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Regular Article

PEG Conjugated PAMAM Dendrimers with a Anti-HIV Drug Stavudine for prolong release

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The present investigation was aimed to design polyamidoamine (PAMAM) dendrimer having polyethylene glycol grafts as novel drug carrier. In this study we successfully synthesized and conjugated PEGylated PAMAM dendrimers with anti HIV drug stavudine. Hemolytic toxicity studies were carried out for PEGylated PAMAM dendrimers which showed that haemolysis of cell was very negligible in PEGylated PAMAM dendrimers. Moreover surface were characterization of PEGylated PAMAM dendrimers was carried out TEM micrographs. Further physiochemical and physiological parameter such as UV, DSC, drug entrapment and drug release were carried out for PEGylated and non PEGylated PAMAM dendrimers. Drug loading, drug release and toxicity are better for PEGylated PAMAM dendrimers.

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

Poly (amidoamine) (PAMAM) dendrimers are a new class of nanomaterials with three-dimensional structure. The highly branched polymers consisted of three basic units including an ethylenediamine core, repeating units, and terminal units (Zeng and Zimmerman 1997; Newkome *et al*. 2001; Crooks *et al*. 2001). PAMAM dendrimers are synthesized through a serial repetition of two reactions: Michael addition reaction of amino groups to the double bond of methyl acrylate, followed by amidation of the resulting methyl ester with ethylene diamine (Zeng and Zimmerman 1997). Polyamidoamine (PAMAM) dendrimers are nanoscale biomaterials with highly controlled branched structures and monodispersed array (Mw/Mn < 1.01); they are recognized as a unique class of synthetic macromolecules replacing traditional synthetic polymers such as linear, cross-linked, and branched polymers with relatively broad molecular weight distributions (Malik *et al.* 2000; Tomalia *et al*. 2002).

The globular structure of PAMAM dendrimers can be divided into two parts: the surface that has primary amine groups and the interior that contains tertiary amine groups, which are closely related with the

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capability of the complex formation with drug molecules and delivery effectively.

The advantage of biodegradable and biocompatible polymers has increased the versatility of polymers in the design of polymeric drug delivery devices for many classes of bioactive agents. These polymers have been used in various macromolecular architectures: linear, cross-linked, and branched (Tomalia *et al.* 2003). A lot of interest has been shown, in recent time, in the use of dendrimers for the design of delivery systems for many classes of bioactive agents. Dendrimers offer a number of advantages compared to other architectural forms of polymers that have been used in drug delivery systems. They have narrow polydispersity; they are in the nanometer size range, which can allow easier passage across biological barriers (e.g., small enough to undergo extravasation through vascular endothelial tissues); host-guest chemistry can take place either in the interior (binding groups in the interior of dendrimers are called endoreceptors) or on the periphery of the dendrimer (groups involved in complexation chemistry on the periphery of the dendrimers are called exoreceptors) (Tomalia *et al.* 1990).

However, PAMAM dendrimers are constrain to limit their applications because of their cytotoxicity, haemolysis and liver toxicity, which are thought that they are to interact with negatively charged cell surface (Neerman *et al*. 2004; Chen *et al*. 2004; Qiu *et al*. 2006; Zinselmeyer *et al*. 2002). Polyethylene glycols (PEG) were conjugated with various dendrimers to reduced cytotoxicity and incorporate the biocompatibility, and improve the physicochemical characteristics of them (Lee *et al*. 2005; Liu *et al*. 1999). Moreover it was verified that the chain length and the molar ratio of PEGs to PAMAM dendrimers could influence their drug-loading capacity (Hedden *et al*. 2003; Jevprasesphant *et al*. 2003; Yang *et al*. 2004).

Acquired immunodeficiency syndrome (AIDS) is a disease of the human immune system caused by the human immunodeficiency virus (HIV) (Broder *et al*. 1984; Bowen *et al*.1985). This condition progressively reduces the effectiveness of the immune system and leaves the individual susceptible to opportunistic infection and tumor. It is transmitted through direct contact of a mucous membrane or the bloodstream with a bodily fluid containing HIV, such as blood, semen, vaginal fluid, pre-seminal fluid, and breast milk (Embretson *et al*. 1993; Price *et al*. 1988). The antiviral therapy has unpleasant side effects, including peripheral neuropathy, acute pancreatitis, abdominal pain, diarrhoea, malaise, nausea, and fatigue. AIDS patients are generally treated with nucleoside or nucleotide reverse transcriptase inhibitors that inhibit reverse transcription by blocking the reverse transcriptase enzyme responsible for conversion from single-stranded RNA to double-stranded DNA in HIV. Zidovudine, didanosine, zalcitabine, stavudine, lamivudine, and abacavir are nucleoside analogs and tenofovir and adefovir are nucleotide analogs used as reverse transcriptase inhibitors for HIV infection (Tavel *et al*. 1999).

In the present study we selected short half-life and poor bioavailability drug stavudine (STV) and PEGylated PAMAM dendrimers as a drug carrier. Here drug stavudine was conjugated with PEGylated PAMAM dendrimers (G4 & G5) for delivery of a drug to overcome the problems of short half-life, poor bioavailability, and strong side effects.

MATERIALS AND METHODS

Materials

MPEG2000 (Sigma, Germany), Ethylene diamine (EDA) and methylmethacrylate (CDH, India), methanol (Rankem, India), 4 dimethyl amino pyridine (sd-fine chemicals, India), Cellulose dialysis bag (MWCO 12-14 Kda, Himedia, India), stavudine was a benevolent gift from Hetro labs Ltd, India. PAMAM dendrimers G4 &G5 were

synthesised by divergent approach and extensively characterized as reported earlier in Prajapati *et al.*2009. All other chemicals were reagent grade and used without further modification.

Synthesis and conjugation of MPEG 4-Nitrophenyl Carbonate

For the synthesis of M-PEG 4- nitro phenyl carbonate, polyethylene glycol was reacted with nitro phenyl carbonate (Kojima *et al.* 2000). Briefly the reaction was carried out by addation of THF (400ml) to M-PEG (0.05mmol) in gradual manner for 1h and continues string for 48 h in room temperature. The resultant reaction mixture was evaporated to yield M-PEG 4 nitro phenyl carbonate. Recrystallization was carried out in chloroform – diethyl ether (10:1, total volume 300- 400ml) to yield purified form M-PEG 4 nitro phenyl carbonate.

Further conjugation of G4 or G5 PAMAM dendrimers with synthesised M-PEG 4 nitro phenyl carbonate to produce PEGylated PAMAM dendrimers (Kojima *et al.* 2000). Briefly in dimethyl sulfoxide (1ml) PAMAM dendrimers G4 or G5 (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 and entrapment efficiency:

Drug loading was carried out by dialysis method. Here PEGylated (G4 & G5) non PEGylated PAMAM dendrimer methanolic solution was taken in dialysis bag (mwco1000da sigma, Germany) and immersed in the aqueous solution of stavudine and incubated for 24 h at 25^{o}C. The entrapment efficiency of dendrimer formulation were measured indirectly by spectrophotometrically estimating the amount of free drug in solution (λmax 266nm) (uv.1601 shimadz japan). Finally lyophilisation of content present in dialysis bag was done to produce drug loaded PEGylated and nonPEGylated dendrimer formulation (Vijayaraj Kumar *et al.* 2007).

Morphology of the Dendrimers

The surface morphology of PEGylated PAMAM dendrimers were charazatized by Transmission electron microscopy (TEM) studies. TEM studies can provide information about particle size and surface nature of stavudine loaded dendrimer nanoparticles. 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

Differential scanning calorimetry studies were carried out to view changes in thermal stability and crystallinty over range of temperature of stavudine loaded PEGylated PAMAM dendrimer, drug and PEGylated PAMAM dendrimer. Briefly known quantity of sample was placed in an aluminium pan and crimped with lid. Further pan was placed 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.

InvitroDrug Release studies

The *in vitro* drug release of STV loaded PEGylated G4 and G5 PAMAM dendrimers were carried out by dialysis method. A known amount of STV loaded PEGylated G4 and G5 PAMAM dendrimers were packed in dialysis bags (MWCO 1000 Da) 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. Finally drug concentrations in formulation determine spectro-photometrically at 266nm λmax.

Hemolytic Toxicity studies

Hemolytic toxicity studies were carried out by earlier procedure reported in Vijayaraj Kumar *et al.* 2007. RBC suspension from human blood with anti-coagulant agent and normal saline were taken separately and incubated for 1hr to undergo hemolytic reaction. Here RBC suspension with hemolytic agents was considering as fully haemolysis of cell solution, as with normal saline no haemolysis cells solution. Hence these solutions were considered as standard solution. Briefly 0.5ml of STV loaded PEGylated and nonPEGylated dendrimer were added with 4.5ml of normal saline and incubated for 1hr 1h with RBC suspension. Further dendrimers solution was centrifugation and supernatants were taken and diluted with an equal volume of normal saline and absorbance was measured at 266nm. 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:

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

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

Stability Studies

Stability studies were carried out for STV 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

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 and entrapment efficiency

The entrapment efficacy percentage of STV in PEGylated PAMAM dendrimer was increased when compared with PAMAM dendrimers (**Table 1**). Since noncovalent interactions with STV and PEGylated PAMAM dendrimer, such as hydrophobic interaction and hydrogen bonding were responsible for binding of drug molecules in interior cavities of dendrimer. Moreover significant increase in entrapment of STV 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	27.32±0.45	15.97±1.17
EDA-PAMAM dendrimer G5	41.67±1.29	23.51±1.35
PEG-PAMAM dendrimer G4	51.87±1.56	1.37±0.82
PEG-PAMAM dendrimer G5	69.94±0.97	1.54±0.75

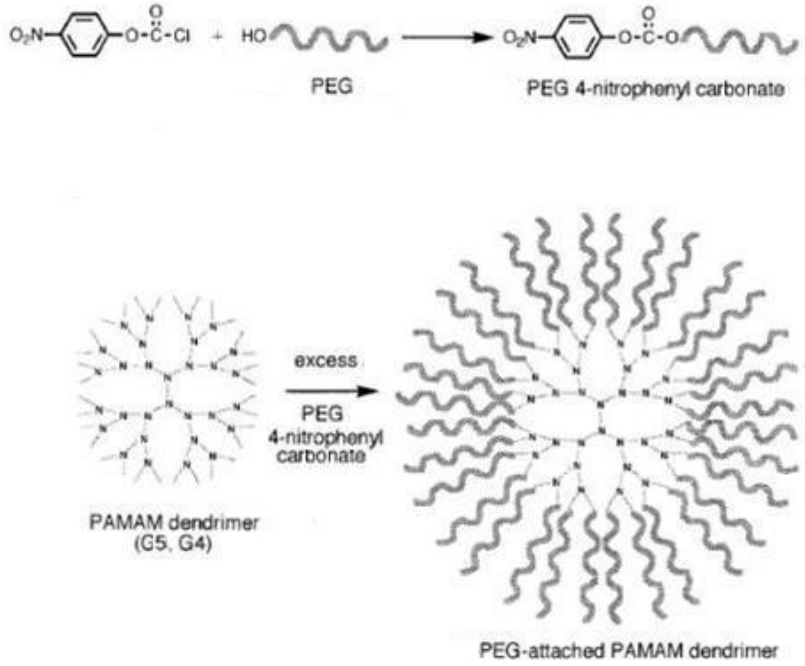
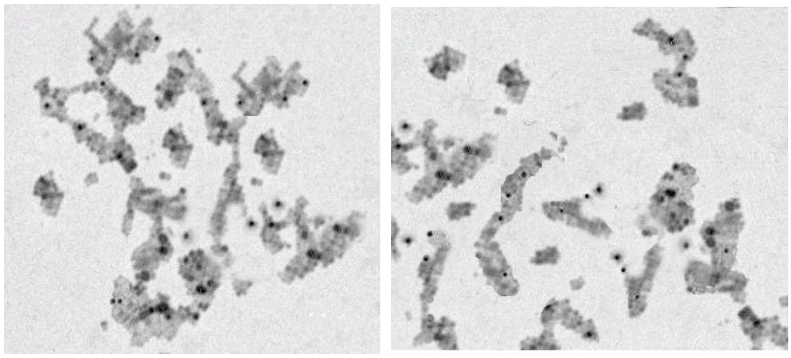


Fig. 1. Synthesis of MPEG attached PAMAM dendrimers



2a **2b**

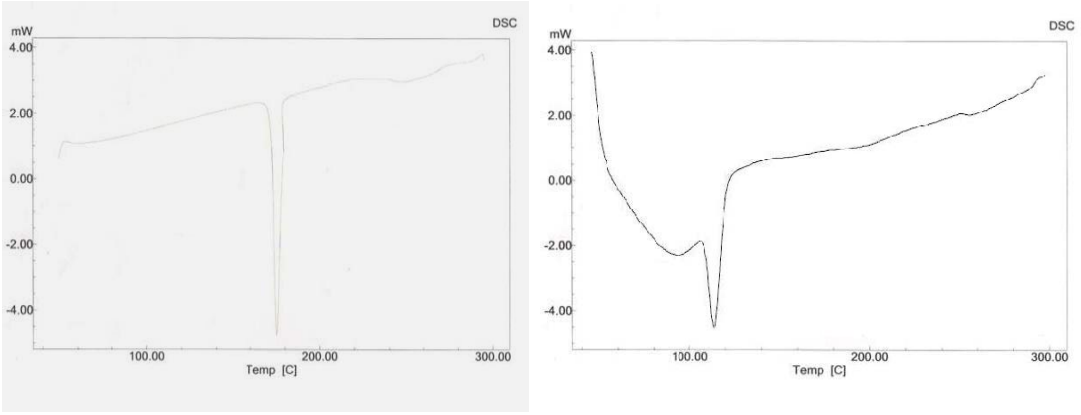
Fig. 2a. Tem image of STV loaded G4 PEG PAMAM dendrimer, **2b.** Tem image of STV loaded G5 PEG PAMAM dendrimer

Surface Morphology

The surface morphology of drug loaded PEGylated PAMAM dendrimers were confirmed by TEM micrographs (**Fig. 2a and 2b**). Micrographs showed that more or less spherical shape dendrimers were formed by agglomeration.

Differential Scanning Calorimetry

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



3a **3b**

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

In vitro Drug Release studies

The invitro drug release profile of STV from PEGylated PAMAM dendrimer and non PEGylated PAMAM dendrimer were presented in **Fig 4**. Here comparative evaluation of the effect of PEGylation on STV release from PAMAM dendrimers is performed. The drug release profile clearly indicates PEGylated PAMAM dendrimer exhibit a significant extended drug release profile compared to that nonPEGylated PAMAM dendrimer. Moreover nonPEGylated PAMAM dendrimer G4 & G5 released the STV in 24 and 36 respectively, while PEGylated PAMAM dendrimers G4 & G5 release the drug in 96h and 120h. The slow release of the drug from PEGylated PAMAM dendrimer is believed to be presence of hydrophobic interaction between drug and core of dendrimers.

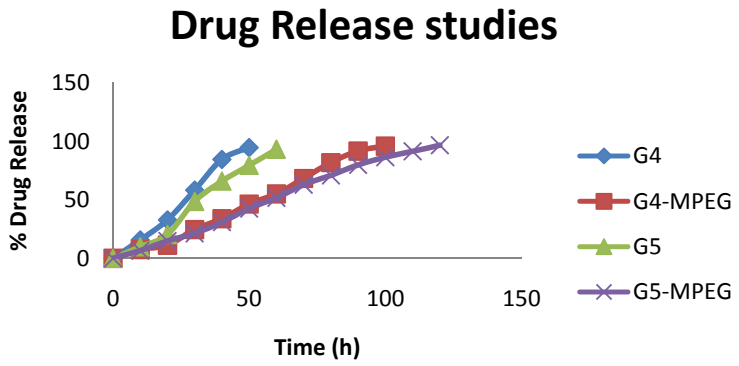


Fig 4. *In vitro* Drug release studies

Hemolytic Toxicity

The result of haemolytic toxicity studies of non PEGylated amine terminated PAMAM dendrimers showed 15-25% toxicity whereas in PEGylated PAMAM dendrimers haemolysis (toxicity) of RBCs were reduced to 2% are present **Table 1**. The surface modification of PAMAM dendrimers by PEGylation reduces the haemolysis of RBCs and toxicity nature. Reduction of toxicity nature in PEGylated PAMAM dendrimers was due to inhibition interaction occurs between RBCs and quaterny ammonium ion.

Stability

Three months storage of STV 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**).

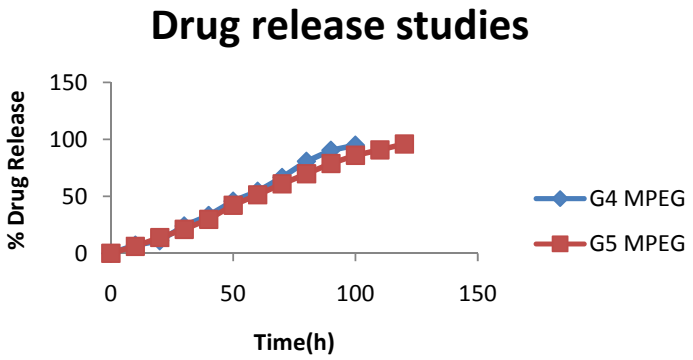


Fig 5. Drug release studies after storage of three months

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

Formulation	Appearance	% Drug release
PEG-PAMAM dendrimer G4	Pale yellow color	50.77±1.56
PEG-PAMAM dendrimer G5	Pale yellow color	68.85±1.75

CONCLUSION

In conclusion we designed PAMAM having PEG grafts as a novel drug carrier. In every chain of the PAMAM dendrimers, PEG was combined to nullify the toxicity and make them compatible carrier. In the encapsulation of anti-HIV drug stavudine in the PEGylated PAMAM dendrimers showed increase amount when compared with non-PEGylated PAMAM dendrimers. *In vitro* drug release studied showed that PEGylated PAMAM dendrimers had controlled and extend drug release. Moreover hemolytic studies proved that PEGylated PAMAM dendrimers had less toxicity compared with nonPEGylated PAMAM dendrimers. Based on above studies we are able to suggest that PEGylated PAMAM dendrimers was one of ideal candidate for anti HIV drug therapy. But further extensive *in vivo* and kinetics studies required stapling them as novel candidate for drug delivery system.

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