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Research Article

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Efavirenz Loaded Novel Citric Acid Dendritic Architecture for Increased Solubility and Sustained Delivery

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Abstract

The present studies were aimed at developing and exploring the use of citric acid dendritic nanostructure for the solubilization and sustained delivery of an anti-viral drug, efavirenz. The citric acid dendritic nanostructure was synthesized and characterized by using IR and NMR spectroscopy. Efavirenz was efficiently loaded into citric acid dendritic nanostructure using dissolution process. Various physicochemical and physiological parameters such as UV, IR, DSC, NMR, SEM, drug loading, solubilization and *in-vitro* release concerning the citric acid dendritic nanostructure were evaluated.

Keywords: Dendrimer; Nanoparticle; Efavirenz; Sustain release; Solubilization

Introduction

Human immunodeficiency virus (HIV) is a lentivirus and a member of the retrovirus family [1-3]. On average, it takes more than 10 years to progress from initial HIV infection to AIDS [4]. The latest statistics of the global HIV and AIDS were published by the Joint United Nations Programme on HIV and AIDS or UNAIDS in November 2011, and refer to the end of 2010. At the end of 2010, an estimated 34 million people [31.6 million–35.2 million] were living with HIV worldwide. This reflects the continued large number of new HIV infections and a significant expansion of access to antiretroviral therapy, which has helped reduce AIDS-related deaths. A total of 2.5 million deaths have been averted in low- and middle-income countries since 1995 due to Antiretroviral therapy being introduced, according to new calculations by UNAIDS [5]. Non-nucleoside reverse transcriptase inhibitors in combination with nucleoside/nucleotide reverse transcriptase inhibitors are highly effective in suppressing HIV replication. In particular, efavirenz is recognized as cornerstones of HIV therapy [6].

Dendrimers are a new class of polymeric materials. The term dendrimer was derived from Greek word *Dendron* (tree) and *meros* (part) and relates to the symmetrical branch-like structure of the polymers. They are highly branched; monodisperse macromolecules. Dendrimer chemistry was first introduced in 1978 by Buhleier et al. [7]. In 1985, Tomalia et al. synthesized the first family of dendrimers [8]. At the same time, Newkome et al. [9] separately reported synthesis of similar macromolecules [10,11]. The synthesis used for dendrimer construction permit almost absolute control over the fundamental molecular design parameters such as size, shape, surface/interior chemistry, flexibility, and topology [12-14]. Biological properties of dendrimers are crucial because of the growing biomedical applications [15-18]. Dendrimers are employed in various pharmaceutical and diagnostic applications which include Targetted and Controlled Release Drug Delivery [12,19,20], Delivery of Anticancer Drugs [12,20], Solubility Enhancers [20,21], Cellular Delivery [21], Nano-Drugs [12], Photodynamic Therapy [12,20], Gene Transfection [12,14], Diagnostics [12,22], and Dendritic Catalysts/Enzymes [23,24].

Efavirenz is a non-nucleoside reverse transcriptase inhibitor approved by the FDA on September 21, 1998, making it the 14th approved antiretroviral drug used for the treatment of a human immunodeficiency virus (HIV) type 1. NNRTIs impede HIV replication within host cells by inhibiting the activity of the HIV reverse transcriptase enzyme [25,26]. Efavirenz was practically insoluble in water at 25°C [24]. Efavirenz was selected for incorporation into citric acid dendritic nanostructure based on its antiviral activity and its hydrophobic nature.

The present studies were aimed at developing and exploring the use of citric acid dendritic nanostructure for the solubilisation and sustained delivery of an anti-viral drug, efavirenz. The citric acid dendritic nanostructure was synthesized and characterized by using IR spectroscopy. Efavirenz was efficiently loaded into citric acid dendritic nanostructure using dissolution process. Various physicochemical parameters such as UV, DSC, drug loading, solubilisation and *in-vitro* release concerning the citric acid dendritic nanostructure were evaluated.

Materials and Methods

Materials

The following chemicals were utilized for synthesis and purification of 3rd generation Citric acid Dendrimer, Polyethylene Glycol 400, Sodium Carbonate, Potassium Permanganate, Ether, Thionyl Chloride, Dimethyl Formide, Pyridine (S.D Fine chem., Mumbai) and Cellulose dialysis bags (sigma, Germany).

Methods

Synthesis of 3rd generation citric acid dendrimer: Novel citric acid dendritic nanostructures were synthesized by previously reported method (Namazi and Adeli) with slight modification [27].

0.175 moles of polyethylene glycol (PEG) 400 was weighed accurately and transferred into a round bottom flask. 3.75 g of sodium carbonate (Na_2CO_3) was dissolved in 38 ml of water and 35.5 g of potassium permanganate ($KMnO_4$) was dissolved in 694 ml of water. Both the solutions were mixed and added to the round bottom flask containing polyethylene glycol. The mixture was stirred vigorously on magnetic stirrer for 3-4 hours at 4-5°C by immersing in an ice bath. The reaction mixture was allowed to reach room

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temperature. The precipitated manganese dioxide was removed by filtration. The obtained filtrate was cooled and heated continuously to get a concentrate filtrate of about 100 ml. The solution of filtrate was cooled and covered with a layer of ether. The solution was kept aside for the separation of ether and aqueous layer. The extraction of aqueous layer was done by two or three portions of ether. The collected aqueous layer was heated on a water bath for removal of ether. The precipitated polyethylene glycol diacid (G1) was filtered. 0.1 mol polyethylene glycol diacid and 0.3 mol thionyl chloride are placed in a round bottom flask attached with a magnetic stirrer and a condenser with drying tube. The reaction mixture is stirred and heated at 70°C. Thus obtained chlorinated PEG (ClOC-PEG-COCl) (G 1.5) was further purified by using column chromatography. 0.1 mol of G 1.5 dendrimer was dissolved completely in dimethyl formide (DMF) kept in an ice bath. 0.2 mol of citric acid was dissolved in DMF completely, to this solution 3 moles of pyridine was added drop wise. It was stirred and kept aside in an ice bath for 24 hours. After 24 hours it was taken and both the solutions were mixed and kept in an incubator for 6 hrs at 55-60°C. Thus obtained 2nd G citric acid dendrimer was further purified by using column chromatography. Citric acid dendrimers up to 3rd generation were prepared by repetition of all the above steps consecutively, with increasing amount of citric acid and thionyl chloride as shown in Scheme-I. The synthesized 3rd generation citric acid dendrimer was further purified by using Sephadex column. IR spectroscopy was carried out using Perkin-Elmer IR spectrophotometer. NMR spectroscopy of the dendrimer sample was carried out at 300 MHz in $CDCl_3$ (Bruker DRX, USA).

Docking studies: Two dimensional structure of 3rd generation citric acid dendrimer, in order to establish their dendritic nature is to be converted into three dimensions and energy minimised (MM2) using Chem3D Ultra 6 (Cambridge soft). 3rd generation Citric acid dendrimer and Efavirenz structures are converted into PDB files. Docking calculations are carried out using Hex software 3rd generation citric acid dendrimer and efavirenz are stored in receptor and ligand files. Docking calculations are carried out by loading a 3rd generation citric acid dendrimer and efavirenz PDB structures into docking algorithm (Hex 4.5 software).

Drug loading in formulations: The citric acid dendrimers so synthesized were dissolved in water and mixed with 100 molar times of efavirenz and allowed the Efavirenz to dissolve. The mixed solution was allowed to incubate with slow magnetic stirring (50 rpm) using teflon bead for 24 h. This solution was dialyzed twice by using Cellulose dialysis bag (MWCO 1000 Da) under strict sink conditions for 10 min to remove free drug from the formulations, which was then estimated spectrophotometrically (λ_{max} 246 nm) to determine indirectly the amount of drug encapsulated with the system. The dialyzed formulations were vacuum dried at 60°C and used for further characterization.

Morphology of the 3rd generation citric acid dendrimer: Chem3D Ultra 10 (CambridgeSoft) was used to study the molecular size and morphology of 3rd generation citric acid dendrimer. The external morphology of the vacuum dried efavirenz loaded citric acid dendrimer complex was analyzed by using a scanning electron microscopy.

Characterization of the formulation: The drug loaded dendrimer was characterized by IR spectroscopy (Perkin-Elmer, USA) and differential scanning calorimetry. IR spectra of drug loaded dendrimer complex, plain drug and plain dendrimer were compared to confirm the loading of drug in dendrimer. Differential scanning calorimetry (DSC) was performed to investigate the thermal stability and changes in crystallinity over a range of temperatures. The citric acid dendrimers and manufactured drug particles and efavirenz loaded citric acid dendrimers were studied by this method. A known mass of powder was placed in an aluminium pan, and a lid was crimped onto the pan. The pan was then placed in the sample cell of a DSC module (DSC TA 6000, TA Instruments, USA). The temperature of the DSC module was equilibrated at 35°C and then increased at a rate of 10°C/min under a N_2 gas purge until the material began to deteriorate. The temperatures were obtained for each peak in the resulting curve and provided indications of temperature stability and phase transitions.

Phase solubility study: Phase solubility study was performed using the Higuchi rotating bottle method with slight modification [25]. The solubility of efavirenz and efavirenz loaded citric acid dendrimers was determined by adding an excess amount of samples into 3 ml of double distilled water in 5 ml screw capped amber colored vials. The vials were rotated at 60 rpm while being kept at room temperature. The current process involved 24 h shaking followed by 6 h equilibration period. Thereafter, samples were filtered through a 0.45 mm polycarbonate filter (Millipore), diluted appropriately with the distilled water and analyzed in a UV spectrophotometer.

***In-vitro* release:** Drug release from known amounts of efavirenz loaded citric acid dendritic nanostructure was determined using the dialysis bags. The medium comprised of a 0.05 mol phosphate buffer solution (PBS) (pH 7.4). The dialysis bags were filled with a known mass of efavirenz loaded citric acid dendritic nanostructure (MWCO 1000 Da) and the dialysis bags were placed in 50 ml of phosphate buffer saline solution (pH 7.4) at 37°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 246 nm.

Results and Discussion

Synthesis of 3rd generation citric acid dendrimer

3rd generation citric acid dendrimer were synthesized by the procedure reported by Namazi and Adeli [27]. IR and NMR data proved the synthesis. IR spectrum (KBr); 1118.71 cm^{-1} for C-O; 1215.15 cm^{-1} for CH_2 rock; 1458.18 cm^{-1} for CH_2 scissor; 1720.50 cm^{-1} for C=O stretch and 2360.87 cm^{-1} and 2339.65 cm^{-1} for CO_2 shown in Figure 1a . ^{1}H NMR spectrum; chemical shifts of protons of citric acid at 2.9–2.6 ppm (CH_2) as a quartet, protons of PEG at 3.8–3.6 ppm (–OCH_2CH_2O–) and 4.1–4.2 ppm (–$COCH_2O$–). The above data confirmed that the compound was citric dendrimer of 3rd generation having COOH group at ppm 9.1 $CDCl_3$ shown in Figure 1b.

Docking studies

The docking studies elucidate the binding pattern of the efavirenz with 3rd generation Citric acid dendrimer. The results of docking studies indicate that efavirenz interacts with polyethylene glycol chain located in dendritic core of 3rd generation citric acid dendrimer. The docking results indicate that efavirenz bound to 3rd generation citric acid dendrimer due to hydrophobic interaction as shown in Figure 2.

Drug loading in formulations

100 ± 0.967 moles of efavirenz were loaded in 50.15 moles of novel 3rd generation citric acid dendritic nanostructure as shown in Table 1.

Morphology of the 3rd generation citric acid dendrimer

The morphology of 3rd generation citric acid dendrimer was determined by using Chem3D Ultra 10 (CambridgeSoft). The average radius of the 3rd generation citric acid dendrimeric molecule was found to be 42.9Å as shown in Figure 3. Atoms are colored as follows: carbon, grey; hydrogen, sky blue; and oxygen, red.

Scanning electron microscopy image (Figure 4) shows that the efavirenz loaded 3rd generation citric acid dendrimers were irregular shape and that the dendrimers were agglomerated.

Characterization of the formulation

Differential scanning calorimetry (DSC) curves suggests that efavirenz loaded 3rd generation citric acid dendrimer were not a physical mixture. Upon heating to 140°C, efavirenz experienced as an endothermic transition. This was previously described as melting of the efavirenz [28-31]. Broad endothermic peaks between 148°C and 246°C were found for 3rd generation citric acid dendrimer. The characteristic peaks of efavirenz and blank 3rd generation citric acid dendrimer almost disappeared in the curve for efavirenz loaded 3rd generation citric acid dendrimer shown in Figure 5.

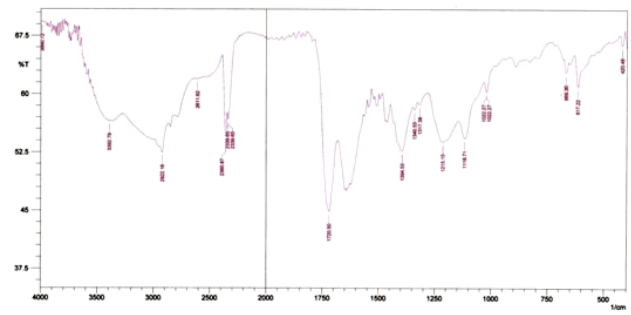

Figure 1a: IR spectrum of 3rd generation citric acid dendrimer.

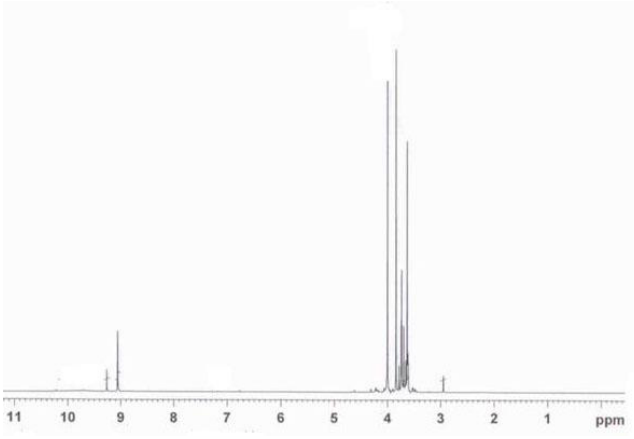

Figure 1b: H1 NMR spectrum of 3rd generation citric acid dendrimer.

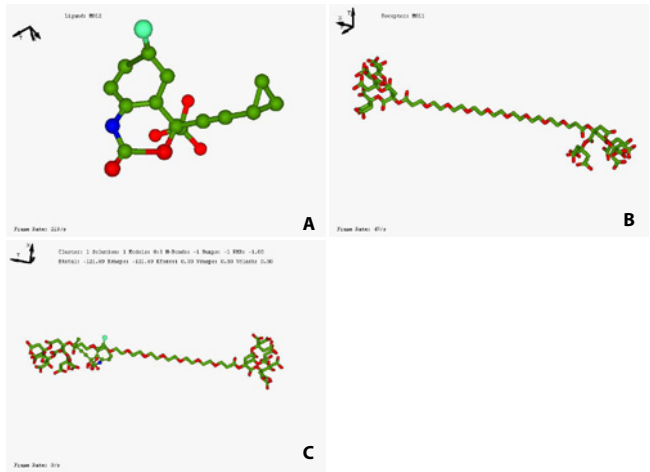

Figure 2: Showing a) 3D structure of Efavirenz b) 3D structure of 3rd generation citric acid dendrimer c) Complex showing docked efavirenz in the polyethylene glycol chain located in dendritic core of 3rd generation citric acid dendrimer.

Table 1: Showing the solubility profile of the efavirenz and percentage drug loading of 3rd generation citric acid dendrimer.

Efavirenz solubility in water*	Solubility	Percentage loading
Without Dendrimer	9.2 ± 0.18 µg/ml	-
Loaded in 3rd generation Citric acid Dendrimer	671.6 ± 0.19 µg/ml	60.01 ± 0.2%

*n=3

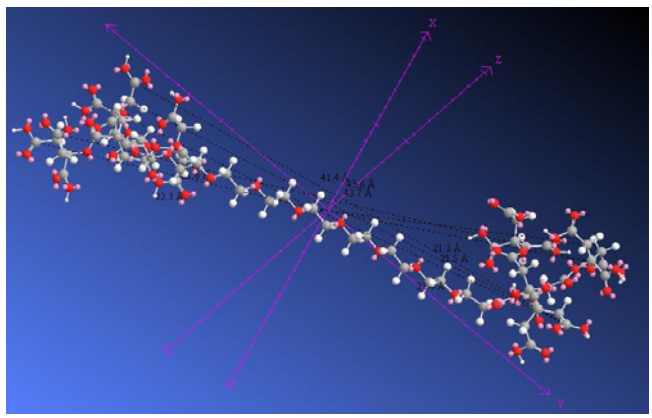

Figure 3: Three dimensional structure of 3rd generation citric acid dendrimer core showing their dendritic nature and size determined by using Chem3D Ultra 10 (CambridgeSoft). Atoms are colored as follows: carbon, grey; hydrogen, sky blue; and oxygen, red).

Phase solubility study

Before the drug release studies were conducted, the solubility of efavirenz and efavirenz in the formulations was determined. The dendrimers could function as solubility enhancer by virtue of electrostatic interactions in addition to hydrogen bonding and molecular encapsulation in the crevices of the dendrimeric network [32-34]. The 3rd generation citric acid dendrimer increased the aqueous solubility of efavirenz by 73 ± 1.817 folds shown in the Table 1.

In vitro release

In vitro release studies of efavirenz loaded 3rd generation citric acid dendrimer was carried out using 7.4 pH phosphate buffer. There was a slow increase in drug release in buffer phase, which has shown 98 ± 0.948% of drug release in 36 hours shown in Figure 6. It was observed that the formulation showed sustained release of the drug. This may be due to greater hydrophobic interaction between the drug and the core of 3rd generation citric acid dendrimer.

Conclusion

The present work establishes suitability of novel citric acid dendritic nanostructure as sustained delivery system for efavirenz. From the present study, it could be concluded that 3rd generation citric acid dendrimer units increased the aqueous solubility of efavirenz by up to 73 folds. The docking results indicate that efavirenz bound to dendrimer due to hydrophobic interaction.

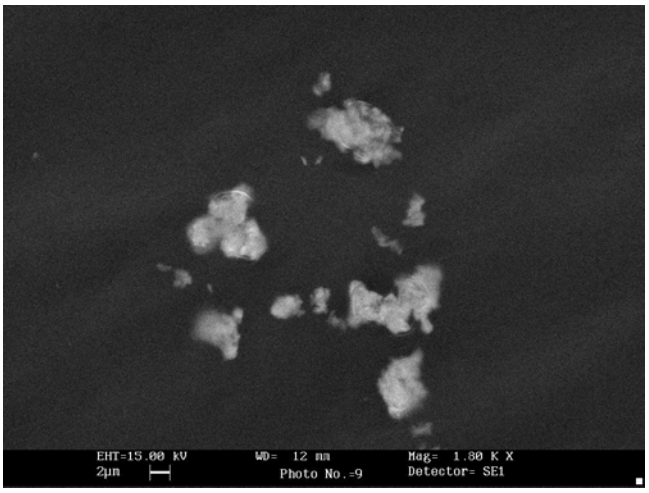

Figure 4: Scanning electron microscopy (SEM) of 3rd generation efavirenz loaded citric acid dendrimer complex.

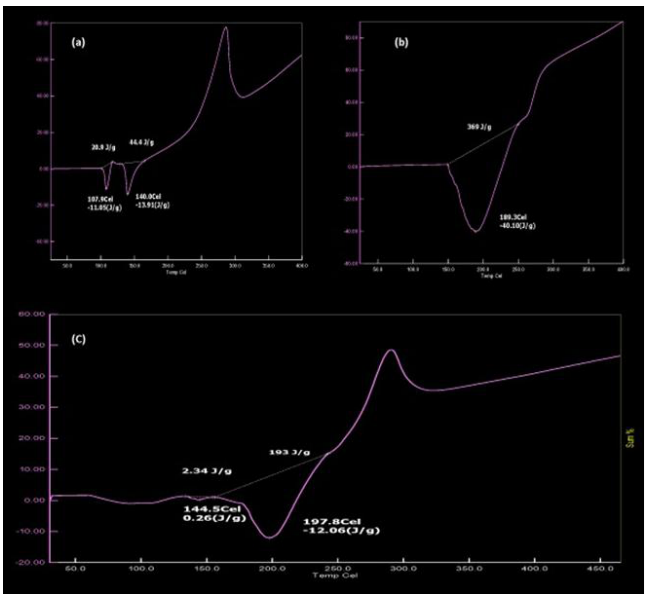

Figure 5: Showing a) DSC analysis of Efavirenz endothermic transition at 140°C b) DSC analysis of 3rd generation Citric acid Dendrimer endothermic peaks between 148°C and 246°C c) DSC analysis of Efavirenz loaded citric acid dendrimer Complex endothermic peaks between 141°C and 246°C.

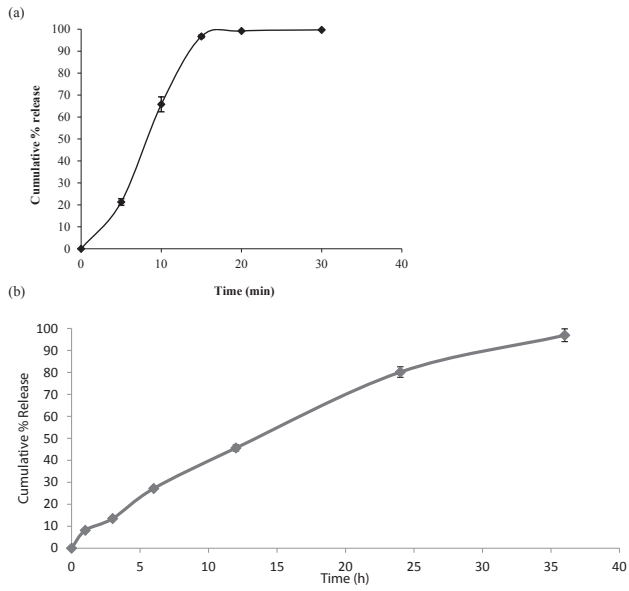

Figure 6: (a) Cumulative percentage efavirenz release in phosphate buffer pH 7.4 at 37°C (n=3). (b) Cumulative percentage efavirenz release from 3rd generation citric acid dendrimer in phosphate buffer pH 7.4 at 37°C. Each bar represents mean ± S.D. (n=3).

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