methods to achieve this sustained/delayed release is via diffusion through a matrix. According to the previous reports, dendrimers are able to pack hydrophobic drugs and these formulations are suitable for different administration routes. These polymeric delivery systems with sustained release behaviors can improve the biocompatibility of drugs loaded in the carriers, simplify the dosing schedule and improve patient compliance [40-
To study the extent of drug release from drug-dendrimer formulations, we investigated the release profiles of PXM from QPPI-NHAc (G2), QPPI-NHAc (G3) and its parent dendrimers PPI (G2) & PPI (G3) and as well as from PEI. The release pattern was plotted as percentage of drug released vs time and the same is shown in Fig. 6. The obtained plot clearly shows that after 1 h, 90% of drug gets released from the control formulations (drug alone) and 70% of drug gets released from the PEI-PXM formulations, whereas in commercial dendrimers 50% of drug was released from PPI-NH\textsubscript{2} (G2)-PXM formulations and it is 40% from PPI-NH\textsubscript{2} (G3)-PXM formulations. Further, in the case of QPPI-NHAc (G2)-PXM formulation the release gets slower to 20% and in the case of QPPI-NHAc (G3)-PXM formulation the release gets sustained to 13% in the same span of time (1 h). That is, As much as 90% drug gets released within 60 mins from the control formulations, and it takes 90 mins to get released from PEI-PXM. Whereas, it takes 110 mins for 90% of drug release from PPI-NH\textsubscript{2} (G2)-PXM formulation and 180 mins for the same amount of drug to get released from the PPI-NH\textsubscript{2} (G3)-PXM formulations.

However, important observation that has to be necessarily highlighted is that the same amount (90%) of drug released from quaternized form of dendrimers QPPI-NHAc (G2)-PXM is extended to 390 mins and for higher generation QPPI-NHAc (G3)-PXM formulation it was extended to 570 mins. These results suggested that the QPPI-NHAc (G3) dendrimer has significantly contributed for the sustained release of PXM than with their control, commercial and PEI formulations. In general, it is well established fact that the sustained/delayed release of a drug by any drug carrier is an indication of formation of stable complex due to the interaction between drug carrier and drug or it is an indication for effective internalization of drug with the drug carrier. In this study, we established that the complexes formed between the quaternary ammonium dendrimer drug carriers and the drugs should facilitate via electrostatic interaction, whose magnitude depends on the ionic strength and structure of QPPI-NHAc (G2)/(G3). The
developed quaternary ammonium dendrimer drug carriers have sustained the drug release significantly, which is due to the formation of stable complexes in the form of HCl (PXM-O⁻)ₙ-(QPPI-NHAc (G2)/(G3)ⁿ⁺. The formation of this stable complex and their internalization within the dendrimer branches greatly contributed towards the sustained/slower release of the drug. However, in this study it is observed that QPPI-NHAc (G2)/(G3) entraps the PXM more stably and thus reflected in the sustained release of PXM. The multi charged cations with hydrophilic character which made to interact well with the anionic drugs. The electrostatic interaction between them are intensively attracted the anionic PXM drug and which in turn favors more drug loading and thus forms stable complex which in turn reflected in the sustained release of respective drug molecule. The prolonged sustain release profile of PXM from the QPPI-NHAc (G2)/(G3) under both generation implied that quaternized poly(propylene imine) dendrimers are extremely important for effective encapsulation and retention of Piroxicam.

3.6 Cytotoxicity Assay

An ideal polymeric drug carrier should be biocompatible by performing its expected functions without the concomitants of toxic and immunologic effects to normal cells [49]. The effect of dendrimers and their complexes with PXM on the viability of Vero cell lines were measured by the MTT assay. The variation of percentage cell viability incubated in Vero cell line with different concentrations of QPPI-NHAc (G3) and PPI-NH₂ (G3) are shown in Fig. 7. From the plot, it is observed that, the modified dendrimer carrier viz., QPPI-NHAc (G3) has proved to be significantly less cytotoxic than its parent dendrimer PPI (G3) and PXM. The presence of PXM with the dendrimer carriers did not reduce cell viability on incubation over the tested concentration. Photographs of Vero cells (100um) shown in Fig. 8a-d, which reflect the cytotoxicity, caused by 0.125 mM PPI-NH₂ (G3) dendrimers on Vero cells after 24 h. The cells got distorted and a significant decrease in cell density can be found (Fig. 8c). However, cells
treated with QPPI-NHAc (G3) dendrimers were observed by retaining the cell structure and also considerable cell density (Fig. 8d), suggesting that modified dendrimers were less toxic on Vero cells compare to PXM. Normally, under in vitro conditions, the degree of cytotoxicity usually been evaluated based on the IC_{50} values (concentrations where 50% of cells are viable) and this value (IC_{50}) increases significantly for QPPI-NHAc (G3) dendrimers. That is, IC_{50} value for PXM (control) and PPI-NH_2 (G3) is less than 0.0625mM, whereas for QPPI-NHAc (G3), it is 0.5mM. These results show that the QPPI-NHAc (G3) dendrimer can effectively increase the tolerance concentration during its administration. Previously reported studies reveal that electrostatic interaction between positively charged polymers and negatively charged cell membranes is responsible for cytotoxicity [21, 32, 50 and 51]. Particularly, cationic dendrimers containing –NH_2 surface group, are prone to attract the negatively charged cell membrane that in turn damaged the cell lines and thus reflected more cytotoxic effect.

Therefore, it is necessary that in any delivery, the cationic surface group of the polymer/dendrimer must be neutralized so as to eliminate the electrostatic interaction of surface group with the negatively charged cell membranes and this in turn reduced the cytotoxicity. On par with this, QPPI-NHAc (G3) is a carrier containing neutral surface group viz -COCH_3 and CH_3 and thereby their electrostatic interaction with Vero cell line was sizably reduced and hence lower cytotoxicity is noticed than its parent dendrimer and plain PXM. This proves that QPPI-NHAc (G3) dendrimer provides a potential platform to reduce the cytotoxicity and thus gives the pathway to increase its biocompatibility.

3.7 In Vivo Anti-Inflammatory Activity

The anti-inflammatory studies were performed using carrageenan-induced paw edema model. It is observed that the inhibition level of plain drug after 12 and 24 h was found to be 29.85% and 26.71% respectively (Fig. 9). But there was a marked observation in the case of
dendrimer-drug formulations as in PPI-NHAc (G2)-PXM 35.96% (12 h) & 31.18% (24 h), in PPI NHAc (G3)-PXM it was found to be 41.37% (12 h) & 37.96% (24 h) and in the modified dendrimer formulations namely QPPI-NHAc (G3)-PXM it was found as 43.82% (12 h) & 41.38% (24 h) and in the case of higher generation viz., QPPI-NHAc (G3)-PXM displayed a maximum inhibition of 79.31% after 4 h and until the 8th h inhibition level was maintained above 50% and even after 12 and 24 h 49.42% and 45.83% inhibition was observed. However, in the case plain PXM drug the maximum inhibition was shown in 4th h with magnitude of 56% and just after 4th h it scored below 50%. The plausible reason for this may be the drug concentration in the body which was maintained for a longer duration in case of QPPI-NHAc (G3)-PXM when compared to plain PXM drug which signifies the localized action of QPPI-NHAc (G3)-PXM formulation in inflamed paw.

3.8 Pharmacokinetics and Biodistribution Studies

Pharmacokinetic results reveal that the blood levels of PXM in edema induced paw tissues were more with QPPI-NHAc (G3)-PXM formulation as compared with other formulations including the plain drug. The area under the plasma concentration time profile with QPPI-NHAc-(G3)-PXM was 298.54±2.07 µg/mL/h which was higher compared to other formulations and plain drug which was 281.28±1.98 (QPPI-NHAc-(G2)-PXM), 278.36±1.38 (PPI-NHAc-(G3)-PXM), 275.24±1.33 and 268.11±1.29 (plain PXM) respectively. The elimination rate constant for PXM observed in QPPI-NHAc-(G3)-PXM formulation was 0.0149 as against QPPI-NHAc-(G2)-PXM, PPI-NHAc-(G3)-PXM, PPI-NHAc-(G2)-PXM and plain drug as 0.0165, 0.0178, 0.0197 and 0.0186 respectively. Also the half-life of the drug PXM with QPPI-NHAc-(G3)-PXM formulation was 38.1h which is significantly higher compared to other formulation and the plain drug (QPPI-NHAc-(G2)-PXM, PPI-NHAc-(G3)-PXM, PPI-NHAc-
(G2)-PXM and plain drug as 33.4, 35.2, 32.6 and 31.7 respectively). All the results collectively suggested the extended plasma level of PXM.

From the organ distribution in the case of plain drug, percentage of drug recovered in paw increased in 4th h (2.32±0.38) compared to 2nd h (1.49±0.24) and diminished slightly after 8th h with 0.30±0.64 (Fig. 10). But in the case of QPPI-NHAc-(G3)-PXM formulation it was observed that a continuous increase in percentage of drug recovered from paw with 3.65±0.80 (2h), 4.58±1.02 (4h) and 4.92±1.24 (8h) respectively. The same trend of drug recovery in paw was also observed in other formulations i.e. in QPPI-NHAc-(G2)-PXM the trend was 3.18±0.62 (2h), 3.97±0.81 (4h) & 4.31±0.94 (8h), in the case of parent formulations PPI-NHAc-(G3)-PXM the trend was 2.87±0.24, 3.36±0.75 and 4.39±1.09 and similarly for PPI-NHAc-(G2)-PXM it was 2.21±0.27, 2.92±0.34 and 3.45±0.78 respectively. Further it was also observed that the percentage of drug recovered in paw with QPPI-NHAc-(G3)-PXM formulation was higher compared to others. It is noted that after 2 h of administration of plain drug, higher concentration of drug was observed in kidney and liver than in paw and also similar observations were made with other formulations. After 4th h, the plain PXM, QPPI-NHAc-(G2)-PXM and their parent dendrimers continued to show higher recovery in liver (3.26±0.92, 2.07±0.28, 2.88±0.49 & 3.02±0.51 respectively) than in paw (2.32±0.38, 2.92±0.34, 3.97±0.81 & 3.36±0.75 respectively). However, it is worth to mention here that, from the formulation QPPI-NHAc-(G3)-PXM, after 4th h higher concentration of PXM was recovered from paw 4.58±1.02 against 3.47±0.84 and 3.98±0.90 in kidney and liver respectively. From the collective results, it is evident that the preferred sites of drug associated with dendrimer formulation are the liver and inflammed paw.

In general, Hydrophobic particles are immediately recognized as “foreign” and are generally covered by plasma proteins known to function as opsonins, which facilitate
phagocytosis, thus in the present strategy, surface characteristics increase hydrophilicity and hence decrease macrophage phagocyte systems (MPS) clearance [52]. Because of this the drug levels observed in edema-induced paw tissues with dendritic formulation were much higher than the free drug at all-time intervals of study. As the dendritic formulation exhibits prolonged retention in blood, it may be proposed that the greater perfusion of blood at the inflamed site could bring a larger amount of PXM from QPPI-NHAc-(G3)-PXM formulation in paw tissues. Also, as there is considerable change in vasculature at the site of inflammation, the chances of QPPI-NHAc-(G3)-PXM localization increase further owing to the enhanced permeability and retention (EPR) effect at such sites as reported for macromolecular polymeric structures like PAMAM [53,54]. The other more plausible reason could be the interaction of PAMAM dendrimer with albumin protein as reported with bovine serum albumin [55].

**Conclusions**

Dendrimer based drug delivery system provides an attractive platform to load and release of poorly soluble drug molecules and thus improves the pharmacodynamic and pharmacokinetic behavior of those drugs. In the present study, for the first time an efficient biocompatible dendrimer based drug carrier viz., QPPI-NHAc (G2) and QPPI-NHAc (G3) were synthesized through simple reactions like surface acetylation and internal quaternization of their commercial PPI (G2) & PPI (G3) dendrimers. The presence of acetyl group on the surface and quaternaryonium in the internal cavities of QPPI-NHAc (G2)/(G3) were established through spectral techniques. The drug carrying potential of these surface acetylated and interior quaternized dendrimers viz., QPPI-NHAc (G2) & QPPI-NHAc (G3) with respect to Piroxicam drug was investigated through aqueous solubility, *in vitro* release and cytotoxicity studies and proved these dendrimers are more active as compared with their PPI (G2), PPI (G3) and PEI. The observed result reveals that the higher generation dendrimer i.e., QPPI-NHAc (G3) has significantly
solubilizing the PXM to the tune of 50 folds compared to its intrinsic solubility and ensures the promising loading of PXM. It is proved that the complexation of PXM with QPPI-NHAc (G3) is responsible for drug loading and in turn for increased solubility. The drug release noticed under \textit{in vitro} conditions have suggested that QPPI-NHAc (G3) is a potential one to release the PXM for long time up to 570 mins and hence it is more suitable for sustained/delayed release. The extent of sustained release observed in this study has reflected the magnitude of interaction between the dendrimer carrier and the drug molecule. Further, QPPI-NHAc (G3) dendrimers have showed increased cell viabilities on Vero cell lines which is correlated with its reduced cytotoxicity over the parent dendrimer as well as plain PXM and hence they are more biocompatible. The increased IC$_{50}$ value observed for QPPI-NHAc (G3) implies that it can effectively increase their tolerance concentration during the preparation of QPPI-NHAc-PXM (G3) dendrimer formulations. To suggest real time applications, the newly synthesized dendrimer based drug carriers were employed for \textit{in vivo} studies namely (i) \textit{in vivo} anti-inflammatory activity and (ii) biodistribution. The pharmacodynamic results in the case of dendrimer-drug formulation QPPI-NHAc-PXM (G3) which displays a maximum inhibition after 4$^{th}$ h (79.31\%) and significant level was observed even after 12$^{th}$ and 24$^{th}$ h (49.42 \% and 45.83 \%). The 2$^{nd}$, 4$^{th}$ and 8$^{th}$ h organ distribution results show higher recovery of PXM in rat paw with QPPI-NHAc-PXM (G3) formulation. In conclusion, dendrimer-based formulation may not only improve the solubility of PXM but also can certainly helpful to localization of drug at the site of inflammation and hence provide better therapeutic efficacy at a lower dose. Therefore, considering all the observed results, it is reported that the surface acetylated and internally quaternized dendrimer, QPPI-NHAc (G3) is undisputedly a promising candidate in the design of polymeric drug delivery system by selectively inhibiting cyclooxygenase (COX) located at the inflammation site.
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Declaration of interest

The opinions expressed are solely those of the author, and the authors state no conflict of interest and have received no payment in preparation of this manuscript.

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Fig. 1a Synthetic route to get QPPI-NHAc (G2)/(G3) dendrimer from PPI (G2)/(G3) dendrimer.

Fig. 1b Structure of Piroxicam (PXM).

Fig. 2 FTIR Spectrum of (a) PPI-NH\textsubscript{2} (G2), (b) PPI-NHAc (G2) and (c) QPPI-NHAc (G2), (d) PPI-NH\textsubscript{2} (G3), (e) PPI-NHAc (G3) and (f) QPPI-NHAc (G3) respectively.

Fig. 3 \textsuperscript{1}H NMR spectrum of (a) QPPI-NHAc (G2), (b) QPPI-NHAc (G3) and (c) QPPI-NHAc (G3)-PXM complex.

Fig. 4 (a) UV-Vis spectra of plain PXM (dotted line) and PXM with different concentrations of QPPI-NHAc (G3) (bold lines) and (b) Solubility behaviour of PXM at various concentrations of PPI-NH\textsubscript{2} (G2), PPI-NH\textsubscript{2} (G3), QPPI-NHAc (G2) and QPPI-NHAc (G3) dendrimer solutions.

Fig. 5 (a) \textsuperscript{1}H-\textsuperscript{1}H NOESY spectrum of the QPPI-NHAc (G3)/PXM/DMSO solution at a mixing rate of 300 ms.

Fig. 5 (b) Expanded region of the \textsuperscript{1}H-\textsuperscript{1}H NOESY spectrum shown in Fig. 5a, Showing the cross-peaks between protons of drug and QPPI-NHAc(G3). The cross-peaks are indicated by rectangles.

Fig. 6 Release behaviour of PXM alone (control) and also in presence of PEI, PPI (G2), PPI (G3) and modified dendrimers QPPI-NHAc (G2) & QPPI-NHAc (G3). PXM and dendrimer concentrations were 1.3mM and 0.2mM in dialysis bag respectively. Each data point represents mean± standard error (S.E) (n=3).

Fig. 7 Cytotoxicity of PXM (control), PPI (G2/G3) and QPPI-NHAc (G2/G3) dendrimers with PXM at different concentrations after 24 h of incubation as determined by MTT assay on Vero cell line. Each data point represents mean± standard error (S.E) (n=3).

Fig. 8 Photographs of (a) Vero cell line and the same cell lines treated with (b) PXM, (c) PPI-NHAc (G3) and (d) QPPI-NHAc (G3)

Fig. 9 Anti-inflammatory activity of PXM and various dendrimer-drug formulations (n=6)

Fig. 10 Percentage of drug recovery in various organs after intravenous administration of the PXM with different formulations (a) paw, (b) kidney, (c) liver and (d) spleen (n=6)
**Fig. 1a** Synthetic route to get QPPI-NHAc (G2)/(G3) dendrimer from PPI (G2)/(G3) dendrimer.

**Fig. 1b** Structure of Piroxicam (PXm).
Fig. 2 FTIR Spectrum of (a) PPI-NH$_2$ (G2), (b) PPI-NHAc (G2) and (c) QPPI-NHAc (G2), (d) PPI-NH$_2$ (G3), (e) PPI-NHAc (G3) and (f) QPPI-NHAc (G3) respectively.
Fig. 3 $^1$H NMR spectrum of (a) QPPI-NHAc (G2), (b) QPPI-NHAc (G3) and (c) QPPI-NHAc (G3)-PXM complex.
Fig. 4 (a) UV-Vis spectra of plain PXM (dotted line) and PXM with different concentrations of QPPI-NHAc (G3) (bold lines) and (b) Solubility behaviour of PXM at various concentrations of PPI-NH$_2$ (G2), PPI-NH$_2$ (G3), QPPI-NHAc (G2) and QPPI-NHAc (G3) dendrimer solutions.
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Fig. 10 Percentage of drug recovery in various organs after intravenous administration of the PXM with different formulations (a) paw, (b) kidney, (c) liver and (d) spleen (n=6)
Table 1 Solubility of PXM in various concentrations of PEI and dendrimer carrier solutions

<table>
<thead>
<tr>
<th>S. No</th>
<th>Carrier $10^{-4}$M</th>
<th>PEI [PXM] $10^{-4}$M</th>
<th>Parent Dendrimers</th>
<th>Modified Dendrimers</th>
<th>PEI Solubility Increment $S_t/S_0^*$</th>
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<td>9.51</td>
<td>3.82 7.783 12.25</td>
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<td>9.31</td>
<td>8.75</td>
<td>16.97</td>
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<td>10.12</td>
<td>12.2</td>
<td>23.54</td>
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<td>15.4</td>
<td>29.46</td>
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<td>2.50</td>
<td>17.18</td>
<td>18.8</td>
<td>36.33</td>
<td>10.98 27.94 24.75</td>
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<td>6</td>
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<td>40.15</td>
<td>12.92 31.79 26.59</td>
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<td>7</td>
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<td>19.18</td>
<td>22.8</td>
<td>45.70</td>
<td>15.48 34.72 27.63</td>
</tr>
</tbody>
</table>

$S_0^*$ is the intrinsic solubility of PXM (0.023mg/ml)
Absorbance measured at $\lambda_{max}$ of PXM