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Drug release characteristics of PAMAM dendrimer–drug conjugates with different linkers

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ABSTRACT

Drug release from polymer–drug conjugates plays a crucial role on the efficacy. This is especially true for dendrimers where there is a steric crowding at the surface. The drug release characteristics of G4-polyamidoamine (PAMAM) dendrimer–ibuprofen conjugates with ester, amide, and peptide linkers were investigated, in addition to a linear PEG–ibuprofen conjugate to understand the effect of architecture and linker on drug release. Ibuprofen was directly conjugated to NH_2-terminated dendrimer by an amide bond and OH-terminated dendrimer by an ester bond. A tetra-peptide-linked dendrimer conjugate and a linear mPEG–ibuprofen conjugate were also studied for comparison to direct linked dendrimer conjugates. Amide-linked conjugates were relatively stable against hydrolysis, whereas the ester-linked conjugates showed pH-dependent release and the extent of release varied with pH from 3% (pH 5) to 38% (pH 8.5) for the 10-day period studied. Direct amide- and ester-linked conjugates did not release ibuprofen enzymatically in cathepsin B buffer and diluted human plasma. In contrast, mPEG conjugate released 65% of its payload within 12 h in diluted plasma by esterase activity, and the peptide-linked dendrimer conjugate released 40% of its payload within 48 h by cathepsin B activity. It is demonstrated that the steric crowding at the surface of PAMAM dendrimer–drug conjugates, along with linking chemistry govern the drug release mechanisms as well as kinetics. Understanding these structural and steric effects on their drug release characteristics is crucial for the design of dendrimer conjugates with high efficacy.

1. Introduction

Polyamidoamine (PAMAM) dendrimers are highly hyperbranched synthetic polymers with well-defined spherical structure and nanometer scale size (Esfand and Tomalia, 2001). Their diameters increase with each generation while molecular weight and number of functional surface groups double (Svenson and Tomalia, 2005). The functional surface groups make them very hydrophilic and highly water soluble (Gillies and Frechet, 2005). These surface functional groups are often used to covalently attach drugs, targeting ligands or imaging agents for targeted delivery, controlled release, or imaging applications (Lee et al., 2005).

Small drug–dendrimer conjugates aim to carry therapeutic agents to specific tissues in order to reduce systemic effects and increase efficacy at the targeted site. For such a strategy to be effective the conjugates should be stable until they reach the site of interest and then release the drugs within the target tissue before they are eventually cleared from the body (Vicent and Duncan, 2006). Premature release or high stability of the conjugate will render the conjugate delivery system ineffective since most small drugs will not be active in conjugated form. Therefore one key challenge in the preparation of dendrimer–drug conjugates is to design systems that can release their payloads specifically at the desired tissue in a predetermined fashion. In order to predict the efficacy of dendrimer conjugates *in vivo*, determination of drug release profiles in conditions that conjugates would go through is necessary. Better understanding of the release mechanisms and profiles of PAMAM dendrimer–drug conjugates will help design effective delivery systems.

Amide and ester bonds are most commonly used for the conjugation of small drugs to polymers whereas other linkages such as disulfide bonds have also been demonstrated (Najlah et al., 2006; Patri et al., 2005; Gurdag et al., 2006; Navath et al., 2008). Preparation and *in vitro* efficacy of PAMAM dendrimer conjugates of ibuprofen, methylprednisolone, and N-acetyl cysteine were recently reported (Khandare et al., 2005; Kolhe et al., 2006; Kurtoglu et al., 2009). One of the objectives of the current work is to determine the release characteristics of such amide-

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and ester-linked G4-PAMAM dendrimer conjugates for prospective intravenous and oral applications. Previously, G5-PAMAM dendrimers conjugated to methotrexate (MTX) with an ester linkage were shown to be stable in PBS buffer (pH = 7.4) for 2.5 h (Patri et al., 2005). In another study, Generation 0 (MW = 517 Da) PAMAM dendrimers were conjugated to Naproxen via ester and amide bonds and their release kinetics were investigated (Najlah et al., 2006, 2007). It was demonstrated that conjugates showed pH-dependent hydrolysis and also enzymatic cleavage. However, reported results do not readily establish the release characteristics of higher generation PAMAM dendrimer conjugates (such as Generation 4, MW = ~14 kDa, diameter = ~4 nm) considering the molecular weight and corresponding size difference.

The 3D spherical nanostructure of dendrimers makes enzymatic release sterically challenging compared to linear polymers such as polyethylene glycol (PEG) and N-(2-hydroxypropyl) methacrylamide (HPMA). Therefore, using linkers that provide spacing from the dendrimer surface and specificity for enzymatic cleavage may be necessary. For this purpose, enzymatically cleavable Gly-Leu-Phe-Gly tetra-peptide is a good candidate as a linker for PAMAM dendrimer conjugates and it was extensively studied for linear polymer conjugates (Kopecek et al., 2000; Duncan, 2003).

In this study, delivery of ibuprofen; a non-steroidal anti-inflammatory drug, widely used to reduce pain, fever, and inflammation, is investigated by conjugation to Generation 4 PAMAM dendrimers via ester, amide and peptide linkages. Combining the targeting capability of PAMAM dendrimers with a better understanding of their release characteristics will improve the ability to design conjugates with high efficacy for inflammatory conditions.

2. Materials and methods

2.1. Materials

Polyamidoamine (PAMAM) dendrimers (Generation 4, with $-NH_2$ and –OH end groups, ethylenediamine (EDA) core) were purchased from Dendritech (Midland, MI, USA) in methanol solution. Polyethylene glycol monomethyl ether (mPEG, MW = 5000 Da, polydisperse) was obtained from Fluka (Steinheim, Germany) Ibuprofen-USP (Racemic form), N,N-dicyclohexyl carbodiimide (DCC) and 4(dimethylamino)pyridine (DMAP) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). Glycine-Leucine-Phenylalanine-Glycine (GLFG) peptide linker was purchased from Biomatik Corporation (Wilmington, DE). Cathepsin B from human liver and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). N-hydroxysuccinimide (NHS) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) was obtained from Pierce (Rockford, IL). Regenerated cellulose (RC) dialysis membrane with molecular weight cut-off of 1000 Da was obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA). Pooled human plasma was purchased from Innovative Research (Southfield, MI). Anhydrous dimethyl sulfoxide (DMSO) was purchased from Acros Organics (Morris Plains, NJ). All other solvents and chemicals used were purchased from Fisher Scientific (Waltham, MA).

2.2. Synthesis of conjugates

2.2.1. Synthesis of G4-NH_2–Ibu

Ibuprofen (57 mg, 276 mmol) and DCC (57 mg, 276 mmol) was dissolved in 2 ml of anhydrous DMSO in a reaction flask and stirred for 2 h at room temperature for the activation of ibuprofen. PAMAM-G4-NH_2 (131 mg, 9.2 mmol) was dissolved in 2 ml of anhydrous DMSO and added to the reaction mixture. 30 M equivalent of ibuprofen was added to the G4-NH_2 dendrimer. The reaction was continuously stirred for 72 h at room temperature under nitrogen purge. The reaction mixture was then filtered through filter paper to remove dicyclohexyl urea (DCU) formed. The filtrate was then purified using dialysis membrane (MWCO = 1000) for 36 h, to remove unreacted ibuprofen and DCC. The dialysis buffer (DMSO) was refreshed every 12 h during dialysis. Dialyzed product was frozen at −40 °C and lyophilized overnight. The final product weighted 86 mg (62% yield) and was analyzed by proton NMR and RP-HPLC. ^{1}H NMR (DMSO-d_6): 0.81 (6H, d, 2XCH_3, Ibu), 1.26 (3H, d, CH–CH_3, Ibu), 1.78 (1H, m, –CH–2XCH$_3$, Ibu), 2.18–3.25 (990H, m, aliphatic proton of G4-NH_2 and CH–CH_2 of Ibu), 3.61 (1H, q, CH–CH$_3$, Ibu), 7.11–7.19 (4H, m, aromatic protons, Ibu), 7.91 (124H, m, CO–NH, G4-NH_2) (Fig. 1).

2.2.2. Synthesis of G4-OH–Ibu

Ibuprofen (47 mg, 228 μmol), DCC (47 mg, 228 μmol) and DMAP (40 mg, 327 μmol) were dissolved in 2 ml of anhydrous DMSO in a reaction flask. G4-OH (107 mg, 7.5 μmol) was dissolved in 2 ml of anhydrous DMSO. After activation of ibuprofen, dendrimer solution was added, the reaction and purification steps were carried as described above. The final product weighted 103 mg (yield 88%) and was analyzed by proton NMR and RP-HPLC. ^{1}H NMR (DMSO-d_6): 0.64 (6H, d, 2XCH_3), Ibu, 1.24 (3H, d, CH–CH_3, Ibu), 1.60 (1H, m, –CH–2XCH$_3$, Ibu), 2.19–3.61 (998H, m, aliphatic protons of G4-NH_2; CH–CH_2 and CH–CH$_3$ of Ibu), 6.81–7.02 (4H, m, aromatic protons, Ibu), 7.75–7.97 (60H, m, CO–NH, G4-NH_2) (Fig. 2).

2.2.3. Synthesis of mPEG–ibuprofen

mPEG (240 mg, 7.5 μmol) polymer was dissolved in 4 ml of anhydrous DMSO. Ibuprofen (122 mg, 592 μmol), DCC (142 mg, 689 μmol) and DMAP (24 mg, 196 μmol) were dissolved in a reaction flask. After activation of ibuprofen, polymer solution was added, the reaction and purification steps were carried as described above. The final product weighted 132 mg (yield 53%) and was analyzed by proton NMR and RP-HPLC. ^{1}H NMR (DMSO-d_6): 0.82 (6H, d, 2XCH_3, Ibu), 1.34 (3H, d, CH–CH_3, Ibu), 1.78 (1H, m, –CH–2XCH$_3$, Ibu), 2.38 (2H, d, CH–CH_2, Ibu), 3.22 (3H, s, OCH_3, mPEG), 3.49 (452H, s, –OCH$_2$–CH$_2$, mPEG), 4.09–4.11 (1H, q, CH–CH$_3$, Ibu), 7.07–7.17 (4H, m, aromatic protons, Ibu) (Fig. 3).

2.2.4. Synthesis of G4-GFLG–Ibu

Ibuprofen (50 mg, 242 μmol), NHS (700 mg, 6086 μmol) and 463 mg EDC (463 mg, 2424 μmol) were reacted in dichloromethane (DCM) for 2 h. The mixture was extracted by deionized water and activated ibuprofen ester was isolated. Gly-Phe-Leu-Gly tetra-peptide linker (100 mg, 255 μmol) was added in anhydrous DMSO and the mixture was reacted overnight. NHS and EDC were added again in the same amounts as above and reacted for 2 h. G4-NH_2 dendrimer (350 mg, 24 μmol) was added to the reaction mixture and reacted overnight. The reaction mixture was then dialyzed against DMSO overnight followed by dialysis against deionized water. The final product weighted 332 mg (yield 88%) and was analyzed by proton NMR and RP-HPLC. ^{1}H NMR (DMSO-d_6): 0.72–0.82 (m, 2XCH$_3$ of Ibu and 2XCH_3 of peptide), 1.24–1.40 (m, CH–CH_3, Ibu and peptide protons), 2.17–3.79 (m, aliphatic protons of G4-NH_2, CH–CH_2 of Ibu and peptide aliphatic protons), 4.20 (m, peptide protons), 4.42 (m, peptide protons), 6.98–7.19 (m, aromatic protons of Ibu and aromatic protons of peptide), 7.90–8.26 (m, CO–NH of G4-NH_2 and amidic protons of peptide) (Fig. 4).

2.3. Characterization of conjugates

PAMAM and mPEG conjugates of ibuprofen were characterized by ^{1}H NMR spectroscopy (Varian 400 MHz). HPLC characteriza-

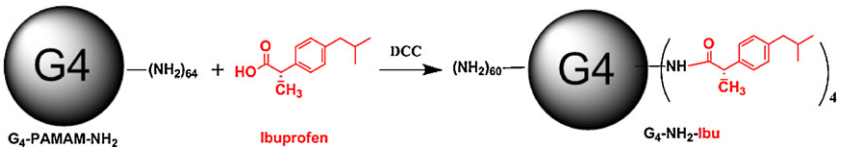

Fig. 1. Synthesis of G4-NH_2–ibuprofen conjugate with amide linkage.

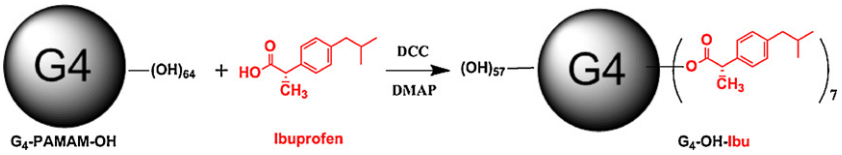

Fig. 2. Synthesis of G4-OH–ibuprofen conjugate with ester linkage.

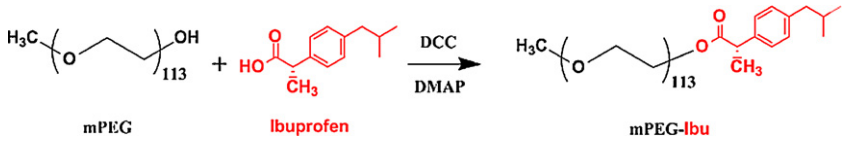

Fig. 3. Synthesis of mPEG–ibuprofen conjugate with ester linkage. Median number of monomer units is shown.

tion was carried out with Waters HPLC Instrument equipped with two pumps, an autosampler and dual UV detector interfaced to Breeze software. The dual UV absorbance detector was used at 210 and 264 nm simultaneously. The mobile phase used was water/acetonitrile (0.14% TFA). Mobile phases were freshly prepared, filtered and degassed prior to the use. Supelco Discovery BIO Wide Pore C5 HPLC Column (5 μm particle size, 25 cm × 4.6 mm length × I.D.) equipped with a C5 Supelguard Cartridges (5 μm particle size, 2 cm × 4.0 mm length × I.D.) was used for the characterization of the conjugates as well as for release studies. Gradient method used for analysis was (100:0) water/acetonitrile to (40:60) in 60 min. The flow rate was set at 1 ml/min.

2.4. Hydrolytic release studies

The hydrolysis of conjugates was studied at pH 1.2 (0.1 M hydrochloric acid buffer), pH 5.0 (0.1 M citrate buffer), pH 7.4 (0.1 M phosphate buffered saline), and pH 8.5 (0.1 M borate buffer). All experiments were performed in triplicate along with controls. 3 mg of each conjugate were added into 3 ml preheated buffer solutions. All the release solutions containing 1 mg/ml dendrimer drug conjugate were stirred continuously and maintained at 37 °C. At appropriate time intervals samples were withdrawn and the reaction was quenched by the addition of methanol solution of sodium salicylate (1.25 mM) at −40 °C. Sodium salicylate was used as internal standard. The samples were stored at −80 °C until HPLC analysis. UV absorbance peak area of ibuprofen was used to derive a calibration and used to determine the concentrations of released ibuprofen in samples.

2.5. Enzymatic release studies

The hydrolysis of PAMAM dendrimer conjugates and mPEG conjugate with esterase activity was studied in pooled human plasma diluted to 80% with 0.1 M phosphate buffer. The conjugates were dissolved in 3 ml preheated plasma solutions at a concentration of 1 mg/ml. The solutions were continuously stirred and maintained at 37 °C. At appropriate time intervals samples were withdrawn and methanol solution of sodium salicylate (1.25 mM) at −40 °C was added to deproteinize the plasma and quench the reaction. The samples were then centrifuged for 5 min at 10,000 rpm and the clear supernatant was collected. The final samples were stored at −80 °C until HPLC analysis. The hydrolysis of conjugates with protease activity was studied in the presence of cathepsin B. Conjugates were dissolved at 1 mg/ml concentration in 0.1 M acetate buffer (pH = 5) solution at 37 °C which contained 0.01 M EDTA, 0.05 M reduced glutathione and 0.5×10^{-6} M cathepsin B. Cathepsin B was replenished every 24 h to maintain protease activity. At appropriate time intervals samples were withdrawn and analyzed immediately by RP-HPLC.

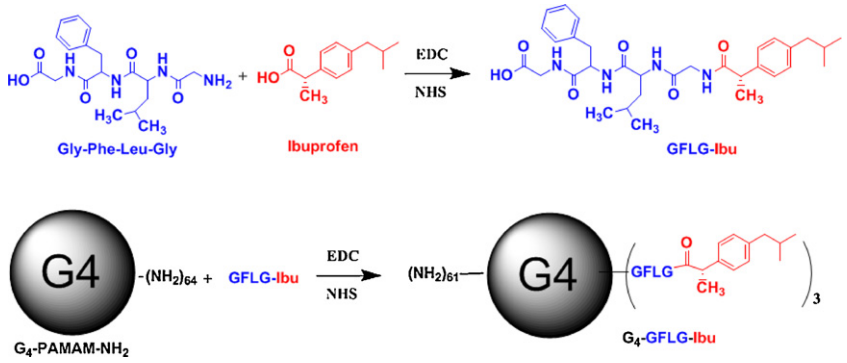

Fig. 4. Synthesis of G4-GFLG–ibuprofen conjugate.

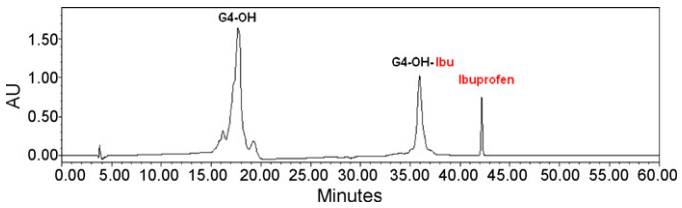

Fig. 5. RP-HPLC, UV absorbance chromatogram at 210 nm, of G4-OH, ibuprofen and G4-OH–Ibu conjugate solution prepared for the demonstration of different elution times.

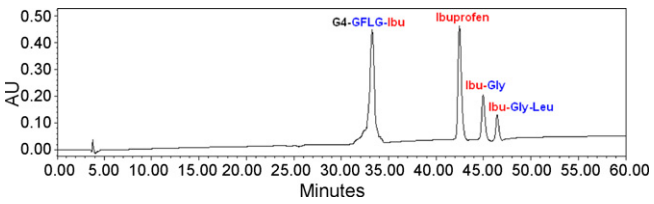

Fig. 6. RP-HPLC, UV absorbance chromatogram at 210 nm, a solution of G4-GFLG–ibuprofen conjugate, ibuprofen and Gly-Ibu, Gly-Leu-ibuprofen intermediates was injected for demonstration of different elution times.

3. Results and discussion

3.1. Synthesis and characterization of conjugates

Amide-linked ibuprofen conjugates of G4-NH_2 dendrimer were prepared by bonding the carboxyl group of ibuprofen and primary amine groups of the dendrimer by a DCC coupling reaction. Peaks at aliphatic region in between 0.5 and 1.5 ppm confirm the formation of the product. Drug payload of the conjugate was determined by ^{1}H NMR spectroscopy proton integration method, using the characteristic aromatic proton peaks of ibuprofen and aliphatic protons of PAMAM dendrimer. The conjugate was also characterized by RP-HPLC. The calculated ibuprofen payload of G4-NH_2–Ibu conjugate was 4.1 per dendrimer molecule (5.6% by weight). Ester-linked ibuprofen conjugates of G4-OH dendrimer was prepared by attaching the carboxyl group of ibuprofen to hydroxyl group of the dendrimer by a DCC/DMAP coupling reaction. Formation of product was confirmed by Peaks at aliphatic region in between 0.5 and 1.5 ppm. Drug payload of the conjugate was determined by ^{1}H NMR spectroscopy proton integration method as 6.7 ibuprofen molecules per dendrimer molecule (8.8% by weight). The conjugate was also characterized by RP-HPLC (Fig. 5). The distinct peaks for free drug, dendrimer and the conjugate were used to confirm that the conjugation reaction was successful, and the product is pure. The hydroxyl group of mPEG and carboxyl group of ibuprofen was used to form ester-linked mPEG conjugates. Two doublets at aliphatic region, 0.8 and 1.3 ppm confirm the formation of the product. Peaks at aromatic region support the formation of the conjugate. The payload of the conjugate was one ibuprofen molecule per mPEG molecule which corresponds to 4% by weight, as determined by RP-HPLC and ^{1}H NMR analysis.

A two-step reaction scheme was used to minimize polymerization of the peptide linker and to increase reaction yield. Activated ester form of ibuprofen was formed by EDC/NHS reaction and this activated ester form was isolated before reacting with the free amine group of the peptide linker. Following the formation of peptide–ibuprofen conjugate, amine groups of the G4-NH_2 dendrimer was reacted with the free carboxyl group of the peptide forming the final G4-GFLG–Ibu conjugate. Two doublet peaks of peptide linker in between 1.3 and 1.6 ppm and one doublet peak of ibuprofen at 0.8 ppm confirm the formation of the product. Multiplets in between 7.0 and 7.2 ppm for aromatic protons of ibuprofen as well as peptide linker support the formation of the conjugate. Two characteristic multiplets at 4.2 and 4.4 ppm of peptide linker also confirm the formation of the G4-GFLG–Ibu conjugate. Drug payload of the conjugate was calculated as three ibuprofen molecules per dendrimer (3.9% by weight) by ^{1}H NMR proton integration method. The conjugate as well as the peptide–ibuprofen fractions that may form were also characterized by RP-HPLC (Fig. 6).

A summary of the four synthesized conjugates is shown in Table 1. The drug payload varied from about 4% to 9% by weight and all the conjugates were soluble in the buffers studied up to 10 mg/ml conjugate concentration (highest concentration tested). The drug payload was kept to these levels in order to enhance the solubility of the conjugates. At very high drug payloads, the solubility in water would be compromised to some extent.

3.2. Release studies

3.2.1. pH dependence

Understanding drug release characteristics of ester and amide bonds is a key step in designing dendrimer conjugates with high efficacy. The drug release mechanism for the conjugates can be enzymatic or pH-dependent hydrolysis. In order to investigate the hydrolysis kinetics, the drug release rates of PAMAM dendrimer conjugates were studied at pH 1.2, 5.0, 7.4, and 8.5. PBS buffer (pH = 7.4) was used to investigate the stability at physiological pH. PAMAM dendrimer conjugates are taken up mainly by endocytosis and spend considerable time in lysosomes (Kannan et al., 2004; Perumal et al., 2008), therefore citrate buffer was used to demonstrate hydrolytic release profile at lysosomal pH (pH = 5.0). HCl buffer (pH = 1.2) and borate buffer (pH = 8.5) were also used in order to analyze the release characteristics in oral routes and for comparison purposes.

Both G4-NH_2–Ibu and G4-GFLG–Ibu conjugates with amide bonds were quite stable against hydrolytic cleavage for the 10-day time period studied and released less than 3% of the conjugated ibuprofen at pH = 1.2 (data not shown). At other pH conditions the release was even slower. This result confirms that for effective drug release, the amide bonds are too stable for hydrolysis and an enzymatic release mechanism should be sought in designing conjugates with this type of linkage. On the other hand, ester-linked G4-OH–Ibu and mPEG–Ibu conjugates were more liable to hydrolysis and the release rates were determined to be pH dependent. The drug release profiles of G4-OH–Ibu conjugate in the buffers studied are shown in Fig. 7. At pH = 7.4 G4-OH–Ibu released 12% of the ibuprofen attached within 10 days. At pH = 8.5 the hydrolysis rate was faster with over 42% of the drug being released at the same time interval. Acidic hydrolysis of G4-OH–Ibu conjugate showed similar trends with basic hydrolysis but it was relatively slower. G4-OH–Ibu released 36% of its payload at pH = 1.2, whereas only 4% of ibuprofen was released at pH = 5 within 10 days. The linear mPEG–Ibu conjugate with ester bond had similar hydrolytic release profile to G4-OH–Ibu conjugate (data not shown). The pH-dependent drug release from the conjugate followed zero order kinetics and the rate constant and the half-life values are tabulated in Table 2. The results indicate that PAMAM dendrimer conjugates with ester linkages can provide sustained release of ibuprofen.

Table 1
Properties of ibuprofen conjugates based on ^{1}H NMR and HPLC analysis.

Conjugate	MW of polymer	MW of conjugate	Ibu/polymer mole ratio	Ibu content (% by weight)
G4-OH–Ibu	14,279	15,659	6.7	8.8
G4-NH_2–Ibu	14,215	15,060	4.1	5.6
mPEG–Ibu	5,000	5,206	1.0	4.0
G4-GFLG–Ibu	14,215	16,009	3.0	3.9

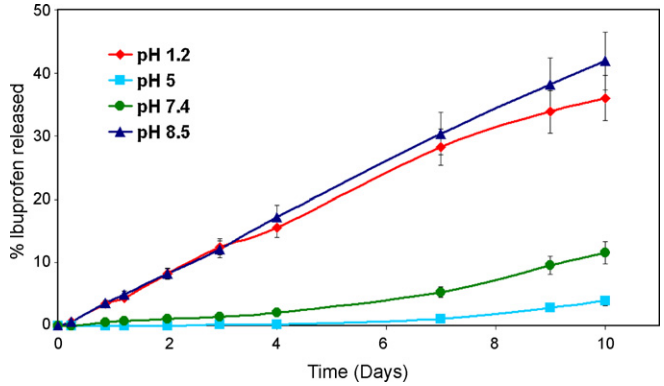

Fig. 7. Ibuprofen release profile of G4-OH–ibuprofen conjugate in four different buffers determined by HPLC analysis. All samples were studied in triplicate for statistical analysis. The cumulative percentage of ibuprofen that has been cleaved from the conjugate at each time point is shown. The release rates are significantly higher at pH 1.2 and 8.5 compared to pH 5.0 and 7.4 while the release rate at pH 5 was the slowest.

Table 2
Zero order release kinetics of G4-OH–ibuprofen conjugates.

	pH 1.2	pH 5	pH 7.4	pH 8.5
k (M/day)	1.65×10^{-5}	1.19×10^{-6}	4.18×10^{-6}	1.83×10^{-5}
$t_{1/2}$ (days)	13.0	179.4	51.1	11.6

3.2.2. Enzymatic release in plasma

Release characteristics of the conjugates were analyzed in 80% diluted human plasma for the demonstration of the drug release by esterase activity. It was previously shown that ester-bonded G0 dendrimer conjugates were hydrolyzed in diluted plasma within 5 h (Najlah et al., 2006). Therefore ester-bonded conjugates G4-OH–Ibu and mPEG–Ibu were expected to release their payload at enzymatic conditions. Surprisingly, while mPEG–Ibu conjugate was a good substrate for esterase enzymes in plasma, G4-OH–Ibu conjugate did not show enzymatic release (Fig. 8). The stability of G4-OH–Ibu against enzymatic hydrolysis suggests that the conjugate structure blocks esterase enzyme activity to cleave the ester linkage. While Generation 0 PAMAM dendrimer conjugates are readily cleaved by esterase enzymes in plasma, 3D spherical nanostructure of Generation 4 PAMAM dendrimer prevents this mechanism. This steric property provides a unique way to prevent premature drug release while in blood circulation. The ester-linked conjugate can be used for sustained delivery of ibuprofen for up to

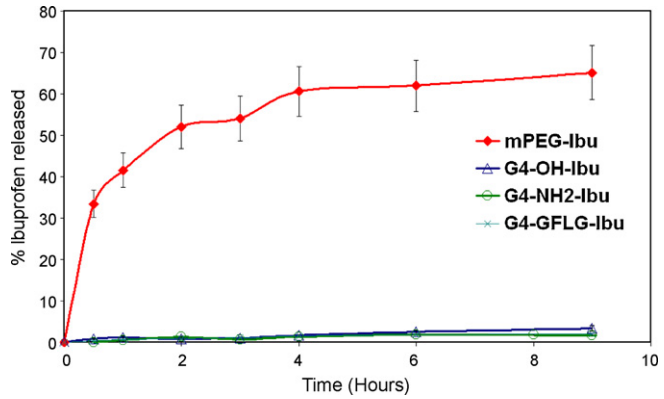

Fig. 8. Ibuprofen release profile of conjugates in 80% diluted human plasma. All samples were studied in triplicate for statistical analysis. The cumulative percentage of ibuprofen that has been cleaved from the conjugates at each time point is shown. Dendrimer conjugates studied were quite stable against esterase activity whereas linear mPEG conjugate was hydrolyzed enzymatically.

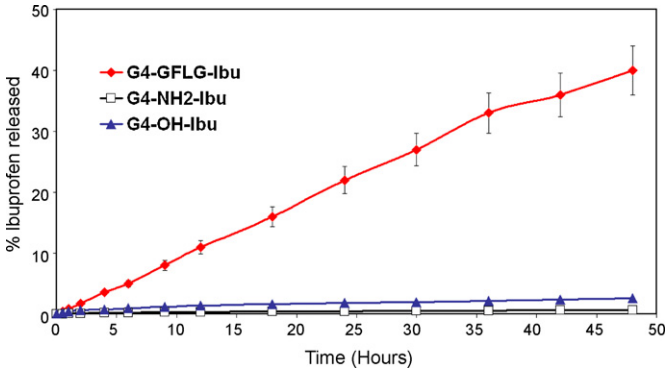

Fig. 9. Ibuprofen release profiles of conjugates in cathepsin B solution. All samples were studied in triplicate for statistical analysis. The cumulative percentage of ibuprofen that has been cleaved from the conjugates at each time point is shown. Peptide linker used clearly gets cleaved enzymatically, whereas the conjugates without linkers were very stable against cathepsin B activity.

a few months, provided the dendrimer–drug conjugate itself is not cleared from the target tissue during this time.

In comparison, mPEG with 5 kDa molecular weight and a similar hydrodynamic radius to the G4-PAMAM dendrimers was able to expose the ester linkage to the catalytic site in the enzyme; probably due to its flexible linear structure. Additionally, the ester linkage is located at the end of the linear polymer chain which may increase the accessibility to the enzyme. The release rates of mPEG–Ibu agreed well with the previously reported enzymatic hydrolysis rates of ester-bonded Generation 0 dendrimer conjugates (Najlah et al., 2006). This result confirms that linear mPEG conjugates as well as the Generation 0 PAMAM dendrimer conjugates were able to expose the ester linkage and therefore able to get hydrolyzed, whereas the G4 dendrimer conjugates were unable to get hydrolyzed by esterase enzymes in plasma. Therefore, ester linkages may give rise to faster release when the conjugate structure is capable of enzymatic release if intravenous route is being considered for administration. Similarly, ester-linked dendrimer conjugates containing long spacers may be susceptible to esterase activity in blood.

3.2.3. Enzymatic release with cathepsin B

Since amide linkages were very stable against hydrolysis at all pH buffers studied, their drug release must rely on enzymatic cleavage. Peptidase enzymes provide such an opportunity for selectively releasing drugs from polymer conjugates in the lysosomal compartment. One enzyme of interest cathepsin B has been extensively studied for its specificity on cleaving various peptide sequences (Etrych et al., 2002). Gly-Phe-Leu-Gly linker was previously used with HPMA copolymer–doxorubicin conjugates for delivering doxorubicin and its favorable release characteristics were demonstrated. For this reason, this peptide linker was selected to facilitate enzymatic cleavage of ibuprofen from dendrimer conjugates in the presence of cathepsin B.

The results of the release studies with cathepsin B are shown in Fig. 9. The direct linked ester (G4-OH–Ibu conjugate) and amide (G4-NH_2–Ibu) conjugates were not hydrolyzed enzymatically. The results confirm that the direct conjugation to dendrimers may not be useful if enzymatic hydrolysis is desired. Alternatively, the introduction of spacer molecules may give access to active site of the enzymes by reducing steric problems. This was evident from the release profile of G4-GFLG–Ibu conjugate as shown in Fig. 9. G4-GFLG–Ibu released 40% of its ibuprofen payload in 2 days and followed pseudo-first order reaction kinetics ($k' = 1.06 \times 10^{-2}\ h^{-1}$, $t_{1/2} = 65.5$ h). This result agreed well with the release rates of the same peptide linker used with linear HPMA

copolymer–doxorubicin conjugates (Etrych et al., 2002). Therefore, use of such linkers makes dendrimer conjugates behave like linear polymers in accessibility of their cleavage site. On the other hand, not all the ibuprofen released from G4-GFLG–Ibu was in free form. The release media contained some ibuprofen containing peptide degradation products such as Gly-Ibu, Gly-Leu-Ibu, whereas these intermediates were further being degraded to free ibuprofen over the period of the release studies. The final released ibuprofen content in the release media was found to be 13% Gly-Leu-Ibu, 22% Gly-Ibu and 65% free ibuprofen at 48 h.

4. Conclusions

Drug release profiles of ester- and amide-bonded conjugates of 4th generation PAMAM dendrimers in various media are reported. The hydrolysis of ester conjugates showed clear pH-dependent rates, whereas the amide conjugates were very stable at all pH buffers. PAMAM dendrimer–ibuprofen conjugates without linkers were found to be stable against enzymatic hydrolysis due to steric effects. The steric blockage of esterase activity in plasma by the dendrimer structure makes ester linkage useful for dendrimer-based sustained release systems. Such ester-linked PAMAM dendrimer conjugates are promising drug carriers that could provide controlled release for various drugs, in addition to their inherent targeting capabilities. Alternatively, amide bonds in peptidyl linkers can be employed for conjugation if an enzymatic cleavage scheme can be identified. Steric hindrance plays a key role in enzymatic cleavage of drugs from dendrimer conjugates, since esterase and protease enzymes are macromolecules of comparable size. Better understanding of such steric effects and appropriate choice of linkage leads to effective drug delivery formulations and have important implications on designing PAMAM dendrimer–drug conjugates.

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