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Synthesis, characterization and stability of dendrimer prodrugs

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Abstract

The design, synthesis and characterization of a series of zero generation (G0) PAMAM dendrimer-based prodrugs for the potential enhancement of drug solubility and bioavailability are described. Naproxen, a poorly water-soluble drug, was conjugated to dendrimers either directly by an amide bond or by ester bonds using either L-lactic acid or diethylene glycol as a linker. All of the prodrugs were more hydrophilic than the parent drug, as evaluated by drug partitioning between 1-octanol and phosphate buffer (pH 7.4). Hydrolysis of the conjugates was measured at 37 °C in hydrochloric acid buffer (pH 1.2), phosphate buffer (pH 7.4), borate buffer (pH 8.5) and in 80% human plasma. The amide conjugate and both ester conjugates were chemically stable at all pHs over 48 h of incubation. Naproxen was enzymatically released from both ester conjugates in plasma; the lactic ester conjugate hydrolyzed slowly with only 25% of naproxen released after 24 h, the diethylene glycol ester conjugate cleaved rapidly following pseudo first order kinetics ($t_{1/2}$ = 51 min). G0 PAMAM dendrimer prodrugs with an appropriate linker (diethylene glycol) show good potential as carriers for oral delivery.

Keywords: Dendrimers; PAMAM; Prodrugs; Solubility

1. Introduction

Polyamidoamine (PAMAM) dendrimers possess a well-defined structure that allows precise control of size, shape and terminal group functionality (Tomalia et al., 1985). Dendrimers have a number of applications in several pharmaceutical fields such as enhancing the solubility of poorly soluble drugs, enhancing the delivery of DNA and oligonucleotides, and as carriers for the development of drug delivery systems (D'Emanuele and Attwood, 2005; D'Emanuele et al., 2003). Dendrimers have been shown to act as potential carrier/delivery systems that cross cell barriers at sufficient rates (Florence et al., 2000; Wiwattanapatapee et al., 2000) by both paracellular and transcellular pathways (El-Sayed et al., 2003; Jevprasesphant et al., 2003a). Furthermore, surface engineering of PAMAM dendrimers (by the addition of lauroyl or polyethylene glycol (PEG) chains) significantly reduced dendrimer cytotoxicity and enhanced transport (Jevprasesphant et al., 2003a,b).

Dendrimers can function as drug carriers either by encapsulating drugs within the dendritic structure (Jansen et al., 1994) or by attaching drugs to their terminal functional groups *via* electrostatic or covalent bonds (prodrug). The covalent linkage of a drug to a dendrimer provides a stable system that is not dependent on an appropriate dynamic equilibrium or thermodynamic factors that apply in matrix systems, e.g. drug encapsulation by dendrimers or micelles (Aulenta et al., 2003). The release of drug from a prodrug occurs via chemical or enzymatic cleavage of a hydrolytically labile bond. Several attempts have been made to utilize dendrimers as drug carriers. For example, Fréchet and coworkers (Ihre et al., 2002) designed and synthesized dendritic polyester systems based on 2,2-bis(hydroxymethyl)propanoic acid. These systems showed good biodegradability and biocompatibility and were evaluated as drug carriers for the anti-cancer drug doxorubicin, which was attached via a pH sensitive linkage. Folate residues have been conjugated to ester-terminated dendrimers and evaluated as potential drug carriers with tumour cell specificity (Kono et al., 1999). It was expected that these folate dendrimer conjugates would exhibit a strong affinity for tumour cells that overexpress the folate receptor on the cell surface. In addition, Zhuo et al. (1999) reported the synthesis of 5-fluorouracil (5FU)-PAMAM dendrimer conjugates (G4 and

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G5) designed to reduce the toxicity of 5FU by releasing it slowly from its conjugate. 5FU-PAMAM conjugates gave a slow release of drug, but an evaluation of the toxicity and the physicochemical properties of this dendritic system were not reported. Battah et al. (2001) described a series of dendritic 5-aminolevulinic acid (ALA) ester prodrugs for use in photodynamic therapy, and in particular examined the possibility of using dendrimers as macromolecular carriers for the delivery of ALA to the tumourigenic cells. In these molecules, ALA residues were attached to the dendritic periphery by ester linkages, with amide bonds connecting the dendrons. The ability of the ALA-dendrimer prodrugs to pass through the cell membrane and release ALA intracellularly was reported; the prodrugs did not induce cytotoxicity in the absence of light. PAMAM dendrimers were also used as a carrier for colonic delivery of 5-aminosalicylic acid (5-ASA) conjugated by using two different spacers containing an azo-bond. The conjugates showed colon specificity and prolonged release of 5-ASA; the results suggested that PAMAM dendrimers have potential for use as colon-specific drug carriers (Wiwattanapatapee et al., 2003).

D'Emanuele et al. (2004) have reported on the development of dendrimer conjugates as potential drug carrier systems that may be used to enhance the transport of propranolol across Caco-2 cells. Propranolol is an insoluble drug and a substrate for the P-glycoprotein (P-gp) efflux transporter. When conjugated to surface modified G3 PAMAM dendrimer, propranolol was shown to bypass the efflux system. Thus, dendrimer nanocarriers may be used to enhance the bioavailability of drugs that are poorly soluble and/or substrates of efflux transporters. More recently, conjugation of methylprednisolone (MP) to PAMAM G2.5-COOH terminal dendrimer and PAMAM G4-OH terminal dendrimer using glutaric acid (GA) as a spacer was reported (Khandare et al., 2005). Fluorescence and confocal microscopy images on A549 human lung epithelial carcinoma cells treated with conjugates showed that the conjugate was mostly localized in the cytosol. MP-GA-dendrimer conjugates showed comparable pharmacological activity to free MP, as measured by inhibition of prostaglandin secretion.

G0 PAMAM dendrimers have been shown to have an apical (A) to basolateral (B) permeability across Caco-2 monolayers that was six-fold higher than the A → B permeability of larger (G3) generation dendrimer, and with nontoxic effects on Caco-2 cells (El-Sayed et al., 2002), suggesting that G0 dendrimers are promising candidates as drug carriers.

The present study focuses on the nature of the covalent linker between dendrimer and drug. It is desirable to utilize a linker that is stable in the gastrointestinal tract and during transit across epithelial cells, but labile once absorbed. Naproxen was selected as a model drug of low aqueous solubility having a carboxylic acid group which facilitated conjugation to G0 PAMAM dendrimer either directly by an amide bond (G0-NAP) or by ester bonds using lactic acid (G0-lact-NAP) or diethylene glycol (G0-deg-NAP) as biolabile linkers. The structures of G0 dendrimer and the naproxen conjugates that have been synthesised and evaluated in this study are shown in the Fig. 1.

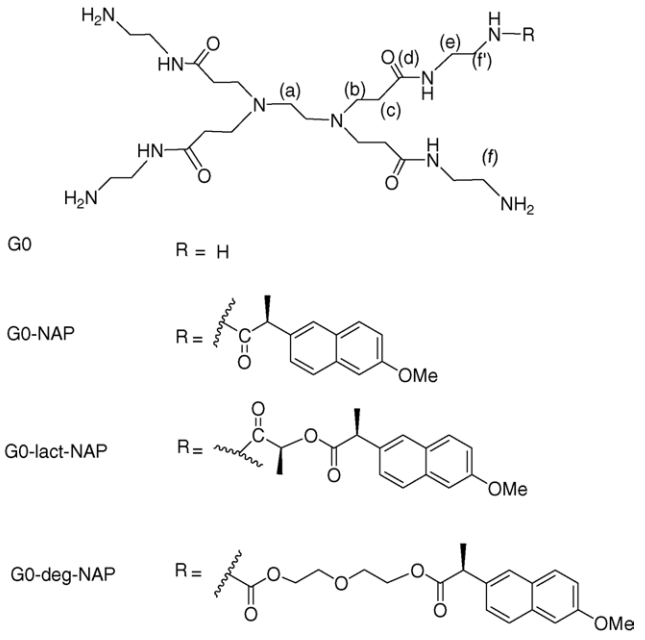


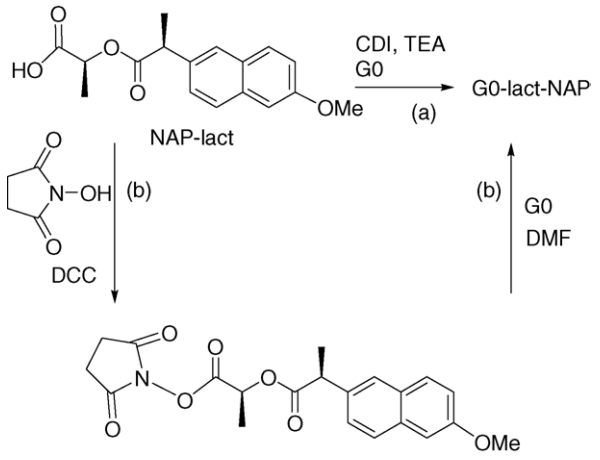
Fig. 1. Structure of G0 PAMAM dendrimer and dendrimer–naproxen conjugates. Letters on G0 PAMAM dendrimer are included to aid NMR assignments.

2. Materials and methods

2.1. Materials

Zero generation PAMAM dendrimers (G0) with ethylenediamine cores were purchased from Dendritech Inc. (Michigan, USA). Anisole, trifluoroacetic acid, (*S*)-(+)-6-methoxy-alpha-methyl-2-naphthaleneacetic acid (naproxen), 4-dimethylaminopyridine (DMAP), L-(+)-lactic acid, paraoxon (diethyl 4-nitrophenyl phosphate), Sephadex LH-20 and Sephadex 15 were purchased from Sigma–Aldrich Co. Ltd. (Poole, Dorset, UK). 2,3,4,5,6-Pentamethylbenzyl chloride, *N*,*N*′-carbonyldiimidazole (CDI) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Lancaster Synthesis (Morecombe, Lancashire, UK). *N*-Hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC) and diethylene glycol were purchased from Fluka (Poole, Dorset, UK). Human plasma was provided by Medeval (Manchester, UK).

Naproxen-dendrimer conjugates were characterised using infrared spectroscopy (AIT Mattson Genesis Series FTIR, Madison, USA) and ^{13}C and ^{1}H NMR spectroscopy (Bruker Avance 300, Bruker, Coventry, UK), ^{13}C NMR spectra were assigned with the aid of DEPT-135. Electrospray ionization mass spectra (ESI) were obtained using Micromass Platform II (Micromass UK Ltd., Altrincham, Cheshire, UK). HPLC analyses were carried out using a Hewlett-Packard Series II 1090 (Germany) instrument equipped with a Luna 5 μm, C18 column (250 mm × 4.6 mm, Phenomenex, Cheshire, UK) with sodium salicylate as an internal standard. The solvent system for characterization of the conjugates was methanol:aq H_3PO_4 (0.05%, w/v), ratio 5:95 for 4 min then 80:20 for the remaining elution time, the flow rate was 1.2 ml/min and UV



Scheme 1. Synthesis of G0-lact-NAP: (a) direct condensation method and (b) active ester method.

detection was at λ = 230 nm. Solvent systems and elution profiles used in the determination of partition coefficients and the chemical and enzymatic stability of conjugates are described below.

Naproxen-lactic acid (NAP-lact) and naproxen-diethylene glycol (NAP-deg) esters were synthesized according to methods previously described by Franssen et al. (1992) and Bonina et al. (2001), respectively.

2.2. Synthesis of G0-NAP and G0-lact-NAP

2.2.1. Method 1

N,*N*′-Carbonyldiimidazole (CDI) (17.8 mg, 0.11 mmol) and triethylamine (TEA) (10.2 mg, 0.10 mmol) were added to naproxen (25.3 mg, 0.11 mmol) or NAP-lact (33.2 mg, 0.11 mmol) in dry *N*,*N*-dimethylformamide (DMF) (3 ml) and stirred for 3 h at room temperature (Scheme 1a) Thereafter, the mixture was added dropwise to DMF (2 ml) containing G0 dendrimer (51.7 mg, 0.1 mmol) and stirred overnight. The resulting solution was diluted with 10 ml water and washed with dichloromethane (DCM) (3 × 3 ml). The aqueous layer was dried (anhydrous Na_2SO_4), filtered and evaporated and the residue was purified by size exclusion (gel filtration) chromatography, using Sephadex 15 (eluted with methanol–water (1:5)). The product was further purified using Sephadex LH 20, eluting with methanol–water (5:1). The yield was 47% for G0-NAP and 30% for G0-lact-NAP.

2.2.2. Method 2

Naproxen (27.6 mg, 0.12 mmol) or NAP-lact (36.2 mg, 0.12 mmol) was dissolved in 3 ml of DCM. DCC (30.9 mg, 0.15 mmol) was added to the solution and stirred for an hour (Scheme 1b). *N*-Hydroxysuccinimide (NHS) (17.25 mg, 0.15 mmol) was added and the mixture was stirred for 24 h. The reaction mixture was filtered and the filtrate was evaporated using a rotary evaporator. The residue was dissolved in DMF (3 ml) and filtered. The filtrate was added dropwise to 2 ml of DMF containing G0 dendrimer (51.7 mg, 0.1 mmol), stirred for 5 days and evaporated under vacuum. The residue was dissolved in 3 ml water and filtered. The filtrate was purified by size exclusion chromatography, using Sephadex 15, eluting with methanol–water (1:5). The product was further purified using Sephadex LH 20 eluting with methanol–water (5:1). The yield was 49% for G0-NAP and 34% for G0-lact-NAP.

2.2.3. G0-NAP NMR data

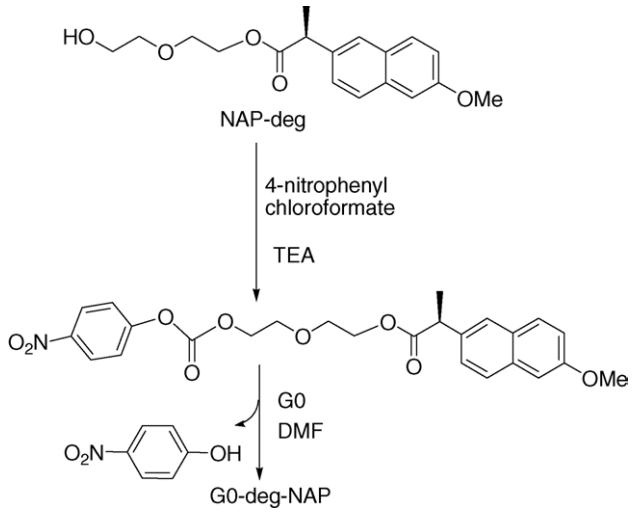
^{1}H NMR (d_4-MeOD): 1.53 (3H, d, J = 7.2, CH_3, NAP), 2.35 (8H, t, J = 6.0, 4 × c-G0), 2.51 (4H, s, 2 × a-G0), 2.73 (8H, m, 4 × b-G0), 2.73 (6H, m, 3 × f-G0), 3.30 (8H, m, 4 × e-G0), 3.30 (2H, m, 1 × f′-G0), 3.87 (1H, q, J = 7.0, CH, NAP), 3.89 (3H, s, CH_3O, NAP), 7.81–7.10 (6H, m, Ar, NAP). ^{13}C NMR (d_4-MeOD): 17.7 (–CH_3, NAP), 37.4 (4 × c-G0), 38.7 (–CH–, NAP), 40.7 (3 × f-G0), 41.2 (f′-G0), 45.0 (4 × e-G0), 48.4 (4 × b-G0), 50.05 (2 × a-G0), 54.5 (–OCH_3, NAP) 105.4 (CH–Ar, NAP), 118.3 (CH–Ar, NAP), 126.4 (CH–Ar, NAP), 126.8 (CH–Ar, NAP), 128.9 (CH–Ar, NAP), 129.3 (CH–Ar, NAP), 133.6 (C–Ar, NAP), 135.2 (2 × C–Ar, NAP), 157.5 (C–Ar, NAP), 174.2 (4 × d-G0), 182.0 (–NHCO–, NAP). (+)-ESI-MS: 730 [M^+ + H].

2.2.4. G0-lact-NAP NMR data

^{1}H NMR (d_4-MeOD): 1.47 (3H, d, J = 7.0, CH_3, NAP), 1.51 (3H, d, J = 7.2, CH_3, lact), 2.27 (8H, m, 4 × c-G0), 2.46 (4H, s, 2 × a-G0), 2.67 (8H, m, 4 × b-G0), 2.77 (6H, m, 3 × f-G0), 3.24 (8H, m, 4 × e-G0), 3.24 (2H, m, 1 × f′-G0), 3.79 (3H, s, CH_3O, NAP), 3.87 (1H, q, J = 6.9, CH, NAP), 4.84 (1H, q, J = 7.0, CH, lact), 7.81–7.10 (6H, m, Ar, NAP). ^{13}C NMR (d_4-MeOD): 18.1 (–CH_3, lact), 19.1 (–CH_3, NAP), 34.4 (4 × c-G0), 36.1 (–CH–, NAP), 38.6 (3 × f-G0), 39.9 (1 × f′-G0), 41.4 (4 × e-G0), 46.5 (4 × b-G0), 49.5 (2 × a-G0), 55.7 (–OCH_3, NAP), 73.6 (–CH–, lact), 106.6 (CH–Ar, NAP), 119.3 (CH–Ar, NAP), 127.2 (CH–Ar, NAP), 127.8 (CH–Ar, NAP), 128.0 (CH–Ar, NAP), 130.3 (CH–Ar, NAP), 135.2 (C–Ar, NAP), 136.4 (C–Ar, NAP), 137.1 (C–Ar, NAP), 159.2 (C–Ar, NAP), 175.4 (4 × d-G0), 178.7 (–NHCO–, lact), 173.8 (–COO–, NAP). (+)-ESI-MS: 801 [M + 1] and 822 [M^+ + Na].

2.3. Synthesis of NAP-deg-(4-nitrophenyl carbonate)

NAP-deg (318 mg, 1 mmol) was dissolved in THF (5 ml) and TEA (204 mg, 2 mmol) was added. The mixture was stirred for 10 min after which 4-nitrophenyl chloroformate (403.5 mg, 2 mmol) was added portionwise and stirred for 24 h at room temperature. THF was evaporated under vacuum and the residue was dissolved in DCM, filtered and purified by silica gel column chromatography, eluting with 20% EtOAc-80% hexane to give NAP-deg-(4-nitrophenyl carbonate) (Rf = 0.12) in a yield of 88%.^{1}H NMR ($CDCl_3$): 1.49 (3H, d, J = 7.2, CH_3), 3.58–3.48 (4H, m, –OCH_2– and –CH_2O–), 3.78 (4H, m, CHCOO– and O–CH_3), 4.15 (4H, m, –$COOCH_2$– and –CH_2OCOO–), 8.1–6.7 (10H, m, Ar). ^{13}C NMR ($CDCl_3$): 17.4 (–CH_3), 44.3 (–CHCOO–), 54.3 (O–CH_3), 62.7 (–$COOCH_2$–), 67.0 (CH_2OCOO), 67.4 (–OCH_2–), 68.1 (–CH_2O–), 156.6–104.5 (Ar), 152.6 (–OCOO–), 173.5 (–COO–) NAP.



Scheme 2. Synthesis of G0-deg-NAP.

2.4. Synthesis of G0-deg-NAP

NAP-deg-(4-nitrophenyl carbonate) (217 mg, 0.45 mmol) in DMF (1 ml) was added dropwise to a stirred solution of G0 dendrimer (200 mg, 0.38 mmol) in DMF (2 ml) (Scheme 2). The reaction mixture was stirred for 5 days. DMF was evaporated under vacuum and the residue was purified by size exclusion (gel filtration) chromatography using Sephadex LH 20 with methanol–water (5:1). The yield of G0-deg-NAP was 79 %. ^{1}H NMR (d_4-MeOD): 1.52 (3H, d, $J = 7.1$, CH_3, NAP), 2.42 (8H, m, 4 × c-G0), 2.68 (4H, s, 2 × a-G0), 2.85 (8H, m, 4 × b-G0), 3.04 (6H, m, 3 × f-G0), 3.24 (2H, m, 1 × f′-G0), 3.33 (8H, m, 4 × e-G0), 3.45 (2H, m, –OCH_2–, deg), 3.59 (2H, m, –CH_2O–, deg), 3.87 (4H, m, CHCOO– and O–CH_3, NAP), 3.99 (2H, m, –CH_2OCONH–, deg), 4.18 (2H, m, –$COOCH_2$–, deg), 7.9–7.65 (6H, Ar, NAP). ^{13}C NMR (d_4-MeOD): 19.4 (–CH_3, NAP), 34.1 (4 × c-G0), 38.9 (3 × f-G0), 41.17 (1 × f′-G0), 41.7 (4 × e-G0), 46.9 (–CH–, NAP), 51.0 (4 × b-G0), 52.0 (2 × a-G0), 56.2 (–OCH_3, NAP), 65.4 (–CH_2OCONH–, deg), 65.5 (–$COOCH_2$–, deg), 70.4(–CH_2O– deg), 70.8 (–OCH_2– deg), 105.5 (CH–Ar, NAP), 118.8 (CH–Ar, NAP), 125.8 (CH–Ar, NAP), 126.0 (CH–Ar, NAP), 127.1 (CH–Ar, NAP), 129.1 (CH–Ar, NAP), 129.2 (C–Ar, NAP), 134.1 (C–Ar, NAP), 135.9 (C–Ar, NAP), 157.7 (NHCOO, deg), 158.0 (C–Ar, NAP) 173.8 (–COO–, NAP), 175.4 (4 × d-G0). (+)-ESI-MS: 862 [M^+ + H] and 884 [M^+ + Na].

2.5. Synthesis of the methyl ester of naproxen (NAP-Me)

Potassium carbonate (K_2CO_3) (207 mg, 1.5 mmol) was added to a solution of naproxen (230 mg, 1 mmol) in DMF (7 ml). After stirring for 5 min, dimethyl sulphate (567 mg, 4.5 mmol) was added and the mixture was stirred overnight at room temperature. The reaction mixture was poured into HCl (2 M, 20 ml). The resulting solid was filtered, washed with H_2O (3 × 4 ml) and dried under vacuum. The yield of the methyl ester of naproxen was 83%, (mp = 87 °C). ^{1}H NMR ($CDCl_3$): 1.50 (3H, d, $J = 7.2$, CH_3), 3.59 (3H, s, $COOCH_3$), 3.78 (H, q, $J = 7.1$, CHCOO–), 3.83 (3H, s, O–CH_3), 7.6–7.0 (6H, m, Ar). ^{13}C NMR ($CDCl_3$): 17.5 (–CH_3), 44.3 (–CHCOO–), 51.0 (COO–CH_3), 54.3 (O–CH_3), 104.6 (CH–Ar), 117.9 (CH–Ar), 124.9 (CH–Ar), 125.1 (CH–Ar), 126.1 (CH–Ar), 127.9 (CH–Ar), 129.2 (C–Ar), 132.7 (C–Ar), 134.7 (C–Ar), 156.6 (C–Ar), 174.1 (–COO–).

2.6. Determination of partition coefficients

The apparent partition coefficients (P_{app}) of naproxen and conjugates between 1-octanol and phosphate buffer (pH 7.4) were determined at 37 °C. Before use, the 1-octanol was saturated with phosphate buffer for 24 h by stirring vigorously. A known concentration of compound in phosphate buffer (pH 7.4, 5 ml) was shaken for 72 h with 1-octanol (5 ml) to achieve equilibrium, and the phases were separated by centrifugation at 10,000 rpm for 5 min. All experiments were performed in triplicate. The concentrations of the compounds in the buffer phase before and after partitioning were determined by HPLC (solvent systems: methanol:aq H_3PO_4 (0.05%, w/v) (80:20) (for the conjugates) and acetonitrile (ACN):aq H_3PO_4 (0.05%, w/v) (40:60) (for naproxen)).

2.7. Chemical stability

The hydrolysis of PAMAM G0-naproxen prodrugs was studied at pH 7.4 (0.02 M phosphate buffer), pH 1.2 (0.06 M hydrochloric acid buffer) and pH 8.5 (0.05 M borate buffer). The reaction was initiated by adding an appropriate amount of conjugate to 5 ml of preheated buffer solution in screw-capped test tubes to give a final concentration of 2×10^{-6} M. The solutions were maintained at 37 °C, and at appropriate intervals samples (50 μl) were withdrawn and the reaction was quenched by adding 200 μl of a methanolic solution of sodium salicylate (1.25 mM) (internal standard). The sample was stored at −20 °C awaiting analysis by HPLC (solvent systems: ACN:aq H_3PO_4 (0.05%, w/v) (40:60)) for NAP-lact and NAP-Me, and methanol:ACN:aq H_3PO_4 (0.05%, w/v) (17.5:30:52.5) for NAP-deg.

2.8. Enzymatic hydrolysis

The hydrolysis of the prodrugs of naproxen was studied in human plasma diluted to 80% with 0.05 M phosphate buffer. The reaction was initiated by adding an appropriate amount of conjugate to 5 ml of preheated plasma solution to give a final concentration of 2×10^{-6} M. The solution was maintained at 37 °C, and at appropriate intervals, samples (50 μl) were withdrawn and 200 μl of a methanolic solution of sodium salicylate (1.25 mM) (internal standard) was added in order to deproteinize the plasma and quench the reaction. After immediate centrifugation for 5 min at 10,000 rpm, the clear supernatant was stored at −20 °C awaiting analysis by HPLC (solvent systems: ACN:aq H_3PO_4 (0.05%, w/v) (40:60)) for NAP-lact and NAP-Me and methanol:ACN-aq H_3PO_4 (0.05%, w/v) (17.5:30:52.5) for NAP-deg. In an esterase inhibition study, the plasma was

preincubated for 15 min (37 °C) with paraoxon, an esterase inhibitor (final concentration 1 mM).

3. Results and discussion

3.1. Synthesis and characterization of dendrimer-NAP conjugates

Amide bonds were formed between the surface amine groups of the G0 dendrimer and naproxen or the naproxen ester of L-lactic acid by using two different approaches; either by (a) a direct condensation using the coupling agent CDI (Scheme 1a shown for the synthesis of G0-lact-NAP) or by (b) the active ester method involving NHS (Scheme 1b). Although both routes gave approximately the same yield, method (b) was faster and gave a cleaner product (traces of CDI could be detected by ^{1}H NMR in the purified product from method (a)).

NAP-deg was attached to the primary amines of G0 dendrimer using 4-nitrophenyl chloroformate activation (Scheme 2). NAP-deg was initially reacted with 4-nitrophenyl chloroformate to obtain NAP-deg-(4-nitrophenyl carbonate) which was purified and characterized by ^{1}H NMR and ^{13}C NMR spectroscopy. G0 dendrimer and NAP-deg-(4-nitrophenyl carbonate) were reacted in an equimolar ratio to form a carbamate bond between the diethylene glycol linker and the dendrimer. Pure sample was obtained in high yield (79%). A comparison between the ^{13}C NMR spectra of NAP-deg-(4-nitrophenyl carbonate) and the conjugate G0-deg-NAP confirmed the formation of a carbamate bond.

The G0 dendrimer conjugates were analyzed by ^{1}H NMR and ^{13}C NMR spectroscopy. The formation of an amide bond between the G0 dendrimer amine group and the carboxyl group of the lactic acid linker or naproxen was demonstrated by an upfield shift in the ^{1}H NMR chemical shift of the methylene group (f) adjacent to the free amine in the dendrimer (2.77–3.24 ppm for G0-lact-NAP and 2.73–3.3 ppm for G0-NAP) (Figs. 2 and 3). In the ^{13}C NMR spectrum, the appearance of the new amide carbonyl peak (at 182.0 ppm for G0-NAP and 178.7 ppm for G0-lact-NAP) and a carbamate carbonyl peak (at 157.7 ppm for G0-deg-NAP) indicates that the conjugated molecule was covalently (rather than electrostatically) bound to the G0 dendrimers.

The IR spectrum of naproxen showed a broad band at 3188 cm^{-1} (acid O–H stretch) and a carboxylic acid C=O stretch at 1728 cm^{-1}. These bands were not visible in the IR spectrum of G0-NAP, indicating successful conjugation. It was not possible to see an absorption peak for the new amide bond, as this was masked by the amide absorption of dendrimer. For G0-lact-NAP, the appearance of the ester C=O stretching at 1722 cm^{-1} and the disappearance of the acid O–H stretch indicates the conjugation of NAP-lact to G0. The IR spectrum of NAP-deg included a broad band at 3453 cm^{-1} (O–H stretch of diethylene glycol unit) and a strong band at 1731 cm^{-1} (ester C=O stretch) (Fig. 4A). Conjugation of NAP-deg to G0 dendrimer via a carabamate bond was confirmed by the disappearance of the O–H stretch and the appearance of an ester C=O stretch at 1725 cm^{-1} (Fig. 4C). In all conjugates, the presence of the G0 dendrimer was confirmed by an N–H stretching band between 3282–3264 cm^{-1} and an amide C=O stretching band between 1667 and 1654 cm^{-1} (Figs. 4B and C).

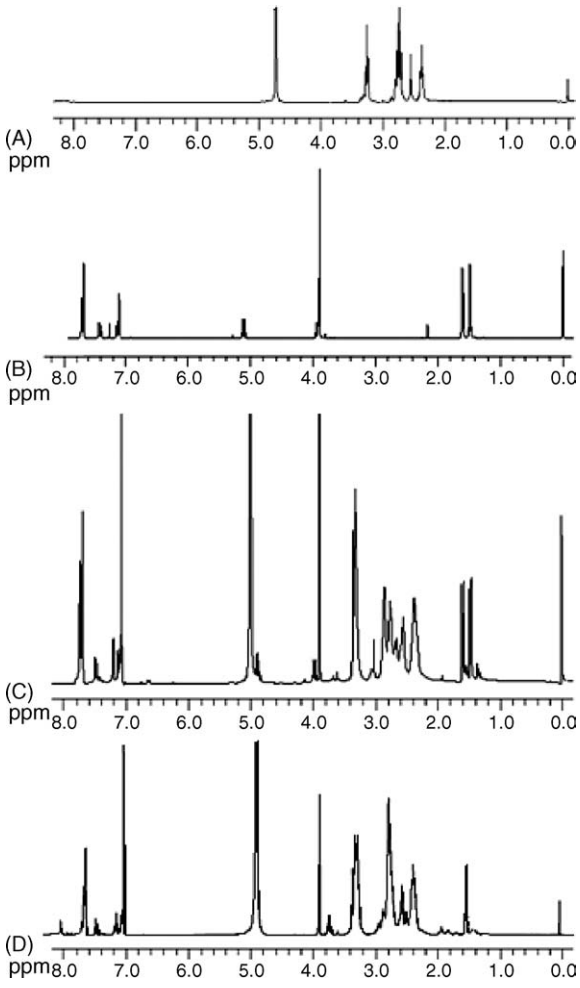


Fig. 2. ^{1}H NMR spectra of (A) PAMAM G0 dendrimer (d_4-MeOD), (B) NAP-lact ($CDCl_3$), (C) G0-lact-NAP and (D) G0-NAP (d_4-MeOD).

The appearance of a new peak for the G0-naproxen conjugates in RP-HPLC chromatograms (Fig. 5) confirms their covalent nature and discriminates against a simple electrostatic interaction of naproxen with G0 dendrimer. The retention times of the dendrimer-conjugates increased with respect to G0 dendrimer alone because of the interaction of the protruding aryl groups on the naproxen moiety with the stationary phase. Slight differences in retention times were reported amongst the conjugates, presumably attributable to either differing interactions of linker with the stationary phase or differences in lipophilicity.

The equimolar ratio of G0 dendrimer to naproxen in the prodrug was confirmed by calculating the relative intensities of ^{1}H NMR peaks originating from the naproxen compared to those of the dendrimer. The mass spectroscopy data confirmed the 1:1 ratio of drug:dendrimer.

The apparent partition coefficients of naproxen and dendrimer prodrugs (Table 1) show the expected decrease in the lipophilicity of naproxen following its conjugation to the highly water soluble G0 PAMAM dendrimer.

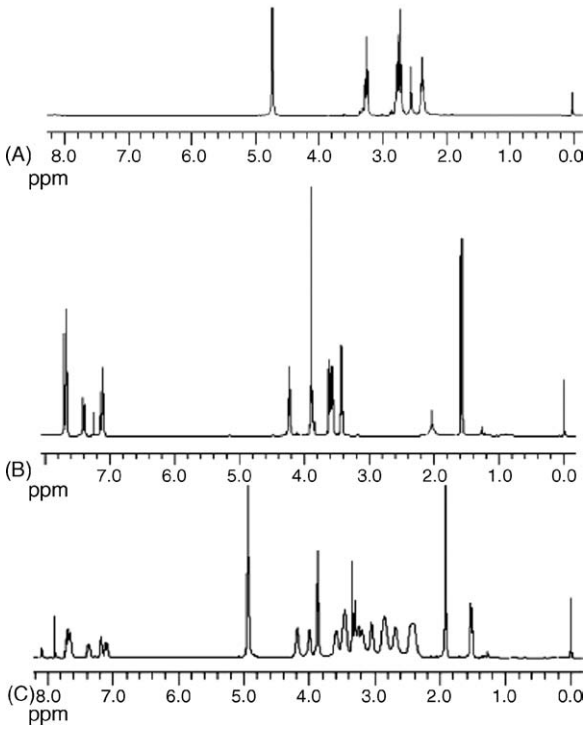


Fig. 3. ^{1}H NMR spectra of (A) PAMAM G0 dendrimer (d_4-MeOD), (B) NAP-deg ($CDCl_3$) and (C) G0-deg-NAP (d_4-MeOD).

3.2. Chemical and enzymatic stability

One of the essential prerequisites for the use of G0 dendrimer prodrugs is that they should be stable over the pH range of the gastrointestinal tract, such that they are absorbed through the gastrointestinal wall intact. Stability studies of the conjugates were performed at pH 1.2, 7.4 and 8.5. The ester linkage present in G0-lact-NAP and G0-deg-NAP was selected due to its potential ability to undergo esterase-catalysed hydrolysis in plasma.

Table 2 shows the percentage of conjugate remaining after hydrolysis of the amide bond in G0-NAP and the ester bonds in G0-lact-NAP and G0-deg-NAP. The direct linkage of naproxen to the G0 dendrimer resulted in a very stable amide prodrug under all pH conditions. Even at pH 1.2 more than 92% of the conjugate remained intact after 10 days. The stabilities of the G0-lact-NAP and G0-deg-NAP ester conjugates were high with approximately 90% of the conjugates remaining at all pH values after 48 h. Similarly, both ester conjugates showed good stability under physiological conditions (pH 7.4 and 37 °C) after 10 days of incubation. Differences in the extent of hydrolysis

Table 1
The log P (pH 7.4) values (±S.D.) of naproxen and conjugates at 37 °C

Compound	Log $P_{o/w}$ (pH 7.4)
NAP	0.11 ± 0.01
NAP-lact	0.19 ± 0.07
NAP-deg	0.15 ± 0.04
G0-NAP	−0.95 ± 0.05
G0-lact-NAP	−0.96 ± 0.16
G0-deg-NAP	−1.07 ± 0.09

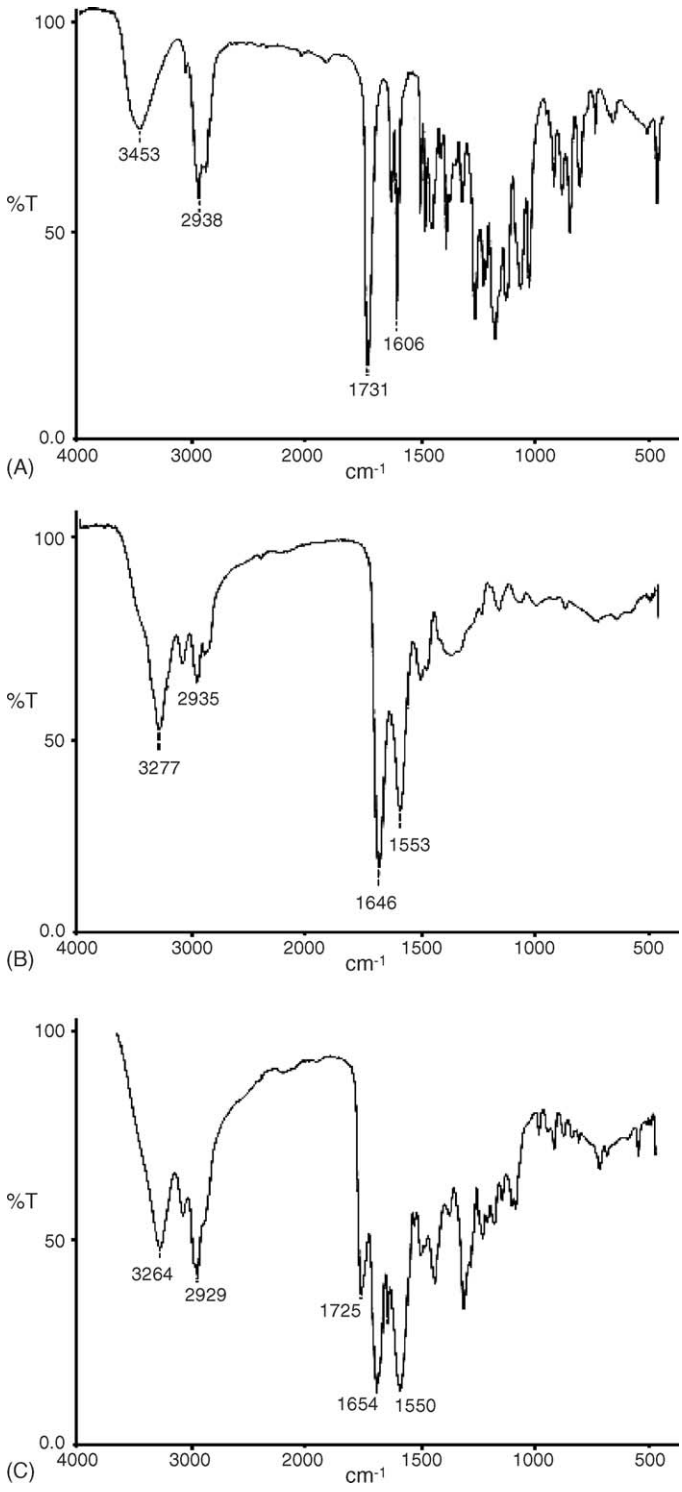


Fig. 4. IR spectra of (A) NAP-deg, (B) PAMAM G0 dendrimer and (C) G0-deg-NAP.

can be observed between the two ester conjugates after 10 days of incubation at all pH values. G0-lact-NAP was more stable than G0-deg-NAP, possibly because the greater spacer length in the diethylene glycol linker prodrug, G0-deg-NAP, resulted in a more readily cleavable ester bond. Alternatively, the primary ester in G0-deg-NAP may be more accessible to hydrolysis than the secondary ester in G0-lact-NAP. However, G0-deg-NAP may

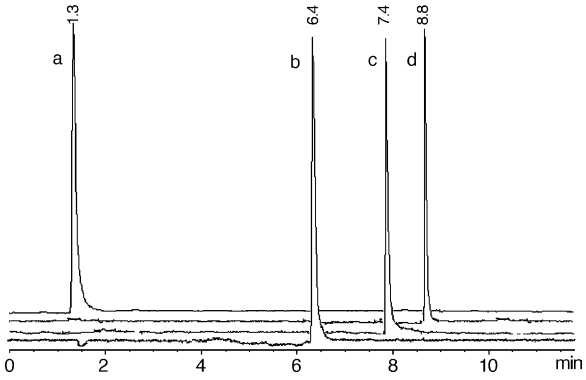


Fig. 5. RP-HPLC chromatograms of (a) PAMAM G0 dendrimer, (b) G0-deg-NAP, (c) G0-lact-NAP and (d) G0-NAP.

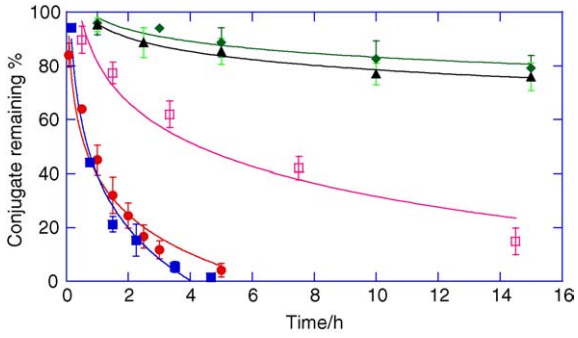


Fig. 6. Stability of PAMAM G0 dendrimer conjugates and naproxen esters in 80% human plasma: G0-lact-NAP (♦), NAP-lact (▲), NAP-Me (□), G0-deg-NAP (●) and NAP-deg (■).

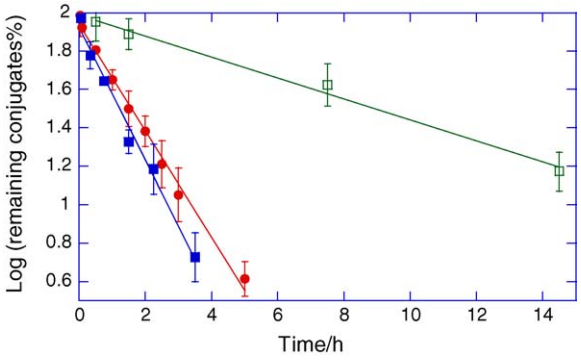


Fig. 7. Pseudo first order plots for the hydrolysis of the ester prodrugs: G0-deg-NAP (●), NAP-deg (■) and NAP-Me (□) in 80% human plasma.

still be considered to be a sufficiently stable ester (only 45% was hydrolysed after 10 days) for use in oral drug delivery.

The stability of all conjugates to enzymatic degradation was examined in 80% human plasma (37 °C). In addition, the enzymatic stability of the drug-linkers (NAP-lact and NAP-deg) was examined to compare the rate of the ester hydrolysis with and without conjugation to G0 dendrimer. The methyl ester of naproxen (NAP-Me) was used as a control esterase substrate. The stability of G0-deg-NAP was also assessed in 80% plasma in the presence of paraoxon, an esterase inhibitor, to determine the action of the plasma esterase towards the diethylene glycol ester linker.

No detectable amounts of naproxen were released from the amide-linked conjugate (G0-NAP, data not shown) over 48 h indicating its stability to hydrolysis in plasma, in agreement with reports that amide bonds of many conjugated drugs are poor substrates for hydrolysis (Franssen et al., 1992). As shown in Fig. 6, the ester conjugate G0-lact-NAP was slowly hydrolyzed in 80% human plasma with 20% release of naproxen after 16 h. There was no significant difference in drug release from G0-lact-NAP and NAP-lact, i.e. conjugating the L-lactic acid linker to G0 dendrimer had no effect on the rate of hydrolysis of the ester. This suggests that using L-lactic acid as a linker yields stable esters that may be used in controlled release delivery or for drug targeting applications. The degradation of NAP-Me was faster than that of G0-lact-NAP with almost complete hydrolysis after 24 h incubation in plasma.

In contrast to the observed incomplete release of drug from the G0-lact-NAP conjugate, it was expected that the use of a diethylene glycol linker would produce an ester bond with naproxen that would be susceptible to enzymatic hydrolysis because of the greater length of this primary alcohol linker compared to lactic acid (secondary alcohol). In addition, its oxyethylene structure should produce prodrugs with good water solubility and rapid enzymatic hydrolysis (Bonina et al., 2002; Bonina et al., 2001). Bonina et al. (2002) have demonstrated that increasing the oligoethylene glycol chain length had no significant impact on the hydrolysis rate. As expected, G0-deg-NAP was cleaved rapidly with pseudo first order kinetics in 80% human plasma ($t_{1/2}$ = 51 min) (Fig. 7). G0-deg-NAP remained intact for 6 h in the presence of paraoxon (Fig. 8), an inhibitor for type B esterase (carboxylesterase), confirming that the diethylene glycol ester was cleaved by carboxylesterase in the plasma. Traces of naproxen could be detected after 15 h due to the chemical hydrolysis of the prodrug.

Fig. 7 shows a lower rate of hydrolysis of the prodrug, G0-deg-NAP compared with that of NAP-deg ($t_{1/2}$ = 38.6 min). Both G0-deg-NAP and NAP-deg exhibited faster degradation than the

Table 2
Chemical stability of G0 conjugates at pH 1.2, 7.4 and 8.5 (37 °C)

Conjugate	% Conjugate remaining					
	pH 1.2		pH 7.4		pH 8.5	
	48 h	240 h	48 h	240 h	48 h	240 h
G0-NAP (amide)	96.7 ± 0.4	92.7 ± 1.1	97.3 ± 0.8	95.2 ± 1.7	96.2 ± 0.8	90.1 ± 2.1
G0-lact-NAP (ester)	96.6 ± 0.5	72.4 ± 1.9	98.5 ± 0.2	89.7 ± 0.8	97.6 ± 0.6	80.1 ± 2.5
G0-deg-NAP (ester)	91.3 ± 0.6	54.9 ± 3.4	97.9 ± 0.1	78.5 ± 0.6	84.4 ± 1.2	56.5 ± 2.9

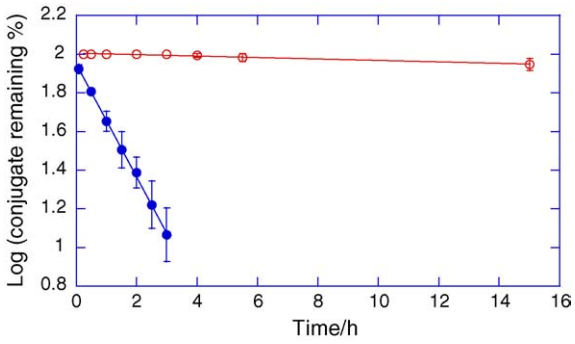


Fig. 8. Pseudo first order plot for the hydrolysis of the ester prodrug, G0-deg-NAP, with paraoxon (◯) and without paraoxon (●).

methyl ester of naproxen NAP-Me ($t_{1/2}$ = 316 min). The results suggest that prodrugs of the type studied here with diethylene glycol linkers may have the ability to release the parent drug at a sufficient rate in plasma once absorbed.

4. Conclusion

The design, synthesis, characterization and stability studies of naproxen conjugates of G0 PAMAM dendrimer using several covalent linkers are reported. Conjugates formed by direct amide linkage of naproxen to the G0 dendrimer were not suitable for the development of prodrugs due to their high chemical and enzymatic stability. In contrast, naproxen was slowly released from the dendrimer conjugate formed using a lactic acid ester linkage, and such conjugates may have potential as controlled release systems or prodrugs for drug targeting. Conjugates in which naproxen was linked to the G0 dendrimer through a diethylene glycol linker showed high chemical stability in buffers, but readily released naproxen in plasma. Such conjugates have potential as carriers for low solubility drugs such as naproxen. The stability of the conjugates in liver homogenates and the permeation of the conjugates through Caco-2 monolayers is currently under investigation.

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References

Aulenta, F., Hayes, W., Rannard, S., 2003. Dendrimers: a new class of nanoscopic containers and delivery devices. Eur. Polym. J. 39, 1741–1771.

Battah, S.H., Chee, C.E., Nakanishi, H., Gerscher, S., Macrobert, A.J., Edwards, C., 2001. Synthesis and biological studies of 5-aminolevulinic acid-containing dendrimers for photodynamic therapy. Bioconjug. Chem. 12, 980–988.

Bonina, F., Puglia, C., Santagati, N.A., Saija, A., Tomaino, A., Tita, B., 2002. Oligoethylene ester derivatives of ketoprofen, naproxen and diclofenac as oral prodrugs: a pharmacological evaluation. Pharmazie 57, 552–555.

Bonina, F.P., Puglia, C., Barbuzzi, T., de Caprariis, P., Palagiano, F., Rimoli, M.G., Saija, A., 2001. In vitro and in vivo evaluation of polyoxyethylene esters as dermal prodrugs of ketoprofen, naproxen and diclofenac. Eur. J. Pharm. Sci. 14, 123–134.

D'Emanuele, A., Attwood, D., 2005. Dendrimer-drug interactions. Adv. Drug Delivery Rev. 15, 2147–2162.

D'Emanuele, A., Attwood, D., Abu Rmaileh, R., 2003. Dendrimers. In: Swarbrick, J., Boylan, J.C. (Eds.), Encyclopedia of Pharmaceutical Technology, vol. 3, second ed., pp. 1–21.

D'Emanuele, A., Jevprasesphant, R., Penny, J., Attwood, D., 2004. The use of a dendrimer-propranolol prodrug to bypass efflux transporters and enhance oral bioavailability. J. Control. Release 95, 447–453.

El-Sayed, M., Ginski, M., Rhodes, C., Ghandehari, H., 2002. Transepithelial transport of poly(amidoamine) dendrimers across caco-2 cell monolayers. J. Control Release 81, 355–365.

El-Sayed, M., Rhodes, C.A., Ginski, M., Ghandehari, H., 2003. Transport mechanism(s) of poly(amidoamine) dendrimers across caco-2 cell monolayers. Int. J. Pharm. 265, 151–157.

Florence, A.T., Sakthivel, T., Toth, I., 2000. Oral uptake and translocation of a polylysine dendrimer with a lipid surface. J. Control. Release 65, 253–259.

Franssen, E.J.F., Koiter, J., Kuipers, C.A.M., Bruins, A.P., Moolenaar, F., Zeeuw, D., Kruizinga, W.H., Kellogg, R.M., Meijer, D.K.F., 1992. Low molecular weight proteins as carriers for renal drug targeting. Preparation of drug–protein conjugates and drug-spacer derivatives and their catabolism in renal cortex homogenates and lysosomal lysates. J. Med. Chem. 35, 1246–1259.

Ihre, H.R., Padilla De Jesus, O.L., Szoka Jr., F.C., Frechet, J.M.J., 2002. Polyester dendritic systems for drug delivery applications: design, synthesis, and characterization. Bioconjug. Chem. 13, 443–452.

Jansen, J.F.G.A., de Brabander van den Berg, E.M.M., Meijer, E.W., 1994. Encapsulation of guest molecules into a dendritic box. Science (Washington, D.C.) 266, 1226–1229.

Jevprasesphant, R., Penny, J., Attwood, D., McKeown, N.L.B., D'Emanuele, A., 2003a. Engineering of dendrimer surfaces to enhance transepithelial transport and reduce cytotoxicity. Pharm. Res. 20, 1543–1550.

Jevprasesphant, R., Penny, J., Jalal, R., Attwood, D., McKeown, N.B., D'Emanuele, A., 2003b. The influence of surface modification on the cytotoxicity of PAMAM dendrimers. Int. J. Pharm. 252, 263–266.

Khandare, J., Kolhe, P., Pillai, O., Kannan, S., Lieh-Lai, M., Kannan, R.M., 2005. Synthesis, cellular transport, and activity of polyamidoamine dendrimer-methylprednisolone conjugates. Bioconjug. Chem. 16, 330–337.

Kono, K., Liu, M., Frechet, J.M.J., 1999. Design of dendritic macromolecules containing folate or methotrexate residues. Bioconjug. Chem. 10, 1115–1121.

Tomalia, D.A., Baker, H., Dewald, J., Hall, M., Kallos, G., Martin, S., Roeck, J., Ryder, J., Smith, P., 1985. A new class of polymers: starburst-dendritic macromolecules. Polym. J. (Tokyo, Japan) 17, 117–132.

Wiwattanapatapee, R., Carreno-Gomez, B., Malik, N., Duncan, R., 2000. Anionic pamam dendrimers rapidly cross adult rat intestine in vitro: a potential oral delivery system? Pharm. Res. 17, 991–998.

Wiwattanapatapee, R., Lomlim, L., Saramunee, K., 2003. Dendrimers conjugates for colonic delivery of 5-aminosalicylic acid. J. Control. Release 88, 1–9.

Zhuo, R.X., Du, B., Lu, Z.R., 1999. In vitro release of 5-flourouacil with cyclic core dendritic polymer. J. Control. Release 57, 249–257.