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Pyrazinamide-Loaded Pegylated Polypropylene Imine Dendritic Architecture for Reducing Haemolytic Toxicity

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ABSTRACT: The purpose of the current study was to construct a Pyrazinamide loaded PEGylated fifth generation (5.0G) Poly (Propylene) Imine (PPI) dendrimer and compare its potential haemolytic toxicity. Pyrazinamide (PZA) is a drug that is used to treat Tuberculosis (TB). The dendrimers exhibit superior performance in targeted drug delivery, yet haemolytic toxicity produced by dendrimers can cause acute renal failure. Henceforth, the present research researches the synthesis of dendrimers along with enhanced biocompatibility that aids in reducing haemolytic toxicity concerning dendrimers-mediated drug delivery. The free 5.0G PPI dendrimer, synthesised Pyrazinamide loaded PEGylated fifth generation PPI dendrimer, and PEGylated 5.0G PPI dendrimer are considered as subjects of haemolytic toxicity investigation. UV-spectrophotometric analysis at 269 nm (n=3) calculated the amount of liberated haemoglobin from RBC, and the haemolysis degree was equated with it. Several research findings have revealed that PEGylation is suitable for modifying the core Ethylene Diamine (EDA)-PPI dendrimers of ethylene diamine initiator. According to comparative haemolytic toxicity experiments, the toxicity of free dendrimer was found to be 20.39 0.82, PEGylated dendrimer was 2.72 1.10, and Pyrazinamide loaded PEGylated 5.0G PPI dendrimer was 1.420.52 each. In this work, synthetic systems' haemolytic toxicity levels are lower and can provide bio-actives. Finally, this study conducted ex vivo and in vivo experiments and proved that haemolytic toxicity is reduced in Pyrazinamide Loaded PEGylated 5.0G PPI Dendrimers and drug circulation time is maximised.

Keywords: PPI dendrimers, Pyrazinamide, drug delivery, Haemolytic toxicity, PEGylation.

INTRODUCTION

The Pyrazinamide inclusion in the drug schedule for patients in the past few decades reduced the duration of TB treatment therapy from twelve months to six months (Gopal *et al.,* 2019). One of the main reasons for deaths across the globe is TB (Gour *et al.*, 2021). The PZA significantly affected the patients who were showing signs of tuberculosis. Around 1952, anti-tuberculosis activity was identified in animal models. Followed by it, it was proven in humans too. The drug showed incredible sterilising activity in the tissues of mice, and its annexation in the treatment of the patients who are at risk of contradicting the TB or TB-affected persons displayed a reduction in the duration of the treatment therapy. Pyrazinamide is a prodrug that the host enzyme will convert into a bioactive form (Lamont *et al.*, 2020). Polymers can be considered drug delivery systems, and it is known that they came into existence after the liposomes. The polymer nanoparticles have several advantages along with the liposomes, like penetration capability of the cell membrane and adeptness to surface modifications to enhance biological activity and physical-chemical properties. Additionally, the polymers provide maximised stability to the carrier, safeguarding the drug from earlier degradation and prolonging the circulation time (Sheikh *et al.*, 2021).

The accomplishment of every drug delivery carrier firmly relies upon the toxicological profile (Seynhaeve *et al.*, 2020). Despite the various benefits provided by the dendrimers as the drug delivery carrier, toxicity remains an issue. The dendrimers are usually nanometres and are composed of definite structures, providing excellent benefits like intracellular drug delivery (Mignani *et al.*, 2019). The polymeric nano-carriers were advanced with the motive of using them as drug carriers. The polymeric nanosystems can be categorised in the nanoparticles poised through a nanocapsule or nanospheres (Farhana *et al.*, 2023 along with a diameter that ranges from 10 to 1000 nm even though most of the polymeric nanoparticles recognised are in the average size of fifty to three hundred and fifty nm. The broad range of drugs can be compressed in the core, overloaded inside a matrix, or be covalently associated with the

carrier polymeric surface. The polymers utilised for the production of those systems can be of natural (Selvaraj *et al.,* 2019), like gelatin, alginate, chitosan and albumin, or they can be of synthetic, like polyamides, polyesters, and polyalkyl-cyanocarbonates and poly amino acids (Sung *et al.,* 2020).

The dendrimers are the class of definite hyperbranched synthetic polymer systems that can be conjoined to several chemical species (Kavand *et al.,* 2020). The word dendrimer originates from Greek words such as Meros, Dendron. Meros means tree, and Dendron means tree (Chis *et al.,* 2020). The highly regular branching units are systematised in layers termed the generations and exhibit the repetitive monomer unit of those synthetic macromolecules. The dendrimers are non-immunogenic and non-toxic, along with an excellent drug-loading capability. Persistent and maintained drug release can be attained with the advancements in controlled release technology (Pandey *et al.,* 2016). There are various approaches for sustained drug release and several reasons contributing to ineffective drug discharge, making the effective drug delivery system essential (Firoz *et al.,* 2020). The controlled drug delivery release is painless (Das & Saha., 2017). The alteration of the dendrimer's surface can improve physicochemical properties and enhance penetrability and biocompatibility in a cellular atmosphere (Irfan *et al.,* 2020). The properties like excellent branching, greater water solubility, polyvalency, nanoscale size and availability in internal cavities, and immunogenicity deficiency make the dendrimers safe. PPI dendrimers are available until the fifth generation and have various uses in material science and biology (Aso *et al*., 2019).

The dendrimers are greatly specified, synthetic polymers, globular, and composed of several features that convert them into beneficial substances in biological systems. The dendrimers can be utilised in several fields like biomedicine (Morgado Benítez *et al.,* 2021), photodynamic therapy (Dhilip Kumar *et al.* 2021), scanning, immunology, siRNA and oligonucleotide conjugation. The dendrimers yield various uses since they can be utilised as scaffolds or diagnoses for rehabilitation and diagnosis. The research on dendrimer-intermediated drug delivery is mainly on the DNA drugs' delivery to the cell nuclei for the antisense therapy or gene. Various reports on the probable use of PPI dendrimer or unmodified amino-terminated PAMAM as non-viral gene transfer agents have been revealed, improving the transfection of DNA into cell nuclei (Mittal *et al.,* 2021). In present times, advanced dendrimers and technologies have been established. The dendrimers are suitable carriers for the delivery and targeting of therapeutic agents due to their distinctive features like flexible surface functionality, internal cavity and monodispersity. The drug molecule can be overloaded within and upon the dendrimer surface also. toxicity ascending from the hemolytic activity of dendrimers impedes their further advancement as drug carrier aspirants. The hemolytic toxicity related to the cationic charge of dendrimers creates an obstacle in its utilisation as the carrier in the drug delivery system. The unique features of dendrimers make them an appropriate drug carrier that involves significant cell absorption, the capability to associate, several functions and maximised circulation time (Chis *et al.,* 2020).

The dendrimers toxicity relies upon surface charge properties like neutral, cationic and anion. The cationic dendrimers mostly display the maximised toxicity levels even though the lower concentration of those substances can be considered secure. Dendrimers have shown significant developments in the medical field, and toxicity research is of great use for the secure application of dendrimers. The toxicity and additional properties of the particular compounds are mainly associated with their structure. Because of their nanoscale size, the dendrimers may interrelate particularly with various nanoscale cellular compounds like nuclei acid, heavy metals, enzymes, organelles ions, vitamins and plasma membranes. These events will lead to membrane rupture, cytokine release, and ROS production, causing cell death and damage. Polyethene glycol is an inert, non-antigenic and non-immunogenic polymer with admirable biocompatibility and water solubility. The dendrimer's PEGylation is generally utilised to shield dendrimers' cationic surface, decrease toxicity, and extend the circulation time of dendrimers (Wang *et al.,* 2022).

The dendrimer toxicity relies upon its size and generation. Higher-generation cationic dendrimers' cytotoxicity is more significant than the low-generation dendrimers that contributed to the maximised number of positive charges (Zenze *et al.,* 2023). The high-generation cationic dendrimers display specific toxicity in vivo (Santos Ramos *et al.,* 2020), and the alteration of dendrimers can decrease it. The modification strategies of dendrimers like folate conjugation, PEGylation, folate conjugation and glycosylation have shown low toxicity in vivo or in vitro. The existing study (Selvaraj *et al.,* 2019) reveals that the in vitro drug release is indirectly proportionate to the polymer concentration. The toxicity of the dendrimers can be decreased by adding components like carbohydrates, amino acids and peptides, which reduce the positive charges upon the surface of the dendrimer. The properties such as stability, drug delivery capability, probability of attaching with the target groups and targeting potential in dendrimers can be enhanced through PEGylation (Dwivedi *et al.,* 2019). The Poly Propylene Imine (PPI) dendrimers remain one of the most recognised dendrimers and comprise amine-terminated hyper-branched macromolecules (Janaszewska *et al.,* 2019). Because of many surfaces' amine groups, the dendrimers display toxicity that limits their clinical uses and applications (Santos *et al.,* 2019).

The present research intends to emphasise dendrimers synthesis along with the enhanced biocompatibility that will establish protection for the dendrimer-mediated drug delivery features. The

folic acid fastening of the PPI dendrimer surface considerably shortened the hemolysis of the Red Blood Cells. The hemolytic toxicity is created due to the PPI dendrimers' polycationic nature. The toxicity level will be reduced because of the fastening of primary amine

groups located on the dendrimer periphery along with the folic acid. (Madaan, K *et al*., 2014). The PPI dendrimers as an efficient nano-carrier for drug delivery is possible through the PPI dendrimer surface modification along with the PEG molecules (Parashar *et al.*, 2019). The reduction of hemolytic toxicity suggested that dendrimers formulation and synthesis can be used as a drug delivery instrument. Hence, the present research intends to reduce the haemolytic toxicity in PPI 5.0G dendrimer through PEGylation and to load the pyrazinamide drug to prove it is an excellent drug carrier.

MATERIALS AND METHODS

Materials: The Reney Nickel (RN), Triethylamine, EthyleneDiamine, N, N dicyclohexyl Cellulose dialysis bag, Pyrazinamide and PEG4000 were the generous offering from the Shasun pharmaceuticals in Chennai, India.

5.0 PPI dendrimers*:* The synthesis of 5.0 G PPI dendrimer takes place by ensuing the procedure stated by Meijer and De Brabender-Van Den Berg by using the initiator core as EDA (EthyleneDiamine) (Pedziwiatr-Werbicka *et al.*, 2019). The acrylonitrile is added to the initiator core in the double Michael addition and reaction process to create the partial generation and, subsequently, the heterogeneous hydrogenation utilising the catalyst RN to make the –NH2 (entire generation dendrimers.). The reaction sequence is cyclic to create the PPI dendrimers until the 5th generation. The overall process involved in synthesising 5.0.

Pyrazinamide loaded 5.0G PPI dendrimer: The N, N dicyclohexyl carbodimide of around 0.64 mmol and Pyrazinamide of 0.64 mmol IN DMSO of about 10 ml were mixed to the solution of 5.0G EDA-PPI dendrimer of 0.01 mmol upon dimethyl sulfoxide of 10 ml. Then, the mixed solution was kept at room temperature for five days. The solution was dialysed in the water to remove the unbound chemicals for one day. The precipitate was created by mixing the dialysed solution with the water. After this process, the pyrazinamide-5.0G PPI precipitate was filtered, and the supernatant lyophilisation occurred. The procedure used to produce the 5.0G pyrazinamide conjugated PPI dendrimer is illustrated in fig. 1.

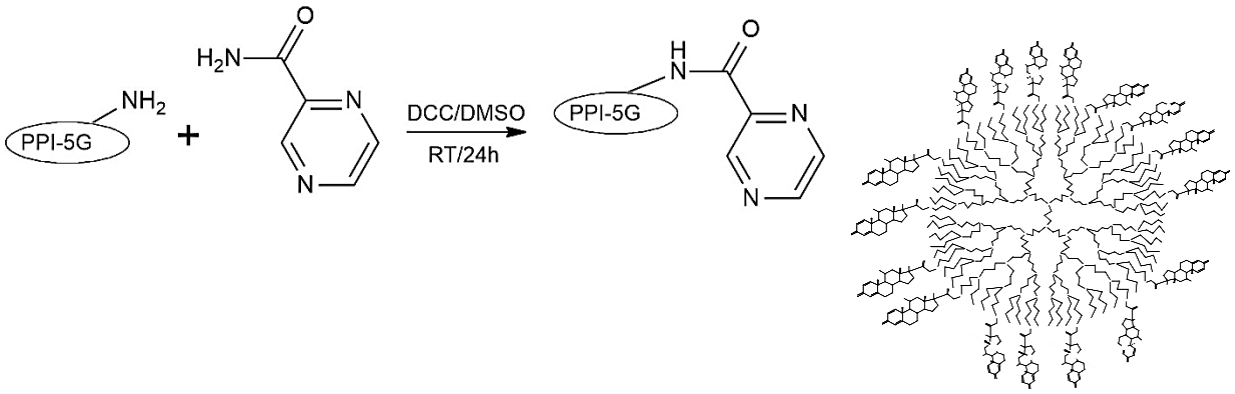

Fig. 1. Structure for the synthesis of Pyrazinamide loaded 5.0GPPI dendrimer.

PEGylated 5.0GPPI dendrimer: The PEGylation (polyethene glycol) derivatisation is executed after the end functional groups of PEG 2000s were initiated. The conversion of Polyethylene glycol 2000 into the derivatives of dicarboxylic acid and marginally varying the procedure. The chloroacetic acid is utilised to make carboxy methyl PEG 2000 di ether. The two carboxylic acid functional groups were formed from the PEG 2000. The 32 mM of potassium tert-butanolate and four mM of PEG 2000 were combined with tert-butanol of 50 ml at 50º Celsius. The mixture was stimulated at the same temperature throughout the entire night. After the evaporation of the solvent took place, 50 ml of dichloromethane was mixed to precipitate. After cleaning and stimulating the mixture using water of 250 cc, the layers are segregated in the separating funnel.

The removal of the lower layer of dichloromethane takes place, which permits the liquid to assemble for an hour; meanwhile, it is strenuous to ten or fifteen ml. Followed by it, it was combined with 200 ml of chilled ether. The precipitation will be developed after being kept inside the refrigerator for one day and night. The cold ether is redissolved in the dichloromethane, which is then used to precipitate the distant precipitate. The precipitate of dicarboxylic acid PEG 2000 is desiccated in a petri dish prior to providing the heat in the oven. The 5.0G PPI dendrimer in DMSO (dimethyl sulfoxide) is mixed into the solution of 0.32mmol in DMSO of 10 ml, and PEG COOH2000 of 0.32 MMOL in DMSO of 10ml and the combined mixture was perturbed for five days in room temperature. In order to remove the COOH, Free PEG 2000, partially PEGylated dendrimers and DCC, the product is dialysed contrary to double distilled water for twenty-four hours. It is then followed by lyophilisation. The precipitation of the product takes place by adding water.

Haemolytic toxicity: The haemolytic studies were performed per protocol sanctioned by the institutional ethical committee of Chalapati Institute of Pharmaceutical Science, Lam Guntur. The blood was collected by using the anticlot blood collecting jars. The centrifugation of blood takes place at 500 rpm for 6

minutes while soaking with the 0.1M phosphate-buffered solution with a pH level of 7.4, and three times the supernatant is pipetted out. The solution of the red blood cells is diluted with 0.1 M PBS to attain a concentration of ten per cent w/v. The RBC suspension of 0.1 ml is combined with 0.9 ml of medication solution, Pyrazinamide-PPI conjugated dendrimer solution, PPI dendrimer solution and PEGylated PPI-5G dendrimer solution in a discrete manner to the three dendrimers. This, in turn, delivers the comparisons of haemolysis data from the above solutions that help understand how conjugation impacts haemolysis. The centrifugation of mixtures takes place at 3000 rpm for ten minutes to eliminate the red blood cells, followed by incubating the systems at thirty-seven degrees Celsius for 30 minutes. The quantity of the liberated haemoglobin is quantified in supernatant utilising the UV-spectrophotometric analysis at 269 nm if n=3 of red blood cells suspension is combined into 0.9 ml of 0.9 per cent NaCl solution, distilled water of 0.9 ml correspondingly to create 0 and 100 per cent haemolysis.

The equation utilised to estimate the haemolytic toxicity.

$$\text{Hemolysis (\%)} = \frac{Abs - Abs_0}{Abs_{100} - Abs_0} \quad ---(1)$$

Where,

$Abs_o, Abs_{100}\ and\ Abs$ Denotes the samples absorbance and the solution with the hundred percent hemolysis and an unhemorrhagic solution.

Ex vivo animal model study*:* The brine shrimp lethality assessment is utilised under the method where brine shrimp nauplii were crosshatched in sterile brine solution beneath constant aeration for forty-two hours. It was created by utilising the sea salt 38g/L and altering the pH to 8.5, utilising the NaOH 1N. Totally ten nauplii were arranged in every vial succeeded by hatching, and with the addition of 25, 50 and 100 of every Pyrazinamide loaded PEGylated PPI dendrimers in the concluding volume of the five ml in each vial, sustained at 37^{O}C for one day beneath the incandescent lamp light and the living larvae are calculated. The experiment was performed with a control element precisely like the test elements. The lethality percentage is determined utilising correlating the mean surviving larvae of control and test tubes. The Fenny probed analysis software is used to calculate the values. The Positive control Podophyllotoxin is associated with the outcomes of the test compound.

Statistical Analysis: The outcomes are statistically investigated using Windows SPSS 10.1, represented in the mean SD (Standard deviation). The five per cent of haematocrit red blood cells incubate for one hour upon the five mg/ml to decide the obtained haemolysis percentage. The unpaired t-test is used to make the pairwise comparisons.

RESULTS AND DISCUSSION

By using the initiator core as ethylene diamine, the 5.0G PPI dendrimer synthesis is carried out using the method explained by De Brabender, Van Den Berg and Mejer. The results were reliable with testified PPI dendrimer synthesis. The use of Pyrazinamide to combine the dendrimers is shown in Fig. 1, which was supported by NMR and IR data. The major stage in the manufacture of PPI pyrazinamide conjugated conjugate is to produce a amide bond.

Pyrazinamide is selected as a linker to diminish the haemolytic toxicity of PPI 5G dendrimer. As stated in the existing study (Nabi *et al.,* 2020), Pyrazinamide is the drug to treat the deadly tuberculosis disease. The motive for forming the amine bonds with PPI dendrimer's amino terminals is that it contains the hydroxylic groups. In the starting phase of production of the amide bond, it is used as the coupling reagent. The quantity of moles of Pyrazinamide conjugated to PPI dendrimer is approximately inferred with UV spectroscopy. The amount of residues of Pyrazinamide and the conjugates drug content is displayed in Table 1. Even though there are various amino terminal groups, the highest ratio is the usual ratio of 3.3 mol per conjugate for PABA spacers. Under specific studies, the regular medication molecules' ideal number is covalently joined to the PAMAM dendrimers. For example, PAMAM G4 and doxorubicin, along with the sixty-four amino terminal groups, have the usual ratio of 2:1; meanwhile, the PAMAM G4 and ibuprofen have the regular balance of 3:1. These recommend that transferring several drug molecules would not need the significant number of terminal groups. On the contrary, the dendrimer's capability to move drugs relies upon several elements, including the drug's chemical temperament, the carrier and spacer, and the reaction engaged.

Table 1: Taxonomy, % haemolysis of normal and PEGylated PPI 5.0G dendrimers.

Formulation code	A definite amount of terminal amine groups	Concentration	Percentage of haemolytic toxicity
5G-PPI dendrimer	64	0.2 µg/ml	20.42±0.82
PEGylated-PPI dendrimer	-	0.2 µg/ml	2.70 ± 1.20
DLDP (1:1)	-	0.2 µg/ml	1.40 ± 0.52

The haemolysis was created utilising 5 mg/ml formulations upon five per cent haematocrit red blood cells on incubation for one hour. The mean SD (n=3).

In the above table 1, the 20.42 0.82 % of 5.0G PPI dendrimer proved that it contains the most significant harmful haemolysis toxicity. Conversely, it is identified that loading the dendrimer with PEG2000 noticeably decreased the Homolysis toxicity of RBCs to the score of 2.701.20 per cent. The dendrimer polycationic nature is accountable for its cytotoxicity, particularly in the circumstance of a complete generation of amine-terminated charged dendrimers, and it is the main reason for the haemolytic toxicity in dendrimer that was adequate to prohibit its usage as the drug delivery system. On the other hand, it is revealed that the dendrimers conjugation decreases the haemolysis of

RBC since it subdues one's interaction with charged quaternary ammonium ions, which are generally available in amine-terminated dendrimers' overall generation. It is also inferred from an existing study. The dendrimers' hemolytic toxicity was enough to constrain their use as a drug delivery system. The toxicity of the findings of the present study also acknowledges it. Pyrazinamide Loaded PEGylated PPI Dendrimers exhibited a ratio of haemolytic toxicity of 1.400.52. Hence. It is revealed that the DLDP (Pyrazinamide Loaded PEGylated PPI Dendrimers) displays the lowest value of haemolytic toxicity.

The ex-vivo experiments were conducted using the Brine shrimp lethality assay, and its outcomes were obtained. The results are tabulated below in Table 2. The mean value in the tables two is calculated using the formula that is the mean of and is also the count of dead shrimps. The denotes the effective dose concentration at 50%.

Table 2: Brine Shrimp Lethality (Cytotoxic) Assay for Drug-Loaded Dendrimers.

Test	Solubility	Control			Shrimps-Live				Mean	ED_{50} (µg/ml)	Degrees of freedom
		Test tube-1	Test tube-2	Test tube-3	Dose µg/ml	Test tube-1	Test tube-2	Test tube-3			
DLDP	DMSO	7	7	8	25	5	8	1	8	41.8	0.0015
					50	4	4	2	12		
					100	1	2	3	16		
STANDARD	DMSO	8	7	6	2.5	4	3	2	12	2.24	0.1267
					5	1	1	1	18		
					10	0	0	0	21		

From the above table 2, it is inferred that the mean value (dead shrimps) in the standard PPI dendrimers obtained from in vivo experiments is higher, indicating that the haemolytic toxicity level is more significant. Meanwhile, the mean value (dead shrimps) in the Pyrazinamide Loaded PEGylated PPI Dendrimers (DLDP) is lower, implying that the haemolytic toxicity is more down. This proves that the conjugation of pyrazinamide in 5.0G PPI dendrimer will reduce the haemolysis toxicity level.

CONCLUSION

Dendrimers emerge as excellent drug carriers, but their toxicity limits their usage in the medical field. Hence, the research on haemolytic toxicity will be of greater significance for the safe application of dendrimers as the drug carrier system. Dendrimers toxicity relies upon various factors such as generation, size, surface charge, etc. Among the classes of dendrimers, PPI dendrimer remains the most recognised one. In addition to it, dendrimers can improve drug efficiency. Several types of research are being conducted to reduce the haemolytic toxicity in dendrimers by introducing various chemical modifications upon the surface. Hence, this research utilises the pyrazinamide drug, which is used for treating tuberculosis and is loaded into the PPI dendrimer to enhance its efficacy and decrease dendrimer toxicity. The PEGylation is discovered as the appropriate surface modification technique to reduce the haemolytic toxicity in 5.0G PPI dendrimer. The study utilises the EDA as the initiator core for synthesising 5.0G PPI dendrimer and finally loaded the Pyrazinamide. Hence, the Pyrazinamide loaded. PEGylated 5.0 G PPI exhibits low toxicity compared to the free fifth-generation PPI and PEGylated PPI dendrimer. Also, the study was conducted *in vivo*. *Ex vivo* experiments revealed lower toxicity levels in Pyrazinamide loaded PEGylated 5.0 G PPI. Through this synthesis.

FUTURE SCOPE

The promising study identified to be exploited commercially to increase PPI Dendrimer-loaded anticancer formulation.

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