Pegylated Lysine Based Copolymeric Dendritic Micelles For Solubilization And Delivery Of Artemether

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ABSTRACT PURPOSE: A newer polymeric amphiphilic micellar system was developed in the present study for solubilsation and controlled delivery of an antimalarial drug, Artemether (ART). Methoxy polyethylene glycol (MPEG) 2000 and 5000 were used as hydrophilic terminal. **METHODS**: The hydrophobic di-Fluorene methoxycarbonyl-l-lysine (di-FMOC-L-lysine) was linked initially to the single reactive end of MPEG, and to the two amino groups of l-lysine by consecutive peptide linkages and deprotection upto 2.5 generations (G). Half-generations are diFMOC-lysine terminated systems and full-generations are deprotected l-lysine terminated systems. The half-generation (0.5G, 1.5 G and 2.5 G) dendritic micelles of MPEG 2000 and 5000 were used to solubilize artemether. IR, NMR and Mass spectroscopy characterized the synthesis of these micellar systems. The CMC of the systems was determined. Then formulations made were characterized for solubility enhancement (i.e. drug loading) and drug-release profile. **RESULTS**: There is considerable solubility enhancement of artemether upto three to fifteen times depending on concentration, generation and type of dendritic micelles used. The size and shape were studied using transmission electron microscopy. The stability of the micellar formulation was also determined by storing the micelles at various temperatures for a definite period of time followed by its successive dilutions. The dendritic carriers were found to form stable micelles at 10-30 μg/ml (lower CMCs) depending on generation and type of MPEG used. The formulations increased the stability of the drug and also prolonged the release of artemether upto 1-2 days *in vitro*. **CONCLUSION**: From all the studies performed, it can be concluded that these micellar systems can be used for the safe and effective delivery of insoluble bioactive.

Abbreviations: IR: infrared; NMR: nuclear magnetic resonance; CMC: critical micelle concentration RES: Reticulo-endothelial system

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INTRODUCTION

Copolymeric micelles are the micelles formed through multimolecular assembly of the block copolymers. They are comprehensively described as novel core–shell typed colloidal carriers for controlled drug delivery and gene targeting. Novel approaches are also used for the formation of such micelles using functionalized poly (ethylene glycols) (PEGs) as hydrophilic outer shell and were focused through PEG-conjugated ligands with minimal non-specific interaction with other proteins. Surface organization of these block copolymer micelles with cross-linking core was also described from a standpoint of the preparation of a new functional surface-coating with some unique macromolecular architecture (1). These have PEG chains attached to one end in a brush configuration, which avoid or reduce interactions with blood proteins and therefore impart RES avoiding properties. To achieve the core shell structure described above, block amphiphilic polymers of the PEG-R types were synthesized. R was chosen from bioerodible polymers such as PLA (poly D, L-lactide), PLGA (polylactideco-glycolide), PCL (poly-caprolactone), Poly (butylene terephthalate) (PBT), poly (ortho ethers) (POE), poly-l-lysine (dendrimers) etc (2).

Earlier, we have synthesized and reported similar PEG coated polyamidoamine (PAMAM) dendrimer based unimolecular micellar system for the delivery of 5-fluorouracil in our laboratory (3, 4). One such similar unimolecular dendritic micelles as solubility enhancers were obtained by coupling polyethylene glycol (PEG) to Starburst PAMAM dendrimers of various generations (5). There was significant change in the solubility of pyrene as was monitored at 334 nm, its maximum absorption

wavelength. A brief survey of such host-guest interactions involving dendritic architectures was also reported (6). The effects of ethylene glycol-based graft, star-shaped, dendritic polymers on solubilization and controlled release of drugs like paclitaxel and nimesulide was also studied (7, 8). The micelles from lipid derivatives of water-soluble polymers as delivery systems for poorly soluble drugs were also studied (9). The solubility of a poorly water-soluble drug, Cyclosporin A, was also increased in aqueous dispersions of dextran-grafted-polyethyleneglycol-alkyl ether (10). The drug release behaviors of nimodipine-loaded poly (caprolactone)–poly (ethylene oxide)–polylactide amphiphilic copolymeric nanoparticles were also studied (11).

Many newer approaches developed for effective antimalarial chemotherapy was reviewed by Bhadra et al. (12). However, in the present approach, poly-l-lysine based peptide dendrimers protected with Fluorene methoxy carbonyl terminal was conjugated at methoxy-PEG-hydroxyl terminals to form an amphiphilic peptide based AB-dendritic copolymeric micelles for solubilization of a potent antimalarial, artemether (an artemisinin derivative). Artemisinin derivatives are active at nanomolar concentrations *in vitro* on both chloroquine-sensitive or -resistant *P. falciparum* strains. ART has been included in the WHO List of Essential Drugs for the treatment of severe multiresistant malaria (13, 14), but the major drawback of artemisinin derivatives is their short half-life (3–5 h). Also, the oral formulations of these drugs were rapidly but incompletely absorbed, and their bioavailability is lower. So there was always some necessity of administration of these derivatives by some alternative parenteral route. The Walter Reed Institute of Research had already patented a stable, water-soluble derivative of this family called artelinic acid (15).

However, the problem associated with such conventional formulations is unavailability of a suitable compatible aqueous base for sustained and controlled delivery of ART. Thus, nanoparticulate depot type carriers were suggested for the delivery of ART compatible intravenous carriers, as used earlier for such bioactives having short half-life (8, 16). This approach could also increase the solubility of ART, similar to water-soluble polymer conjugates of the anti-malarial drug, artelinic acid, developed using water soluble and non-peptidic polymer backbones, such as poly(ethylene glycol), mPEG, bifunctional PEG and multi-arm PEG (18). In the present study, however, much stable MPEG-Lysine-diFMOC based dendrimeric nanoparticulate carriers were selected for solubilization of artemether, and prolonging its release and stability, which can enable sustained and controlled delivery of ART in solubilized systems by i.v. route as aqueous solution.

MATERIALS AND METHODS

Materials: The drug ART was a generous gift sample from M/s Ipca Laboratories, Mumbai, India. Lysine, Fluorenemethoxy carbonyl succinimide (FMOC-Su), N,N'-Dicyclohexyl carbodiimide (DCC), and cellophane dialysis membrane bag of 2.4nm, were purchased from HiMedia Laboratories Ltd., Mumbai, India. 1-Hydoxybenzotriazole (HOBT), Dimethylamino pyridine (DMAP), Sodium azide, and 10% Pd catalyst adsorbed on charcoal were purchased from M/s Spectrochem Pvt. Ltd., Mumbai, India.

Synthesis of MPEG-lysine-diFMOC dendrimers: The MPEG-lysine-diFMOC dendrimers were synthesized using MPEG-amine 2000D and 5000D as core and protected diFMOC-lysine for progressive linking on terminal amino groups of prior generations consecutively by liquid phase peptide synthesis as discussed in our earlier work (19, 20) upto 2.5G. MPEG amine was synthesized firstly by stepwise synthesis scheme as suggested by Zalipsky et al. (21). For the synthesis of dendrimers, protected l-lysine was required for allowing uniform branching. This was carried out using FMOC-Su (Fluorenyl methoxy carbonyl succinimide) to form di-FMOC-lysine by method suggested by Lapatsanis et al (22). Finally, the MPEG terminated lysine-di-FMOC micellar systems of various generations were synthesized by the well-known DCC-HOBt coupling procedure in DCM: DMF (1:1) solvent system. Deprotection of diFMOC groups from the micelles was carried out by piperidine-based hydrolysis for synthesis of further higher generations (19, 20, 22).

The products were separated, dried and stored in a vacuum desiccator. The protection and de-protection steps were repeated alternately with subsequent increase in reactants (Fig. 1) for every consecutive generations upto 2.5G. The half generations of dendrimers of 0.5G, 1.5G and 2.5G of each MPEG2000 and 5000 types were used in the present studies for formulations and solubilization of artemether (Table 1).

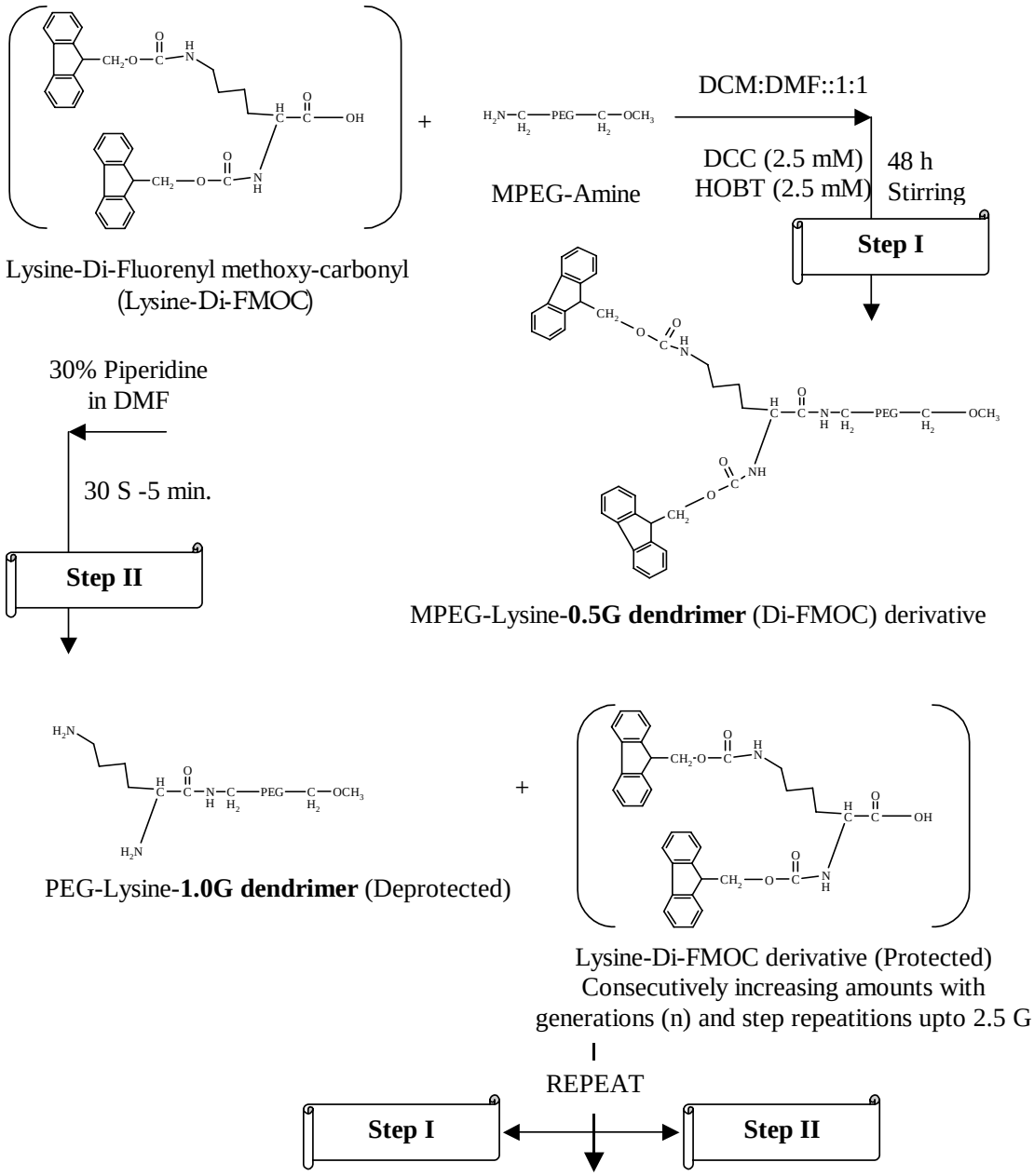

Fig. 1. Structural scheme for the synthesis of MPEG-Lysine-Di-FMOC amphiphilic dendrimeric micellar carriers.

Table 1. Code and various physical parameters determined for dendrimeric micellar formulations selected in the present studies

Code	Generations	PEG taken	MALDI-TOF Mass (D)		CMC in Molarity (μM)
			Theoretical	Actual	
MPL2K05G	0.5 G	MPEG 2000D	2590	2598.12	20.50±0.12
MPL2K15G	1.5 G	MPEG 2000D	3326	3318.94	14.22±0.23
MPL2K25G	2.5 G	MPEG 2000D	4798	-	10.15±0.17
MPL5K05G	0.5 G	MPEG 5000D	5590	-	25.30±0.14
MPL5K15G	1.5 G	MPEG 5000D	6326	6328.50	19.60±0.21
MPL5K25G	2.5 G	MPEG 5000D	7798	7784.11	13.20±0.19

The dendrimeric amphiphiles were characterized by IR, NMR, MALDI-TOF mass spectroscopy and Kaiser Test for completion of reaction and structural elucidation as described earlier (19).

Determination of Critical Micelle Concentration: Amphiphilic block copolymers like MPEG-lysine systems of half generation tend to form micelles at concentrations above CMC, hence determination of CMC is required for formulation of drug-loaded carriers. Amongst the various methods used to determine CMC, $\log_{10}$(concentration) vs. absorbance plot of half generation systems (0.5G, 1.5G and 2.5G systems), at its absorbance maxima (λ_{max}, 258 nm), was used in the present study. The half generations of dendrimers were dissolved in water, vortexed (Superfit), sonicated and shaken on mechanical incubator shaken at 37°C for 2 h to prepare a stock solution of 1000μg/ml. The aliquots of the stock solution were further diluted to prepare different dilutions and kept overnight for equilibration and formation of micelles for the study.

In the curve where there was a change in the slope of curve at CMC. The concentration corresponding to the point of intersection of the slopes of lower and upper curve denotes the CMC of the copolymers. This method was based on sudden changes in absorbance due to aggregation at CMC (23). However, the whole-generation PEG-lysine systems have no CMC, and they actually form unimolecular nanoparticulate dendrimeric carrier systems for loading drug by complexation or hydrotropic solubilzation within its structure by steric hindrance and group complexation.

Phase solubilization of artemether: The studies were conducted using continuous variation method of Higuchi and Connors (24). Briefly, an excess of the drug was added to various concentrations of dendrimeric micellar solutions, followed by vortexing and equilibration. Half-generations of PEGylated-lysine-diFMOC dendrimers at concentration range 1000 μg/ml to 5000 μg/ml (i.e. above CMC values) were used for solubilization of water insoluble ART. The final suspensions of drug

and dendrimers (half-generations) were vortexed and sonicated. The final mixture was shaken in mechanical incubator shaker at 37°C overnight, filtered, lyophilized and stored until further use.

Characterization of formulations: The final dendritic micellar formulations with and without drug prepared and dialyzed were used for electron microscopic studies. The Transmission Electron Microscopic 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% Uranyl acetate at various magnifications on Moragagni 268D with digital TEM image analysis system of Soft Imaging System, GmbH (Germany) at 50-60kV.

Drug loading was performed by phase solubilization of the drug in different concentration of half-generation dendrimeric micelles as entrapment of drug in micelles can also increase its solubility.. For the determination of entrapment efficiency of the systems, the drug molecules were partitioned out from 1ml portions of aqueous formulations by shaking with 5ml portions of dichloromethane (DCM). This led to breaking of micelles, thereby releasing the entrapped drug. The DCM layer was dried under vacuum and methanol was used to solubilze the residue. The methanol solubilized portion was hydrolyzed using 1 ml of 5 M HCl by heating for 15 min. The amount of drug was determined spectrophotometrically at 254 nm after proper dilution (14, 25). The amount of drug solubilized by various concentrations of dendrimers was used to determine amount of drug (g) entrapped by per gram of dendrimeric micelles (w/w) and also as molar ratio of drug, in moles of drug per moles of dendrimeric micelles.

The drug release studies were carried out by dialysis using cellophane tubes (Pore 2.4 nm, Himedia). 100 mg of lyophilized drug-dendrimer system was dissolved and taken in cellophane tubes and immersed in the aqueous medium (20 ml) under magnetic stirring. Samples were withdrawn from it at every 1 h for 8 h. After that samples were withdrawn at 24 h. and every day thereafter. The cumulative amount of drug coming out of the micellar carriers was plotted against time to determine the release pattern of the systems.

Stability studies: The dendrimer-drug micellar formulations were kept in tightly closed vials and stored at 0°C, room temperature (25°C) and 50°C (controlled oven) for a period of 15 days. The samples were analyzed initially and periodically after every week for any precipitation, turbidity, crystallization, change in colour, consistency, drug leakage and chemical nature of formulations. The data obtained was used to predict the stability, the required storage conditions, and the precautions required during storage.

Effect on chemical nature of preparation was ascertained by comparison of the intensity of colour developed by Kaiser Test with 1ml of formulation, spectrophotometrically (λ_{max}=570nm) (26). The percentage change in the intensity of the color produced was used for the determination of free amino groups available at the periphery of dendrimeric formulation. The drug leakage was determined by checking for increase in the release rate of drug from the formulations after storage at accelerated conditions. The formulation samples (2ml) were kept in cellulose tubing and dialyzed across the tubing. The external medium (10ml) was analyzed for content of drug, spectrophotometrically. The procedure was repeated every week for upto five week. The percentage increase in drug release from the formulation was used to analyze the effects of accelerated conditions of storage on the formulation. The amount of residual insoluble drug (ART) present in formulations were also analyzed for all formulations by filtration and analyzing similar as for drug entrapment in micelles every 5 days.

Hydrolytic attack on artemether and degree of stability provided by the micellar formulations were determined by mixing 0.5 g of ART-loaded micellar preparation of 2.5G of MPEG 5000D with 1.0 ml of 1 M HCl producing a stock solution of 5000μg/ml. This stock was equally divided and stored at room temperature (25°C), for determination of effect of HCl on encapsulated ART in separate ten 10 ml vials. Similarly, diluted methanolic aqueous ART solution was also kept with 1M HCl, for comparison. This study was designed to determine the protection efficacy of micelles on the encapsulated drug.

The effect of dilution on stability of formulations was determined by diluting 1 ml micellar formulations (5000 μg/ml) with 1-10 ml of water. These dilutions were monitored for any crystallization after 2 h. The amount of drug remaining solubilized in the formulations was determined by filtration followed by drug estimation in the micellar solutions.

RESULTS AND DISCUSSION

In the present study, amphiphilic poly-l-lysine based peptide dendrimers, having PEG at hydrophilic ends and di-FMOC at other hydrophobic end was used for the aqueous solubilization of artemether, an artemisinin derivative. Peptide dendrimers are radial or wedge-like branched macromolecules consisting of a peptidyl branching core and/ or covalently attached surface functional units. The multimeric nature of these constructs, the unambiguous composition, and the ease of production make this type of dendrimer well suited to various biotechnological and biochemical applications e.g. diagnostic reagents, protein mimetics, carriers for drugs, vaccines and genes. Earlier, Sadler & Tam (27) reviewed extensively such peptide dendrimers, their synthesis and applications. Choi et al. (28, 29) also synthesized one such barbell-like ABA-type triblock copolymer, poly(L-lysine) dendrimer-poly(ethylene glycol)- poly(L-lysine) dendrimer (PLLD-PEG-PLLD) by liquid-phase peptide synthesis, similar to the present system.

Synthesis of MPEG-lysine-diFMOC dendrimers. Lysine-diFMOC was used to synthesize the proposed peptide dendrimers by amide linkages using DCC-HOBT techniques following scheme given in Fig 1. The RasMOL representation (Fig 2) gives the evidence of basic linear configuration and closeness of structure as generation increases. The reaction was allowed to complete with further addition of DCC (taken 10% molar excess quantity) to the solution of protected lysine and HOBT (taken equimolar to DCC). Lysine-diFMOC was taken 10-50% molar excess of stoichiometric amounts depending on generations.

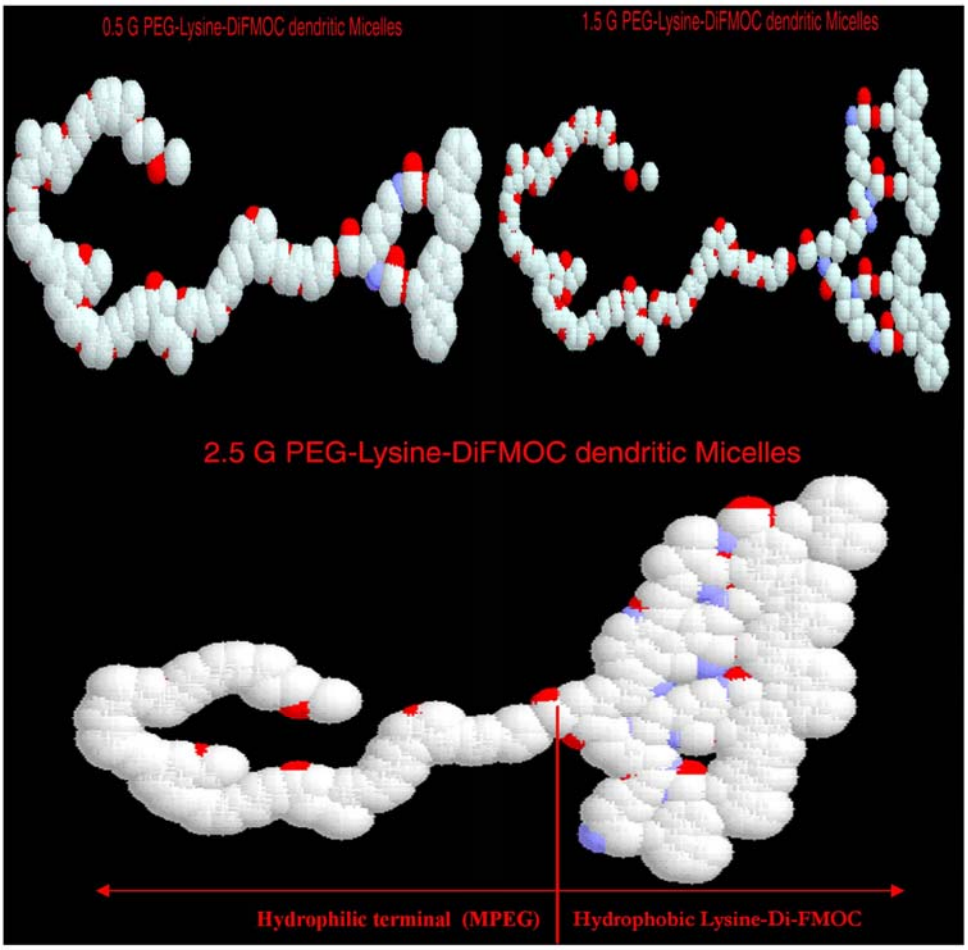

Fig. 2. Rasmol version 2.5 representations of MPEG-Lysine-diFMOC micelles of various dendrimeric generations, where a) 0.5G, b) 1.5G, c) 2.5G MPEG-Lysine di-FMOC dendrimers.

The reaction of amide linkages with the protected lysine-diFMOC took 1-5 days, depending on generations. In the case of higher generations, the time required for completion of reactions was increased. Intermittent checking for completion of reaction at amine termination of lysine was done by negative Kaiser Test.

The progress of generations was confirmed by Kaiser Tests giving Ruhemann's purple blue chromophore absorbing at 570 nm. This absorbance gives the number of amine groups of amino acids (Table 1). The positive Kaiser test and absorbance of the dendrimeric generations (full-generation) was further used for quantitative estimation of terminal lysine on each dendrimer molecules (26). On protection by di-FMOC no such formation occurred and only yellow colour was obtained in the reaction mixture. The test was used to determine equivalent amino groups in each generation and completion of reaction until there is no blue colour development in aliquots of reaction mixtures tested for completion of reaction.

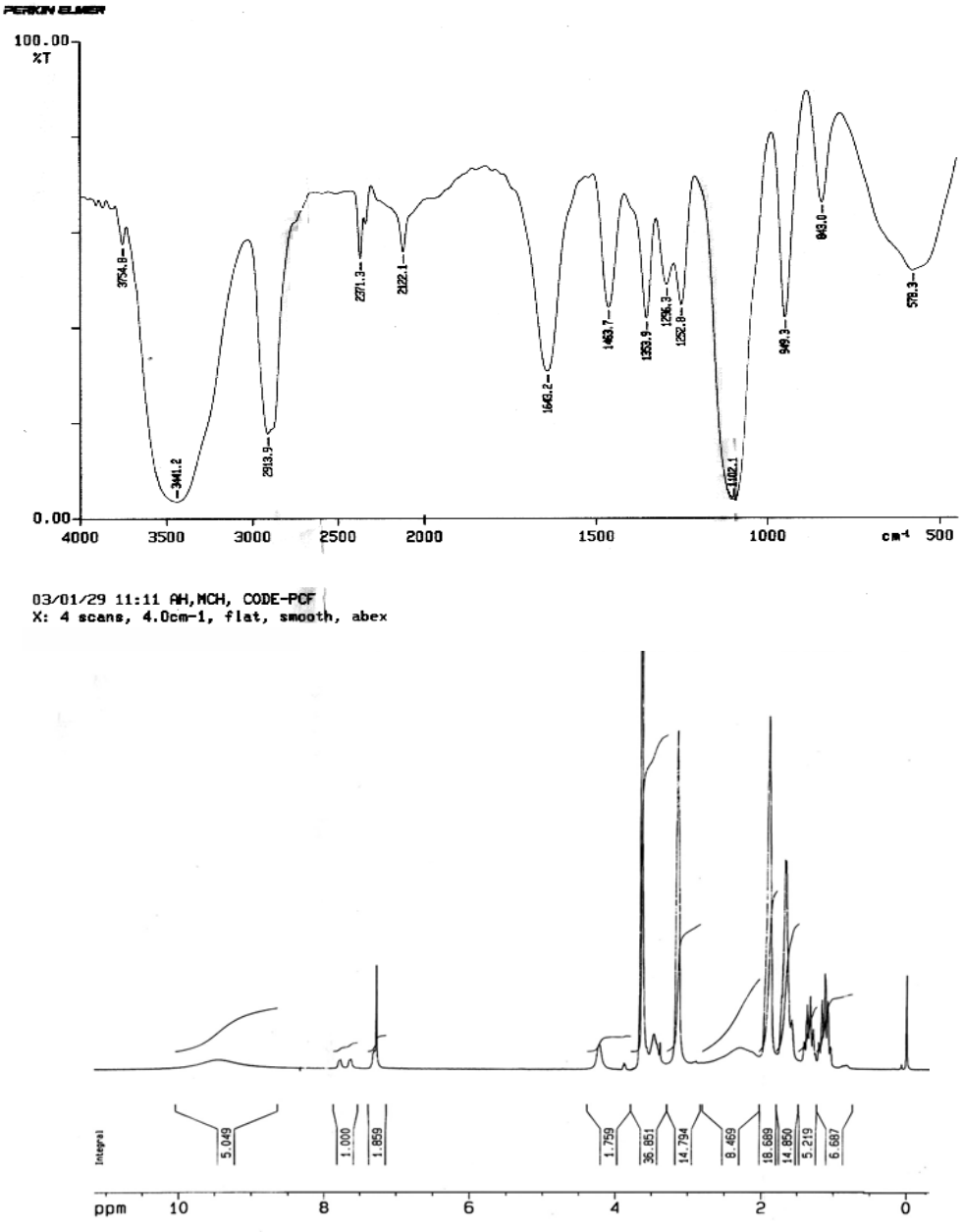

Fig. 3. IR and ^{1}H-NMR Spectrum of MPEG-Lysine-DiFMOC type of half generation peptide dendrimeric micelles.

Finally, IR, NMR and MALDI-TOF mass spectroscopy confirmed the completion of synthesis. The IR spectrum (Fig. 3) showed some distinct peaks in half-generation of dendrimers with peaks of PEG and lysine consecutively. For example, 2931.7 & 2832.8 cm^{-1} for –C-H stretch of methyl groups; 1592.2 cm^{-1} for N-H bends due to amine gr. of lysine; and 1120.5 cm^{-1} for C-O-C str. of PEG. Peak at 1629.7 cm^{-1} for C=O str. and at 3043.6 cm^{-1} for N-H stretch confirms the formation of amide linkage. Other important peaks were at 1360.5; 1162.5; 773.4 cm^{-1}.

The NMR spectrum showed some distinct peaks in half generations of dendrimers. The major shifts are at 0.9-1.1 ppm for C-H protons of other lysine; 1.3-1.4 ppm for methylene protons of PEG; 1.5-1.8 ppm for α-, β, and γ- methylene protons of poly-lysine; 1.9-2.1 ppm for -N-H of amine of poly-l-lysine at δ position; 2.2-2.6 for NH proton near COOH gr. of lysine; 3.0-3.3 ppm for ether groups of PEGs; 3.5-3.8 ppm for ether groups of terminal portion methoxy and residual diethyl ether left; 4.2-4.4 ppm for amide linkage at δ position; 7.1-7.4 ppm for amide linkage at α position; 7.6-7.8 ppm for aromatic fluorene; and 8.9-10.2 ppm for carboxyl groups of lysine. The ratios of NMR peak intensity for the ethylene protons of PEG segment (δ=3.0-3.3 ppm) and the α-, β, and γ- methylene protons of PLL dendrimers (δ=1.4-1.8 ppm) were further used to determine the ratio of lysine to PEG chains in dendrimers. The experimental ratios of the peaks are more or less equivalent to the theoretical ratios of the peaks in the spectrum. The MALDI-TOF-mass spectroscopy was used to determine the mass of the dendrimers formed in each generation, protected and unprotected types. Average mass of the systems were determined from the peaks of parent molecular ion. It matched to a significant extent with the actual theoretical mass (Table 1).

Determination of Critical Micelle Concentration (CMC). The CMC values were determined by the method of changes in turbidity and absorbance at 258nm (λ_{max}) associated with it at concentration equal to or more than CMC due to aggregation of unimers at that concentration leading to an abrupt increase in absorbance (23).

The CMC values were in the range of micromolar concentration for such polymeric micellar carriers that were found to be decreasing with increase in generations due to in increase in the hydrophobicity of the ends (Table 1). No general trend was observed with increase in molecular weight of the carriers but with the increase in molecular weight of PEGs (hydrophilic terminal), the CMC values were significantly higher than that of the same generations made from lower molecular weight PEGs.

This conforms to the predictions for the CMC based on structures of the surfactants in a series of surfactants (30). The lower CMC values indicate that the systems could be used as stable and sustained drug delivery carriers without much effect on physicochemical stability of formulations on dilution and at the same time, can protect the drug molecules from degradation due to external environment (31). By the Rasmol representation of molecular orientation and structure, it can also be ascertained that the structures at higher generations are suitable as unimolecular micelles. The hydrophobic end is well structured and voluminous that could well load the hydrophobic molecules within their hydrophobic environment and at the same time remain solubilized due to the presence of PEG at the periphery (Fig. 4).

So it can be concluded that the higher generations have more entrapment capacity and stabilizing potential.

Electron microscopy. The particles were not ordinarily viewable by normal microscopy or dynamic light scattering technique as they are in nanometric size-range. Such nanoparticulate carriers are more easily and suitably focused by Transmission Electron Microscopy (TEM). All formulations are in nanometric size range, spherical and uniform shaped (Fig. 5).

The micellar carriers were stained negatively by 4% uranyl acetate, which stained the background more prominently and leave the particles in unstained state. The drug-loaded micelles were seen as dark dots. This might be due to the positive staining of drug-loaded carriers considerably due to presence of drug within such carriers. The size of micelles was found to be 5-25 nm (as evident by scale below shown by digital image analysis system Soft imaging system version 3.1) with increase in generations from 0.5G to 2.5G for MPEG-lysine-diFMOC carriers of 5000D.

Drug Entrapment and solubilization. The drug loading in dendrimers were carried out by equilibrium dialysis method leading to drug loading by adsorption and physical interaction like protein binding onto the carriers.

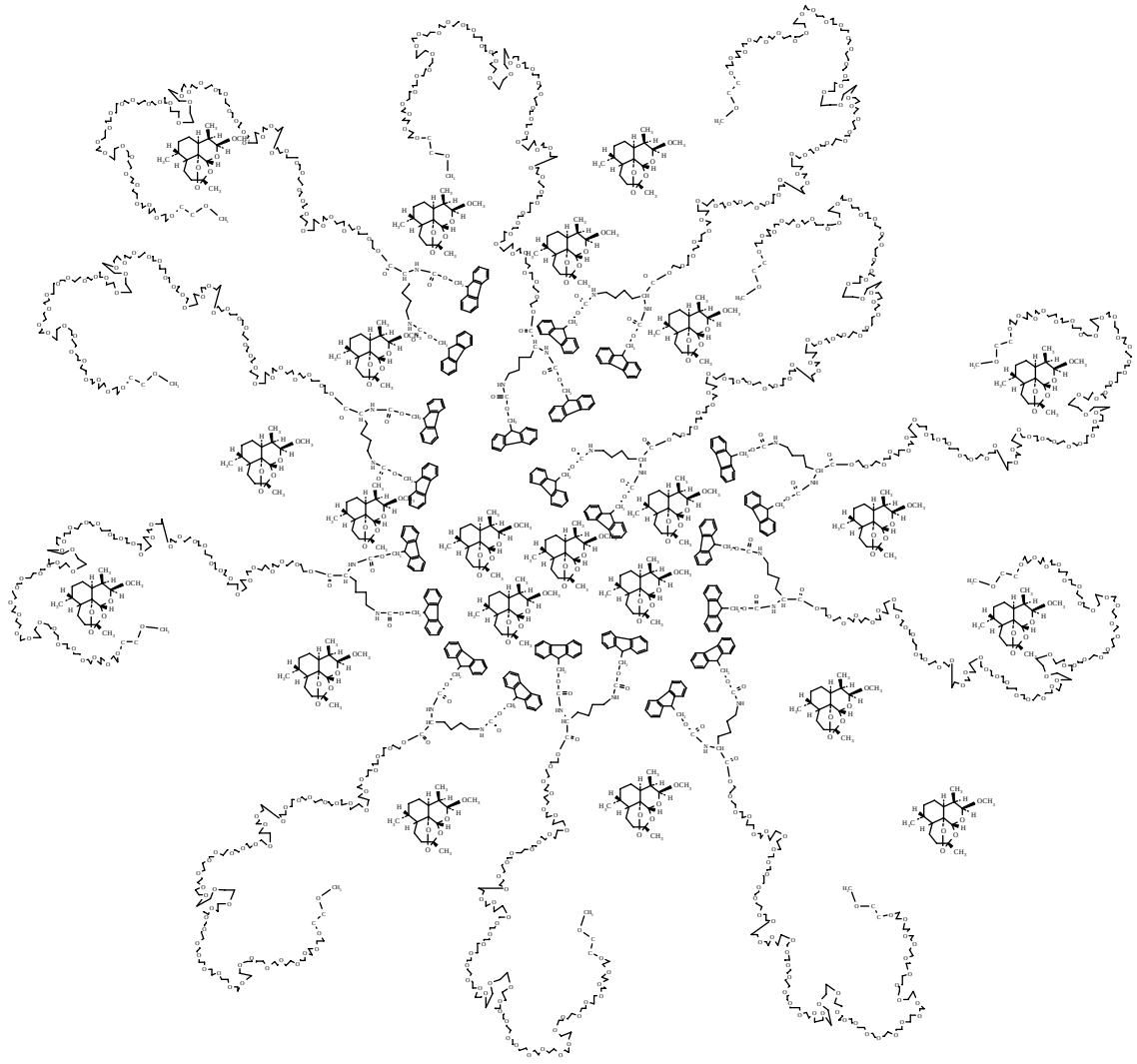

Fig. 4. Representative structure of insoluble artemether loaded in MPEG-Lysine-FMOC Micellar carrier of 0.5G dendrimeric generations.

The entrapment was expressed in terms of weight of drug loaded per gram of dendrimers (Table 2). This was used to calculate stoichiometrically molecular entrapment using theoretical molecular weights of the carriers to determine possible number of drug molecules loaded within one molecule of dendrimer. The micellar entrapment of ART also followed a definite trend, where increase in micellar generations causing increase in weight-by-weight and molar drug content. This occurred because with increase in generation of micelles, there was a distinct increase in hydrophobic tail length and volume, which caused entrapment of drug molecules in micellar aggregates and in tails with FMOC groups by hydrophobic interactions. Thus, entrapment in micelles occurred both by hydrophobic and hydrotropic complexation based interactions. The entrapment was upto 5 to 16 molecules per molecules of unimers. These values did not undergo significant changes with increase in molecular weight of MPEGs from 2000D to 5000D. The entrapment of ART in weight terms undergoes significant changes with increase in molecular weights of MPEGs (hydrophilic tails) and also with increase in generations of dendrimeric micelles.

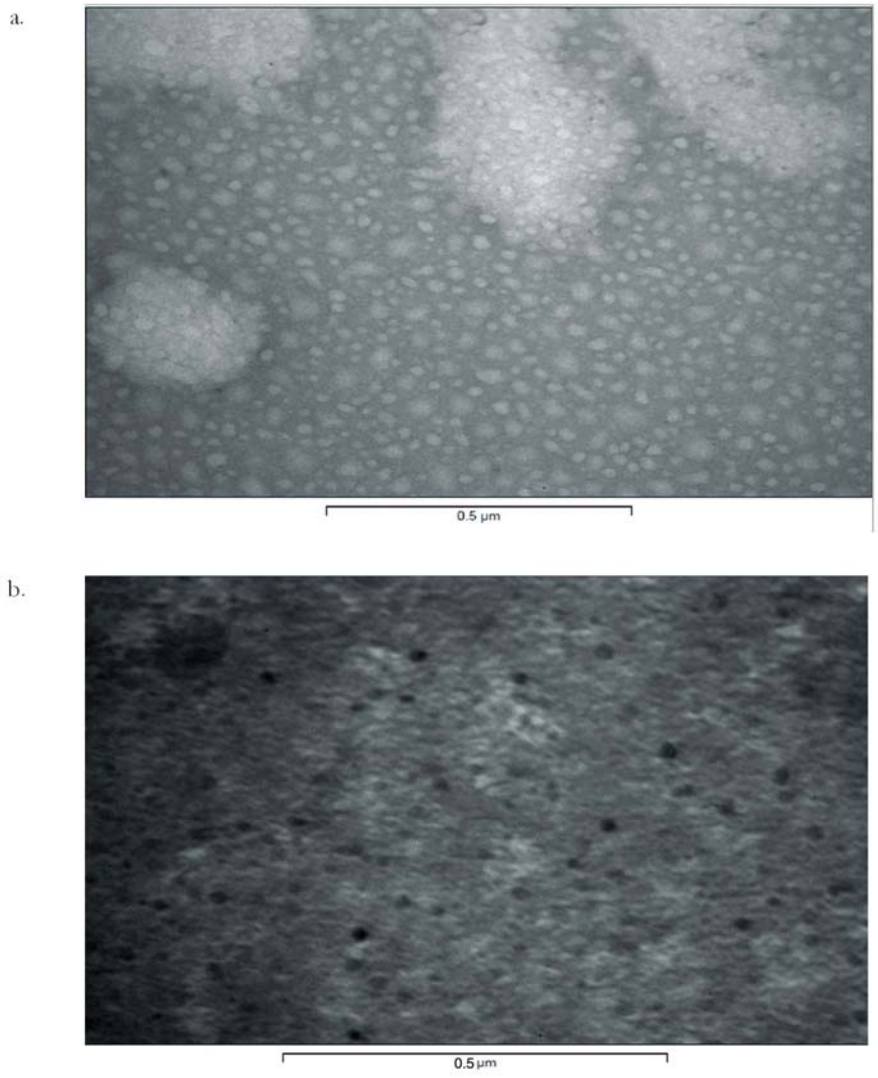

Fig. 5. TEM photomicrograph representations of 2.5G dendritic micelles after negative staining with uranyl acetate where a) represents the empty micelles; b) represents drug loaded micelles

As the CMC increases with molecular weight of MPEG, there was an increase in the requirements of number of molecules for micellization or aggregation for the drug loading. This led to significant decrease in weight of drug loaded per gram of dendrimer with increase in molecular weights of MPEGs significantly. The drug entrapment reduced significantly from 0.98±0.06g/g for MPL2K25G to 0.620±0.10g/g for MPL5K25G, which in molar times was however, non-significant (Table 2). However, the weight-by-weight drug loading increased from 0.58±0.07 g/g for 0.5G generations of 2000D micelles to 0.98±0.06 g/g for 2.5G MPEG 2000D species.

Drug Release Profile. The release of drugs from the dendrimeric formulations was determined across dialysis cellulose tubing of 2.4nm and estimated spectrophotometrically, after appropriate dilution. The drug release was estimated in terms of % cumulative drug released using the average amounts of drug loaded in the dendrimeric carriers (Table 2). The effect of mass & generation of dendrimeric carriers on initial burst release from carriers; release patterns and release rate constants were also analyzed.

Table 2. Drug Solubilzation and release rate profile of Artemether from various dendritic micellar carriers

Formulation	Drug loading (w/w)	Molar loading (M/M)	% Initial Burst Release	Release rate constant (initial phase)*	Correlation Coefficient (Initial release phase; r^2)**
MPL2K05G	0.58±0.07	5.03±0.61	29.5±1.2	13.10	0.997
MPL2K15G	0.72±0.08	8.03±0.90	24.8±1.5	10.05	0.994
MPL2K25G	0.98±0.06	15.76±0.97	22.9±1.5	07.45	0.987
MPL5K05G	0.34±0.08	6.37±1.50	32.1±2.1	13.56	0.997
MPL5K15G	0.50±0.13	10.6±2.81	26.8±1.2	10.30	0.994
MPL5K25G	0.620±0.10	16.20±2.61	23.5±1.2	08.34	0.989

* Release rate constant and **correlation coefficients are calculated from the slope of the initial portions (before 24 h portions) of drug release curve and correlations of that portions of first order release curve.

The micelles on dialysis get diluted and displace drug comparatively rapidly being multi-molecular in nature. The release rate of MPL2K05G, MPL2K15G and MPL2K25G was 13.10%, 10.05% and 7.45% per h, respectively. For higher molecular weights of MPEGs (i.e. 5000D series), the release rate was slightly higher. It was 13.56%, 10.30% and 8.34% per h for MPL5K05G, MPL5K15G and MPL5K25G, respectively. Similar trend was observed in case of burst release from such carriers, which reduced with increase in generations of such carriers viz. 29.5±1.2%, 24.8±1.5% and 22.9±1.5% respectively for 0.5G, 1.5G and 2.5G of MP2000 series and 32.1±2.1%, 26.8±1.2% and 23.5±1.2% respectively for 0.5G, 1.5G and 2.5G of MP5000 series. This was only due to the fact that higher CMCs of micellar carriers of 5000D made the carriers more vulnerable for drug release (Fig. 6). With the increase in generations of micelles, there was significant decrease in CMCs, also there is an increase in hydrophobic fluorene groups (FMOC) in the micelles for hydrophobic interactions leading to increase in drug loading (Table 2). With increase in generations of dendrimers, increase in groups for complexation and additional binding caused increased steric hindrance, causing reduction in drug leakage. The higher drug release from the micelles of higher molecular weights of MPEG may also be contributed to the lower wt fraction of hydrophobic core in these polymers other than the effects of the CMC values.

Stability Studies. Micelles of high molecular weight MPEGs are comparatively more stable as to lower molecular weight micellar carriers. There was increase in turbidity in formulations at lower temperature more due to lower solubility of carriers that occurs highly in lower molecular weight PEG carriers as these carriers have lower solubility because of lesser number PEG ethereal linkages, which additionally increases the stability of such carriers. The decrease in solubility of such carriers at lower temperature additionally caused crystallization of drug because of displacement of ART from such carriers. The percentage degradation of ART as measured by appearance of absorbance on hydrolysis at 256 nm for the formulations stored at various conditions for testing integrity and protective nature of formulations, also proved that higher molecular weight MPEG carriers were more stable from degradations as compared to lower molecular weight carriers. This occurred due to increased steric hindrance by higher molecular weight PEG that prevents physicochemical degradation and losses of structural integrity of such carriers.

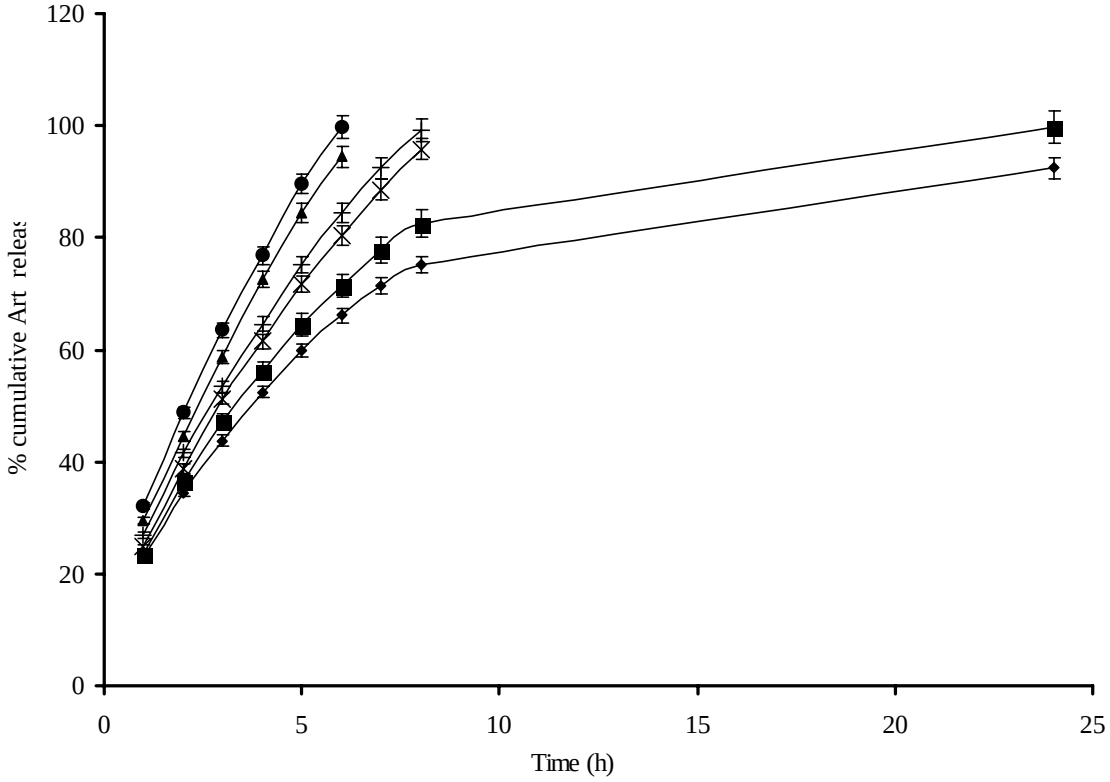

Fig. 6. Cumulative release pattern of artemether from the various generations of different MPEG-Lysine peptide dendritic micelles, where ▲ represents release from MPL2K05G; X from MPL2K15G; ◆ from MPL2K25G; ● from MPL5K05G; + from MPL5K15G and ■ from MPL5K25G generations of micelles.

The various micellar carriers were tested for hydrolytic attacks by HCl by keeping in mild HCl at room temperature for a definite period of ten days and observed for drug degradation pattern every day upto ten days. For micellar carriers low molecular weight MPEG carriers (MPL2K25G) were found less stable as compared to high molecular weight MPEG carriers. There was appearance of ART crystallization from such carriers in 5-6 days and much heavy crystallization was observed by 10^{th} day.

The higher molecular weight micellar carriers were more stable chemically even at lower temperature and at room temperature as evident from change in chemical nature by percentage increase in developed color intensity by Kaiser reagent (Table 3). The changes in chemical nature as determined by percentage increase in developed colour intensity by Kaiser test showed more colour development for lower MWt. carriers (MPL2K25G) as to MPL5K25G, which was by 10^{th} day only 4.7% as compared to 14.2% for MPL2K25G. This could be attributed to molecular weight of MPEG, where increase in number of molecular groups causes increased stability by its steric hindrance from the attack of HCl (Fig. 7).

Encapsulation of ART in micelles also stabilized the drug from outer environment as evidenced by HCl hydrolytic attack for the entrapped drug. The HCl induced hydrolytic attack on encapsulated drug after incubation showed that there was increased amount of hydrolyzed drug in aqueous milieu from free drug solutions as compared to the drug entrapped in micellar dendrimers (Fig. 7). The studies correlated well with the reports on stabilizing DNA within dendrimers against nuclease attack carried out by Rackstraw et al (31).

The various ART loaded micellar carrier were tested for stability on dilution with water. More drug crystallization was found from higher molecular weight MPEG micelles MPL5K25G on dilution because of higher CMC values so at increased dilutions they were producing drug crystals.

Table 3. Accelerated Stability studies of Artemether loaded MPEG-Lysine (MPL5K25G) dendritic micelles of different molecular weights of MPEG tails

Parameters		MPL2K25G			MPL5K25G		
		0°C	RT	50°C	0°C	RT	50°C
Turbidity (after 15 days)		++	+	+++	+	+	++
Precipitation (after 15 days)		++	+	+++	+	+	++
Crystallization (after 15 days)		++	+	++	+	-	+
Change in chemical nature (% increase in developed colour intensity)	5 days	1.6%	0.6%	8.5%	1.2%	0.4%	6.8%
	10 days	2.2%	0.8%	16.8%	1.7%	0.7%	13.2%
	15 days	2.9%	1.2%	27.9%	2.2%	0.9%	18.5%
% Increase in drug leakage	5 days	0.9%	0.5%	9.1%	0.6%	0.4%	7.5%
	10 days	1.4%	0.8%	13.9%	0.8%	0.6%	11%
	15 days	1.9%	1.1%	18.7%	1.1%	0.9%	15%
% Degradation of ART (By appearance of absorbance on hydrolysis at 256 nm)	5 days	1.2%	4.3%	9.6%	0.7%	2.2%	6.3%
	10 days	10.7%	18.6%	30.3%	7.4%	12.3%	21.5%
	15 days	18.2%	35.7%	65.2%	14.3%	29.7%	54.3

'-' indicates no change; '+' indicates smaller change; '++' considerable change; '+++' enough change as compared to initial.

This could be attributed to the intrinsic solubility of drug freed from the micelles in water, after disruption of micelles, as the concentration of micelles falls below CMC that was earlier preventing the drug from precipitation or crystallization from lower molecular weight carriers on dilution. The lower CMC values and micellar aggregates reduced drug crystallization by its release on dilution upto some extent but after a definite dilution there is a breakdown of aggregates and release of drug causes the drug concentration to exceed its solubility in aqueous milieu.

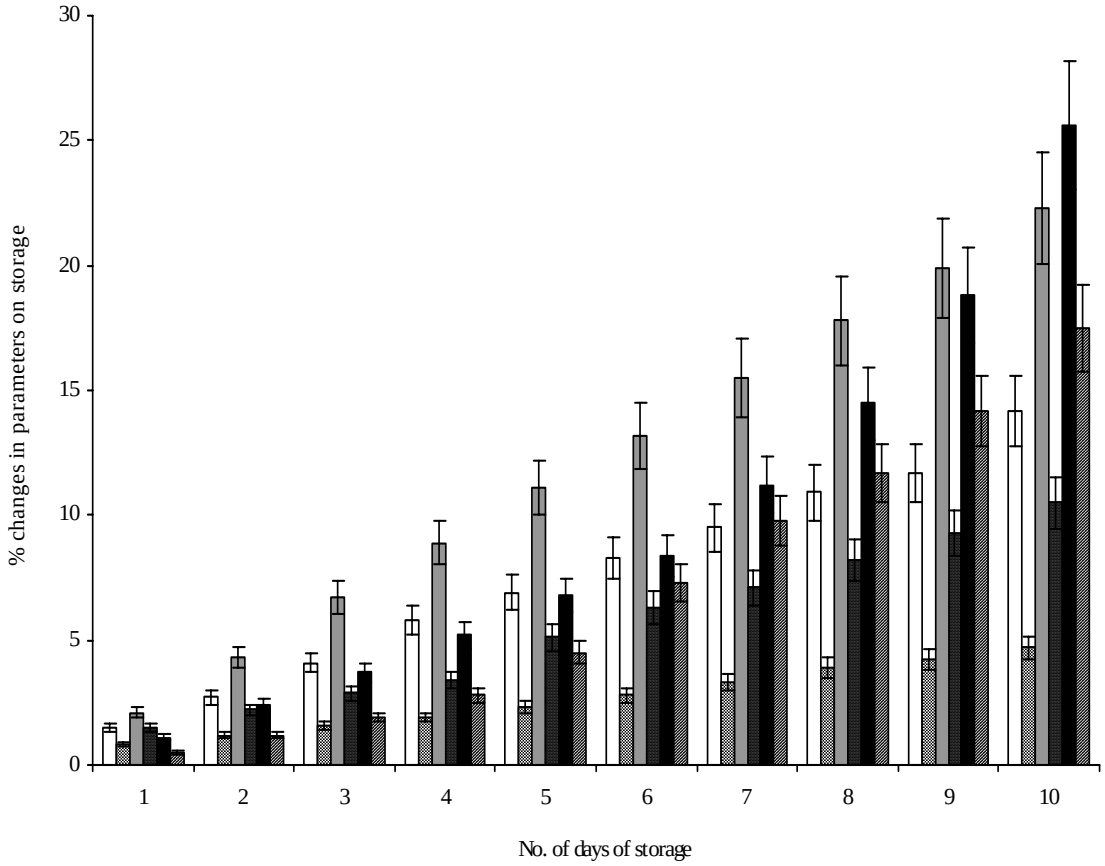

Fig. 7. Hydrolytic stability profile of various Artemether loaded dendrimeric micellar carriers on storage for various time intervals of storage, where □ is related to percentage changes in chemical nature of MPL2K25G and ▨ MPL5K25G dendrimeric micelles; ▒ is related to percentage changes in entrapment of artemether in MPL2K25G and ▓ in MPL5K25G; and ■ is percentage degradation of artemether with time intervals of storage when present in MPL2K25G and ▨ when present in MPL5K25G.

It can thus be concluded that micellar carriers of lower generations and lower molecular weight carriers can simply be well diluted and administered intravenously as compared to higher generations and higher molecular weight MPEG carriers. The work is only representation of a newer and novel type of amphiphilic micellar carrier having at one end PEG and other end is hydrophobic due to the presence of FMOC termination of protected essential amino acid l-lysine. The systems were found very suitable for solubilization and encapsulation of hydrophobic drugs like artemether, by hydrophobic interactions within their FMOC terminus that is in dendrimeric form as branched structure as shown in RasMol representations. The toxicity of such systems might be very less due to their organization in the form of densely clubbed hyper-branched micellar structures and possibly slow rate of degradation, which would be further studied. All this can also be attributed to PEG ends which can reduce toxicity of many toxic drugs, when coupled with them, by the control of bioavailability.

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REFERENCES

[1] Otsuka, H.; Nagasaki, Y. and Kataoka, K., PEGylated nanoparticles for biological and pharmaceutical applications. *Adv Drug Deliv Rev*, 55(3): 403-419, 2003.

[2] Bhadra, D.; Bhadra, S.; Jain, P. and Jain, N. K., Pegnology: a review of PEG-ylated systems. *Pharmazie*, 57(1): 5-29, 2002.

[3] Bhadra, D.; JAIN, S.; Bhadra, S.; Jain, R. and Jain, N. K., PEGylated Dendrimers for delivery of 5-fluorouracil. *Control. Rel. Soc. Proc.*, San Diego, USA, 2001.

[4] Bhadra, D.; Bhadra, S.; Jain, S. and Jain, N.K., A PEGylated dendritic nanoparticulate carrier of fluorouracil. *Int J Pharm*, 257: 111–124, 2003.

[5] Yang, H.; Morris, J.J. and Lopina, S.T., 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(1): 148-154, 2004.

[6] Moorefield, C.N. and Newkome, G. R., Unimolecular micelles: supramolecular use of dendritic constructs to create versatile molecular containers. *Comptes Rendus Chimie*, 6(8-10): 715-724, 2003.

[7] Ooya, T.; Lee, J. and Park, K., Effects of ethylene glycol-based graft, star-shaped, and dendritic polymers on solubilization and controlled release of paclitaxel. *J Control Rel.*, 93(2): 121-127, 2003.

[8] Bhadra, S.; Bhadra, D. and Agrawal, G.P., Amphiphilic copolymeric micelles for delivery of nimesulide: Preparation, Optimization and Characterization. *Ind J Pharm Sc*, 65(2): 139-145, 2003.

[9] Lukyanov, A.N. and Torchilin, V.P., Micelles from lipid derivatives of water-soluble polymers as delivery systems for poorly soluble drugs. *Adv Drug Deliv Rev*, 56(9): 1273-1289, 2004.

[10] Francis, M. F.; Lavoie, L.; Winnik, F. M. and Leroux, J.C., Solubilization of cyclosporin A in dextran-g-polyethyleneglycolalkyl ether polymeric micelles. *Eur J Pharm Biopharm*, 56(3): 337-346, 2003.

[11] Huo, F.; Xu, H.; Zhang, L.; Fu, Y.; Wang, Z. and Zhang, X., Hydrogen-bonding based multilayer assemblies by self-deposition of dendrimer. *Chem Commun (Camb)*, 7: 874-875, 2003.

[12] Bhadra, D.; Bhadra, S.; Jain, N. K. In Jain, N.K. (Ed.) *Progress in Controlled and Novel Drug Delivery Systems*, CBS Publishers and Distributors, New Delhi, 1st ed, pp 209-247, 2004.

[13] The use of artemisinin & its derivatives as anti-malarial drugs world health organization. WHO, WHO/MAL/98/1086 Geneva, Malaria Unit Division of Control of Tropical Diseases, World Health Organization, 1998.

[14] *International Pharmacopoeia.* World Health Organization, Avenue Appia, Geneva, Switzerland, 3rd ed. Vol. 5, pp187-198, 2003.

[15] Li, Q.; Peggins, J.O.; Fleckenstein, L.L.; Masonic, K.; Heiffer, M.H. and Brewer, T.G., The pharmacokinetics and bioavailability of dihydroartemisinin, arteether, artemether, artesunic acid and artelinic acid in rats. *J Pharm Pharmacol*, 50: 173–182, 1998.

[16] Kolhe, P.; Misra, E.; Kannan, R.M.; Kannan, S. and Lieh-Lai, M., Drug complexation, in vitro release and cellular entry of dendrimers and hyperbranched polymers. *Int J Pharm*, 259: 143-160, 2003.

[17] Bhadra, D.; Bhadra, S.; Jain, S. and Jain, N.K., A PEGylated dendritic nanoparticulate carrier of fluorouracil. *Int J Pharm*, 257(1-2): 111-124, 2003.

[18] Bentley, M. D.; Zhao, X. and Clark, J. L., Water-soluble polymer conjugates of artelinic acid. *US Patent*, 6,461,603., 2002.

[19] Bhadra, D.; Bhadra, S. and Jain, N. K., Pegylated Peptide Based Dendritic Nanoparticulate Systems for Delivery of Artemether. *STP Pharm. Sc.* Theme issue 2005 (in Press).

[20] Bhadra, D.; Bhadra, S. and Jain, N. K., PEGylated-poly-l-lysine dendrimers for delivery of Chloroquine phosphate. International Conference on MEMS, NANO, and Smart Systems. Alberta, Canada, 2004.

[21] Zalipsky, S.; Gilon, C. and Zilkha, A., Attachment of drugs to polyethylene glycols. *Eur Polym J*, 19(12): 1177-1183, 1983.

[22] Lapatsanis, L.; Milias, G.; Froussios, K.; Kolovos, M., Synthesis of N-2,2,2-(Trichloroethoxycarbonyl)-l-Amino acids and N-(9-Fluorenylmethoxycarbonyl)-l-amino acids involving succinimidoxy anion as a leaving group in amino acid protection. *Synthesis*, 671-673,1983.

[23] Goni, F. M. and Alonso, A., Spectroscopic techniques in the study of membrane solubilization, reconstitution and permeabilization by detergents. *Biochim Et Biophys Acta*, 1508: 51-68, 2000.

[24] Higuchi, T.; Connors, K.A. in: Reilly, C.N. (Ed.), Advances in analytical chemistry and instrumentation. Vol. 4, Interscience, New York, 117-212, 1965.

[25] Thomas, C.G.; Ward, S.A. and Edwards, G., Selective determination in plasma, of artemether and its major metabolite. Dihydroartemisinin, by High Performance Liquid chromatography with ultraviolet detection. *J. Chromatograph*, 583: 131-136, 1992.

[26] Sarin, V.K.; Kent, S.B.H.; Tam, J.P. and Merrifield, R.B., Quantitative monitoring of solid phase peptide synthesis by the Ninhydrin reaction. *Anal Biochem*, 117: 147-157, 1981.

[27] Sadler, K. and TAM, J. P., Peptide dendrimers: applications and synthesis. *Rev Mol Biotech*, 90: 195-229, 2002.

[28] Choi, J. S.; Lee, E. J.; Choi, Y. H.; Jeong, Y.J. and Park, J. S., Poly(ethylene glycol)-block-poly(L-lysine)

dendrimer: novel linear polymer/dendrimer block copolymer forming a spherical water-soluble polyionic complex with DNA. *Bioconjug Chem,* 10(1): 62-65, 1999.

[29] Choi, J S.; Joo, D. K.; Kim, C. H.; Kim, K. and Park, J. S., Synthesis of a Barbell-like Triblock Copolymer, Poly(L-lysine) Dendrimer-*block*-Poly(ethylene glycol)-*block*-Poly(L-lysine) Dendrimer, and Its Self-Assembly with Plasmid DNA. *J Am Chem Soc,* 122*:* 474-480, 2000.

[30] Huibers, P. D. T.; Lobanov, V. S.; Katritzky A. R.; Shah, D. O. and Karelsonr, M., Prediction of Critical Micelle Concentration Using a Quantitative Structure-Property Relationship Approach. 1. Nonionic Surfactants *Langmuir*, 12, 1462-1470, 1996.

[31] Rackstraw B.J.; Stolnik, S.; Davis, S.S.; Bignotti, F. and Garnett, M.C., Development of multi-component DNA delivery systems based upon poly(amidoamine)-PEG-co-polymer. *Biochem et Biophys Acta*, 1575, 269-286, 2002.