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Glycoconjugated peptide dendrimers-based nanoparticulate system for the delivery of chloroquine phosphate

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Abstract

Dendrimers consisting of different molecules of metabolic pathways such as amino acids can greatly reduce the toxicity associated with amine-terminated dendrimers e.g. polyamidoamine (PAMAM) and polypropylene imine (PPI) dendrimers. In the present study, poly-L-lysine dendrimers having polyethyleneglycol (PEG-1000) as core, were synthesized upto fourth generation. Dendrimers were synthesized by alternating protection and deprotection steps of L-lysine by di-BOC (di-tertiary butyl pyrocarbonate) till the formation of 4.0G peptide dendrimer took place. D-galactose was selected as model sugar for peripheral conjugation (coating) of these peptide dendrimer. The complete formation of uncoated and galactose-coated poly-L-lysine dendrimers was characterized by transmission electron microscopy (TEM), IR, NMR and MALDI TOF mass spectroscopic studies. Chloroquine phosphate (CP)-loaded uncoated and coated dendrimers were evaluated for *in vitro* drug release rate, hemolytic toxicity and stability studies. *Ex vivo* cellular uptake studies of uncoated and coated drug dendrimer formulations in macrophages revealed almost 5 times reduced phagocytosis due to galactose coating ($p<0.0001$). *In vitro–in vivo* release behavior indicated possibilities of galactose-coated drug dendrimers formulation in controlled drug delivery of CP. Galactose coated formulations drastically reduced hemolytic toxicity compared to uncoated poly-L-lysine formulation as well as plain drug. Hematological data suggests that galactose-coated formulations are less immunogenic compared to uncoated formulations. Finally, it can be concluded that galactose-coated polylysine dendrimers can be utilized for controlled delivery of CP more safely compared to its uncoated formulation both *in vitro* and *in vivo*.

Keywords: Chloroquine phosphate; Controlled delivery; Peptide dendrimer; Glycoconjugation

1. Introduction

Polymers today have become an indispensable part of the drug delivery systems be it a conventional drug delivery or novel drug delivery. Polymeric drug carriers such as nanoparticles, microspheres, polymeric micelles have well proven their applications in sustained and targeted drug delivery [1–4]. In the last two decades of the scientific research, dendrimers have been visualized well as a drug delivery module [5–10]. Dendrimers are known for their three-dimensional, monodispersed, highly branched, macromolecular nanoscopic architecture with number of reactive end groups [11]. Apart from drug delivery, dendrimers have recently been used successfully in field of biomedicine (DNA and gene delivery, cancer diagonosis) [12–15], catalysis [16,17], as light-harvesting devices, optical sensors [18,19], and in desalination [20], etc.

Dendrimers have been reported to host both hydrophilic and hydrophobic drugs. Different types of dendrimers, which have shown to exhibit such potential, include polyamidoamine (PAMAM), polypropylene imine (PPI), polylysine dendrimers [21]. In recent years, monomers which are either intermediates or products in various metabolic pathways are being used in dendrimer synthesis to render them biodegradable [22]. Peptide dendrimers (containing peptide bonds) based on different amino acids such as lysine, exhibited promising vaccine, antiviral and antibacterial properties. Peptide dendrimers are frequently utilized as protein and liposomal mimetics as well as biomaterials in life sciences and biomedical applications.

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Similar to other dendrimers, synthesis of peptide dendrimers is tightly controlled with products of consistent size, architecture and composition. The poly-valency of the peptide dendrimers enhances the affinity of peptide specific interactions with peptides, proteins and carbohydrates [23]. Recently, citric acid–polyethylene glycol based biodegradable dendrimers have shown controlled release of mefenamic acid, diclofenac and 5-amino salicylic acid [24]. A number of polyester dendrimers incorporating glycerol, succinic acid, phenylalanine and lactic acid have also been prepared and explored for their possible use in tissue engineering [22]. However, in spite of their broad applicability, toxicity associated due to the terminal $-NH_2$ groups and cationic charge, limits these candidatures for their clinical applications [6]. One approach to reduce amine termination associated toxicity is to modify the terminal $-NH_2$ groups of dendrimers with neutral or anionic moieties to avoid the toxicity and liver accumulation associated with these cationic dendrimers. Earlier, PAMAM dendrimers with PEG grafts on surface exhibited improved biocompatibility and solubility of anticancer drugs adriamycin, methotrexate and 5-fluorouracil [5,6,25].

Glycodendrimers have shown immense potential as drug carrier in this context. The term 'glycodendrimer' is used to describe dendrimers that incorporate carbohydrates into their architecture. Carbohydrate dendrimers can be classified as carbohydrate-centred, carbohydrate-based and carbohydrate-coated dendrimers.

Carbohydrate coating on the dendrimeric system can reduce the hemolytic toxicity by neutralizing the cationic charges due to the presence of NH_2 groups on the periphery of dendrimers [6,26–28]. It can also help in targeting the system to the active sites of action suitably, with reduced immunogenicity, anti-genecity and toxicity by shielding the system from recognition by the macrophages. Starburst glycodendrimers offer the potential to serve as high-affinity ligands for clinically relevant sugar receptors [6,29]. Among the various ligands, galactose has been found to be suitable for liver targeting because of the asialo-glycoprotein receptors present on liver parenchymatous cells, which recognize galactose units on chemically conjugated drug carriers. In a recent study, galactose-coated polypropylene imine dendrimers (5.0G) have been used to target primaquine phosphate, a liver schizonticide, directly to liver cells [6]. These peripherally galactose-coated PPI dendrimers were found to be safer and suitable for sustained drug delivery [30].

Malaria is still one of the most dreadful protozoan diseases. An ideal antimalarial agent must be able to exert an effect on two fronts simultaneously, namely; to eradicate the microzoan from the blood and also from the tissues, in order to produce an effective 'radical cure.' Chloroquine is extensively employed for the suppression and treatment of malaria (Fig. 1). It exerts a quick schizontocidal effect and seems to affect cell growth by interfering with DNA. Malaria caused by chloroquine sensitive *P. falciparum* is generally treated by slow

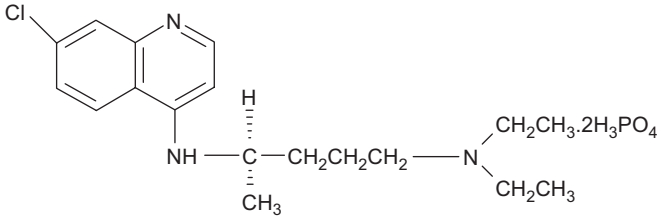


Fig. 1. Chemical structure of chloroquine phosphate.

parenteral infusion. Rapid administration of therapeutic or high dose by parenteral route leads to acute chloroquine toxicity necessitating a sustained chloroquine delivery [31]. Formulating chloroquine phosphate (CP) as a parenteral dosage form for sustained realease, we have selected it as a model drug in present study.

In the present study, we have designed, characterized and compared galactose-coated and -uncoated peptide dendrimers as novel drug carriers for delivery of CP. Peptide dendrimers consisting of L-lysine as the dendrimeric branch, di-tertiary butyl-pyro carbonate (di-BOC) as the branching agent and polyethyleneglycol 1000 (PEG-1000) as the central core were synthesized and well characterized up to fourth generation through divergent synthetic scheme. Peripheral coating of these dendrimers was performed using D-galactose. The effect of galactose coating on poly-L-lysine dendrimers was compared for their safety and delivery of CP *in vitro* and *in vivo*.

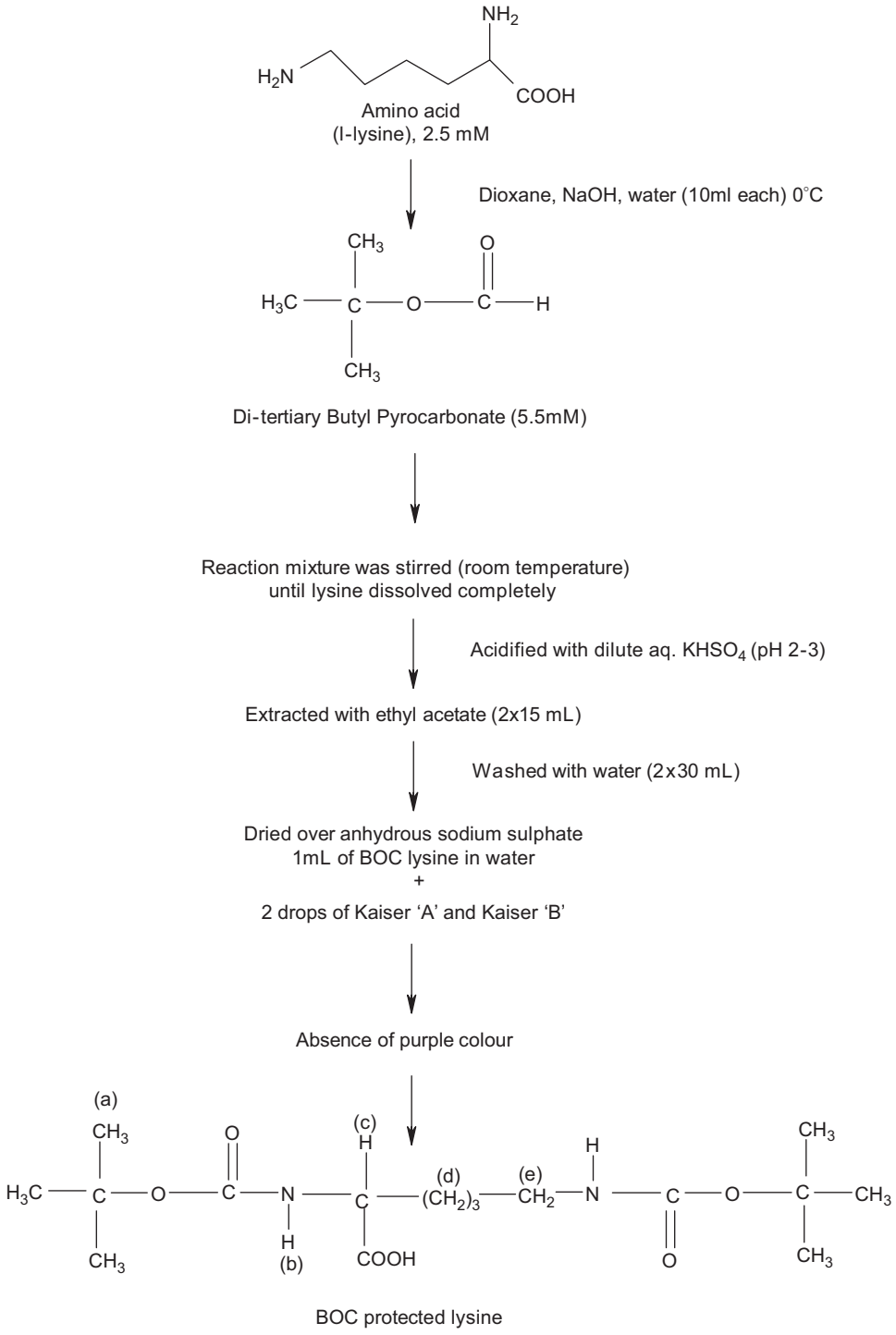
2. Materials and methods

2.1. Materials

PEG-1000 was obtained from Wilson Laboratories, Mumbai, (India) while L-lysine pure (free crystalline) was purchased from Himedia, India. Di-tertiary butyl pyrocarbonate (di-BOC), 1-hydroxybenzotriazole (HoBt), *n,n*-dicyclohexyl carbodiimide (DCC), dimethylaminopyridine (DMAP) were purchased from Spectrochem, India. D-galactose was procured from CDH (P) Ltd. India. CP was obtained as a gift sample from Broshell Remedies, India. All the other chemicals used were of analytical grade.

2.2. Protection and characterization of amino acid (L-lysine) by di-BOC

Preparation of homogeneous dendrimers was initiated with the protection of the functional groups on amino acid, L-lysine. Protection of L-lysine with di-BOC was accomplished under basic conditions (Scheme 1). The amino acid, L-lysine (410 mg) was dissolved in a mixture of solvents containing 2.5 mL of dioxane, 2.5 mL of distilled water and 2.5 mL of 1 M sodium hydroxide. Di-tertiary butyl pyrocarbonate (4.8 g, 22 mM) was added to the lysine mixture with constant stirring (Room temp., 30 min). The solution was concentrated on a rotary evaporator to about 10–15 mL, and was further cooled in an ice water bath. The mixture was then covered with a layer of ethyl acetate (30 mL) and acidified with dilute aqueous potassium hydrogen sulfate ($KHSO_4$) solution to pH 2–3. The aqueous phase was extracted with ethyl acetate (2×15 mL) and the ethyl acetate extract was cooled, washed with water (2×30 mL) and dried over anhydrous Na_2SO_4 and evaporated in vacuum. The compound



Scheme 1. Schematic representation showing protection of L-lysine di-Boc.

obtained i.e. di-BOC protected lysine was subjected to IR spectroscopy using KBr pellet method (Perkin Elmer, USA); 3428.1 cm^{-1} (OH stretch), 2934.8 cm^{-1} (symmetric CH stretching), 2829.9, 2722.2 cm^{-1} (aliphatic CH stretch), 1595.8 cm^{-1} (–C═O stretch due to ester linkage), 1360 cm^{-1} (asymmetric and symmetric CH bending), 1169.1 cm^{-1} (CO stretch), 1113.1 cm^{-1} (CH bending), 773.4 cm^{-1} (CC stretching), 619.5 cm^{-1} (CH rocking). ^{1}H-NMR (D_2O, Bruker DRX300, Germany) δ 5.893 (s, Hb), δ 4.143 (d, Ha) δ 1.234–1.276 (m, Hd), δ 3.127 (s, Hc), δ 2.968 (s, He) (Scheme 1). A qualitative analysis was also performed to confirm the protection of L-lysine by di-BOC. Aqueous solution of di-BOC protected lysine 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) and color was observed [32].

2.3. Synthesis and characterization of poly-L-lysine dendrimer

di-BOC–lysine (2.5 mM; 0.865 g), PEG-1000 (1 mM; 1.0 g), dicyclohexyl carbodiimide (DCC) (2.5 mM; 0.515 g) in a flat bottom flask were dissolved

Table 1
Theoretical details of the reactants and intermediates in the synthesis of poly-L-lysine dendrimer

Generation number	Number of moles of reactant	Molecular weight of intermediate	Number of terminal functional groups
0.0G	1.0 M of PEG-1000	—	—
0.5G	1.0 M of PEG-1000 2 mM Boc lysine 2.5 mM DCC	1948	4
1.0G	Deprotection of 0.5G	1256	4
1.5G	0.04 mM, 0.5G 4 mM Boc lysine	2284	8
2.0G	Deprotection of 1.5G	1768	8
2.5G	0.056 mM, 1.5G 8 mM Boc lysine	4536	16
3.0G	Deprotection of 2.5G	2792	16
3.5G	0.036 mM, 2.5G 16 mM Boc lysine	5536	32
4.0G	Deprotection of 3.5G	4840	32

in a solution containing 10 mL of 1:1 solution of dichloromethane (DCM) and dimethyl formamide (DMF) and 10% of dimethylamino pyridine (DMAP) and 1-hydroxybenzotriazole (HoBt) acting as a catalyst (Table 1). The mixture was allowed to stir under perfect ice-cold conditions for 24 h using magnetic stirrer (Scheme 2).

After 24 h, the precipitate was filtered, and the reaction mixture was stirred again; the concentrated mixture was added in quantity 6 times the volume of the reaction mixture and was allowed to precipitate by keeping it in deep-freezer for one day. The precipitate than obtained (0.5G) was collected and was subjected for further reaction preceded by its deprotection with 1:1 triflouroacetic acid (TFA): dichloromethane (DCM) (1 mL) (usually <2 h). The deprotection of the mixture gave the whole generations i.e. 1.0G, 2.0G, etc. [23]. The reaction steps were repeated alternately till the formation of 4.0G (Scheme 2). ^{1}H-NMR (D_2O, Bruker DRX300, Germany) obtained at 300.1299709 MHz, δ 3.70 [s, O–$(CH_2)_n$–O], δ 2.7–2.89 (m, NH_2–CH–X substituted alkyl group), δ 3.07 (m, $NCH_2CH_2CH_2CH_2NH_2$), δ 5.26 (br, NH). MALDI TOF MS was also performed by running samples on a MI cromass Tof Spec 2E instrument using a nitrogen 337 nm laser (4 ns pulse). Atleast 40–50 shots were summed up. The matrix used was 2,5-dihydroxy benzoic acid dissolved in acetonitrile (ACN). Sample and matrix solution were mixed together and 1 μl spotted on MALDI target. Transmission electron microscopy (TEM) of the prepared dendrimers was done to characterize the systems after drying on 3 mm forman (0.5% plastic powder in amyl acetate)-coated copper grid (300 mesh) at 60 kV (Morgani, 268D; Holland) after staining negatively using uranyl acetate (4%) and photomicrographs were taken at suitable magnifications. Kaiser test was also performed as explained before.

2.4. Coating and characterization of D-galactose on poly-L-lysine dendrimers

Galactose coating was performed by the method reported elsewhere [33]. D-galactose was chosen as model sugar to conjugate with dendrimer. Galactose (0.33 mM; 60 mg) was dissolved in 0.1 M sodium acetate (NaOAc) buffer (pH 4.0) to a concentration of 0.1 M. This solution was then added to the deprotected poly-L-lysine dendrimer, 4.0G (0.002 mM; 10 mg), agitated to ensure dissolution of the dendrimer and allowed to stand at ambient temperature for 2 days. ^{1}H-NMR (D_2O, Bruker DRX300, Germany) obtained at 300.1299709 MHz, δ 2.597–2.949 (m, alcohol of carbohydrate), δ 7.486 (s, aldehydic residue). MALDI TOF and TEM were performed in the same manner as for uncoated dendrimers.

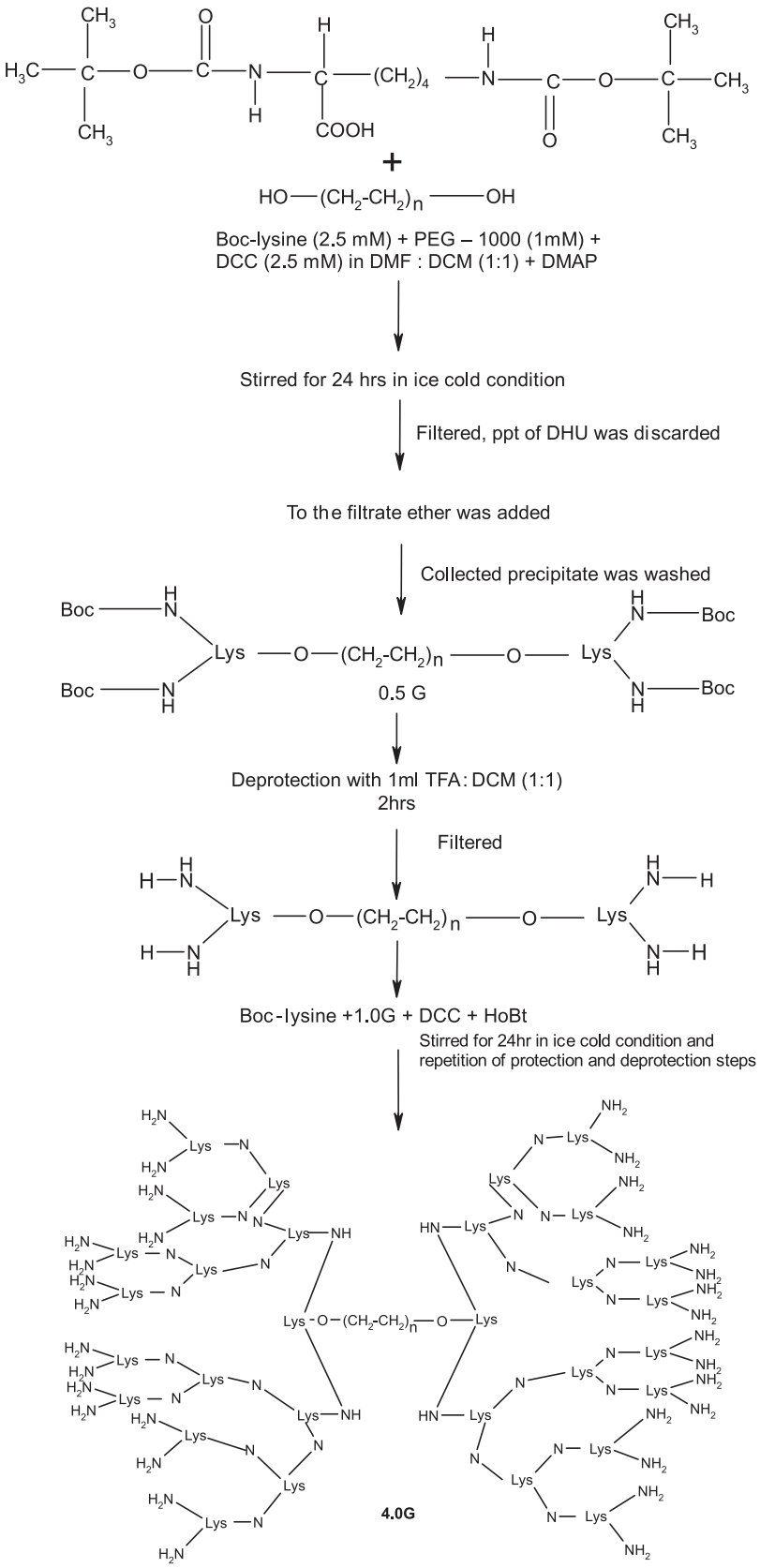
2.5. Drug encapsulation and in-vitro release of CP by uncoated and galactose-coated poly-L-lysine dendrimers

Drug loading was achieved according to the equilibrium dialysis method reported earlier [34,35]. Aqueous solution of CP was prepared (10 mL; 1000 μg/mL) by dissolving 10 mg of CP in 10 mL of double distilled water, 1 mL of this solution was incubated with 100 mg of dendrimer. The volume was made upto 10 mL with double-distilled water. The mixed solution was incubated with slow magnetic stirring (50 rpm) using Teflon bead for 24 h. Similarly, drug was loaded in galactose-coated peptide dendrimer of 3.0G and 4.0G. Percent entrapment of drug was determined by equilibrium dialysis method in which 5 mL of drug-dendrimer solution was taken in a cellulose dialysis bag (MWCO 12,000–14,000, Sigma, Germany). This solution was dialyzed twice against double-distilled water under strict sink conditions for 10 min to remove free drug from the formulation, which was then estimated spectrophotometrically at 343 nm (UV-1601, Shimadzu, Japan) to determine indirectly the amount of drug loaded within the system (Table 2).

In the *in vitro* release studies, 5 mL of dendrimer drug complex of both coated and uncoated (UPD3-CP, GCPD3-CP, UPD4-CP, GCPD4-CP) dendrimers were taken in a dialysis bag separately and were dialyzed under sink conditions. One mL of aliquots were collected after every 1 h interval for 24 h of both coated and uncoated systems and drug release was estimated indirectly using UV-Visible spectrophotometer (Shimadzu UV-1601, Japan) and double-distilled water as blank.

2.6. Hemolytic toxicity

Whole human blood was collected in HiAnticlot blood-collecting vials (Hi media, India). The RBCs were separated by centrifugation and were resuspended in normal saline solution to have 10% hematocrit value [36]. The RBC suspension was mixed with distilled water (considered as producing 100% hemolysis) and normal saline (taken as blank for spectrophotometric estimation). One milliliter of suitably diluted, coated and uncoated dendrimer drug formulations (UPD3-CP, GCPD3-CP, UPD4-CP and GCPD4-CP) were added separately to 4.5 mL of normal saline and allowed to interact with RBC suspension. Similarly, 0.5 mL of drug solution and 0.5 mL of dendrimer solution were mixed with 4.5 mL of normal saline and interacted with RBC suspension. The drug and dendrimers in separate tubes were taken in such amount that the resultant final concentrations of drug and dendrimer were equivalent in all the cases. The coated dendrimeric formulations were taken in amount such that the resultant final concentrations of drug and dendrimer were equivalent to that in uncoated formulations. Thus the hemolysis data of



Scheme 2. Synthesis of 4.0G poly-L-lysine dendrimer.

Table 2
Percent entrapment of drug in galactose coated and uncoated dendrimer

Formulation code	Molar ratio of drug to dendrimer	% Entrapment of CP
UPD3-CP	2.24 ± 0.068^{a}	41.4 ± 0.052^{b}
GCPD3-CP	6.17 ± 0.044^{a}	64.32 ± 0.038^{b}
UPD4-CP	4.33 ± 0.038^{a}	46.0 ± 0.015^{b}
GCPD4-CP	15.27 ± 0.084^{a}	78.0 ± 0.047^{b}

$n = 6$ tabulated value represents mean ± S.D.
$^{ab}p < 0.05$ (Comparison of GCPD3-CP with GCPD4-CP).
$^{ab}p < 0.05$ (Comparison of UPPD3-CP with UPD4-CP).
$^{ab}p < 0.05$ (Comparison of UPD4-CP with GCPD4-CP).
UPD3-CP: uncoated poly-L-lysine dendrimer (3.0 G) -chlorquine phosphate complex; GCPD3-CP: galactose-coated Poly-L-lysine dendrimer (3.0 G) -chlorquine phosphate complex; UPD4-CP: uncoated poly-L-lysine dendrimer (4.0 G) -chlorquine phosphate complex; GCPD4-CP: galactose-coated poly-L-lysine dendrimer (4.0 G) -chlorquine phosphate complex.

Table 3
Percent phagocytosis by macrophages

Formulation code	% phagocytosis after			
	1 h	2 h	4 h	6 h
UPD4-CP	28 ± 3.14^{a}	42.2 ± 3.24^{a1}	78 ± 4.24^{a2}	96.4 ± 3.24^{a3}
GCPD4-CP	8.54 ± 1.22^{a}	9.1 ± 2.24^{a1}	12.4 ± 2.12^{a2}	18.4 ± 1.44^{a3}

$n = 6$ albino rats per group, tabulated values represent mean ± S.D.
$^{a,a1,a2,a3}p < 0.0001$ (Comparison of UPD4-CP with GCPD4-CP).

plain drug, dendrimer, uncoated dendrimer formulations and coated formulations were compared. After centrifugation, supernatants were taken and diluted with equal volume of normal saline and absorbance was taken at 540 nm against supernatant of normal saline diluted similarly as blank [37]. Percent hemolysis was thus determined for each sample by taking absorbance of water as 100% hemolytic sample, using the following equation:

$$\%\ \text{hemolysis} = \left[\frac{\text{ABs}}{\text{AB}_{100}}\right] \times 100.$$

AB_s is the absorbance of the sample and AB_{100} the absorbance of control without formulation.

2.7. Stability studies/leakage studies

The samples (5 mL) of GCPD4-CP formulations were kept in dark in amber colored vials and in colorless vials at 0 °C, room temperature (25–30 °C) and 50 °C in controlled oven for a period of five weeks. The samples were analyzed after every week for upto 5 weeks for any precipitation, turbidity, crystallization, change in color, consistency and drug leakage. Drug leakage was determined by checking for increase in the release of drug from the formulation after storage at accelerated condition (50 °C). The formulation samples (2 mL) were dialyzed across cellulose tubing. The external medium (50 mL) was analyzed for content of the drug, spectrophotometrically. The procedure was repeated every week for up to 5 weeks [5].

2.8. Macrophage uptake studies

Male albino rats (Sprague Dawley strain), of similar weight and size were taken for the present *ex vivo* cellular uptake study. The lung and liver macrophages were obtained by extraction and washing of lung and liver from sacrificed rats by Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (1000 μg/mL) and streptomycin (100 μg/mL). The macrophage suspension was incubated with galactose coated (GCPD4-CP) and uncoated (UPD4-CP) formulations in equal ratio for 6 h at 37 ± 1 °C. At different time intervals of incubation (1, 2, 4 and 6 h) smears were prepared, washed with distilled water and examined at 100 × under optical microscope (Leica, DMLB, Switzerland). Percent phagocytosis (Table 3) was determined by counting number of macrophages undergone phagocytosis out of 100 macrophages observed [38,39].

2.9. Blood level studies

On the basis of *in vitro* characteristics and percent drug encapsulation, formulations of uncoated and coated poly-L-lysine dendrimers (UPD4-CP and GCPD4-CP) were selected for *in vivo* performance evaluation. Male albino rats (Sprague Dawley strain) of uniform body weight with no prior drug treatment were used for the studies and were maintained on standard diet and water. The protocol was duly approved by the Institutional Animal Ethical committee, by letter number Animal Eths. Comm./DB/304 of Dr. H.S. Gour University, Sagar, M.P., India.

Albino rats (130 ± 10 g) taken for present study were divided into four groups with three rats in each group. Animals were fasted overnight before administration of dose. To the first group aqueous solution of CP in a concentration of 250 μg/mL was administered through caudal vein of the animal. Uncoated and galactose coated poly-L-lysine dendrimeric formulations (UPD4-CP, GCPD4-CP) were administered to each rat of the second and third groups, respectively in an equivalent concentration of CP (250 μg/mL). The fourth group was kept as control.

One rat from each group was sequentially taken and 0.1 mL of blood was withdrawn from retro-orbital plexus in 0.9% saline after 0.25, 1.0, 1.5, 2.0, 2.5,…, 12 h, respectively from each group and drug concentration in each group was determined spectrophotometrically (Shimadzu UV-1601).

2.10. Hematological study

The male albino rats (SD strain) of uniform weight and size were taken for the study. The animals were divided into four groups comprised of

three rats in each group. Plain drug (CP), galactose coated (GCPD4-CP) and uncoated dendrimeric formulation (UPD4-CP) containing 250 μg/mL equivalent CP were administered intravenously into first, second and third groups of animals, respectively every day up to 7 days. The fourth group was kept as control, which was maintained on same regular diet for 7 days. After 7 days blood samples were collected from the animals and analyzed for RBC count, WBC count and differential count of monocytes, lymphocytes and neutrophils by the pathology lab (Table 4).

3. Results and discussion

3.1. Protection and characterization of amino acid (L-lysine) by di-BOC

The di-BOC ptotection was confirmed by IR and NMR spectroscopy. The peak at 3428.1 cm^{-1} confirmed the presence of O–H stretch while peak at 1595.8 cm^{-1} confirmed of –C═O stretching of ester linkage between di-BOC and L-lysine molecule. The peaks at 2934.8, 2829.9, 2727.2 cm^{-1} were all due to C–H stretching, while peak at 1360 cm^{-1} was possibly due to the asymmetric and symmetric C–H bend. The peak at 1169.1 cm^{-1}(C–O stretch) confirmed the presence of ether group. Also the absence of doublets around 3400 confirmed the masking of $–NH_2$ groups of L-lysine, indicating total protection of L-lysine by di-BOC. Various shifts in NMR spectra (Scheme 1) also supported di-BOC protection of L-lysine. Negative Kaiser test (absence of purple color) further confirmed di-BOC protection of L-lysine [32].

3.2. Synthesis and characterization of uncoated and galactose-coated poly-L-lysine dendrimer

Poly-L-lysine peptide dendrimers of 3.0G and 4.0G were synthesized using PEG-1000 as core by coupling with the di-BOC protected lysine followed by deprotection till the formation of 4.0G. (Scheme 2). Galactose in sodium acetate buffer reacted with the amino groups of dendrimers with the formation of Schiff base ($–N═CH_2–$) followed by reduction ($–NH–CH_2–$). Uncoated carbohydrate and other impurities were removed easily by dialysis across the cellulose tubing (1200–1400 MWCO). TEM photographs of dendrimers prove their nanometric-sized vesicles as well as increase in particle size due to galactose (Fig. 2). The theoretical details of reactants and products for the synthesis of peptide dendrimer are given in Table 1.

The synthesis of uncoated and galactose-coated poly-L-lysine dendrimers was well confirmed by NMR and MALDI TOF mass spectroscopic methods. The various peaks and shifts obtained were analyzed and interpreted.

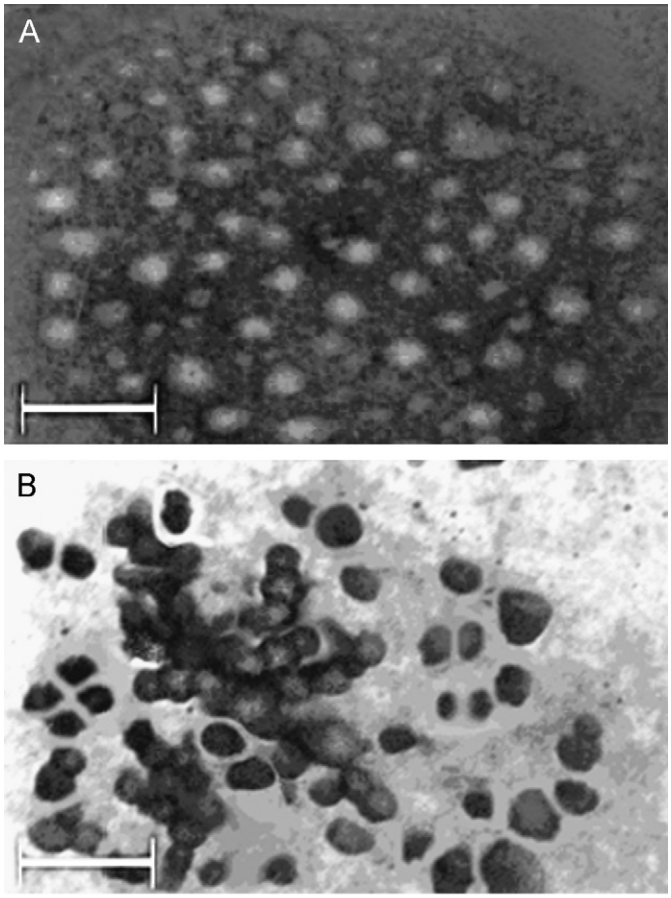


Fig. 2. Transmission electron micrograph of (A) uncoated poly L-lysine dendrimeris 4.0G at 300,000× where bar shows 30 nm on negative staining with 4% uranyl acetate, and (B) galactose-coated poly L-lysine dendrimers 4.0G at 150,000× where bar shows 50 nm on negative staining with 4% uranyl acetate.

Table 4
Hematological study of drug dendrimer formulations on albino rats

Formulations	RBC count ($\times 10^6$/μl)	WBC count ($\times 10^3$/μl)	Differential count ($\times 10^3$ μl)		
			Monocytes	Lymphocytes	Neutrophils
Control	9.1±0.3	10.8±0.6	1.5±0.3	8.2±0.5	1.8±0.5
Drug	8.4±0.2[a]	10.7±0.5[b]	1.3±0.2[b1]	9.4±0.6[b2]	1.6±0.2[b3]
UPD4-CP	7.5±0.3[a]	15.2±0.4[b]	1.5±0.3[b1]	12.4±0.2[b2]	2.3±0.2[b3]
GCPD4-CP	8.8±0.2[a]	11.3±0.4[b]	1.3±0.4[b1]	9.1±0.9[b2]	2.7±0.4[b3]

$n = 6$ albino rats per group, tabulated values represent mean±S.D.
[a,b,b1,b2,b3] $p<0.05$ (comparison of drug with UPD4-CP).
[a,b,b1,b2,b3] $p<0.05$ (comparison of drug with GCPD4-CP).
[a,b,b1,b2,b3] $p<0.05$ (comparison of UPD4-CP with GCPD4-CP).

NMR spectra of 4.0G uncoated poly-L-lysine revealed peaks at δ 3.70 [s, O–$(CH_2)_n$–O], δ 2.7–2.89 (m, NH_2–CH–X substituted alkyl group), δ 3.07 (m, $NCH_2CH_2CH_2CH_2NH_2$), δ 5.26 (br, NH). All these chemical shifts were in accordance with the proposed structure of poly-L-lysine dendrimers (Scheme 2). The relative changes in chemical shifts also further confirmed coating of galactose. Chemical shift of imide between 7.0 and 8.0 ppm i.e. at 7.486 and peak of alcohol of carbohydrate were obtained between 2.597 and 2.940 ppm. MALDI TOF mass spectra of uncoated peptide dendrimer 4.0G was 4829.2734 which is very close to theoretically calculated mass of 4840. Also, increase in mass after carbohydrate coating was well revealed by MALDI TOF mass spectra (Table 1). The obtained results are comparable with the reported procedure of β-alanine-based dendritic peptides [40].

3.3. Drug encapsulation and in vitro release

Drug loading in both uncoated and coated dendrimers of 3.0G and 4.0G dendrimers was confirmed spectrophotometrically. This was evident by a sufficient change in the values of original λ_{max} of CP (343.0 nm in water), and for CP entrapped uncoated and galactose-coated poly-L-lysine dendrimer of 3.0G and 4.0G, when estimated against dendrimer of same concentration. Observed results were in accordance with that illustrated for eriochrome black-T with poly (propyleneimine) dendrimers reported earlier [41].

The drug loading increased with higher generations, which was determined by dialysis through cellulose dialysis membrane (MWCO 12,000–14,000) and estimating the amount of unentrapped drug in external sink (Table 2). The increased entrapment was possibly due to increased polymeric architecture in higher generations. Coating with galactose further increased complexation and sealing of the open structure leading to increase in the drug entrapment. A similar trend was reported for PEGylated and glyco-conjugated dendritic nanoparticulate carrier of fluorouracil and primaquine phosphate, respectively as well as with other dendrimers [5,21].

In vitro release rate of drug from coated dendrimeric formulations was found to be less compared to their corresponding uncoated formulations. *In vitro* release rate was slowest for the formulation GCPD4-CP, which corresponds to its possible larger, compact and peripherally sealed structure (Fig. 3). Among similar generations *in vitro* release rate of CP from coated dendrimers was observed to be slower and sustained compared to corresponding uncoated formulations. Among different generation formulations, however, fourth generation formulations demonstrated a significantly slower ($p<0.05$) *in vitro* drug release compared to third generation formulations. Difference may be possibly due to more compact and sealed architecture of higher generations compared to lower generations [6]. Drug release pattern from galactose-coated formulations can be exploited for sustained release delivery of CP.

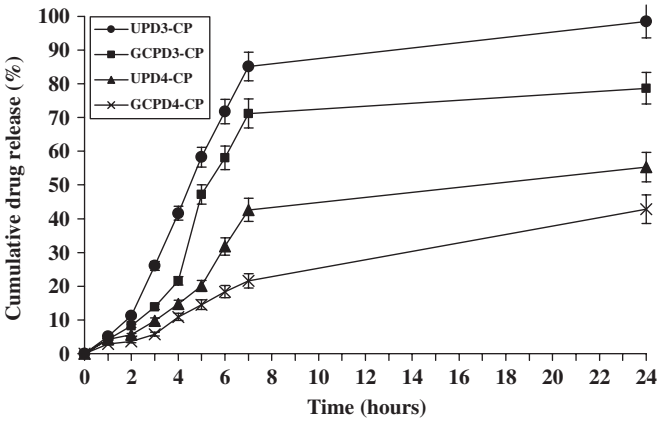


Fig. 3. *In vitro* release studies ($n=4$). Values represent mean ± S.D.

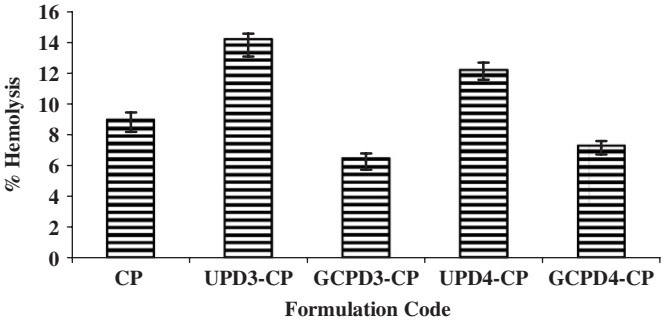


Fig. 4. Percentage hemolytic toxicity of drug and different formulations ($n=6$). Values represent mean ± S.D.

3.4. Hemolytic toxicity

Polycationic charge associated with amine groups limits the possible use of dendrimers in drug delivery [42]. Synthesis of dendrimers with biocompatible material such as lysine drastically reduces RBC hemolysis/hemolytic toxicity compared to other synthetic dendrimers [7]. It was found that uncoated dendrimeric formulations i.e. UPD3-CP and UPD4-CP, exhibited about 12.22 ± 2.4% and 14.1 ± 1.02% RBC hemolysis, respectively. Galactose conjugation further reduced RBC hemolysis to 6.5 ± 3.2% and 7.3 ± 2.8%, respectively (Fig. 4) for GCPD3-CP and GCPD4-CP. Hemolytic toxicity was found to be 2.53 times less (for UPD4-CP) and 5.2 times less (for GCPD4-CP) as compared to 35.7% RBC hemolysis of similar generation of polypropyleleimine dendrimers reported by Bhadra et al. [6]. Further reduction in the hemolytic toxicity due to coating was possibly due to hindrance of cationic charge of NH_2 groups associated with uncoated dendrimers. In case of GCPD3-CP and GCPD4-CP formulations hemolytic toxicity was even less compared to plain drug CP possibly

due to the shielding of drug in a biocompatible dendrimeric environment [6,42].

3.5. Stability studies

The stability data of galactose-coated formulation (GCPD4-CP) was evaluated at various conditions of temperature (0 °C, RT and 50 °C) after keeping both in dark (amber color glass vials) and light (colorless vials) for a period of 5 weeks. The formulation was found to be most stable in dark, at room temperature (Fig. 5), which was also in conformity with earlier reports [5,6].

The drug leakage was found to be minimum at room temperature as compared to that at 0 °C (Fig. 5), which may be due to the shrinking of the dendrimeric architecture leading to decrease in cavity enclosing drug molecules. Release of drug from the coated formulations was found to be greater at higher temperature (50 °C) as compared to room temperature. However, the possible reason behind such variable release could not explored. It was observed that the drug leakage was higher in formulations stored in light than those stored in dark, which may be attributed to structure cleavage at higher temperature and light leading to bond breakage due to higher reaction kinetics at higher temperature. From above it can be concluded that the carbohydrate-coated dendrimeric formulations are more stable in dark and at room temperature than at 0, 50 °C [6].

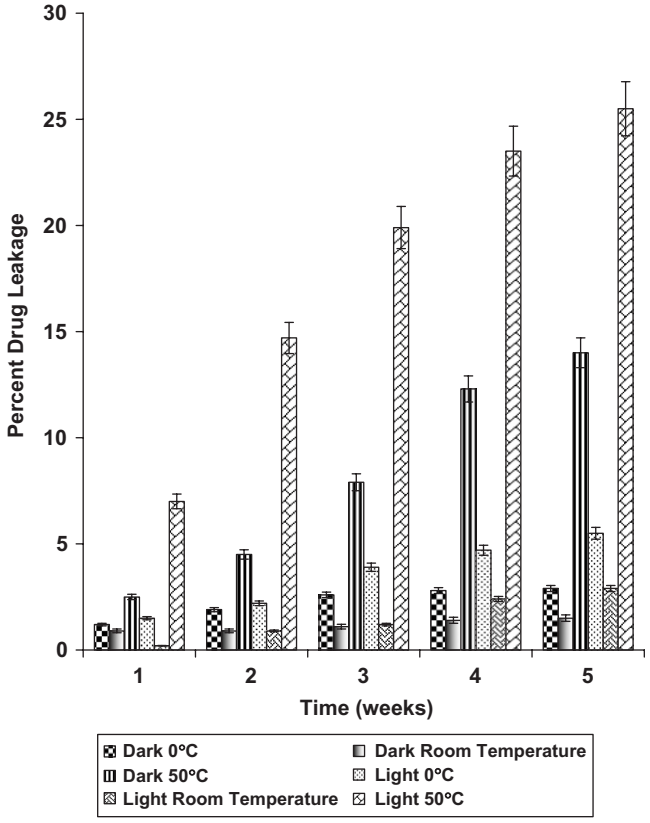


Fig. 5. Percent increase in drug leakage from GCPD4-CP formulation at accelerated conditions. Values represent mean ± S.D. ($n = 4$).

3.6. In vivo studies

The blood serum was used to determine the concentration of drug in blood samples at various time intervals. The formulations were found to be sustained release *in vivo* also. The release rate in case of uncoated formulation however, was found to be increased (Fig. 6) possibly due to open structure and metabolism by the enzymes and hydrolysis in body. The blood level of the drug was sustained in case of coated formulations than that of uncoated system due to slower release rate of CP, which was in accordance to the trend found in *in vitro* drug release studies. The initial drug level was also observed to be lower and decreasing at slower rate than that for the free drug administered intravenously.

3.7. Hematological studies

The blood parameters RBC count, WBC counts and differential lymphocytes count were evaluated (Table 4). The RBC count was found to have decreased below normal values in case of uncoated systems than that of galactose-coated formulation (GCPD4-CP). The WBC count of uncoated dendrimer–drug formulation (UPD4-CP) increased significantly ($p < 0.05$) as compared to normal values. However, for GCPD4-CP, the increase was less as compared to that of uncoated formulations and also less than the normal count in controlled group. Similarly, a relative increase in lymphocyte count was observed with dendrimer–drug complex. This was similar to the reported blood toxicity and cytotoxicity effects of acrylate nanoparticulates [43].

3.8. Macrophage uptake study

In *ex vivo* macrophagic uptake studies, many macrophagic cells were found to have increased in size and loaded with some dense foreign materials (Fig. 7). Reduced macrophage uptake with galactose-coated formulation (GCPD4-CP) was observed. Coated formulation exhibited approximately 5 times less ($p < 0.0001$) macrophage-mediated phagocytosis

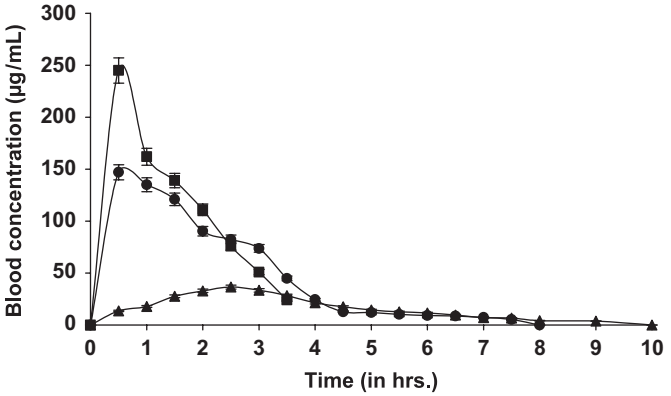


Fig. 6. *In vivo* blood level studies ($n = 6$) of plain drug (-■-), UPD4-CP (-●-), GCPD4-CP (-▲-). Values represent mean ± S.D.

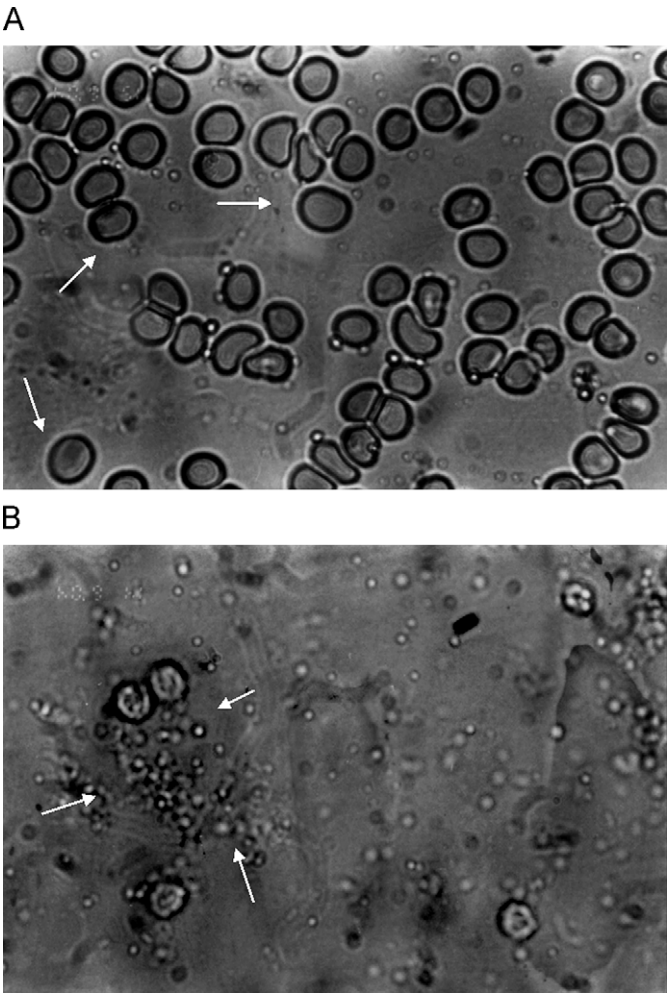


Fig. 7. Macrophage view (unstained) after treatment with GCPD4-CP (A) and UPD4-CP (B) as seen at 100× (without oil immersion).

compared to uncoated formulation (UPD4-CP) (Table 3). Macrophage cells mostly got ruptured on contact with the uncoated dendrimer formulation than that in contact with galactose-coated formulations (Fig. 7), which gives an indication of lesser immunogenicity [44,45].

4. Conclusion

Dendrimers in controlled and sustained drug delivery is still an area today be explored for its clinical applicability. However, some recent investigations have well-proved dendrimers capabilities in this area [46,47]. Approaches to synthesize a biocompatible dendrimer can further be advantageous in this regard. Present study results conclude that a dendrimer having biocompatible framework exhibits reduced toxicity and at the same time provide a sustained drug release behavior *in vitro* as well as *in vivo*. Galactose conjugation can be utilized to reduce the toxicity associated with uncoated poly-L-lysine dendrimers. Such conjugation (coating) of biocompatible framework dendrimer is even less toxic over galactose coated dendrimers of other organic compounds (e.g. PPI). The results of the present study suggests that this approach of galactose coating on peptide dendrimers can be used as safe, effective and stable sustained release system for chloroquine phosphate. The potential of this delivery vehicle as a sustained release delivery system for other drugs appears worth for further exploitation.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biomaterials.2007.04.004

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