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Effect of pH on the solubility and release of furosemide from polyamidoamine (PAMAM) dendrimer complexes

Bharathi Devarakonda [a,b], Daniel P. Otto [a], Anja Judefeind [a], Ronald A. Hill [b], Melgardt M. de Villiers [a,*]

[a] *School of Pharmacy, University of Wisconsin-Madison, Madison, WI 53705, USA*
[b] *Department of Basic Pharmaceutical Sciences, College of Pharmacy, The University of Louisiana at Monroe, Monroe, LA 71209, USA*

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Abstract

The complexation of the practically insoluble drug furosemide (acidic pK_a 3.22) with lower generation PAMAM dendrimers showed a significant release dependence on the ionization state of the drug. UV and FTIR studies suggested that the drug was localized in the interior of the dendrimer. The dendrimer amine, amide and ester groups, demonstrated pH-dependent ionization as did the drug carboxylic acid group and it was proven that the most efficient drug complexation was achieved in slightly acidic conditions (pH 4.0–6.0). At this pH, amide groups in the dendrimer cavities were at least partially ionized to expose a positive charge whilst the furosemide carboxylic acid ionized to great extent (pH > pK_a) resulting in electrostatic complexation. Conversely, higher release rates were observed in acidic conditions (pH 1.2) where furosemide was virtually unionized, emphasizing the importance of the drug ionization state in the determination of drug release. Despite the complex interactions between the dendrimer and drug and its effects on release kinetics, the dendrimers resulted in higher solubility of the drug and contributed significantly to the array of available techniques to increase the solubility of poorly water-soluble drugs that are very abundant in industry today. Complexation with low generation PAMAM dendrimers (<generation 4) could provide opportunities to both increase drug solubility and tuning of the release profile for practically insoluble drugs.

Keywords: PAMAM dendrimer; Furosemide; Complexation; Ionization; Solubility; Release

1. Introduction

Furosemide, 5-(aminosulfonyl)-4-chloro-2-((2-furanylmethyl)amino) benzoic acid (Fig. 1) is a loop diuretic that is used orally in the treatment of edematous states associated with cardiac, renal and hepatic failure and the treatment of hypertension (Al-Obaid et al., 1989; Reynolds, 1989; Murray et al., 1997). Since furosemide is a weak acid (reported acidic pK_a 3.48) with a carboxylic acid functional group, its aqueous solubility increases as function of medium pH from 0.18 mg/ml (pH 2.3) to 13.36 mg/ml (pH 10.0) (Rowbotham et al., 1976). The major problem associated with the formulation and effectiveness of the furosemide is its variable oral absorption (11–90%, Jackson, 1996) due to insufficient aqueous solubility at gastrointestinal pH, thus making solubility the rate-determining step in the gastric absorption of furosemide (Hammarlund et al., 1984).

Several techniques have been used to enhance the solubility of drugs in solid dosage forms i.e. solubilization by surfactants, co-solvents (Shihab et al., 1979), crystal modification, pH-control (Doherty and York, 1989), solid dispersions (Shin and Kim, 2003) and prodrug formation (Suescun et al., 1998; Mombru et al., 1999). Among these techniques, complexation with cyclodextrins has been widely investigated to improve the solubility and dissolution properties of furosemide (Özdemir and Ordu, 1998; Ammar et al., 1999; Spamer et al., 2002; Vlachou and Papaioannou, 2003). These studies reported 1.4–11-fold increases in solubility depending on the type as well as percentage of the included cyclodextrin to produce the complexes.

Recently the use of dendrimers, Fig. 1, as drug delivery systems has gained much attention in the pharmaceutical literature

* Corresponding author. Tel.: +1 608 890 0732; fax: +1 608 262 5345.
E-mail address: mmdevilliers@pharmacy.wisc.edu (M.M. de Villiers).

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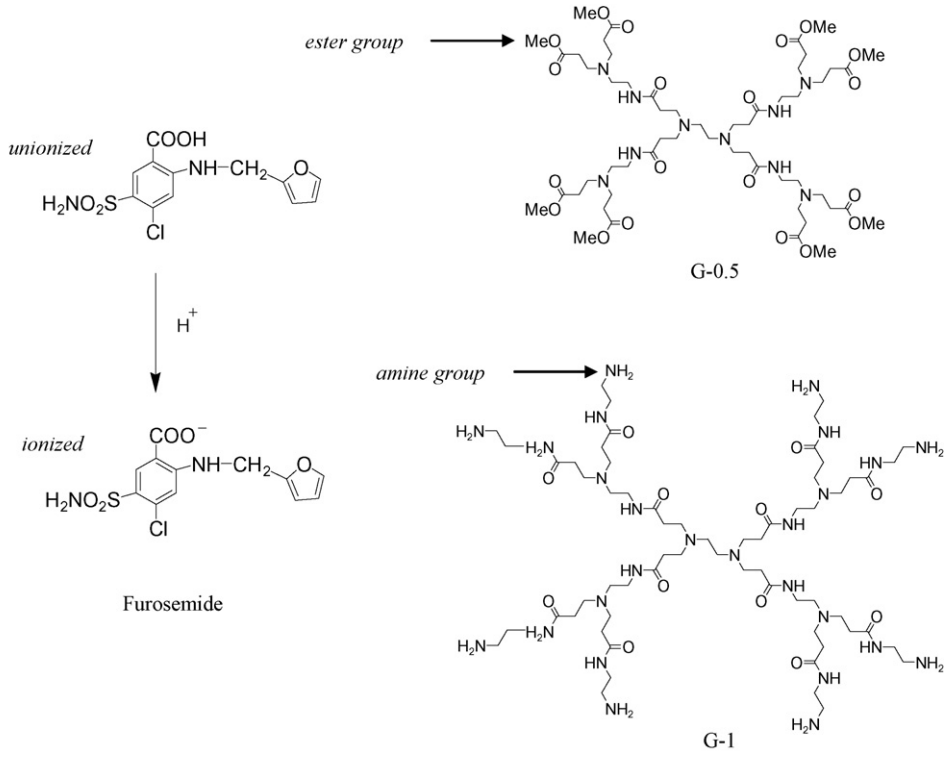

Fig. 1. Molecular structures of furosemide and PAMAM dendrimers with ester- (G0.5) and amine-terminated (G1) surface functional groups.

and applications include increasing the solubility (Devarakonda et al., 2004, 2005a,b) and bioavailability of drugs with poor water solubility (Wiwattanapatapee et al., 2000; Milhem et al., 2000; Kolhe et al., 2003; Chauhan et al., 2004), the delivery of DNA and oligonucleotides across cell barriers (Poxon et al., 1996), and as carriers for gastrointestinal drug delivery (Wiwattanapatapee et al., 2000).

The aim of this study was to investigate whether EDA core polyamidoamine (PAMAM) dendrimers could be used to enhance the aqueous solubility of furosemide. The solubility of furosemide at different pH was measured in the presence of amine- and ester-terminated PAMAM dendrimers evolved to different generations. In addition, the in vitro release of the drug from complexes at pH's commonly found in the gastrointestinal tract is also reported.

2. Materials and methods

2.1. Materials

Furosemide USP (a white to slightly yellow, odorless, crystalline powder that is practically insoluble in water), ethylenediamine, methyl acrylate, methanol (HPLC grade), sodium dihydrogen phosphate, citric acid, tromethamine, sodium borate, potassium chloride, and standard pH buffers were obtained from the Spectrum Chemical Company (Gardena, CA, USA). Double deionized water was used for solubility studies and HPLC analysis. PAMAM dendrimers were synthesized as described previously (Esfand and Tomalia, 2001) or purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Synthesis of PAMAM dendrimers

Ethylenediame (EDA) core PAMAM dendrimers were synthesized using Tomalia's divergent growth approach (Esfand and Tomalia, 2001). The synthesis involves two consecutive chain-forming reactions, the exhaustive Michael additions reaction, and the exhaustive amidation reaction, repeating alternatively. Michael addition of methyl acrylate to ethylenediamine in methanol gives the ester terminated half generation dendrimers designated, G*n*.5. The exhaustive amidation reaction of ester-terminated dendrimers with large excess of ethylenediamine in methanol produce amine terminated full generation dendrimers referred to as G*n*. Repetition of Michael addition and amidation reactions produces the next, higher generation dendrimers.

In the present study, both amine-terminated full generation (G0–G3) and ester-terminated half-generation (G0.5–G2.5) PAMAM dendrimers were used in the solubilization studies of furosemide. Since the dendrimers are highly hygroscopic, they were stored as 10% (w/w) solutions in anhydrous methanol.

2.3. HPLC analysis of furosemide

The amount of furosemide in the solubility test samples were analyzed by high performance liquid chromatography as previously reported (Devarakonda and de Villiers, 2005). The HPLC used consisted of a Spectrum System, AS 1000 autosampler and P2000 pump, (Thermo Separation Products, Waltham, MA) equipped with a multiple wavelength UV detec-

tor (UV 3000 detector) set at a wavelength of detection 272 nm. Chromatographic separation was performed with a Supelco® Discovery RP Amide C_{16} column (250 mm × 4.6 mm, 5-μm particles, Bellefonte, PA, USA) using a mobile phase of H_2O: acetonitrile: acetic acid (60:40:1, v/v); flow rate 1.0 ml/min; injection volume 20 μl. The retention time for furosemide was approximately 7 min and the limits of detection 1.0 ng/ml. Results represent the mean of three analyses, and the solutions were protected from light to prevent photodegradation of furosemide.

2.4. Preparation and characterization of furosemide–dendrimer complexes

Furosemide was dissolved in methanol and then the dendrimer was added. The initial molar ratios of furosemide to dendrimer were 10:1, 20:1, 40:1 and 50:1. The reaction mixtures were stirred for 24 h in the dark and then evaporated using a rotating evaporator to remove the methanol (Kolhe et al., 2003). The precipitates were dried under vacuum in order to remove methanol completely, followed by addition of deionized water. Subsequently, this aqueous solution was stirred in the dark for 24 h to extract the drug–dendrimer complex since the dendrimer and drug–dendrimer complex is soluble in water whilst furosemide is not. The solutions were filtered through a 0.2 μm hydrophilic PTFE membranes (25 mm diameter, Omnipore, Millipore, Bedford, MA, USA) and then lyophilized to remove water. The drug–dendrimer complex obtained was in the form of off-white powders. The amount of furosemide in the complexes was determined by HPLC (Devarakonda and de Villiers, 2005).

Appropriate quantities (based on the furosemide content) of the various complexes were dissolved in methanol to produce a concentration of 6 μg/ml furosemide. The UV-spectra (200–400 nm) of these solutions were obtained with a Shimadzu MultiSpec spectrophotometer (Shimadzu, Kyoto, Japan). The wavelengths of maximum absorption of furosemide in methanol are at 226, 276 and 336 nm. The lower generation PAMAM dendrimers (<G4), shows strong UV absorbance between 200–240 nm and some weaker absorbance for higher concentrations (>100 μg/ml) between 240–280 nm (Devarakonda and de Villiers, 2005). Any shift or suppression of the strong absorption peaks at 276 and 336 nm (furosemide UV absorption) was ascribed to the formation of complexes.

G2.5 PAMAM-furosemide and G3 PAMAM-furosemide complexes in the ratio of 1:1 (w/w, dendrimer/drug) were prepared by dissolving furosemide and dendrimers for 24 h in methanol followed by evaporation of the solvent. The drug–dendrimer complexes, furosemide (alone) and G2.5 as well as G3 PAMAM dendrimers (alone) were subjected to ATR-FTIR analysis. The analysis was conducted using a Bruker Equinox 55/S FTIR spectrophotometer (Bruker Optics, Inc., Billerica, MA, USA) equipped with a HeNe laser light source and an ATR sampling accessory with ZnSe crystal. FTIR spectra were obtained from 32 scans at 4 cm^{-1} resolution in a wavenumber range of 4000–650 cm^{-1}. Processing of spectra was performed with OPUS™ 5.5 software.

2.5. pH-solubility profile of furosemide

The solubility of furosemide was measured in TRIS-buffer with pH 2.0–8.0 and pH 10.0–12.0 at 30 ± 1.0 °C. The ionic strength of the buffers was maintained at 0.5 M with potassium chloride. From the linear portion of the pH-solubility profile (Fig. 5a), the intrinsic solubility and acidic pK_a of furosemide was calculated using the Henderson–Hasselbalch equation for a weak acid (Eq. (1)) (Sinko, 2006).

$$\frac{1}{[H_3O^+]} = \frac{S}{K_a S_0} - \frac{1}{K_a} \quad (1)$$

where $[H_3O^+]$ is the hydronium ion concentration, K_a the dissociation constant for weak acid, S the observed molar solubility of furosemide, and S_0 is the intrinsic molar solubility of furosemide. By plotting $1/[H_3O^+]$ versus S (Fig. 5b), K_a and S_0 can be calculated from the slope ($1/K_aS_0$) and intercept ($-1/K_a$) of the linear regression curve, respectively.

2.6. Solubility measurements

Solubility studies were performed using the Higuchi rotating bottle method (Higuchi and Connors, 1965). An excess amount of furosemide was added to 5 ml amber colored vials containing 3 ml of TRIS buffer (pH 2.0 and 4.0–6.0) with increasing concentrations of the dendrimers and sealed. The vials were rotated at 60 rpm while maintained at 30 ± 1.0 °C. Preliminary experiments indicated that 24 h provided sufficient time to achieve equilibrium. After 24 h, samples were filtered through 0.45 μm cellulose acetate filters (Osmonics Inc., Minnetonka, MN, USA), diluted appropriately with methanol and analyzed by HPLC. Phase solubility diagrams were constructed by plotting the molar concentration of furosemide (solubility) versus molar concentration of dendrimers.

Mathematical analysis of the phase solubility diagrams provided estimates of the apparent equilibrium stability constants. It was assumed that a 1:1 furosemide–dendrimer complex was formed at those dendrimer concentrations where the total solubility versus dendrimer concentration curve was linear. The apparent equilibrium stability constant, $K_{1:1}$, was estimated by regression analysis using the following equation (Higuchi and Connors, 1965):

$$S_t = \frac{K_{1:1} S_0}{1 + K_{1:1} S_0} L_t + S_0 \quad (2)$$

where S_t is the observed molar solubility of furosemide, $K_{1:1}$ is the equilibrium stability constant, S_0 is the intrinsic molar solubility of furosemide, and L_t is the total molar dendrimer concentration.

If the total solubility versus dendrimer concentration curve was parabolic, it was assumed that higher-order complexes were formed. These data were analyzed by non-linear regression analysis using Eq. (3), assuming that only 1:1 and 1:2 complexes

were present.

$$S_t = S_0 + S_0 K_{1:1} L_t + S_0 K_{1:2} L_t^2 \quad (3)$$

where $K_{1:2}$ is the 1:2 equilibrium complexation constant.

Additional studies on the pH-dependent solubility and the formation of furosemide–dendrimer complexes were conducted by adding an excess of furosemide to each of the aqueous solutions having a defined pH (2.0 and 7.0) and dissolving it (Chauhan et al., 2004). After equilibration, the suspensions were centrifuged at 8000 rpm for 10 min. The supernatant was filtered through a membrane filter to obtain saturated furosemide solutions at the respective pH values. The pH of the solutions was noted, then 5 ml aliquots from the solutions were placed in SpectraPor Float-A-Lyzers (5 ml, Spectrum Laboratories, Rancho Dominguez, CA, USA) with biotech cellulose ester membrane tubing (MWCO = 2000) that was pre-sealed at one end and attached to a floatable cap at the other. Before loading the samples the dialysis tubes were rinsed in DI water to remove the preservative.

The molecular weight cut-off of the dialysis membrane was selected because the dendrimers have a significantly higher molecular weight, ensuring that the dendrimers would remain inside the dialysis membrane, whereas the small molecular weight drug would readily diffuse out of the dialysis bag. The floatable cap facilitated easy loading of the samples to the dialysis bags. The filled dialysis tubes were transferred to 50 ml glass cylinders containing the saturated drug solutions which were agitated in the dark using a shaker bath for 24 h to reach equilibrium (concentration inside and outside the dialysis tube was the same). Subsequently, 1 ml of the drug solution was removed from the dialysis tube and replaced with 1 ml of either G2.5 or G3 PAMAM dendrimer stock solutions in water so that the dendrimer concentration inside the tubes was either 0.1%, 0.2% or 0.5% (w/v). The solutions were agitated in the dark (to prevent photochemical degradation) for an additional 24 h preceding the HPLC analysis of the concentration of furosemide inside and outside phases of the dialysis tubes (Devarakonda and de Villiers, 2005).

2.7. In vitro release studies

In vitro release of uncomplexed furosemide (control) and from drug–dendrimer complexes was performed by the dialysis technique. The dialysis technique made use of SpectraPor Float-A-Lyzers (5 ml) with biotech cellulose ester membrane tubing (MWCO = 2000) that was used after being rinsed in deionized water to remove the preservative. Furosemide was dissolved in methanol (2 mg/ml) and used as a control and furosemide complexes were dissolved in deionized water at a concentration equivalent to 2 mg/ml furosemide. After samples were taken from the control and dendrimer solutions (5 ml), they were transferred immediately to the dialysis tubes. The tubes were promptly placed in 500 ml tall form glass beakers containing 400 ml of the dissolution medium maintained at 37 °C. The outer phase was stirred continuously with a magnetic stirrer and sampling (1 ml) was made at specific time intervals followed by replenishment with 1 ml fresh buffer.

The amount of drug in the samples withdrawn from the outer phase was determined over a 12 h period and analyzed by HPLC to characterize the release of furosemide. The release studies for both the control and drug–dendrimer complexes were repeated in simulated gastric fluid (SGF composed of an aqueous solution containing 0.2% sodium chloride and 0.7% hydrochloric acid without pepsin, pH 1.2), simulated intestinal fluid (SIF composed of an aqueous solution containing 0.68% monobasic potassium phosphate and sodium hydroxide without pancreatin, pH 7.4) and the USP dissolution medium for furosemide (phosphate buffer pH 5.8). The dialysis apparatus was completely covered with aluminum foil to prevent photochemical degradation of furosemide.

2.8. Statistical analysis

To study the effect of generation size of amine-terminated full-generation dendrimers and pH of the aqueous medium on the solubility of furosemide, a two-factor factorial design (De Muth, 1999) with $n = 3$ replicates was used where the aqueous solubility of furosemide was measured for four levels of factor A (pH of the medium; 2.0, 4.0, 5.0, and 6.0) and four levels of factor B (generation size; G0, G1, G2, and G3). Similarly, the effect of generation size of ester-terminated half-generation dendrimers was measured for four levels of factor A (pH) and three levels of factor B (generation size; G0.5, G1.5, and G2.5). The effect of the two factors on the aqueous solubility of furosemide was evaluated at a probability level of $p = 0.05$ (95% confidence interval) using a commercial software package (Student Statistix7.0, Analytical Software, Tallahassee, FL, USA). In the presence of significant interaction among the factors, a one-way ANOVA was used. Finally, differences between two sample means were determined by pair-wise comparisons using a least significant difference (L.S.D.) test performed with SPSS 10.0 for Windows™ (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Structural characterization of the PAMAM dendrimers

The PAMAM dendrimers were characterized structurally via ^{1}H- and ^{13}C-NMR, and mass spectral analysis (Table 1) (Devarakonda et al., 2004, 2005a,b). Examples of the molecular structures of the ester (G0.5) and the amine (G1) terminated PAMAM dendrimers are shown in Fig. 1 (See also Esfand and Tomalia, 2001). As shown (Table 1) molecular weight and number of peripheral groups of dendrimers increase exponentially with each generation, whilst the diameter demonstrated an approximately linear increase (Esfand and Tomalia, 2001). This implied that with each ensuing generation, the surface density of peripheral moieties (primary amines in full generation dendrimers and ester groups in half generation dendrimers), increased.

Full generation PAMAM dendrimers have primary amine groups at each branch end and tertiary amine groups at each

Table 1
Selected characteristics of ethylenediamine core PAMAM dendrimers taken from Esfand and Tomalia (2001) and the maximum number of furosemide molecules incorporated per dendrimer molecule determined experimentally (see also Fig. 1)

G*n*	Molecular weight (Da)	Diameter (nm)	No. of tertiary nitrogens	No. of surface groups	No. of furosemide molecules/ dendrimer molecule
NH_2-terminated					
0	517	1.4	2	4	3
1	1430	1.9	6	8	5
2	3256	2.6	14	16	12
3	6909	3.6	30	32	20
$COOCH_3$-terminated					
0.5	1207	–	6	8	3
1.5	2809	–	14	16	6
2.5	5978	–	30	32	12

–: not available.

branching point. As the dendrimer generation n is increased, Table 1 shows that the number of primary amines, N, grows as $N = cm^n$ and the number of tertiary amines grows as $N = cm^n - 2$, where c is the number of branches of the core and m is the multiplicity of monomer. For EDA core PAMAM, $c = 4$ and $m = 2$. The PAMAM-Gn.5-$COOCH_3$ dendrimers have $N = cm^n$ ester groups and $N = cm^n - 2$ tertiary amines.

3.2. Furosemide encapsulation with PAMAM dendrimers

Either furosemide can be encapsulated in the interior or surface bound with the PAMAM dendrimer amine groups. Encapsulation/complexation ability of different amounts of drug in dendrimer (PAMAM-Gn-NH_2 and PAMAM-Gn.5-$COOCH_3$) was studied to estimate the maximum number of furosemide molecules that can be incorporated in a dendrimer molecule. The initial molar ratios of furosemide to dendrimer were 10:1, 20:1, 40:1 and 50:1. These values were selected on the assumption that there should be a strong interaction between the amine (both internal tertiary groups and surface groups) functional groups of PAMAM and the COOH groups of furosemide.

The results in Fig. 2 show that the encapsulation/complexation of furosemide in PAMAM-dendrimers was successfully carried out and most likely occurred in the interior of the dendrimer. The number of furosemide molecules incorporated into the dendrimers increased with an increase in dendrimer size for both NH_2 and $COOCH_3$ terminated dendrimers. However, the number of furosemide molecules associated with a single dendrimer molecule did not correspond with the number of tertiary or surface amine groups present in PAMAM-G3-NH_2 and PAMAM-Gn.5-$COOCH_3$ (values listed in Table 1).

With the PAMAM-G3 that had 30 tertiary and 32 surface NH_2, 20 drug molecules were incorporated per dendrimer molecule to yield the highest amount of bound furosemide molecules. Correspondingly, only 12 drug molecules were incorporated into the PAMAM-G2.5 that had 30 tertiary and no surface NH_2 groups. This indicated that there was a stronger interaction between furosemide molecules and NH_2 group terminated dendrimers compared to the $COOCH_3$ terminated dendrimers. However, based on the number of furosemide molecules that was associated with each dendrimer molecule the complexation was most probably an interaction between interior dendrimer amide groups and carboxylate ions of furosemide.

Since shifting and suppressions of UV-absorption and fluorescence quenching have been reported as evidence of complexation for other drug PAMAM dendrimer complexes the UV-spectra of the drug, dendrimers and drug–dendrimer complexes in methanol were recorded (Kleinman et al., 2000; Kolhe et al., 2003). Evidence of complexation was observed as there were significant changes in die UV-spectrum of furosemide in the complexes as shown in Fig. 3. In addition to the peak maxima at 276 nm and 330 nm being suppressed in the complexes, the peak maximum at 276 nm was also shifted slightly to 271 nm.

Furthermore, ATR FTIR analysis supported the complex formation between PAMAM dendrimer and furosemide. FTIR spectra (2000–650 cm^{-1}) of furosemide, G2.5 PAMAM, furosemide-G2.5 PAMAM are shown in Fig. 4a and for G3 PAMAM and furosemide-G3 PAMAM in Fig. 4b. The characteristic absorption bands of furosemide, C=O stretching of the carboxylic acid group at 1672 cm^{-1}, N–H bending at 1592 cm^{-1} and 1564 cm^{-1} as well as the S=O stretching of the sulfonamide

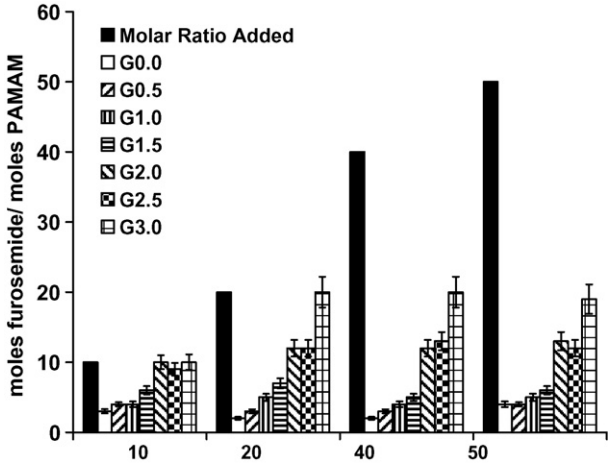

Fig. 2. Furosemide encapsulation or complexation with PAMAM dendrimers. Molar ratios of furosemide to dendrimer added were 10:1, 20:1, 40:1 and 50:1. Maximum number of furosemide molecules incorporated was 20 per 1 molecule G3 PAMAM dendrimer.

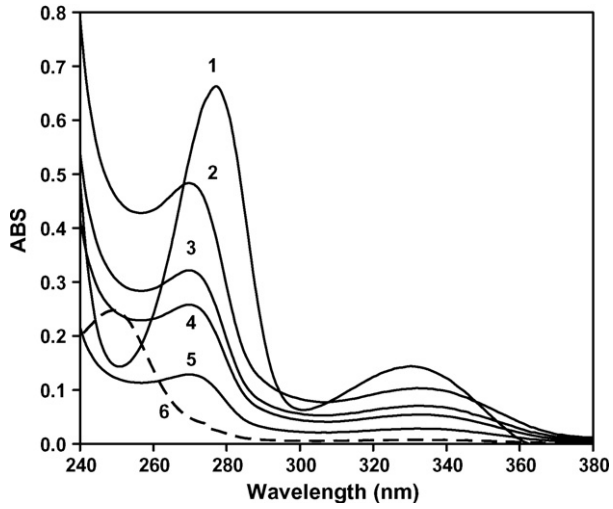

Fig. 3. UV-spectra of methanolic solutions: (1) furosemide, 6 μg/ml; (2) furosemide: G1.5 complex; (3) furosemide: G2 complex; (4) furosemide: G2.5 complex; (5) furosemide: G3 complex; (6) 100 μg/ml PAMAM G3.

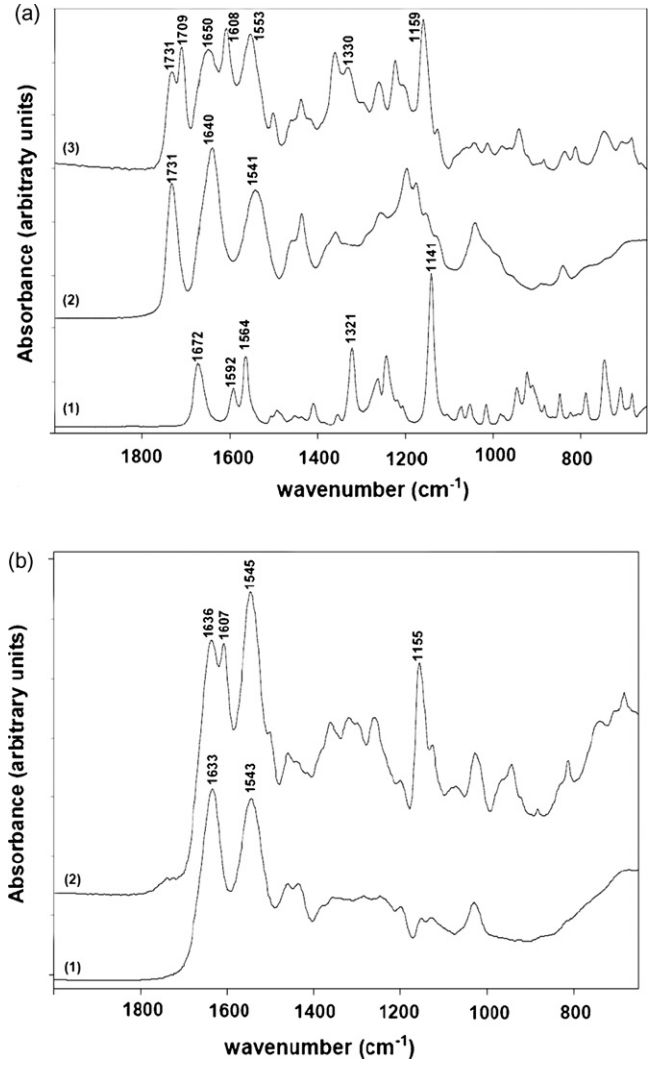

Fig. 4. (a) Expanded ATR FTIR spectra of (1) furosemide (2) G2.5 PAMAM and (3) furosemide-G2.5-PAMAM. (b) Expanded ATR-FTIR spectra of (1) G3 PAMAM and (2) furosemide-G3-PAMAM.

group at 1321 cm^{-1} (asymmetric) and 1141 cm^{-1} (symmetric) (Doherty and York, 1987; Al-Obaid et al., 1989) shifted to different wavenumber positions in the furosemide-G2.5 PAMAM complex spectrum. The C=O and N–H absorption bands shifted to lower frequencies (1650 cm^{-1} and 1553 cm^{-1}, respectively) that could be explained by intermolecular hydrogen bonding between dendrimer groups and drug.

In contrast the frequency of both S=O absorption bands shifted to higher frequency values (1330 cm^{-1} and 1159 cm^{-1}, respectively). The reason might be the interruption of intermolecular hydrogen bonding between furosemide molecules (Doherty and York, 1987) due to interaction of furosemide with the dendrimers. Furthermore, shifts to lower frequencies were also obtained for the C=O vibration of the ester groups (1731–1709 cm^{-1}), the amide I band (C=O stretching, 1640–1608 cm^{-1}) and amide II band (N–H bending/C–N stretching, 1541–1553 cm^{-1}) of the G2.5 PAMAM dendrimers (due to hydrogen bonding between drug and dendrimers). Moreover, the occurrence of the C=O absorption band (ester group of the G2.5 PAMAM) at the same wavelength (1731 cm^{-1}) in the furosemide-G2.5 PAMAM complex spectrum as in the pure G2.5 PAMAM spectrum led us to postulate that not all ester groups at the surface of the G2.5 PAMAM dendrimers were involved in hydrogen bonding.

A similar pattern was observed for the complexation of furosemide and G3 PAMAM dendrimers (Fig. 4b). As seen with the furosemide-G2.5 PAMAM complex, the complexation of G3 PAMAM dendrimers and furosemide yielded frequency shifts to lower frequencies for the C=O vibration (1636 cm^{-1}) and N–H vibration (1545 cm^{-1}) of furosemide due to hydrogen bonding to the dendrimers. Again, the symmetric S=O stretching band was obtained at a higher wavenumber (1155 cm^{-1}) indicating a reduction in the intermolecular hydrogen bonding between furosemide molecules. The asymmetric S=O stretching band could not be allocated precisely in the furosemide-G3 PAMAM complex spectrum as an overlapping in absorption with the pure G3 PAMAM occurred. The amide I band of G3 PAMAM also absorbed at a lower wavenumber (1607 cm^{-1}) when complexed with furosemide indicating that the amide group is involved in hydrogen bonding with furosemide. The characteristic absorption bands of PAMAM dendrimers (amide I, amide II and C=O stretching of ester group) were confirmed by data in literature (Liu et al., 2004; Popescu et al., 2006).

From the ATR-FTIR results it can be concluded that changes occurred in a series of intermolecular hydrogen bonds (furosemide–furosemide, dendrimer–dendrimer, furosemide–dendrimer) and that a complexation of furosemide and dendrimer occurred by hydrogen bonding.

3.3. Effect of pH on the solubility of furosemide in PAMAM dendrimer solutions

Additional insight into the furosemide–dendrimer interaction was sought after the increase in solubility was noted upon further addition of dendrimer to the complex. Therefore, an investigation was launched to investigate the effect of pH level on the

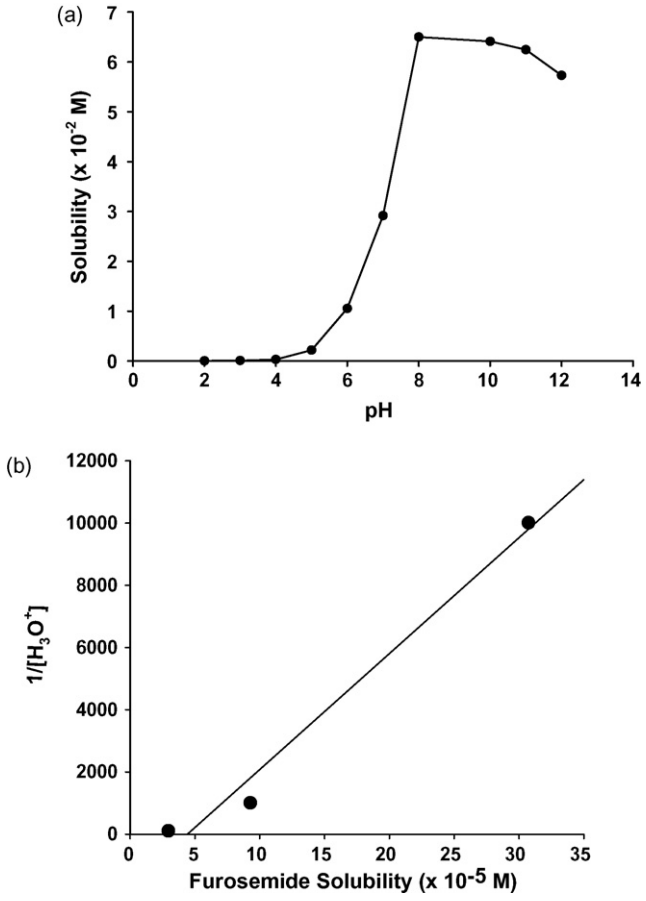

Fig. 5. (a) pH-solubility profile of furosemide in TRIS buffers at 30 ± 1 °C. (b) 1/[H_3O^+] vs. solubility plot of furosemide plotted according to the Henderson–Hasselbalch equation (1). ($y = 3.7 \times 10^6\, x - 1.6 \times 10^3$, $r^2 = 0.9815$; $K_a = 6.1 \times 10^{-4}$ M; p$K_a = 3.22$; $S_0 = 4.4 \times 10^{-5}$ M (14.6 μg/ml)).

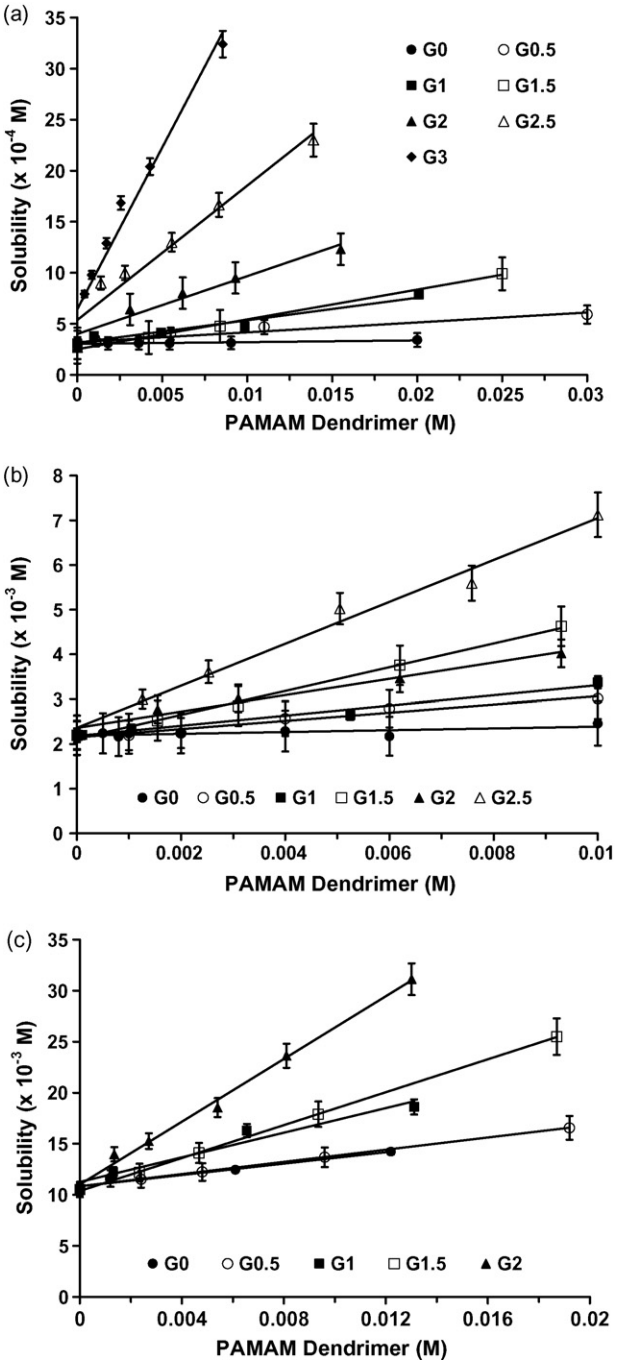

Fig. 6. Linear increase in the aqueous solubility of furosemide in the presence of increasing concentrations of amine- (closed symbols) and ester-terminated (open symbols) dendrimers at (a) pH 4.0, (b) pH 5.0 and (c) pH 6.0. Symbols represent experimentally determined values and lines best fits using Eq. (2).

solubility. Fig. 1 shows the unionized and the ionized forms of furosemide. The formation of the anion is responsible for the increase in solubility (stronger polar interaction with water) with increasing pH of the aqueous media as shown in Fig. 5. The solubility showed a minimum of 10 μg/ml at pH 2.0 and a maximum of 21.9 mg/ml at pH 8.0 (2000-fold increase), followed by a marginal decrease in solubility (∼18 mg/ml) as increased above pH 8.0. The curve demonstrated linearity in the region pH 2.0–4.0 and the intrinsic solubility and acidic pK_a of the furosemide calculated with the Henderson-Hasselbalch Eq. (1), were 14.62 and 3.22 μg/ml, respectively (Fig. 5) (Sinko, 2006).

Solubility measurements of furosemide–dendrimer complexes in pH-controlled media showed a positive, linear correlation between solubility (Higuchi A_L-type diagrams, Fig. 6) and dendrimer concentration except at pH 5.0 with G3 and at pH 6.0 with G2.5 and G3 dendrimers that showed Higuchi A_p-type diagrams (Fig. 7). A summary of the increase in solubility, calculated as the percentage increase in the aqueous solubility of furosemide per mM of added dendrimer, is given in Table 2. Furosemide is practically insoluble in water, $S_0 = 4.4 \times 10^{-5}$ M. The increased solubility of furosemide in the presence of PAMAM dendrimers could be due to non-covalent interactions between the drug and the macromolecules involving a variety of driving forces such as hydrogen bond formation, electrostatic interactions, and hydrophobic bonding (Higuchi and Connors, 1965).

The solubility Higuchi A_L-type diagrams shown in Fig. 6 indicates that soluble complexes between furosemide and the dendrimers have 1:1 stoichiometries (Higuchi and Connors, 1965). In the presence of G3 at pH 5.0 and pH 6.0 and G2.5 at pH 6.0 (Fig. 7) Higuchi A_p-type diagrams ($r^2 = 0.991 \pm 0.055$) were observed indicating that these dendrimers form multiple

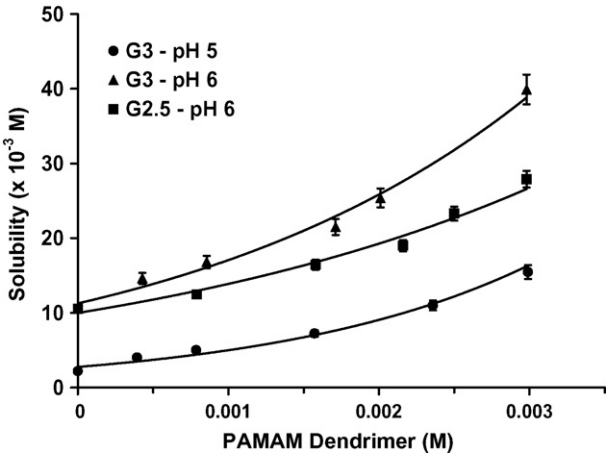

Fig. 7. Higuchi A_p-type solubility profiles of furosemide in the presence of increasing concentrations of dendrimers: G3 at pH 5.0; G2.5 at pH 6.0; G3 at pH 6.0. Symbols represent experimentally determined values and lines best fits using Eq. (3).

complexes with furosemide. Table 2 shows the equilibrium stability constants for the complexation between the drug and these dendrimers calculated using Eqs. (2) and (3). The fits for Eq. (2) (Higuchi A_L-type diagrams) were excellent with a mean $r^2 = 0.989 \pm 0.042$ for all profiles. At a given pH, the stability of the complexes increased with increasing generation size with amine-terminated dendrimers also proving superior to the ester-terminated dendrimers with the same number of surface functional groups.

In addition, the stability constants for complexes of furosemide with G1 and G0.5 dendrimers were similar to those reported for furosemide–cyclodextrin complexes which ranged the same orders of magnitude as the dendrimer complexes reported here (Özdemir and Ordu, 1998; Ammar et al., 1999; Vlachou and Papaioannou, 2003). Significantly higher stability constants were observed in the presence of G2, G3, G1.5, and G2.5 dendrimers indicating the formation of more stable dendrimer complexes. The fact that no burst release effects were observed, Fig. 8, additionally suggested that the drug molecules were encapsulated and not bound to a large extent to amines at the periphery of the dendrimers.

Table 2
Percentage increase in the solubility of furosemide per mM of added dendrimer and equilibrium stability constants (1:1 and 1:2) for dendrimer-furosemide complexes

pH	G*n*	Increased solubility (% per mM dendrimer added)	$K_{1:1}$ (M^{-1})	$K_{1:2}$ (M^{-1})
4	0	0.76 ± 0.02	6.38	–
	1	7.72 ± 0.02	83.74	–
	2	19.35 ± 0.9	222.3	–
	3	111.3 ± 1.6	1720	–
	0.5	3.87 ± 0.04	35.94	–
	1.5	10.62 ± 0.5	111.5	–
	2.5	27.92 ± 1.0	255.4	–
5	0	1.93 ± 0.01	12.21	–
	1	5.12 ± 0.02	55.24	–
	2	11.28 ± 0.06	133.8	–
	3	102.8 ± 0.9	–	241689
	0.5	3.70 ± 0.05	45.63	–
	1.5	8.63 ± 0.06	114.7	–
	2.5	21.88 ± 0.7	389.5	–
6	0	2.87 ± 0.02	36.67	–
	1	5.84 ± 0.02	144.6	–
	2	15.01 ± 1.0	4205	–
	3	98.39 ± 2.0	–	191528
	0.5	2.96 ± 0.04	40.24	–
	1.5	7.59 ± 0.08	394.0	–
	2.5	20.83 ± 0.3	–	199791

Full-generation PAMAM dendrimers have primary amines on the surface and tertiary amines in their internal cavities whereas half-generation dendrimers expose ester groups on their surface with internal tertiary amines (Fig. 1). The reported pK_a values of the primary amines (surface groups) are 7.0–9.0 and for the interior tertiary amines 3.0–6.0 (Tomalia et al., 1985; Ottaviani et al., 1996; Chen et al., 2000; Kleinman et al., 2000; Sideratou et al., 2000; Niu et al., 2003; Maiti et al., 2004, 2005). At physiological pH 7.4, most of the primary amines are protonated, and at pH 4.0 all of the tertiary amines are protonated. Therefore, the protonation level of the PAMAM could be altered by changing the solution pH, which in turn significantly affected the ability of the PAMAM dendrimer to interact with furosemide.

Furosemide is a weak acid with an ionizable carboxylic acid group with an experimentally determined pK_a of 3.22 and amino sulfonyl, chlorine, and furonyl methyl amino groups (Fig. 1). The COOH ionizable functional group might act as a counterion for the dendrimer amine groups thereby participating in the interaction between furosemide and the dendrimers. This was substantiated because at pH 2.0, no significant increase ($p > 0.05$) in the solubility of furosemide was observed because furosemide is in the unionized form at this pH and hence could not interact electrostatically with the ionized dendrimer moieties. Conversely, at pH 4.0–6.0 the furosemide carboxylic group would be in its ionized form and therefore, the increase in the solubility of furosemide in the presence of dendrimers between pH 4.0–6.0 would probably be due to the electrostatic interactions between the positively charged tertiary amines of the dendrimers and the negatively charged carboxylate anion of the furosemide (Figs. 6 and 7). In this pH range most of the surface NH_2 groups of the full generation PAMAM's will be protonated with the interior tertiary amines at least partially protonated.

For a given generation size, the solubility of furosemide did not increase significantly ($p > 0.05$) when the pH of the aqueous medium was increased from pH 4.0–6.0 (Table 2), since the dendrimers would have the same low surface charge density in this pH-range. At a given pH; however, the solubility of furosemide increased significantly with an increase in PAMAM dendrimer generation size.

This could be due to an increase in the number of interior binding sites available with an increase in generation size (Tables 1 and 2). The solubility of furosemide also increased significantly ($p < 0.05$) in the presence of ester-terminated half-generation dendrimers (Table 2). Conversely, at a given pH, when compared to the full-generation dendrimers, the increase in the solubility of furosemide was significantly lower in the

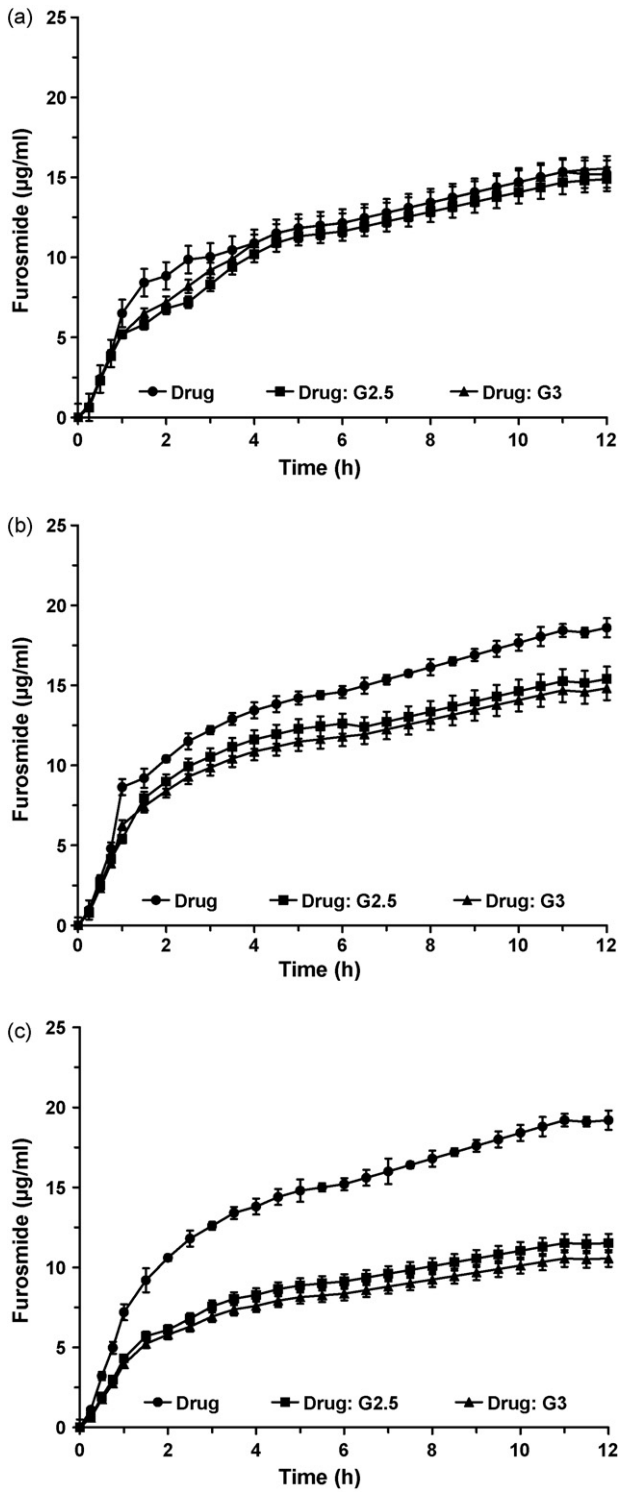

Fig. 8. In vitro release of furosemide from furosemide: PAMAM dendrimer complexes compared with the diffusion of a furosemide solution: (a) SGF, pH 1.2; (b) furosemide tablet dissolution medium (USP) pH 5.8; (c) SIF pH 7.4 ($n = 3$).

presence of the half-generation dendrimers. This was because these dendrimers have non-reactive surface groups and the interior tertiary amines of the half-generation dendrimers are less susceptible to protonation (Ottaviani et al., 1996). Therefore, these dendrimers would interact with furosemide mainly via hydrogen bond formation and only to a diminished extent by electrostatic interactions.

Maiti et al. (2005) found that in the presence of a polar solvent like water, significant penetration of water throughout the interior of the dendrimer causes the dendrimer structure to swell. The extent of swelling depends on the extent of protonation. At high pH > 12.0 there was no protonation, however the mere presence of a good solvent such as water increased the size of the dendrimer by almost 10–15% (Maiti et al., 2005). This trend was observed in all the generations reported in this paper. At neutral pH, all the primary amines are protonated and there was no significant change in the dendrimer size as was evident from very small increase in radius of gyration, indicating a limited degree of swelling (Maiti et al., 2004, 2005).

As the pH was lowered further (pH < 4.0), almost all tertiary amines were protonated and at that protonation level, the dendrimer size increased almost 30–40% in water compared to the case when no solvent is present. This results from the favorable interaction of the solvent with the primary and tertiary amines. It was concluded that, depending on the degree of protonation, a significant portion of the counterions condensed within the dendrimer, residing very close to the protonated sites with some moieties solvated by the water. It is therefore possible to assume that for ionized furosemide as the protonation level of the dendrimer increased, more furosemide molecules would reside inside the dendrimer since the dendrimer swelled more at higher protonation levels (Kleinman et al., 2000; Maiti et al., 2005).

In another study, Beezer et al. (2003) observed that all drug/dendrimer complexes were unstable at pHs less than pH 7 and the bound substrate begins to precipitate after only 10 min at pH 6. This suggests that it is the nitrogens within the dendrimer that are important with respect to binding, for as they are protonated (at pH 6 and below), the ability of the dendrimer to bind its guest is lost. This implied that the binding was probably due to a simple ion-pairing mechanism. The internal tertiary nitrogens are strongly basic, the pK_a of an aqueous solution of PAMAM dendrimers was measured as 9.5, and are therefore capable of deprotonating the acidic guest molecules. The ensuing quaternized nitrogens can then bind to the resulting carboxylate counter-ions. From the observed pH dependence of binding, we concluded that it was only possible for acidic guest molecules to bind within the dendrimers interior, as nifedipine and other small non-polar molecules could not be retained within the dendrimer (Beezer et al., 2003; Devarakonda et al., 2004).

Further studies on pH-dependent furosemide solubility were conducted by preparing saturated solutions of furosemide in aqueous medium at pH 2.0 and 7.0 and the effect of G2.5 and G3 PAMAM addition was observed. The solubility of furosemide was increased from 9.9 to 9640 μg/ml (1000-fold increase) by increasing the pH from 2.0 to 7.0. A contrasting trend was observed when different concentrations of the dendrimers were added to the saturated solutions (devoid of excess undissolved drug) (Table 3), i.e. furosemide solubility was decreased with increasing PAMAM dendrimer concentrations due to the interaction between the drug and dendrimer to form complexes that did not diffuse out of the dialysis tubes. This effect resulted in an

Table 3
Effect of the addition of G2.5 and G3 PAMAM dendrimers to saturated aqueous furosemide solutions at different pH on the solubility of furosemide

pH	Solubility (μg/ml)	Dendrimer added (%)	G2.5 PAMAM		G3 PAMAM	
			pH[a]	Solubility (μg/ml)	pH[a]	Solubility (μg/ml)
2	9.9 ± 1.2	0.1	3.0	8.8 ± 0.4	3.1	8.4 ± 0.4
		0.2	3.4	8.5 ± 0.5	3.6	8.1 ± 0.2
		0.5	3.6	7.9 ± 0.2	3.7	7.5 ± 0.1
7	9640 ± 78	0.1	8.3	2345 ± 21	8.4	878 ± 13
		0.2	8.2	1141 ± 11	8.5	439 ± 8
		0.5	8.0	469 ± 8	8.6	177 ± 4

[a] Change in pH upon addition of dendrimer.

increase in the concentration of furosemide inside the dialysis tube whilst the concentration outside the tube decreased.

For both dendrimers the relative decrease in furosemide solubility was significantly higher at pH 7.0 (75–98%) than at pH 2.0 (11–24%) since more drug molecules dissolved in the pH 7.0 solution (more ionized), resulting in more pronounced phase-separation after addition of dendrimer solution. This observation was consistent with the change in solubility seen with a change in the protonation level of the drug and dendrimer as a function of pH seen in Figs. 6 and 7. The NH_2 terminated PAMAM dendrimer at pH ~ 8.5 (pH changed from 7 to 8.5 after the addition of the dendrimer) interacted strongly with the ionized drug because the amount of free drug in solution decreased by 90–98% depending on the amount of dendrimer added (0.1–0.5%) as seen in Table 3.

At pH ~ 8 the $COOCH_3$ terminated G2.5 dendrimer also significantly interacted with ionized furosemide because the solubility decreased by 75–95% with the addition of the same concentrations of dendrimer. However, the decrease in furosemide solubility, Table 3, was significantly greater for the G3 compared to the G2.5 dendrimer.

3.4. Release studies

The in vitro release of furosemide from the furosemide–PAMAM dendrimer complexes was determined in SGF without pepsin (pH 1.2), SIF without pancreatin (pH 7.4) and the USP dissolution medium for furosemide (phosphate buffer pH 5.8). The results are shown in Fig. 8. The release profiles of the furosemide–dendrimer complexes were compared with that for pure furosemide using a similarity factor (Eq. (4)) (Moore and Flanner, 1996) to see whether the rate and extent of furosemide release was different from that of the solutions.

$$f_2 = 50 \log \left\{ \left[1 + \left(\frac{1}{n} \right) \sum_{i}^{n} (R_i - T_i)^2 \right]^{-0.5} 100 \right\} \quad (4)$$

where n is the number of time points, R_t is dissolution value of the reference sample at time t, and T_t is the dissolution value of the test sample at time t. The similarity factor is a simple model independent approach using mathematical indices to define differences and similarities between dissolution profiles. These factors are divided from Minkowski-differences (average absolute differences) and mean-square difference respectively. When $f_2 = 100$ then the test and reference mean profiles are identical. The test and reference products are not equivalent when there is larger than 10% difference between dissolution profiles, indicated by a similarity factor $f_2 < 50$ (Moore and Flanner, 1996).

The f_2 values of the control solutions were different at the different pH values. The order of release was pH 7.4 ≥ pH 5.8 > pH 1.2. Although release at pH 1.2 seemed not to be the same as shown in Fig. 8(a) calculated f_2 values ranged from 53 to 66 indicating that the release profiles were within 10% over the extent of the release profiles. Therefore, the ranking of the control solution, drug-G2.5-PAMAM and drug-G3-PAMAM were equivalent at pH 1.2. This was perhaps not surprising considering that furosemide would be virtually unionized at this pH.

At pH 5.8 f_2 values indicated that the release from the G2.5 complex was similar to that of the G3 complex ($f_2 = 65$), however these profiles differed by more than 10% from that of the control solution ($f_2 = 36$ and 32). The release from the G2.5 ($f_2 = 16$) and G3 ($f_2 = 13$) complexes at pH 7.4 was significantly slower than from the control solution with the release from the G2.5 and G3 complex showing very similar release profiles ($f_2 = 61$). In addition, the release from the complexes at pH 7.4 was even slower than from the complexes at pH 5.8 and pH 1.2 ($f_2 < 50$).

The release results indicate that in addition to solubilizing furosemide the PAMAM dendrimers could be utilized to control the release of this drug. At low pH, the dendrimers release the drug very fast and the diffusion out of the dialysis tube is the same as for the control solution of the drug. In contrast at neutral pH the interaction between the drug and dendrimer was significantly enhanced and decelerated the release of the drug. Again the differences in release rate can be correlated to a combination effect of the ionization state of the drug and the PAMAM dendrimers.

4. Conclusion

In this study, the increase in the solubility of the practically insoluble drug furosemide in combination with low generation water-soluble polyamidoamine (PAMAM) dendrimers ($G < 4$) was demonstrated for the first time. FTIR and solubility studies of the complexes suggested that furosemide was encapsulated in the dendrimer cavity. The increase in solubility of the drug by the dendrimers was determined by the pH of the aqueous medium, generation size, and type and number of internal ter-

tiary amine groups. The increase in the solubility of furosemide in the presence of dendrimers was primarily due to the electrostatic interactions between the positively charged tertiary amines of the dendrimers and negatively charged carboxylate anion of furosemide, resulting in a favorable solubilization effect.

Overall, the results showed that the PAMAM dendrimers could be exploited to improve the solubility and dissolution of furosemide, however that the enhancement depended on the choice of dendrimer, generation size, and surface functional group of the dendrimer. Careful selection of the counterion species exposed by the dendrimer markedly influenced the drug–dendrimer interaction during the drug release process at a given pH. In acidic dissolution media, both drug and dendrimer were fully protonated and abolished the electrostatic association with a resultant high drug release. Conversely at neutral pH, the ionization states of both the drug and dendrimer favored electrostatic interaction with resultant slower release.

From this study we conclude that the ionization state of the drug molecule dominated the solubility and release properties of the drug–dendrimer complex and could be exploited in future for the formulation of pharmaceutical products containing poorly water-soluble drugs. In addition, to provide even more effective encapsulation, higher generations of dendrimers could be employed to compare it to the dendrimers studied here.

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