Hyperbranched Polymer-Drug Conjugates with High Drug Payload for Enhanced Cellular Delivery

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Purpose. To synthesize and evaluate hyperbranched polymer (HBP)-drug conjugates with high drug payload for enhanced cellular delivery.

Methods. Polyol- and polyglycerol-ibuprofen conjugates with or without imaging agent fluorescein isothiocyanate (FITC) were synthesized using dicyclohexilcarbodiimide (DCC) as a coupling agent. Drug-polymer conjugates were characterized using ^{13}C NMR, ^{1}H NMR, and gel permeation chromatography (GPC). Stability of the drug-conjugates was studied using free drug release through a dialysis membrane. Cellular entry of FITC-labeled HBP conjugates was studied using fluorescence activated cell sorter (FACS), and cell supernatant was analyzed by UV-visible spectrophotometer. The intracellular localization of FITC-labeled conjugates in A549 lung epithelial cells was imaged using fluorescence microscopy. Anti-inflammatory activity of the HBP-ibuprofen conjugates was estimated *in vitro* by measuring the concentration of prostaglandin (PGE_2) using an ELISA kit.

Results. The average number of ibuprofen molecules conjugated per molecule of HBP was estimated to be 50 for polyol and 53 for polyglycerol. The HBP-drug conjugates did not release the drug up to 72 h in methanol, indicating the presence of stable ester bonds. Both the polymer-drug conjugates entered the cells rapidly. The conjugates were localized in the cell cytosol as evidenced by fluorescence microscopy. Within 30 min, the HBP-drug conjugates showed rapid suppression of PGE_2 synthesis, whereas free ibuprofen did not show any activity. At later times, the conjugates showed comparable activity.

Conclusions. For the first time, we report HBP conjugates with a high drug payload. HBP-drug conjugates entered the cells rapidly and produced the desired pharmacological action. This study demonstrates that hyperbranched polyol and polyglycerol are promising nanovehicles for achieving enhanced cellular delivery of drugs.

KEY WORDS: cell entry; dendrimers; hyperbranched polymers; ibuprofen; polyglycerol; polyol.

INTRODUCTION

The efficacy of a drug can be greatly improved by delivering it to the target site with minimal systemic exposure. Different degrees of enhanced and targeted drug delivery are now possible due to the significant advancements in polymer science with regard to versatility in chemistry and the ability of the delivery vehicle to interact with the host over various length scales ranging from nanometer to micrometer (1–3). Ever since the first report of dendrimer preparation through controlled synthetic methods (divergent and convergent), they have emerged as attractive drug delivery vehicles (4–6). Dendrimers have generated a great deal of interest for various applications due to their unique structural properties such as monodispersity (~1.0), high density of functional groups at periphery, well defined globular shape (~20 nm), and multivalency (7–8). These salient features make dendrimers potential alternatives to traditional polymers in a wide range of applications especially as nanodevices for controlled, enhanced, and targeted delivery of therapeutic compounds. A variety of dendritic polymers have been synthesized containing a hydrophobic core or a hydrophilic shell for diverse applications. Conjugation of drugs to polyethylene glycol attached dendrimers was shown to increase the circulation time (9–10). In the past, various strategies have been devised to load dendrimers with drug molecules, genetic materials, targeting agents, dyes and imaging agents, either by encapsulation or conjugation (11–20). By conjugating appropriate targeting moieties, drugs and imaging agents to dendritic polymers, 'smart' drug delivery nanodevices can be developed.

On the other hand, hyperbranched polymers (HBPs) such as polyol and polyglycerol possess relatively higher polydispersities than dendrimers. Nevertheless, due to their highly branched architecture and chemical tunability, they can be used as effective drug delivery vehicles, especially when drugs are covalently attached to the surface. The general term of hyperbranched polymer includes both "perfectly-branched" dendrimers and "imperfectly branched" polymers. Typically, hyperbranched polymers comprise of a randomly branched structure, having precisely one focal unit and at least two branching points. Such polymers are generally prepared via AB_m-type monomers. In this paper, we refer to hyperbranched polymers, which are one step synthesized with some "control" over the branching architecture. HBPs used in this research are typically synthesized by one pot self-condensation of multifunctional AB_x type monomers (where A is a functional group that reacts with B groups and $x \geq 2$) (21). These polymers have attracted attention as potential alternative for perfectly branched dendrimers, which are usually prepared by a tedious stepwise procedure. Although, HBPs possess relatively high polydispersity, the synthetic procedure could eventually make them cost effective for clinical applications. Here, we explore two challenging aspects of HBP in drug delivery: 1) achieving high drug payload, and 2) evaluating the therapeutic effectiveness of HBP-conjugated drugs *in vitro*, especially for non-cancer applications. It has been widely recognized that achieving high drug payloads in HBPs has been a challenge. A recent patent by Duncan *et al.* reported drug payload of as much as 25% for cisplatin using dendrimers (22). Recently, HBPs have been considered as attractive materials vehicles for controlled drug release, solubilization of inorganic compounds in organic media, and dispersion of polar dyes in hydrophobic polymers (23,24). We investigate the potential of HBPs such as polyol and polyglycerol as drug delivery vehicles for carrying high drug payload. In literature, most of the dendrimer-drug conjugate therapies have focused upon cancer therapy, perhaps due to the fact

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that *in vitro* effectiveness of the conjugates is easier to monitor through cell toxicity. (25). Arguably, these nanovehicles offer tremendous potential for cancer therapeutics. At the same time, the HBPs can be promising vehicles in other therapeutic areas, where the therapeutic target is intracellular. Therapeutic areas like asthma, pain and inflammation remain unexploited, where rapid drug delivery and high intracellular drug levels would significantly improve therapeutic efficacy. In the present study, ibuprofen is used as a model drug to prepare HBPs conjugates for evaluating the cell entry dynamics and pharmacological activity of the conjugated drug. Ibuprofen is a commonly used high dose non-steroidal anti-inflammatory drug and acts by inhibiting enzyme cyclooxygenase 2 (COX-2). Intracellular delivery of ibuprofen would help to reduce its systemic side effects, such as renal dysfunction and gastrointestinal hemorrhage. Further, with a high payload drug delivery system, it would also be possible to reduce the dose by effectively delivering the drug inside the cell.

In our previous work, we reported PAMAM–NH_2 dendrimer-ibuprofen *complexes* that were formed by ionic interactions between amine groups of dendrimer and carboxyl groups of ibuprofen. It was demonstrated that the complexation and encapsulation of ibuprofen enhances the cellular uptake of ibuprofen compared to free ibuprofen. The complexes thus formed were able to exert a greater therapeutic efficacy by suppressing COX-2 gene (26–27). In this work, we designed hyperbranched polymer-drug *conjugates* that may be more stable than the polymer-drug complex *in vivo* for prolonging drug circulation and enhancing cellular uptake. We report conjugation of polyol and polyglycerol containing "high payload" of ibuprofen, characterized using ^{1}H NMR, ^{13}C NMR, and GPC. The stability of the HBP-drug conjugates was evaluated using HPLC assay. FITC-drug-HBP conjugates were synthesized for studying cellular entry in A549 lung epithelial cells using flow cytometry and fluorescence microscopy. The therapeutic efficacy of HBP-ibuprofen conjugates was evaluated by estimating the percent inhibition of prostaglandin release.

MATERIALS AND METHODS

Hyperbranched polymers, polyol and polyglycerol were obtained from Perstorp polyol (Perstorp, Sweden) and Hyperpolymers Inc. (Freiberg, Germany), respectively. Polyglycerol is synthesized by single step, controlled anionic ring-opening polymerization of glycidol using partially deprotonated triol as alkoxide initiator. Hyperbranched polyglycerol has an M_w = 7,095, M_n = 4,785 and polydispersity (PDI) = 1.48 (28). Perstrop hyperbranched polyol (PDI ~1.6) is synthesized by direct esterification of bis-dimethyl propionic acid (Bis-MPA) with a trimethylolpropane (TMP) or pentaerythritol (PE) as a core, of certain generation. The resulting polymer has a main core/shell fraction and may have a trace fraction consisting of –COOH functional branches (29). In the present polyol sample, the acid groups were absent in NMR spectra. Simple, relatively easy 'one pot polymerization process' is the key distinguishing feature of hyperbranched macromolecules, as opposed to the controlled, stepwise, intricate synthesis of dendrimers (30). The *average* number of end groups of polyol and polyglycerol were estimated by end group analysis method. The structural features of hyperbranched polymers used in this study are summarized in Table I. Fluoroisothiocynate (FITC), ibuprofen-USP, and dicyclohexylcarbodiimide (DCC) (Aldrich Chemical, St. Louis, MO), interleukin (IL-2β), lipopolysaccharide (LPS) (Sigma, St. Louis, MO, USA), and ELISA kit for prostaglandin estimation (Cayman Chemical Company, Ann Arbor, MI, USA), were purchased. The dialysis membrane of molecular weight cut off of 3500 Da was obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA). Dimethyl sulphoxide (DMSO), dimethyl formamide (DMF), methanol and diethyl ether were purchased from Fischer Scientific (Fairlawn, NJ, USA). All other chemicals used were of analytical grade.

Synthesis of Polyol or (Polyglycerol)-Ibuprofen Conjugates

Hyperbranched polyol (0.75g, 0.05 mM) and ibuprofen (1.5 g, 7.28 mM) were dissolved in anhydrous DMF. This corresponds to an average molar ratio of 1.4 drug molecules to one –OH terminal group of polyol (40% excess). (The average molar ratio of the drug to the polyol was calculated on the basis of average molecular weight and average number of end groups of polyol). To this solution DCC (1.5g, 7.28 mM) was added as a coupling agent and the reaction was stirred continuously for 3 days at room temperature. The reaction mixture was filtered to remove dicyclohexylurea (DCU) formed during the reaction to obtain a clear filtrate. The solution was further dialyzed (dialysis membrane of molecular weight cut off 3500 Da) against DMF for 24 h to remove free ibuprofen and DCC. Excess solvent was removed under vacuum at room temperature to obtain polyol-ibuprofen conjugates. Repurification was carried out using diethyl ether to remove any unreacted ibuprofen. The number of ibuprofen conjugated to HBP was estimated using ^{1}H NMR (Table I). The conjugates are highly soluble in several solvents (~10 mg/ml), and do not show gelation in water,

Table I. Hyperbranched Polymer-Ibuprofen Conjugates

Material	Average molecular weight (g/mol)	Average no. of end groups[a]	Polydispersity (PDI)	Average mol% of ibuprofen in HBPs conjugate	Average weight% of ibuprofen in HBP conjugate	Average no. of ibuprofen per molecule of HBP conjugate
Polyol	15,000[b]	120	1.60[b]	98[c]	42[c]	50[c]
Polyglycerol	4,785[d]	68	1.48[d]	98[c]	70[c]	53[c]
Ibuprofen	206	1	—	—	—	—

[a] Reported by manufacturer using end group analysis method.
[b] From Perstorp Polyol literature.
[c] Estimated from ^{1}H NMR.
[d] Reported by Hyperpolymers Inc.

ethanol methanol, DMF, and DMSO indicating absence of cross-linking between the polyol molecules.

A similar reaction procedure was used for synthesizing polyglycerol-ibuprofen conjugates, where polyglycerol (0.75g, 0.15 mM), ibuprofen (1.9 g, 10.2 mM) and (1.9 g, 9.6 mM) DCC were used. This corresponds to a ratio of one drug molecule for every –OH end group on polyglycerol, on an average.

Synthesis of Polyol or (Polyglycerol)-FITC Conjugates

Polyol (or polyglycerol) and FITC (20 molecules of FITC for one molecule of polyol) were dissolved in dry dimethylformamide. The resultant solution was stirred at room temperature in dark and equimoles of dicyclohexylcarbodiimide was added as a coupling agent. The reaction mixture was stirred for 48 h at room temperature. *N, N′*-dicyclohexylurea formed in the reaction was removed by filtration. The solution was dialyzed for 24 h in DMF with continuous stirring to remove unreacted FITC and DCC. The solution was dried in vacuum to obtain hyperbranched polyglycerol- FITC conjugate. Absence of free FITC was verified by TLC method using chloroform and methanol (1:1) as solvents.

A similar procedure was used to synthesize polyol-FITC conjugates.

Synthesis of Polyol-Ibuprofen-FITC Conjugates

The polyol-ibuprofen conjugates (0.5 g, 0.0019 mM) and FITC (0.078 g, 0.019 mM) were dissolved in anhydrous DMF and DCC (0.04 g, 0.0019 mM) was added to it. (The molar ratio of the polyol/polyglycerol-ibuprofen to FITC was calculated on the basis of molecular weight of the conjugate and unreacted end groups of the polymer). The reaction mixture was stirred for 5 days at room temperature and filtered to remove *N, N'*-dicyclohexylurea. The solution was filtered and dialyzed against DMF for 24 h to remove unreacted FITC and DCC. The contents inside dialysis bag were removed and the solvent was evaporated in vacuum to obtain polyol-ibuprofen-FITC conjugates. Absence of free FITC in the conjugate was verified by TLC method using chloroform and methanol (1:1) as solvents.

A similar procedure was used to synthesize polyglycerol-ibuprofen-FITC conjugates.

Gel Permeation Chromatography

GPC analysis was carried out on Waters GPC instrument (Milford, MA, USA) equipped with manual injector and UV detector interfaced to Breeze software. The mobile phase used was 0.05 M $NaHCO_3$ / 0.1 M NaOH / deionized water (50:23:27) with a pH of 11. The pKa of polyglycerol is between 9 and 10. Below a pH of 9, it may be ionized and may interact with the column packing. To avoid this, a pH of 11 was used. Because the elution times of the polymer and the conjugate are compared, the same pH was used for the conjugates also. Mobile phase was freshly prepared, filtered and degassed prior to use. Ultrahydrogel 1000 (7.8 × 300 mm dimensions; Waters (Milford, MA, USA)) column was used and flow rate was maintained at 0.6 ml/min. For hyperbranched polyol, same column and mobile phase were used with a flow rate of 1 ml/min. The polyol was dissolved in methanol and the solution was injected. For the polyol-ibuprofen conjugate, because of solubility problems GPC could not be performed. The injection volume was 20 μl and the absorbance of ibuprofen and polyglycerol were monitored at 280 nm, whereas 210 nm was used for polyol.

In Vitro Release of Ibuprofen from the Conjugates

Polymer-drug conjugates (10 mg) were dissolved in methanol and transferred to dialysis bag (molecular weight cut off 3500 Da) and the bag was placed in a beaker containing 25 ml of methanol. The solutions were clear and there was no evidence of precipitation during the experiment. Sample (500 μl) was removed at specific time intervals (0–24 h) and the outer phase was replenished with 500 μl of methanol. The quantitative analysis of samples collected was performed using HPLC assay (26).

Cell Culture

Human lung epithelial carcinoma cell line (A549) was obtained from Children's Hospital of Michigan Cell Culture Facility and used for the cell entry and drug activity studies. Cells were grown in 75 mm^2 culture flasks using RPMI 1640 (In Vitrogen, Grand Island, NY, USA) cell culture medium supplemented with 10% fetal calf serum (FCS; Invitrogen, Grand Island, NY, USA) and 1% penicillin-streptomycin at 37°C with 5% CO_2 in an incubator. The cells were subcultured every 48 h and harvested from sub confluent cultures (60 to 70%) using 0.05% trypsin (Sigma, St. Louis, MO, USA).

Flow Cytometry Analysis

A549 cells (3.3×10^5 cells /ml) were grown overnight on 60×15 mm^3 cell culture plates using RPMI 1640 cell culture medium supplemented with 10% FCS and 1% penicillin-streptomycin. Next day, the medium was removed and replaced with serum free medium, followed by treatment with HBP-FITC/ HBP-Ibuprofen-FITC conjugates for 5, 10, 15, 30, 45, 60, 120, and 240 min. The cells were washed with phosphate buffered saline (PBS), trypsinized, and centrifuged at 1500 rpm for 5 min to obtain a cell pellet. The cells were then rinsed with PBS, spun down twice, resuspended in PBS. Analysis was carried out in flow cytometer (FACS caliber, Becton Dickinson, San Jose, CA, USA) and 10,000 cells were counted. The data was expressed as histogram plots with number of counts vs. fluorescent intensity and the mean fluorescence intensity was calculated at each time point. During FACS analysis, only those cells seen both in forward and side scatter were used for counting, hence eliminating artifacts.

Cell Supernatant Analysis

The cell supernatant from the above study was collected and the concentration of fluorescent-tagged HBP or HBP-drug conjugates in the supernatant was estimated by measuring the UV/Vis. absorbance of FITC at 496 nm. The pH of the supernatant over the period of the experiment was the same (7.74). Quantification was done using calibration curve generated in serum free medium using FITC-labeled HBP-drug conjugate and the cell supernatant was used as blank solution.

Fluorescence Microscopy

The procedure for cell culture and drug treatment was same as described in previous section. After treatment with the conjugate for 4 h, the cells were washed with phosphate-buffered saline (pH 7.4). A few drops of the buffer was added

before observing under the fluorescence microscope (Leica DM1L inverted microscope, Bannockburn, IL, USA). Images (400×) were captured and stored using SensiCam-QE 12 bit monochrome camera and Camware 3.1 software, respectively.

Pharmacological Activity of HBP-Ibuprofen Conjugates

A549 lung epithelial cells (2×10^5 cells/ml per well) were seeded in 24-well plates and allowed to grow overnight in RPMI 1640 medium supplemented with 10% FCS and 1% penicillin. On the next day, the medium was removed and fresh serum free medium was added. The cells were exposed to lipopolysaccharide (LPS) and interleukin (IL-β2) for 30 min to induce prostaglandin (PGE_2) production. Cells were then treated with 10 μg of free ibuprofen or HBP-ibuprofen conjugates (10μg equivalent of ibuprofen) dissolved in ethanol. Control treatments with solvent, polymers, positive control with no treatment, and negative controls with no PGE_2 induction were also studied. The supernatant was removed at specific time intervals of 30, 60, and 360 min and analyzed for PGE_2 concentration using a commercial ELISA kit. Results were represented as percent inhibition of PGE_2 compared to positive control.

RESULTS AND DISCUSSION

Synthesis and Characterization of HBP-Drug Conjugates

We report the conjugation of hyperbranched polymers to ibuprofen using DCC as a coupling agent. High payload of ibuprofen was obtained with polyol and polyglycerol using DCC method (Fig. 1). The resulting conjugates do not exhibit gelation even in a wide range of solvents such as methanol, ethanol, DMF, and DMSO, indicating absence of crosslinking between the HBPs and drug. After removal of free drug using dialysis, the conjugates were repurified using diethyl ether.

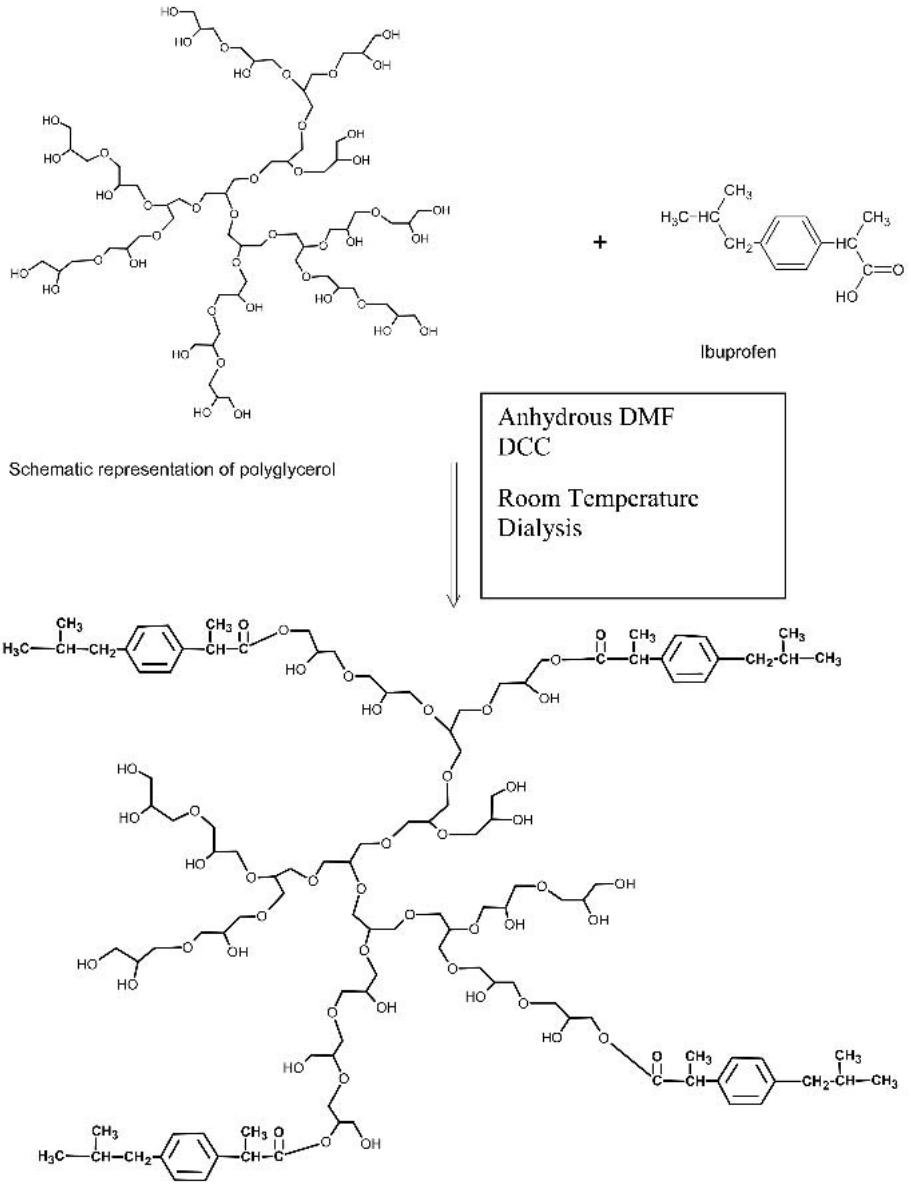

Fig. 1. Schematic synthesis method of polyglycerol-ibuprofen conjugates.

HPLC analysis for the presence of nonconjugated drug in the conjugates revealed that only a small fraction is present (less than 4% for polyol-ibuprofen conjugate), as discussed later.

Conjugation of ibuprofen in HBPs was characterized by ^{1}H NMR, and the conjugation ratio was estimated using proton integration method (31). ^{13}C NMR spectrum was used to confirm the formation of ester bond in the polymer conjugates. Figure 2a shows ^{1}H NMR spectrum for polyglycerol-ibuprofen conjugate. Two doublets at 7.062 and 7.21 ppm correspond to aromatic ring of ibuprofen, which account for 4 protons. Multiplets between 3.5 to 4 ppm, correspond to presence of 100 protons of CH in polyglycerol. The integration

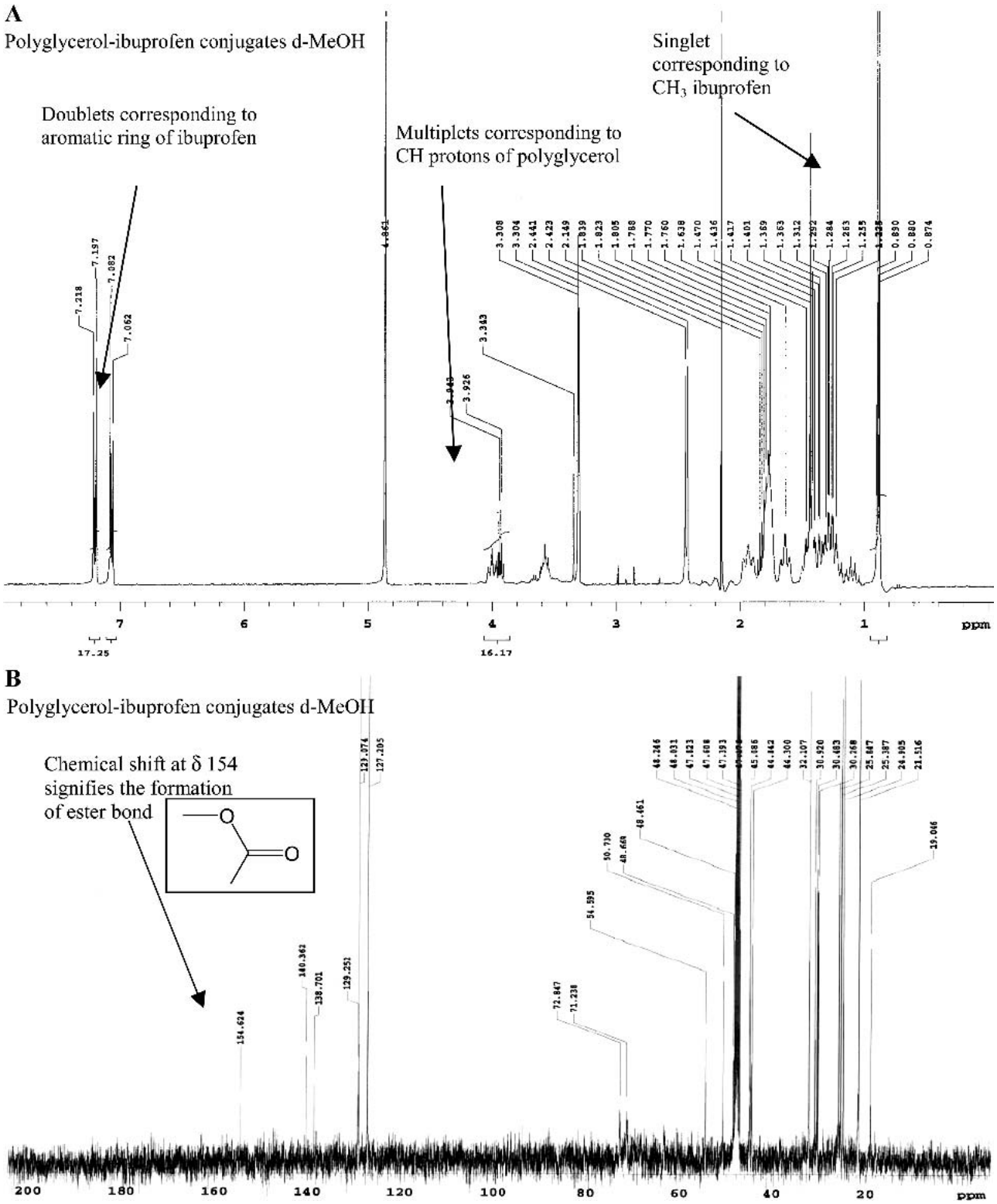

Fig. 2. (a) ^{1}H NMR spectrum polyglycerol-ibuprofen conjugate. (b) ^{13}C NMR spectrum polyglycerol-ibuprofen conjugate.

ratio of these doublets to multiplets is 2.14, which suggests 214 (2.14 × 100 = 214) protons of ibuprofen in the conjugate. Therefore, average of 53 molecules of ibuprofen are attached to a single molecule of polyglycerol, corresponding to a drug payload of 70%, based on total conjugate weight % (Table I). In case of polyol-ibuprofen conjugate (spectra not shown), a broad singlet at 4.2 ppm corresponds to the presence of 120 total protons of CH_2 in polyol branches. The integration ratio of doublets to singlet is 1.68, implying that 201 (1.68 × 120 = 201) protons of ibuprofen are present in the conjugate. Because each ibuprofen molecule has 4 protons, average of 50 molecules of ibuprofen are attached to one molecule of polyol. ^{1}H NMR indicates about 42% ibuprofen loading in the polyol conjugates, based on percent weight of the conjugate (Table I). The higher reactivity of ibuprofen with –OH groups in HBPs is attributed due to the presence of reactive –COOH group in the drug.

The conjugation of ibuprofen to polyol was further analyzed by ^{13}C spectra. Pure ibuprofen showed chemical shift at 177 ppm corresponding to the carbon arising from carboxyl groups. Whereas, in ^{13}C NMR spectra of conjugates, the signal at 177 ppm *disappeared* and instead a new signal at 155 ppm appeared, indicating the formation of ester bond between carboxyl group of ibuprofen and OH end group of polyol. The presence of 155 ppm signal for ester bond is consistent with literature reports (32). Similarly, ^{13}C NMR confirmed the conjugation of ibuprofen to polyglycerol.

In addition, FITC was conjugated to HBPs using DCC as a coupling agent. Unreacted FITC was removed by membrane dialysis using DMSO for 24 h. Moreover, free FITC was removed by additional repurification step using excess acetone. The absence of free FITC in the conjugate was verified by thin layer chromatography (TLC), using chloroform and methanol (1:1) as solvents. Similar method of dialysis and TLC is reported for characterizing free FITC from dendrimer-FITC urea based conjugates (33). The proton integration ratio of ibuprofen-polyol conjugate was compared to the additional protons arising from FITC molecules in ibuprofen-polyol-FITC conjugate. ^{1}H NMR reveals the conjugation of average of 8 FITC molecules in polyol-ibuprofen conjugate.

Gel Permeation Chromatography

HBP-ibuprofen conjugates were further characterized by GPC. pH 11 was used as the mobile phase to avoid the ionization of polyglycerol with column packing. The chromatogram of polyglycerol suggests polydispersity due to hyperbranched architecture of polymer (Fig. 3a). The dominant peak at 21.02 min is indicative of the average polyglycerol molecule, while a shoulder peak at 23.05 min indicates the presence of lower molecular weight polyglycerol fractions. Three distinct peaks at 22.2 min, 19.7 min and 18.23 min were observed for polyglycerol-ibuprofen conjugate (Fig. 3a, inset), perhaps signifying the presence of three distinct molecular weight conjugate fractions. From the ^{1}H NMR estimation, on an *average* 53 ibuprofen molecules are conjugated to 68 OH end groups on polyglycerol. Therefore, it is unlikely that there are polyglycerol molecules with no ibuprofen molecules (Peak at 22.2 is unlikely to be unreacted polyglycerol). Therefore, the three distinct peaks at 18.23, 19.7 and 22.2 min for the conjugate indicate different molecular weight fractions with varying amounts of ibuprofen. The GPC trace of the conjugate does suggest a "polydispersity" in polyglycerol, and/ or in the conjugation ratios. Quantitative estimation of free ibuprofen using HPLC assay revealed that 4% of non-conjugated ibuprofen was present in the conjugate. The GPC trace for polyol showed that the polymer is nearly monomodal even though it is not mondispersed, with a elution time of 11.1 min at 1 ml/min (Fig. 3b). For polyol, methanol was used as a solvent before injection into the column, since it is not soluble in water. The GPC chromatogram could not be obtained for polyol-ibuprofen conjugate, because of precipitation of the conjugate when used with the mobile phase.

To summarize, we successfully synthesized HBPs conjugates containing ibuprofen, with high payload by. The molecular weight distribution of hyperbranched polymers-ibuprofen conjugates appears to be relatively broad and trimodal in case of polyglycerol. Achieving high payload of drug molecules in conjugates is critical, as it can potentially increase the local concentration of drug at the target site and therefore result in improved efficacy.

In Vitro Stability of Polymer-Ibuprofen Conjugates

Stability of the conjugates was investigated by performing *in vitro* release in methanol using dialysis membrane having a molecular weight cut off of 3500 Da. Methanol was used due to the low aqueous solubility of conjugates. The low solubility of conjugates in PBS is expected due to the high payload of ibuprofen per HBP molecule (ibuprofen is insoluble in PBS). Current efforts are focused on improving the aqueous solubility of these high payload conjugates. Pure ibuprofen completely diffused (100%) through the dialysis membrane within 5 h, whereas only 4% ibuprofen was released from the HBP conjugates over a period of 27 h (Fig. 4). The results demonstrate that the conjugates are stable and did not release ibuprofen. However, the conjugates are expected to release ibuprofen through the cleavage of the ester bond by lysosomal enzymes, once they are inside the cell.

Cell Entry of Conjugates in A549 Lung Epithelial Cells

Cellular entry of FITC-labeled HBPs and HBP-drug conjugates were evaluated using flow cytometry. Results are represented as the log of fluorescence intensity plotted against the number of events. Polyglycerol-FITC (Fig. 5a) shows an "exponential" increase in fluorescence intensity within 5 min, indicating rapid entry of polymer in the cell. Over the next 120 min the intensity did not change significantly. However, for polyglycerol-ibuprofen-FITC *conjugates* (Fig. 5b), increase in intensity was gradual over a period of 240 min, with a gradual shift in peak along the fluorescence intensity axis.

The flow cytometry results correlated well with UV/Vis analysis of cell supernatant treated with conjugates. Figures 6a and 6b show the decrease in the concentration of FITC-labeled drug conjugates in the cell supernatant and the corresponding increase in fluorescence intensity inside the cell (from flow cytometry) for both the HBP-ibuprofen conjugates. More than 50% of the conjugates are inside the cell within 30 min for both polyglycerol and polyol- ibuprofen conjugates. From flow cytometry it was observed that the cell uptake peaked at 30 min for both the conjugates. There was no statistical difference ($p > 0.05$) in the cell entry dynamics of both the conjugates. Fluorescence microscopy images (taken

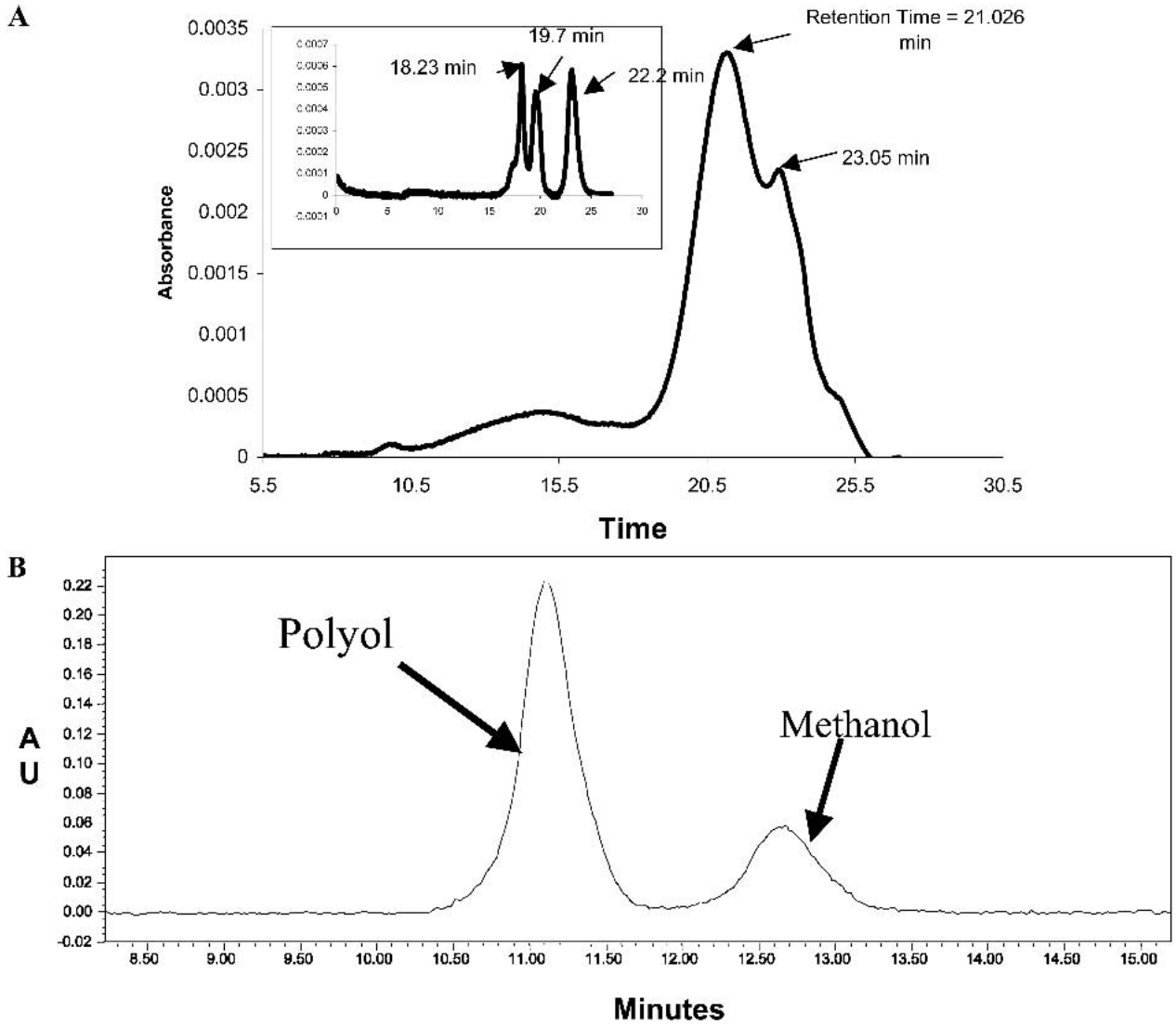

Fig. 3. (a) GPC chromatogram of pure hyperbranched polyglycerol and polyglycerol-ibuprofen conjugate (inset). (b) HPLC chromatogram of hyperbranched polyol.

after 4 h) of A549 cells treated with FITC labeled conjugates provide insights into the location and distribution of the conjugates inside the cells (Figs. 7a–7d). The cells without any treatment show no fluorescence as expected (Fig. 7a). The photomicrographs for the FITC-labeled ibuprofen (Fig. 7b), the polyol-ibuprofen conjugate (Fig. 7c), and polyglycerol-ibuprofen conjugate (Fig. 7d) show the presence of FITC-labeled conjugate primarily in the cytosol. The fluorescence intensity for ibuprofen-FITC conjugate is higher than that associated with HBP conjugates, since each ibuprofen molecule contains one FITC, whereas in the HBP conjugates the relative FITC molecule ratios are much smaller. It appears that (at 4 h) there is an appreciable distribution of conjugates inside the cell. Further studies with confocal microscopy would identify the exact localization of the conjugates. From the above results, it appears that HBP-drug conjugates are transported inside the cells efficiently, perhaps by endocytosis mechanism.

Previously, we have reported (29) cell entry dynamics of HBP polymers, where it was found that hyperbranched polyol

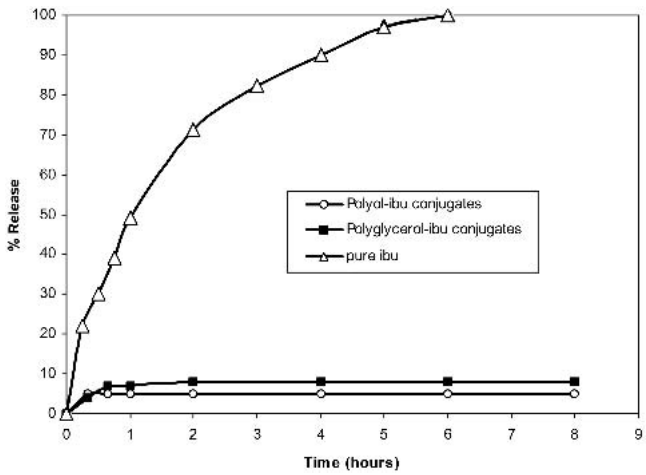

Fig. 4. *In vitro* release kinetics of ibuprofen from polyol-ibuprofen conjugates. Minimal release of drug (~4%) was observed. The data are the average of three experiments.

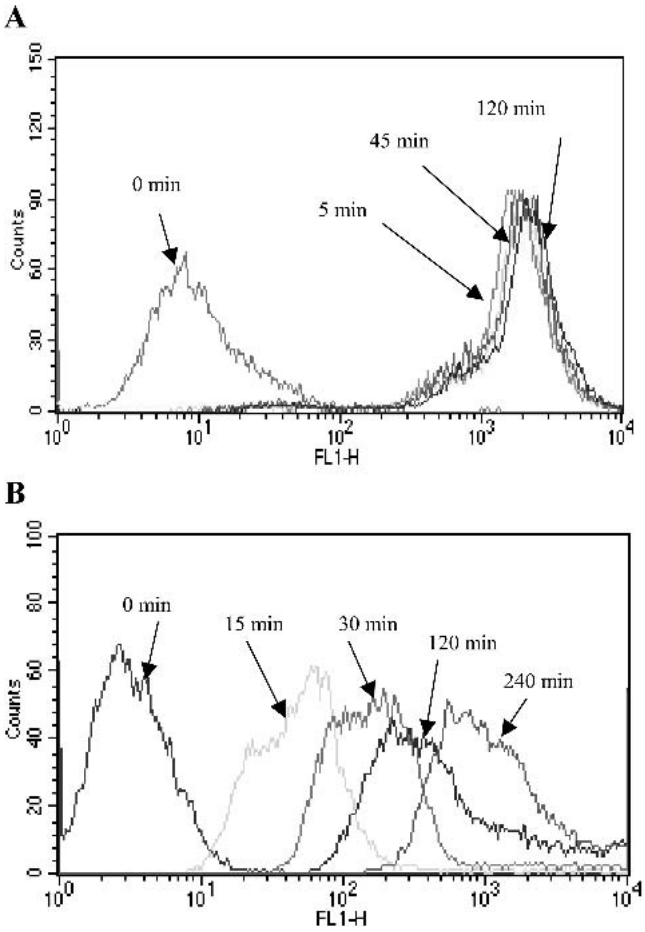

Fig. 5. (a) Flow cytometry of the cell entry dynamics of pure polyglycerol in A549 lung epithelial cell line. The log of FITC absorption intensity (FL1-H on x-axis) is plotted against the number of cells (counts on y-axis). The exponential increase is the cellular uptake of polymer within few minutes is evident. (b) Flow cytometry of the cell entry dynamics of polyglycerol-ibuprofen conjugates in A549 lung epithelial cell line. Hyperbranched polyglycerol-conjugate enters in cells in about 4 h. The cell entry of the conjugate is presumably lower due to the high payload of drug.

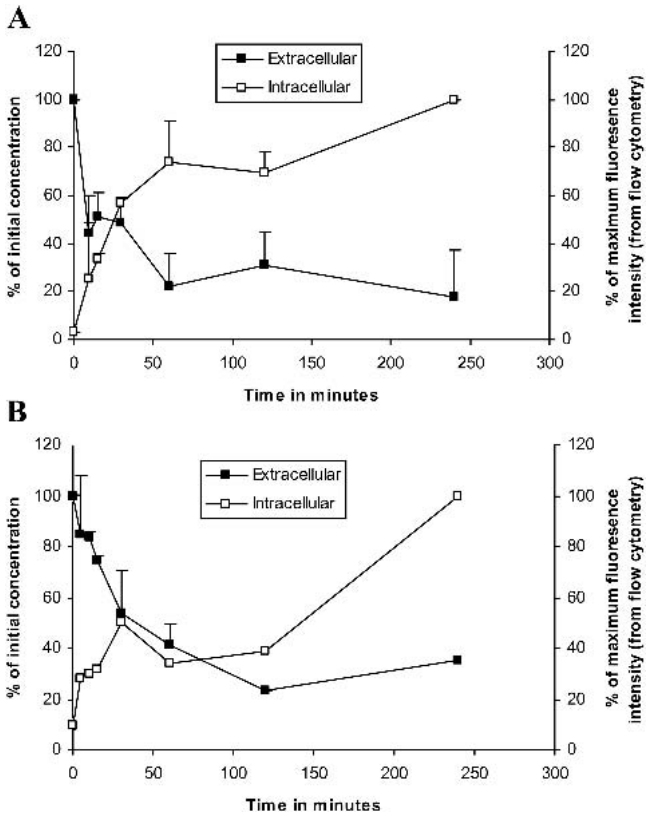

Fig. 6. Decrease in concentration of FITC-labeled HBP-drug conjugate in the cell supernatant (by UV analysis; left-hand y-axis indicated by closed squares) and the corresponding increase in fluorescence intensity inside the cell (from flow cytometry; right-hand y-axis indicated by open squares for: FITC-labeled (a) polyol-ibuprofen conjugate and (b) polyglycerol-ibuprofen conjugate. Each data point is represented as mean with error bars (n = 3). The values at different time points were statistically significant ($p < 0.05$) from initial values for cell supernatant analysis and final values for flow cytometry data, respectively.

enters A549 lung epithelial cells gradually over a period of 2 h. The cell entry was attributed to the adsorptive endocytosis, as polyol does not carry any charge and possess hydroxyl surface groups at the terminals. Because epithelial cells are known to possess anionic charge, there might be no significant interaction between epithelial cells and hydroxyl groups of polyol. Similar hypothesis would apply for hyperbranched polyglycerol, which possesses hydroxyl surface groups. Recently Jevprasesphant *et al.* have shown direct evidence using transmission electron microscopy that surface modified dendrimers are transported by endocytosis in Caco-2 cells (34). Hence, it appears that HBP nanocomposites are primarily transported by endocytosis.

Anti-inflammatory Activity of Conjugates

Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) that acts by inhibiting cyclooxygenase enzyme. This enzyme catalyzes the conversion of unsaturated fatty acids to prostaglandins. COX-1 and COX-2 are two iso forms of cyclooxygenase (COX) enzyme. We evaluated the efficiency of the HBP-ibuprofen conjugates to suppress COX-2 by measuring the prostaglandin (PGE_2) in the cell supernatant. Figure 8 demonstrates the percent inhibition of PGE_2 secretion by conjugates compared to control with no treatment. At 30 min, ibuprofen did not show any inhibition, while polyol (~5%) and polyglycerol-ibuprofen conjugates (~30%) showed inhibition of PGE_2 synthesis. Polyglycerol-ibuprofen conjugates demonstrated higher inhibition ($p < 0.05$) than polyol-ibuprofen conjugates. At 60 min and 360 min, there was no significant difference ($p > 0.05$) in PGE_2 inhibition between free ibuprofen and HBP-conjugated ibuprofen. Percent inhibition increased for both free ibuprofen and HBP-ibuprofen conjugates with increase in incubation time ($p < 0.05$). The HBPs and solvent did not show any inhibition of PGE_2 secretion. From these results, it is evident that HBP-ibuprofen conjugates are able to produce a pharmacological action, which is rapid compared to the free ibuprofen.

After intracellular uptake, the contents of endocytic vesicle are delivered to lysosomes for degradation (35). The low pH and the hydrolytic enzymes in the lysosomes are expected to hydrolyze the ester bond and release free ibuprofen from the HBP-drug conjugates. The difference in the activity

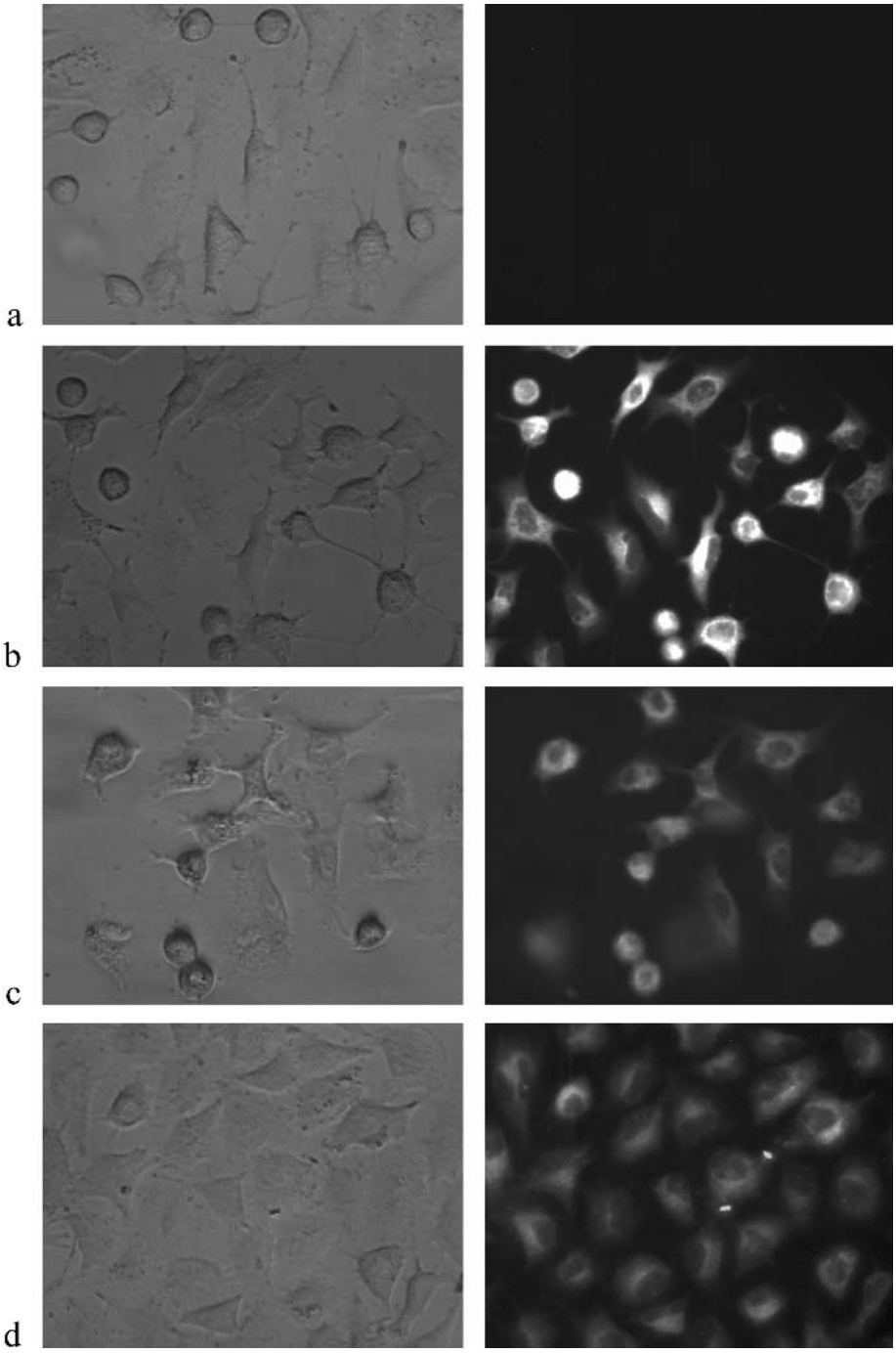

Fig. 7. Phase contrast and fluorescence microscopic images (400×) after 4 h of treatment with (a) control, (b) FITC-labeled ibuprofen, (c) FITC-labeled polyol-ibuprofen conjugate, and (d) FITC-labeled polyglycerol-ibuprofen conjugate.

of the polyol-ibuprofen and polyglycerol-ibuprofen conjugates can be attributed to the difference in the kinetics of hydrolysis of the conjugates dictated by steric hindrance, hydrophobicity and other factors. It is also pertinent to note that the percent loading of ibuprofen in polyglycerol is much higher than in polyol conjugate (70% vs. 42%), which would also account for the difference in the activity of the conjugates. In order to understand the factors affecting the release of ibuprofen from the conjugates, further *in vitro* enzymatic studies at various pH are required. On the other hand it is not clear at this point, as to why free ibuprofen shows lesser activity than polyglycerol-ibuprofen conjugate in 30 min. Probably, the concentration of free ibuprofen at 30 min was not sufficient enough to inhibit COX-2. Unlike the free ibuprofen the ibuprofen-polyglycerol conjugate is expected to produce a high local concentration inside the cell, thereby resulting in a higher pharmacological response. This is conceivable from the fact that the COX-2 inhibition increased with increase in treatment time, which in other words imply an increase in drug concentration inside the cell.

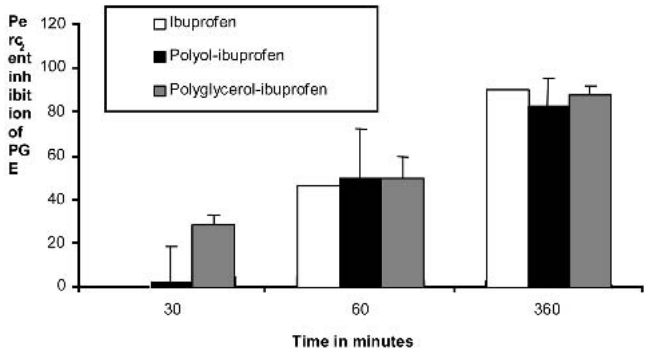

Fig. 8. Percent inhibition of prostaglandin (PGE_2) of ibuprofen and HBP-ibuprofen conjugates with increasing treatment time. Values are represented as mean with error bars (n = 4). For each treatment, the values are significantly different at the three time points ($p < 0.05$). At 30 min, polyglycerol-ibuprfen conjugate showed significantly higher ($p < 0.05$) inhibition than polyol-ibuprofen and free ibuprofen. The latter two did not differ significantly ($p > 0.05$).

Never the less, by achieving a high local drug concentration with conjugates at the target site one could overcome the systemic adverse effects of free ibuprofen and improve the therapeutic efficacy significantly with a reduced dose. Furthermore, as the conjugates are expected to be stable in blood, it would achieve more sustained drug levels leading to reduced dosing frequency.

CONCLUSIONS

The potential of hyperbranched polymers as drug delivery vehicle has been evaluated. High ibuprofen payloads were achieved in polyol (~42%) and polyglycerol (~70%), using DCC as coupling agent. The synthetic methodology for conjugation of drugs to HBPs is relatively simple and yet results in high drug payload. HBP-ibuprofen conjugates were able to deliver the drug rapidly inside the cells, and conjugates appear to be distributed primarily in the cytosol. Therapeutic efficacy of conjugates was investigated by monitoring prostaglandin inhibition. In spite of higher polydispersity compared to dendrimers, the HBP conjugates exhibited rapid cell entry and significant therapeutic effect. The therapeutic effectiveness appears to be comparable to that of dendrimer conjugates, as discussed in a separate publication (36). Because HBPs are significantly less expensive compared to dendrimers, they have potential applications as economical intracellular drug delivery vehicles.

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