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Research Paper

PEGylated Peptide Dendrimeric Carriers for the Delivery of Antimalarial Drug Chloroquine Phosphate

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Purpose. The present study was aimed at developing and exploring the use of uncoated and chondroitin sulfate A (CSA) coated PEGylated poly-L-lysine-based dendrimers for controlled and sustained delivery of a blood schizonticide, chloroquine phosphate (CQ).
Methods. The poly-L-lysine-based peptide dendrimers with PEG amine core prepared and coated with CSA were used to encapsulate the drug molecules by dialysis method. Effect of CSA coating on the surface characteristics, drug entrapment, drug release, stability, hemolytic toxicity, macrophageal interactions, and cytoadherence were determined and compared with those of uncoated systems.
Results. The CSA coating of the carriers was found to increase size and drug loading capacity, and reduce drug release rate and hemolytic toxicity. Transmission electron microscopic study revealed the surface properties of the systems. Stability studies had shown increased stability of the formulations on CSA coating. There was a significant reduction in hemolytic toxicity and cytotoxicity of CQ by the present dendrimeric carriers, which became more prominent on further CSA conjugation of the equivalent drug-loaded dendrimeric carriers. There were also significant reduction in levels of ring and trophozoite stages of *Plasmodium falciparum* in liquid culture when treated with CSA coated dendrimers because of the expression of similar carbohydrate receptors as that by placental and cerebral barriers for infected red blood cells. The systems were also found suitable for prolonging and controlling the blood level of drug as indicated by blood level and organ distribution studies in albino rats on intravenous administration, precluding any significant hematological or toxicological manifestations.
Conclusion. Thus it can be said that CSA coating can improve drug-loading capacity, control and sustain the release of CQ from such carriers, and can suitably act as safer and effective carriers for intravenous CQ administration.

KEY WORDS: antimalarial; chloroquine; chondroitin sulfate; dendrimers; peptide.

INTRODUCTION

Due to numerous side effects associated with prolonged conventional antimalarial chemotherapy, there is an increased necessity for administration of antimalarial drug candidates via some newer sustained release dosage forms/formulations, i.e., novel drug delivery systems. There are many newer emerging strategies and novel drug delivery systems, such as liposomes, nanoparticles, microparticles, hydrogels beads, prodrugs, etc., that have attempted to enhance antimalarial therapy, the details of which were reviewed in our past work (1). For the purpose of safe, sustained, and localized delivery of commonly used antimalarial, chloroquine phosphate (CQ) in blood plasma by intravenous injection, nanoparticulate carriers are envisaged as successful drug delivery aid. Among the various nanoparticulate carriers recently reported, dendrimers were selected in the present study as a drug delivery vehicle, because of their relatively higher chemical and biological stability, purity, efficacy, and longer shelf life.

Boas and Heegaard (2) earlier reviewed the use of various dendrimers in drug research. Fuchs *et al.* (3) had also prepared surface-modified dendrimers for potential application and described their synthesis, *in vitro* toxicity, intracellular localization, and application as drug delivery vehicles. Teertstra and Gauthier (4) reviewed dendrigraft polymers, branched macromolecules engineered on a mesoscopic scale. Multiple branching levels characterized the architecture of these molecules, in analogy to dendrimers and hyperbranched polymers. The size of dendrigraft polymers is typically 1–2 orders of magnitude larger than their dendritic counterparts, ranging from around 10 nm to a few hundred nanometers. Dendrigraft polymers exhibit many of the physical properties and characteristics of dendritic molecules on a mesoscopic scale. Interesting properties were reported and discussed for such dendrigraft polymers with some suggestions for potential applications. Yang *et al.* (5) synthesized unimolecular dendritic micelles designed as solubility enhancers obtained by coupling polyethylene glycol (PEG) to Starburst polyamidoamine (PAMAM) dendrimers. Abdullah *et al.* (6) synthesized peptide dendrimers based on a β-cyclo-

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dextrin core with guest binding ability. Beezer *et al.* (7) described the synthesis of three neutral water-soluble PAMAM dendrimer derivatives and their ability to bind small acidic hydrophobic molecules. Imae *et al.* (8) investigated binding of polyamidoamine dendrimer to sodium hyaluronate (NaHA) in aqueous 0.25 M NaCl solution by static light scattering. Sadler and Tam (9) had also reviewed peptide dendrimers, their applications, and synthesis.

In our earlier studies, we used PAMAM dendrimers for delivery of drugs such as 5-fluorouracil and indomethacin (10,11). Recently, we used the PEGylated poly-L-lysine-based dendrimers for the delivery of insoluble drug such as artemether (12) and polypropylene imine glycodendrimers for the targeted hepatic delivery of primaquine phosphate (13). In the present study we envisaged the localized delivery of another potential blood schizonticide, chloroquine, in blood plasma preferably by chondroitin sulfate A (CSA) coated and uncoated poly-L-lysine-based peptide dendrimers. We have also tested the potential toxicity of such dendrimeric candidates in rats and tried to determine the safety and efficacy of such drug delivery candidates.

MATERIALS AND METHODS

Materials

The drug, chloroquine phosphate (CQ), was a generous gift sample from Bayer Pharmaceuticals (Thane, India). Lysine, fluorenemethoxy carbonyl succinimide (FMOC-Su), polyethylene glycol (PEG-4000, 1500), *N*,*N*′-dicyclohexyl carbodiimide (DCC), cellophane dialysis membrane bag of 2.4 nm, RPMI-1640, and fetal bovine serum were purchased from Himedia Laboratories Ltd. (Mumbai, India). 1-Hydoxybenzotriazole (HOBT), dimethylamino pyridine (DMAP), sodium azide, 10% Pd catalyst adsorbed on charcoal, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and 1-(3-dimethyl aminopropyl) 3-ethyl carbodiimide hydrochloride (EDCl) were procured from Spectrochem (Mumbai, India). Chondroitin sulfate A (CSA, sodium salt, from bovine trachea), was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Formulation of Dendrimeric Carriers

CSA coated and uncoated PEG-lysine dendrimers were synthesized using PEG amine as core. Protected di-FMOC-lysine was used for progressive linking on side amino groups of prior generations consecutively by liquid-phase peptide synthesis, as previously described (14), by dicyclohexyl carbodiimide (DCC) coupling strategy. Equilibrium dialysis method was used for loading of drug in uncoated and coated PEG-lysine dendritic carriers. The aqueous dendritic systems of various generations (10 mL of 1% w/v solution) were dialyzed by placing it in cellulose tubing (Himedia Laboratories; pore size, 2.4 nm) in CQ solution (10 mg mL^{-1}) and incubated for 24 h. The final dendrimer solution in dialysis bag was lyophilized. The amount of drug remaining in outer dialysis medium was estimated and used for indirect estimation of amount of drug loaded in the dendrimers by complexation or binding (15).

Characterization of Formulations

Transmission electron microscopic (TEM) studies were carried out using 3 mm Forman (0.5% plastic powder in amyl acetate) coated copper grid (300 mesh) at 60 kV using negative staining by 4% phosphotungstic acid (PTA) for whole generations of dendrimers, at various magnifications on Philips CM-10 TEM (at 50–60 kV). The unentrapped drug found in the dialysis medium on dialysis after 24 h with coated and uncoated dendrimers of whole generations was used to indirectly estimate the amount of drug entrapped during loading of CQ (soluble drug) by adsorption, binding, or complexation. The known amount of drug was taken for dialysis, and the amount of drug remaining unentrapped in the medium was used for estimation of drug loaded within the dendrimers. The value of drug so loaded was converted to molar terms to estimate molar amount of drug loading for a particular system (Table I).

The drug release studies were carried out by dialysis through cellophane tubes (Himedia Laboratories; pore size, 2.4 nm). Lyophilized drug–dendrimer complex (100 mg) was dissolved in double-distilled water and transferred to cellophane tubes. This dialysis bag was immersed in the aqueous medium (20 mL) under magnetic stirring. Samples were withdrawn from it at 1-h intervals up to 8 h, then at 24 h, and thereafter at 24-h intervals. The amount of drug dialyzed out of the complex/gels was used to determine release pattern of the systems.

Stability Study

The coated and uncoated dendrimer-drug formulations (5.0 G) were kept in tightly closed vials. These formulations were kept in colorless vials (type I) in the refrigerator at 2–8°C, room temperature (25°C), and 50°C (controlled oven)

Table I. Significant Physicochemical Properties of the Synthesized Dendrimer Formulations

Formulation code	Generation (G)	Core taken	Comments	Theoretical molecular weight	No of $-NH_2$ terminal by Kaiser test	Entrapment efficiency (% w/w)	Entrapment efficiency (m/m)
PL15K4G	4.0	PEG 1500D	Uncoated	5,880	33.2 ± 1.9	0.263 ± 0.09	3.0 ± 1.03
PL15K5G	5.0	PEG 500D	Uncoated	10,552	65 ± 1.8	0.450 ± 0.07	9.3 ± 1.43
PL4K4G	4.0	PEG 4000D	Uncoated	8,380	37.5 ± 4.5	0.308 ± 0.06	5.0 ± 0.97
PL4K5G	5.0	PEG 4000D	Uncoated	13,052	66.9 ± 5.7	0.671 ± 0.011	16.9 ± 0.28
PL4K4GCS	4.0	PL4K4G	CSA-coated	–	6.8 ± 1.2	0.597 ± 0.08	9.7 ± 1.3
PL4K5GCS	5.0	PL4K5G	CSA-coated	–	11.5 ± 1.5	1.087 ± 0.09	27.5 ± 2.3

for a period of 5 weeks. Any physical change in the samples was analyzed initially and periodically after every week for up to 5 weeks. The samples were observed for any precipitation, turbidity, crystallization, and change in color, consistency, and drug leakage. Data obtained were used for the determination of the conditions and precautions required for storage. The samples were initially colorless solutions.

Change in the chemical nature of the preparation was ascertained by comparison of intensity of color developed in 1-mL formulation by Kaiser test, spectrophotometrically at 570 nm (12,14,16). The percentage change in the intensity of the color produced was used for the determination of free amino groups available at the periphery of coated dendrimeric formulation, complexed with the drug. The data were recorded and analyzed accordingly for any instability of internal bonds of dendrimers and bonds with CSA. Effect of storage condition on drug leakage was determined by checking the increase in release of drug from the formulations after storage at accelerated conditions. The formulation samples (2 mL) were dialyzed across cellulose tubing. The external medium (10 mL) was analyzed spectrophotometrically for drug content. The procedure was repeated every week for up to 5 weeks. The percentage increase in drug release from the formulation was used to analyze the effects of accelerated conditions of storage on drug leakage from the formulation.

Hemolytic Toxicity of Drug–Dendrimer Formulations

Red blood cell (RBC) suspension from human blood was used for hemolytic studies (10,12). To 1 mL RBC suspension, taken in centrifuge tube of 5 mL, distilled water was added, which was considered to produce 100% hemolysis. Similarly, 5 mL normal saline was added to 1 mL RBC suspension in another tube producing no hemolysis, hence acting as blank. A 0.5-mL aqueous solution of drug, dendrimers, and drug-loaded dendrimer formulations was placed in separate tubes and mixed with 4.5 mL normal saline and 1 mL RBC suspension. The drug and dendrimers were taken in separate tubes in such amounts that the resulting final concentration of drug (1 mg mL^{-1}) and dendrimer (5 mg mL^{-1}) of each generation were equivalent in all cases. The tubes were allowed to stand for half an hour with gentle, intermittent shaking.

Similar procedure was used for CSA coated drug–dendrimer complexes. The CSA coated carriers of drug were taken in such amounts that the resultant final concentration of drug and dendrimers of each generation were equivalent to that of uncoated systems. This allows the comparison of hemolysis data of the drug, dendrimers, drug–dendrimer formulations, and CSA coated systems to help us understand the effect of drug entrapment and coating on hemolytic toxicity.

The tubes were centrifuged for 15 min at 4,000 rpm. The supernatants were taken and diluted with an equal volume of normal saline, and absorbance was taken at λ_{max} (540 nm), against supernatant of blood sample similarly diluted with normal saline, as blank. Percent hemolysis was calculated for each sample by taking absorbance of blood diluted with water as 100% hemolytic sample. The values so calculated were compared.

Macrophage Interaction Study

For this study, macrophages were collected from the peritoneal cavity of C3H mice via the methods that we have previously described (12). Washed cells were resuspended in fresh supplemented RPMI-1640 culture medium (penicillin, 10 units mL^{-1}; 10% fetal bovine serum; 100 μg mL^{-1} streptomycin; 1 mM sodium pyruvate; 10 mM HEPES). Cells were diluted to 5×10^5 cells mL^{-1} after the number of viable cells was calculated by staining (17). Cells were then transferred to 96-well microtiter plates and incubated at 37 ± 1°C for 2 h using candle jar method (in 5% CO_2), allowing the cells to adhere onto wells. The supernatant was drained off.

To multiple wells, various coated and uncoated dendrimeric formulations (equivalent to 100 μL of 200 μg mL^{-1}), with and without drug (equivalent to 20 μg mL^{-1}), were added, and the volume was made up with supplemented RPMI-1640 medium. One series of wells with supplemented medium only was taken as blank. The formulations were incubated for 24 h for toxicity as well as drug uptake studies. The supernatant was drained off partially, and to every well 10 μL of 5 mg mL^{-1} dendrimer formulations per 100 μL medium was added and allowed to incubate at 37 ± 1°C for 4 h. To every well, 100 μL acid–DMSO (0.04 N HCl in DMSO) was added to dissolve dark blue crystals and allowed to stay for half an hour at room temperature (25°C). A portion (150 μL) of the supernatant was taken out from every well in 2-mL Eppendorff tubes, and volumes were made up with acid–DMSO and absorbance was taken at 570 nm (18). The percentage of viable cells was determined by taking absorbance of untreated cells as 100%. IC_{50} values were also determined taking various concentrations of dendrimers of coated and uncoated types in each well. Macrophage uptake of drug was also determined after lysis by trypsin for the amount of drug using HPLC method as discussed in *in vivo* studies. IC_{50} values (concentration of formulations producing 50% cell death), reduction in macrophageal toxicity as compared to blank, and macrophageal drug uptake of the formulations were compared.

CSA Cytoadherence Study

This study was performed to determine the targeting propensity of CSA coated PEG-lysine dendritic formulations. Here, a well-established and reported protocol for *Plasmodium falciparum* culture was used. The parasitemia of NF54 strain was raised to 6–8% in 8% hematocrit in supplemented RPMI-1640 medium (with RBCs and serum). Cultures were synchronized using 5% sorbitol (19).

Two milliliters of final cultures was transferred to tissue culture Petri dish and incubated with 100 μL of 200 μg mL^{-1} of dendrimeric formulations, CSA coated and uncoated types without drug and simple medium (control). These were incubated at 37 ± 1°C for 24 h using the candle jar method (20). The samples were withdrawn from the Petri dishes, and parasitemia and degree of freely available rings and trophozoites were determined in each dish and compared with standard blank medium of untreated cells after 24 h. The plates were allowed to incubate for additional 2–3 days and evaluated for freed schizonts on rupture of parasitized

Table II. *Ex Vivo* Interaction Profile of Different Formulations of Chloroquine Phosphate

S. no.	Dendrimer formulations	% Reduction of hemolysis ($n = 3$)[a]	Macrophageal IC_{50} (μM; $n = 3$)	% Reduction of circulating infected RBCS ($n = 3$)	% Drug uptake by macrophages ($n = 3$)	% Inhibition of cytotoxicity[b] ($n = 3$)
1	PL15K4G	4.5 ± 0.21	97.5 ± 14.2	11.0 ± 1.2	52.31 ± 2.54	2.3 ± 0.56
2	PL15K5G	7.4 ± 0.64	39.7 ± 11.2	15.8 ± 1.7	64.22 ± 3.12	4.6 ± 0.71
3	PL4K4G	10.5 ± 0.56	135.7 ± 9.2	18.5 ± 1.4	57.43 ± 2.31	9.8 ± 0.54
4	PL4K5G	12.4 ± 0.52	80.5 ± 11.4	24.6 ± 1.9	64.24 ± 2.12	13.4 ± 1.2
5	PL4K4GCS	28.4 ± 1.7	Above 3 mM	50.7 ± 2.1	15.12 ± 2.13	40.2 ± 1.2
6	PL4K5GCS	34.1 ± 1.8	Above 2 mM	67.2 ± 2.3	19.85 ± 2.35	48.2 ± 1.3

[a] Reduction of hemolysis produced by the chloroquine in formulations as compared to equivalent amount of free chloroquine.
[b] Reduction of macrophageal cytotoxicity produced by the chloroquine in formulations as compared to equivalent amount of free chloroquine.

RBCs upon maturity. The final medium was treated with equal volume of 15% saponin solution for 20 min (20) with intermittent shaking to free the malarial parasites from RBCs after lysis and concentrated by centrifugation at 10,000 rpm. These were freshly cultured in fresh complete RPMI-1640 medium (5–6% hematocrit) from each well and observed for parasitemia on incubation for few days at 37 ± 1°C for determination of viability of malarial schizont stages so liberated (Table II).

Blood Level Study

Healthy albino rats (Sprague–Dawley strain) of either sex (100 ± 20 g) with no prior drug treatment were used for the present *in vivo* studies. The rats were maintained on standard diet and water under controlled temperature, humidity, and light cycling conditions. The protocol was duly approved by the Institutional Animal Ethics Committee of the University (registration no. 379/01/ab/CPCSEA, India).

CSA coated and uncoated 5.0 G PEG-lysine (4000D) dendrimers were used to deliver CQ intravenously. Blood levels of the drugs were determined at various time intervals against blank of pure PEG-lysine dendrimers without drug. Albino rats were divided into groups composed of three rats each.

One group of rats was kept as control. Aqueous solution of CQ (equivalent to 1,000 μg drug) were injected intramuscularly in thighs of separate group of rats. CSA coated and uncoated dendrimers of 5.0 G of CQ having equivalent drug content were dissolved in triple-distilled HPLC-grade water and administered i.v. to the remaining groups.

From each rat, 100-μL blood samples were withdrawn every 15 min for up to 2 h, then half hourly up to 7 h, followed by sampling at hourly intervals. All blood samples were obtained via tail prick method using 50-μL graduated capillaries (Himedia Laboratories). Blood samples were clotted and washed by vortexing with normal saline, and the washings were centrifuged at 2,000 rpm for 15 min. Serum was deproteinized by acetonitrile (1 mL mL^{-1} of serum). Samples were centrifuged, then supernatants were filtered by 0.45-μm membrane filter and analyzed for drug contents by HPLC for respective drugs using Luna C_{18} 5 μ (250 × 4.6 mm) column of Phenomenex (USA) by photo diode array detector (SPD-M10A; Shimadzu, Tokyo, Japan) at various wavelengths and ambient temperature.

The treated serum samples were injected into HPLC sample injector. This was eluted by pumping (LC10 AT) methanol–sodium dihydrogen phosphate monohydrate (58 mM) mobile phase containing 6 mM heptanesulfonic acid (Spectrochem) and estimated at 256 nm at a retention time of 13 min (Table III). The blood level of drug so obtained was analyzed by noncompartmental statistical moment theory to determine the area under curve (AUC), area under first moment curve (AUMC), and mean residence time (MRT), etc.

Biodistribution Study

Albino rats (100 ± 20 g) were used to study the biodistribution of CQ. Rats were divided into groups of three and given plain drug solutions; drug-loaded CSA coated dendrimer formulation and uncoated drug–dendrimer complex

Table III. Changes in Various *in Vivo* Parameters on Administration of Different CQ-Loaded Uncoated and CSA-Coated Dendrimeric Formulations

Formulation	AUC (μg h mL^{-1})	AUMC (μg h^2 mL^{-1})	MRT (h)	C_{max} (μg mL^{-1})	T_{max} (h)	% Changes in various values of organ function test compared to standard untreated control (mean ± SD; $n = 3$)		
						ALT/SGPT	BUN	Bilirubin
Drug (CQ)	74.72	121.89	1.63	50.23	0.5	52.5 ± 2.12	35.3 ± 1.52	45.2 ± 2.25
Drug–dendrimer (uncoated)	187.75	1,110.59	5.91	19.85	4	25.5 ± 1.25	20.5 ± 1.03	25.7 ± 1.25
CSA coated drug–dendrimer formulation	120.58	777.74	6.47	13.85	6	10.5 ± 0.85	8.2 ± 0.42	10.4 ± 0.52

Table IV. Hematological Parameters of Drug–Dendrimer Formulations in Rats After 7 Days of Continuous Administration ($n = 3$)

Formulation	Hemoglobin count (g 100 mL)$^{-1}$	RBC count $\times 10^6/\mu L$	WBC count $\times 10^3/\mu L$	Differential count ($\times 10^3/\mu L$)		
				Monocytes	Lymphocytes	Neutrophils
Control	13.55 ± 0.51	9.21 ± 0.22	14.82 ± 0.54	1.15 ± 0.18	11.63 ± 0.19	2.82 ± 0.41
Drug	12.84 ± 0.32	8.74 ± 0.23	14.95 ± 0.61	1.28 ± 0.07	10.82 ± 0.14	3.121 ± 0.51
Drug–dendrimer (uncoated)	12.98 ± 0.21	8.9 ± 0.23	14.81 ± 0.84	1.21 ± 0.21	11.12 ± 0.17	2.98 ± 0.23
CSA-coated drug–dendrimer formulation	13.45 ± 0.31	9.15 ± 0.34	14.78 ± 0.59	1.17 ± 0.19	11.24 ± 0.21	2.97 ± 0.12

were administered intramuscularly, whereas one group of rats served as control. After 2 h animals from each group were sacrificed and the organs (liver and spleen) were excised and homogenized in PBS. The homogenates were deproteinized with acetonitrile, then centrifuged, filtered, and estimated for drug content using HPLC as described above. Finally, we compared blood levels in the samples.

Hematological and Toxicological Study

Albino rats of uniform weight, size, and sex were divided into four groups ($n = 3$ per group). The first group was administered (i.m.) simple plain drug. The other groups were given uncoated and CSA coated drug–dendrimer complex (5.0 G) containing 1,000 μg of CQ, everyday. One group, which served as control, was maintained on regular diet for 7 days in standard living conditions. After 7 days, blood samples were obtained and analyzed for hemoglobin (hb) level, RBC, WBC, differential monocytes, lymphocytes, and neutrophils in pathology laboratory, to study the changes in hematological parameters on chronic dosing (Table IV). Blood urea nitrogen (BUN) and serum glutamate pyruvate transaminase (SGPT/ALT) were estimated for the determination of systemic toxicity.

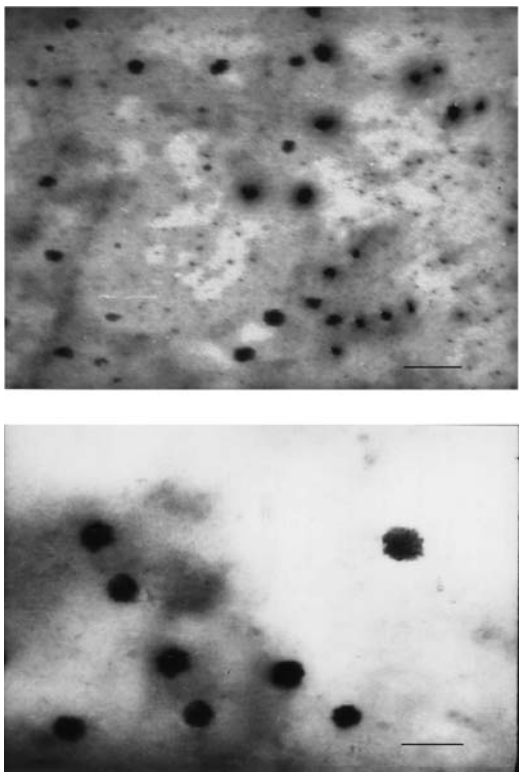

Fig. 1. TEM of (a) uncoated and (b) CSA coated 4.0 G dendrimers.

RESULTS AND DISCUSSION

Formulation of Dendrimeric Carriers

The synthesis and physicochemical characterization of unloaded carriers for the delivery of artemether were reported in our previous study (12). The CQ-loaded carriers were analyzed for effects of CSA coating on shape, size, drug loading, and release of drug from dendrimers. Kaiser test was used to determine the level of suppression of amine groups of dendrimers by CSA conjugation. This test showed that CSA coated (1:1) 4.0 G dendrimers of PEG4K type (PL4K4GCS) contain an average of 6.8 ± 1.2 terminal amines, whereas coated (1:1) 5.0 G dendrimers (PL4K5GCS) contain 11.5 ± 1.5 of terminal amines, similar to that reported in our previous work (12).

The particles are not ordinarily viewable as they are molecular particles in nanometric ranges. Such nanoparticulate carriers are more easily and suitably focused by TEM. The particle surfaces of uncoated dendrimers were found to be rather smooth and uniform compared to CSA coated systems as evidenced by TEM studies at 100,000× for coated formulations (Fig. 1). All formulations were in nanometric size range (as evident by scale shown by Soft Imaging System version 3.1), and were of uniform spherical shape. On CSA coating, there was a significant increase in particle size.

The dendrimers were focused by 4% PTA staining, which could stain the coated and uncoated nanoparticulate carriers. Sizes of the systems were calculated by the degree of magnification, scale, and size of the particles. Particle size of the carriers was found to be in the range of 10–30 nm. The size increased with increase in generations from 3.0 to 5.0 G (Fig. 1) from around less than 10 to 40 nm. The CSA has a carbohydrate of molecular weight more than three times that of the present dendritic carriers, thus it could increase the particle size on coating significantly. We confirmed this fact by coating the dendrimers with CSA. On CSA coating the size was found to have increased significantly, with generations, up to 100 nm for 5.0 G.

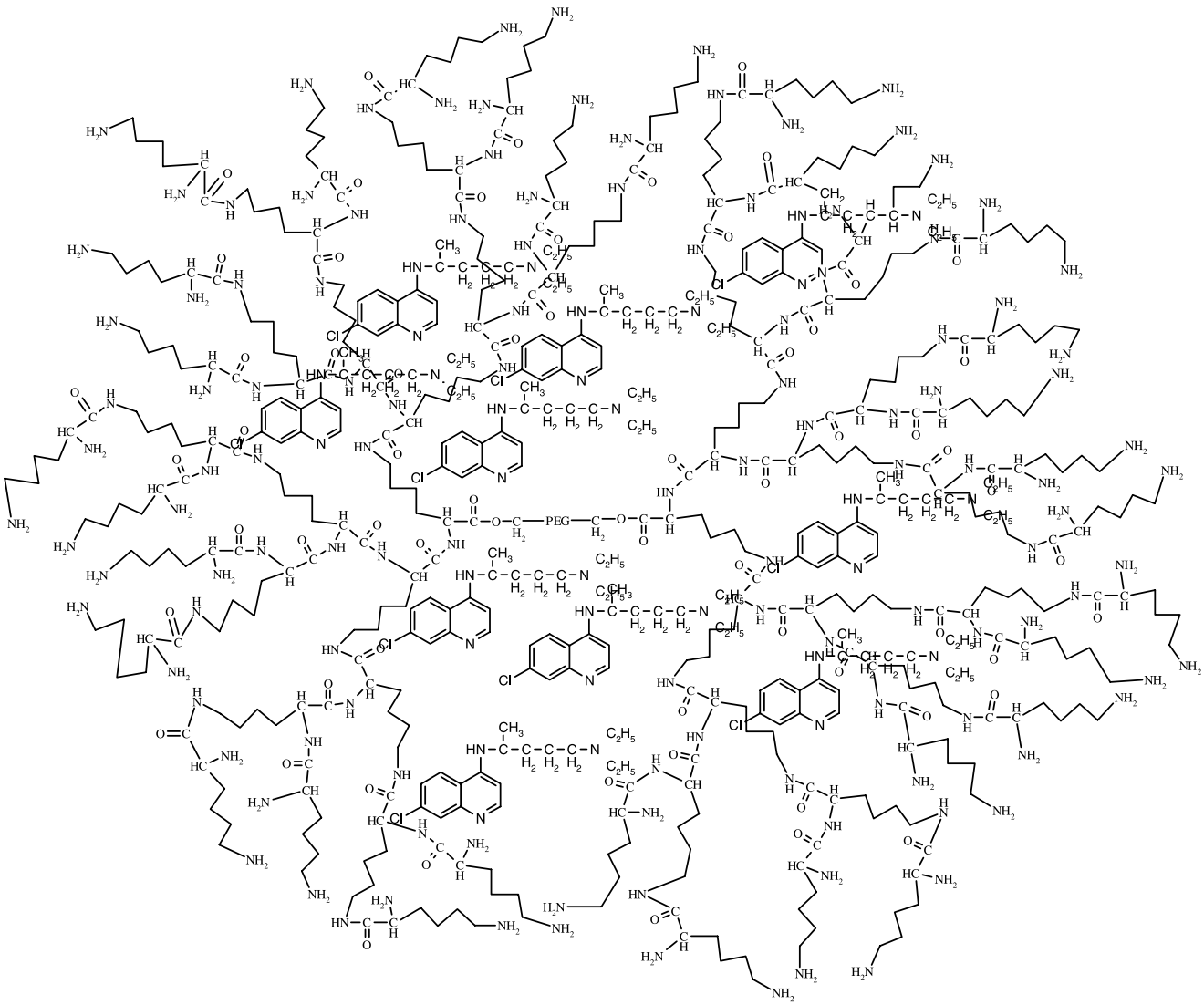

Fig. 2. Pictorial representation of chloroquine phosphate loaded in a molecule of PEG-lysine 5.0 G dendrimer.

Drug Entrapment and Release Profile

The entrapment of CQ increased for PEG 4000D as compared to that of PEG from 1500, increase in generations of dendrimers (from 4.0 to 5.0 G), and CSA coating on dendrimers (Table I). There was a significant increase in molar drug loading with increase in molecular weight of PEG. The loading increased from around 3 to 5 molecules for 4.0 G and from 9 to 17 molecules per molecule of dendrimers for 5.0 G. This may be attributed to an increase in molecular spacing for drug encapsulation and increase in ethereal groups for drug complexation. The increase in molecular weight of PEGs also attributes fluidity to structure so that it can spread and load an increasing number of drug molecules. With CSA coating present in the coat, there was a significant increase in drug loading of dendrimers as a result of the increase in sealing of periphery of the dendrimers, so that it could hold an increasing number of drug molecules by hydrotropic interaction, complexation, and steric hindrances. The amount of drug molecules loaded in dendrimeric nanoparticles had doubled on CSA coating from 5 to 17 molecules and from 9 to 27 molecules of CQ per molecule for 4.0 and 5.0 G dendrimers, respectively. CSA coating makes the dendrimers a well-closed container for higher amounts of drug loading as evident from structural (Fig. 2) and RasMol molecular interpretations of 4.0 G (Fig. 3).

The release pattern of CQ from dendrimers of various generations suggested that the dendrimers acted as prolonged release carriers. The release of drug from dendrimers was initially of first order, and a linear plot was found in the first 8 h. Release rate was prolonged with increase in molecular weights of PEG in the structure. This was significantly high for higher molecular weights of PEG, i.e., 4000D, as compared to 1500D. The release rate in the initial phase as evident from the slope of the curve was 23.50% per hour for lower molecular weight PEG (1500D) as compared to 12.76% h^{-1} for PEG 4000D. This showed that the release rate had nearly halved with increase in molecular weights of PEGs. This may be due to the increase in steric hindrance, increased availability of groups for complexation, and increased hydrotropic solubilizing capacity of dendrimers for the drug molecules that precludes the drug from coming

Table V. Release Profile of CQ from Various Drug Dendrimeric Formulations

Time (h)	Cumulative % drug release (mean ± SD) by various formulations (n = 3)					
	PL15K4G	PL15K5G	PL4K4G	PL4K5G	PL4K4GCS	PL4K5GCS
1	28.8 ± 0.26	25.6 ± 0.24	22.3 ± 0.21	18.8 ± 0.12	16.6 ± 0.15	14.8 ± 0.21
2	52.3 ± 0.55	45.6 ± 0.43	39.5 ± 0.42	33.5 ± 0.32	31.2 ± 0.31	28.9 ± 0.34
3	78.2 ± 0.80	67.8 ± 0.64	54.2 ± 0.45	47.5 ± 0.45	43.1 ± 0.43	39.7 ± 0.42
4	98.5 ± 1.01	85.2 ± 0.82	68.5 ± 0.75	58.5 ± 0.54	54.2 ± 0.54	49.7 ± 0.51
5	–	98.7 ± 1.1	80.5 ± 0.75	67.2 ± 0.65	64.7 ± 0.64	57.8 ± 0.61
6	–	–	90.5 ± 1.02	75.8 ± 0.72	72.3 ± 0.72	66.5 ± 0.65
7	–	–	98.6 ± 1.2	83.5 ± 0.82	77.4 ± 0.78	69.8 ± 0.72
8	–	–	–	87.5 ± 0.85	81.5 ± 0.80	74.5 ± 0.78
24	–	–	–	99.2 ± 0.98	90.3 ± 0.94	83.5 ± 0.83
48	–	–	–	–	99.2 ± 1.04	92.5 ± 0.95
72	–	–	–	–	–	99.4 ± 1.08

out in the release medium. This is as also evident from the initial burst release of the drug from dendrimers, which got significantly reduced from 28.8 ± 0.26 and 25.6 ± 0.24% for PL15K4G and PL15K5G to 22.3 ± 0.21 and 18.8 ± 0.12% for PL4K4G and PL4K5G, respectively (Table V).

There was also a significant increase in time for complete drug release with increase in generations of PEG1500D dendrimers. Similar trends were found for increase in generation of dendrimers of PEG4000. There was a significant decrease in release rate and initial burst release from dendrimers. With increased generations of PEG-poly-L-lysine dendrimers, there was again similar increase in groups for complexation and binding, causing increased steric hindrance and consequent reduction in drug leakage.

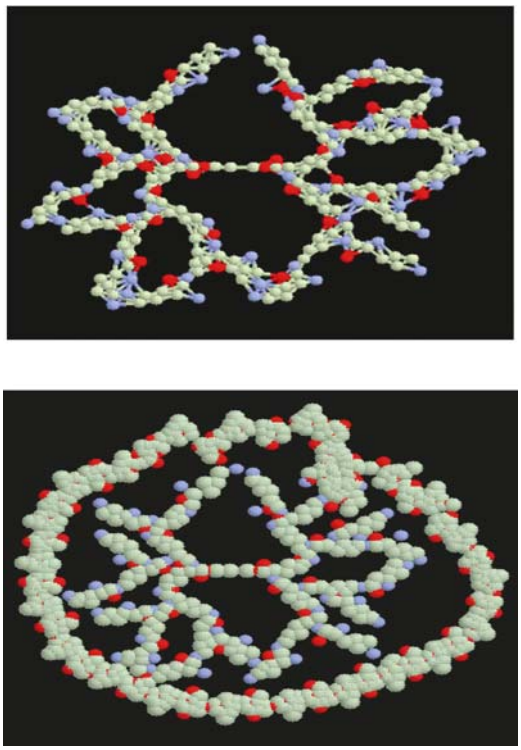

Fig. 3. RasMol representations of (a) uncoated and (b) CSA coated 4.0 G dendrimers.

With CSA coating on the dendrimers, there was further sealing of dendrimeric periphery and increased steric hindrance and viscosity causing reduction in drug leakage. In CSA coated dendritic nanoparticulate carriers, there was a significant reduction in initial drug leakage from 22.3 ± 0.21 and 18.8 ± 0.12% for PL4K4G and PL4K5G to 16.6 ± 0.15 and 14.8 ± 0.21% for PL4K4GCS and PL4K5GCS, respectively. A similar trend was observed for the release rate, which decreased from 12.76 and 9.82% per hour for PL4K4G and PL4K5G to 9.33 and 8.46% per hour for PL4K4GCS and PL4K5GCS, respectively (Table V). This also showed generation-dependent effects, as with increased generations of dendrimers there was increased CSA conjugation, which increased sealing of periphery and compactness of structure (Fig. 3).

Stability

CQ-loaded uncoated and CSA coated PL4K5G dendrimers were selected for study. The formulations were stored in a refrigerator (at 2–8°C), at room temperature (RT = 25°C), and at 50°C, and monitored for any turbidity, precipitation, and crystallization of drug up to 5 weeks. Turbidity, precipitation, and crystallization occurred due to changes in solubilities and properties of drug and systems on storage.

CSA coated PL4K5GCS was more stable at refrigerated conditions and at room temperatures. However, at room temperature there was only slight development of turbidity in CSA coated carriers due to CSA in coating. The CSA coated carriers do not degrade physically, hence there was also no crystallization in formulations at RT, whereas considerable crystallization occurred at 50°C. CSA coated carriers and dendrimers were more stable at 2–8°C. Coating imparts additional stability to drug carriers even at higher temperatures as compared to uncoated carriers.

The change in chemical nature was determined by Kaiser test. The % increase in the color intensity was determined and compared with the initial color developed. These results also suggest that CSA coating imparted additional physicochemical stability to the carriers. Furthermore, CSA coating of the carriers reduced the percentage increase in drug leakage as compared to the original release

Table VI. Accelerated Stability Study of Uncoated and CSA-Coated PEG-Lysine (PL4K5G) Dendritic Carriers of CQ

			CSA-coated (PL4K5GCS)			Uncoated PL4K5G		
S. no.	Parameters		Fridge	RT	50°C	Fridge	RT	50°C
1	Turbidity (after 5 weeks)		−	+	++	−	+	+++
2	Precipitation (after 5 weeks)		−	+	++	−	+	+++
3	Crystallization (after 5 weeks)		−	−	+	−	+	++
4	Change in chemical nature (% increase in developed color intensity)	1 week	0.9%	0.5%	4.7%	2.9%	1.6%	10.8%
		2 weeks	1.8%	0.7%	5.8%	3.7%	1.8%	15.2%
		3 weeks	2.2%	0.9%	6.9%	4.4%	2.9%	20.5%
		4 weeks	2.8%	1.2%	8.5%	5.2%	3.1%	25.2%
		5 weeks	3.2%	1.5%	9.8%	6.8%	3.8%	30.5%
5	% increase in drug leakage	1 week	1.1%	0.5%	6.3%	1.1%	0.8%	11.2%
		2 weeks	1.6%	0.8%	6.9%	1.8%	1.2%	14.5%
		3 weeks	1.8%	0.9%	7.7%	2.4%	1.5%	17.7%
		4 weeks	1.9%	1.2%	8.2%	2.8%	1.9%	23.5%
		5 weeks	2.1%	1.4%	8.8%	3.2%	2.5%	26.9%

−: No change; +: smaller change; ++: considerable change; +++: enough change as compared to initial.

rate of the coated as well as uncoated carriers even at higher temperatures. This may be attributable to the presence of bulky CSA coating that further stabilizes the dendrimers against any degradation (Table VI).

Hemolytic Toxicity

Antimalarial bioactives have significant hemolytic toxicity because of their multiple cationic charges in the drug molecules (21). Similar results were obtained for the present dendrimeric carriers. There was a significant decrease in hemolytic toxicity of CQ by 4.5 ± 0.21, 7.4 ± 0.64, 10.5 ± 0.56, and 12.4 ± 0.52% for PL15K4G, PL15K5G, PL4K4G, and PL4K5G, respectively. On CSA coating, there was further reduction in the hemolytic toxicity of the drug—28.4 ± 1.7 and 34.1 ± 1.8% for PL4K4GCS and PL4K5GCS for CQ, respectively.

The reduction in toxicity may be attributed to the masking of free groups in drugs and charges in drug molecules on complexation and encapsulation. The masking or reduction in hemolytic toxicity was further enhanced upon CSA coating of the carriers as CSA conjugation further masks the residual polycationic nature of the dendrimeric carriers. Reduction in toxicity was again generation-dependent, causing additional encapsulation of more drug molecules and thus, reducing hemolytic toxicity of the known amount of drug samples in dosage form. Similar results were found in the case of 5-fluorouracil in PAMAM dendrimers (10).

However, some initial alarming reports on the residual hemolytic toxicity of polycationic dendrimers (22,23) prompted us to test the effects of CSA conjugation on hemolytic toxicity of all drug carriers under study. We performed this test so as to make the systems safer for drug delivery, in addition to increased drug loading and reduction of drug leakage via the steric hindrance and charge neutralization of free amines on the dendrimers. The charges in lysine of the carriers are more significant as compared to free amino acids because of the absence of the zwitterionic structure of lysine in dendrimers, as a result of the nonavailability of free carboxylic acid groups in polylysine structures.

Hemolytic toxicity due to same amount of dendrimeric formulations was significantly reduced up to less than 5% (Table II). CSA conjugation leads to masking of all polycationic terminal amines on the final dendrimers. CSA conjugation can thus significantly reduce the cellular interactions of dendrimers. RBCs are anionic in nature, and reduction in cationic charges and steric hindrance produced by CSA can be credited as the cause of a significant decrease in hemolytic toxicity. This is comparable and similar to the effects produced by surface modifications in PAMAM dendrimers and similar cationic carriers in earlier studies (10,24).

Macrophage Interaction

Results of the preliminary hemolytic toxicity study prompted us to undertake a macrophage interaction study that could be used to determine the efficacy of coating and IC_{50} values of CSA coated and uncoated dendrimers. This can further reveal the cytotoxicity and cellular uptake of dendrimers. The higher the IC_{50} values of the carriers, the greater the amount of carriers that can be safely used for drug administration.

The cytotoxicity of CQ was reduced by dendrimeric carriers under study, and was significantly decreased upon further CSA conjugation of the equivalent drug-loaded dendrimeric carriers. CSA coating helped in reducing the cytotoxicity of dendrimers on mice peritoneal macrophages by more than five times for an equivalent amount of dendrimers. IC_{50} values on CSA coated drug-loaded dendrimers exceeded 1 mM of resultant dendrimers as compared to much lower values for uncoated dendrimers. These results were found to be in agreement with the studies of Fischer *et al.* (23). CSA coating of the systems is thus assumed to decrease the interaction of cationic dendrimers with the cells, which, in turn, could increase the safety and efficacy of such dendrimers and could make the system a prolonged release drug delivery carrier via reduction in its cellular phagocytosis as well as any toxic manifestation.

Reduction in drug uptake by macrophages on incubation with coated and uncoated drug–dendrimers complex is the

supporting evidence for possible reduction of drug dumping on administration, due to phagocytosis or cellular interactions of dendrimers. CSA conjugation significantly reduced drug uptake by macrophages. Drug uptake by uncoated drug–dendrimers complex was significant in 2 h and comparable to incubation with plain drug solution, indicating dose dumping from dendrimers. However, on CSA conjugation CQ uptake can be significantly reduced, which was least up to 24 h, more or less equivalent to the amount of drug leached from the systems, which further supports the stabilizing effects of drug molecules by CSA-conjugated dendritic carriers on interaction with cellular constituents.

These carriers of various generations (PEG series) of dendrimers were found to be safer for delivery of antimalarials at higher doses (as evidenced by its IC_{50} values). This could also be attributed to the presence of PEG at the terminal interaction sites for interactions with drug molecules. Also, there was a significant reduction in drug uptake by macrophages on interaction because of the presence of PEG chains, which made such systems stealth carriers. Percentage drug uptake was also dependent on the ratio of PEG to hydrophobic interiors, which decreased at higher generations, hence increasing the drug uptake significantly.

Cytoadherence

Hyaluronic acid (HA) and closely related glycosaminoglycan, chondroitin sulfate A (CSA), in the extracellular matrix of placenta has been described as new receptors for parasitized RBCs (PRBCs) (25). These are responsible for placental or maternal malaria among primigravidae or women carrying their first pregnancy in endemic areas (26). CSA conjugation on dendrimeric nanoparticulate carriers without drug was carried out to sequester the circulating ring stages of infected RBCs on incubation with formulations devoid of drug to preclude the effects of CQ.

The study showed that CSA conjugation can express similar carbohydrate receptors as those by placental and cerebral barriers, for infected RBCs. In the case of uncoated dendritic carriers, there were significant levels of ring and trophozoite stages in liquid culture. Therefore, in the case of treatment by CSA-conjugated systems, schizont stages reappear from the degrading RBCs on incubation, but ring stages do not appear. Moreover, incubation of the excess medium leads to increase in parasitemia in both coated and uncoated carriers, indicating lack of any antimalarial action of dendrimers. This proved that the disappearance of schizont stages from the medium was attributable to adherence with circulating carriers, hence showing no visible schizont stages.

The results are in agreement with the established fact that in infected individuals there are changes in the antigenic and adhesive properties of PRBCs during maturation, eventually interfering normal erythrocyte circulation. Infected cells attach to the endothelial cells of small vessels (sequestration) from late ring stage until schizonts rupture (27). Saponin lysis of infected RBCs produced a number of free schizonts in both formulations, which has a similar reinfection possibility. Surface morphology of infected erythrocytes also changes dramatically from about 10 to 30 h postinvasion. The PRBCs acquire pleomorphic, irregular shape with small numbers of knoblike protrusions instead of characteristic smooth biconcave disks of RBCs. Absence of any such cell in media incubated with CSA conjugated dendritic formulations is definitely the evidence of sequestration with CSA coat.

Thus, the CSA coated carriers were found to be more suitable than uncoated carriers for safer drug delivery —devoid of any significant hemolytic toxicity, toxic cellular interaction, and, at the same time, targetable to PRBCs—and can effectively reduce such parasitized RBCs. The present carriers were therefore taken up for further *in vivo* studies.

Blood Level

The formulations were finally tested for their *in vivo* efficacy for drug delivery by various animal studies. For the present study, only uncoated and coated 5.0 G PEG-lysine dendrimer formulations with chloroquine phosphate (CQ) were taken up. The formulations were delivered by various suitable routes and studied for their blood level, tissue distribution, hematological toxicity, and tissue toxicity.

The blood concentrations of drugs on administration by various formulations were determined and compared. The blood level of drug was determined for up to 13 h only due to ethical restrictions (in terms of blood collection for the allowed number of blood sampling from one rat and the maximum volume of withdrawal possible). However, these data were inadequate and meaningless for the calculation of K_e and $t_{1/2}$, as originally there are no definite changes in the drug metabolism anticipated on drug delivery by various vehicles. Hence these data were compared by noncompartmental analysis: AUC, AUMC, and MRT for various delivery carriers.

CQ loaded in various dendrimeric formulations was delivered intravenously, and blood level data were compared with those obtained for intramuscular administration of plain CQ solution. The blood level of CQ on i.m. dosing was found to have reached the peak (50.23 μg mL^{-1}) slowly at 0.5 h, whereas from PL4K5G dosing there was a slower attainment of C_{max} and lower levels of drug in blood. C_{max} was attained at 3–4 h with uncoated (19.8 μg mL^{-1}) and around 13.8 μg mL^{-1} at 6 h for CSA coated carriers. CSA coating of the carriers was found to sustain the availability of drug in blood, where the drug (CQ) is most required.

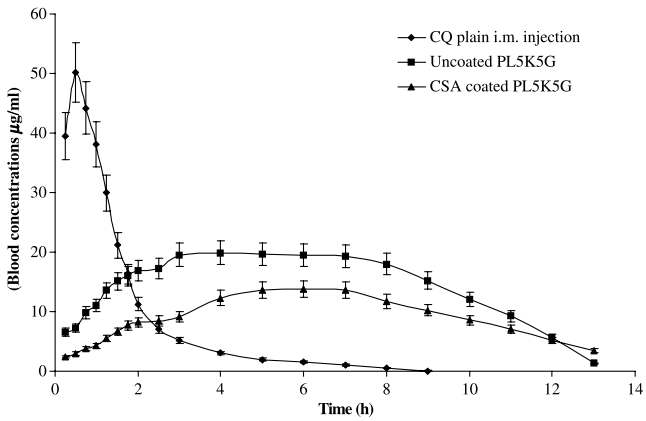

Fig. 4. Blood level profile of CQ after i.m. administration of plain drug solution (◆), and i.v. administration of uncoated PL5K5G (■) and CSA coated PL5K5G (▲).

Both AUC and MRT of CQ on administration by dendrimeric formulations increased significantly. However, the C_{max} values had declined to somewhat safer and constant levels. AUC had increased by more than 2 to 2.5 times. The MRT of drug in uncoated and CSA coated dendrimers had increased by 3.5 to 4 times than that of the free drug. The coating of the carriers had definitely retarded the drug release *in vivo* and made the systems long circulating carriers for CQ delivery up to 13 h in an almost sustained manner as compared to uncoated carriers (Fig. 4).

Biodistribution

The distribution of antimalarial bioactives is generally more for liver and spleen as compared to blood, which was in the range of 10,000 times the level in the blood. But dendrimers, because of its sustained drug delivery and long-circulatory nature, make the drug molecules more available in blood than in liver and spleen for a longer period. The extent of drug available in blood plasma after 3 h is significantly more as compared to that in liver and spleen, when delivered using some uncoated and CSA coated dendrimeric carriers. The changes were relatively much higher when delivered using CSA coated carriers than from uncoated carriers. Such an increased availability of bioactives in blood plasma than in liver was due to the slow release pattern of drug from formulations and time for the drug to attain a level in liver cells and rate of metabolism in liver cells (Fig. 5).

Hematological and Toxicological Studies

The dendrimers can thus sustain and prolong the bioavailability of antimalarial bioactives. So, in the final step, we carried out the hematological and toxicological assays of the present carriers to determine their safety and potential hazards in drug delivery as recently reported by many authors. In toxicological evaluations, the antimalarials such as CQ were found to disturb the normal functional tests of the blood as studied after delivery of plain drug to rats for 7 days.

However, the uncoated and CSA coated carriers were found to reduce the side effects of organ functionality to a greater extent as the % changes in ALT/SGPT, BUN, and bilirubin levels were reduced to half and one-fourth by uncoated and CSA coated carriers, respectively. Such carriers reduce the disturbance of organ functionality because of the controlled and sustained release of drug molecules from such carriers. CSA coated carriers were more protective because of a more sustained mode of drug delivery from the formulations and reduction of drug leakage and residual dendrimer-related toxicity.

A hematological study was also carried out for the formulations, and similar trends were found for the formulation used for the delivery of antimalarial bioactives. There were less hematological disturbances by drug-loaded CSA coated dendrimeric carriers than by uncoated 5.0 G dendrimers. Micellar carriers were also found to be equally safer than free drug. All these are mainly due to the slower and sustained availability of drug causing minimum organ and hematological disturbances in comparison to free drugs (Table IV).

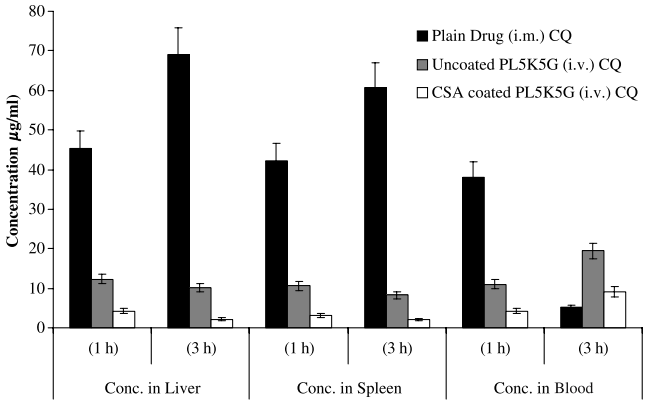

Fig. 5. *In vivo* organ distribution of CQ after i.m. administration of plain drug solution (■), and i.v. administration of uncoated PL5K5G (▩) and CSA coated PL5K5G (□).

CONCLUSION

Peptide dendrimers were intended for exploring the possibilities of designing chemically, physically, and biologically stable drug delivery systems, which is the characteristic of dendritic macromolecular nanoparticulate systems in contrast with other biological carrier-based systems. In the present study, PEG-lysine-based system was synthesized and was found highly effective for stabilizing drug molecules and sustaining the release of bioactives from its uniformly branched architecture. The systems were found also highly suitable for sustaining drug release both *in vitro* and *in vivo*.

Dendritic carriers can sustain the blood level of a drug for a much longer period, in significant therapeutic concentration, for prolonged duration, at steady state levels. They are mildly hemolytic and possess some cytotoxicity, due to their polycationic nature, causing interactions with anionic biological constituents. They were therefore modified by CSA coating, which contributed to their targeting capacity by acting as ligands for PRBCs as proved by *in vitro* interaction studies. Moreover, hemolytic toxicity and cytotoxicity were also reduced. The systems acted as highly closed and coated, protected systems, reducing drug leakages and sustaining and controlling drug release up to 10 h at steady state levels of bioactives in albino rats. Thus, the CSA coated or uncoated systems both can be appropriately regarded as suitable and safe drug delivery carriers for the selected antimalarial CQ, via the proposed PEGylated nanoparticulate circulating carriers.

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REFERENCES

1. D. Bhadra, S. Bhadra and N. K. Jain. In N. K. Jain (ed)., *Progress in Controlled and Novel Drug Delivery Systems*, 1st ed., CBS Publishers and Distributors, New Delhi, India, 2004, pp. 209–247.
2. U. Boas and P. M. Heegaard. Dendrimers in drug research. *Chem. Soc. Rev.* **33**:43–63 (2003).
3. S. Fuchs, T. Kapp, H. Otto, T. Schoneberg, P. Franke, R. Gust, and A. D. Schluter. A surface-modified dendrimer set for potential application as drug delivery vehicles: synthesis, *in vitro* toxicity, and intracellular localization. *Chemistry* **10**(5):1167–1192 (2004).
4. S. J. Teertstra and M. Gauthier. Dendrigraft polymers: macromolecular engineering on a mesoscopic scale. *Prog. Polym. Sci.* **29**:277–327 (2004).
5. H. Yang, J. J. Morris, and S. T. Lopina. Polyethylene glycol-polyamidoamine dendritic micelle as solubility enhancer and the effect of the length of polyethylene glycol arms on the solubility of pyrene in water. *J. Colloid Interface Sci.* **273**:148–154 (2004).
6. M. A. Abdullah, E. Muhanna, Ortiz-Salmerón, L. García-Fuentes, J. J. Giménez-Martíneza, and A. Vargas-Berenguel. Synthesis of peptide dendrimers based on α-cyclodextrin core with guest binding ability. *Tetrahedron Lett.* **44**:6125–6128 (2003).
7. A. E. Beezer, A. S. H. King, I. K. Martin, J. C. Mitchel, L. J. Twyman, and C. F. Wain. Dendrimers as potential drug carriers; encapsulation of acidic hydrophobes within water soluble PAMAM derivatives. *Tetrahedron* **59**:3873–3880 (2003).
8. T. Imae, T. Hirota, K. Funayama, K. Aoi, and M. Okada. Binding of poly(amido amine) dendrimer to sodium hyaluronate in aqueous NaCl solution. *J. Colloid Interface Sci.* **263**:306–311 (2003).
9. K. Sadler and J. P. Tam. Peptide dendrimers: applications and synthesis. *Rev. Mol. Biotechnol.* **90**:195–229 (2002).
10. D. Bhadra, S. Bhadra, S. Jain, and N. K. Jain. A PEGylated dendritic nanoparticulate carrier of fluorouracil. *Int. J. Pharm.* **257**:111–124 (2003).
11. A. S. Chauhan, S. Sridevi, K. B. Chalasani, A. K. Jain, S. K. Jain, N. K. Jain, and P. V. Diwan. Dendrimer-mediated transdermal delivery: enhanced bioavailability of indomethacin. *J. Control. Release* **90**:335–343 (2003).
12. D. Bhadra, S. Bhadra, and N. K. Jain. PEGylated peptide based dendritic nanoparticulate systems for delivery of artemether. *J. Drug Deliv. Sci. Technol.* **15**(1):65–73 (2005).
13. D. Bhadra, A. K. Yadav, S. Bhadra, and N. K. Jain. Glycodendrimeric nanoparticulate carriers of primaquine phosphate for liver targeting. *Int. J. Pharm.* **295**(1–2):221–233 (2005).
14. D. Bhadra, S. Bhadra and N. K. Jain. PEGylated-poly-L-lysine dendrimers for delivery of chloroquine phosphate. The 2004 International Conference on MEMS, NANO, and Smart Systems. Aug 25–27, Banff, Alberta, Canada, (2004).
15. A. Martin, J. Swarbrick and A. Cammerata. *Physical Pharmacy,* 3rd ed. (Indian ed.) Varghese Publishing House, Bombay, India, 1991.
16. V. K. Sarin, S. B. H. Kent, J. P. Tam, and R. B. Merrifield. Quantitative monitoring of solid phase peptide synthesis by the Ninhydrin reaction. *Anal. Biochem.* **117**:147–157 (1981).
17. T. Cruz, R. Gaspar, A. Donato, and C. Lopes. Interaction between polyalkylcyanoacrylate nanoparticles and peritoneal macrophages: MTT metabolism. NBT reduction and NO production. *Pharm. Res.* **14**:73–79 (1997).
18. T. Mosman. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**:55–63 (1983).
19. C. Lambros and J. P. Vanderberg. Synchronization of *P. falciparum* erythrocytic stages in culture. *J. Parasitol.* **65**:418–420 (1979).
20. W. Trager and J. B. Jensen. Human malaria parasites in continuous culture. *Science* **193**:673–675 (1976).
21. D. M. Domanski, B. Klajnert, and M. Bryszewska. Influence of PAMAM dendrimers on human red blood cells. *Bioelectrochemistry* **63**:189–191 (2004).
22. N. Malik, R. Wiwattanapatapee, R. Klopsch, K. Lorenz, H. Frey, J. W. Weemer, E. W. Meijer, W. Paulus, and R. Duncan. Dendrimer: relationship between structure and biocompatibility *in vitro* and preliminary studies on the biodistribution of I^{135}-labelled polyamidoamine dendrimers *in vivo*. *J. Control. Release* **65**:133–148 (2000).
23. D. Fischer, Y. Li, B. Ahlemeyer, J. Krieglstein, and T. Kissel. *In vitro* cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* **24**:1121–1131 (2003).
24. R. Jevprasesphant, J. Penny, R. Jalal, D. Attwood, N. B. McKeown, and A. D'Emanuele. The influence of surface modification on the cytotoxicity of PAMAM dendrimers. *Int. J. Pharm.* **252**:263–266 (2003).
25. B. M. Cooke and R. L. Coppel. Cytoadhesion and falciparum malaria: going with flow. *Parasitol. Today* **11**:282–287 (1995).
26. J. G. Beeson, G. V. Brown, M. E. Molyneux, C. Mhango, F. Dzinjalamala, and S. J. Rogerson. *Plasmodium falciparum* isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. *J. Infect. Dis.* **180**:464–472 (1999).
27. G. B. Nash, B. M. Cooke, K. Marsh, A. Berendt, C. Newbold, and J. Stuart. Rheological analysis of the adhesive interactions of red blood cells parasitized by *Plasmodium falciparum*. *Blood* **79**:798–807 (1992).