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Prolonged Drug Delivery System of PEGylated PAMAM Dendrimers with a Anti-HIV Drug

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Polyamidoamine (PAMAM) dendrimers is a new non viral drug carrier. However their high surface toxicity limits PAMAM dendrimers application in drug delivery. The purpose of present work was aimed to developing and exploring PEGylated G4 and G5 PAMAM dendrimers for anti HIV drug lamivudine. In this study we successfully prepared G4 and G5 PAMAM dendrimers with ethylene diamine core and PEGylated with MPEG for surface modifications. Further physiochemical and physiological parameter such as UV, IR, TEM, DSC, drug entrapment, drug release and hemolytic toxicity of both PEGylated and non PEGylated PAMAM dendrimers were determined and compared. Here the PEGylation of PAMAM dendrimers reduce the surface toxicity and increase the drug loading capacity of PAMAM dendrimers. Moreover PEGylated PAMAM dendrimers had released then drug in controlled and prolonged time. Hence the PEGylated PAMAM dendrimers were found as suitable drug delivery carrier for anti HIV drug lamivudine.

Key words: PAMAM, Dendrimers, Anti-HIV, PEGylation.

Dendrimers are new class of artificial macromolecules which have attracted much interest because of their unique structures and properties (Bosman *et al* 1999; Tomalia *et al* 2002; Newkome *et al* 1999; Hawker *et al* 1993; Jansen *et al* 1994.). Their size, structure, and surface properties are highly controllable. In addition, their interiors cavities can encapsulate small drug molecules (Liu *et al* 1999; Stiriba *et al* 2002; Boas *et al* 2004; Haba *et al* 2007.). Considering these features, dendrimers are highly attractive materials for application in the biomedical field for tasks such as drug delivery and diagnosis (Tomalia 2005; Lee *et al* 2005; Medina *et al* 2009; Wolinsky *et al* 2008; Tekade *et al* 2009.). Among the numerous dendrimers used for drug delivery, poly amido amine (PAMAM) and poly propylene imine (PPI) dendrimers are the two commercially available and most investigated ones. They were reported to effectively improve solubility, stability, and deliver efficacy, decrease side-effects, and tailor pharmacokinetic and pharmacodynamic behaviours of several families of drugs, which reveal the promising future of dendrimer-based drug delivery systems. Dendrimers biodistribution and toxicity of dendrimers are strongly affected by surface functionalities. Moreover, modification of their surfaces with poly(ethylene glycol) (PEG)

markedly decreases their toxicity and improves their circulation time, which might contribute to their accumulation at target sites, associated with angiogenesis through so-called enhanced permeation and retention effects (Haba *et al* 2007). In addition, attachment of PEG might increase its ability to encapsulate drugs by enhancing hydration around the dendrimer periphery (Umeda *et al* 2010; Kojima *et al* 2000.). Acquired immunodeficiency syndrome (AIDS) is a degenerative infectious disease of the immune system caused by the human immunodeficiency virus (HIV) (Broder *et al* 1984; Price *et al* 1988.). The HIV infection, which targets the monocytes expressing surface CD4 receptors, eventually produces profound defects in cell-mediated immunity. Overtime infection leads to severe depletion of CD4 T-lymphocytes (T-cells) resulting in opportunistic infection (OIs) like tuberculosis (TB), fungal, viral, protozoal and neoplastic diseases and ultimately death (Bowen *et al* 1985; Tavel *et al* 1999; Simon *et al* 2006; Grossman *et al* 2006.). The search for an effective chemotherapeutic treatment against HIV infection has led to the development of agents that target specific and critical events in the HIV replicative cycle. The best known and the most intensively studied active drugs against HIV are reverse transcriptase (RT) inhibitors, viral protease inhibitors, entry inhibitors and, more recently, integrase inhibitors(Calogeropoulou *et al* 2003; Piacenti 2006; De Clercq 2007; Klivanov *et al* 2009; Dau *et al* 2009; De Clercq 2007). Lamivudine (LMV) is an important antiretroviral drug belonging to the category of reverse transcriptase inhibitors. Lamivudine has been shown to be somewhat less toxic than other nucleoside reverse transcriptase inhibitors (NRTIs) and active against zidovudine-resistant HIV isolates (Perry 1997; Soudeyns 1991; Chu 1991; Coates 1992.)[29-32]. Intracellularly, LMV is phosphorylated to its active triphosphate derivative (lamivudine triphosphate), which inhibits HIV reverse transcription via viral DNA chain termination. In addition, lamivudine triphosphate inhibits both then RNA and DNA dependent DNA polymerase activities of reverse transcriptase, and is a weak inhibitor of mammalian α, β, and γ DNA polymerases. Several studies in HIV-1 infected patients have shown that treatment failure and adverse effects are associated with low and high plasma concentrations of antiretroviral bioactives including LMV. Hence administration of LMV directly to the HIV infected cells is highly desirable. Decreasing the required drug doses and preventing or minimizing their action on non-infected cells would also reduce the harmful side effects (Product Monograph GlaxoSmithkline Shire Biochem 2004; Thomas 2004.).

The present study was aimed at developing and exploring the use of PEGylated newer (ethylene diamine) EDA-PAMAM dendrimers for delivery of anti-hiv drug, lamivudine (LMV). Here selection of lamivudine in PEGylated (EDA)- PAMAM dendrimers was based on its anti HIV activity, short biological half-life and solubility characteristics. PEGylation of EDA- PAMAM dendrimers establishes suitability of PEGylated dendrimer as a drug delivery system for LMV. It was observed from the hemolytic study that this delivery system could be safely administered through i.v. route. We envisaged that current approach will improve the management of drug therapy in HIV patients by delivering the drug at a controlled rate for a prolonged period of time.

MATERIALS AND METHODS

Materials

Ethylene diamine (EDA) and methylmethacrylate (CDH, India), methanol (Rankem, India). MPEG2000 (Sigma, Germany), Cellulose dialysis bag (MWCO 12-14 Kda, Himedia, India), 4 dimethyl amino pyridine (sd-fine chemicals, India), Lamivudine was a benevolent gift from Ranbaxy labs Ltd, India. All other chemicals were reagent grade and used without further modification.

Synthesis of PAMAM Dendrimers

PAMAM dendrimers of 4th and 5th generations (G4 and G5) were prepared through reported Michal addition and amidate reaction (Prajapati *et al* 2009). Briefly in alight resistance environment EDA was reacted with methnolic solution of methyl acrylate (5% molar excess) to form ester terminated dendrimer. Then excess of methnolic solution of EDA (10% excess) for 55h in dark. PAMAM dendrimer G4 and G5 were produced by repeated above mentioned reaction sequence. Copper sulphate color reaction was carried out to confirm the completion of the each step. Further synthesised PAMAM dendrimer were characterized by FTIR and H-NMR.

Synthesis of MPEG 4-Nitrophenyl Carbonate

M-PEG 4- nitro phenyl carbonate was proposed by reacting M-PEG with nitro phenyl carbonate (Kojima *et al* 2000). In THF (400ml) M-PEG (0.05mmol) were added to above solution in gradual manner for 1h followed by string in room temperature for 48 h. Finally the reaction mixture was evaporated to yield M-PEG 4 nitro phenyl carbonate. Further recrystallizations of M-PEG 4 nitrophenyl carbonate mixture for chloroform – diethyl ether (10:1, total volume 300- 400ml) to produced purified form M-PEG 4 nitro phenyl carbonate.

Conjugation of MPEG to PAMAM

Synthesised G4 or G5 PAMAM dendrimers were reacted with M-PEG 4 nitro phenyl carbonate to undergo PEGylation (Kojima *et al* 2000). Briefly in dimethyl sulfoxide (1ml) PAMAM dendrimers G3 or G4 (0.5µmol) was dissolved and solution was stirred to react at room temperature for 3 to 6 days (based on the generation of PAMAM dendrimers and PEG modification ratio). Then resulted reaction mixture was dialyzed against distilled water for 72h. Lyophilisation of above solution will yield PEG-PAMAM dendrimer.

Drug Loading in Formulations

PEGylated (G4 & G5) non PEGylated PAMAM dendrimers (G4 & G5) were dissolved separately in methnol and mixed with aqueous solution of lamivudine (100mol). [37] Further incubation of above solution was continued with string for 24 h at 25°C. Then removal of free drug from the formulation was carried out by dialyzing in cellulose dialysis bag (mwco1000da sigma, Germany)against double distilled water for 10 min. Spectrophotometrically estimation was done for above solution (λmax 272nm) (uv.1601 shimadz japan) to determine indirectly amount of drug loaded in the formulation. Further lyophilization of formulation was done and used for further characterization.

Morphology of the Dendrimers

Transmission electron microscopy (TEM) was performed to investigate particle size and provide information on nanoparticle morphology. Prepared and dialyzed lamivudine loaded dendrimer formulations were used for Transmission electron microscopic studies. The TEM studies were carried out using 3mm Forman (10.5% plastic powder in amyl acetate) coated copper grid (300 mesh) at 60 Kv using negative staining by 2% phosphotungstic acid (PTA) for whole generation of dendrimers at 150,000X magnification on Philips CM-10 TEM and Fei-Philips Morayagni 268D with digital TEM image analysis system at 50-60 Kv.

Differential Scanning Calorimetry

Thermal stability and crystallinty changes over range of temperature of LMV loaded PEGylated PAMAM dendrimer, drug and PEGylated PAMAM dendrimer were studied by differential scanning calorimetry. In aluminium pan known quantity of sample was placed and crimped with lid further pan was analyzed in the sample cell for DSC module. (DSC

Q10 V9.0 Build 275, TA Instruments, USA). Temperature in the DSC module was increased by 10°C/min from 35°C equilibrated temperature under a N_2 gas purge. Temperature stability and phase transition of sample were obtained from peak in the resulting curve.

Drug Release studies

Drug release from known amounts of LMV loaded PEGylated G4 and G5 PAMAM dendrimers were determined by dialysis method (Vijayaraj Kumar *et al* 2007). The dialysis bags were filled with a known mass of LMV loaded PEGylated dendritic architectures (MWCO 1000 Da) and the dialysis bags were placed in 50 ml of PBS (pH 7.4) at 37^{o}C with slow magnetic stirring under sink conditions. Aliquots of 1 ml were withdrawn from the external solution and replaced with the same volume of fresh PBS. The drug concentration was detected in a spectrophotometer at 272nm λmax.

Hemolytic Toxicity of Dendrimer–Drug Systems

Briefly, in HiAnticlot blood collection vials (Himedia Labs, India) RBC suspension (5% hematocrit) of the human blood was collected (Vijayaraj Kumar *et al* 2007). In normal saline (4.5ml), LMV encapsulated PEGylated, non PEGylated formulations, drug solution and dendrimers solution (0.5ml) was added in incubated for 1h with RBC suspension. The drug and dendrimers in separate tubes were taken in such amount that the resultant final concentrations of drug and dendrimer were equivalent in all the cases. The PEGylated system of dendrimer–drug complex was taken in amount such that the resultant final concentrations of drug and dendrimer were equivalent to that in non-PEGylated systems. This allowed comparison of the hemolysis data of the drug, dendrimer, LMV loaded PAMAM dendrimers and PEGylated dendrimers to assess the effect of PEGylation on hemolysis. After centrifugation, supernatants were taken and diluted with an equal volume of normal saline and absorbance was measured at 272nm. To obtain 0 and 100% hemolysis, RBC suspension was added to 5 ml of 0.9% NaCl solution (normal saline) and 5 ml distilled water, respectively. The degree of hemolysis was determined by the following equation:

$$\textit{Hemolysis (\%)} = \textit{Abs-Abso / Abs100-Abso} \times 100$$

Where *Abs, Abs100,* and *Abso* are the absorbance of sample, a solution of 100% hemolysis, and a solution of 0% hemolysis; respectively.

Stability Studies of PEGylated Dendrimer Formulations

Stability studies were carried out for LMV loaded PEGylated G4 and G5 PAMAM dendrimers. Here sample was stored in 40^{o}c for three months. Drug content and drug release studies were carried out to analyze the stability of the formulation.

RESULTS AND DISCUSSION

Synthesis of PAMAM Dendrimers

PAMAM dendrimers of G4 and G5 were synthesized employing ethylenediamine (EDA) as initiator core and methylacrylate in appropriate molar ratio was confirmed with copper sulphate chelation reaction in which half-generations gave a deep blue colour while a full generation dendrimers gave a purple color. Further confirmation of synthesized dendrimer was carried out by FT-IR, 1H-NMR and microscopic studies. In case of FT-IR spectrum of G4 PAMAM dendrimer, the presence of peaks at 3438.5 cm^{-1} (N-H stretching of primary amine); 1731.1 cm^{-1} (C=O stretching of ester); 1650.6, 1583.2 cm^{-1} (-NH-CO stretching of amide); 1439.9, 1387.8 cm^{-1} (N-H bending of N substituted); 1208.5, 1030.2 cm^{-1} (C-O stretching) and at 2670 cm^{-1} (C-H bending peaks) confirmed the synthesis. Similarly in the FT-IR spectrum

of G5 PAMAM dendrimer, peaks at 3350.2 cm^{-1} (N-H stretching of primary amine); 3190.0 cm-1 (N-H stretch anti-symmetric of substituted primary amine); 2890.0 cm-1 (C-H stretch); 1641.3 cm^{-1} (C=O stretch of carbonyl group); 1566.0 cm^{-1}, 1327.9 cm^{-1} (N-H bending of N substituted amide); and 1198.5 cm^{-1} (C-C bending) confirmed the synthesis.

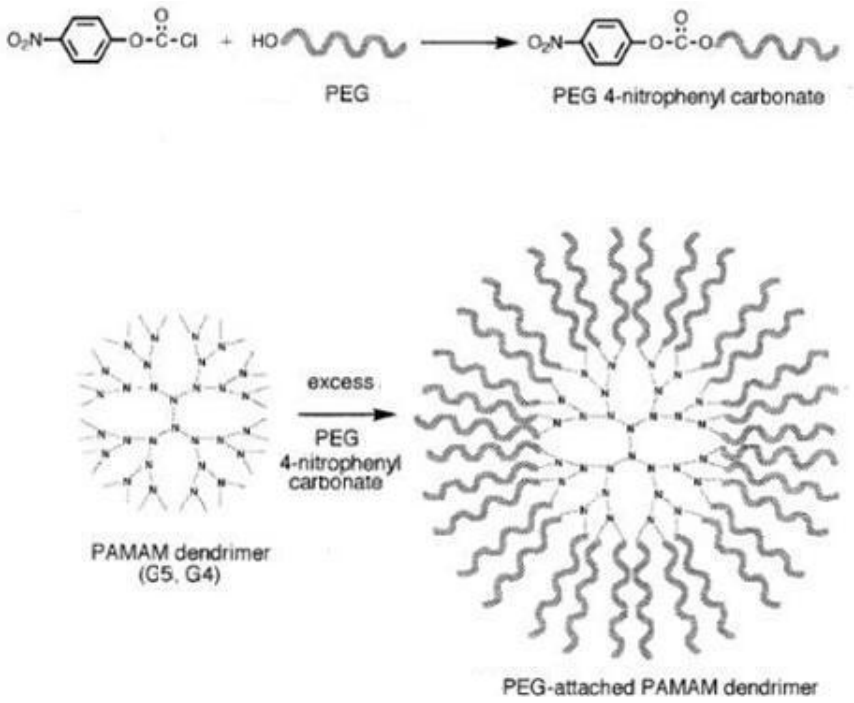


Fig. 1. Synthesis of MPEG attached PAMAM dendrimers

Conjugation of MPEG-PAMAM Dendrimers

The Conjugated synthesis of MPEG-PAMAM dendrimers was confirmed by out by FT-IR, ^{1}H-NMR and microscopic studies. In IR spectra, the MPEG-PAMAM dendrimers showed peaks at 3439 cm^{-1} for N-H stretching and 1379 cm^{-1} for N-H bending confirmed the Conjugation synthesis of MPEG-PAMAM dendrimers. The recorded IR spectra of representative MPEG 4-Nitrophenyl Carbonate showed nitro group band at 1432 cm^{-1} and aromatic band at 3012 cm^{-1}. This peaks are missing in MPEG-PAMAM dendrimers, it's clearly envisages that the nitro group and aromatic ring of MPEG 4-Nitrophenyl Carbonate is converted into secondary NH. The proton magnetic resonance spectra of MPEG-PAMAM dendrimers and their corresponding derivatives have been recorded in $CDCl_3$. In this NH signal of MPEG-PAMAM dendrimers appear at 7.27-7.92 (s) ppm respectively. The position and presence of NH signal in the ^{1}H-NMR spectra of final compounds conforms the secondary NH proton in MPEG-PAMAM dendrimers. All these observed facts clearly demonstrate that the MPEG 4-Nitrophenyl Carbonate is converted into secondary amino group as indicated and conforms the proposed structure of MPEG-PAMAM dendrimers.

Drug Loading

Since PEGylated PAMAM dendrimer has basic and hydrophobic interior. The entrapment efficiency of LMV in PEGylated PAMAM dendrimer is driven by non covalent interaction. The entrapment efficacy of LMV in PEGylated PAMAM dendrimer was increased when compared with PAMAM dendrimers (**Table 1**). The significant increase in entrapment of

LMV in PEGylated PAMAM dendrimers with respect to that of PAMAM dendrimers might due to more interaction of drug and mpeg at peripheral portion of dendrimers.

Table 1. Drug Entrapment and Hemolytic Studies

Formulation	% Drug Entrapped	% Hemolytic studies
EDA-PAMAM dendrimer G4	28.21±1.45	15.21±1.56
EDA-PAMAM dendrimer G5	43.45±1.86	23.21±2.24
PEG-PAMAM dendrimer G4	54.36±1.23	1.27±0.34
PEG-PAMAM dendrimer G5	71.54±1.56	1.94±0.56

Morphology of the Dendrimers

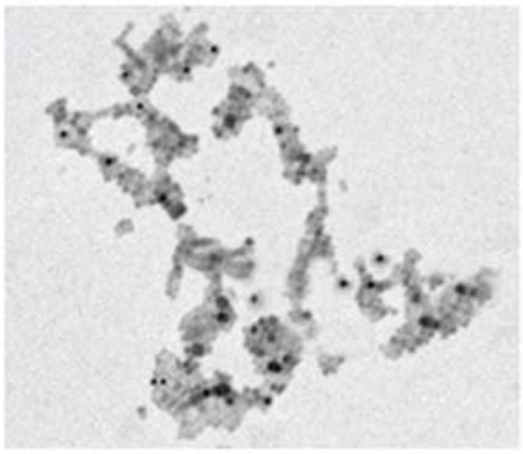
Agglomeration of drug loaded dendrimers lead to form spherical shape dendrimers were confirmed by the TEM micrographs (**Fig. 2a and 2b**).

Differential Scanning Calorimetry

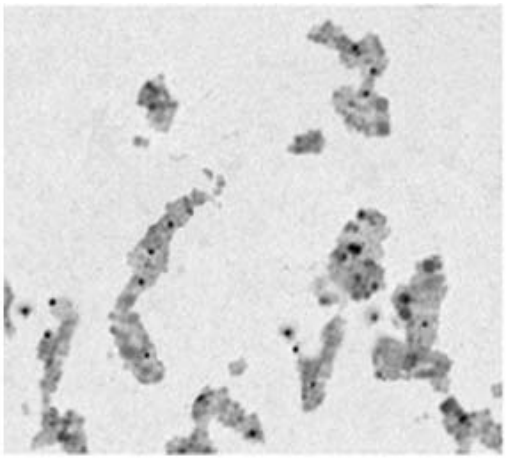
DSC curves (**Fig 3b**) showed that LMV loaded PEGylated PAMAM dendrimers was not a physical mixture by endothermic and exothermic transition. DSC graph (**Fig 3a**) of Lamivudine showed their characteristic peak at 179^0c. Absence of characteristic peak of LMV in the DSC of PEGylated PAMAM G5 dendrimer (**Fig 3b**) confirmed the drug encapsulation in PAMAM dendrimers.

Drug Release studies

The drug release profile of LMV from non PEGylated PAMAM dendrimer and PEGylated PAMAM dendrimer were shown in **Fig 4**. The drug release profile showed that release of LMV from PEGylated PAMAM dendrimer was significant slower drug release when compared with non PEGylated PAMAM dendrimers. While non PEGylated PAMAM dendrimers release the drug was 24 h and 36 h respectively and PEGylated PAMAM dendrimer release the drug were 96h and 120h. The fact that shows slow release of drug by PEGylated PAMAM dendrimers was due hydrophobic interaction between drug and core of dendrimer. Moreover difference in the number of terminal peg groups also contributes to the slow release of drug.

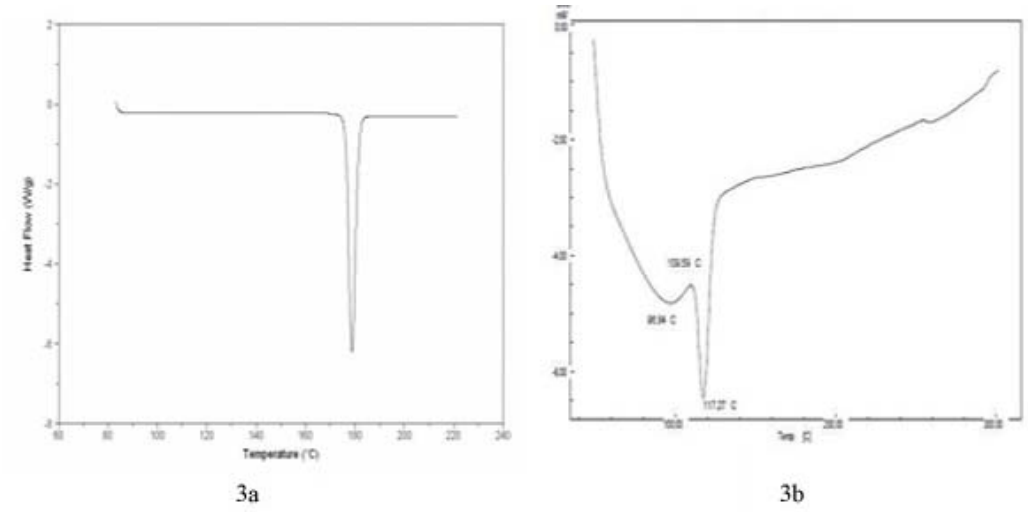


2a



2b

Fig. 2a. Tem image of LMV loaded G5 PEG PAMAM dendrimer, **2b.** Tem image of LMV loaded G5 PEG PAMAM dendrimer



3a 3b

Fig. **3a.** DSC of pure Lamivudine, **3b.** DSC of drug loaded G5 PEG- PAMAM dendrimer

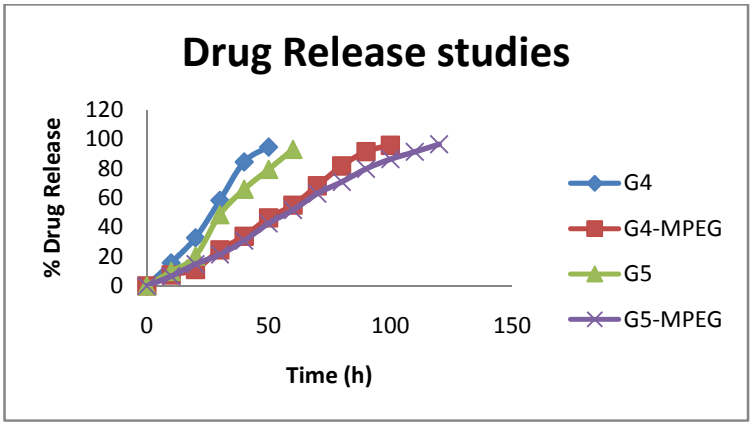


Fig 4. Drug release studies

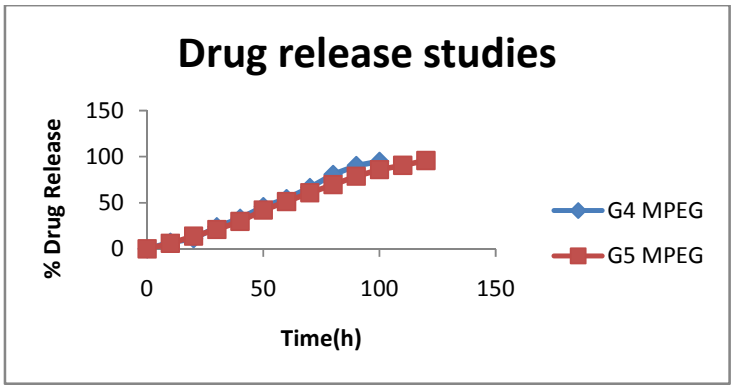


Fig 5. Drug release studies after storage of three months

Hemolytic Toxicity

Hemolytic toxicity of non PEGylated amine terminated PAMAM dendrimers showed 15-25% toxicity. But in PEGylated PAMAM dendrimers haemolysis (toxicity) of RBCs were reduced to 2%. Reduction of haemolysis (toxicity) might be due to surface modification of PAMAM dendrimers by PEGylation. In PEGylation inhibition interaction between RBCs and quaterny ammonium ion occurs which reduces cytotoxicity nature of PAMAM dendrimers.

Stability

Three months storage of LMV loaded PEGylated PAMAM dendrimers at 40±2^{0}C showed no change in appearance and redispersing ability. Moreover there was no significant difference in potency and cumulative % drug release (**Table 2** and **Fig 5**).

Table 2. Stability studies of Drug loaded PEG-PAMAM dendrimers

Formulation	Appearance	% Drug release
PEG-PAMAM dendrimer G4	Pale yellow color	53.57±2.34
PEG-PAMAM dendrimer G5	Pale yellow color	70.35±1.54

CONCLUSION

In this work we designed PAMAM having PEG grafts as a novel drug carrier. PEG were combined to essentially every chain of dendrimers with the generation G4 and G5. Moreover we prepared the PEGylated PAMAM dendrimers encapsulating the anti-HIV drug lamivudine. While encapsulating ability of PEGylated PAMAM dendrimers are increased when compared with non-PEGylated PAMAM dendrimers. Performed drug release studies of PEGylated PAMAM dendrimers showed prolong drug release for longer time when compared with non-PEGylated PAMAM dendrimers. Hemolytic toxicity studies revealed that PEGylated PAMAM dendrimers are relatively low toxicity and safer with non-PEGylated PAMAM dendrimers. Considering the features of PEGylated PAMAM dendrimers, such as highly controlled molecular size, biocompatible surface, encapsulation capacity of drug and prolong drug release made them ideal candidate for anti-HIV drug therapy. Thus finding obtained in this study provided important information for design PAMAM dendrimers as drug carrier for anti-HIV drugs. But further Pharmacokinetic and pharmacodynamic aspect of PEGylated PAMAM dendrimer required making them as novel drug carriers. So drug therapy management of HIV patient are expected to be improved by this approach.

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