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Glycodendrimeric nanoparticulate carriers of primaquine phosphate for liver targeting

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Abstract

In the present study it was intended to deliver primaquine phosphate (PP), a liver schizonticide directly to liver cells using polypropyleneimine (PPI) dendrimers-coated peripherally with galactose. PPI dendrimers were synthesized by consecutive Michael double addition reaction (using ethyelenediamine as core), followed by hydrogenation reaction. Galactose conjugation was carried out by ring opening reactions, followed by Schiff's reaction and reduction to secondary amine in sodium acetate buffer (pH 4.0). IR, NMR, MASS spectroscopy were used for the confirmation of synthesis of uncoated and coated dendrimers. The formulations were made by equilibrium dialysis of dendrimers with the solution of PP. Then the formulations were characterized by TEM for size and shape. Release rate, hemolytic toxicity; bio-distribution and blood level studies were also performed on lyophilized formulations. The results obtained indicated that galactose coating of PPI systems increases the drug entrapment efficiency by 5–15 times depending upon generations. Galactose coating prolonged release up to 5–6 days as compared to 1–2 days for uncoated PPI systems. The hemolytic toxicity, blood level and hematological studies proved these systems to be safer and suitable for sustained drug delivery. Blood level studies proved the suitability of the systems for the prolonged circulations and delivery of PP to liver. The galactose coating of PPI dendrimers can therefore make the PPI systems more effective and suitable for targeted delivery of Primaquine phosphate to liver.

Keywords: Polypropyleneimine; Dendrimers; Primaquine; Glycodendrimer; Targeting; Malaria

1. Introduction

The term 'glycodendrimer' is a general term and has been used to describe wide range of architecture of dendrimer, which incorporate carbohydrate into their structure. These classes of dendrimer have immense potential for drug encapsulation and delivery. Glycodendrimers can be classified basically as carbohydrate coated, carbohydrate-centered and carbohydrate-based types. Carbohydrate coated dendrimers involve the divergent modification of pre-existing dendrimers in a convenient way to make polyvalent glycoconjugate in minimal number of steps, assuming that the

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starting dendrimer is commercially or otherwise readily available. This type of coating is largely focused on two classes of dendrimers, namely the polyamidoamine (PAMAM) dendrimers (Starburst™ dendrimers) (Tomalia et al., 1985) and polypropylene imine dendrimers (Astramol™ dendrimers) (De Brabander-Van den Berg and Meijer, 1993). Both types of dendrimer have tertiary amine-based skeletons, displaying primary amine groups around their peripheries to which other functional group or molecules can be attached by means of urea, thiourea and amide bonds. The later approach had been employed (Ashton et al., 1997) to make a fifth generation series of galactose and lactose bearing glycodendrimer with 4- to 64-saccharide units per dendrimer. This coupling involved reaction between primary amine-bearing dendrimers with equivalent NHS-active glycosyl ester in dichloromethane to form the corresponding protected glycodendrimer (93–100% yield) (Wooley et al., 1991; Lee, 1978; Jayaraman and Stoddart, 1997; Aoi et al., 1995).

Carbohydrate centered dendrimers have monosaccharides as multifunctional building blocks and as such, they had been found use as cores for nondendritic glycoclusters (Hanessian et al., 1985; Kieburg et al., 1997). Lindhorst was the first person to realize their potential in dendrimer synthesis in which it had been used as core for PAMAM-type compounds (Dubber and Lindhorst, 1998). An impressive example of a designed multivalent ligand for a lectin system was reported recently by Kitov et al. (2000), who used Lindhorst's dendrimer core to achieve pseudo five-fold symmetry, which matched the pentameric structure of the lectin subunit of the Verotoxin from *E. coli* 0157. The multifunctional nature of monosaccharides, in combination with the inherent stereochemistry of glycosidic linkages, greatly increased the potential structural diversity that was possible for oligomers of these building blocks, relative to peptides or nucleotides (Turnbull and Stoddart, 2002).

Primaquine phosphate, an antimalarial drug that was active against exo-erythrocyte forms of Plasmodium that is *P. vivax*, *P. ovale* and the early preerythrocytic form of *Plasmodium falciparum*, was used to induce "radical cures" of relapsing malarias. It has main action as liver schizonticide but many serious side effects were reported with its use. The larger dose may cause nausea, headache, and disturbance of visual accommodation, pruritis and abdominal cramps and sometimes severe reactions were reported in case of high-dose therapy like leucopoenia and methaemoglobinaemia that causes cyanosis. Patients on primaquine therapy are instructed to promptly report sign of hemolysis like reddening or darkening of urine. G-6-PD deficiency in erythrocytes can make the individual sensitive in varying degrees to primaquine-induced hemolysis. The amount of hemolysis was primarily dependent on various factors like degree of G-6-PD deficiency; age of red cell population; and the dose size and hypersensitivity (Foye et al., 1995), etc. So it was envisaged to target PP directly to liver cells, where its activity was required the most, using nanometric molecular drug delivery systems.

For the present study, nanometric molecular drug carriers like PPI dendrimers with specific ligands for the corresponding receptors on the cell surface can be useful for localization of drug in specific areas. Among various ligands investigated so far, galactose had been shown to be a promising ligand for hepatocyte (liver parenchymal cells) targeting because the liver cells possess a large number of the Asialo-glycoprotein (ASGP) receptors that can recognize the galactose units on the oligosaccharide chains of glycoproteins or on chemically galactosylated drug carriers (Ashwell and Harford, 1982). The receptor–ligand interaction was known to be showing a significant 'cluster effect' in which a multivalent interaction results in extremely strong binding of ligands to the receptors (Lee et al., 1983).

2. Materials and methods

2.1. Materials

PPI dendrimer system was synthesized by divergent method using ethylenediamine as core. Ethylenediamine and Raney Nickel were purchased from Merck, India. Acrylonitrile was purchased from CDH, India. Galactose was purchased from Siscochem Industries, India. Methanol was purchased from Ranbaxy Chemical Division, India. Dialysis tubing of Molecular weight pore size 2.4 nm was procured from Himedia, India. All the other chemicals were purchased from Himedia, Lab., India. The drug primaquine phosphate was a generous gift sample from M/s IPCA Laboratories, Ratlam (MP) India.

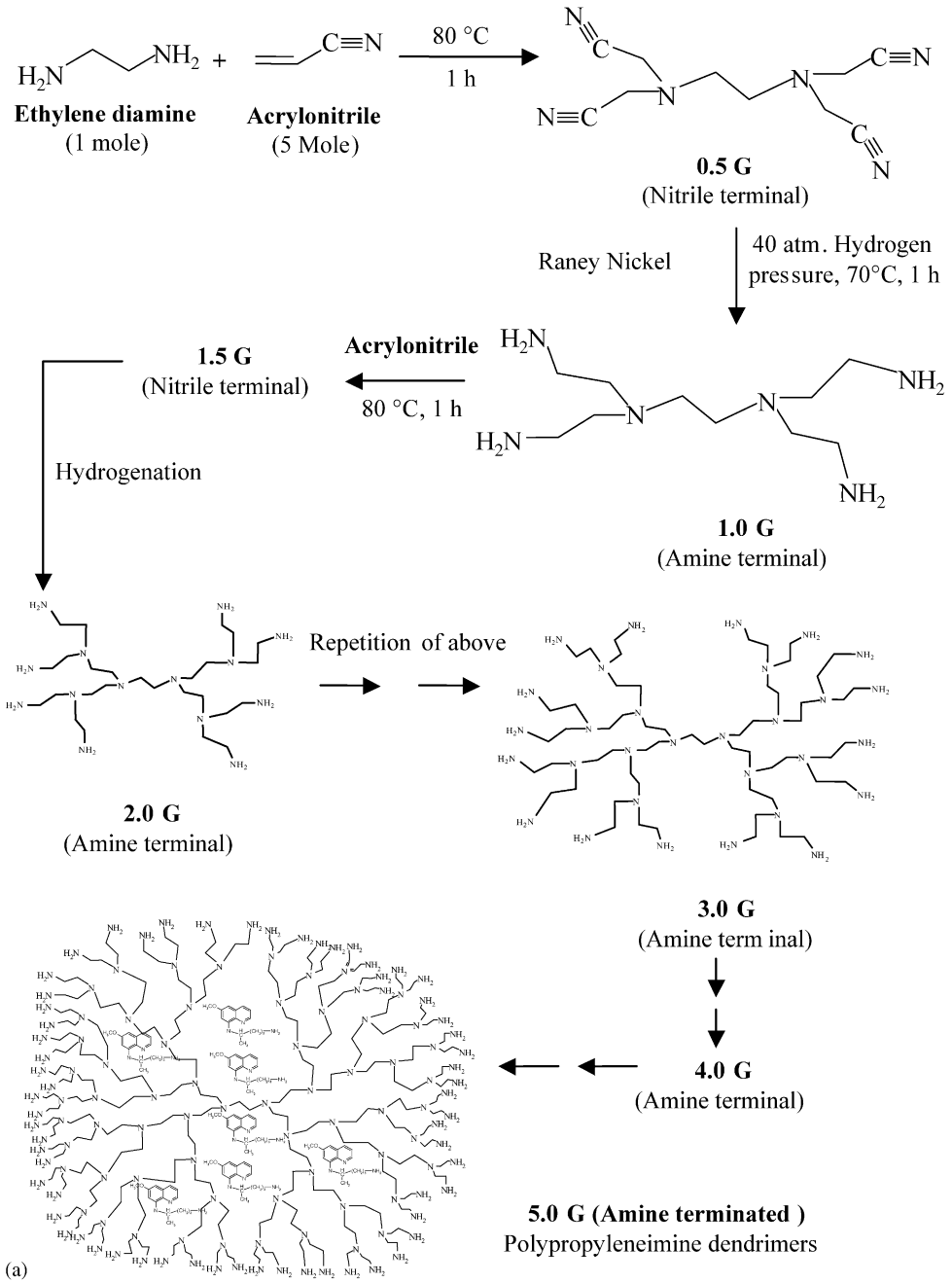

Fig. 1. Flow chart for synthesis of (a) uncoated polypropyleneimine dendrimer and (b) galactose-coated PPI dendrimers, loaded with primaquine (drug) molecules.

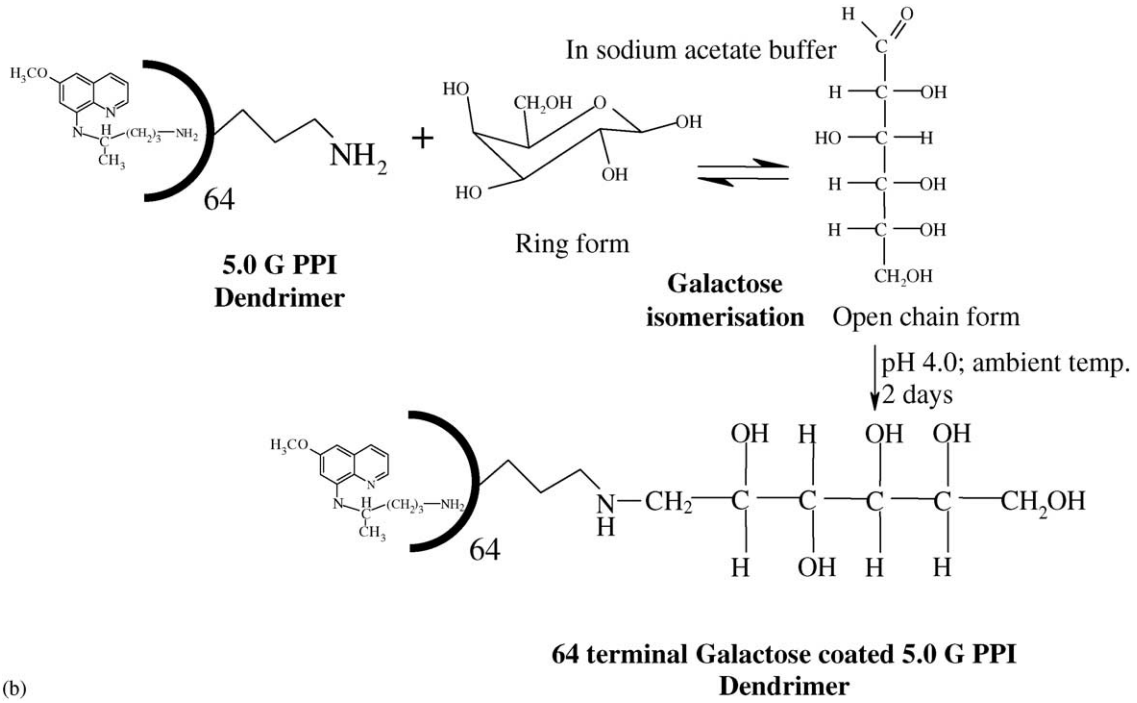

Fig. 1. (*Continued*).

2.2. Synthesis and characterization of dendrimer

Polypropyleneimine (PPI) dendrimers were synthesized using the scheme given by De Brabander-Van den Berg and Meijer (1993) taking ethylenediamine as core. The half generation EDA-dendr-$(CN)_{4n}$ (where n is generation of reaction or reaction cycle) was synthesized by double Michael addition reaction between acrylonitrile (2.5 molar times per terminal NH_2 group of core amine moiety) and aqueous solution of ethylenediamine or previous full generation dendrimers. After the initial exothermic phase, the reaction mixture was heated at 80 °C for 1 h to complete the addition reaction. The excess of acrylonitrile was then removed by vacuum distillation (16 mbar, bath temperature 40 °C). The Full generation EDA-dendr-$(NH_2)_{4n}$ was obtained by hydrogenation in methanol at 40 atm hydrogen pressures and 70 °C for 1 h with Raney Nickel (pretreated with hydroxide and water) as catalyst. The reaction mixture was cooled, filtered and the solvent was evaporated under reduced pressure. The product was then dried under vacuum. PPI dendrimers up to 5.0 G were prepared by repetition of all the above steps consecutively, with increasing quantity of acrylonitrile (De Brabander-Van den Berg and Meijer, 1993).

Galactose coating of PPI dendrimer was carried out by the method (Fig. 1) reported by Mitchell et al. (1999). Briefly, galactose (8 μM) was dissolved in 0.1 M sodium acetate buffer (pH 4.0) and added to lyophilized PPI dendrimers (0.1 μM). The mixture was agitated at ambient temperature for 2 days to ensure completion of reactions. Resulting solution was concentrated under vacuum at 60 °C. Coated dendrimer was purified by dialyzing against water in dialysis tubing (2.4 nm pore size), which allowed free carbohydrate to diffuse out into water and final glycodendrimer was retained inside. IR spectroscopy was carried out using KBr pellet method after adsorption of smaller amounts of substance on KBr using Perkin-Elmer IR spectroscope. NMR spectroscopy of the dendrimers samples were carried out at 300 MHz, after dissolving in D_2O (Bruker DRX 300 MHz, USA). The molecular weights of the dendrimers were determined

by electrospray ionization (set at 3.5 KV and cone voltage of 40 V) of the samples dissolved in water. It was recorded using triple quadrupole mass spectrometer (Micromass Quattro II) as an average spectra of 6–8 scans. This also indicated an increase in molecular weight on galactose-coating of the PPI dendrimers.

Color reaction was also performed using Ninhydrin Kaiser test to confirm the degree of coating by galactose of the coated PPI dendrimers. Briefly, very diluted dendrimeric aqueous solution was mixed with two drops of Kaiser A (0.5 ml of aqueous KCN solution 0.065%, w/v, with 24.5 ml of dry pyridine and 2.5 ml of 400%, w/v, phenol/ethanol mixture) and Kaiser B (5%, w/v, ethanolic Ninhydrin solution) in microcentrifuge tube and heated on a pre-heated block for 15–30 s (Sarin et al., 1981).

2.3. Dendrimer–drug formulation

Loading of drug was carried out by Equilibrium Dialysis method (Martin et al., 1991). Briefly, 5 ml of 10% (w/v) dendrimer (500 mg) solution was taken in a dialysis bag (cellulose dialysis tubing of 2.4 nm pore size, Himedia, India) and immersed in 10 mg/ml aqueous solution of PP and incubated for 24 h at 25 °C. The content of dialysis bag mixture was then lyophilized. The entrapment efficiency of the system was indirectly measured by the decrease in the amount of drug in dialyzing medium spectrophotometrically (Rao et al., 1989) after appropriate dilution. The blank taken was the dialyzing medium of dendrimers in distilled water containing no drug. Briefly, to the aliquots, 0.5% of 4-aminoantipyrine and 0.1% sodium periodate were added to produce blue color and absorbance was taken at 579 nm.

Five hundred milligrams of lyophilized drug-dendrimer complex of various generations was filled in cellulose tubing and dialyzed against 10 ml of water reservoir by equilibrium dialysis methods (Martin et al., 1991). The amount of drug released was determined in aliquots spectrophotometrically, after appropriate dilutions. Transmission electron microscopy was carried out to determine the surface characteristics of the resultant dendrimers in aqueous medium using 3 mm Forman (0.5% plastic powder in amyl acetate) coated copper grid (300 mesh) at 60 KV using negative staining by 2% phosphotungastic acid (PTA) at various magnifications (Philips CM-10 TEM).

2.4. Hemolytic toxicity of drug–dendrimer system

Whole human blood was collected in HiAnticlot blood collection vials (Himedia Labs, India). The red blood cells were collected by centrifugation and resuspended in normal saline solution (10% hematocrit). One milliliter of the RBC suspension was incubated with the distilled water (taken as 100% hemolytic standard); normal saline (taken as blank for spectrophotometric estimation); drug; uncoated and coated dendrimeric formulations after making up the volume to 10 ml with normal saline. For the studies pre-dialyzed, uncoated lyophilized dendrimers (5 mg/ml) and coated dendrimers of respective generations in equivalent amounts were taken in separate tubes. The tubes were allowed to stand for 1 h at 37 °C with intermittent shaking. The tubes were centrifuged for 15 min at 3000 rpm and the absorbance of supernatants at 540 nm was used to estimate % hemolysis against absorbance for supernatant of 100% hemolytic standard (distilled water) diluted similarly.

2.5. Stability studies

The glycodendrimer–drug (5.0 G) formulations were kept in various amber colored and colorless vials at 0 °C, room temperature (25 °C) and 50 °C for a period of 5 weeks. The samples were analyzed initially and periodically after every week for up to 5 weeks for increase in drug leakage. The samples were analyzed for any precipitation, turbidity, crystallization, changes in color and consistency after storage. The data obtained was used for the analysis of any physical or chemical degradation at different storage conditions. The drug leakage was indirectly determined by checking for increase in release rate of drug from the formulation after storage. The percentage increase in drug released from the formulations was used to analyze the effect of accelerated conditions of storage on the stability of the formulations and for determination of the precaution required for storage.

2.6. Hematological study

Healthy male albino rats (Sprague–Dawley strain) of uniform body weight (100 ± 20 g) with no prior drug treatment were used for all the present in vivo studies. The rats were maintained on standard diet and water.

The protocol was duly approved by the Institutional Animal Ethics Committee of the University (Registration Number 379/01/ab/CPCSEA, India).

The animals were divided into four groups having three rats in each group. Plain drug solution (PP), carbohydrate coated and uncoated dendrimer–drug complex, each equivalent to 250 μg of PP was administered intravenously to the first, second and third group every day. Fourth group was kept as control, which was maintained on same regular diet for 7 days. After 7 days blood was withdrawn from the animals and was analyzed for hemoglobin (Hb) content, RBC, WBC, differential monocytes, lymphocytes and neutrophils in pathology lab.

2.7. Bio-distribution studies

Albino rats (100 ± 20 g) were used to study biodistribution of drug (PP). The rats were divided into four groups of three animals each. To the first group plain drug solution (PP), to the second group drug-loaded carbohydrate coated dendrimer formulation and to the third group uncoated dendrimer–drug complex were administered through the caudal vein, while the fourth group served as control. After 2 h the animals from each group were sacrificed and the organs (liver and spleen) were excised and homogenized in PBS. The homogenates were deproteinized with acetonitrile. The homogenates were finally centrifuged, filtered and estimated for the drug contents by HPLC (Dua et al., 1996).

Briefly, reversed phase HPLC method without any internal standard was used, using acetonitrile: methanol: 1 M perchloric acid: water (30:9:1:95) as mobile phase passed at a flow-rate of 1–5 ml/min by LC10 AT, pump on a 5μ-Luna C_{18} column (Phenomenex, USA) with UV detection at 254 nm using photo diode array detector (SPD-M10A).

2.8. Intrahepatic disposition

The rats were divided into four groups with three animals in each. To the first group plain drug solution (PP) was injected intravenously. To the second and third group, respectively, coated and uncoated drug-loaded dendrimer formulations were injected through the caudal vein. Whereas, the fourth group served as control. Rats were anesthetized in chloroform chamber. The body temperatures of the rats were kept at around 37 °C with a heat lamp during the whole experiment. Two hours post-administration, the liver was perfused first with Ca^{2+}, Mg^{2+}-free perfusion buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 137 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4 and 0.4 mM Na_2HPO_4, pH 7.2) for 10 min, and then with a perfusion buffer supplemented with 5 mM $CaCl_2$ and 0.05% (w/v) collagenase (type I) (pH 7.5) for 10 min. As soon as the perfusion started, the vena cava and aorta were cut and the perfusion rate was maintained at 3–4 ml/min. At the end of the perfusion, the liver was excised and its capsular membranes were removed. The cells were dispersed in ice-cold Hank's–HEPES buffer containing 0.1% BSA by gentle stirring. The dispersed cells were separated by centrifugation at 5000 rpm for 1 min. The pellets containing hepatocytes were washed twice with Hank's–HEPES buffer by centrifuging at 500 rpm for 1 min. The supernatant of this washing containing non-parenchymal cells (NPC) was similarly centrifuged at least twice at 2000 rpm for 2 min. Hepatocytes and NPC were resuspended separately in ice-cold Hank's–HEPES buffer (Murao et al., 2002). These cells were lysed with water and deproteinized with acetonitrile. The mixture was centrifuged and the drug content was determined by HPLC as described prior (Dua et al., 1996).

2.9. Blood level studies

The formulated products (carbohydrate coated and uncoated dendrimer–drug complex) were used to deliver PP and blood level of drug was determined at various time intervals and compared against that from pure PP injections of equivalent strength, in albino rats. Twelve albino rats were divided into four groups having three rats per group. Fifth generation (5.0 G) dendrimer was taken as formulation for analysis. One group of rats was kept as control. Another group of rats were given 250 μg drug (PP) intravenously (i.v.) through caudal vein. Dendrimer–drug complex of 5.0 G carbohydrate coated and uncoated dendrimer having equivalent drug content of 250 μg were dissolved in PBS (pH 7.4) and given i.v. to third and fourth group, respectively. 0.1 ml of blood samples were withdrawn from retro-orbital plexus in 0.9% normal saline every 15 min for up to 2 h, followed by every half hour up to 7 h, and then at hourly intervals up to 12 h. The blood samples were

clotted and washed by vortexing with normal saline and the washings were centrifuged at 2000 rpm for 15 min. Serum was deproteinized by acetonitrile (1 ml/ml of serum). The samples were centrifuged and supernatants were analyzed for drug content against similarly treated blood sample of control rats by HPLC (Dua et al., 1996).

3. Results and discussion

3.1. Synthesis and characterization of dendrimers

Polypropyleneimine dendrimers were synthesized taking ethylenediamine as core. Synthesis of 0.5 G PPI was confirmed by IR peaks, mainly of nitrile at 2249 cm^{-1}. All the nitrile terminal 0.5 G PPI got converted into EDA-$(NH_2)_4$, which was confirmed by IR of PPI (1.0 G), that give major peak at 3432.8 cm^{-1} of amine (N–H str.). Synthesis of 5.0 G PPI dendrimer was similarly confirmed by I.R. peaks for C–C bend (1103.2 cm^{-1}); C–N stretch (1230.5 cm^{-1}, 1326.9 cm^{-1}); C–H bend (1423.4 cm^{-1}, 1477.4 cm^{-1}); N–H deflection of amine (1643.2 cm^{-1}) and N–H stretch of primary amine at 4176.3 cm^{-1}, confirming most of the nitrile terminal groups of dendrimer were converted to amine terminals. The results matched with the reported synthesis of PPI dendrimers (De Brabander-Van den Berg and Meijer, 1993).

Galactose (carbohydrate) coating was done by ring opening reactions followed by reaction of aldehyde groups of galactose in 0.1 M sodium acetate buffer (pH 4.0) with the amino groups of dendrimers (Mitchell et al., 1999). This leads to formation of Schiff's base (–N=CH–), which may then get itself reduced to secondary amine (–NH–CH_2–) and remain in equilibrium with Schiff's base at basic pH. The uncoated carbohydrate and other impurity were removed easily by dialysis. There was a moderate relative change in peaks of coated 5.0 G PPI dendrimer on coating with galactose (Table 1). Appearance of the major peaks like for –C=N stretch of imine at 1639 cm^{-1}, broad strong intense O–H stretch of carbohydrate around 3382 cm^{-1}, N–H deformation of secondary amine at 1407 cm^{-1}, confirmed Schiff's base formation and some amine formation in linkage between carbohydrate and amine termination of dendrimer.

Proton NMR peaks and shifts also further confirmed the synthesis. Peaks of alkane between 0.75 ppm and 1.25 ppm and peaks of alkyl amine were obtained between 1.3 ppm and 2.0 ppm. Substituted alkyl (C–CH_2–X) exhibited peak between 3.25 ppm and 3.5 ppm. The relative changes in peaks (Table 2) also confirmed galactose coating. The peaks of imine and some reduced amine were obtained between 6.0 ppm and 7.5 ppm and peak of –OH group of carbohydrate was obtained between 3.0 ppm to 5.0 ppm. ESI mass of uncoated PPI 5.0 G was 7004 (Weener et al., 1997) and of coated PPI 5.0 G was found to be 18,660 (theoretical mass—18,524). The coating by galactose was also confirmed by using Kaiser Ninhydrin test, which showed the disappearance of Ruhemann's purple blue chromophore peaks (absorbing at 570 nm) on scanning of coated dendrimer solution (Sarin et al., 1981).

3.2. Dendrimer–drug formulation

The drug loading in PPI dendrimers was achieved by equilibrium dialysis of drug with dendrimer solution (Martin et al., 1991; Miklis et al., 1997; Bhadra et al., 2003). The drug loading was effectively determined indirectly by drug remaining in dialysis medium, in which known concentration of drug solution was taken and dialyzed with dendrimers. The dendrimers were retained in the membrane due to their higher molecular

Table 1
Data of Infrared spectrum of coated 5.0 G PPI dendrimers

S. no.	Wave number (peak) in cm^{-1}	Spectrum characteristics	Functional group
1	1083, 1188	Weak	C–H deflection of alkane
2	1249	Medium	–OH deflection of primary alcohol of carbohydrate
3	1407	Weak	N–H deflection of secondary amine
4	1639	Strong	C=N stretch of imine
5	3382	Wide and strong	O–H and N–H stretch over lapping

Table 2
Major NMR peaks (absent in spectrum of uncoated dendrimer) interpreting the galactose coating in spectrum of coated 5.0 G PPI dendrimer

S. no.	Peak range (in ppm)	Integral values	Interpretation
1	1–2	15.53	Primary alkane C─H coupled with secondary amines ─NH─CH_2
2	2.5–5.5	307.95	Alcoholic group of carbohydrate (major proof)
3	6–8	1.87	─CH═N─ (Schiff's base) intermediate formed during reaction between galactose and PPI dendrimer

weight and being branched structures having greater than 2.4 nm, and only free drug can easily permeate across the dialysis tube (pore size 2.4 nm). There was also an increase in the drug entrapment by 5–15 times with increase in generations (data not shown). The entrapment of PP varied from 0.18 ± 0.05 g of PP/g of dendrimer (four to five molecules of PP per molecules of uncoated 5.0 G dendrimers) to nearly 1.1 ± 0.25 g (for coated 4.0 G) and 2.2 ± 0.52 g (for coated 5.0 G) of PP per g of dendrimer. This when converted to molar quantities become 30–60 molecules per molecule of coated systems depending upon generations. The drug entrapment for coated systems thus had increased with generations of dendrimers and further with coating.

This might be due to the fact that with increase in generations the structures become more compact and peripherally closer for higher generations and coating with galactose further increased number of functional groups for complexation and provided a steric hindrance, preventing the drug release from open structure, hence can increase drug entrapment. There was also a significant decline in release rate of PP from coated dendrimers as compared to uncoated dendritic carriers, which get prolonged up to 5–6 days from 24 h for uncoated 3.0–5.0 G dendrimers (Table 3). The reasons for decline in release rate by galactose coating may be the same as the effect of coating on drug entrapment. The Transmission Electron Photomicrograph at various magnifications had shown an increase in size in nanometric particulate size range of uniform spherical shape for the coated dendritic carriers (Figs. 2 and 3).

3.3. Hemolytic toxicity

The hemolytic toxicity of the dendrimer was a major limitation for the use of such polycationic dendrimeric drug delivery systems. All cationic dendrimers were lytic at concentration of 1 mg/ml, but diaminobutane and ethylenediamine core dendrimers showed no generation-dependency in such activity (Malik et al., 2000). This was found true in our case of amine terminated PPI dendrimers also but not in case of coated PPI dendrimer. Carbohydrate coating drastically reduced

Table 3
In vitro percentage cumulative drug release from dendrimer system ($n = 3$)

S. no.	Time interval (h)	Uncoated PPI dendrimer			Coated PPI dendrimer		
		3.0 G	4.0 G	5.0 G	3.0 G	4.0 G	5.0 G
1	1	6.9 ± 0.86	5.2 ± 0.63	4.43 ± 0.36	5.11 ± 0.41	4.89 ± 0.85	3.86 ± 0.86
2	2	18.94 ± 1.2	16.4 ± 1.2	5.16 ± 1.1	6.67 ± 0.72	5.28 ± 0.58	4.35 ± 0.45
3	3	29.7 ± 2.5	28.9 ± 1.1	15.96 ± 1.3	8.37 ± 0.81	6.31 ± 0.86	4.99 ± 0.75
4	4	40.49 ± 2.8	40.26 ± 2.1	27.63 ± 1.6	9.94 ± 0.45	8.37 ± 0.92	6.01 ± 0.98
5	5	51.83 ± 2.7	42.46 ± 2.3	36.49 ± 2.4	10.91 ± 0.78	9.30 ± 0.82	8.09 ± 1.1
6	6	64.57 ± 3.5	58.73 ± 2.7	43.04 ± 2.1	13.5 ± 1.1	11.64 ± 1.1	10.76 ± 0.84
7	7	80.29 ± 3.3	66.93 ± 2.6	52.55 ± 2.8	16.4 ± 1.2	14.40 ± 0.97	12.22 ± 1.1
8	24	94.36 ± 3.8	82.80 ± 3.4	63.09 ± 3.4	46.4 ± 1.6	45.87 ± 2.1	32.02 ± 2.1
9	48			84.70 ± 3.7	57.43 ± 2.2	55.02 ± 2.3	40.84 ± 2.4
10	72				68.87 ± 2.5	64.85 ± 2.7	48.63 ± 2.7
11	96				88.25 ± 3.1	76.48 ± 2.6	57.57 ± 2.5
12	120				95.43 ± 3.2	88.54 ± 2.7	69.75 ± 3.1
13	144					96.32 ± 3.1	89.53 ± 3.4

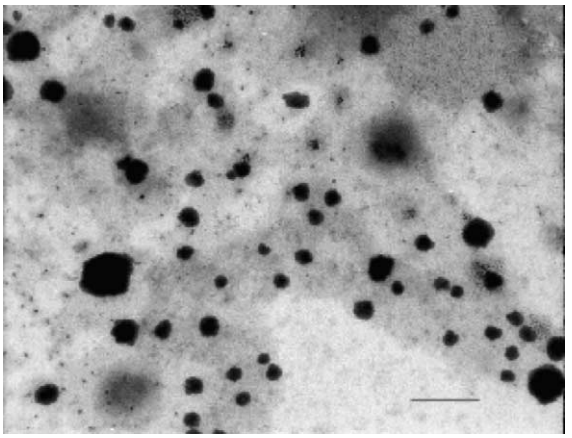

Fig. 2. Transmission electron micrograph of uncoated PPI dendrimers at 200,000× (large clumps are dendrimeric aggregates), where bar represents 50 nm.

the hemolysis of RBC, due to covering of the cationic amine terminations, which was responsible for hemolysis with carbohydrates. Percentage of hemolysis increased with generations from 35.7% (4.0 G) to 49.2% (5.0 G) uncoated dendrimers. There was approximately 3.5–7 times reduction of hemolysis on coating with galactose, which was only 10% to 7.1% of residual hemolysis for coated 4.0 G and 5.0 G, respectively, as compared to higher values for similar amounts of uncoated dendrimers. The suppression of hemolytic toxicities correlated well with other similar studies of dendrimer coatings (Bhadra et al., 2003).

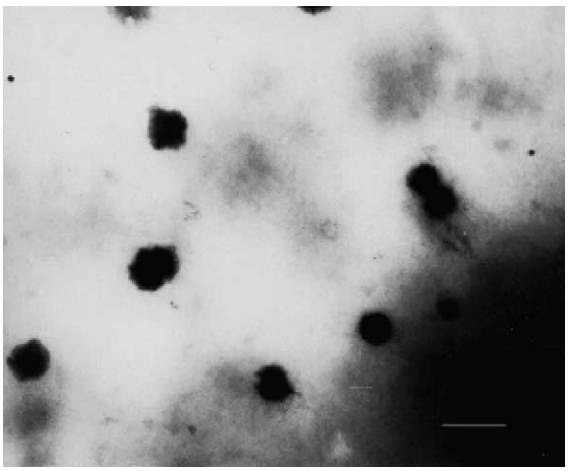

Fig. 3. Transmission electron micrograph of coated PPI dendrimers at 200,000×, where bar represents 50 nm.

3.4. Stability studies

The stability of carbohydrate coated drug–dendrimer formulation (5.0 G) was evaluated at various conditions of temperature (0 °C, 25 °C and 50 °C) after keeping both in amber colored and colorless vials. These were evaluated every week, for a period of 5 weeks. The formulations were found to be most stable in dark, at room temperature (25 °C). The drug leakage was found to be minimum at room temperature as compared to that at 0 °C, which may be due to hypothetical shrinking of the dendrimeric structure because of reduction in energy levels that lead to decrease in energy for efficient interaction with the drug molecules. There was further a decrease in the leakage of the drug at lower temperatures as compared to higher temperature (50 °C). At higher temperature the uniform ring like structure is destabilized by increase in free energy level of molecules and carbohydrate linking may break, which could make the structures wide open, causing release of the drug to a greater extent than at room temperature. It was also found that the drug leakage was more in formulations stored in light than those stored in dark. These may be attributed to the cleavage at higher temperatures and in light leading to bond breakage due to higher molecular kinetic at higher temperature. By all this, it can be concluded that the carbohydrate coated dendrimeric formulations are more stable at room temperature in dark (Table 4).

The physical changes like turbidity arising at lower temperature was attributed to the decrease in solubility of coated dendrimers like high molecular weight polymers in water so it become somewhat cloudy and turbid at lower temperature. At higher temperature due to polymerization tendency of free groups by degeneration of structure there is appearance of precipitation and turbidity and could lead to increased leakiness of dendrimers as evident from increase in drug leakage. This polymerization tendency at higher temperature was accelerated by light. The desolubilzation at lower temperature and precipitation in adverse conditions causes changes in consistency of the formulations on storage as observed after a period of 5 weeks.

Table 4
Stability profile of 5.0 G galactose-coated PPI dendrimer

Parameter	Dark			Light		
	0 °C	RT	50 °C	0 °C	RT	50 °C
Turbidity	+	–	+	+	+	++
Precipitation	–	+	++	–	++	+++
Color change	+	+	++	–	++	+++
Change in consistency	+	–	++	++	–	+++
Increase in rate of drug leakage as to initial fresh formulations						
1 Week	1.2%	0.9%	2.5%	1.5%	0.2%	7.0%
2 Weeks	1.9%	0.9%	4.5%	2.2%	0.9%	14.7%
3 Weeks	2.6%	1.1%	7.9%	3.9%	1.2%	19.9%
4 Weeks	2.8%	1.4%	12.3%	4.7%	2.4%	23.5%
5 Weeks	2.9%	1.5%	14.%	5.5%	2.9%	25.5%

(–) Indicate no change; (+) indicate smaller change; (++) indicate greater change; (+++) indicate considerable change.

3.5. Hematological studies

The hematological study was undertaken to assess the relative effects of the carbohydrate coated and uncoated systems as compared to the plain drug on various blood parameters. The blood parameters undergoing major changes were RBC counts, WBC counts and differential lymphocytes count. The RBC count was found to have decreased below normal values in case of uncoated systems than that of carbohydrate coated dendrimer system by $1.1 \pm 0.05 \times 10^6$ RBCs/μl. This was mainly due to hemolytic toxicity and cytotoxicity of similar positive charged or amine coated acrylate type nanoparticulates (Duncan et al., 1996). The uncoated dendrimers also stimulated the macrophage level and WBC count in rats more as compared to coated systems. The WBC count of uncoated PPI dendrimer increased by $2.7 \pm 0.3 \times 10^3\ \mu l^{-1}$ cells as compared to normal values. However, for coated PPI dendrimer complex the increase was by $1 \times 10^3\ \mu l^{-1}$ cells to $1.5 \times 10^3\ \mu l^{-1}$ WBCs as compared to normal count in controlled group. The statistical analysis showed there was a significant stimulation of WBCs count and decrease in RBC count by uncoated dendrimers as to normal and by coated PPI dendrimers ($P > 0.05$ by Student's *t*-test comparison) Further, there were no significant changes in other hematological parameters in case of drug delivery using coated PPI systems as compared to uncoated PPI ($P < 0.05$), which was found in conformity with the other prior studies for the coated dendrimers (Veronese et al., 1989; Bhadra et al., 2003).

3.6. Bio-distribution studies

Organ distribution study was undertaken to assess the amount of drug reaching liver and spleen. The free primaquine accumulates progressively in liver, where up to $30.7 \pm 2.6\%$ of dose is localized there 20 min after administration (Pirson et al., 1982) but after 120 min only $2.3 \pm 0.05\%$ of primaquine concentration were found in liver and $1.5 \pm 0.04\%$ and $18.5 \pm 0.89\%$ were in found in spleen and blood, respectively. In case of uncoated PPI drug formulation drug concentration were found to be $25.7 \pm 2.89\%$ of initial dose of primaquine in liver after 2 h of administration, and $3.4 \pm 0.36\%$ and $21.8 \pm 0.89\%$ primaquine concentration was found in the spleen and blood. But in case of carbohydrate coated PPI primaquine formulation, $50.7 \pm 5.9\%$ of primaquine was found in liver, whereas only $5.5 \pm 0.05\%$ and $7.8 \pm 0.76\%$ was found respectively in spleen and blood after 2 h of administration of the formulation (Fig. 4).

The amount of primaquine in body depends upon its release, its distribution, metabolism and excretion from body. Initially, the free PP content in liver was highest and after 2 h it declined in case of free drug injection to least. This might be due to rapid elimination of PP from liver, the prime site of its necessity and action. By the coated PPI dendrimers the % PP level was stabilized to significant amounts of requirement. These were facilitated due to galactose coating because galactose specific receptors were found in liver, which slowly localized the coated systems in liver and sustained release of drug locally from the

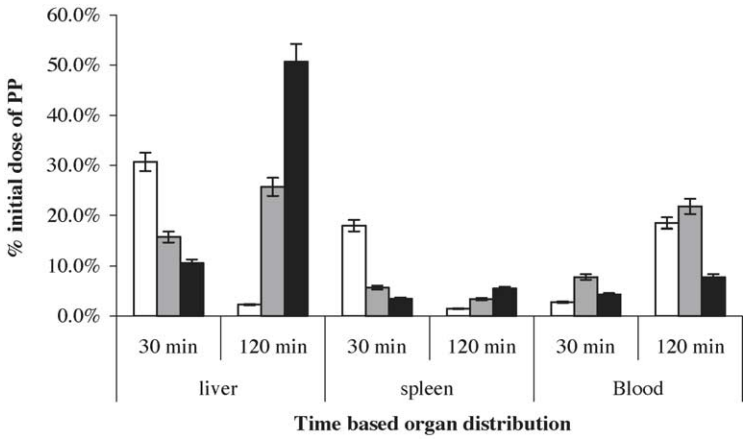

Fig. 4. Time based organ distribution of primaquine phosphate (PP), where the white bars represent plain PP injection dosing; gray bars are for uncoated PPI-PP dendrimer dosing and dark bars represent coated PPI-PP dosing and each bar represents mean % initial dose of PP found in various organs after a definite period of time ($n = 3$).

dendrimers in liver and maintained a significant level of PP in liver and spleen up to 2 h (more than blood concentration). The data correlated well with similar studies using liposome by Pirson et al. (1982) for such entrapped PP in liposomes for disposition of drug to liver only.

Intrahepatic disposition study was also undertaken to assess the amount of drug reaching parenchyma cells (p.c.) of liver. Using galactose-coated PPI 5.0 G dendrimer formulation $29.5 \pm 2.56\%$ of drug was found in p.c. whereas, in case of uncoated PPI 5.0 G dendrimer formulation only $19.2 \pm 1.25\%$ drug was found in p.c. of liver because of galactose specific receptor present in p.c. of liver. The results correlated well with studies of Murao et al. (2002) for galactosylated liposomes. It was found that different liver cells express galactose specific receptors and interact with different albeit specific ligand class. This was found because hepatocytes could avidly take up molecular and small sized particulate ligands (up to 8 nm) via Asialo-glycoproteins receptors, whereas endothelial cells could take up smaller and intermediate sized galactose-terminating ligand. There exists a clear difference in the liver homing of the small or molecular ligands as compared against large, particulate and cellular ligand, although the initial recognition always involves terminal galactosyl groups. A similar pronounced altered intrahepatic distribution was also observed by Meijer et al. (1996), when Naproxen (NAP) was coupled to lactosaminated and mannosylated human serum albumin (Lac-HSA and Man-HSA, respectively). Coupling of Nap to LaC_{27}-HSA and Man_{10}-HSA resulted in a major shift in intrahepatic distribution from endothelial cells to the hepatocytes and Kupffer cells, respectively.

However, further studies of the anti-malarial activity of the drug PP on liver stages of parasites by using such systems is anticipated from the present studies and would be taken up subsequently to prove the efficacy of such targeted molecular delivery systems in nanometric range for effective and safer treatment of liver stages or cellular stages of malaria parasites for radical cure from such dreaded disease as malaria, instead of prolonged exposure to PP.

3.7. Blood level studies

Blood level studies of these sustained release formulations were finally taken up to determine the release and performance of formulations in vivo. The formulations were predialyzed and lyophilized and formulations having equivalent to that of free drug (250 μg) was administered i.v. to the rats. The concentration of drug was determined in blood serum samples at various time intervals. The present method was validated prior for the peaks of free PP in deproteinized serum by the HPLC method (Dua et al., 1996) and the concentration of drug in blood samples were measured (without

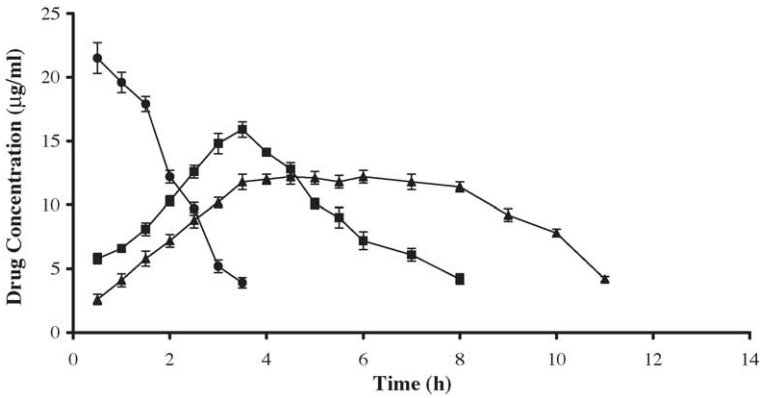

Fig. 5. Blood level studies of formulations ($n = 3$), where solid squares represent primaquine phosphate PPI dendrimer complex: solid triangles represent primaquine phosphate-coated PPI dendrimers and solid circles represent primaquine phosphate intravenous injection.

extraction of drug) in the serum by the corresponding area of peak for PP. The 5.0 G drug–dendrimer formulations were used for this study. The formulations were found to be following sustained release characteristics (Fig. 5).

The peak blood concentration of the drug was found to be lower in case of carbohydrate coated systems than that of uncoated system due to lower rate of release of the drug, similar to the trend observed in vitro for the drug release. The initial drug level was found to be lower and decreasing at lesser rate than that found for the free drug in body injected intravenously. The pharmacokinetic parameters such as $t_{1/2}$, K_e, etc. of the drug do not generally undergo large changes, because the system was designed to effect release of drug only, and there would be no enzyme or metabolism suppression by the dendrimers. The blood mean residence time (MRT) and area under blood level curve (AUC) values had undergone major changes as compared to normal drug injections. AUC values for the blood drug concentrations from uncoated dendrimers had almost doubled (i.e. 72.2 μg h/ml as compared to 38.65 μg h/ml for plain drug injections) and found more than tripled (100 μg h/ml) for coated formulations. MRT had increased four folds for administration by uncoated formulations (4.18 h) and six folds for coated formulations (6.11 h) as compared to administration of plain drug (1.68 h) injections. Thus, there was prolonged blood level of PP after 12 h of observations when coated dendrimeric formulations were used for administration. The observations were taken for a period of 12 h due to ethical limitations of possible number blood sampling from rats.

4. Conclusion

In conclusion, galactose-coated PPI dendrimers were found to be suitable for delivery of PP. As compared to vesicular and other nanoparticulate types of novel drug delivery systems dendritic systems are more stable because of molecular or chemical or non-biological nature. This drug delivery system could not only reduce the hemolytic properties of primaquine phosphate by controlling its release but also could selectively deliver the PP to liver parenchyma cells, which in turn could help in reduction of toxicity to other organs and at the same time could increase its efficacy for radical cure from malaria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2005.01.026.

References

Aoi, K., Itoh, K., Okada, M., 1995. Globular carbohydrate macromolecule sugar balls. 1. Synthesis of novel sugar-persubstituted poly(amidoamine) dendrimers. Macromolecules 28, 5391–5393.

Ashton, P.R., Boyd, S.E., Brown, C.L., Nepogodiev, S.A., Meijer, E.W., Peelings, H.W.I., Stoddart, J.F., 1997. Synthesis of glycodendrimer by modification of poly (propylene imine) dendrimers. Chem. Eur. J. 3, 974–983.

Ashwell, G., Harford, J., 1982. Carbohydrate specific receptor of liver. Annu. Rev. Biochem. 51, 531–554.

Bhadra, D., Bhadra, S., Jain, S., Jain, N.K., 2003. A PEGylated dendritic nanoparticulate carrier of fluorouracil. Int. J. Pharm. 257, 111–124.

De Brabander-Van den Berg, E.M.M., Meijer, E.W., 1993. Poly (propyleneimine) dendrimers: large-scale synthesis by heterogeneously catalyzed hydrogenations. Angew. Chem. Int. Ed. Engl. 32, 1308–1310.

Dua, V.K., Kar, P.K., Sarin, R., Sharma, V.P., 1996. High performance liquid chromatographic determination of primaquine and carboxyprimaquine concentration in plasma and blood cells in *Plasmodium vivax* malaria cases following chronic dosage with primaquine. J. Chromatogr. Biomed. Appl. 675, 93–98.

Dubber, M., Lindhorst, T.K., 1998. Synthesis of chiral carbohydrate-centered dendrimers. Chem. Commun., 1265–1266.

Duncan, R., Dimitrijevic, S., Eyagororou, E.G., 1996. The role of polymer conjugate in the diagnosis and treatment of cancer. STP Pharm. Sci. 6, 237–263.

Foye, O., William Thomas, L.L., David, W.A., 1995. Principal of Medicinal Chemistry, fourth ed. B.I. Wavley Pvt. Ltd., New Delhi, pp. 563–564.

Hanessian, S., Hoornaert, C., Pernet, A.G., Nadzan, A.M., 1985. Design and synthesis of potential megacaloric parenteral nutrients. Carbohydr. Res. 137, C14–C16.

Jayaraman, N., Stoddart, J.F., 1997. Synthesis of carbohydrate-containing dendrimer 5. Preparation of dendrimers using unprotected carbohydrates. Tetrahedron Lett. 38, 6767–6770.

Kieburg, C., Dubber, M., Lindnorst, T.K., 1997. A new type of carbohydrate clustering: synthesis of a pentavalent glycocluster based on a carbohydrate core. Synlett, 1447–1449.

Kitov, P.I., Sadowska, J.W., Mulvey, G., Armstrong, G.D., Ling, H., Pannu, N.S., Read, R.J., Bandle, D.R., 2000. Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. Nature 403, 669–672.

Lee, Y.C., 1978. Synthesis of some cluster glycosides suitable for attachment to protein or solid matrices. Carbohydr. Res. 67, 509–514.

Lee, Y.C., Townsend, R.R., Harely, M.R., Lonngren, J., Arnaep, J., Haraldson, M., Lonn, H., 1983. Binding of synthetic oligosaccharide of hepatic Gal/GalNac lectin. Dependence on fine structure feature. J. Biol. Chem. 258, 199–202.

Malik, N., Wrwattanapatapee, R., Klopsch, R., Lorenz, K., Frey, H., Weenee, J.W., Meijer, E.W., Paulus, W., Dunean, R., 2000. Dendrimers: relation between structure and biocompatibility in vitro. J. Control Rel. 65, 133–148.

Martin, A., Swarbrick, J., Cammerata, A., 1991. Physical Pharmacy, third ed. (Indian ed.). Varghese Publishing House, Bombay, India, pp. 337–338.

Meijer, D.K.F., Molema, G., Moolenaae, F., de Zeeuw, D., Swaet, P.J., 1996. Glyco-protein drug carriers with an intrinsic therapeutic activity: The concept of dual targeting. J. Control Rel. 39, 163–172.

Miklis, P., Cagin, T., Goddard, W.A., 1997. Dynamics of Bengal Rose encapsulated in the Meijer dendrimer box. J. Am. Chem. Soc. 119, 7458–7462.

Mitchell, J.P., Roberts, K.D., Langley, J., Koentegen, F., Lambert, J.N., 1999. A direct method for the formation of peptide and carbohydrate dendrimers. Biol. Med. Chem. Lett. 9, 2785–2788.

Murao, A., Nishikawa, M., Managit, C., Wong, J., Kawakami, S., Yamastuta, F., Hashida, M., 2002. Targeting efficiency of galactosylated liposomes to hepatocytes in vivo: effect of lipid composition. Pharm. Res. 19, 1808–1813.

Pirson, P., Steiger, R., Trouct, A., 1982. The disposition of free and liposomally encapsulated antimalarial's primaquine in mice. Biochem. Pharmacol. 31, 3501–3507.

Rao, J.V., Prasad, T.N.V., Sastry, C.S.P., 1989. New spectrophotmetric method for the estimation of primaquine using 4-aminophenazone. Ind. Drug 26, 194–195, Anal. Abst. 51 (6), 6E83.

Sarin, V.K., Kent, S.B.H., Tam, J.P., Merrifield, R.B., 1981. Quantitative monitoring of solid phase peptide synthesis by the Ninhydrin reaction. Anal. Biochem. 117, 147–157.

Tomalia, D.A., Baker, H., Dewald, J., Hall, M., Kalles, G., Martin, S., Roeck, J., Smith, P., 1985. A new class of polymer: starburst-dendritic macromolecules. Polym. J. 17, 117–138.

Turnbull, W.B., Stoddart, J.F., 2002. Design and synthesis of glycodendrimer. Rev. Mol. Biotech. 90, 231–255.

Veronese, F.M., Calicetti, P., Pastorino, A., Schiavon, O., Sartore, L., 1989. Preparation, physicochemical and pharmacokinetic characterization of methoxy polyethylene glycol derivitized superoxide dismutase. J. Control Rel. 10, 145–154.

Weener, J.W., Van Hest, J.L.W., Hummelen, J.C., Meijer, E.W., 1997. Fragmentation studies of poly(propyleneimine) dendrimers in the gas-phase by using eletrospray ionization mass spectrometry. ACS PMSE Prepr. 77, 147–148.

Wooley, K.L., Howker, C.J., Frechet, J.M.J., 1991. Hyperbranched macromolecules via a novel double stage convergent growth approach. J. Am. Chem. Soc. 113, 4252–4261.