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Drug complexation, in vitro release and cellular entry of dendrimers and hyperbranched polymers

Parag Kolhe [a], Ekta Misra [a], Rangaramanujam M. Kannan [a,*], Sujatha Kannan [b], Mary Lieh-Lai [b]

[a] *Department of Chemical Engineering and Material Science, Wayne State University, 5050, Anthony Wayne Drive, Detroit, MI 48202, USA*
[b] *Pediatric Critical Care, Children's Hospital of Michigan, Wayne State University, Detroit, MI 48201, USA*

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Abstract

Highly branched, functionalized polymers have potential to act as efficient drug carrier systems. Dendrimers are ideal candidates among model hyperbranched polymers because of their well-defined structure and high density of functional groups. Using ibuprofen as a model drug, we studied the interaction between the drug and Polyamidoamine (PAMAM) dendrimers (generations 3 and 4 with $–NH_2$ functionality) and Perstrop Polyol (generation 5, hyperbranched polyester with –OH functionality). FTIR and NMR studies suggest that ibuprofen predominantly forms a *complex* with PAMAM dendrimers because of the ionic interaction between the $–NH_2$ end groups and the carboxyl group of ibuprofen. On an average, up to 78 molecules of ibuprofen could be incorporated into one molecule of PAMAM-G4-NH_2 with 64 end groups. This complex is stable in deionized water and methanol. The in vitro release of ibuprofen from drug–dendrimer complex is appreciably slower compared to pure ibuprofen. The complexed drug enters A549 cells much more rapidly than pure drug suggesting that dendrimers may be able to carry the complexed drug inside cells efficiently. Hyperbranched Polyol (with 128 –OH end groups) appears to *encapsulate* approximately 24 drug molecules. Perhaps the lack of strong interactions between the –OH end groups and the drugs prevents complex formation.

Keywords: Ibuprofen; PAMAM; Polymer drug complexation; Hyperbranched polymer; Dendrimer

1. Introduction

Efficacy is the most important characteristic of any drug. However, efficacy of the drug may often be reduced because of the inability to deliver the drug to the specific cells or tissues. After administration, the drug may pass through different physiologic barriers and/or pathways, decreasing the actual amount of drug that reaches the site. Tissue specificity, product stability and solubility are all desirable characteristics of drug, but are not always attained (Shultz and Zimmerman, 1999). Therefore, the need to develop a drug carrier system with such characteristics is of great importance. In the last 20 years, there have been numerous efforts focused on the development of the drug carrier systems. Investigators have made attempts to develop a specific drug carrier system, which can maintain continuous drug levels in a desired range, reduce side effects by improved tissue or organ specificity (Langer, 1998).

* Corresponding author. Tel.: +1-313-577-3879; fax: +1-313-577-3810.
E-mail address: rkannan@che.eng.wayne.edu (R.M. Kannan).

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Current efforts have been directed towards the possible use of synthetic polymers for drug carriers. In order to be a key component in a carrier system, the polymer should be inert, biocompatible, and should have a definite physical structure that can be processed with ease (Brannon-Peppas, 1997). Some classes of polymers used include Polylactides (PLA), Polyglycolides (PGA), Poly(lactide-co-glycolides) (PLGA), Polyanhydrides, Polyorthoesters, and more recently dendrimers. Using a polymer system, the drug can be delivered by diffusion through the system, chemical cleavage, and solvent activation (Langer, 1998). From a practical point of view, several polymer-based drug delivery technologies have been developed. Some of the examples include a silicon device containing medication (Jacoby, 2001), microspheres of Poly(anhydride-co-imide) (Hanes et al., 1998) and a glucose sensitive hydrogel (Dorski et al., 1997). Recently, liposomes are also being evaluated as drug delivery systems. It has been found that surface modification of liposomes with peptides can mediate targeted drug delivery (Lestini et al., 2002).

In the search for an ideal carrier system, the dendrimers may have significant potential. Dendrimers are synthetic macromolecules with a well-defined globular structure (Esfand and Tomalia, 2001). Because of the step-by-step synthesis, precise control of size, shape, and end-group functionality can be achieved (Liu and Frechet, 1999). Examples of these synthesis methods include divergent approach developed by Tomalia et al. (1985) and Newkome et al. (1985) along with convergent approach developed by Hawker and Frechet (1990). With increasing generation number, the size and molecular weight of dendrimer increase. The dendritic core can act as a reservoir, encapsulating drug molecules (Kojima et al., 2000). In addition, the free surface functional groups can form complexes or conjugates with drug molecules or ligands (Liu and Frechet, 1999). This ability makes dendrimers attractive vehicles for targeted drug delivery. Polyamidoamine (PAMAM) dendrimers have attracted interest because of their dimensional stability and controlled methods of synthesis and wide availability. PAMAM dendrimers have been shown to exhibit minimum cytotoxicity up to generation 5 (Malik et al., 2000). Attempts have been made to design novel drug delivery systems using dendrimers as a drug carrier (Liu and Frechet, 1999; Yoo and Juliano, 2000). Studies have been carried out with non-ionic folate conjugated PAMAM dendrimers labeled with fluorescein isothiocyanate (FITC) for targeting tumor cells which are known to express hFR (Baker et al., 2001). Cyclic core dendritic polymer was covalently attached to 5-flurouracil, an anti-cancer drug (Zhuo et al., 1999).

Attaching Polyethylene Glycol (PEG) grafts to the terminal amine groups of the dendrimer can enhance biocompatibility of higher generation PAMAM dendrimer. PEG is known to be a non-toxic, hydrophilic polymer. Recently, Kojima et al. (2000) found that it is possible to synthesize PAMAM dendrimers with a hollow core for encapsulation of drug along with biocompatible surface comprised of Polyethylene grafts. Using this technique, they successfully encapsulated methotrexate (MTX; ∼25 molecules) and adriamycin (ADR; ∼6 molecules) (Kojima et al., 2000). Kono et al. (1999) designed a dendritic carrier system, which contained folate or MTX residues. Recent work has shown that dendrimers can enter the Caco-2 cell *monolayers* (El-Sayed et al., 2002) and A549 lung epithelial cells readily (Kannan et al., submitted).

The other polymer used in our study is Perstorp Polyol which belongs to the class of hyperbranched polymers. It is a generation 5 aliphatic hyperbranched Polyester with peripheral hydroxyl groups. Polyol is expected to be more polydispersed since no protection groups are employed for dendritic growth. The branching structure closely resembles a dendrimer, but is less well defined. Polyol consists of tetrafunctional neopentylic alcohol followed by 5 pseudo-generations of Polyester structure from 2,2-dimethylolpropionic acid and estimated to have 128 –OH end groups (Petterson, 1996; Fig. 1). To our knowledge, the potential use of hyperbranched Polyol as drug carrier system has not been explored.

We investigate the potential of dendrimers and hyperbranched polymers as drug carriers using ibuprofen as a model drug, since the methodologies for evaluation of the cellular activity of ibuprofen are well known. Ibuprofen is an acidic non-steroidal anti-inflammatory drug belonging to the class of propionic acid and is used to reduce pain, fever, and inflammation (Fig. 2). The anti-inflammatory activity of ibuprofen is due to the inhibition of cyclooxygenase-2 (COX-2), an enzyme crucial to the production of inflammatory mediators. Possible side effects of ibuprofen are renal dysfunction and gastrointestinal

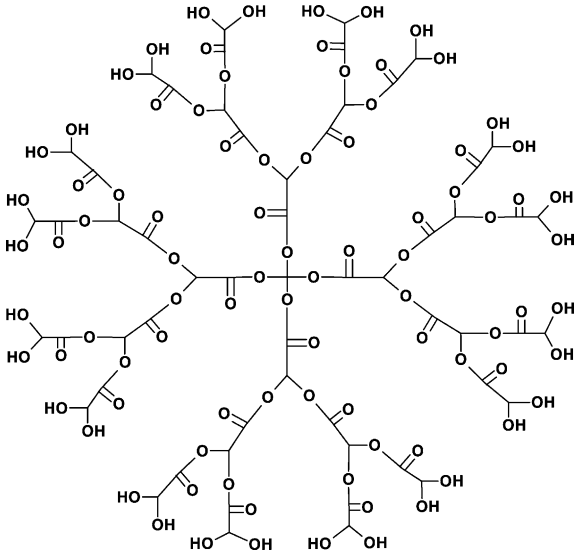


Fig. 1. Structure of the generation 3 hyperbranched Polyol (Petterson, 1996).

alteration of hemorrhage. Targeted delivery of ibuprofen may help avoid adverse side effects. In this paper, we explore the interaction of the drug with dendrimers (their encapsulation or complexation) with different cores, generation numbers and end group functionality. The nature of the interaction was characterized by FTIR and NMR spectroscopy. The in vitro release of ibuprofen from drug–dendrimer complex was evaluated using UV-Vis spectroscopy and HPLC. The cellular entry of the dendrimer, drug, and drug–dendrimer complex was evaluated using UV-Vis spectroscopy into A549 lung epithelial cells.

2. Materials and methods

2.1. Materials

PAMAM-G3-NH_2 (molecular weight = 6909 g/mol, 32 amine end groups) and PAMAM-G4-NH_2 (molecular weight = 14,215 g/mol, 64 amine end groups) were purchased from Aldrich Chemical Company. Hyperbranched Polyol-G5-OH (molecular weight = 15,000 g/mol, 128 hydroxyl end groups) was purchased from Perstorp Polyol, Sweden. FITC was purchased from Fluka. Ibuprofen (molecular weight = 206 g/mol) was provided by Farmacon Pharmaceuticals. Internal standard for HPLC experiments, 5-ethyl-5-tolybarbituric acid (ETBA), was purchased from the Aldrich Chemical Company.

RPMI 1640 culture medium (purchased from Invitrogen Corporation) was used for in vitro and cell entry studies. In addition to several amino acids and vitamins, the following salts were present in this medium: $Ca(NO_3)_2$ (0.42 mM), KCl (5.3 mM), $MgSO_4$ (0.4 mM), NaCl (103 mM), $NaHCO_3$ (23.8 mM), Na_2HPO_4 (5.63 mM).

2.2. Encapsulation of ibuprofen in dendrimer

Ibuprofen was dissolved in methanol following which the dendrimer was added. The reaction mixture was stirred for 24 h in the dark, then evaporated using rotaevaporator to remove methanol. The traces were dried under vacuum in order to remove methanol completely. To these traces, deionized water was added. This solution was stirred in the dark for 24 h.

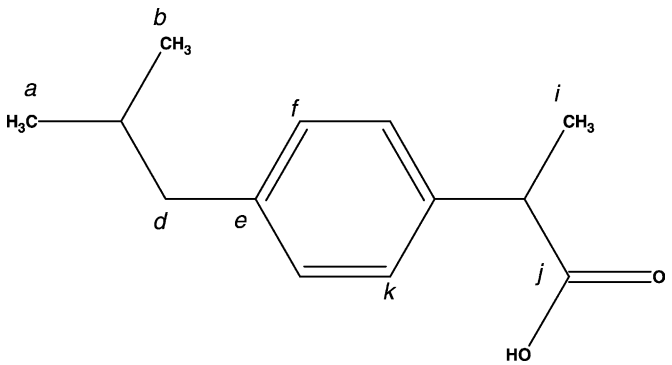


Fig. 2. Structure of ibuprofen (small alphabets represents the carbon atoms; note the COOH end group).

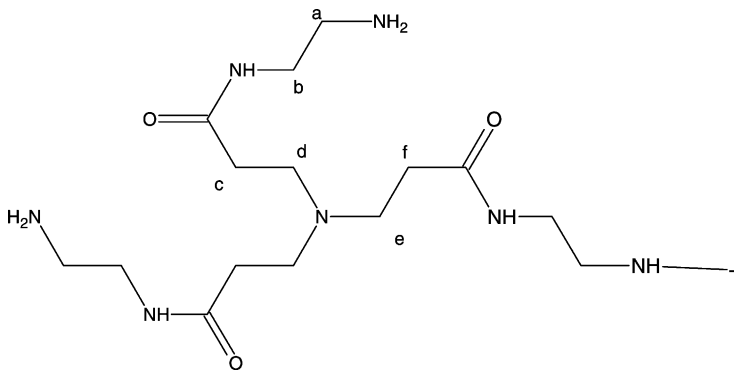


Fig. 3. Part of a PAMAM dendrimer.

This was to extract the drug–dendrimer complex, as dendrimer is soluble in water while ibuprofen is not. The solution was then filtered through PTFE membrane (Millex Millipore) of pore size 200 nm, and then lyophilized to remove water. The drug–dendrimer complex obtained was in the form of a white powder.

2.3. Fourier transform infrared spectroscopy (FTIR)

In order to investigate the structural changes in drug–dendrimer complex, FTIR spectroscopy was used. The drug, dendrimer, and drug–dendrimer complex were dissolved in methanol individually and the FTIR spectra were measured in solution. In the case of drug–dendrimer complex, the measurements were performed within 10 min after dissolution. Perkin-Elmer spectrometer (1600 series) that enables liquid IR measurements was used. Sixteen scans were averaged with resolution of 4 cm^{-1}.

2.4. NMR analysis

NMR analysis was carried out on QE-300 spectrometer.^{13}C-NMR for PAMAM dendrimer (CD_3OD; Fig. 3; Kojima et al., 2000): δ_{PAMAM} 33.6 (C_c), δ_{PAMAM} 37.4 (C_f), δ_{PAMAM} 40.8 (C_b), δ_{PAMAM} 41.8 (C_a), δ_{PAMAM} 49.9 (C_d), δ_{PAMAM} 52.3 (C_e), δ_{PAMAM} 173.5, and 173.9 (CONH). ^{13}C-NMR for ibuprofen (CD_3OD; Fig. 2): δ_{ibu} 17.9 (C_i), δ_{ibu} 21.5 (C_a and C_b), δ_{ibu} 30.2 (C_c), δ_{ibu} 44.8 (C_h), δ_{ibu} 45 (C_d), δ_{ibu} 127 (C_k), δ_{ibu} 129.1 (C_f), δ_{ibu} 138.5 (C_g), δ_{ibu} 140.3 (C_e), δ_{ibu} 177.3 (C_j). For PAMAM-G3-NH_2–ibuprofen complex in CD_3OD: $\delta_{complex}$ 182.2 (COO^-).

2.5. UV-Vis spectroscopy

Perkin-Elmer UV-Vis spectrometer was used to estimate the amount of drug incorporated in the dendrimer. Ibuprofen in phosphate buffer solution (PBS) of K_2HPO_4/KH_2PO_4 (pH = 7.4, 500 mM) gives maximum absorbance in UV region at $\lambda_{max} = 264$ nm corresponding to the vibrational frequency associated with the benzene ring. A calibration curve of ibuprofen in PBS at different concentrations was prepared using the specific absorbance peak of ibuprofen at 264 nm. The calibration curve was linear over a concentration range of 0.1–1 mg/ml with r^2 value of 0.981. The drug–dendrimer complex was dissolved in PBS at pH = 7.4. Since the dendrimers and hyperbranched polymers give weak or no absorbance at this wavelength, the absorbance obtained from drug–dendrimer complex would be solely from ibuprofen. This absorbance was correlated with the calibration curve and amount of ibuprofen was determined. Since the amount of dendrimer was known, we expressed the results as no. of ibuprofen molecules incorporated per dendrimer molecule.

2.6. In vitro release studies

In vitro release of pure ibuprofen and ibuprofen from drug–dendrimer complex in RPMI 1640 culture medium solution was investigated. Ibuprofen and ibuprofen–dendrimer complex were dissolved in RPMI 1640 culture medium. This solution (2 ml in volume) was transferred to a dialysis bag (molecular weight cut off = 3500 Da) immediately. This molecular weight cut off was used because the dendrimers

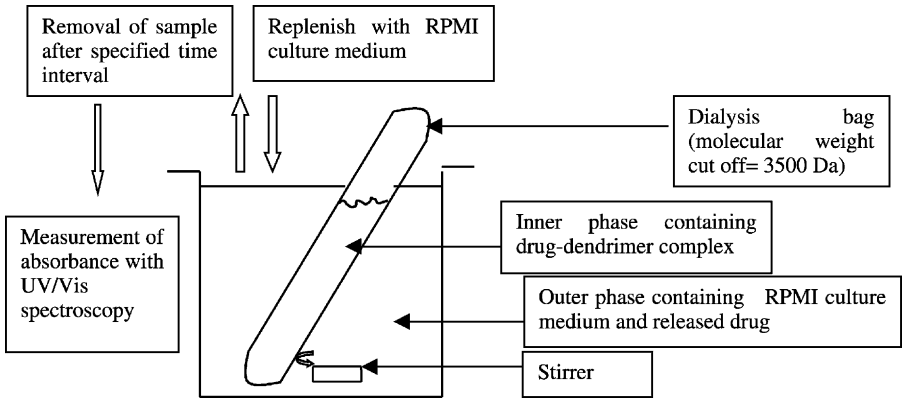


Fig. 4. Schematic diagram of in vitro release study experiment set up.

have a significantly higher molecular weight, so that they would stay inside the dialysis membrane, whereas the small molecular weight drug would readily diffuse out of the dialysis bag. The dialysis bag was placed in a beaker containing 20 ml RPMI 1640 culture medium. The outer phase was stirred continuously. After a specific interval of time, 500 μl of sample was withdrawn from the outer phase, and the outer phase was again replenished with 500 μl of RPMI 1640 culture medium. The absorbance of the outer phase was monitored at 264 nm over a period of time in order to characterize the release of ibuprofen. A schematic diagram of this process is illustrated in Fig. 4. For the drug–dendrimer complex, in vitro release study was also carried out in deionized water and methanol.

2.7. HPLC studies

HPLC analysis was performed on a Perkin-Elmer series HPLC instrument that had UV-Vis detector interfaced with Sigma 1.0 data system with manual injection. The mobile phase used was 35% acetonitrile in 0.1 M sodium acetate. The mobile phase was freshly prepared and degassed under vacuum just prior to use. The column used was Symmetry shield RP_{18} with particle size of 5 μm with 3.9 mm × 150 mm as dimensions. The flow rate maintained was 1.1 ml/min.

The sample obtained from in vitro release was lyophilized and dissolved in 1 ml methanol. To this sample, 50 μl of internal standard, ETBA, was added (Aravind et al., 1984). The internal standard nullifies any possible error due to variation in volume of injected sample, thereby allowing for better quantitative analysis. Under the conditions specified above, ETBA and ibuprofen had retention times of 2.70 and 4.38 min. The absorbance was measured at 220 nm.

2.8. Fluorescence labeling of PAMAM dendrimer and drug–dendrimer complex

FITC was predissolved in acetone (solution of concentration < 5 mg/ml). The molar ratio of PAMAM-G4-NH_2 and FITC taken was 1:20. Dilute solution of PAMAM-G4-NH_2 dendrimer with a factor of 100 (v/v) was prepared in PBS of pH = 7.4. FITC solution was added to unlabelled PAMAM-G4-NH_2 dendrimer solution. The solution was allowed to stand overnight at room temperature with occasional stirring. The acetone carrier solvent was removed by sparging with air. The labeled PAMAM-G4-NH_2 solution was extensively dialyzed against deionized water through sterilized and rinsed membrane tubing (molecular weight cut off = 3500 Da). The solution was filtered through a 0.2 μm gelman Polytetrafluroethylene filter (prewetted with alcohol, then rinsed with water) to remove any trace amount of FITC that may be present (Keunok and Paul, 1996). The degree of labeling was estimated by measuring absorbance of labeled PAMAM-G4-NH_2 dendrimer in PBS (0.1 M, pH = 8.0) at wavelength of 492 nm and by correlating the absorbance with the previously prepared calibration curve. PAMAM-G4-NH_2 was labeled with approximately 8 molecules of FITC.

The PAMAM-G4-NH_2–ibuprofen complex was prepared from this labeled dendrimer with the procedure explained earlier.

2.9. Cellular entry of drug, dendrimer, and the complexed drug

A549 lung epithelial cells, obtained from the established cell line (American Tissue Cell Culture), were incubated with DMEM cell culture medium (Sigma, St. Louis, MO) with 10% fetal calf serum (Gibco BRL, Life Technologies, Frederick, MD), and subcultured every 48 h. A549 carcinoma lung epithelial cells are used in this study, because they are known to endogenously express COX-1 and COX-2 genes. Prostaglandin E_2 (PGE_2) secreted into the cell culture medium is a product of these gene activities. Since ibuprofen targets COX-1/COX-2 expression and blocks the production and secretion of PGE_2, this would allow us to monitor and compare the efficacy of ibuprofen when it is incorporated in dendrimers. Also since these are cancer cell lines, they will also be used in the future studies on the delivery of cancer drugs.

These cells were treated with FITC-tagged PAMAM-G4-NH_2, PAMAM-G4-NH_2–ibuprofen complex and pure ibuprofen for 6 h in RPMI 1640 culture medium. The cells were fixed with 4% formaldehyde. Supernatant and cell lysate were collected at different intervals of time and the absorbance was monitored with UV-Vis spectrophotometer at 264 nm for ibuprofen and 492 nm for fluorescent-labeled dendrimer. At fixed intervals, the supernatant was removed, and fresh RPMI medium is added to the cells, and the cells are shaken for more than 1 min. The cells are subsequently trypsinized, and the cell lysate was prepared. This was done to ensure the complete removal of dendrimer and/or drug present (if any) on cell surface. Cell lysate analysis was not done for ibuprofen because the drug may get metabolized in the cells. Furthermore, a mass balance on ibuprofen was done based on the initial concentration, and the supernatant concentration as a function of time, and most of the drug was accounted for. Therefore, it is unlikely that appreciable amount of drug was just on the cell surface.

3. Encapsulation and characterization study on ibuprofen–dendrimer complex

3.1. Drug encapsulation with dendrimer

Drug can be either encapsulated or complexed to the dendrimer. Encapsulation/complexation ability of different amounts of drug in dendrimer (PAMAM-G3-NH_2, PAMAM-G4-NH_2 and hyperbranched Polyol-OH) was studied to estimate the 'maximum' number of molecules that can be incorporated in a dendrimer molecule. The initial molar ratios of ibuprofen to PAMAM-G3-NH_2 were 22, 45, and 70. When molar ratios were greater than 32, only 32 moles of ibuprofen could be incorporated (Fig. 5a). Similarly for PAMAM-G4-NH_2, initial molar ratios used in feed were 40, 48, and 175. When molar ratio were greater than 80 it can be seen that only 78 molecules of ibuprofen were incorporated (Fig. 5b).

In the case of Polyol-OH, the initial molar ratios used were 100, 225, 424. Despite the increase in molar ratio, there was only a small change in the amount of ibuprofen in Polyol-OH (Fig. 5c). The estimated number of ibuprofen molecules incorporated to dendrimers and hyperbranched Polyol is summarized in Table 1.

Table 1
Estimated amount of ibuprofen incorporated into dendrimers

Dendrimer type	No. of end groups	No. of ibuprofen molecules incorporated per dendrimer molecule
PAMAM-G3-NH_2	32[a]	32
PAMAM-G4-NH_2	64[a]	78
Polyol-OH	128[b]	24

[a] Reported by Tomalia et al. (1990).
[b] Reported by Petterson (1996).

The encapsulation/complexation of ibuprofen in PAMAM-G3-NH_2 and PAMAM-G4-NH_2 was successfully carried out. The number of ibuprofen incorporated into PAMAM-G3-NH_2 and PAMAM-G4-NH_2 were 32 and 78, respectively. These values are consistent with the number of free amine groups present in PAMAM-G3-NH_2 (32) and PAMAM-G4-NH_2 (64). It appears that there is a strong interaction between the NH_2 functional groups of PAMAM and the COOH groups of ibuprofen. In the PAMAM-G4-NH_2, the number of drug molecules incorporated is 78, 14 more compared to the 64 free amine groups available. Previous studies showed that the drugs which do not interact with the end groups can be encapsulated in the interior of dendrimer and the extent of encapsulation can be increased by increasing the generation number, or by attaching long chain molecules to the end groups of dendrimer (Kojima et al., 2000). Since PAMAM-G4-NH_2 molecule is bigger in size

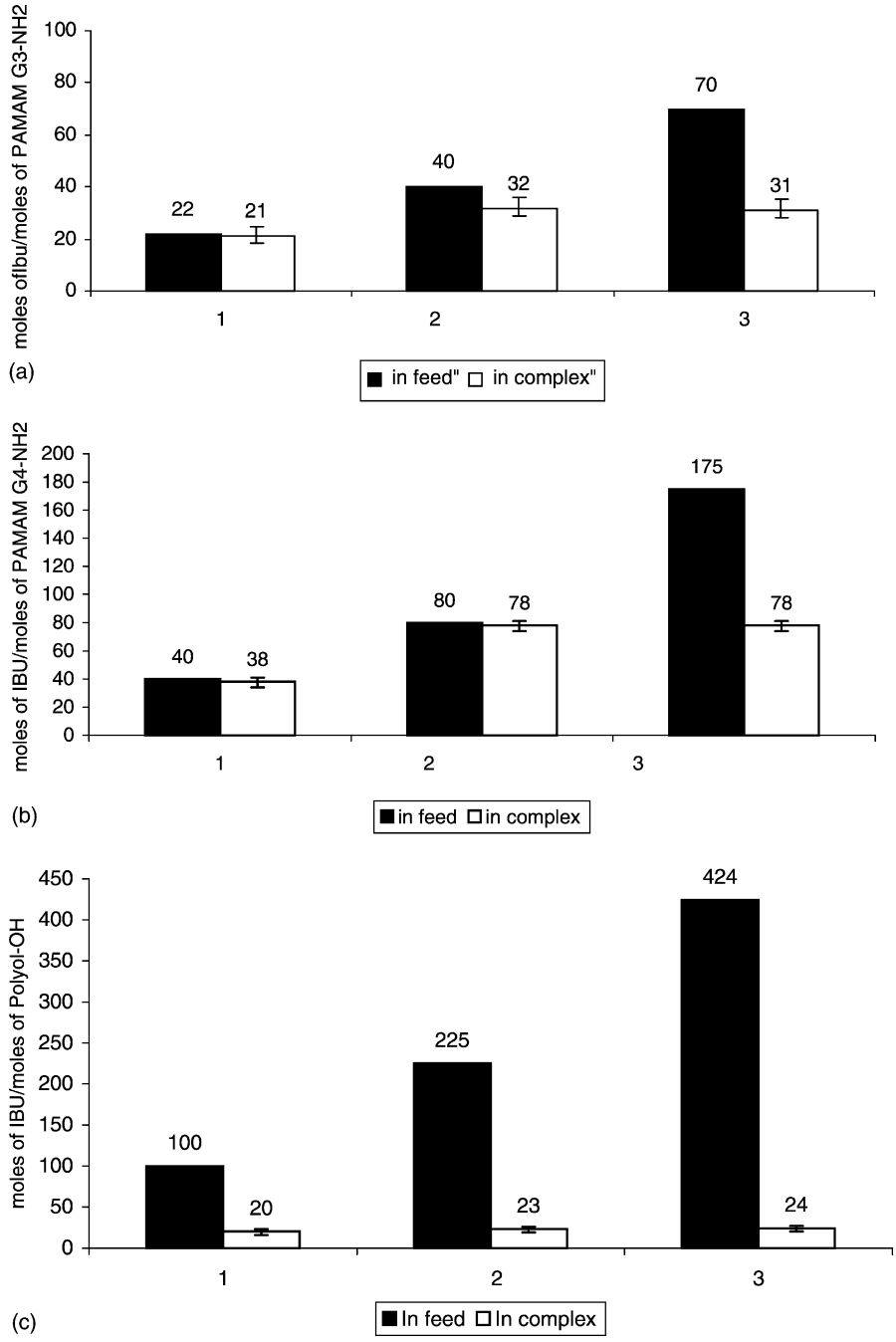


Fig. 5. (a) Ibuprofen incorporation data for PAMAM-G3-NH_2. Molar ratios of ibuprofen to PAMAM-G3-NH_2 in feed were 22, 40, and 70. When molar ratio in feed are greater than 32, only 32 ibuprofen molecules were incorporated. (b) Ibuprofen incorporation data for PAMAM-G4-NH_2. Maximum number of ibuprofen molecules incorporated are 78, when molar ratio in feed was greater that 78. (c) Ibuprofen incorporation data for hyperbranched Polyol. Despite using higher molar ratios of ibuprofen to Polyol-OH in feed, the maximum number of ibuprofen incorporated is 24.

compared to PAMAM-G3-NH_2, some ibuprofen molecules could have been encapsulated in the core of the dendrimer in addition to complexation with the surface end groups. This encapsulation could be a result of weak interactions between the dendrimer core and the drug, as pointed out previously (Kojima et al., 2000). The hyperbranched Polyol-OH was able to incorporate only 24 ibuprofen molecules despite the 128 available hydroxyl end groups. One would not expect strong interactions between the –OH surface groups and the drug. Therefore, we suggest that the drug molecules are encapsulated inside the large generation-5 polymer, through weak interactions.

3.2. Investigation of the nature of the drug–dendrimer complex

The ability of the dendrimer to form a complex with drugs depends on the nature of the core-surface groups of dendrimer (hydrophobic versus hydrophilic), electrostatic interactions between the dendrimer and the drug, and the ability of the drug to form a conjugate with the dendrimer through chemical bonding. Therefore, it is possible to manipulate the incorporation process for a given drug by appropriate selection of the dendrimer, and the surface functionality. One might expect that the ibuprofen with the carboxylic group may form a complex with surface NH_2 groups of PAMAM dendrimer, whereas the Polyol, hyperbranched polymer with OH surface groups may physically encapsulate the drug. This was investigated using FTIR, NMR, and in vitro release studies.

FTIR spectrums of PAMAM-G3-NH_2, PAMAM-G4-NH_2, and ibuprofen were obtained as shown in Fig. 6a–c, respectively. Pure PAMAM-G3-NH_2 and PAMAM-G4-NH_2 show N–H deformation vibration at 1626 and 1631 cm^{-1} (Socrates, 1994). These peaks are assigned to the free N–H groups present on the periphery of the dendrimer. The peak at 1556 cm^{-1} is assigned to C–N stretching vibrations, which correspond to C–N bond inside the core. Peaks in the region 2800–3200 cm^{-1} are assigned to N–H stretching and C–H stretching vibrations (Fig. 6a and b). Pure ibuprofen shows a strong carbonyl band absorbance at 1703 cm^{-1}, which corresponds to the carboxyl acid group (COOH) present in ibuprofen. Other smaller peaks in the region 1200–1000 cm^{-1} are contributions from the benzene ring (Fig. 6c; Socrates, 1994). FTIR spectrum for PAMAM-G3-NH_2–ibuprofen complex shows the disappearance of strong carbonyl absorbance at 1703 cm^{-1} of ibuprofen and 1626 cm^{-1} peak of PAMAM-G3-NH_2. Instead, a new peak at 1637 cm^{-1} is observed and the 1556 cm^{-1} peak remains (Fig. 6d).

Similar behavior was observed for PAMAM-G4–ibuprofen complex in which a new peak appears at 1650 cm^{-1} (Fig. 6e). This new peak may be attributed to the asymmetric stretching vibration of COO^-, since carboxylic acid salts exhibit strong characteristic COO^- asymmetric stretching band in the region of 1650–1550 cm^{-1} (Socrates, 1994). No other changes in drug–dendrimer complex were seen. Contributions from ibuprofen and dendrimer can be easily observed in complex from 2953 cm^{-1} peak and the 3298 cm^{-1} peak, respectively.

From ^{13}C-NMR spectra of pure ibuprofen, PAMAM-G3-NH_2 and PAMAM-G3-NH_2–ibuprofen complex are presented in Fig. 7a–c. The peak assignments are discussed in Section 2.4 and the indexing of the carbon atoms for ibuprofen and PAMAM-G3-NH_2 are discussed in Figs. 2 and 3, respectively. The ^{13}C-NMR spectrum of the complex contains the signals from *both* PAMAM-G3-NH_2 and ibuprofen. The PAMAM-G3-NH_2 shows chemical shifts at 41.8, which corresponds to the carbon next to terminal amino group and a shift at 40.8, which corresponds to carbon next to this carbon. Also ibuprofen spectrum exhibits a shift at 44.8 that corresponds to carbon atom next to carboxyl group. However, in the PAMAM-G3-NH_2–ibuprofen complex spectrum, the 41.8 and 40.8 shifts from dendrimer, and the 44.8 shift from ibuprofen completely disappeared and a new shift at 39.5 appeared. Also the shift at 17.9 for ibuprofen, which corresponds to the carbon atom of side chain (as shown in Fig. 2), shifted to higher value of 19.068. Theoretical calculations attribute this shift to the presence of carboxylate ion (Pretsch et al., 1989).

The ^{13}C-NMR data gives further insight into the interaction between the terminal amine groups of dendrimer with carboxyl group of ibuprofen. Pure ibuprofen shows a shift at 177.3 ppm, which corresponds to the carboxyl group. In the ^{13}C-NMR spectrum of the complex, this shifts to 182.2. If it has been a conjugation reaction, this would shift to *lower* values than 177.3 as observed earlier for PAMAM–PEG conjugation (Kojima et al., 2000). Theoretical calculations

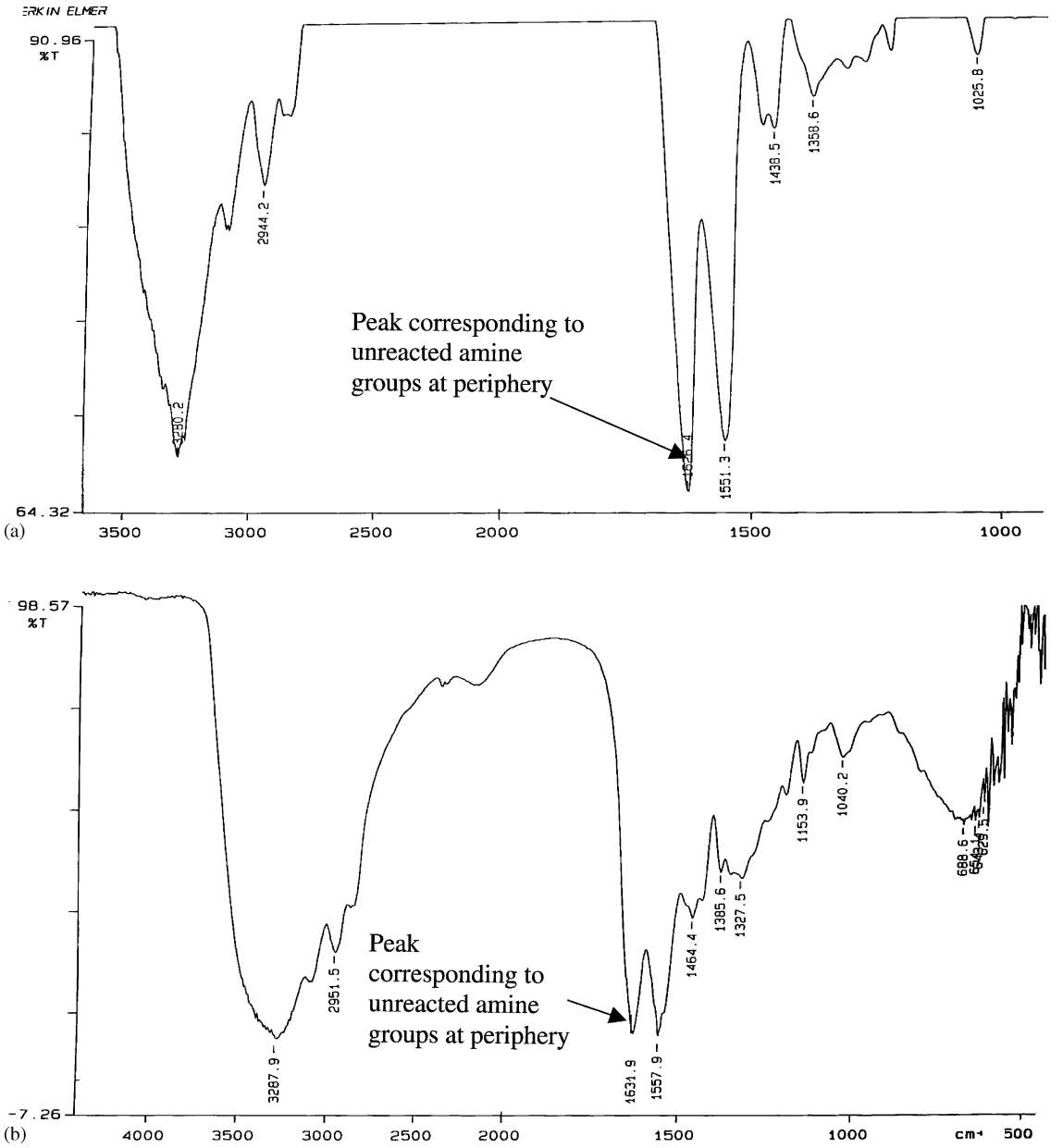
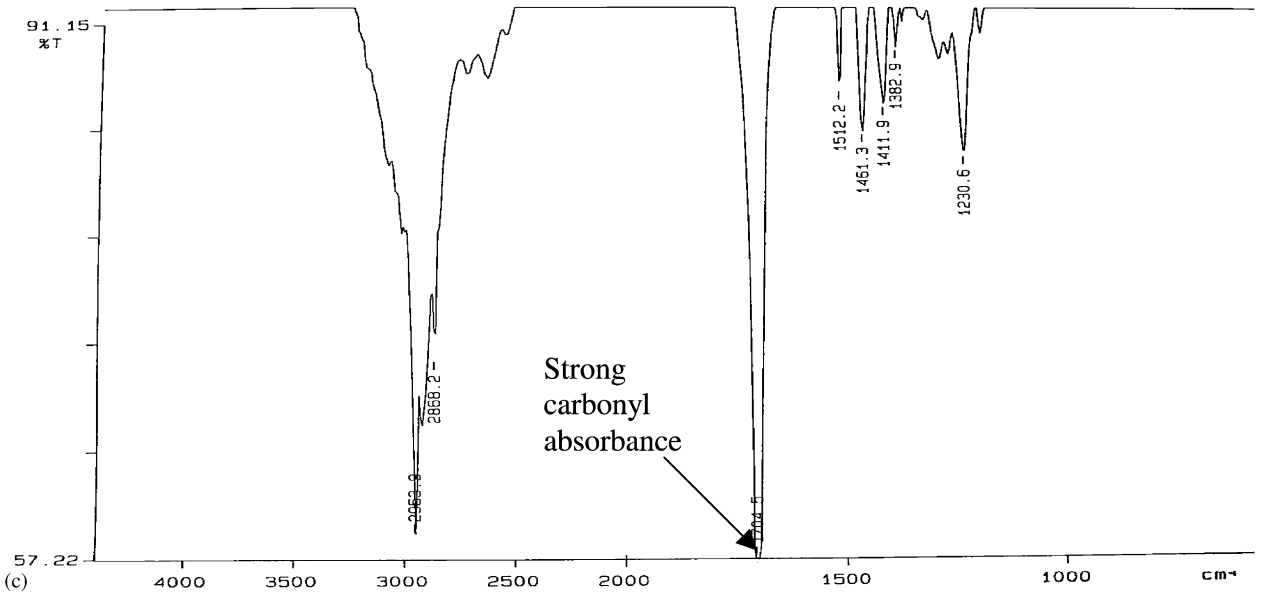
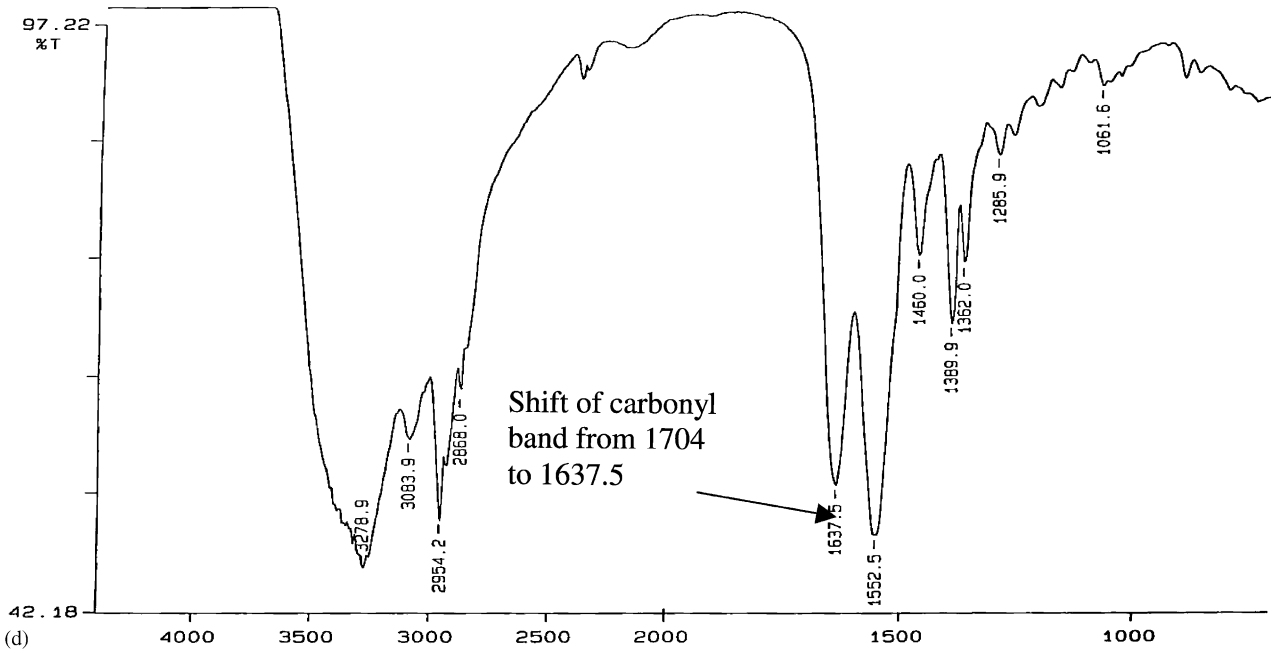


Fig. 6. (a) IR spectrum of pure PAMAM-G3-NH_2. Strong N–H deformation vibration corresponding to peripheral amine groups can be seen at 1626 cm^{-1}.(b) IR spectrum of pure PAMAM-G4-NH_2. N–H deformation corresponding to peripheral amine groups can be seen at 1631 cm^{-1}.(c) IR spectrum of pure ibuprofen. Strong carbonyl band occurs at 1703 cm^{-1}, which corresponds, to COOH functional group of ibuprofen. (d) IR spectrum of PAMAM-G3-NH_2–ibuprofen complex. Disappearance of peaks at 1703 and 1626 cm^{-1} and appearance of new peak at 1637 cm^{-1} corresponds to the carboxylate ion of ibuprofen. (e) IR spectrum of PAMAM-G4-NH_2–ibuprofen complex. Absence of peak at 1703 and 1631 cm^{-1} and appearance of new peak at 1650 cm^{-1} may signify the formation the carboxylate ion resulting from the interaction between the free surface amine groups of dendrimer and carboxyl group of ibuprofen.



(c)



(d)

Fig. 6. (*Continued*).

for carboxylate ion gave the estimate to be 183 that agrees well with the value observed in our study (Pretsch et al., 1989). Therefore, we suggest the formation of a drug–dendrimer complex with ionic interaction, which agrees with the previous findings (Milhem et al., 2000).

In summary, our FTIR, NMR, and UV-Vis spectroscopy results suggest:

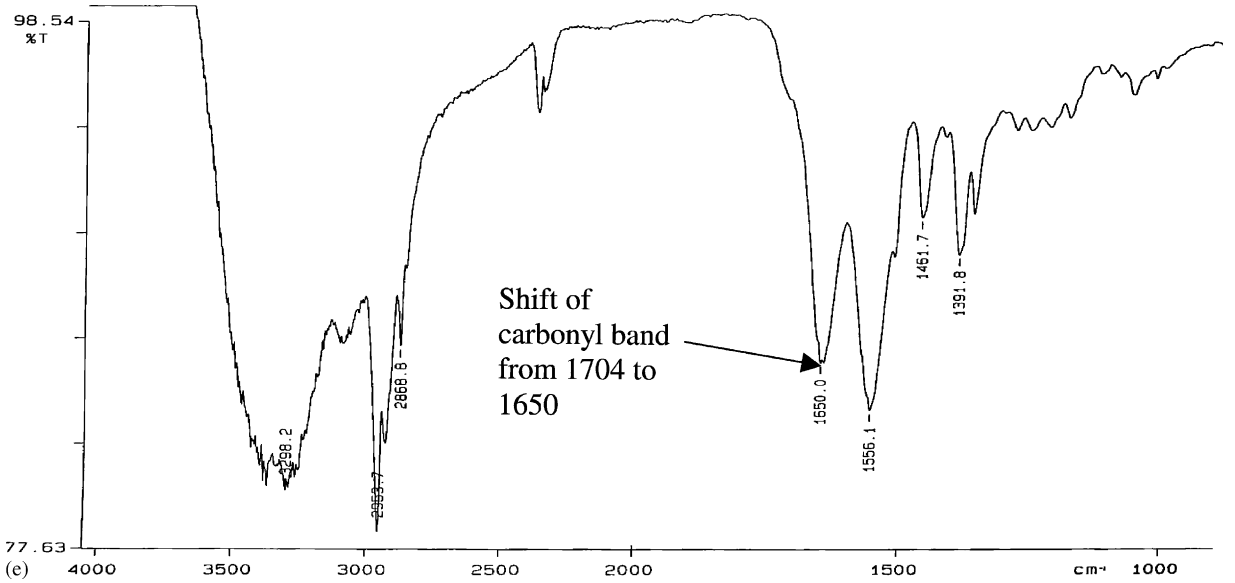


Fig. 6. (*Continued*).

(i) The number of drug molecules incorporated with the dendrimer correlated strongly with the number of surface amine groups,
(ii) FTIR of the complex shows a 1650 cm^{-1} peak suggesting the peak of carboxylate ion,
(iii) ^{13}C-NMR shows peak shift from 177.3 to 182.2 ppm, consistent with the presence of carboxylate ions.

Therefore, we suggest that the carboxylate ion may be forming a complex with the amine surface group of the cationic PAMAM. The smaller interior space in PAMAM-G3-NH_2 does not allow any perceptible encapsulation whereas the relative bigger interior of PAMAM-G4-NH_2 may encapsulate a small number of additional ibuprofen molecules (approximately 14 molecules), on top of the 64 ibuprofen molecules (approximately) that form complex with 64 amine surface groups. The strength and stability of this complex will be discussed in the next section.

On the other hand, encapsulation of ibuprofen in Polyol-OH was relatively less efficient. The average number of ibuprofen molecules incorporated per Polyol-OH was 24. The IR data did not show any combination peaks, further suggesting physical encapsulation. The Polyol-OH core may have a relatively weak electrostatic interaction with ibuprofen, and there may be no end group interaction with the -OH groups. It must be pointed out that the number of drug molecules incorporated in PAMAM in this study through the simple process of complexation, is much higher than observed previously for other drugs.

3.3. In vitro release rate studies

The strength and stability of the drug–dendrimer complex was examined using in vitro release studies in RPMI 1640 culture medium, deionized water, and methanol. UV-Vis absorbance measurement and HPLC were carried out for all the experiments. HPLC and UV-Vis results agreed very well and the HPLC results are shown in this section.

Pure ibuprofen was released in 5 h in RPMI culture medium. In methanol and deionized water, less than 15% ibuprofen was released from the PAMAM-G4-NH_2–ibuprofen complex even after 9 h (Fig. 8). This suggests that the drug–dendrimer complex is relatively very stable in methanol and water. The release of ibuprofen from drug–dendrimer complex was appreciably slower compared to pure ibuprofen. After 1 h, ~50% of the pure drug is released, whereas only ~20% is released from the PAMAM–ibuprofen complex. In 8 h, 84% release was obtained for PAMAM-G4-NH_2–ibuprofen complex while 78%

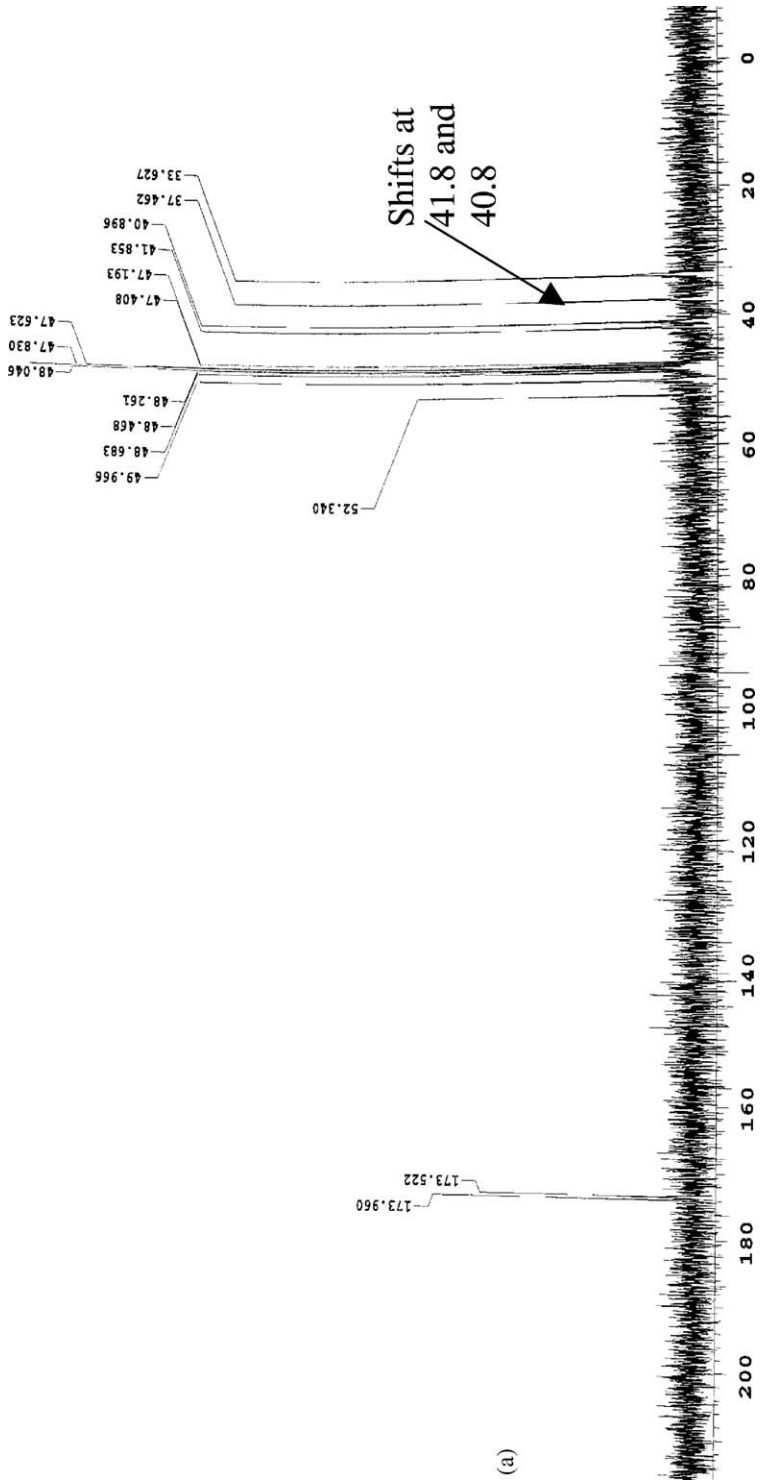


Fig. 7. (a) NMR spectrum of PAMAM-G3-NH_2. (b) NMR spectrum of ibuprofen. (c) NMR spectrum of PAMAM-G3-NH_2–ibuprofen complex.

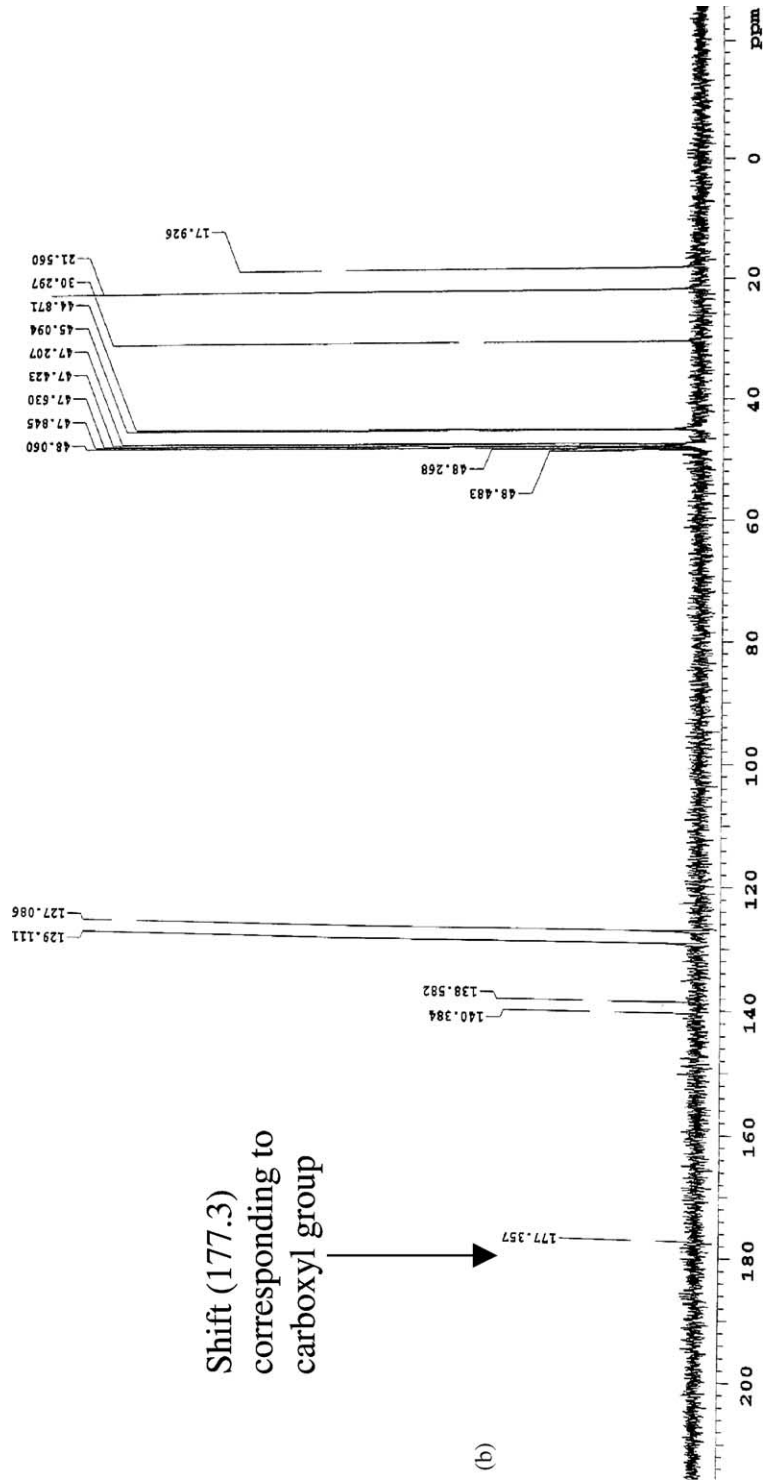


Fig. 7. (*Continued*).

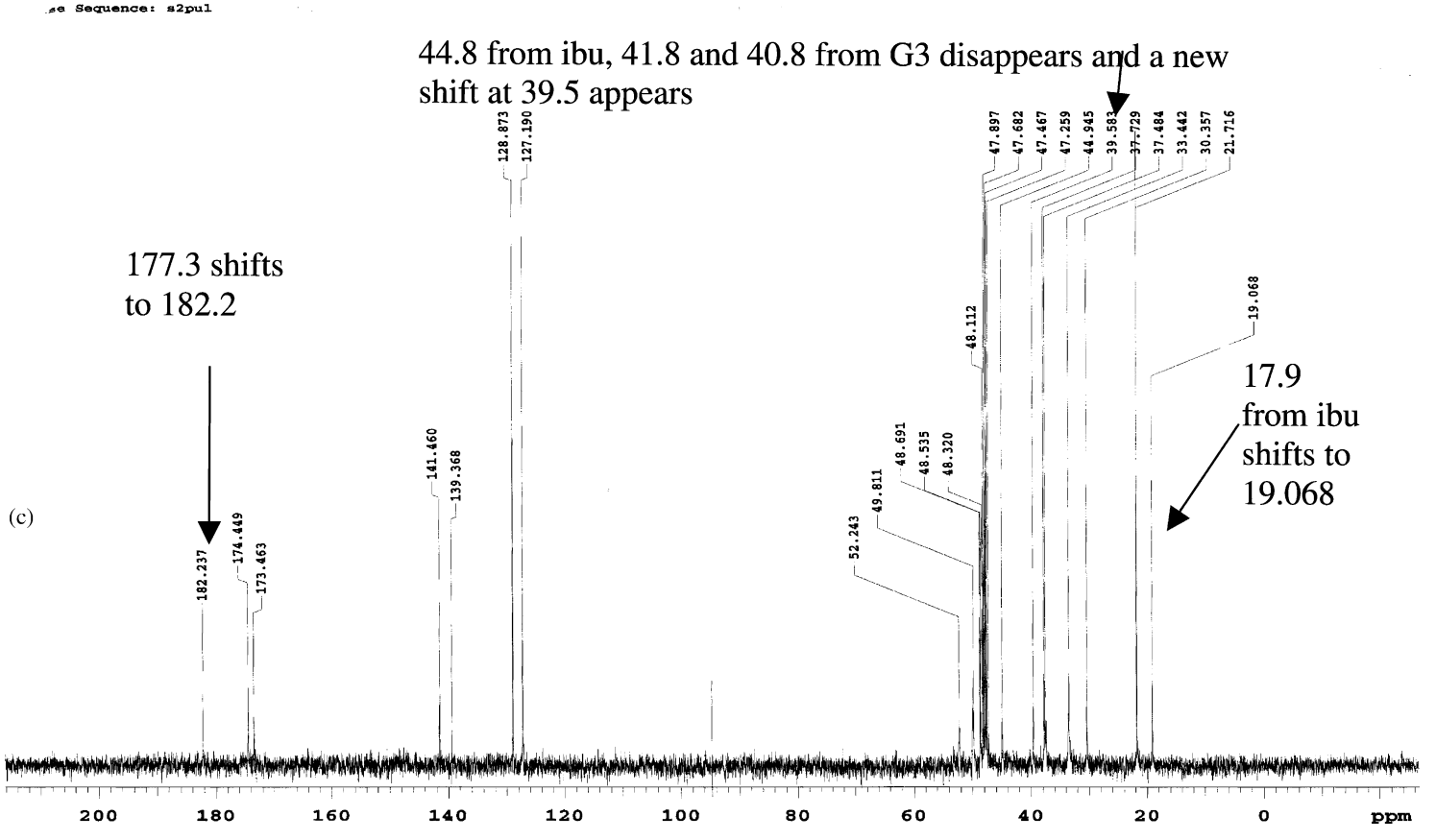


Fig. 7. (*Continued*).

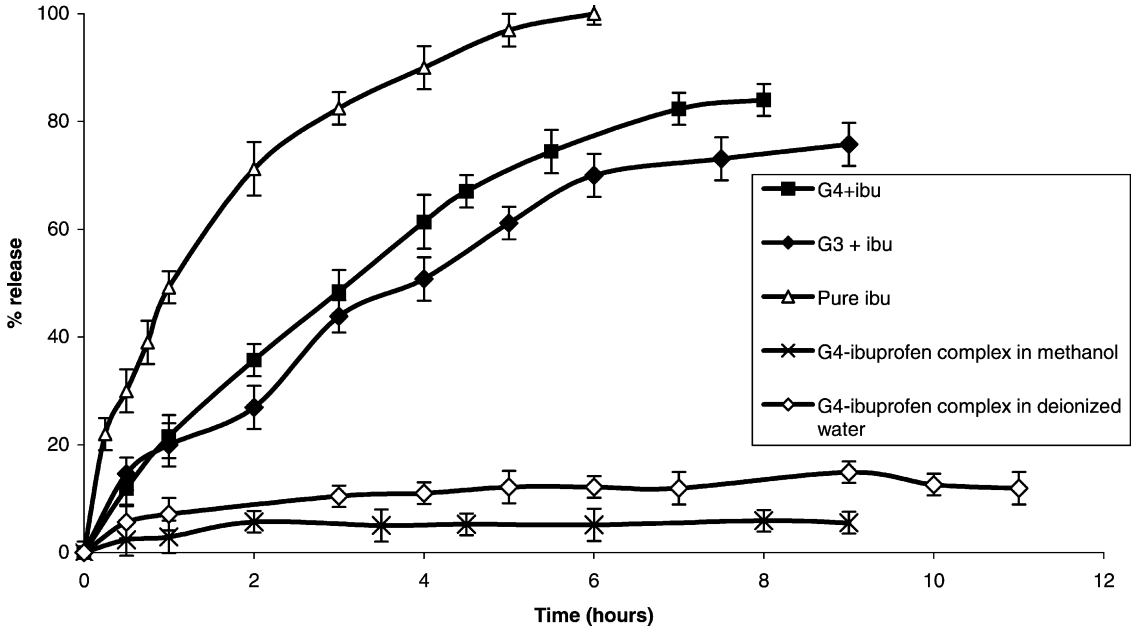


Fig. 8. Release of ibuprofen from drug–dendrimer complex in different solvents.

release was obtained for PAMAM-G3-NH_2–ibuprofen complex in 9 h. The release of the drug from the dendrimer complex depends on the strength of the ionic interactions, and the salt concentration. This will be explored in detail in future studies.

3.4. Cellular entry of PAMAM dendrimer

A549 lung epithelial cells were treated with pure FITC-labeled PAMAM-G4-NH_2, pure ibuprofen, and FITC-labeled PAMAM-G4-NH_2–ibuprofen complex. The UV-Vis analysis using ibuprofen and FITC calibration curves, of the supernatant and the cell lysate for pure PAMAM-G4-NH_2 suggests that most of dendrimer (∼90%) enters the lung epithelial carcinoma cells within 1 h (Fig. 9a). This is seen by a decreasing FITC content in the supernatant and a simultaneous increase in the FITC content in the cell lysate. This may be because of the cationic nature of the dendrimer (El-Sayed et al., 2002). On the other hand, for pure ibuprofen, it takes 4 h. The drug–dendrimer complex was also analyzed for cell entry and the analysis showed that the cellular entry profile of the complexed dendrimer was comparable to the pure dendrimer, but the ibuprofen entry is significantly speeded up (Fig. 9b). In the complexed state, ibuprofen appears to enter the cells at rates comparable to the pure dendrimer and significantly faster than the free drug (more than 80% of complexed ibuprofen inside the cell in 1 h, whereas it takes about 3 h for 80% pure ibuprofen to get inside the cell).

The PAMAM-G4-NH_2–ibuprofen complex contains ∼50% of dendrimer and ∼50% ibuprofen by weight. Therefore, if the dendrimer were carrying the drug inside the cells, we would expect comparable cell entry profiles (Fig. 9b). Even though the profiles are nearly identical, there is a small difference at 30 min and 1 h. We believe that this may be due to a small amount of ibuprofen that may be released in the cell culture medium during the time the complex is dissolved and poured into the plates (this may take between 5 and 10 min) The amount of free ibuprofen present in supernatant at early time points was analyzed with HPLC, which showed that approximately 12–14% of free ibuprofen was present in the supernatant. If this were taken into account, the dendrimer and ibuprofen in the complex would have nearly identical profiles, as one would expect if the complex

were entering the cell as a unit. Time-resolved fluorescence microscopy also revealed the similar cellular entry profile for the dendrimer. Preliminary results on the cellular delivery of ibuprofen using dendrimers, based on RT-PCR analysis of the COX-2 suppression, suggest that the complexed ibuprofen is more efficient and fast-acting, compared to the free drug. The effect of generation number and end functionality of the dendrimer on cellular entry will be discussed elsewhere (Kannan et al., submitted).

These results suggest that the dendrimer enters the A549 lung epithelial cells within 1 h, and that the complexation with dendrimer may enhance the cell entry rates of ibuprofen significantly. The subsequent cellular release and the performance of the drug inside the cell are being investigated using COX-2

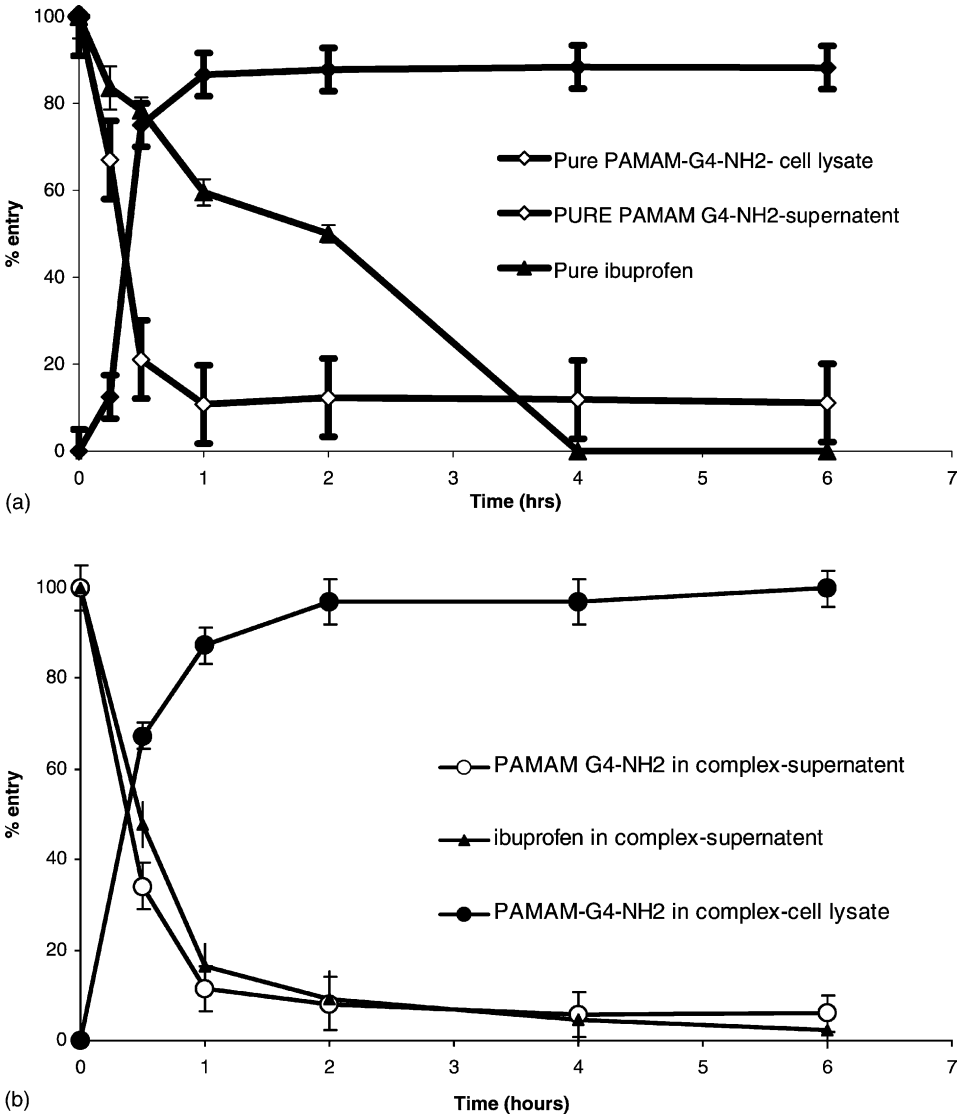


Fig. 9. (a) Cellular entry profile of PAMAM-G4-NH_2 dendrimer and pure ibuprofen. (b) Cellular entry profile of PAMAM-G4-NH_2 dendrimer and ibuprofen in complex. (c) Cellular entry profile of pure ibuprofen and ibuprofen in the drug–dendrimer complex. The drug–dendrimer complex enhances the cellular entry of ibuprofen.

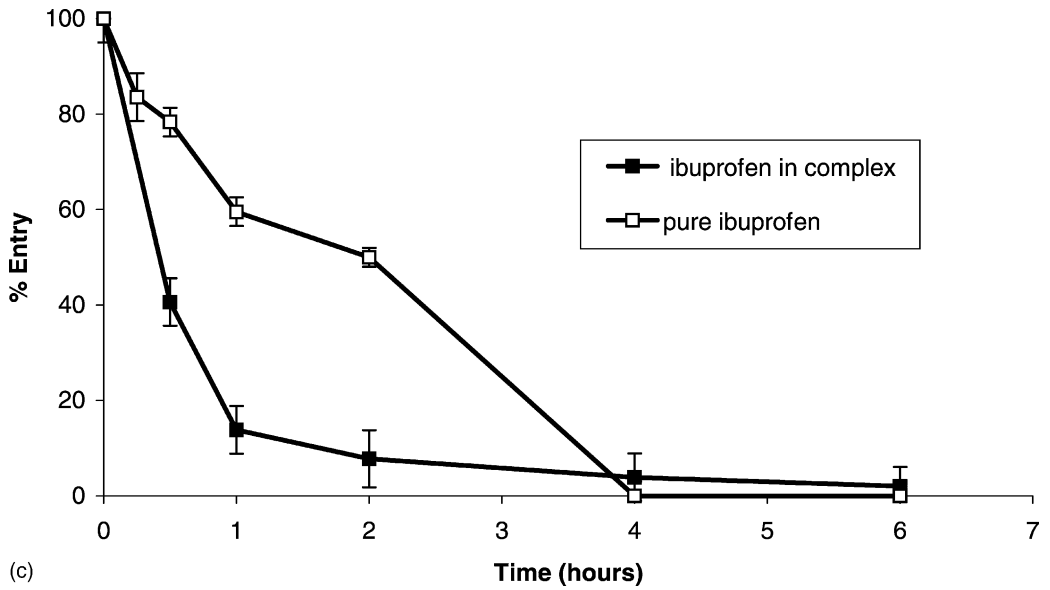


Fig. 9. (*Continued*).

gene suppression by pure ibuprofen, and the ibuprofen complexed with the dendrimer. These will be discussed in a future publication.

4. Conclusions

The ability of dendrimer to form complex or encapsulate drug molecules was explored using ibuprofen as model drug, with PAMAM dendrimers and hyperbranched Polyol. The nature of drug–dendrimer interaction, the in vitro drug release, and cellular entry were explored using a combination of UV-Vis, HPLC, FTIR, and NMR spectroscopy. Our studies suggest that:

(i) The amine-terminated PAMAM-G3-NH_2 and PAMAM-G4-NH_2 may predominantly form a complex with the carboxylate ion from the ibuprofen because of the ionic interactions, as shown by FTIR spectroscopy and NMR. For the generation 4 PAMAM, some ibuprofen may be encapsulated in the interior also. On the other hand, the –OH-terminated Polyol appears to encapsulate ibuprofen.

(ii) A large number of ibuprofen molecules could be incorporated into the dendritic polymer, with PAMAM-G4-NH_2 incorporating approximately 78 ibuprofen molecules.

(iii) This complex is stable in deionized water and methanol, showing minimal release even after 8 h. In RPMI 1640 culture medium, the release rate of ibuprofen from drug–dendrimer complex was slower compared to pure ibuprofen.

(iv) The PAMAM-G4-NH_2–ibuprofen complex enters in the lung epithelial carcinoma cells readily and ibuprofen can be delivered within 1 h through this drug–dendrimer complex. In comparison, pure ibuprofen appears to take more than 3 h to enter the cell completely. The cellular activity of the released ibuprofen is being investigated using the COX-2 gene suppression.

Current studies are exploring the complexation/conjugation ability of these dendrimer/hyperbranched polymers to a wide variety of drugs such as ADR and MTX (for chemotherapy), epinephrine (for treatment of shock), and solu-medrol (for asthma). The dynamics of the cellular entry of these dendrimers and drug–dendrimer complexes are being investigated.

Acknowledgements

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