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Prednisolone Conjugated Polypropylene Imine Dendritic Architecture Confers Reducing Hemolytic Toxicity- A Comparative Study

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Abstract

The present study was aimed to design Prednisolone conjugated-5.0G PPI dendrimer and evaluate comparative preliminary hemolytic toxicity . The hemolytic toxicity study was conducted among free 5.0G PPI dendrimer, designed Prednisolone conjugated-5.0G PPI dendrimer and PEGylated- PPI dendrimer. The degree of hemolysis was compared with the amount of released hemoglobin from RBC was estimated by UV-spectrophotometric determination at 540nm (n=3). PEGylation has been found to be suitable for modification of ethylene diamine initiator core EDA-PPI dendrimers in various research outcomes. The results of comparative hemolytic toxicity studies observed that the toxicity viz Prednisolone conjugated-5.0G PPI dendrimer is 1.42 ± 0.52, PEGylated dendrimer 2.72 ± 1.10 and free dendrimer found to be 20.39±0.82 respectively. In this study hemolytic toxicity of synthesized systems are also relatively safer and hold potential to deliver some bioactive.

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Polypropyleneimine dendrimer, Prednisolone, PEGylation, Hemolytic toxicity.

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INTRODUCTION

Dendrimers have generated a great deal of interest as controlled and targeted drug delivery system due to their exceptional structural properties such as low polydispersity (z1), high density of peripheral functional group, well defined globular shape (z2.0

nm) and multivalency [1,2]. Dendrimers are synthesized from branched monomer units in a stepwise manner so it is possible to conduct precise control on molecular size, shape, dimension, density, polarity, flexibility and solubility by choosing different building/branching units and surface functional groups [1, 3]. Till now dendrimers have been widely explored as carrier system in drug delivery [4]. Poly amido amine (PAMAM), Poly propylene imine (PPI), Polylysine and Triazine are most widely explored dendrimers for drug delivery and their major limitation is hematological toxicity and imperfect organ accumulation properties [5,6] . Due to this reason, no commercial dendrimer based formulation for systemic administration is available. The present investigation was aimed at studying the possibility of using a novel dendrimer carrier system. PPI dendrimer synthesized as a carrier for sustained delivery of drug. PPI dendrimer was found to have good stability as evaluated in the previous study. In the present study, an attempt was made to prepare drug-dendrimer conjugate using Prednisolone as an ant leukemic drug and attempt was also made for evaluating the hemolytic toxicity of free dendrimer, PEGylated dendrimer and conjugation of prednisolone with dendritic nanostructure.

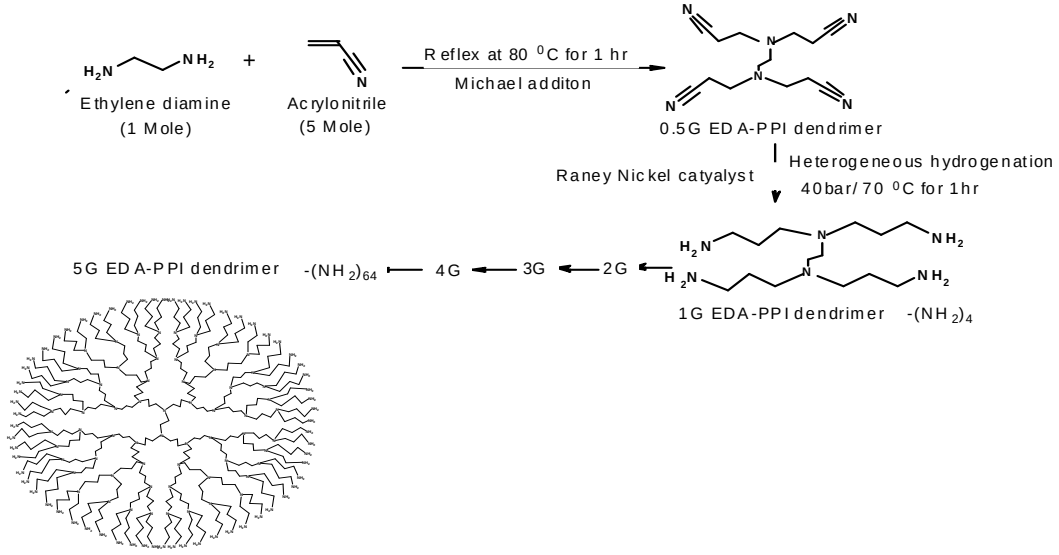
MATERIALS AND METHOD

Materials

Poly ethylene glycol-2000(PEG-2000), Raney Nickel (Merck, India), Ethylene diamine, Acrylonitrile (CDH, India), N, N dicyclohexyl carbodiimide (DCC), Cellulose dialysis bag (MWCO 12-14 Kda, Himedia, India), 4- dimethyl amino pyridine (sd-fine chemicals, India), DMSO(sd-fine chemicals, India) , Prednisolone was a benevolent gift from shasun chemicals.(Chennai) , India. All other chemicals, reagents were purchased from CDH, India.

Synthesis of 5.0G PPI dendrimer

5.0G PPI dendrimer was synthesized following the procedure reported by (De Brabender-Van Den Berg and Meijer) [7] using EDA as initiator core [8]. Briefly, ethylenediamine (EDA) was used as initiator core and acrylonitrile was added to it in a double Michael addition-reaction method to produce half generation (-CN terminated), followed by heterogeneous hydrogenation using Reney Nickel as catalyst to produce full generation (-NH_2) dendrimers. The reaction sequence was repeated cyclically to produce PPI dendrimers up to fifth generation (PPI-5.0G) as shown in Figure 1.

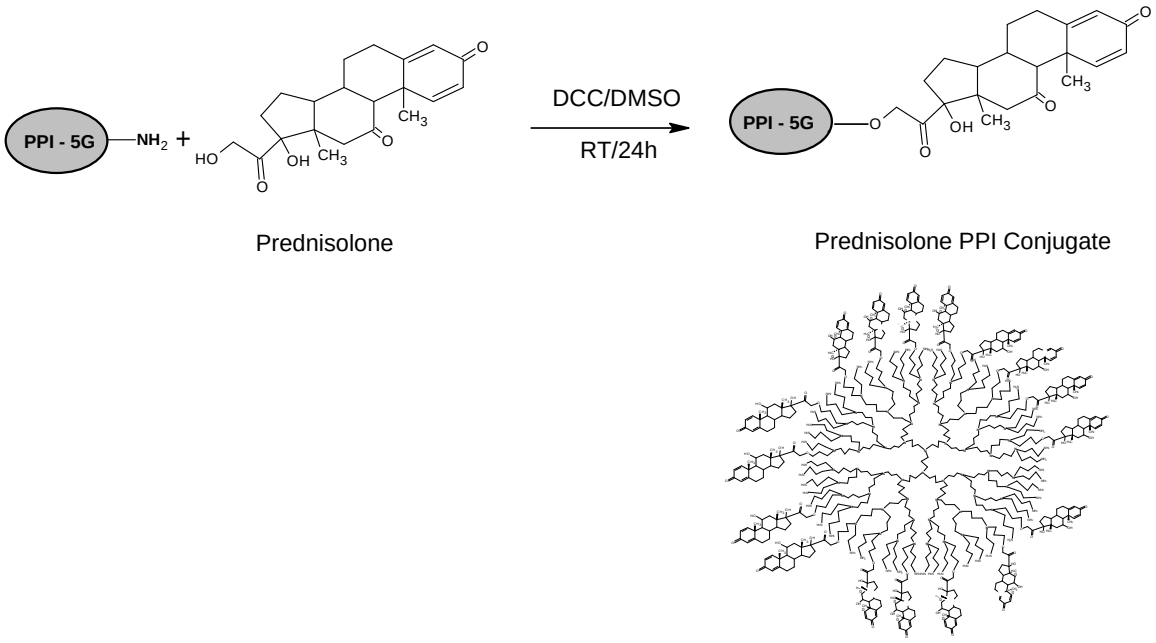


MALDI TOF Peak at 7036.21
Calculated Molecular weight 7042

Figure 1: Schematic diagram for synthesis of PPI-5G dendrimer

Synthesis of Prednisolone conjugated - 5.0GPPI dendrimer

The Prednisolone conjugated 5.0G PPI dendrimer were synthesized via the procedure shown in Figure 2. To a solution of 5.0G EDA-PPI dendrimer (0.01 mmol) in dimethyl sulfoxide (10 ml), Prednisolone (0.64 mmol) in DMSO (10 ml) and N, N dicyclohexyl carbodimide (DCC) (0.64 mmol) in DMSO (10 ml) was added and the solution was kept for 5 days at room temperature. The solution was further dialyzed (MWCO 12-14 Kda, Himedia, India) against water for 24 h to remove unreacted compounds. The dialyzed solution was then added to water to get a precipitate. The Prednisolone-5.0GPPI precipitate was filtered and the filtrate was then lyophilized (Heto drywinner, Germany).



MALDI TOF Peak at 18,566.08
Calculated Molecular weight be 18, 576.08

Figure 2: Schematic diagram for synthesis of Prednisolone – PPI- 5G conjugate

Synthesis of PEGylated 5.0GPPI dendrimer

The polyethylene glycol derivatization (PEGylation) was performed subsequent to activation of end functional groups of PEG 2000. The activation of PEG 2000 was carried out by converting it to dicarboxylic acid derivatives with slight modification of method to Veronese et al., (1989) [9].Chloro acetic acid was used to prepare dicarboxy methyl PEG 2000 diether. This generated two carboxylic acid functional groups on PEG 2000. 4Mm of PEG 2000was dissolved in 50 ml of tert-butanol at 50°C and 32mM of potassium tert-butanolate was added. The mixture was stirred at the same temperature throughout the night. The solvent was evaporated and 50ml of dichloromethane was added to the precipitate.It was washed with the 250ml of water by stirring and the layers were separated in a separating funnel. The lower layer of dichloromethanewas removed allowing the mixture to stay for 1 hr and concentrated to 10-15ml.This was added to cold 200ml ether Keeping in refrigerator for overnight the excess of ether has further increased the precipitation. The precipitate was removed and redissolved in dichloromethane and reprecipitated by cold ether. The precipitate of dicarboxylic acid PEG 2000 was dried in a Petri dish and slightly heated in an oven. To a solution of 5.0G - PPI dendrimer (0.01 mmol) in dimethyl sulfoxide (DMSO) (10 ml), PEG COOH2000 (0.32 mmol) in

DMSO (10 ml) and N, N dicyclohexyl carbodiimide (DCC) (0.32 mmol) in DMSO (10 ml) were added and the solution was stirred for 5 days at room temperature. The product was precipitated by addition of water, filtered and dialyzed (MWCO 12-14 Kda, Himedia, India) against double distilled water for 24 h to remove free PEG 2000 , COOH, DCC and partially PEGylated dendrimers followed by lyophilization (Heto dry winner, Germany).

Hemolytic toxicity

The Hemolytic studies were conducted in accordance with the protocol approved by the institutional ethical committee of Vignan pharmacy college, Vadlamudi (Registration No 1499/PO/a/11/CPCSEA).Blood was collected in Hi anticlot blood collection vials (Himedia Labs, India). The blood was washed with 0.1M phosphate-buffered solution (PBS) (pH 7.4), centrifuged at 600 rpm for 5 min, and the supernatant was pipette off repeatedly (three times). The red blood cells (RBC) suspension was diluted with 0.1M PBS to obtain a concentration of 10% w/v. RBC suspension (0.1ml) was added separately to 0.9 ml of drug solution, PPI dendrimer solution, Prednisolone-PPI conjugated dendrimer and PEGylated PPI-5G dendrimer solution respectively. This gives comparative results of the hemolysis data of the above solutions which helps to understand the effect of conjugation on hemolysis. After incubating the systems at 37°C for 30min, the mixtures were centrifuged at 3000 rpm for 10 min to remove non-lysed RBCs. The supernatant was collected and analyzed to determine the amount of released hemoglobin by UV-spectrophotometric determination at 540nm (n=3). To obtain 0 and 100% hemolysis, 0.1 ml RBC suspension was added to 0.9 ml of 0.9% NaCl solution (normal saline) and 0.9 ml distilled water, respectively. The degree of hemolysis was determined by the following equation:

$$\text{Hemalysis (\%)} = \frac{\text{Abs} - \text{Abs}_0}{\text{Abs}_{100} - \text{Abs}_0} \times 100$$

where Abs, Abs100, and Abs0 are the absorbance of samples, a solution of 100% hemolysis, and a solution of 0% hemolysis, respectively.

Stastical Analysis

The results are expressed as mean ± standard deviation (S.D) and stastical analysis was performed with SPSS 10.1 for windows (SPSS, Chicago, USA). % hemolysis produced by 5 mg/ml conjugates on 5% hematocrit RBCs on incubation for 1h were observed by pair wise comparisons using unpaired t test..

RESULTS

5.0G PPI dendrimer were synthesized by the procedure reported by De Brabender-Van Den Berg and Meijer [7] using ethylene diamine as initiator core [8]. The results matched with the reported synthesis of PPI dendrimers . The synthesized dendrimers were conjugated using prednisolone, IR and NMR data proved the synthesis shown in Figure 1 & 2. Synthesis of PPI–Prednisolone conjugate consists of single step, amide bond formation.

Characterization of the 0.5G EDA-PPI dendrimer and its conjugates

Synthesis of 0.5G EDA-PPI dendrimer was confirmed by IR peak of nitrile group at 2249 cm^{-1}. The peak observed at 3432 cm^{-1} (N—H stretch) revealed that nitrile terminated 0.5G PPI dendrimer converted to amine terminated 1.0G EDA-PPI dendrimer in the subsequent step. Synthesis of 5.0G EDA-PPI dendrimers were similarly confirmed by IR peaks for C—C bend (1101 cm^{-1}); C—N stretch (1217 cm^{-1}, 1352 cm^{-1}); C—H bend (1461 cm^{-1}, 1531 cm^{-1}); N—H deflection of amine (1645 cm^{-1}) and primary amine at 3429 cm^{-1}(N—H stretch). 5.0G EDA-PPI dendrimer was further characterized by ^1H NMR spectroscopy. Multiplets (m) between δ 0.8 to 1.2

ppm corresponds to methylene (—CH_2—) groups of EDA, δ = 1.6-1.75 (m, —CH_2— CH_2—), δ = 2.3 (—NH_2, amine), δ = 2.4- 2.6 (m, —N[C] C) and δ = 2.7-2.9 (m, —CH_2—NH_2). The present result matched with the reported data.

The synthesis of drug – dendrimer conjugate shown in figure 2.Then the synthesized conjugate were confirmed by IR peaks via, nitrile group at 2249 cm^{-1} C=C stretching(cm^{-1}) -1456,1596; C=O Stretching(cm^{-1})-1711;O-H bending free(cm^{-1})-1050,1136; C=H Stretching(cm^{-1})- 2957.Further IR and NMR data proved the synthesis of PEGylated PPI - 5.0G dendrimer via, C=O stretch of carbonyl group (cm−1)- 1626.11; C---H stretch (cm−1)- 2928.22, 2850.41; Secondary amide (cm−1)- 3326.65. Selected δ Values of 1H NMR Spectroscopic Data (300 MHZ in DMSO) for dendrimer (s, CONH)- 7.52; (s, CH2-CH2-0)- 3.64, 3.47; (m, correspond to the presence of CH2 of EDA-PPI dendrimer)- 1.94 to 1.07. Then the molecular weight of the dendrimer, PEGylated dendrimer and Prednisolone conjugated - dendrimer was determined by MALDI TOF Analyzer (Applied Biosystems Inc., Framingham, MA) equipped with a Nd: YAG 355-nm laser.The matrix used for mass spectra was 2,5-di hydroxyl benzoic acid, which is soluble in $CHCL_3$. In the mass spectrum of plain dendrimer, the m+1 peak was observed at 7036.21 were as calculated mass number of 5G PPI dendrimer was found to be at 7042.The plain dendrimer having molecular weight 7042 and 32 groups of Prednisolone(MW 360.44) were attached in drug dendrimer conjugation reaction. Conjugated system showed the peak at 18,566.08 and molecular weight was found to be18, 576.08.In case of PEGylated system showed the peak at 71,031 and the molecular weight was found to be 71,042. Here one or two molecules of matrix may cause interference in the spectra of synthesized compounds. Hence there is expected change in mass of compounds respectively after the reaction, which indicated prednisolone conjugation and PEGylation was done and only 32 groups were attached.

DISCUSSION

Prednisolone was selected as the linker to reduce the haemolytic toxicity of PPI 5G dendrimer. They contain hydroxylic groups for amide bond formation with the amino terminals of PPI dendrimers. In the first step, in amide bond formation, N, N dicyclohexyl carbodimide was used as a coupling reagent. The number of moles of Prednisolone conjugated to the PPI dendrimer was estimated indirectly by UV spectroscopy. The number of Prednisolone residues and the drug contents in the conjugates are shown in Table 1. Although the molecule of 5.0G PPI dendrimer consists of amino terminal groups, the average ratio of 3.3 mol per conjugate for PABA spacers, respectively, was found to be the maximum ratio. Some researchers have reported the average optimum number of drug molecules covalently attached to the PAMAM dendrimers, for example, the average ratio of doxorubicin to PAMAM G4 with 64 amino terminal groups was 2:1 [10] and ibuprofen to PAMAM G4 was 3:1 . These indicate that the large number of terminal groups may not be necessary for carrying more drug molecules. In contrast, the drug-carrying capacity of the dendrimer depends on other factors such as the chemical structure of the drug, spacer and the carrier, and also the reaction used.

Hemolytic toxicity

Hemolytic toxicity was found in 5.0G PPI dendrimer 20.39±0.82 percent. However, conjugation of the dendrimer with PEG2000 was found to have decreased the hemolysis of the RBCs very significantly to less than 2.72 ± 1.10 % (Table 1). The hemolytic toxicity of the dendrimers was enough to preclude its use as drug delivery system and may be attributed to the polycationic nature of the dendrimers, which was also responsible for their

cytotoxicity, particularly in case of whole generation of amine-terminated charged dendrimers. However, conjugation of the dendrimers was found to have decreased the hemolysis of the RBCs due to inhibition of interaction of RBCs with the charged quaternary ammonium ions that are generally present in the whole generations of amine terminated dendrimers [10]. This is comparable and similar to the effects produced by surface modification of PAMAM dendrimers and similar to cationic dendrimers in earlier studies. In this study hemolytic toxicity observed with the prednisolone conjugated system 1.42 ± 0.52%.. Hence, this is comparable and similar to the effects produced by the surface modification of PAMAM and cations in earlier study.

Table 1: Nomenclature, Physiochemical and Biological Characterization of Prednisolone Conjugated Formulations

Formulation code	No.of Prednisolone conjugated mol/mol	Actual number of terminal amine groups	%hemolytic toxicity
5G-PPI dendrimer	-	64	20.39±0.82
PEGylated-PPI dendrimer	-	-	2.72 ± 1.10
Prednisolone-Conjugated 5G-PPI dendrimer	1:3.3	-	1.42 ± 0.52

[a] % hemolysis produced by 5 mg/ml formulations on 5% hematocrit RBCs on incubation for 1h. mean ±SD. (n = 3).

CONCLUSION

5.0G-PPI dendrimer were conjugated using Prednisolone. Conjugation has been found to be suitable for modification of ethylene diamine initiator core of PPI dendrimer to reduce hemolytic toxicity produced by the charged quaternary ammonium ions in amine terminated dendrimer. The result of hemolytic toxicity studies revealed that these systems are relatively safer and hold potential as delivery system for Prednisolone.

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